Filariasis elimination in Egypt: impact of low microfilaraemics as sources of infection for mosquitoes

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ABSTRACT The elimination strategy for lymphatic filariasis aims at reducing blood microfilaraemia to levels at which vector transmission cannot be sustained. We aimed to determine whether patients with pre-treatment low or ultra-low microfilaria (MF) counts could be a reservoir of infection after mass drug administration (MDA) with a combined regimen. Laboratory-reared mosquitoes were fed on 30 volunteers after 2 rounds of MDA. Microfilaria uptake, infectivity rates and number of Wuchereria bancrofti L3 per mosquito were assessed. One year after MDA-1, 6 subjects transmitted MF, but up to 9 months after MDA-2 transmission failed. Six months after MDA-2 > 90% had clear MF smears and either failed to transmit MF or transmitted MF that did not develop to L3. We conclude that the transmission cycle is seriously weakened after MDA-2.

Elimination de la filariose en Egypte : impact sur des sujets faiblement microfilariémiques comme sources d’infection pour les moustiques

RESUME La stratégie d’élimination de la filariose lymphatique vise à réduire la microfilarémie dans le sang à des niveaux auxquels la transmission vectorielle ne peut être maintenue. Notre but était de déterminer si les patients avec des numérations de microfilaries faibles ou ultra-faibles avant traitement pourraient constituer un réservoir d’infection après distribution massive de médicaments associés. Des moustiques élevés en laboratoire ont été nourris sur 30 volontaires après 2 campagnes de distribution massive de médicaments. L’absorption de microfilaries, les taux d’infectiosité et le nombre de larves L3 de Wuchereria bancrofti par moustique ont été évalués. Un an après la première distribution massive de médicaments, 6 sujets ont transmis des microfilaries, mais jusqu’à 9 mois après la deuxième distribution massive de médicaments il n’y avait plus de transmission. Six mois après la deuxième distribution, plus de 90 % présentaient des frottis clairs, et soit ne transmettaient pas des microfilaries soit transmettaient des microfilaries qui n’évolueraient pas vers le stade L3. Nous en concluons que le cycle de transmission est sérieusement affaibli après la deuxième distribution massive de médicaments.

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Introduction

Lymphatic filariasis is a major cause of clinical morbidity and an impediment to socioeconomic development affecting some 80 countries in the tropics and subtropics. Recently, improved diagnostic methods [1,2] and therapies [3] have led the World Health Organization to develop a strategy for worldwide elimination of the disease that is based on repeated annual mass drug administration (MDA) of single-dose combination drug regimens, albendazole with either diethylcarbamazine or ivermectin, to endemic populations [4].

The ultimate goal of the WHO Global Programme to Eliminate Lymphatic Filariasis is to reduce blood microfilaraemia to levels at which transmission by the vector mosquito cannot be sustained, thereby arresting the cycle of the disease. The programme has rapidly expanded, with 80 million people from 34 countries treated in 2002 [5]. Egypt is one of the first countries to have implemented a widescale national filariasis elimination programme based on the recommended strategy. In September 2000, it launched a 5-year intervention programme in 161 filaria-endemic villages, based on annual MDA with a single dose of diethylcarbamazine (6 mg/kg) and albendazole (400 mg). Some 2.3 million people in 178 villages were retreated in 2001 and 2002 [5] (Ministry of Health and Population, unpublished data, 2003).

Prior to the initiation of MDA, nocturnally periodic lymphatic filariasis caused by *Wuchereria bancrofti* infection was known to be focally endemic in villages of the Nile delta with levels of endemicity exceeding 20% in some areas [6]. However, most endemic villages had pretreatment low rates and intensities of infection [7]. Epidemiological studies conducted by our group revealed that about 60% of untreated people identified as microfilaraemic by venous blood filtration had low microfilaria (MF) counts (< 100 MF/mL) and of these 65% had ultra-low counts (< 30 MF/mL) (R.M.R. Ramzy, unpublished data, 1990). Culicine vectors of filariasis exhibit limitation or proportionality and therefore ingest and develop low-density MF more efficiently than anophelines, which exhibit facilitation [8]. Extensive experimental infection studies carried out by our group revealed that the main filariasis vector mosquito, *Culex pipiens*, readily ingests and develops MF from low-density MF carriers. This suggests that ultra-low and low MF carriers could be a significant reservoir of infection capable of sustaining transmission [9]. Threshold MF levels needed for transmission are largely undetermined. We have recently reported, however, that rates of mosquito MF uptake and development resulting from exposure to MF carriers with negative blood smears were extremely low [10]. Therefore, we proposed that, although zero MF count in blood smears is not an absolute threshold, it is a practical goal for the purposes of filariasis elimination programmes.

