PARASITOLOGICAL RESPONSES OF *ONCHOCERCA VOLVULUS* TO IVERMECTIN TREATMENT AND GENETIC ANALYSIS OF BETA TUBULIN GENE

BY

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DECLARATION

I do hereby declare that all supervised experimental works described in this thesis were carried out by myself with the exception of references made to other people's works published or not and have all been duly acknowledged. This thesis has never been submitted anywhere else for the award of similar or different degree neither in whole nor in part.

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DEDICATION

TO

My dearest mother, Georgina Sarkodie, for her love, care, support and for giving me a reason to go to bed and wake up each day; My late father for challenging me to aspire for a higher education; My wife, Linda Gyan, for her perpetual love, review and support; and My boss, Dr. Mike Yaw Osei-Atweneboana, for being a father in supporting me in all aspects of my life and for mentoring me in the field of medical parasitology and molecular epidemiology.

Thanks to you all for giving me a foundation for a brighter future

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LIST OF ABBREVITION

ABC	Adenosine Tri-Phosphate Binding Cassette
APOC	African Programme for Onchocerciasis Control
ATP	Annual Transmission Potential
CDC	Centers for Disease Control and Prevention
CDTI	Community Directed Treatment with Ivermectin
CMFL	Community Microfilarial Load
DALY	Disability-Adjusted Life-Years
DEC	Diethylcarbamazine
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EPND-LAC	Epidemiological Profiles of Neglected Diseases and other infections related to poverty in Latin America and the Caribbean
GABA	Gamma Amino Butyric Acid
GDP	Guanosine Di-Phosphate
GHA-COM	Ghanadistricts.com
GluCl	Glutamate-gated Chloride Channels
GPELF	Global Programme for the Elimination of Lymphatic Filariasis
GTP	Guanosine Tri-Phosphate
HCl	Hydrochloric Acid
IVM	Ivermectin
L_3	Third Stage Larvae of *Onchocerca volvulus*
MDA	Mass Drug Administration
MDR	Multidrug resistance
Mf	Microfilaria(e)
MFD	Microfilarial Density

MFU	Medical Field Units
$MgCl_2$	Magnesium Chloride
MMDA	Metropolitan, Municipal and District Assembly
MMWR	Morbidity and Mortality Weekly Report
MOH	Ministry of Health
NTDCP	Neglected Tropical Diseases Control Programme
OCP	Onchocerciasis Control Programme
OEPA	Onchocerciasis Elimination Programme for the Americas
OSD	Onchocercal Skin Disease
PAHO	Pan American Health Organization
PCC-OEPA	Program Coordinating Committee and Onchocerciasis Elimination Programme for the Americas staff
PCR	Polymerase Chain Reaction
Pgp	Permeability Glyco-Protein (P-glycoprotein)
SIZ	Special Intervention Zone
SNP	Single Nucleotide Polymorphism
SOR	Sub-Optimal Response(s)
SSA	Sub-Saharan African countries
TEP	Transmission Elimination Phase
TH1	T Helper cell 1
TH2	T helper cell 2
TH3	T helper cell 3
TIP	Transmission Interruption Phase
Trt	Treatment
TSP	Transmission Suppression Phase
UV	Ultra-Violet
WHO	World Health Organization

ABSTRACT

Ivermectin (IVM) still remains the only safe drug for the mass control of onchocerciasis, and the continued success of the control programmes depends on its efficacy. However, recent reports show that there are populations of adult *Onchocerca volvulus* responding sub-optimally to IVM. This requires assessment of parasitological response profile of *O. volvulus* to IVM and genetic analysis of Beta-tubulin gene known to be associated with IVM selection, to determine the association between worm phenotypes and genotypes. A fifteen month longitudinal study involving skin snipping, three rounds of bi-annual IVM treatment and nodulectomies was carried out in seven selected endemic communities. A total of 584 subjects were assessed for microfilaria (mf) load at the beginning of the study and then treated with IVM. Out of these, 87 subjects who were mf positive at pre-treatment assessment were then followed-up on days 180, 270 and 360 after the first IVM treatment study and nodulectomies conducted at day 452 (90 days after the third IVM treatment). Nodules were digested, adult worms removed and embryogram analysis performed. A total of 59 worms were selected from Jagbengbendo and Takumdo communities for genetic analysis on a 684 bp DNA fragment of Beta-tubulin gene. Out of the 584 subjects assessed, we observed an overall nodule and mf prevalence and community microfilarial load (CMFL) of 20.9%, 14.9% and 3.1 mf/s respectively. The majority of subjects responded well to IVM treatment, except 3.3% from Chabon, 5.3% from Kojobone and 8.3% from Agblekeme II who responded poorly or sub-optimally to IVM. Genetic analysis showed four Single Nucleotide Polymorphisms (SNPs) at genomic positions 1183 (T/G), 1188 (T/C), 1308 (C/T) and 1545 (A/G), with the homozygote genotypes of the latter three SNPs showing significant association ($p<0.05$) to the poor response phenotype worms. A 364bp DNA sequence of beta-tubulin gene, encompassing the four SNPs, has been proposed for developing a molecular

marker to detect IVM resistance. This study shows that IVM resistance is emerging in some endemic communities and requires monitoring.

CHAPTER ONE

1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

Onchocerca volvulus (Filarioidea: Onchocercidae) is the causative agent of onchocerciasis, commonly known as river blindness and it is transmitted by blackflies of the genus *Simulium* (Diptera: Simuliidae) (Blanks *et al*., 1998). The only known natural reservoirs are humans (Udall, 2007). It is estimated that 37 million people are infected worldwide with the parasite, of these 99% live in Africa and over 90 million (as at year 2005) are at risk in 27 Sub-Saharan African countries (SSA) (WHO, 1995; APOC, 2005; Remm *et al.*, 2006) with the at risk population of infection rising to 123 million within 38 endemic countries as at year 2013 (MMWR, 2013). Over 400,000 people are infected in Central and South America and the disease is endemic in six Latin American countries (Brazil, Colombia, Ecuador, Guatemala, Mexico and Venezuela), with a rare hyper-reactive clinical manifestation of the disease called 'Sowda' found in smaller foci of the endemic region of Yemen (Gasparini, 1964;WHO, 1995). The transmission foci in the Americas are relatively smaller and geographically delimited when compared to the localities of transmission in Africa (Thylefors and Alleman, 2006).

Some of the consequences of infection with *O. volvulus* include severe itching, blindness, disfiguring skin lesions, epilepsy due to heavy infection, abandonment of fertile river valleys, social ostracism, decreased life expectancy among sighted individuals with high microfilarial load and considerably excess loss of mortality of the blind (Prost, 1986; Vlassoff *et al.*, 2000; Boussinesq *et al.*, 2002; Pion *et al.*, 2002; Little *et al.*, 2004b). Onchocerciasis causes annual loss of approximately 1.5 million disability-adjusted life-years (DALYs), which is the healthy life-years lost as a result of disability and mortality. More than half of this result from skin

diseases and the disease diminishes income-generating capacity, increases health expenditure to cause negative socioeconomic impact on infected individuals and their habitations (Evans, 1995; Oladepo *et al.*, 1997; Remme, 2004b; Remme *et al.*, 2006).

The World Health Organization (WHO) launched the Onchocerciasis Control Programme (OCP) of West Africa in 1974 with the aim of eliminating onchocerciasis as a disease of public-health importance with core activities involving aerial spraying of rivers with larvicides whiles treating infected individuals with Diethylcarbamazine (DEC) (Benton, 1998; Basáñez and Boussinesq, 1999). Treatment with DEC had some setbacks and could not be used safely for mass treatment. It had intrinsic toxic side effects (Hawking, 1979). Moreover, as described by Mazzotti (1948), DEC treatment usually resulted in serious systemic, dermal, and ocular side effects. Although the drug could kill microfilariae (Mf) in vivo, it had no significant demonstrable effect on adult worms (Taylor *et al.*, 1980). There was therefore the need to find and rely on an effective drug that could safely be used for mass treatment of onchocerciasis.

Various governments, policy and decision makers at the forefront of the battle against onchocerciasis have continuously made efforts to control and eliminate the disease, presently relying largely on IVM. The predominant programmes include the Onchocerciasis Elimination Program for the Americas (OEPA), the African Programme for Onchocerciasis Control (APOC), and the former Onchocerciasis Control Programme (OCP) (Sturchio, 2001).

IVM, registered in 1987 for human use in the treatment of onchocerciasis, has still proven to be the only safe drug for mass-treatment of onchocerciasis (Cupp *et al.*, 2011). The recommended single annual dose of 150 µg/kg body weight of IVM is capable of eliminating Mf in the skin and eyes to prevent further progression of the severe pathologies that are associated with the disease (Green *et al.*, 1989). IVM is capable of maintaining very low skin Mf loads for nine to 12 months (Awadzi *et al.*, 1985; Awadzi *et al.*, 1989; Brown and Neu,

1990; Duke *et al*., 1992; Alley *et al*., 1994; Kläger, Whitworth and Downham, 1996; Abiose, 1998; Brieger *et al*., 1998). Administering the drug at the standard dose does not kill substantial numbers of the adult parasites but it temporarily blocks the release of intrauterine Mf by the adult female worms (Greene *et al*., 1985; Schulz-Key and Karam, 1986).

More than 800 million doses of IVM have been administered to over 80 million people within the past 25 years through the development of mass drug distribution programmes due to the safety profile of the drug, thus establishing a standard for other organizations to follow (Sturchio, 2001; Amazigo *et al*., 2002a). Consequently, there has been significant reduction in transmission of onchocerciasis in more than 25 countries with transmission being interrupted in a minimum of 10 countries to such an extent that the disease is no more observed in children in many formerly endemic countries (Sturchio, 2001). Moreover, there have been significant successes in onchocerciasis control programmes, with OCP covering 1,200,000 square kilometers to protect 30 million people in 11 countries, prevention of 600,000 blindness, making 25 million hectares of land safe for resettlement and lightening the burden of millions of the world's poorest people with some task left to claim final victory in the battle against river blindness (Harlem, 2002; Hopkins, 2005).

Thus, these successes of the onchocerciasis control and elimination programmes worldwide are mainly due to the efficacy of IVM as a microfilaricide used widely in Africa and Latin America (Richards *et al*., 2004).

A lot of rural communities in Latin America and Africa have greatly benefited from the infrastructure and distribution systems developed for these programs to such an extent that it has become a model for health care. Thus, the successes of the IVM-based programmes, policies and plans have demonstrated that it is possible to put forth activities and endeavours targeting chronic diseases in remote and poor localities of the world to effect significant improvements in productivity, morbidity and long-term mortality (Hotez *et al*., 2007).

If a drug is used frequently for longer periods, it increases the tendency for suboptimal response and even resistance. There has been the consideration of the possibility that *O. volvulus* could develop sub-optimal response (SOR) and even resistance to IVM (Awadzi, 1993; Shoop, 1993; Boussinesq and Gardon, 1999; Dadzie, Neira, and Hopkins, 2003), thus resulting in WHO urging for the establishment of methods and procedures to detect the development of such resistance as well as studying the underlying mechanisms (WHO, 1995).

Following the introduction of IVM in 1981 for treatment of parasites of livestock, there was a report of the first case of parasite resistant to IVM in 1985 (Carmichael *et al*., 1987). The production of ruminants was faced with the major threat of resistance to the avermectin-milbemycin class of drugs (Wolstenholme *et al*., 2004). There were many factors influencing IVM resistance development, these include treatment frequency, the use of treatment strategies that restrict *refugia* and the genetics of the worm (Wolstenholme *et al*., 2004). The proportion of *O. volvulus* parasite population in the insect vector and the parasites in untreated members of a given locality make up the population in *refugia.*

In some foci in Ghana, there have been reports of suboptimal response to annual IVM treatment, which was defined as "a higher than normal rate of skin Mf repopulation by *O. volvulus* adult female worms" (Awadzi *et al*., 2004a; Awadzi *et al*., 2004b). In that report, there was an observation in 1997 survey that individuals with at least 10 Mf per skin snip at days 90 and 365 post-treatment despite the fact that they received at least 9 annual IVM treatments, the rapidly repopulating Mf were capable of infecting vector black flies and developed to infective stage larvae (L_3). Although this suggested that they were developmentally competent, the progeny parasites however remained sensitive to subsequent treatment with IVM, a clear indication that a small proportion of adult female worms had

probably become insensitive to the efficacious paralyzing effect of IVM on preventing them from releasing intra-uterine Mf.

Another report from Ghana indicated that there were population of *O. volvulus* responding poorly to IVM treatment, evident by the rapid repopulation of skin with Mf by the adult female worms and suggesting possible development of IVM resistance (Osei-Atweneboana, 2007). This conclusion was however questioned by other authors (Cupp *et al.*, 2007; Mackenzie, 2007) who believed that the repopulation of skin Mf was either caused by poor treatment coverage (Cupp *et al*., 2007; Mackenzie, 2007), or that young female worms that were naturally highly fecund were reproducing at a higher rate (Remme *et al*., 1989; Remme *et al.*, 2007). In addition, there was a noticeable observation of two of the three river basins studied having annual transmission potential (ATP) of at least 45 infective larvae per person per year (Awadzi, 2004a). Since an average transmission of 8 L_3 per person per year is deemed sufficient to sustain a parasite population (Basáñez, 2002), the authors believed that there was a possibility that many of the reported suboptimal responders live in localities having ATP levels that are five times greater than necessary to maintain the populations of *O. volvulus.*

APOC carried out a retrospective survey and an in-depth analysis of the IVM coverage in 122 villages located within the 20 km of the vector flight range of the study communities (Osei-Atweneboana, 2007) and pointed out that villages with worms that are responding well had all received regular annual treatments during the seven years prior to the Osei-Atweneboana study. However, there were noticeable coverage problems with two communities that showed rapid Mf repopulation (APOC, 2008). However, the survey could not provide adequate justification for their results.

