Phenotypic Evidence of Emerging Ivermectin Resistance in *Onchocerca volvulus*

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Abstract

**Background:** Ivermectin (IVM) has been used in Ghana for over two decades for onchocerciasis control. In recent years there have been reports of persistent microfilaridermias despite multiple treatments. This has necessitated a reexamination of its microfilaricidal and suppressive effects on reproduction in the adult female *Onchocerca volvulus*. In an initial study, we demonstrated the continued potent microfilaricidal effect of IVM. However, we also found communities in which the skin microfilarial repopulation rates at days 90 and 180 were much higher than expected. In this follow up study we have investigated the reproductive response of female worms to multiple treatments with IVM.

**Methods and Findings:** The parasitological responses to IVM in two hundred and sixty-eight microfilaridermic subjects from nine communities that had received 10 to 19 annual doses of IVM treatment and one pre-study IVM-naïve community were followed. Skin snips were taken 364 days after the initial IVM treatment during the study to determine the microfilaria (mf) recovery rate. Nodules were excised and skin snips taken 90 days following a second study IVM treatment. Nodule and worm density and the reproductive status of female worms were determined. On the basis of skin mf repopulation and skin mf recovery rates we defined three categories of response—good, intermediate and poor—and also determined that approximately 25% of subjects in the study carried adult female worms that responded suboptimally to IVM. Stratification of the female worms by morphological age and microfilarial content showed that almost 90% of the worms were older or middle aged and that most of the mf were produced by the middle aged and older worms previously exposed to multiple treatments with little contribution from young worms derived from ongoing transmission.

**Conclusions:** The results confirm that in some communities adult female worms were non-responsive or resistant to the anti-fecundity effects of multiple treatments with IVM. A scheme of the varied responses of the adult female worm to multiple treatments is proposed.

Introduction

Ivermectin (Mectizan) has been in operational use for the control of human onchocerciasis in the former Onchocerciasis Control Programme in West Africa (OCP) areas since 1987 and is still the drug of choice for the African Programme for Onchocerciasis Control (APOC) and the Onchocerciasis Elimination Programme for the Americas (OEPA). A single dose of ivermectin rapidly kills skin microfilariae (mf) and inhibits microfilarial release by adult female worms, possibly because of paralysis of the lower uterus [1]. These effects result in a rapid decline in skin microfilarial counts, an accumulation of degenerating intra-uterine microfilariae and a long-lasting suppression of *Onchocerca volvulus* microfilaridermias [2]. However, the inhibition of mf release is reversible by six months resulting in the reappearance of microfilariae in the skin (repopulation) and after a further six months a restitution of a proportion of the initial load (recovery) [2]. A proportion of ivermectin exposed female worms do not resume reproductive activity even after one year [1] and this can persist for up to 18 months after treatment [3]. The prolonged microfilarial suppressant effect of ivermectin has beneficial effects on morbidity and on parasite transmission.

Several studies [1,4,5,6,7,8] have demonstrated that multiple treatments with ivermectin have marked suppressive effects on embryogenesis consistent with some cumulative effect of ivermectin treatments on microfilarial production. Quantitative estimates have ranged from an irreversible decline in microfilarial production of ~30% after five annual treatments [9], a reduction in the productivity index of 90% or more after 10 six-monthly doses over 6 years [10], to arrest of development at the single cell stage after four or five six-monthly doses [11]. Recently, the cumulative effect of ivermectin on microfilarial production by *O.
Onchocerciasis, commonly known as river blindness, is caused by the filarial nematode Onchocerca volvulus and is transmitted by a blackfly vector. Over 37 million people are thought to be infected, with over 90 million at risk. Infection predominantly occurs in sub-Saharan Africa. Foci also exist in the Arabian Peninsula and Central and South America. Ivermectin, the sole pharmaceutical available for mass chemotherapy, has been used on a community basis for annual or semi-annual treatment since 1987. Multiple treatments with ivermectin kill the microfilariae that are responsible for the pathology of onchocerciasis. More importantly, ivermectin suppresses the reproductive activity of the adult female worms, thus delaying or preventing the repopulation of the skin with new microfilariae and thereby reducing transmission. This study extends earlier reports of sub-optimal responses to ivermectin by examining repopulation levels of microfilaria one year after treatment, worm burdens per nodule, the age structure of adult female worms recovered from nodules, and the reproductive status of adult female worms 90 days after ivermectin treatment. In some communities which have shown a pattern of sub-optimal response to treatment, the data is consistent with an emergence of ivermectin non response or resistance manifested by a loss of the effect of ivermectin on the suppression of parasite reproduction.