This study addresses issues that are important to filariasis elimination efforts in Egypt and around the world. We have examined the effects of 2 cycles of community-based MDA of diethylcarbamazine/ albendazole in subjects with low pretreatment MF counts and whether such treatment reduces MF levels below the threshold needed for transmission by mosquitoes. A separate study of the effects of diethylcarbamazine/albendazole on MF in subjects with high MF counts and on parameters of mosquito infection will be presented elsewhere (H.A. Farid et al., unpublished report, 2004).
Methods

To investigate whether Cx. pipiens may acquire and develop W. bancrofti MF after feeding on pretreatment low-level microfilaraemic subjects (< 100 MF/mL venous blood) administered with a single annual dose of diethylcarbamazine/albendazole, we exposed mosquitoes to infected subjects 12 months after they were treated with the first cycle of this regimen (MDA-1) and 3, 6, 9 and 12 months after the second cycle of treatment (MDA-2).

Selection of W. bancrofti carriers

Primary selection of low-level MF carriers was through a large survey we carried out in a filaria endemic village in Giza governate, within 6 months prior to the initiation of the national elimination programme. We selected 20 females and 14 males (13–45 years of age). The criteria for inclusion in the study were: pretreatment low-level microfilaraemia (< 100 MF/mL venous blood) and participation in the national lymphatic filariasis elimination programme (had received the first dose of treatment). Intake of drug (diethylcarbamazine: 6 mg/kg, albendazole: 400 mg) was observed by health workers.

We obtained written informed consent for night blood collection and exposure to mosquitoes from all participants. The study was approved by the institutional review board at Ain Shams University and by the Egyptian Ministry of Health and Population.

Effects of therapy on W. bancrofti infection

To estimate the impact of treatment on blood microfilaraemia, 1 mL venous blood samples were collected from study participants between 22:00 and 24:00 hours at the specified post-treatment time points. Membrane-filtered blood samples, 5 µm pore size (Nucleopore, Pleasanton, California), were Giemsa stained and examined by microscopy for MF. One week after each blood collection, Cx. pipiens females (about 300 females/volunteer) were exposed (22:00 to 24:00 hours) to selected individuals. For this, field-collected Cx. pipiens larvae reared to maturity in our insectary were transported to the field site and allowed to feed on treated MF carriers as described in Farid et al. [10]. Before exposure to mosquitoes, 50 µL finger-prick blood samples were thick-smear on a glass slide for further assessment of microfilaraemia.

Uptake of MF (% uptake and MF/mosquito) by mosquitoes was estimated in an aliquot of blood-engorged females (50 mosquitoes) that were cold-killed immediately after blood feeding and stored at −70 °C until they were dissected and microscopically examined for the presence and number of MF. The other females were maintained on a carbohydrate diet for 12 days (the extrinsic incubation period of the parasite), then surviving females were cold-killed and dissected for the presence and number of infective W. bancrofti (L3/mosquito).

Relative MF levels by filter (expressed as % of the pretreatment level) were calculated for each volunteer. Changes in MF counts by smear and mosquito uptake and infectivity were calculated relative to baseline values obtained just prior to MDA-2. In cases where MF counts, MF uptake or infectivity increased after treatment, relative values were considered to be 100% (i.e. no reduction). Clearance was defined as the percentage of treated subjects who had negative blood MF counts, or from whom blood-fed mosquitoes failed to ingest MF or failed to produce L3.
Statistical analysis
Proportions were tested by chi-squared or Fisher exact test. Analysis of effects of treatment was done using a customized general linear model. Repeated measurements of mean blood MF count, mean % MF uptake, % infectivity, MF/mosquito and L3/mosquito were compared by analysis of variance of positively skewed data transformed as square roots. Homogeneity of variances was determined by Levene’s test of equality. Pairwise comparisons were based on estimated marginal means in the general linear model procedure or on the Mann–Whitney test for non-parametric data. Database management and analysis were performed with SPSS, version 11.0.1 (SPSS Science, Chicago, Illinois, USA).