On the other hand, Osei-Atweneboana and colleagues provided additional phenotypic evidence to support the emergence of IVM resistance in *O. volvulus* IVM (Osei-

Atweneboana *et al.*, 2011). This was considered as SOR of *O. volvulus*, described as a reduction in the duration of anti-fecundity effect of IVM on the adult *O. volvulus* or the reduction in the microfilaricidal action of IVM (Grant, 2000).

Furthermore, Osei-Atweneboana and colleagues in 2012 carried out a 21 month epidemiological study to find out the response of *O. volvulus* to IVM in 10 Ghanaian endemic communities as well as carrying out molecular analysis to elucidate the genetic profile of the worms and demonstrated the occurrence of higher reproductive activity in worms obtained from poor response communities. In addition, genotyping of the full length genomic DNA sequence of ß-tubulin gene revealed an association between worm phenotypes and specific genetic changes that demonstrated that IVM resistance is being selected for in *O. volvulus* (Osei-Atweneboana *et al.*, 2012). Various workers have also demonstrated similar selection of IVM SOR on beta tubulin, resulting in changes in allele frequencies (Eng and Prichard, 2005; Eng *et al*., 2006; Bourguinat *et al*., 2007b; Nana-Djeunga *et al*., 2012).

1.2 JUSTIFICATION

Onchocerciasis is endemic in 9 of the 10 regions of Ghana. The use of IVM for mass drug administration (MDA) for over two decades, coupled with the change from annual to bi-annual treatment heightens the likelihood that drug resistance may develop to jeopardize the success of control programmes. SOR and resistance to various therapeutic agents are known to occur in many organisms including bacteria and parasites, and the need to confirm that SOR or resistance to IVM has occurred in *O. volvulus* is of utmost importance.

Presently, it is not clear if the possible emergence of IVM resistance could also be occurring in communities close by and those with similar treatment history. It may also be interesting to find out if communities with similar prevalence and intensities and transmission indices would exhibit similar parasitological response profile to IVM treatment.

There is the need to carry out further studies to investigate the possibility of emerging IVM resistance in *O. volvulus*. This study will also assess the worm profile in some of the communities in the previous studies to determine if any change has occurred and also to survey other *O. volvulus* communities to assess their parasitological response profile to IVM treatment, and investigate the association between the worm phenotypes and genotypes.

1.3 OBJECTIVES OF THE STUDY

The main objective of the study is to determine the prevalence and intensity of onchocerciasis, assess the parasitological response profile of *O. volvulus* to IVM treatment and identify genetic changes in beta tubulin gene associated with SOR to IVM.

The specific objectives are:

1. To determine the prevalence and intensity of *O. volvulus* in five onchocerciasis endemic communities in Ghana.
2. To assess the parasitological response profile of *O. volvulus* to IVM treatment in five endemic communities
3. To assess the genetic differences in beta tubulin gene of *O. volvulus* from individuals showing good and sub-optimal responses
4. To determine genetic changes in beta tubulin gene associated with sub-optimal response to IVM
5. To propose specific DNA sequence of beta tubulin gene for developing genetic marker for early detection and monitoring of IVM resistance.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Major Control Programmes of Onchocerciasis and Progress Made

2.1.1 The Need for Control Programmes

To be able to prevent, manage, control and eliminate onchocerciasis at the local community level, there is the need for organized effort from control programmes like the OCP (Remme, 2004b), APOC (Remme, 1995) and OEPA (Cupp *et al*., 1992; Blanks *et al*., 1998) to harness resources and coordinate activities towards a common goal.

2.1.2 Onchocerciasis Control Programme (OCP)

The OCP was launched in 1974 due to the health and socioeconomic impact associated with onchocerciasis (Remme, 2004b) to eliminate the disease, first in seven and then in 11 West African countries, through aerial application of larvicides to the breeding sites of blackflies (WHO, 1997; Boussinesq & Hougard 1998). The successes of OCP include more than 40 million people in the 11 countries of the programme being free from infection and eye lesions, at least 1.5 million people are no more infected, over 200 000 cases of blindness prevented by 1995 -and in year 2005, about 600,000 blindness cases prevented (Harlem, 2002; Hopkins, 2005)- at least 16 million children born since the launch of the programme are free of the disease, 25 million hectares of fertile land in river valleys have become available for agriculture and resettlement, and the net value for the OCP over its lifespan of existence from 1974 to 2002 is estimated by a cost-benefit analysis to be US$485 million (Kim and Benton, 1995).

2.1.3 African Programme for Onchocerciasis Control (APOC)

African Programme for Onchocerciasis Control was launched in 1995 with the objective of extending the gains of OCP to 19 other endemic African countries not covered by the OCP (Remme, 1995). The WHO Expert Committee on Onchocerciasis estimated that 123 million people were at risk of contracting the disease with 17.7 million people already infected in Africa, Central and South America, of whom about 270,000 were blind and 500,000 were severely visually impaired (WHO, 1995; Murray & Lopez 1996). However, information from rapid epidemiological mapping of onchocerciasis (Noma *et al.*, 2002) by APOC indicated that the number of those infected was twice as high with about 37 million people infected in 1995 (to 2005) and an estimated 1.99 million DALYs lost because of onchocerciasis in 1995. Onchocerciasis presently, being the second cause of infectious blindness after trachoma, still affects 37 million people living in 34 countries with 99% of the reported cases in SSA and the DALY of the disease is currently estimated at 1-1.5 million (Basáñez *et al*., 2006; Remme *et al*., 2006; WHO, 2012b) and 123 million persons at risk of infection within 38 endemic countries as at year 2013 (MMWR, 2013).

Unlike OCP, APOC is based on the strategy of community directed treatment with IVM (CDTI), whereby local communities direct the treatment process with the health workers providing only the necessary training and supervision (Remme, 1995; Dadzie, 1997; Amazigo *et al*., 2002b). Communities have always responded with great interest to this strategy (Seketeli *et al*., 2002), and interest continue to grow in using this approach for interventions against other diseases (Homeida *et al*., 2002).

Following a major significant reduction in the prevalence and intensity of onchocerciasis infection to low levels from localities that have taken IVM for over 15 years (Borsboom *et al*., 2003), and the prediction from computer simulations that the disease would not be of public health problem for 10-20 years if treatment stopped (Remme, Alley and Plaisier,

1995), the following deduction was made. The deduction was that supposing 70% of endemic areas are covered by community-directed treatment using IVM and 80% of those areas are capable of maintaining annual treatment at 65% coverage for a period of not less than 15 years, then a minimum of 26 million DALYs would be prevented over a period of 25 years (Remme *et al*., 2006). This knowledge must guide IVM treatment duration and coverage in deciding the inclusion criteria of IVM studies.

2.1.4 Onchocerciasis Elimination Programme for the Americas (OEPA)

The Onchocerciasis Elimination Programme for the Americas (OEPA) was launched in 1991 in response to a 1991 resolution of the Pan American Health Organization (PAHO) that called for the elimination of the morbidity of onchocerciasis from the Americas by 2007 (Blanks *et al*., 1998). Thus, the OEPA was setup in 1993 to eliminate onchocerciasis morbidity in the six-affected Latin American Country foci (Cupp *et al*., 1992). In 2009, a PAHO resolution called for the elimination or control of 12 neglected infectious diseases that were related to poverty in the Americas by 2015 and it incorporated the elimination of onchocerciasis as one of its targets (Sauerbrey, 2008). The primary approach used by OEPA to eliminate onchocerciasis from the Americas has been through the process of health education, community mobilization and administering biannual mass IVM treatment in all affected communities located in the 13 endemic foci within the six affected countries (Blanks *et al*., 1998; MMWR, 2013; Sauerbrey, 2008) because it was shown that offering IVM treatment every six month has higher impact on transmission (Cupp *et al*., 1992) and great effect on adult female worm fecundity (Duke *et al*., 1991).

The IVM MDA for the Americas has the aim to achieve at least 85% coverage of the population at risk that are eligible for treatment and the target communities are divided by baseline onchocerciasis prevalence into three categories known as Hyperendemic (≥60%), Mesoendemic (≥20%, but <60%), and Hypoendemic (prevalence <20%) (MMWR, 2013). It

is difficult to break transmission in hyperendemic localities and it may require administering MDA every three months to achieve it (WHO, 2012a). The six areas in Latin America and the Caribbean that onchocerciasis remains endemic (Basáñez *et al*., 2006) had about 500,000-510,000 people at risk of infection, eight of these focal areas demonstrated transmission interruption before 2007 (Ault, 2007; EPND-LAC, 2009). In the period of 1993 to 2012, a total number of 11,069,285 MDA IVM treatments were administered and transmission of infection was interrupted or eliminated by the end of 2012 in four of the six countries (MMWR, 2013). Following this achievement by OEPA, the interventions to limit transmission were halted in Colombia in 2008, in Ecuador in 2010, Guatemala in 2012, and in Mexico in 2012 (CDC, 2013b). Since active transmission is found in only two foci among the indigenous populations of Yanomami in the adjacent border areas of southern Venezuela and northern Brazil (MMWR, 2013), interventions to limit transmission has not ceased in these two localities. In this year 2013, only 4% (23,378) of the 560,911 individuals originally at risk in the Americas have been targeted for IVM MDA partly due to the fact that ocular morbidity has been detected only in southern Venezuela and no new blindness has been attributed to onchocerciasis in the Americas from 1995 up till now (MMWR, 2013). The three most remarkable achievements of OEPA are the reduction in the population under mass drug treatments in the Americas, without any transmission consequences, from an estimated 500,000 (in 1991) to about 23,000 (in 2013), the interruption of transmission of *O. volvulus* in 11 of the 13 foci (with only 4% of the population at risk in need of MDA), and Colombia has successfully eliminated onchocerciasis (MMWR, 2013; WHO, 2012a; WHO, 2012b). The current approach of OEPA to speed up the elimination process is to maximize IVM MDA to quarterly administration in the most highly endemic localities around the border foci, and to identify and adequately treat any unknown endemic community that may be detected (MMWR, 2013)

2.1.5 WHO Guidelines for Transmission Interruption Determination

A set of technical guidelines was set up in 2001 by WHO to enable onchocerciasis programmes determine whether transmission of the disease has been interrupted and if MDA using IVM could be halted (WHO, 2001; Lindblade *et al*., 2007). Three major phases were included: (1) Transmission Suppression Phase (TSP), whereby vectors no longer introduce infective stage larvae (L3) into the human population but there is the maintenance of the capacity by the parasite population in the human reservoir to recover upon withdrawal of IVM treatment; (2) Transmission Interruption Phase (TIP), that occurs at the point that the parasite population is unable to recover, thereby enabling the cessation of IVM treatment; and (3) Transmission Elimination Phase (TEP), that occurs such that post-treatment surveillance duration of at least three years confirms the inability of the parasite population to recover in the absence of intervention (PCC-OEPA, 2012). By the guidelines, ocular morbidity can be declared eliminated if there is less than 1% prevalence of acute eye lesions that is attributable to onchocerciasis (Lindblade *et al*., 2007). The WHO will set up an independent team of international experts to carry out a final verification of a country subject to the condition that all foci in the country have reached the elimination stage (MMWR, 2013)

2.2 Etiology and Distribution of Onchocerciasis

Onchocerciasis in humans is caused by the filarial parasitic nematode called *Onchocerca volvulus* and it is transmitted by blackflies of the genus *Simulium* (Blanks *et al*., 1998). An infected blackfly introduces third-stage filarial larvae on the skin of the human host during a blood meal, where they penetrate into the bite wound to develop into adult filariae commonly found in nodules in subcutaneous connective tissues for approximately 15 years, and fertilized females can produce millions of Mf during an average of 9-10 years to cause the morbidity associated with the infection (CDC, 2013a; WHO, 2013a). The Mf are sometimes found in sputum, urine and peripheral blood but are usually found in the lymphatic and skin

of the connective tissues, where they can be ingested by a blackfly taking a blood meal from the skin, to migrate from the blackfly's midgut through the hemocoeal to the thoracid muscles to develop into first-stage larvae and subsequently into third-stage infective larvae (Figure 2.1). These third-stage infective larvae then migrate to the proboscis of the blackfly and may infect another person during a blood meal by the blackfly (CDC, 2013a).

There are many simuliid species that have been incriminated in the transmission of *O. volvulus* (Crosskey and Chichester, 1990) and together with the parasite, influence transmission patterns in endemic areas. About 95 percent of the global disease cases are attributed to the *Simulium damnosum* sensu lato (s.l.) species complex usually found in Africa (including the 27 SAA shown in Figure 2.2) and includes about 60 cytoforms (Crosskey and Chichester, 1990; Crosskey and Howard, 2004). The main vectors of the Latin America endemic areas in Brazil and southern Venezuela (about 20,000 people at risk), northern Venezuela (104,500), Ecuador and Colombia (24,600), and Guatemala and Mexico (approximately 360,000) are respectively *S. guianenses*.l., *S. metallicums*.l., *S. exiguums*.l., and *S. ochraceums*.l (Onchocerciasis, 2005; WHO, 2005).