Ivermectin Suboptimal Response in O. volvulus

Author Summary

Onchocerciasis, commonly known as river blindness, is caused by the filarial nematode Onchocerca volvulus and is transmitted by a blackfly vector. Over 37 million people are thought to be infected, with over 90 million at risk. Infection predominantly occurs in sub-Saharan Africa. Foci also exist in the Arabian Peninsula and Central and South America. Ivermectin, the sole pharmaceutical available for mass chemotherapy, has been used on a community basis for annual or semi-annual treatment since 1987. Multiple treatments with ivermectin kill the microfilariae that are responsible for the pathology of onchocerciasis. More importantly, ivermectin suppresses the reproductive activity of the adult female worms, thus delaying or preventing the repopulation of the skin with new microfilariae and thereby reducing transmission. This study extends earlier reports of sub-optimal responses to ivermectin by examining repopulation levels of microfilaria one year after treatment, worm burdens per nodule, the age structure of adult female worms recovered from nodules, and the reproductive status of adult female worms 90 days after ivermectin treatment. In some communities which have shown a pattern of sub-optimal response to treatment, the data is consistent with an emergence of ivermectin non response or resistance manifested by a loss of the effect of ivermectin on the suppression of parasite reproduction.

Methods

Study Design

This was a randomized, open 21 month longitudinal study involving two annual ivermectin treatments, serial skin snipping and nodulectomies in the communities under a study that was carried out between October, 2004 and June, 2006. Skin microfilarial profiles were assessed in onchocerciasis affected patients at various time-points pre- and post-treatment to determine skin mf repopulation and, at one year post treatment, the microfilaria recovery rates. At the end of the study (90 days after a second annual treatment during the study), nodules containing adult Onchocerca volvulus were surgically removed and digested to extract adult worms. Embryogrammes were constructed to assess the reproductive status of the adult female worms. A summary of the study design and conduct is shown in Figure 1.

Study Population and Area, Subject Eligibility and Selection

The study was carried out in 10 onchocerciasis endemic communities located in three Districts in Ghana; Kintampo and Atebu Districts in the Brong-Ahafo Region, and Gonja East District in the Northern Region. The criteria for selection of the communities have been detailed previously [15] and for the 9 communities that had received multiple treatments include: good written documentation on annual community treatment coverage, good community and individual treatment history, at least one survey over the previous 6 yrs (prevalence, community microfilarial load or both), average treatment coverage ≥50% in the previous 5 yrs, uninterrupted annual treatment over the previous 6 years and ivermectin treatment within the previous 10–12 months. Additionally, communities had to be accessible by road throughout the entire study period, especially during the period for nodulectomies. One community was ivermectin-naïve prestudy.

Ethical approval for the study was obtained from the Institutional Review Board of Noguchi Memorial Institute for Medical Research, Ghana and the Ethical Review Board of McGill University, Canada. The informed consent procedure involved a durbar (meeting with the chief, elders and the entire community) with the study population at which the study design, investigative procedures and the risks and benefits of participation were outlined by the principal investigator in English and in the local language through an interpreter. Unlimited time was allowed for questions and explanations. After the durbar individuals who were interested in participation met with the investigator and interpreter individually and the contents of the consent form were explained in detail. After further questions and explanations, each subject signed or thumb-printed an informed consent form that testified to the fact that they had been told the details of the study, any questions they had asked had been answered to their satisfaction and that they freely consented to participate in the study.

Investigative Procedures

Subjects were aged between 18-65 years, had lived in the communities for at least ten years and, with the exception of the prestudy ivermectin-naïve, had received between 10 and 19 annual doses of IVM confirmed by interviews of participants and community ivermectin distributors, and examination of written
treatment records, and had been skin snip positive for *O. volvulus* at the beginning of the study.

All subjects were examined in detail for Onchocerca nodules and the locations of the nodules were recorded on anatomical diagrams. One year after the last ivermectin treatment (364 days), one skin snip was taken from each iliac crest using a 2 mm Holth-type corneo-scleral punch, by the same member of the study team, who was experienced with conducting skin snips, to maintain consistency. A second annual ivermectin treatment was then given during the study, as part of Ghana Onchocerciasis Control Program, to all subjects at 150 mg/kg body weight. The final skin snips were then taken by the same operator only from subjects who were nodulectomised 90 days after the second IVM treatment. Skin snips were placed in 96-well microtitre plates containing a few drops of physiological saline solution, incubated for 24 hours and microfilariae that had emerged were counted using a dissecting microscope. The average of the microfilarial counts from the two sites was taken as the intensity of infection for each subject expressed as microfilariae/snip.

Nodulectomies were performed 90 days following the second ivermectin treatment, i.e. day 455 after the first study treatment. Using local anesthesia, all palpable Onchocerca nodules were aseptically excised from 140 patients from the 10 endemic communities. Nodules were stored in liquid nitrogen until ready for digestion. Nodules were dressed free of extraneous tissue and placed in 50 ml tubes containing 10 ml of 0.5% collagenase (in sterile medium 199 solution) for digestion at 37°C in a shaking water bath for 10–24 hours. Adult worms were harvested after washing with sterile normal saline solution, and under a dissecting microscope, the viability and morphological age of worms at the time of nodulectomy were scored and intact/viable female worms prepared for embryogramme analysis. Worms were classified as alive prior to nodulectomy based on intact internal morphology, motility of the worms, and the condition of the uterine musculature. Broken or ruptured worms were not examined for embryogrammes. The age of the worms was estimated based on the morphology, including the color and size of the female worms, the prominence of cuticular ridges and the degree of inclusions [10,11,25]. In addition to the above criteria, small and transparent worms were scored as young, opaque and yellowish as middle aged, and large and brown as older [25,26]. Each intact female worm was cut into small pieces, two millilitres of fresh sterile medium 199 was added and worms homogenized using a toughened glass test tube mortar and pestle. By turning the pestle gently, the embryonic stages were squeezed out of the pieces of worms and embryogrammes constructed [27]. The homogenate was transferred to a Fuchs-Rosenthal counting chamber and all embryonic stages assessed as described previously [26,27]. Quantitative assessment of normal and abnormal forms of each embryonic stage up to stretched microfilariae was done to determine the reproductive status and microfilarial content of each individual female worm [28].