Results

Effect of therapy on venous blood
The study included a total of 34 participants with pretreatment filter blood counts of 1–74 MF/mL. We aimed, however, to test 30 participants at any post-treatment time point since it was not always possible to test all of them. Mass treatment with a single annual dose of diethylcarbamazine/albendazole induced sustained reductions in MF filter counts (Table 1 and Figure 1).

One year after MDA-1, 24 of 30 MF carriers had significantly lower filter counts (2.1 ± 5.9, \(P < 0.001\)). At each time point after MDA-2, ≥ 29 patients had significantly reduced filter counts relative to pretreatment (\(P = 0.002\)) and relative to counts 12 months after MDA-1 (\(P = 0.06\)). Reductions were dramatic 1 year after MDA-1 and 3 months after MDA-2 (Figure 1), but further decreases were not significant (\(P = 0.392\)). Clearance of MF by filter was moderate 1 year after MDA-1. Three months after MDA-2, more than 80% of the participants were filter-negative and this rose steadily to about 95% 12 months after. One year after MDA-1, the 13 filter-positive subjects had mean filter counts of

<table>
<thead>
<tr>
<th>Time post-treatment</th>
<th>No. tested</th>
<th>No. positive</th>
<th>MF/mL venous blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SDa</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>34</td>
<td>34</td>
<td>23.1 ± 22.4</td>
</tr>
<tr>
<td>MDA-1 12 months</td>
<td>30</td>
<td>13</td>
<td>15.4 ± 34.7</td>
</tr>
<tr>
<td>MDA-2 3 months</td>
<td>30</td>
<td>5</td>
<td>2.2 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4</td>
<td>2.5 ± 11.9</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>3</td>
<td>1.0 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>2</td>
<td>1.6 ± 8.4</td>
</tr>
</tbody>
</table>

MF = microfilaria.
MDA-1 = first cycle of mass drug administration.
MDA-2 = second cycle of mass drug administration.
SD = standard deviation.
*All post-treatment values were significantly lower than pretreatment, \(P < 0.001\).
35.5 ± 46.2 MF/mL (median 8.0), whereas 1 year after MDA-2, 2 filter-positive subjects had values of 45.0 and 1.0 MF/mL blood respectively. Filter levels of smear-positive subjects were, however, comparable to pretreatment levels ($P = 0.678$).

**Mosquito feeding studies**

Mosquitoes were exposed to selected volunteers 1 year after MDA-1 and at 3-month intervals after MDA-2 (Table 2). Up to the 9-month time point after MDA-2, 4980 mosquito blood meals from 120 feeds were dissected for MF uptake, with 41.5 ± 10.9 (10–50, median 50) females dissected per feed. Likewise, 14 501 females were dissected for infectivity, with 120.8 ± 44.5 mosquitoes dissected by subject-treatment (24–259, median 115). Uptake and infectivity data obtained at the 12-month time point after MDA-2 were, however, from only 118 and 360 mosquitoes fed on 6 and 4 volunteers respectively, and were excluded from statistical analysis. In fact, the weather was exceptionally hot and humid, severely affecting our studies at this time point. Repeat measurements for smear

<table>
<thead>
<tr>
<th>Time post-treatment</th>
<th>No. tested</th>
<th>Smear count (mean ± SD)</th>
<th>MF uptake (%)</th>
<th>Mosquito infection (mean ± SD)</th>
<th>L3/100 mosquitoes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MF/50 µL finger prick thick smear taken immediately before exposure to mosquitoes.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Six subjects tested.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Four subjects tested.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2 Effect of mass drug administration with an annual single dose of diethylcarbamazine and albendazole on Wuchereria bancrofti infection of Culex pipiens exposed to treated microfilaraemic patients**

Figure 1 Mean microfilaria (MF) counts after 2 cycles 12 months apart of mass drug administration (MDA) of a single dose of diethylcarbamazine and albendazole
counts and indices of filarial transmission by mosquitoes were obtained from 27 MF carriers. Mean MF smear counts and rates and intensities of MF uptake after MDA-2 were significantly lower than those observed after MDA-1 ($P = 0.020$, $P = 0.013$, and $P = 0.012$, respectively) (Figure 2). For subjects who remained smear-positive ($n = 8$) or transmitted MF to mosquitoes ($n = 9$) after MDA-1, relative levels of smear MF counts and rates of MF uptake were low 3 months after MDA-2, and completely clear at later time points. Three months after MDA-2, mean rates and intensities of infectivity were extremely low, but did not vary from mean values observed after MDA-1 ($P = 0.089$ and $P = 0.083$ respectively) (Table 2). These parameters were, however; significantly lower at later time points ($P = 0.043$ and $P = 0.045$ respectively). Six subjects (20%) who transmitted MF that developed to L3 in mosquitoes 1 year after MDA-1 failed to transmit MF to mosquitoes up to 9 months after MDA-2.