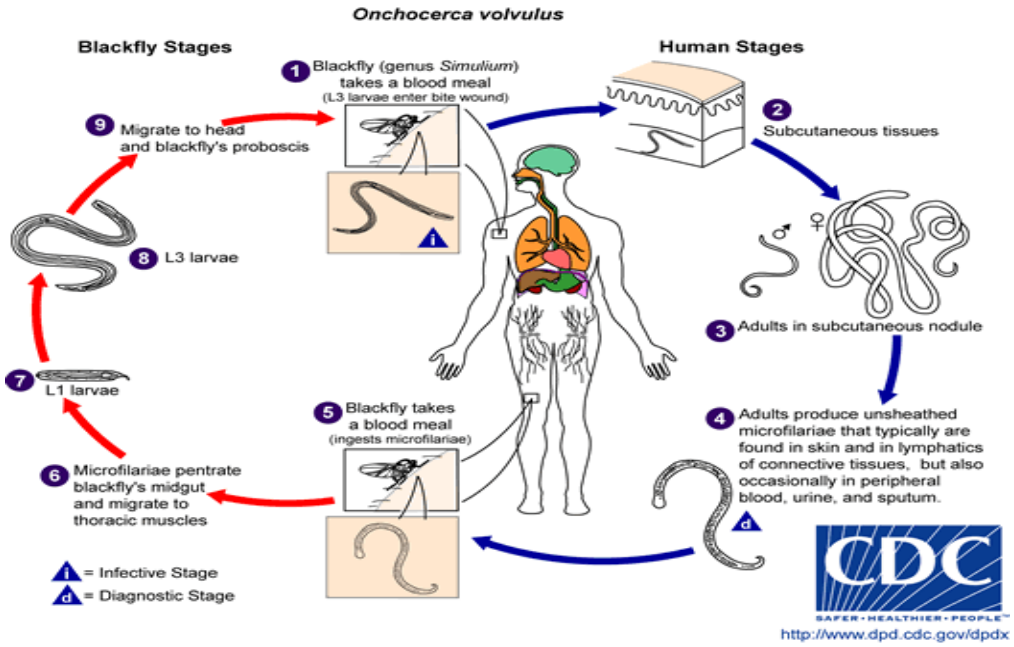

Figure 2.1: Life Cycle of *O. volvulus* (Accessed from: http://www.cdc.gov/parasites/onchocerciasis/biology.html)

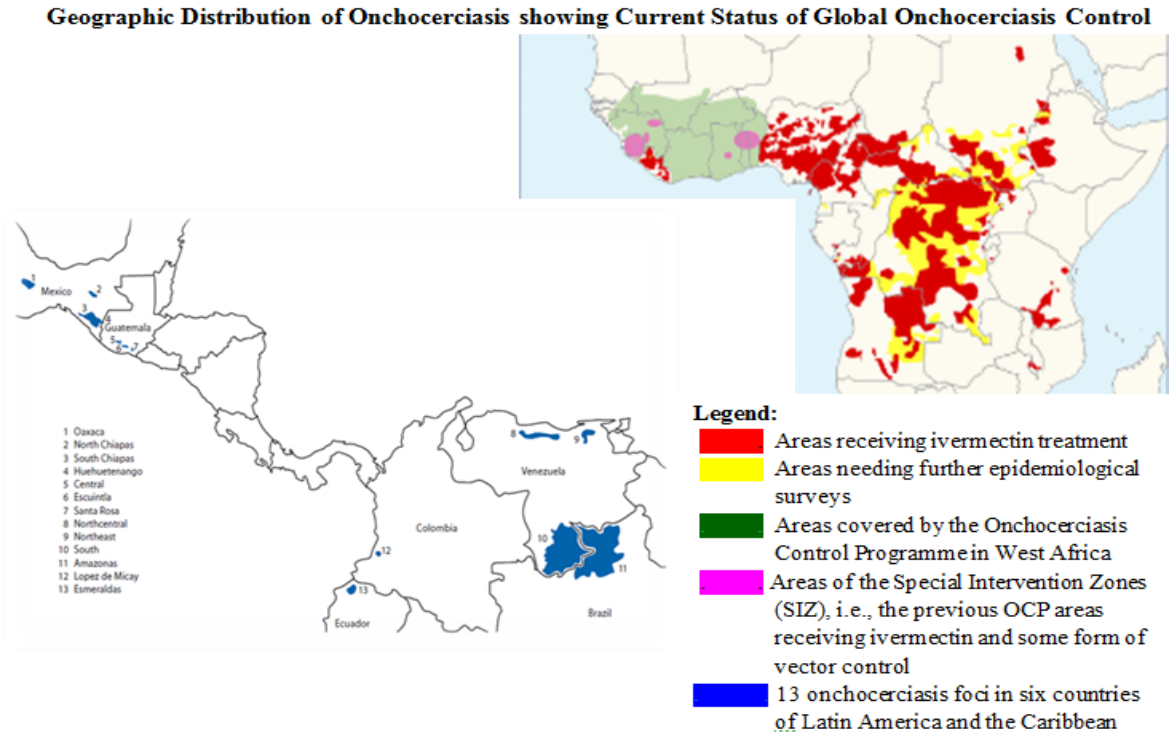

Figure 2.2: Geographic Distribution of Onchocerciasis Showing Current Status of Global Onchocerciasis Control (Map redrawn from WHO, 2002; APOC, 2004; Richards *et al.*, 2004; Basáñez *et al.*, 2006; MMWR, 2013)

2.3 Onchocerciasis Pathology and Clinical Signs

2.3.1 Complications of Onchocerciasis

The main complications of onchocerciasis are the severe eye disease that can result in blindness and the severe skin disease with intense itching and lesions (WHO, 1995). The disease is often known as “River Blindness” because the larvae and pupae of the *Simulium* vector develop in rapidly flowing, well-oxygenated rivers and streams (Basáñez *et al.*, 2006). The thousands of Mf that fail to reach blackfly vectors die in the human body to provoke inflammatory reactions either in tissues of the eyes to cause the irreversible ocular lesions, vision impairment and total blindness (WHO, 1995), or in tissues of the skin to result in the intense itching, depigmentation, dermatitis, and skin atrophy (Murdoch *et al.*, 2002). A less common inflammatory response from the death of Mf is lymphadenitis, that can lead to hanging groin and elephantiasis of the genitals, and evidence suggest that onchocerciasis is a

risk factor for both epilepsy and hyposexual dwarfism in certain areas (Boussinesq *et al*., 2002).

2.3.2 Onchocerciasis Disease Pattern

Onchocerciasis disease pattern relating to ocular and dermal disease severity differs considerably between geographical zones with ocular blindness being extensive in hyper-endemic localities of the West African Savannas, whiles the forest communities are characterized by a comparable intensity of skin manifestation of the disease (Dadzie *et al*., 1989; Murdoch *et al*., 2002). These differences result from the presence of different vector-parasite complexes with strains of *O. volvulus* possessing variations of pathogenicity (Zimmerman *et al*., 1992) and the important role played by Onchocercal Skin Disease (OSD) in contributing to the disease burden of onchocerciasis has been acknowledge quite recently (Murdoch, 2010). The differences in the ocular disease between the savannah and forest strain result from variations in the numbers of *Wolbachia* as a significant positive correlation has been found between ocular onchocerciasis and the quantities of *Wolbachia* in the severe forms of savannah strains than in the mild forms of forest strains (Higazi, Filiano and Katholi, 2005). Factors that may influence different patterns of entomological and epidemiological surveys include individual compliance, CDTI coverage and the likelihood that IVM efficacy is compromised (Taylor *et al*., 2009).

The people living near streams and rivers are most at risk of infection with onchocerciasis because of the proximity to the breeding sites of the blackfly *Simulium* vector and many bites are required before being infected thereby making travellers to endemic localities for relatively short periods of time (normally less than three months) have low chance of getting infected with the *O. volvulus* parasite (CDC, 2013b; MMWR, 2013)

There is a direct positive relationship between infection symptoms and the prevalence and intensity of infection making the increase in prevalence and intensity of infection to result in

increases in visual impairment and blindness (Dadzie *et al*., 1989). Although the relationship between transmission intensity and prevalence infection as well as that between transmission intensity and infection prevalence follow similar patterns in both Latin America and Africa (Basáñez *et al*., 2002), individual communities vary in infection prevalence that can be as high as 80-100% in some localities by age 20 and blindness being at its climax at age 40-50 (Murdoch, 2012).

2.3.3 Onchocercal Skin Disease

The disturbance of itching associated with dermal onchocerciasis cause difficulties working, studying or interacting socially (Vlassoff *et al*., 2000; Murdoch *et al*., 2002) and it accounted for 60% of DALYs lost in the 1990s and early 2000 (Remme, 2004a). In addition, OSD diminishes the income-generating capacity of infected individuals and there is twice as high school dropout rate among children from households that has the head of the family affected by onchocercal skin disease (Benton, 1998). The medical and socio-economic problems associated with onchocerciasis have given it a great public importance (Duke, 1990; Workneh *et al*., 1993; Evans, 1995; WHO, 1995).

2.3.4 Lesions Pathogenesis

All eye tissues may be affected by ocular lesions from punctate and sclerosing keratitis of the anterior segment to optic nerve atrophy of the posterior eye segment (Basáñez *et al*., 2006). Blindness incidence is associated with past microfilarial load in infected individuals (Little *et al*., 2004a) thus leading to the progressive deterioration of onchocercal eye disease following parasite exposure (Basáñez *et al*., 2006). Basically, lesions of the anterior chamber are believed to result from a cascade of inflammatory processes triggered by components of Mf (Hall and Pearlman, 1999). A recent accepted hypothesis suggests that the pro-inflammatory events that continuously worsen corneal opacity are stimulated by the endosymbiotic *Wolbachia* bacteria when they are released by dead Mf (Saint-André *et al*., 2002; Brattig,

2004). However, the retinal lesions are caused by autoimmune events provoked by the cross-reactivity of *O. volvulus* proteins with human retinal antigen (McKechnie *et al*., 2002).

2.3.5 Pathogenesis Mechanisms

The clinical lesions are attributed to infiltrates around dead Mf being attacked by neutrophils, eosinophils and macrophages (Pearlman *et al*., 1999); and in the case of the cornea, the inflammation may be caused by the products of the *Wolbachia* bacteria (Brattig, 2004). Consequently, the effector reactions of the T helper cell 1 (TH1) and the T helper cell 2 (TH2) are suppressed by the T helper cell 3 (TH3) or T regulatory cell 1 (Hoerauf and Brattig, 2002). Through mediations of interleukin 10, there is the down-regulation of the immune system thereby enhancing the survival of parasite (Brattig, 2004). Host genetic factors may affect the outcome of host immune response to degenerating parasites (Meyer *et al*., 1994), and in some cases may require repeated cycles of inflammation, infiltration by neutrophils, eosinophils and macrophages, as well as the degradation of live and dead Mf present in individuals with low Mf loads (Ali *et al*., 2003).

2.4 Onchocerciasis Control in Ghana

2.4.1 Onchocerciasis Events in Ghana

Onchocerciasis remains endemic in nine of the ten regions of Ghana and at least 3204 communities from 66 districts are endemic in these regions with 247 communities in the Ashanti and Brong-Ahafo regions designated as Special Intervention Zones (SIZ) (Taylor *et al*., 2009). The SIZ are characterized by hyperendemicity within the Pru River basin and serve as foci of the CDTI, that was introduced in 1998 after the commencement of IVM distribution in Ghana by the use of mobile teams in 1987 (Taylor *et al*., 2009). The history of onchocerciasis control in Ghanaian communities however dates back to the colonial period when deliberate organized effort was made by the Medical Field Units (MFU) of the Ministry of Health (MOH) of Ghana (former Gold Coast) from 1953 to 1955 to control the ocular form

of onchocerciasis around the northern localities of the Ghana using Suramin (formerly called Antrypol) (McLean, 1959). Albendazole and IVM combined treatment was started in five pilot districts in 2001 and eventually reached 61 endemic districts in 2005 (Taylor *et al*., 2009) as a result of the initiative of the Global Programme for the Elimination of Lymphatic Filariasis (GPELF) (WHO, 2008).

2.4.2 Onchocerciasis Distribution, Morbidity and Treatment in Ghana

Onchocerciasis causes visual impairment and blindness in the savannah area (McLean, 1959) and OSD along the forest bioclimatic zones (Debrah *et al*., 2006). About one million of DALYs is lost annually from the blinding and OSD forms of onchocerciasis in other endemic localities (Remme, 2004a; Basáñez *et al*., 2006) with one million people presently blind or have sever visual impairment globally (Crump, Morel and Õmura, 2012).

The OCP started vector control in West Africa, including Ghana, in 1975 as a result of the socioeconomic importance of the disease (Remme, 2004b) and successfully achieved considerable reductions in the populations of the vectors that was believed to be low enough not to sustain transmission (Cheke and Garms, 1983). However, transmission occurred later on and the disease in Africa (including Ghana) cannot be eliminated with existing tools (Dadzie, Neira and Hopkins, 2003), leaving a total of approximately 3.2 million population at risk in Ghana (Taylor *et al*., 2009).