**Data Management and Statistical Analysis**

After data verification, the mean microfilarial density (mf/snip) at each time point and the density as a percentage of the initial count were determined for each community. The number of subjects in each community whose skin mf densities were greater than, the same as, or less than the initial density, or who were skin snip negative, was defined, as well as the number of subjects with greater than 10 mf/snip at day 364. Based on skin mf repopulation (early reappearance of microfilariae in the skin at day 90 and/or day 180 post-IVM treatment determined in the initial study), the mf recovery rate (skin mf density, at day 364, as a percentage of the initial count) was calculated for each community.

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**Figure 1. Study design.** Design for the entire study, showing time points used for examination of various parameters and the study populations at each phase of the study.
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Differences were considered as significant at p<0.05. The Chi-square test or Fisher’s exact test where appropriate. Female worms and embryogramme analysis were carried out using categories regarding prevalence, reproductive activity of adult and pair-wise comparisons by the non-parametric Mann-Whitney log transformed data using the Kruskal-Wallis non-parametric test.

Comparisons of microfilarial densities, mf recovery at day 364 and mf repopulation at day 455 (90 days after the second study IVM treatment) between the 10 communities were carried out on log transformed data using the Kruskal-Wallis non-parametric test and pair-wise comparisons by the non-parametric Mann-Whitney test. Comparison between the 10 communities in terms of nodule characteristics and worm density and between the IVM response categories regarding prevalence, reproductive activity of adult female worms and embryogramme analysis were carried out using the Chi-square test or Fisher’s exact test where appropriate. Differences were considered as significant at p<0.05.

Results

Two hundred and sixty eight of the 301 microfilaridermic subjects from the 10 communities who had fully participated in the previous phase of the study [15] were available and agreed to continue in the follow up study. The results for pretreatment and microfilaria assessment up to day 180 have already been reported [15]. In that report, the microfilariae in all communities responded readily to ivermectin with reductions of 99.3% to 100% being achieved at day 30. However, we found that while in five of the nine communities that had received multiple doses of ivermectin (Senyase, Beposo, Hiampe, Baaya) the mf skin recovery rates of more than 110%; the exception was Asubende which had a recovery rate of 103.8%. Three of the four communities previously classified as poor responders, had microfilaria recovery rates of more than 110%; the exception was Wiae with 99.1%.

We compared mf skin recovery rate at day 364 post IVM treatment (Table 1) between all the 10 communities. Pair-wise comparisons showed that at day 364 there were no differences in mf skin recovery between the four multi-dosed communities (Senyase, Beposo, Hiampe, Baaya). However, Begbemdo, the pre-study IVM naïve community, had significantly lower (p<0.05) mf recovery rate than the four communities. These five communities had significantly lower mf recovery than Kyingakrom, Jagbenbendo and New Longoro (p<0.01) and Wiae and Asubende (p<0.05).

The distribution pattern of mf recovery rates of individuals in the communities is summarized in Table 2. The good ivermectin response communities had 0–6% of subjects with an mf recovery rate of more than 100% of pretreatment counts compared with 40.3%–51.9% of the subjects in the poor ivermectin response communities. Between these two groups were Asubende (33.3%)

### Table 1. Densities of *O. volvulus* microfilaria before, and at 364 days after treatment, in each community.

| Community      | No. of subjects examined on day -7 N = 268 | Microfilaria/snip at Day -7 Density % of day -7 * (recovery rate) | Day 364 post-treatment Density % of day -7 * (recovery rate) |
|----------------|--------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Senyase        | 18                                         | 10                                                           | 1.69                                                         | 1.21                                                         | 71.6 |
| Beposo         | 18                                         | 10                                                           | 2.24                                                         | 1.62                                                         | 72.3 |
| Hiampe         | 17                                         | 17                                                           | 3.03                                                         | 2.37                                                         | 78.2 |
| Baaya          | 18                                         | 20                                                           | 1.38                                                         | 0.91                                                         | 65.9 |
| Asubende       | 19                                         | 12                                                           | 2.40                                                         | 2.49                                                         | 103.8% |
| Wiae           | 10                                         | 22                                                           | 3.42                                                         | 3.39                                                         | 99.11 |
| Kyingakrom     | 17                                         | 27                                                           | 6.40                                                         | 7.41                                                         | 115.8% |
| New Longoro    | 17                                         | 62                                                           | 5.73                                                         | 6.32                                                         | 110.3% |
| Jagbenbendo    | 12                                         | 51                                                           | 8.17                                                         | 9.22                                                         | 112.9% |
| Begbemdo       | 1                                          | 37                                                           | 30.74                                                       | 12.89                                                         | 41.9% |

Geometric mean densities (mf/skin snip) in the ten Ghanaian communities participating in the study.