**Clearance rates**

One year after MDA-1, clearance rates for MF by smear and MF uptake were moderate, but were 80% for mosquito infectivity (Figure 3). Clearance rates for MF counts by smear, MF uptake and infectivity increased to over 90% following MDA-2 (Figure 3). Complete clearance of smear and complete failure of mosquitoes from subjects to ingest MF and develop L3 were observed 6 months after MDA-2; but 3 months later, 3 of 3337 mosquitoes produced one L3 each. All 3 mosquitoes were fed on 1 subject, who was, however, MF-negative by both filter and smear.

**Smear-positive subjects**

Ten of 30 treated subjects were still MF positive by smear after MDA-1 (Table 3). Mosquito MF uptake and development
were reduced after feeding on all retreated patients. Three months after MDA-2, MF reduction of 3 smear-positive subjects was 31.3% ± 81.9% of the level observed 1 year after MDA-1, but MF uptake and infectivity did not change significantly ($P = 0.440$). At later time-points, only 1 subject remained smear-positive, but failed to transmit MF to mosquitoes (Table 3).

**Smear-negative subjects**

One year after MDA-1, smear-negative subjects infected some mosquitoes, but MF ingested by females did not produce L3 (Table 3). Three months after MDA-2, both the rate and intensity of MF uptake were 11.6% of the respective values observed after MDA-1, but infectivity was resumed. Observed differences, however, were not significant ($P = 0.070$). Between 3 and 9 months after MDA-2, MF uptake and development were either extremely low or completely suppressed. Fluctuations between these time points were not significant ($P = 0.133$).

**Table 3**

<table>
<thead>
<tr>
<th>Time post-treatment</th>
<th>No. tested</th>
<th>MF uptake (%)</th>
<th>MF/100 mosquitoes</th>
<th>Infectivity (%)</th>
<th>L3/100 mosquitoes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Smear-positive subjects</strong></td>
<td>12 months</td>
<td>10</td>
<td>3.85 ± 4.05</td>
<td>4.78 ± 5.17</td>
<td>1.15 ± 2.05</td>
</tr>
<tr>
<td></td>
<td>15 months</td>
<td>3</td>
<td>2.00 ± 2.00</td>
<td>2.00 ± 2.00</td>
<td>0.64 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>18 months</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>21 months</td>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Smear-negative subjects</strong></td>
<td>12 months</td>
<td>20</td>
<td>1.29 ± 2.92</td>
<td>1.30 ± 2.97</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>15 months</td>
<td>27</td>
<td>0.15 ± 0.77</td>
<td>0.15 ± 0.79</td>
<td>0.15 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>18 months</td>
<td>30</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>21 months</td>
<td>29</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04 ± 0.21</td>
</tr>
</tbody>
</table>

*Subjects were retreated after 12 months. MF = microfilaria.

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المجلة الصحية للشرق المتوسط، منظمة الصحة العالمية، المجلد التاسع، العدد 4، 2003.
After MDA-1, the rate of MF uptake by mosquitoes fed on smear-negative subjects was significantly lower ($P = 0.044$) than for mosquitoes fed on smear-positive ones, but the difference in infectivity was not significant ($P = 0.422$). After MDA-2, rates of MF uptake and infectivity did not differ significantly for mosquitoes fed in the smear-negative or smear-positive groups ($P = 0.086$ and $P = 0.509$ respectively).

Discussion

Our study aimed to determine whether the transmission cycle of the filarial parasite by mosquitoes could be interrupted by MDA of annual single doses of a combined regimen of diethylcarbamazine/albendazole to low microfilaraemic residents of endemic villages in Egypt. This group of MF carriers represented a significant proportion of residents with microfilaraemia in endemic localities in Egypt. This is the first formal study to assess transmission of *W. bancrofti* by *Cx. pipiens* mosquitoes following MDA based on diethylcarbamazine/albendazole. Our human and mosquito data clearly demonstrated that administration of 2 rounds of drug treatment resulted in clearance of microfilaraemia from more than 90% of the patients we studied, and mosquitoes failed to ingest MF and develop L3 from these persons 18 months post-MDA. Because the volunteers who participated in our study had extremely low pretreatment MF counts (1–74 MF/mL) by membrane filtration, some of them might have cleared microfilaraemia spontaneously, as observed in a recent longitudinal study we carried out in filarial endemic villages of the Nile delta [7]. However, the clearance rates observed are important, and although they might have been overestimated, we believe that they mostly resulted from the first round of MDA.