Meanwhile, the national programme for the control of the disease has been monitoring recrudescence by means of entomological and epidemiological surveillance efforts such that many communities have been treated since 1987 to such extent that 3.4 million people have been treated from 2002 to 2007 by means of CDTI with coverage of 48.4-79.1% (Taylor *et al*., 2009). From 2006 till now, onchocerciasis control has been implemented in the context of the Neglected Tropical Diseases Control Programme (NTDCP), whose implementation officially started on a pilot basis in five Regions of Ghana in April 2007 (Taylor *et al*., 2009).

Despite mass treatment of communities using IVM alongside various research works, onchocerciasis still remains endemic in all nine of the ten Regions of Ghana as observed from prevalence and intensity studies. Taylor *et al*. (2009) found Mf positive prevalence of 8.9% in 2004. Thirteen of the 18 villages studied showed prevalence above 5%, and about 2.8% and 0.75% of individuals studied had visual impairment and total blindness respectively. In 2005, they found high infectivity rates of 1.82 per 1000 parous flies from Asubende (Pru River basin). They further found in 2006 that there were high infectivity rates of 0.556 to 1.01 per 1000 parous flies within the Pra, White Volta, Kulpawn and Anum River basins; and more than 5% prevalence in 9 of 24 communities studied in Ashanti Region. Osei-Atweneboana *et al*. (2007) found high Mf prevalence of 2.2-51.8% from 19 endemic communities in the districts of Kintampo (Lower Black Volta River basin), Atebubu and Nkoranza (Pru River basin) (Figure 2.3), and Gonja East (Daka River basin). A follow-up study in the Brong Ahafo Region (such as Tain District) and the Northern Region (e.g. Kpandai District: Figure 2.4) needs to be carried out for assessment of skin onchocerciasis and ocular onchocerciasis respectively.

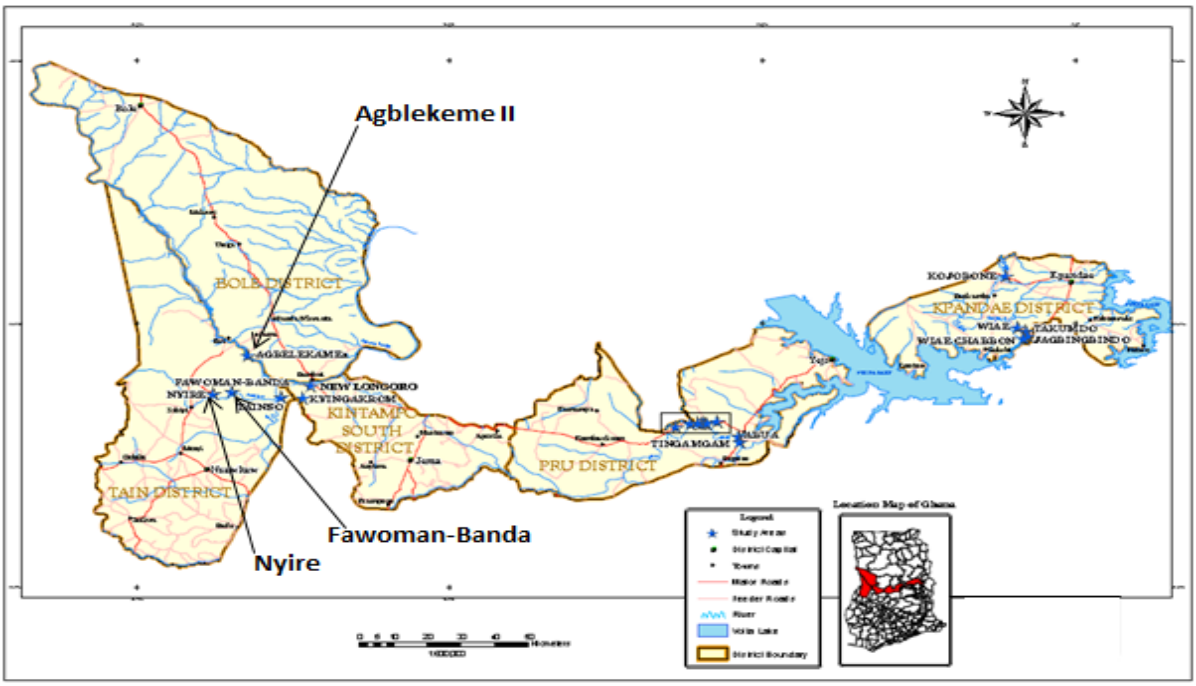

Figure 2.3: Map of Study Areas (represented by symbol ★) in the Tain District (Lower left) in relation to those in the Kpandai District (Extreme right)

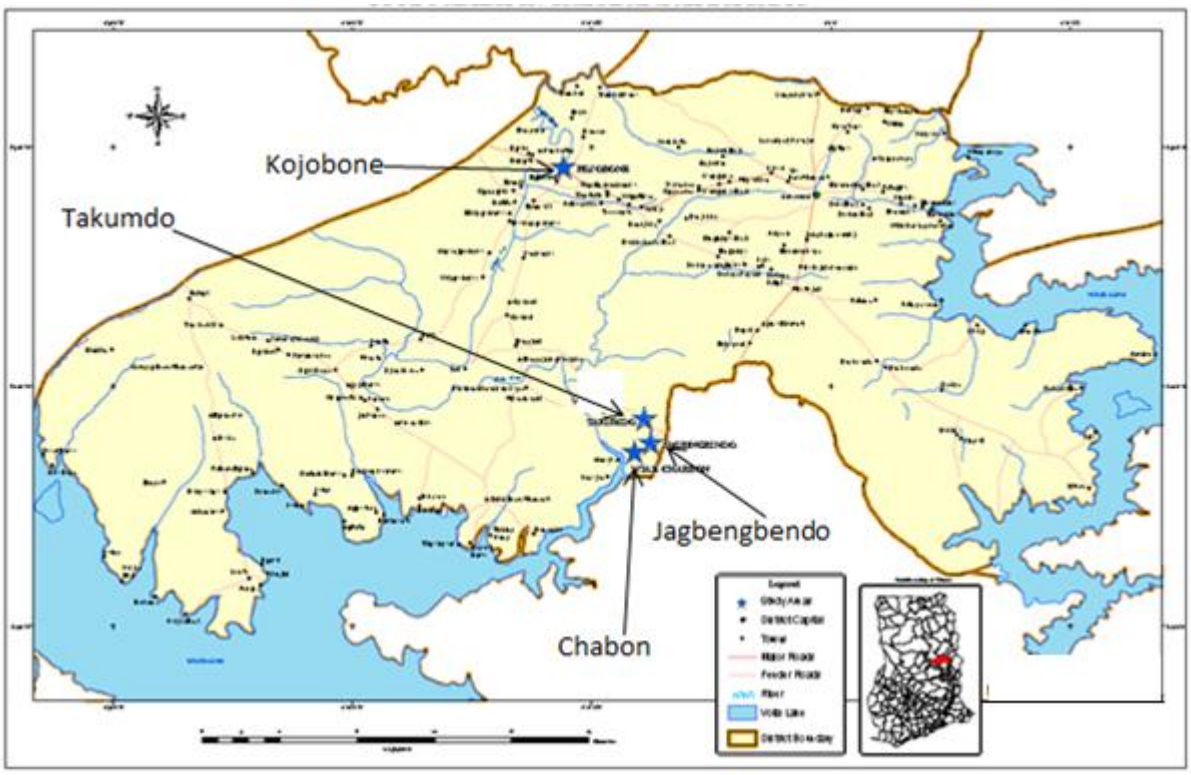

Figure 2.4: Map of Kpandai District Showing Study Areas with the Symbol ★

2.5 Ivermectin, Uses and Genes Involved In Its Selection/Resistance

2.5.1 Ivermectin and Its General Use

Ivermectin (22, 23-dihydroavermectin B_{1a} + 22, 23-dihydroavermectin B_{1b}) is known to be a broad-spectrum antiparasitic avermectin drug originally assigned with the code name MK-933 by Merck whiles in development but it is currently sold under the brand names of Mectizan in Canada by Merck, Stromectol in the United States, Ivomec in Europe by Merial Animal Health, and Ivexterm in Mexico by Valeant Pharmaceuticals International (Pampiglione *et al*., 1985). Although the drug is globally used in the control and elimination of onchocerciasis and to prevent the transmission of lymphatic Filariasis (TCC, 2013a; TCC, 2013b; WHO, 2013b), recent evidence support its efficacious use against arthropods like mites (TCC, 2013b; Victoria and Trujillo, 2001; Strong and Johnstone, 2007), lice (Dourmishey, Dourmishey and Schwartz, 2005; Strycharz, Yoon and Clark, 2008) and bed bugs (Donald and McNeil, 2012). The use of IVM in the treatment of scabies is normally

limited to cases that are resistant to topical treatments or in the advanced state like that of Norwegian scabies (Strong and Johnstone, 2007). A study that used IVM lotion (0.5%) approved by the Food and Drugs Administration for individuals aged six months and older, after a 10-minute single application found that 78% of the patients were free of lice after two weeks (David *et al*., 2012). Thus, this level of efficacy is equivalent to other lousicide treatments that need a minimum of two applications (LAT, 2012). Studies have demonstrated that IVM taken at the normal recommended dose enters the blood stream, and when the host is bitten by a bed bug, result in the death of the bed bug few days later (Donald and McNeil, 2012). IVM is administered alone or in combination with other medications for the treatment of a broad spectrum of animal parasites in the area of veterinary medicine, such as its efficacious usage in some dog breeds that do not respond to their usual recommended treatments due to mutation in their Multidrug resistance protein 1 (MDR1) gene (ASHGI, 2013).

2.5.2 Ivermectin Pharmacodynamics, Pharmacokinetics, Toxicity and Interactions

IVM and other avermectins are members of the macrocyclic lactones derived from the bacterium *Streptomyces avermitilis* and usually exert its paralyzing or killing effect by interfering with muscle and nervous system function such as its ability to inhibit neurotransmission (Õmura and Crump, 2004). It can bind and activate glutamate-gated chloride channels that are members of the Cys-loop family of ligand-gated ion channels found in neurons and myocytes (GluCls) (Yates and Wolstenholme, 2004). IVM, when administered by mouth or injection, does not normally cross the blood-brain barrier of mammals at the normal recommended dosage due to the presence of Permeability glycoprotein (Pgp), hence can be used in mammals against invertebrate parasites that either lack the Pgp, or if present may permit IVM to reach all aspects of their neurons (Borst and Schinkel, 1996).

This implies that humans and other mammals with defect in their Pgp gene can be poisoned by IVM and it is contraindicated in being used together with drugs that inhibit Cytochrome P450 3A4 (CYP3A4) enzymes (such as statins, many calcium channel blockers, HIV protease inhibitors, and glucocorticoids like benzodiazepine) because they can also inhibit Pgp transport to intensify the risk of increased absorption of IVM across the blood-brain barrier (Brunton, Lazo and Parker, 2005). IVM is also contraindicated in children below the age of five or to people whose weight are below 15kg (33 lb) (Dourmishey, Dourmishey and Schwartz, 2005). The drug is to be used with care in veterinary purposes as kittens are susceptible to its toxicity (Frischke and Hunt, 1991). The dung of animals treated with IVM persists longer and can support significantly reduced diversity of invertebrates.

2.5.3 Genes Involved in Ivermectin Selection

It has been reported that genes that express in the formation of functional proteins of P-glycoprotein (Xu *et al*., 1998), Gamma-Amino Butyric Acid receptor sub-unit (GABA) (Blackhall *et al*., 2003), Glutamate-gated Chloride Channels (GluCl) (Njue *et al*., 2004) and beta-tubulin (Mottier and Prichard, 2008) are associated with IVM selection and resistance in veterinary nematodes. In the human parasitic nematode, *O. volvulus*, genetic selection has been reported to occur in beta-tubulin of worms in human populations who have been administered repeated IVM treatments (Eng and Prichard, 2005; Eng *et al*., 2006; Bourguinat *et al*., 2007b). This is not surprising because there is the possibility for a limited number of resistance genotypes to be present in worms from individuals not under IVM selection because alleles that confer resistance can already be found in low frequencies in anthelmintic-naïve populations (Kelly *et al*., 1978; Prichard *et al*., 1980; Jackson, 1993). Thus, the selection of already existing alleles in the IVM-naïve population could result in IVM resistance (Anderson *et al*., 1998). When IVM selection occurs, *O. volvulus* worms carrying genotypes that are susceptible to the efficacious actions of the drug becomes selected against, thereby leaving only worms that carry genotypes associated with the resistance phenotypes to

successfully continue to reproduce and contribute their gene pool to those of the next generation (Prichard, *et al*., 1980). There is the tendency for human hosts demonstrating signs of SOR to IVM treatment to exhibit reduced proliferative responses to the antigens of *O. volvulus* as a result of host immunocompetence issues (Ali *et al*., 2002), hence the need for studies investigating sub-optimal and/or resistance responses in *O. volvulus* to include studying host immunity before and after IVM administration.