*Data for % of day -7 are based on the subjects that participated at all sampling times up to day 364.

*p<0.05 when compared to the good responders.

*p<0.01 when compared with good responders or prestudy IVM-naïve.

*p<0.05 when compared to good responders. Good responders = Senyase, Beposo, Hiampe, Baaya. Intermediate responders = Wiae, Asubende. Poor responders = Kyingakrom, New Longoro and Jagbenbendo.

*Ivermectin naïve at study start.*

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and Wiae (27.3%). Additionally, in the good response communities, between 55–80% of the subjects had reductions in microfilarial counts, with 12–20% being amicrofilaridermic one year after treatment, while in the poor response communities only 26–32% of subjects had reductions and very few to zero. When the three variables (proportion of individuals having greater than pretreatment mf density, same as pre-treatment mf density, and less than pre-treatment mf densities) were compared together between the ten communities, Kyingakrom, New Longoro and Jagbenbendo (poor responding communities) were significantly higher ($p<0.001$) than the five communities (Senyase, Beposo, Baaya, Hiampe and Begbomdo) that responded well to IVM treatment. The three poor responding communities were also higher than Wiae and Asubende (intermediate response communities) but the differences were not significant.

A further comparison examined the number of subjects with more than 10 mf/s at day 364 across the communities (Table 2). There were 3/57 (5.3%) for Baaya, Beposo, Senyase, Hiampe and Begbomdo, the five communities (Senyase, Beposo, Baaya, Hiampe and Begbomdo) that responded well to IVM treatment. The three poor responding communities were also higher than Wiae and Asubende (intermediate response communities) but the differences were not significant.

When the results of the indices of skin repopulation by mf are taken together they show a gradation of response to multiple doses of ivermectin that permits the re-classification of the responses as good (Senyase, Beposo, Baaya, Hiampe and Begbomdo), intermediate (Asubende and Wiae), and poor (Kyingakrom, New Longoro and Jagbenbendo). The response to ivermectin in the previously treated naı¨ve community. In each of the three poor response communities the level of skin mf repopulation was greater after the second study treatment (day 455) than after the first study treatment (day 90), but the differences were not significant.

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Table 2. Distribution pattern in each community of microfilarial densities one year after ivermectin treatment.

| Community     | Total No. of subjects | % (No.) of subjects with day -7 mf density >10 mf/s | % (No.) of subjects with day 364 mf density: |
|---------------|-----------------------|-------------------------------------------------|---------------------------------------------|
|               |                       | % (No.) of subjects with day -7 mf density >10 mf/s | % (No.) of subjects with day 364 mf density: |
|               |                       |                                                 | > 100% of pre-treatment | The same as pre-treatment (100%) | Less than pre-treatment mf density (0-99%) | Reduced to zero (0%) | > 10 mf/s |
| Senyase       | 10                    | 10 (1)                                          | 0                                           | 20.0 (2) | 80.0 (8) | 20.0 (2) | 0          |
| Beposo        | 10                    | 10 (1)                                          | 0                                           | 20.0 (2) | 80.0 (8) | 20.0 (2) | 0          |
| Hiampe        | 17                    | 17.6 (3)                                        | 5.9 (1)                                     | 23.5 (4) | 70.6 (12) | 11.8 (2) | 11.8 (2)  |
| Baaya         | 20                    | 5 (1)                                           | 5.0 (1)                                     | 35.0 (7) | 55.0 (11) | 20.0 (4) | 5 (1)      |
| Asubende      | 12                    | 16.7 (2)                                        | 33.3 (4) n/s                                | 16.7 (2) | 50.6 (6) | 16.7 (2) | 16.7 (2)  |
| Wiae          | 22                    | 22.7 (5)$^a$                                    | 27.3 (6) n/s                                | 27.3 (6) | 45.5 (10) | 13.6 (3) | 22.7 (5)$^n$ n/s |
| Kyingakrom    | 27                    | 40.7 (11)$^a$                                   | 51.9 (14) $^a$                              | 22.2 (6) | 25.9 (7) | 3.7 (1)  | 48.1 (13)$^a$ |
| New Longoro   | 62                    | 25.8 (16)$^a$                                   | 40.3 (25) $^a$                              | 24.2 (15) | 32.3 (20) | 3.2 (2)  | 30.6 (19)$^a$ |
| Jagbenbendo   | 51                    | 37.3 (19)$^a$                                   | 43.1 (22) $^a$                              | 25.5 (13) | 31.4 (16) | 3.9 (2)  | 43.1 (22)$^a$ |
| Begbomdo      | 37                    | 81.1 (30)$^a$                                   | 2.7 (1)                                     | 0        | 97.3 (36) | 2.7 (1)  | 70.3 (26)$^a$ |

Three variables, proportions of individuals having (a) greater than pretreatment mf density, (b) same as pre-treatment mf density, and (c) less than pre-treatment mf density, were compared together between the ten communities. Kyingakrom, New Longoro and Jagbenbendo (poor responding communities) were significantly higher ($p<0.001$) than the five communities (Senyase, Beposo, Baaya, Hiampe and Begbomdo) that responded well to IVM treatment. The three poor responding communities were also higher than Wiae and Asubende (intermediate response communities) but the differences were not significant.