Of special interest is our observation that mosquitoes exposed to a volunteer who had 115 MF/mL 11 months post-MDA-1 failed to ingest and develop infective larvae throughout the study period, suggesting that ingested MF, although morphologically intact, were considerably affected by the drug. Also of importance is our observation that at 21 months post-MDA-1, mosquitoes dissected after feeding were free of MF, but mosquitoes blood-fed on the same volunteer at the same time point, when dissected after the extrinsic incubation period of the parasite had elapsed, could contain L3. This indicates that MF uptake was limited and not detectable in the 50 mosquito samples dissected. It was difficult to increase the sample size because other fed mosquitoes were needed to assess infectivity. These findings suggest that, as the MDA programme progresses, we should aim only to detect L3 in mosquito vectors, as they are the end product for transmission. Given the fact that thousands of wild-caught mosquitoes should be tested as the MDA programme approaches an end, this could be better achieved using molecular diagnostic tools, which are more sensitive than (and superior to) the traditional dissection method [11]. In any event, since the currently available molecular tools (polymerase chain reaction assays) cannot differentiate between the larval stages of the parasite (MF, L1, L2 and L3) in mosquitoes, there is an urgent need to develop a sensitive polymerase chain reaction assay that is specific for detection of L3.

Our data show that some of the patients with low MF counts remained microfilaraemic after the first-round of MDA. For instance, 13 subjects (43.3%) had residual MF by filter after MDA-1. *Culex pipiens* present in lymphatic filariasis endemic areas are considered efficient vectors as mosquitoes fed on 6 of 30 low microfila-
raemic subjects (20%) 12 months post-MDA-1 were still capable of ingesting MF and supporting their development to L3. This demonstrates the need for at least 1 more cycle of MDA.

After MDA-2, 3 or fewer of 30 participants remained microfilaraemic by thick smear. Mosquito feeding data obtained from such a small number of smear-positive subjects cannot be conclusive. In contrast, mosquitoes which had blood-fed on 29 smear-negative subjects 9 months after MDA-2 produced only 1 L3. This finding strongly supports our previous recommendation that filarial elimination programmes should aim at reducing MF counts by smear to zero [10]. It also suggests that 2 cycles of MDA with diethylcarbamazine/albendazole are probably sufficient to interrupt filarial transmission in low-endemicity settings. However, 40% of the MF carriers in filarial endemic villages of the Nile delta had pretreatment microfilarial counts > 100 MF/mL, highlighting the necessity for pursuing the elimination programme for as long as necessary. In this respect, xenomonitoring the impact of elimination programmes could be a powerful tool for estimating the number of cycles necessary to interrupt transmission.

We conclude that the transmission cycle of the filarial parasite by mosquitoes is seriously impaired by MDA of annual single doses of a combined regimen of diethylcarbamazine/albendazole. If efforts to achieve high MDA coverage rates can be sustained for the planned 5–6 years, our data suggest that the Egyptian national lymphatic filariasis elimination programme is likely to diminish filariasis as a public health problem in the country. We are currently xenomonitoring the impact of MDA in villages with low prevalence and intensity of infection, and data accumulating from these studies support our conclusion.

Acknowledgements

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Lymphatic filariasis

In 1997, the World Health Assembly, having considered the problem of lymphatic filariasis, resolved to eliminate the disease as a public health problem. That decision is embodied in resolution WHA50.29. This signalled the start of the Global Programme to Eliminate Lymphatic Filariasis. Thus WHO, with support from organizations including international development agencies and foundations, the private sector, NGOs, academia and research institutions began developing a coalition to eliminate the disease. This coalition has continued to grow and in 2000 was named the Global Alliance to Eliminate Lymphatic Filariasis.

There are two principal goals of the Programme to Eliminate Lymphatic Filariasis: to interrupt transmission of infection; and to alleviate and prevent both the suffering and disability caused by the disease. Further information about filariasis and the strategies to eliminate the disease can be obtained on line at: http://www.who.int/topics/filarasis/en/ and http://www.who.int/tdr/diseases/lymphfil/