2.6 *O. volvulus* Responses and Issues of Sub-optimal Responses/Resistance to Ivermectin

2.6.1 Adult Worm and Its Microfilariae Identification and Classification

To determine the effect of IVM on a particular *O. volvulus* adult female worm, the worm has to be extracted from a nodule, identified, characterized and the status of its embryonic stages (including oocytes, horseshoe, morifold, coiled and stretched Mf) assessed. After nodulectomy and worm extraction, a worm is classified as being alive at the time of nodulectomy if its internal structures are intact and may also depend on worm movement and the state of the uterine morphology. It is possible to see embryos in the uteri through the body structures of the adult female worms. Estimation of worms age depend on body structure, colour and size of the female worms, the prominence of cuticular ridges as well as the extent of inclusions (Chavasse *et al*., 1992; Kläger, Whitworth and Downham, 1996; Specht *et al*., 2009). To augment these criteria, a worm that is small in size with transparent body structure is usually scored as young, opaque and yellowish ones are accepted as middle aged, and large and brown types classified as older worms (Schulz-Key, 1988; Specht *et al*., 2009). Each female worm that is not mutilated in any way is cut into small pieces into about 2ml fresh sterile medium 199 and the worms homogenized by the help of a toughened glass test tube mortar and pestle by turning the pestle gently to squeeze out the embryonic stages from the pieces of worms and the embryogrammes constructed (Schulz-Key, Albiez and Buttner, 1977). The homogenate is usually transferred to a counting chamber (Fuchs-Rosenthal) and the embryonic stages assessed (Schulz-Key, Albiez and Buttner, 1977; Schulz-Key, 1988).

Finally, the quantitative assessment of normal and abnormal types of the embryonic stage up to the stretched Mf is carried out to determine the Mf content and reproductive status of the adult female worms (Schulz-Key, Jean and Albiez, 1980)

2.6.2 Epidemiological, Embryonic and Phenotypic Responses

Resistance and SOR of *O. volvulus* to IVM is demonstrated by responses that are not compatible with previous experience following multiple treatments, parasite exposure to adequate concentrations of IVM , control of on-going transmission effects and evidence of individuals receiving the appropriate treatments under consideration (Taylor *et al*., 2009). SOR becomes implicated by realizing a higher than expected of the prevalence and/or infection intensity by skin Mf through skin Mf assessment and adult female worm examination. A single standard dose of 150 µg/kg body weight treatment of IVM results in the reduction of Mf counts by half after 24 hours, 74% after 48 h, approximately 85% after 72 h, 94% after one week, 98% after two weeks, and by 99% after one to two months (Basáñez *et al*., 2008). Basáñez and workers (2008) further predicted that Mf repopulate the skin at a rate that result in baseline load percentages of 6%, 17% and approximately 40% at six, 12 and 24 months following the single standard dose of IVM treatment (150 µg/kg). Multiple treatments using IVM result in significant effects on embryogenesis (Gardon *et al*., 2002). Estimates from quantitative assessments, following multiple treatments, range from irreversible decline in Mf production of approximately 30% for each treatment (Plaisier *et al*., 1995), 83% decrease in productive index (Kläger *et al*., 1993) to cessation of development at the single cell stage (Chavasse *et al*., 1992). Hence, observed responses can be compared with these expected responses to determine if SOR or resistance has occurred.

There was an open case control hospital based investigation (Awadzi *et al*., 2004b) of microfilaridermic and amicrofilaridermic individuals from two river basins located in Ghana that assessed the effect of multiple IVM treatments and determined the presence of non-

responsive adult female worms despite the fact that Mf were generally sensitive to IVM, and this observation was confirmed in a 30-month follow-up study (Awadzi *et al*., 2004a).

A two-phase epidemiological investigation (Osei-Atweneboana *et al*., 2007) that assessed the efficacy of IVM in 19 onchocerciasis endemic communities from three river basins in Ghana showed an unusually high repopulation rate at day 90 IVM post-treatment that was confirmed at day 180 IVM post-treatment and indicated the emergence of resistant adult parasite population that are not responding as expected to IVM. This finding is also demonstrated by the rapid return of skin Mf at four to six months in individuals in non-endemic area after receiving single dose of IVM (Ali *et al*., 2002).

In a recent study (Pion *et al*., 2013), the dynamics of *O. volvulus* skin Mf densities following IVM treatment in two cohorts were compared with one receiving 13-years repeated treatment whiles the control did not have any known large-scale treatment. The study showed that worms from the multi-treated area recovered Mf productivity earlier but were less productive than the worms obtained from the IVM-naïve locality between 80 and 180 days after IVM treatment but they did not support any strong cumulative effect of repeated treatments on female worm fecundity. Moreover, the Mf repopulation rate had association with host factors, positively with number of nodules but negatively with age. This is an indication of host immunity playing a significant role in the mechanism of IVM action.

A phenotypic investigation (Osei-Atweneboana *et al*., 2011) on the parasitological responses of *O. volvulus* to IVM was carried out in 268 microfilaridermic individuals from nine communities and one pre-study IVM-naïve community. After determining the intensity of nodules and Mf, assessing the reproductive status of female worms and stratifying the female worms by morphological age and Mf content, it was realized that adult female worms in some communities were non-responsive or resistant to the anti-fecundity actions of multiple IVM treatments. These phenotypic manifestations may have genetic basis.

2.6.3 Genetic Events of Ivermectin Resistance

Drug resistance is defined as the loss of normal response to a particular treatment with the possibility of being inherited (Prichard *et al*., 1980). IVM exerts its effect on onchocerciasis parasites at two different morphological stages; it firstly kills the Mf, clearing them from the skin and then secondly paralyzes the uteri of the female worms for a temporary period of time. IVM SOR and resistance is common in veterinary parasites, such as *Haemonchus contortus*, and may have genetic basis with associations to selection on genes of Beta-tubulin, Gamma Amino Butyric Acid (GABA) and Adenosine Tri-Phosphate (ATP)-binding cassette (ABC) transporters like Permeability-glycoproteins (Bourguinat *et al*., 2007a; Taylor *et al*., 2009).

A phenotypic and genotypic study (Eng *et al*., 2006) was carried out to assess the responses of *O. volvulus* and *H. contortus* to IVM from IVM-naïve and naturally infected hosts who had taken IVM and located in at least 36 communities from Ghana, Sierra Leone, Cote d'Ivoire, Guinea, Senegal and Uganda. It was realized that IVM selects on beta-tubulin in both species of nematodes. Eng and Prichard (2005) had earlier on reported that IVM selects for specific SNPs in the beta-tubulin gene of *O. volvulus* that results in three amino acid changes in the H3 helix of the beta-tubulin gene and causes deletions in associated intron.

A comparison of samples from individuals who have received many rounds of IVM treatment from localities in Cameroon and Ghana with worms taken from treatment-naïve subjects or the same subjects prior to treatment revealed significant changes in genes of beta-tubulin, P-glycoproteins and other ABC transporters (Bourquinat *et al*., 2007; Prichard, 2007).

A 21 month epidemiological investigation (Osei-Atweneboana *et al*., 2012) was carried out on ß-tubulin gene to determine the response of *O. volvulus* to IVM in 10 onchocerciasis endemic communities in Ghana by analysing worm DNA for association between genotype and IVM response phenotype. Embryonic assessment revealed higher reproductive activity in

female worms from the poor response communities when compared with that of the good response communities, IVM resistant genotypes were selected for and the genotype (1183GG/1188CC/1308TT/1545GG) showed strong association with the resistant phenotype.

2.7 Beta-Tubulin and its Role in Ivermectin Selection/Resistance

Beta (ß) tubulin is a member of the tubulin family with a molecular weight of about 55 kiloDaltons, isoelectric point between 5.2 and 5.8, and it is slightly acidic (Williams *et al*., 1999). ß-tubulin forms dimer with α-tubulin, assemble together to form microtubules by binding to Guanosine Tri-phosphate (GTP) and assemble onto the (+) ends of microtubules while they are in the GTP-bound state (Heald and Nogales, 2002). The ß-tubulin subunit becomes exposed on the microtubule's (+) end whiles the α-tubulin subunit gets exposed on the (-) end, the incorporated molecule of GTP bound to ß-tubulin becomes hydrolysed into Guanosine Di-Phosphate (GDP) via inter-dimer contacts along the microtubule protofilament to form the actual microtubule (Howard and Hyman, 2003). Furthermore, the ability of ß-tubulin to bind to either GTP or GDP determines the stability of the dimer in the microtubule because the formed dimers bound to GTP are able to assemble into microtubules but those that bind to GDP fail to assemble into microtubules.

The microtubules formed from the αß-tubulin dimer, along with a number of associated motor proteins, are involved in cellular functions such as cell motility, vesicle movement, and chromosome segregation during cell division (Keskin *et al*., 2002). Hence, the microtubules form key target for cancer therapy, just as agents of tubulin-binding form a therapeutic class of compounds with broad activity against solid and hematologic neoplasias (Chabner and Longo, 1996).

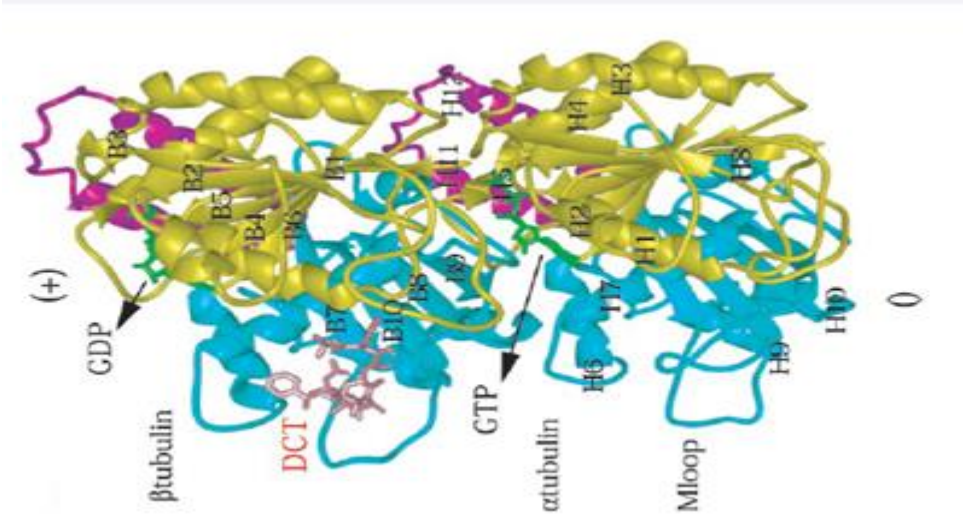

Figure 2.5: Ribbon diagrams of αß tubulin dimmer (Nogales, Wolf and Downing, 1998).

In Figure 2.5 above, the αß tubulin dimmer protein is made up of two subunits: a GTP-bound α-tubulin (- end) and a GDP-bound ß-tubulin (+ end), and the ß-tubulin monomer contains a pair of central ß-sheets that are surrounded by α-helices (Nogales *et al*., 1998). H1-H12 (helices) and B1-B10 (ß-strands) represent the secondary structural elements. The ß-tubulin monomer consists of three domains: a drug binding or intermediate domain (D2, residues 206-381, blue colour) where IVM binds, a nucleotide-binding or N-terminal domain with a Rossman fold (D1, residues 1-205, yellow colour), and a mitochondria-associated protein-binding or C-terminal domain (D3, residues 382-440, magenta colour).

When IVM binds to the drug binding domain, it changes the structural conformation to prevent the polymerization of the αß-monomers and consequently inhibit the formation of microtubules. This prevents the vital functioning of a cell causing the cell to die and subsequently preventing the normal functioning of the cell, tissue or organs they constitute. However, a mutation in the genes that express the drug binding domain will change the structure of the domain thereby preventing IVM from binding to it and then confer IVM-resistance characteristics to the *O. volvulus* containing it.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Area and Population

The study was conducted in seven onchocerciasis endemic communities located in two districts in Ghana; the Tain and Kpandai Districts in the Brong Ahafo and Northern Regions respectively. The study communities were Agblekeme II (N 08^o 23'14.0" W 000^o 20'58.6"), Fawoman-Banda (N 08^o 02'12.7'' W 001^o 02'42.5'') and Nyire (N 08^o 06'43.7'' W 002^o 17'45.4'') located in the Tain District (Figure 2.3) and Chabon (N 08^o 19'21.6'' W 000^o 09'12.0''), Kojobone (N 08^o 29'09.4'' W 000^o 11'10.0''), Takumdo (N 08^o 18'55.3'' W000^o 07'53.3'') and Jagbengbendo (N08^o 17'39.8'' W000^o 07'32.1'') located in the Kpandai District (Figure 2.4).

The Tain District has vegetation cover that spans the moist semi-deciduous forest as well as the Guinea Savannah woodland vegetation zones and represents an eco-climatic zone. The climatic conditions of the district have effect on development as it influences the quality and quantity of land cover. There is a generally high temperature averaging 24.5^{o}C throughout the year with average maximum temperature of 30.9^{o}C and minimum temperature of 21.2^{o}C. The hottest months occur from February to April. Sufficient rainfall and moisture content are important factors for existing potential resources of this district. The rainy season is between April and October with an average annual rainfall of 1140mm-1270mm, followed by a short duration of dry period in August. The district is located within Latitudes 7^o 5` N and 8$^o$ 45` N and longitudes 2^o 52` W and 0$^o$ 28` East. It covers 4,125 sq. kilometers of land area. The topography is mainly undulating with gentle slopes of less than 1% inclination with the land rising 30m above sea level to over 61m in the North West and high elevation of 592.2m. The Tain River Basin and parts of the Black Volta River Basin are located in the Tain District.