$^a$Ivermectin naïve at start of study. n/s = not significantly different from good responders. The superscript in the table e.g., (5)$^a$, represents the number of subjects that had >20 mf/s.

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Table 3. Densities and microfilaria repopulation rates after first (day 90) and second ivermectin treatments (day 455).

| Community       | No. of treatments at study end | No. of subjects nodulectomized and skin snipped day 455 | Microfilaria* /snip at |
|-----------------|-------------------------------|---------------------------------------------------------|------------------------|
|                 |                               | N = 140                                                  | Day -7     | Day 90 mf/s (% day -7) | Day 364 | Day 455 mf/s (% day 364) |
| Senyase         | 18                            | 8                                                       | 1.9        | 0 (0)                  | 1.3     | 0 (0)                     |
| Begbomdo        | 18                            | 8                                                       | 2.4        | 0 (0)                  | 1.7     | 0 (0)                     |
| Hiampe          | 17                            | 7                                                       | 3.3        | 0.06 (0.02)            | 2.2     | 0 (0)                     |
| Baaya           | 18                            | 6                                                       | 1.9        | 0 (0)                  | 1.2     | 0 (0)                     |
| Asubende        | 19                            | 5                                                       | 3.2        | 0 (0)                  | 3.38    | 0.08 (0.02)               |
| Wiie            | 10                            | 10                                                      | 4.1        | 0.28 (6.8)             | 4.0     | 0.2 (5.0)                 |
| Kyingakrom      | 17                            | 20                                                      | 7.9        | 1.7 (21.5)†            | 9.1     | 2.1 (23.1)†               |
| New Longoro     | 17                            | 20                                                      | 7.2        | 0.63 (8.8)†            | 8.1     | 0.99 (12.2)†              |
| Jagbenbendo     | 12                            | 39                                                      | 8.2        | 0.94 (11.5)†           | 9.16    | 1.1 (12.0)†               |
| Begbemdo ¶      | 2                             | 20                                                      | 44.2       | 1.4 (3.2)              | 17.3    | 0.5 (2.9)                 |

Geometric mean densities (mf/skin snip) of O. volvulus microfilaria and repopulation rates observed at day 90 following the first study IVM treatment (day 90) and second IVM treatment (day 455) in the 10 onchocerciasis endemic communities studied in Ghana.

*All data are based only on the subjects that were nodulectomized. At days 90 after the first study treatment and day 90 after the second study IVM treatment (i.e., day 455), there were no differences in skin mf repopulation (mf count as a % of pre-treatment) between five communities (Senyase, Begbomdo, Hiampe, Baaya and Begbomdo – good response or naive communities). However, three communities (New Longoro, Jagbenbendo and Kyingakrom) had significantly higher († p < 0.05) skin mf repopulation rates (%) than the good response communities.

¶ Previously naïve had received two study treatments.
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repopulation and recovery rates. The nodule characteristics and distribution of adult worm populations are shown in Table 4. Overall, 100 out of 140 subjects (71.4%) had nodules containing at least one viable worm. The features compared were the number of subjects with nodules containing viable worms, number of nodules with viable worms and the average number of male and female worms per nodule. The prestudy ivermectin naïve (Begbomdo), and the poor response communities were significantly higher (p < 0.0003) than the good response communities in terms of nodules with viable worms and the average number of male and female worms per nodule. The prestudy IVM treatment had only one third of the worms were older (52–78%) and approximately one quarter were middle aged (22–30%). The proportion of young worms was only 5.1% and 8.1% in the poor response communities only in the average number of male and female worms per nodule.

The age structure and the state of reproductive activity of adult female worm populations in endemic communities give important information on parasite transmission and the contribution of each parasite age group to the repopulation of the skin with microfilariae after IVM treatment. The adult female worms were grouped into older, middle aged and young worms as described previously and are presented by response category in Tables 5A and B. In all three categories of response to multiple doses of ivermectin at least half of the worms were older (52–78%) and approximately one quarter were middle aged (22–30%). The proportion of young worms was only 5.1% and 8.1% in the poor response communities.

Table 4. O. volvulus nodule characteristics and worm densities in the IVM response/treatment categories.

| Response/ treatment categories | IVM treatment history | No. of subjects nodulectomised | No. of nodules removed | No. (%) of subjects with nodules containing viable worms | No. (%) of nodules with viable worms | No. of male worms | No. of female worms | Average No. of males per nodule | Average No. of females per nodule |
|-------------------------------|-----------------------|-------------------------------|------------------------|----------------------------------------------------------|------------------------------------|--------------------|----------------------|-----------------------------|-------------------------------|
| Begbomdo¶                    | 2                     | 20                            | 46                     | 19 (95.0)†                                               | 42 (91.3)                         | 68                 | 104                  | 2.26†                       | 1.48†                         |
| Good                          | 18–19                 | 26                            | 51                     | 7 (26.9)                                                 | 12 (23.5)                         | 8                  | 18                   | 0.35                        | 0.16                          |
| Intermediate                  | 11–20                 | 15                            | 29                     | 10 (66.6)                                                | 16 (55.2)                         | 19                 | 36                   | 1.24*                       | 0.66*                         |
| Poor                          | 13–18                 | 79                            | 167                    | 64 (81.0)†                                               | 130 (77.8)                        | 180                | 258                  | 1.54†                       | 1.08†                         |
| Total                         | 140                   | 293                           | 100                    | 200                                                     | 275                               | 416                | 416                  | 1.42                        | 0.94                          |