About 49.4% of the total population (112,939 as at 2009 with a growth rate of 2.6%) in the Tain District are females whiles 50.6% are males. The male – female ratio of 1:0.9 is similar to that of the Brong Ahafo regional sex ratio of 1:1.008 (GHA-COM, 2012; DPCU, 2012).

The Kpandai District lies in the Tropical Continental Climatic Zone with temperatures ranging between 29^{o}C and 40^{o}C and having maximum temperature usually in April but minimum temperature around December-January. There is irregular rainfall pattern with annual rainfall ranging between 1150mm to 1500mm. It is found at the South-Eastern part of the Northern Region of Ghana and lies between latitudes 8^{o} 0` N and 9$^{o}$ 29` N and longitudes 0^{o} 29` W and 1$^{o}$ 26` W. It is found in the transitional zone between the Northern Savannah and the moist semi deciduous forest. The district possesses a total surface area of 1772.04 sq. km with 5% water coverage. The White Volta, Oti and Daka rivers are the three main rivers found in the district. The water bodies serve as resources for the people because they depend on them for domestic purposes, transportation and fishing. The Kpandai District has current population estimate of at least 96,291 people and a 2.1% growth rate (MMDA, 2012).

3.2 Community Selection and Inclusion Criteria

Information about the study population, and IVM treatment history was obtained from the Disease Control Officers of the Ghana Health Service in the Tain and Kpandai Districts to guide the selection of the study communities. Community accessibility was a factor for the selection of a community. The main inclusion criteria were for an adult to be permanent residents of the community for a period of not less than five years, have well documented information on IVM treatment history for at least the previous five years, have received IVM treatment within the previous 9-12 months, and have no known contraindication for IVM. Further inclusion criteria for the study community selection involved at least 55% average

IVM treatment coverage over the past 5 years and continuous yearly IVM treatment over the past 6 years. These selection and inclusion criteria were adhered to before choosing the sample size.

3.3 Sample Size and Sampling Methods

Sample size calculation was based on the population size of each community, a 1.96 Z-score and a minimum of 70% statistical power of the statistical test such that there was 95% confidence interval and a 5% significance level. To ensure a statistically representative sample size, individuals (≥ 20 years) were randomly selected from communities with a population size greater than 50. For communities with population size of less than 50, all individuals (≥ 20 years) were sampled.

Strategies for community entry was slightly different for larger (>50) and smaller communities (<50). For large communities, census data was acquired followed by a meeting with the chief and elders of the community to inform and explain the proposed study to them. After informed consent, households were randomly selected and sequential numbers were assigned to households with at least one adult aged 20-60 years. A computer-based random-number generator was used to create a list of household numbers from a database of all the households with the sequential numbering created earlier. Finally, households were sequentially recruited from the randomized list created. Confirmation and consistency check was done. The treatment history of all participating individuals from the recruited households was confirmed by checking the IVM treatment records as well as carrying out interviews of the participants and the community IVM distributors. Individuals who met the inclusion criteria were enrolled after assessing the adults aged 20-60 years from recruited households who had the desire to participate in the study.

3.4 Study Design

A fifteen month longitudinal study was carried out in seven onchocerciasis endemic communities in the Tain and Kpandai Districts of Ghana. The study involved serial skin snipping, two rounds of semi-annual IVM treatment and nodulectomies. One week before IVM treatment, skin snips were taken from the iliac crest of each subject using 2mm-Holth type corneoscleral punches to assess skin microfilarial load. Each subject was then treated with IVM at a standard dose of 150 µg/kg body weight. All subjects who were Mf positive at pre-treatment were followed-up and Mf assessment done at 3 months and 6 months after IVM treatment. As a result of the paradigm shift from onchocerciasis control to elimination, onchocerciasis control strategy in Ghana has changed in some communities from annual to semi-annual treatment. Consequently, IVM treatment was given again at 6 (and 12) months. The number of Mf per skin snip was counted and recorded for each person in each of the communities. The nodule infection intensity was determined by counting the number of palpable nodule of *O. volvulus* whiles the prevalence and intensity of Mf were determined for each community by counting the number of individuals per community with Mf and the average number of Mf in each skin snip respectively (WHO, 1995).

At 90 days after the third IVM treatment (day 452 after the first IVM treatment), nodulectomies were carried out in six communities: Agblekeme II, Chabon, Jagbengbendo, Kojobone, Nyire and Takumdo and nodules stored for adult *O. volvulus* isolation and embryogram analysis before selecting 59 worms from Jagbengbendo and Takumdo for genetic analysis.

3.5 Ethical Consideration

Prior to the commencement of the study, approval (Ethical Clearance) for the research study protocol was obtained from the Institutional Review Board of the Council for Scientific and Industrial Research (Appendix 5). Informed consents were obtained from the study subjects

before recruitment to participate in the study (Appendix 4). The study participants were made to know of the benefits and discomfort of their participation.

3.6 Nodulectomies, Nodule Digestion and Adult *O. volvulus* Isolation

Onchocerca volvulus nodules were removed from onchocerciasis patients using local anesthesia, under aseptic conditions. The nodules were preserved in liquid nitrogen in the field and transported to Accra where they were kept frozen at -80^{o}C in Ultra Low Freezer until ready for nodule digestion.

Any excess host tissue from the nodules were removed and each nodule was placed in a 50ml conical tube containing 0.5% collagenase in Medium 199 supplemented with Earl's salt, L-glutamine, and sodium bicarbonate to adjust the pH to 7.0 (Gibco BRL0). The nodules were incubated at 37^{o}C in water bath (Thermo Scientific Lindberg Blue M) for 10 to 18 hours before the adult worms were extracted. Worms were harvested from the nodules by washing with 0.9% saline to expose the worms. The individual worms were separated by sex. All worms were kept in RNAlater solution and stored at -20°C until ready for classification and DNA extraction.

3.7 Classification of *O. volvulus* IVM Response Status

There was a parasitological assessment of *O. volvulus* response to IVM treatment through epidemiological study. Samples characterizing different responders were obtained. The IVM responders were generally classified into two main groups: (1) Good-IVM Responders, represented samples from individual subjects responding well to IVM treatment over the past 6 years, characterized by not having skin Mf repopulation in 3 months after IVM treatment, with the female worms ceasing egg production and having no intra-uterine Mf; and (2) Poor-IVM Responders, represented samples from individual subjects who had treatment for at least 6 years and had skin Mf repopulation in 3 months after IVM treatment, as well as having female worms with active embryogenesis and still producing intra-uterine Mf in 3 months

after IVM treatment (Trt). The classified responses formed the basic grouping for the DNA extraction.

3.8 DNA Extraction

DNA was extracted from 59 worms in Jagbengbendo and Takumdo. By using sterilized scalpel, 1-2cm of the anterior or posterior end of the adult *O. volvulus* free from intra-uterine Mf (Figure 4.5.4), was cut into small pieces. The sample was homogenized in 50 microliter (µl) of Buffer TL (Tris Sodium Dodecyl Sulfate (SDS) and Ethylenediaminetetraacetic acid (EDTA). This was mixed by pulsed-vortexing for 5 seconds (sec) to obtain a homogeneous solution, followed by the addition of 20µl of Proteinase K and gentle mixing. An amount of 12µl Lysis Enhancer was added and mixed immediately by pulsed-vortexing. The mixture was incubated in a shaking waterbath at 65^{o}C overnight to ensure thorough digestion of the sample. An approximate amount of 560µl of Buffer TB (54g Tris base and 27.5g Boric acid) was added and mixed thoroughly by pulsed-vortexing and incubated for 10 minutes (min) at 65^{o}C. An amount of 200µl of ethanol (96%) was added and mixed immediately by pulsed-vortexing to prevent any uneven precipitation of nucleic acid due to high local ethanol concentrations. The sample was centrifuged at a speed of 15000 x *g* for 10 sec with the Labnet Prism^{TM} Microcentrifuge.

The column was washed with 750µl Wash Buffer containing ethanol and centrifuged at 5000 x *g* for 1 min. To elute DNA, the column was placed into a clean 1.5 ml microcentrifuge tube. An amount of 200µl of preheated sterile water was added onto the column membrane and allowed to stand at room temperature for 2 min. It was finally centrifuged at 5000 x *g* for 2 min to elute DNA and the DNA stored at -20^{o}C.

3.9 Amplification of Fragment of ß-Tubulin Gene by Polymerase Chain Reaction (PCR)

The region 1012-1695bp of the 3696bp full length (Appendix 3) of the beta-tubulin gene was flanked and amplified with the following set of PCR primers: Ov IVM Tub-F2 (5^1-GAGATGGATAATATGGACTAG-3^1) and Ov IVM Tub-R2 (5^1-GATCCACAAATTGCACCTG-3^1). Amplification of the fragment of 684bp of beta tubulin gene was carried out with the Universal Gradient PeQSTAR (PeQlab) PCR Machine for each sample. The beta tubulin gene of *O. volvulus* was amplified in a 50µl reaction solution mix containing final volumes and concentrations of 5µl of 1X Taq Buffer (containing 1.5mM $MgCl_2$), 1µl of 0.2mM dNTP, 2.5µl of 0.5µM each of the forward and reverse primers,5 units of Taq DNA polymerase, 36µl of water and 2µl of genomic DNA template. To avoid contamination, PCR mix was prepared in a UV3 HEPA PCR Work station. The PCR mix was subjected to 35 amplification cycles, with each cycle consisting of 45 sec denaturation at 94 ^oC, 45 sec annealing at 52 ^oC, 1 min extension at 72 ^oC and 5 min final extension at 72 ^oC. There was a 1 hour holding at 20 ^oC for the final PCR products before storing at -4 ^oC.

3.10 Agarose Gel Electrophoresis

Electrophoresis of PCR products was done using 1.5% agarose gel stained with ethidium bromide. For each gel run, 5µl of PCR products was mixed with 1µl of 6X DNA loading buffer/dye (Bromophenol blue). All gel runs included a 100bp DNA ladder (Invitrogen, U.S.A.). Gel electrophoresis was done at a voltage of 100v and run for 1 hour. Following electrophoresis, the gel was visualized on a benchtop Ultra-violet trans-illuminator (BioDoc-It^{Tm} Imaging System). The bands of PCR positive samples (Plate 7) were cut with a clean sharp scalpel, on a Trans-illuminator (PeQlab Biotechnologie GmbH).

3.11 Gel extraction Protocol (Quick-Start protocol)

Three volumes of Buffer QG (5.5 M Guanidine thiocyanate (GuSCN) and 20 mM Tris HCL pH 6.6) were added to 1 volume of gel (100 mg ~ 100 µl). It was ensured that the maximum

amount of gel per spin column was 400 mg. For more than 2 % agarose gels, 6 volumes of Buffer QG were added. The gel was incubated at 50^{o}C for 10 minutes with intermittent vortexing to completely dissolve it. A QIAquick spin column was placed in a 2 ml collection tube, the sample was applied to the QIAquick column and centrifuged for 1 min and, the flow-through was discarded.

To elute the DNA, 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the column and centrifuged for 1 min, allowed to stand for 1 min and then centrifuged for 1 min. The 59 extracted DNA were then sent for sequencing.

3.12 Genetic Diversity of *O. volvulus*, Indices and Statistical Analysis

Following the molecular study, the DNA sequences obtained from different samples of *O. volvulus* adult worms from the two communities (Jagbengbendo and Takumdo) were analyzed to look at genetic differences, including polymorphisms and allelic changes. This was achieved by using SEQUENCHER® 5.1 software (Gene Codes Corporation, U.S.A). Fisher exact tests and chi-square test from the SPSS 17 software (SPSS Inc., U.S.A.) were used to determine significant differences in allele and genotype frequencies between good-IVM response and poor-IVM response phenotype worms.

The Linear Correlation of Pearson's Product Moment Correlation Coefficient from the SPSS 17 software was used to test for associations between the nodules and Mf parameters under study. The Spearman's rho correlation test was explored before deciding on the appropriate relationship model. A Likert scale was developed for the worm phenotype and treated as ordinal data whiles treating the worm genotype as nominal data before testing for associations between worm phenotypes and genotypes. The five likert scale category in order of IVM response from good responding to poorest responding was good, moderate, poor, poorer and poorest.

Furthermore, the Hardy-Weinberg expected frequency and inbreeding coefficient were determined. In addition, a Hardy-Weinberg equilibrium test was performed to determine whether or not the genotype frequencies were constant from generation to generation.

There were 3 parasitological indices calculated to assess the degree of endemicity in the communities studied. The infection intensities in the communities were determined with the Community Microfilarial Load (CMFL). This is the reference index adopted by the OCP and it is calculated as the geometric mean of individual microfilarial loads (including zero counts) in people who are 20 years or above similar to that described by other workers (Remme *et al*., 1986), with that age limit partly chosen because studies have shown that there is a stable number of Mf over time in individuals aged 20 years and above (Duke and Moore, 1968; Basáñez *et al*., 2002; Duerr *et al*., 2004). The log (x+1) transformation, with x being the individual microfilarial load was used for the calculation. A comparison of the microfilarial densities examined in the second part of the study was done by using the Kendall Tau non-parametric test meant for related samples, which is most appropriate for our dependent sample. Pair-wise comparisons of the microfilarial densities were however carried out with the non-parametric Wilcoxon Signed Rank test for related or dependent samples. The 95% confidence interval was used whiles regarding differences as significant at $p<0.05$.

CHAPTER FOUR

4.0 RESULTS

Out of the 584 patients randomly selected from the five study communities, 122 (20.9%) had nodules (Table 4.1) with the individual community nodule prevalence being 12.8% (Kojobone), 17.5% (Nyire), 20.7% (Fawoman-Banda), 35.8% (Agblekeme II) and 36.2% (Chabon) in order of increasing magnitude (Figure 4.1).

Table 4.1: A summary of the number of patients assessed for nodules and microfilaria

Sample	Frequency	Percentage (%)	Sample	Frequency	Percentage (%)
Total Nodule Positive Patients	122	20.89	Total Pre-Treatment Mf Positive Patients	87	14.90
Total Nodule Negative Patients	462	79.11	Total Pre-Treatment Mf Negative Patients	497	85.10
Total	584	100	Total	584	100

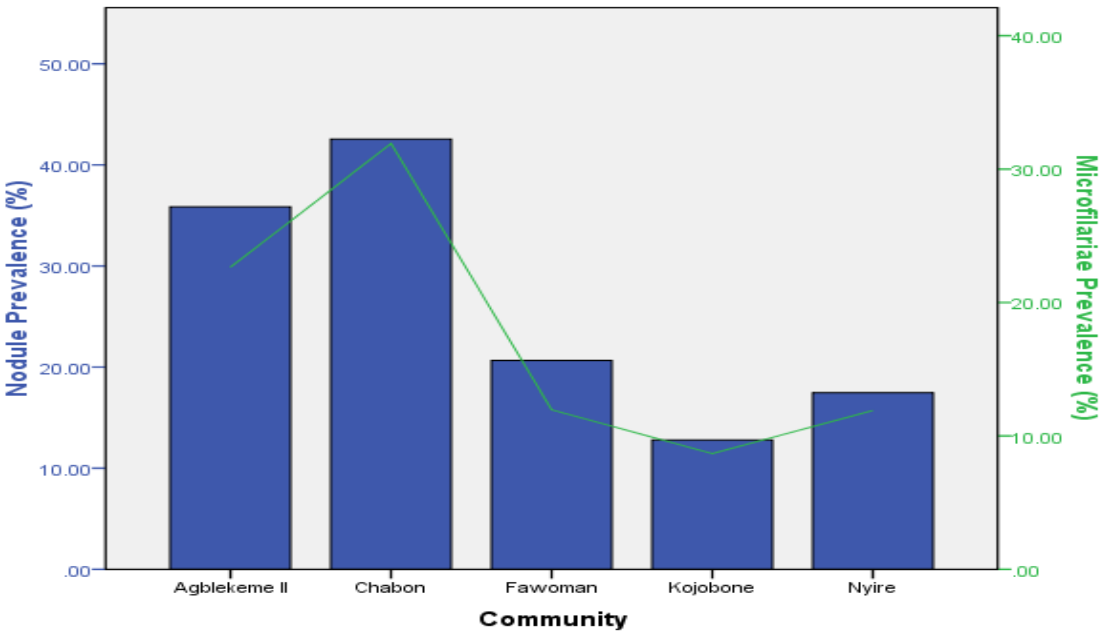

Figure 4.1: Comparison of nodule prevalence and microfilaria prevalence

Of the 584 patients assessed for skin Mf at pre-treatment, 87 were positive resulting in an overall Mf prevalence of 14.9% (Table 4.1) with the individual Mf prevalence being 8.7% (Kojobone), 11.9% (Nyire), 12.0% (Fawoman-Banda), 22.6% (Agblekeme II) and 31.9% (Chabon) in order of increasing magnitude (Figure 4.1). There was a general pattern of infection being highest at pre-treatment, followed by day 180 post-treatment, 360 post treatment and lowest at day 270 post-treatment across the five communities. Thus, there was mean nodule prevalence of 20.9% with individual communities ranging 12.8-36.2%; mean Mf prevalence of 14.9% with individual communities falling between 8.7-31.9%; mean CMFL of 3.1 (1.4-4.4) Mf per snip; mean Mf density at day 180 post-treatment of 1.4 (0.6-2.1) Mf per snip; average Mf density at day 360 post-treatment of 0.3 (0.1-0.4) Mf per snip; and average Mf density at day 270 post-treatment of almost zero (0.0-0.1) shown in Tables 4.2-4.4 and Figure 4.2 (Additional details are in Appendix 1).

Table 4.2: Descriptive statistics of nodule and Mf prevalence, CMFL and MFD at days 180-, 270 and 360 post-treatment

Parameters	Mean	Std. Deviation	Minimum	Maximum
Nodule Prevalence	20.90	12.72	12.79	36.17
Infection Intensity at Pre-Treatment (CMFL)	3.11	1.14	1.42	4.43
Mf Density at day 180 Post Treatment	1.44	0.57	0.67	2.11
Microfilariae Prevalence	14.90	9.67	8.68	31.91
Mf Density at day 270 Post Treatment	0.04	0.02	0.00	0.06
Mf Density at day 360 Post Treatment	0.26	0.15	0.08	0.39

Table 4.3: Descriptive statistics of Mf at pre-treatment and at days 180-, 270 and 360 post-treatment across the 5 communities

Parameters	Mf at Pre-Treatment	Mf at day 180 Post-Treatment	Mf at day 270 Post-Treatment	Mf at day 360 Post-Treatment
Mean	5.65	2.32	0.06	0.45
Std. Error of Mean	1.05	0.53	0.03	0.11
Median	2.50	1.00	0.00	0.00
Range	65.50	43.50	1.50	5.00
Minimum	0.50	0.00	0.00	0.00
Maximum	66.00	43.50	1.50	5.00
Sum	491.50	202.00	5.50	39.50

Table 4.4: Wilcoxon Signed Ranks Test Statistics of Mf at pre-treatment and at days 180-, 270 and 360 post-treatment across the 5 communities

Statistic	Mf at Pre-Treatment - Mf at day 180 Post-Treatment	Mf at Pre-Treatment - Mf at day 270 Post-Treatment	Mf at Pre-Treatment - Mf at day 360 Post-Treatment	Mf at day 180 Post-Treatment - Mf at day 360 Post-Treatment	Mf at day 360 Post-Treatment - Mf at day 270 Post-Treatment
Z	-7.06[a]	-8.11[a]	-7.97[a]	-7.26[a]	-4.06[a]
Asymp. Sig. (2-tailed)	0.00	0.00	0.00	0.00	0.00

a. Based on negative ranks.

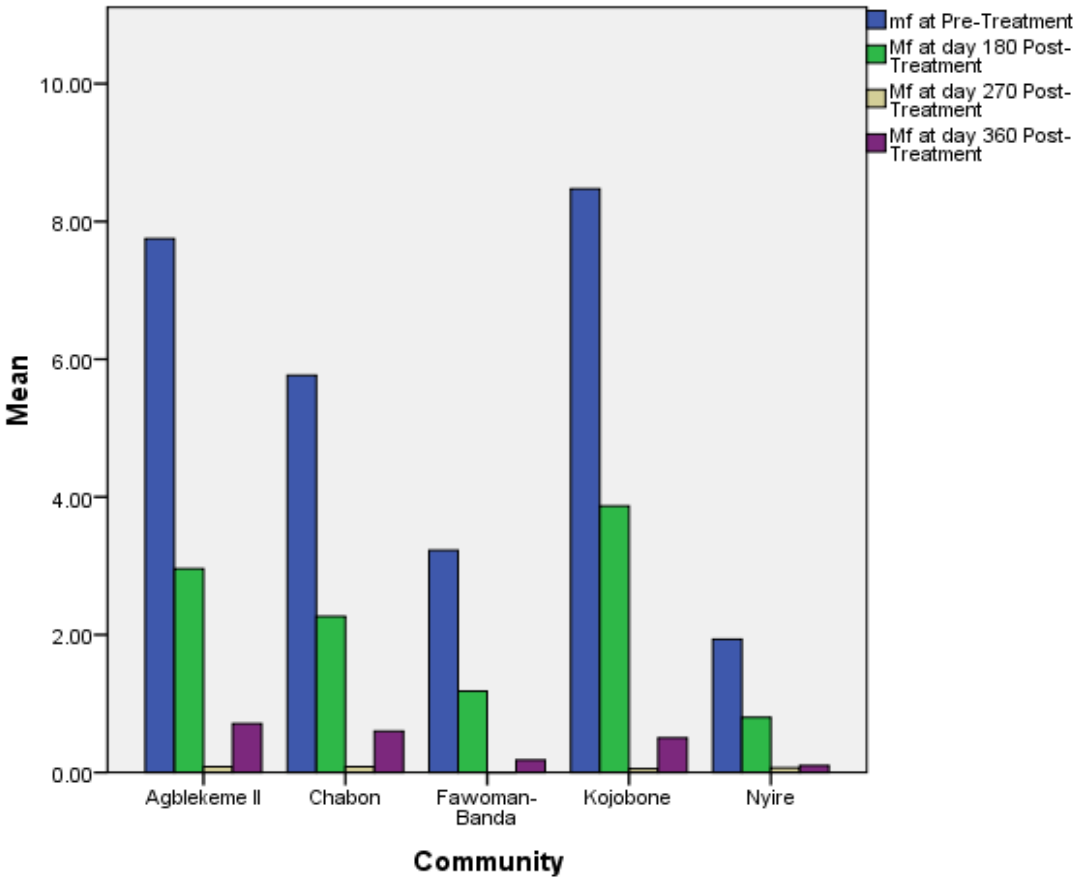

Figure 4.2: Average (mean) microfilaria intensity at pre-treatment and at days 180-, 270- and 360 post-treatment

The assessment of Mf densities at days 90 and 180 post-treatment in the five study communities were used as a measure of skin Mf repopulation by the adult female worms through the determination of the percentage of pre-treatment counts. The assessment of Mf at 90 days after the second study IVM treatment (i.e. Mf at day 270 after the first study treatment) showed 100% clearance of Mf in more than 99% of people in two communities (Fawoman-Banda and Kojobone) (Table 4.5).

Table 4.5: Community Microfilaria Load (CMFL) and geometric mean densities (microfilaria per skin snip) at different times in the five study communities

Community	Mf Infection Intensity at Pre Treatment (*CMFL)	MfD at day 180 Post Trt§	MfD at day 270 Post Trt§	MfD at day 360 Post Trt§
Agblekeme II	3.80	2.11 (55.53%)	0.06 (1.58%)	0.39 (10.26%)
Chabon	3.19	1.60 (50.16%)	0.06 (1.88%)	0.37 (11.60%)
Fawoman-Banda	2.72	1.09 (40.07%)	0	0.11 (4.04%)
Kojobone	4.43	1.75 (39.50%)	0.04 (0.90%)	0.33 (7.45%)
Nyire	1.42	0.67 (47.18%)	0.05 (3.52%)	0.08 (5.63%)

* = Microfilaria density per snip at day 7 before treatment

§ = Percentage of pre-treatment (i.e. referring to the values indicated in brackets)

During this period, three of the five communities (Agblekeme II, Chabon and Nyire) had considerable Mf repopulation, from 1.6% to 3.5% of pre-treatment counts, rising to 10.3% (in Agblekeme II) by day 180 after the second IVM treatment (Microfilarial Density-MFD at day 360 post-treatment). The percentage increases in skin Mf repopulation within the five communities (less than 4% of pre-treatment counts) at day 90 assessment is within expectations (less than 6% of pre-treatment count is acceptable) (Awadzi *et al*., 2004b). There was no subject with Mf repopulation in Fawoman-Banda at 90 days after the second study IVM treatment, but the proportion of subjects with Mf repopulation in the other communities varied from as low as 6.7% in Nyire to as high as 10.5% in Kojobone (Appendix 1: Table A7).

However, the day 180 assessment after the first IVM treatment (MFD at day 180 post treatment) showed an alarming pattern of unexpectedly large increases in skin Mf repopulation in all the five study communities, with repopulation densities far higher than 30% of pre-treatment counts (39.5% - 55.5% of pre-treatment counts), which is unacceptable. Nevertheless, the day 180 assessment after the second IVM treatment (MFD at day 360 post treatment) showed increases within expectations in all the five study communities (4.0% - 10.3% of pre-treatment count). There was no individual in Fawoman and Nyire with either pre-treatment or day 180 post-treatment Mf densities above 10 Mf per snip. Although the proportion of subjects in Agblekeme II, Chabon and Kojobone with Mf densities greater than 10 Mf per snip at pre-treatment (16.7-21.1%) showed considerable decrease at day 180 pre-treatment, 3.3-8.3% of the individuals still had Mf intensities above 10 Mf per snip (Table 4.6). These results indicate that IVM when used as an annual treatment is not effective in maintaining skin Mf clearance but effective when used bi-annually.

Table 4.6: Subjects' microfilaria density responses in the five study communities

Community	Total No. of subjects (follow-ups)	% (No.) of subjects with pre-treatment mf densities >10 mf/snip	% (No.) of subjects with day 180 mf densities >10 mf/snip
Agblekeme II	12	16.70 (2)	8.30 (1)
Chabon	30	20.00 (6)	3.30 (1)
Fawoman-Banda	11	0.00	0.00
Kojobone	19	21.10 (4)	5.30 (1)
Nyire	15	0.00	0.00

There was a very strong significant positive correlation (p=0.003, R=0.982) between nodule prevalence and Mf prevalence but no such association was observed between the prevalence and CMFL (p>0.05) (Table 4.7 and Figure 4.3).