The number of subjects with nodules containing viable worms, number of nodules with viable worms, and average number of males and females per nodule were compared between the different IVM response categories (see Table 2 for allocation of communities to response categories) and the IVM naïve community. Begbomdo and poor response communities were significantly higher (¶ p < 0.0003) than the good response communities in terms of number of nodules with viable worms and average number of male and female worms per nodule, as well as the number of subjects containing viable worms († p < 0.01). The intermediate response communities were significantly higher (* p < 0.002) than the good response communities in the average number of male and female worms per nodule.

¶ Ivermectin naïve at start of study.
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Table 5. Age structure of all female *O. volvulus* worms sampled in IVM response/treatment categories.

| Community response/treatment category | No. of years of IVM treatment | % (No.) older female worms | % (No.) middle aged female worms | % (No.) young female worms | Total number of female worms |
|--------------------------------------|-------------------------------|-----------------------------|---------------------------------|---------------------------|----------------------------|
| Begbomdo †                          | 2                             | 51.9 (54)                   | 26.9 (28)                       | 21.2 (22)§                | 104                        |
| Good                                 | 18–19                         | 77.8 (14)                   | 22.2 (4)                        | 0                         | 18                         |
| Intermediate                         | 11–20                         | 62.2 (23)                   | 29.7 (11)                       | 8.1 (3)                   | 37                         |
| Poor                                 | 13–18                         | 70.4 (181)                  | 24.5 (63)                       | 5.1 (13)                  | 257                        |
| Total                                | -                             | 65.4 (272)                  | 25.5 (106)                      | 8.1 (9.1)                 | 416                        |

The IVM naïve community had significantly higher numbers of young worms (§ *p* < 0.002) than each of the IVM community response categories. The worm age distribution was not significantly different between the good, intermediate and poor response categories.

† Begbomdo was IVM naïve at the start of the study.

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and intermediate response categories respectively and there were no young worms found in the good response category. This proportional distribution by age (52–78% for the older worms and 22–30% for the middle aged worms) was similar in all response categories except the prestudy ivermectin naïve that had a significantly higher proportion of young worms (21.2% *p* < 0.002) than all multidosed response categories (0–8%). When all the worms were pooled and compared by age group there were significantly more older (65.4%; *p* = 0.0001) than middle aged (25.5%; *p* = 0.0002) and young worms (9.1%; *p* < 0.0001), and more middle aged worms than young worms (*p* = 0.0001) (Table 5).

An examination of the live stretched mf production by the female worms showed clear differences between the 3 response groups. In the good response category, none of the worms examined was producing mf. In the intermediate response category 5% of the old worms and 10% of the middle aged worms were producing mf but none of the young worms. In the poor response category 15.1% of the old worms, 22.2% of the middle aged worms and only 0.8% of the young worms (1 worm) were producing mf. Overall, 12.2% of the older worms, 17.2% of the middle aged worms and only 1.1% of the young worms were producing mf (Table 6). The differences between the older and middle aged aged worms combined and the young worms were highly significant (*p* < 0.0001) but not that between the older and middle aged worms.

In order to more precisely quantify the contribution of the various age groups of adult worms to the skin microfilariae, the various age categories were pooled across all response groups and the mean production of stretched microfilariae calculated. The results are summarized in Table 7. The mean of the production of total (live and degenerate) stretched microfilariae/female worm was significantly higher in the middle aged worms as compared to the young worms (*p* = 0.039), but the older worms were not significantly different from the young worms (*p* = 0.21). The middle aged worm production of live stretched microfilariae/female worm was higher than those of the older (*p* = 0.042) and the young worms (*p* = 0.033). There was no significant difference between the numbers of live stretched microfilariae in the young worms compared with the older worms.

Table 6. Prevalence and reproductive activity of young, middle-aged and older female worms in the response/treatment categories.

| Response/treatment category | No. female worms Embryogrammed | No. (%) older female worms | No. (%) middle aged female worms | No. (%) young female Worms |
|-----------------------------|---------------------------------|-----------------------------|---------------------------------|---------------------------|
|                            | Total (live stretched mf)       | Total (live stretched mf)   | Total (live stretched mf)       |
| Begbomdo †                 | 20                             | 9 (45.0)                    | 6 (30.0)                        | 5 (25.0)                  |
| Good                       | 14                             | 10 (71.4)                   | 4 (28.6)                        | 0                         |
| Intermediate               | 20                             | 10 (50.0)                   | 7 (35.0)                        | 3 (15.0)                  |
| Poor                       | 126                            | 66 (52.4)                   | 48 (38.1)                       | 12 (9.5)                  |
| Total                      | 180                            | 95 (52.8)                   | 65 (36.7)                       | 20 (11.1)                 |

In each IVM response category, the proportion of female worms in each age group, producing intra-uterine live stretched mf, were compared. In the poor response communities, the middle aged worms had a significantly higher († *p* < 0.05) proportion of female worms producing intra-uterine live stretched mf than the older and young worms. Pooling all response groups together, the middle aged worms had significantly higher (‡ *p* < 0.03) proportions of female worms producing intra-uterine live stretched mf than the older and young worms.

† Begbomdo was naïve at the start of the study and had received two study treatments by time of nodulectomy.

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Studies have shown that irrespective of the initial levels of pretreatment microfilarial load, repeated annual treatment with IVM for five years or more results in a reduction of microfilarial loads to a mean microfilaria density of less than 10 mf/s one year after the fourth or fifth treatment [9,29,30]. The classical observation is the study by Alley and others [29] who, using skin snip methodology similar to that employed in this study, consistently followed up more than 260 subjects every year after each treatment for five years. They observed a dramatic reduction in the mean microfilaria counts from >90 mf/s to <3 mf/s. Our results for the good response communities are consistent with these reported findings. In contrast, the poor response communities had mean mf counts of >10 mf/s to as high as 16.54 mf/s. Furthermore, considering the distribution of microfilarial loads of individual subjects in each community (Table 2) we observed significantly higher proportions (30–48%) of subjects in poor response communities having 10 mf/s as compared to 16–23% in the intermediate response communities and 0–12% in the good responding communities despite repeated ivermectin treatments over 10 years. The use of the 10 mf/s criterion in assessing recovery rates is particularly useful as it is independent of initial mf densities and of percentage change in initial densities. Additional skin snip data obtained 90 days (day 455) after a second dose of IVM treatment during the study, confirmed the earlier observation of rapid skin mf repopulation in the poor response communities.

The skin snip data and their analysis permit the conclusion that a higher than expected return of mf to the skin occurred in some communities. The explanation for this finding is that either the adult female worms have become unresponsive or resistant to the suppressive effects of multiple doses of IVM or that new infections from ongoing transmission could account for the higher than expected rate of repopulation of the skin with microfilariae. This latter possibility was statistically analyzed [31] and the conclusion was reached that the patterns in the communities showing sub-optimal responses could not be explained by new infections. Furthermore, the distinction between the higher than expected microfilarial repopulation rates due to new infections or due to resistance of the adult worms to the effects of ivermectin on reproduction can also be made on the basis of the examination of the age distribution and embryogrammes of the adult worms. On the basis of the age distribution of the adult female worms, the embryogrammes and the statistical analysis [31], the only explanation for the rapid repopulation rates seen in the poor response communities is that individual worms exist in these communities which do not respond as expected to ivermectin.

Repeated IVM treatment has marked effects on nodule numbers, morphology and composition, on adult female worm fertility and a less marked effect on adult worm vitality. Additionally, a reduction in transmission results in a decrease in new infections [3,5]. Klager and others [11] reported a marked tendency towards smaller nodules and fewer live females per nodule with increasing length of exposure to ivermectin. They also showed that the geometric mean number of worms per nodule and total live female and male worms per nodule were significantly reduced after 10 doses of IVM. For our study, all treated communities had received at least ten years of annual IVM treatment and we expected a similar effect of ivermectin on the viability of both nodules and adult worms. However, our results showed significant differences between the good and poor response communities.

| Female worm age group | No. worms embryogrammed | Total stretched microfilaria | Live stretched microfilaria |
|------------------------|--------------------------|-----------------------------|----------------------------|
|                        |                          | Mean/ worm                  | Range                      | % degenerate | Mean/worm | Range |
| Older aged             | 95                       | 2829.0                      | 0–14080                    | 84.5%        | 437       | 0–6400 |
| Middle aged            | 65                       | 3141.3                      | 0–13440                    | 70.4%        | 930.61    | 0–8960 |
| Young                  | 20                       | 1874.2                      | 0–12800                    | 80.5%        | 365.7     | 0–5120 |

Reproductive status of multiple IVM treated female worms 90 days after the second ivermectin treatment during the study, showing mean microfilarial numbers of total and active (live) stretched microfilariae by age of female worm. The middle aged worms had a significantly higher († p≤0.05) number of microfilariae in the uterus than the young worms. Also the middle aged worms had a significantly higher († p≤ 0.05) number of live stretched mf than both young and older worms.
communities. In the good response communities, more than 65% of nodules were calcified and contained a mean of 0.35 female and 0.16 male worms per nodule (Table 4). On the other hand the average numbers of male and female adult worms per nodule in the poor response communities were similar to those in the pre-study IVM-naive community. In view of the fact that there were few young worms which could suggest recent infection, this result suggests a non response or resistance to multiple doses of IVM.

The proportions of older (62–78%) and middle aged worms (22–30%) in all three community response categories were similar. There were only 5% and 8% of young worms in the poor and intermediate response communities, respectively, and none in the good response communities. Additionally, the most reproductively active group was the middle aged worms followed by the older and then the young female worms. We conclude that the major contributors to the skin mf population are not the young adult worms. They must of necessity be the worms that are a few to many years older than the young worms. Since these are the worms that must have been exposed to multiple treatments with IVM in their lifetime (and two IVM treatments during the study itself) they represent non responders or worms that are resistant to the suppressive effects of multiple doses of IVM on mf production. However, the high proportion of degenerate stretched mf in utero found in all communities indicates the retention of the ability of IVM to prevent the release of the mf in some worms.

It has been suggested [32,33], that an alternative explanation for the high skin microfilarial repopulation rates is the occurrence of repeated re-infections due to poor coverage in the study communities and in surrounding communities. However, all of the communities categorized as poor or intermediate responders had good records of treatment coverage. While treatment coverage in some communities in the East Gonja district (where the pre-study treatment naıve community is found) may have been poor, coverage in surrounding communities in the Atebubu and Kintempo districts was generally good (Osei-Atweneboana and Prichard, unpublished). The mechanism underlying the alternate hypothesis must be the recruitment of significant numbers of new adult worms that would account for the skin mf load. Notably, the embryogrammes from the multi-dosed communities showed a dearth of young adult worms at a modest level of reproductive activity. These findings do not support the alternative hypothesis, nor does the analysis of the annual transmission rate that would be required to account for the skin mf repopulation rates observed in the poor responder communities [31]. However, support for the alternate hypothesis can be found in the pre-study IVM-naive community where coverage was poor and where there were a relatively high proportion of young adult female worms (21%) as compared to 5% in the poor responding communities. It is also of interest that in this example of poor coverage in the treatment naıve community, the recovery rate of skin mf count 364 days after IVM treatment was only 41.6% of pre-treatment mf count, in contrast to the situation seen in the poor responder communities where the recovery rates were all well in excess of 100% of pre-treatment mf counts.

The evidence from the initial and follow up studies enable a description of the non response or resistance phenotype to multiple treatment with IVM as follows: a) the microfilaricidal response is normal, b) there is early skin microfilarial repopulation, high microfilarial recovery rates one year post ivermectin treatment and many subjects with more than 10 mf/snip, and c) many live microfilariae are present in utero in female worms recovered 90 days after treatment. The discovery of such a phenotype should lead to a reevaluation of control strategies in order to prevent the spread of the phenomenon, and a search for the genotypic correlate that would unequivocally confirm the development of resistance.

From our studies and evidence from other studies [13,14,34], we propose differing patterns of adult female O. volvulus responses to repeated rounds of ivermectin treatment. The
three response patterns observed are: fully responsive, partial or incomplete response, and non-responsive. The fully responsive is manifested as female worms showing complete cessation of embryogenesis leading to amicrolariademia for prolonged periods; any microfilariae detected in a subject harboring only such parasites must originate from an external source (e.g., migration of infected individuals into the community). These parasites fall into “category 1”. The second response pattern is the partial or incomplete response. This involves incomplete cessation of embryogenesis in some female worms, resulting in intermittent low level microfilaridemiass beginning six or more months after IVM treatment. These parasites fall into “category 2”. The third response pattern, non-responders, shows minimal interruption in embryogenesis with active intra-uterine mf production associated with rapid repopulation of skin and high recovery rates (“category 3” responders). Of these, a subgroup (category 3a in Figure 2) retained the ability to sequestrate mf in utero. Because of this effect, skin mf do not rise precipitously and abnormally levels of repopulation are only detected three or more months after treatment. The embryogramme shows high levels of microfilarial production associated with the accumulation of a high proportion of degenerate intrauterine mf. In category 3b, a high level of mf production is associated with a low proportion of degenerate intra-uterine mf because the block to their release has been lost and the ivermectin effect is reduced to being only a microfilaricide. There are massive increases in the skin mf soon after one month post-treatment (Awadzi, unpublished). Category 3 responses are likely to allow for parasite transmission for much of the year following ivermectin treatment. It is possible that all of these patterns of response may occur in different communities and the community response category is determined by the proportional distribution of the various adult worm response patterns. The proposed patterns of O. volvulus response have been summarized in Figure 2. It is likely that the mechanism of microfilaricidal action of IVM is distinct and separable from the inhibition of the release of mf from the uterus and the suppressive effects of ivermectin on the adult female worm.

At the moment the prevalence of functional blindness, as described by Kennedy and other [30], is almost absent in the study communities, except for a few blind older members of the communities. However, since the risk of developing ocular lesions and blindness is directly related to the intensity of infection [35,36] it is important to establish strict monitoring of onchocerciasis pathology. Unfortunately, due to a lack of monitoring, the extent of the non response of the adult female worms and the influence it may have on the control of onchocerciasis in areas subjected to annual treatments are at present unknown.

Supporting Information

Checklist S1 STROBE checklist. Found at: doi:10.1371/journal.pntd.0000998.s001 (0.08 MB DOC)

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Author Contributions

Conceived and designed the experiments: MY-OA RKP. Performed the experiments: MY-OA KA SKA DAB. Analyzed the data: MY-OA RKP. Contributed reagents/materials/analysis tools: JOG. Wrote the paper: MY-OA KA DAB JOG RKP.

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