Table 4.7: Correlation test of nodule prevalence, Mf prevalence and CMFL in the 5 communities

Correlation between	**Pearson's R**	***p*-value**	**Spearman's rho R**	***p*-value**
Nodule prevalence & Mf prevalence (in 5 Communities)	0.982	0.003	1.000	NA
Nodule prevalence & CMFL (in 5 Communities)	0.123	0.844	-0.100	0.873
Mf prevalence & CMFL (in 5 Communities)	0.105	0.866	-0.100	0.873

NA= Not applicable (as the perfect correlation gave no *p*-value)

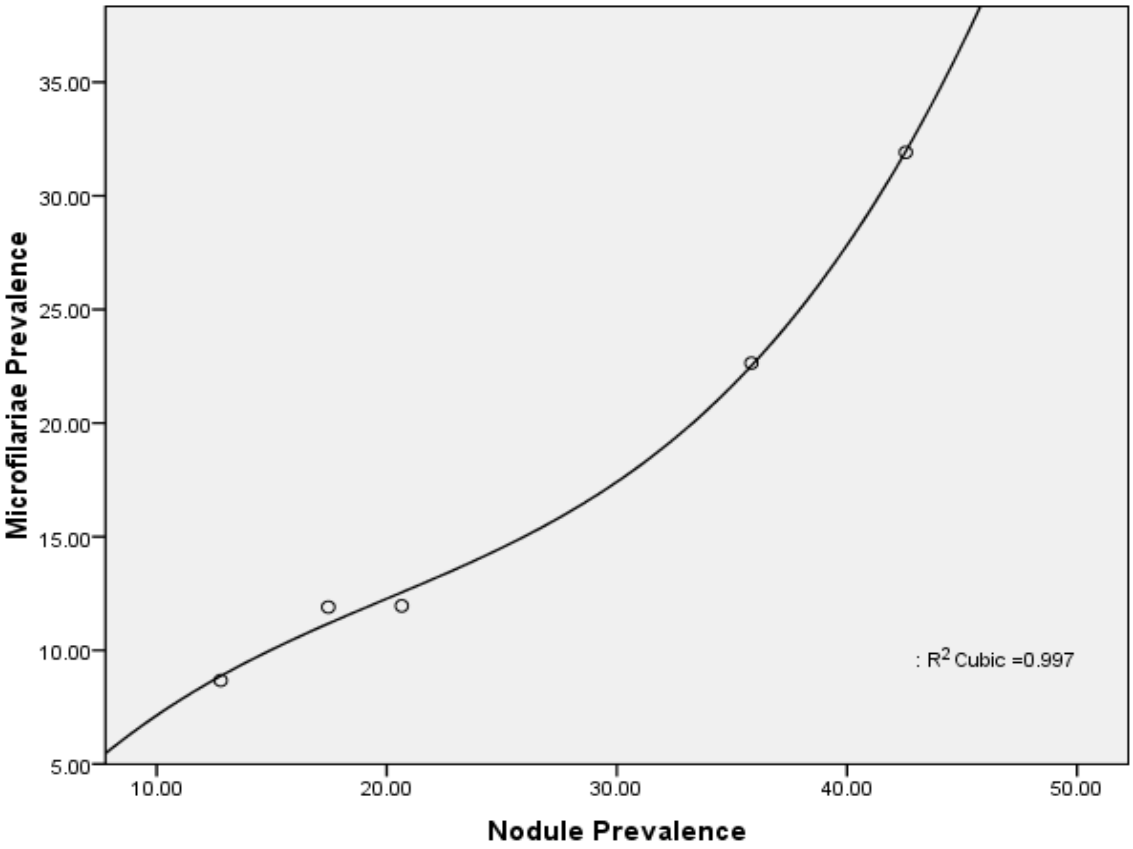

Figure 4.3: Microfilariae prevalence against nodule prevalence in the 5 communities with cubic line fit

Similar significant positive association was observed between: nodules and nodule sites in each of the five communities; day 180 post-treatment Mf and number of nodule sites in Chabon and Fawoman-Banda; and day 360 post-treatment Mf and number of nodules in Fawoman-Banda ($p<0.05$). In all the correlations, the cubic model (Figures 4.4-4.12 and Tables 4.8-4.25; the rest are shown in Appendix 2) demonstrated better fit than the quadratic model, and the quadratic model also demonstrated better fit than the linear model (R^2=0.431-1). Moreover, the differences in nodule and Mf prevalence at pre-treatment as well as the observed differences in Mf densities at the various treatment days were all significant ($p<0.05$). Similar pattern was also observed within each of the five communities (Tables 4.8-4.16).

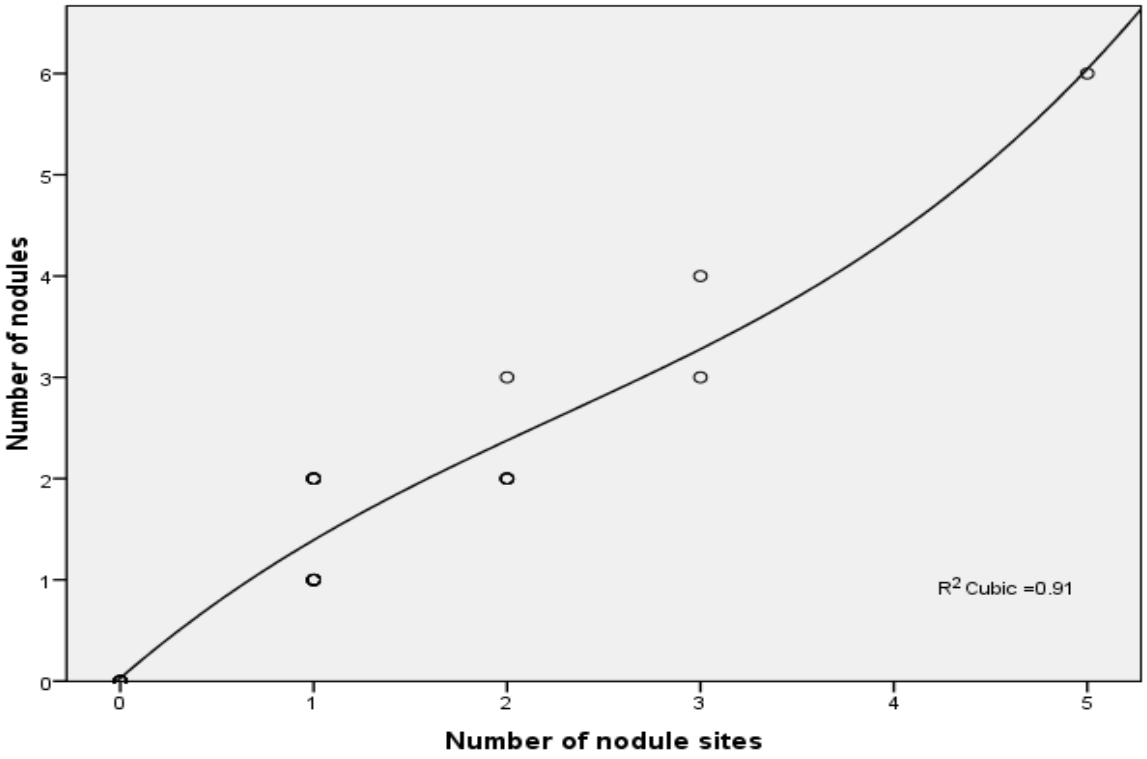

Figure 4.4: Correlation of number of nodules and nodule sites in Agblekeme II with cubic line plot

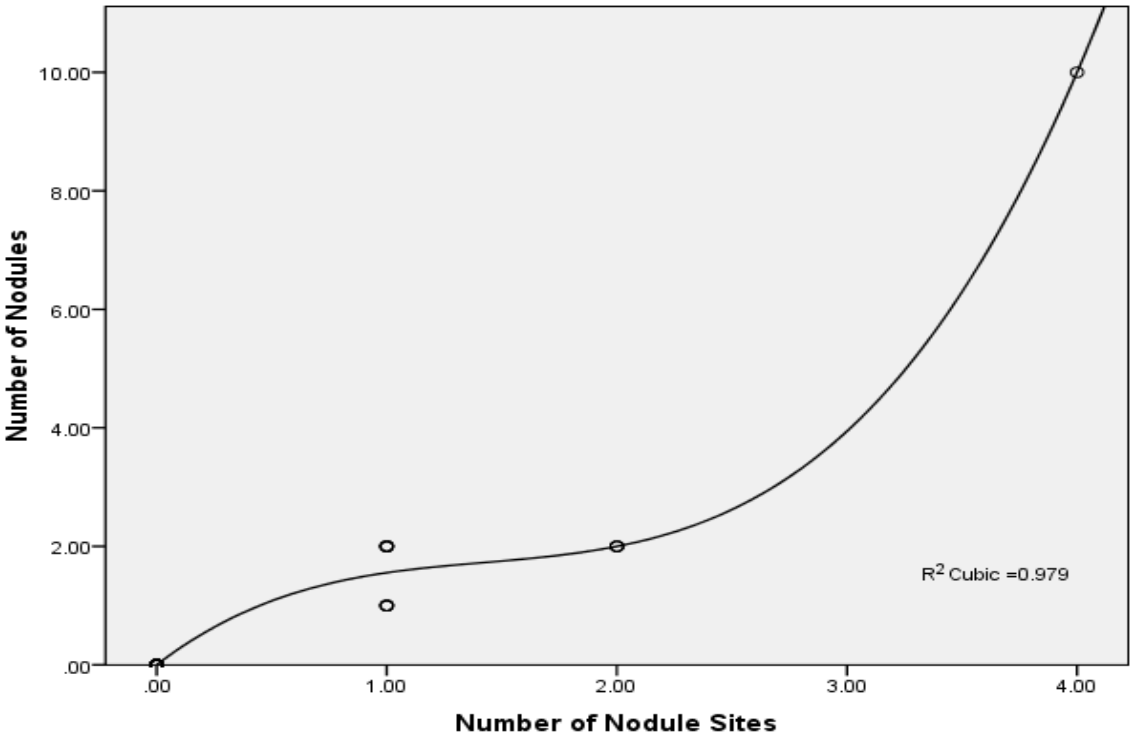

Figure 4.5: Correlation of number of nodules and nodule sites in Chabon with cubic line fit

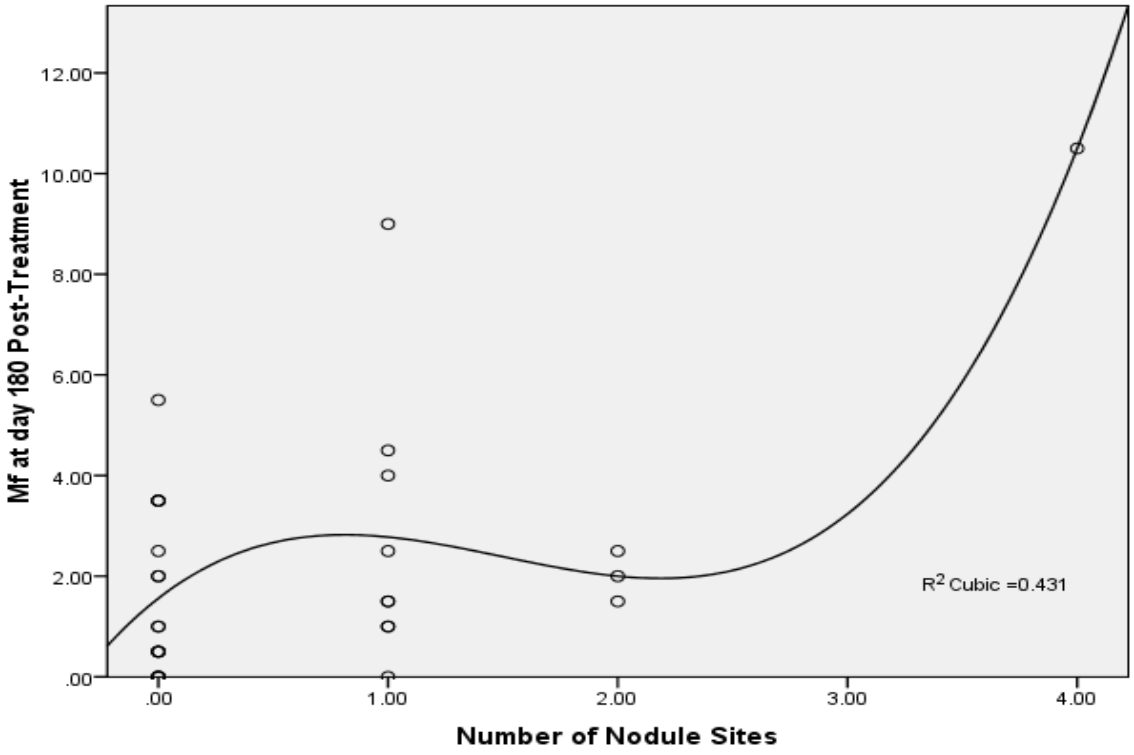

Figure 4.6: Correlation of Mf at day 180 post-treatment and number of nodule sites in Chabon with cubic line fit

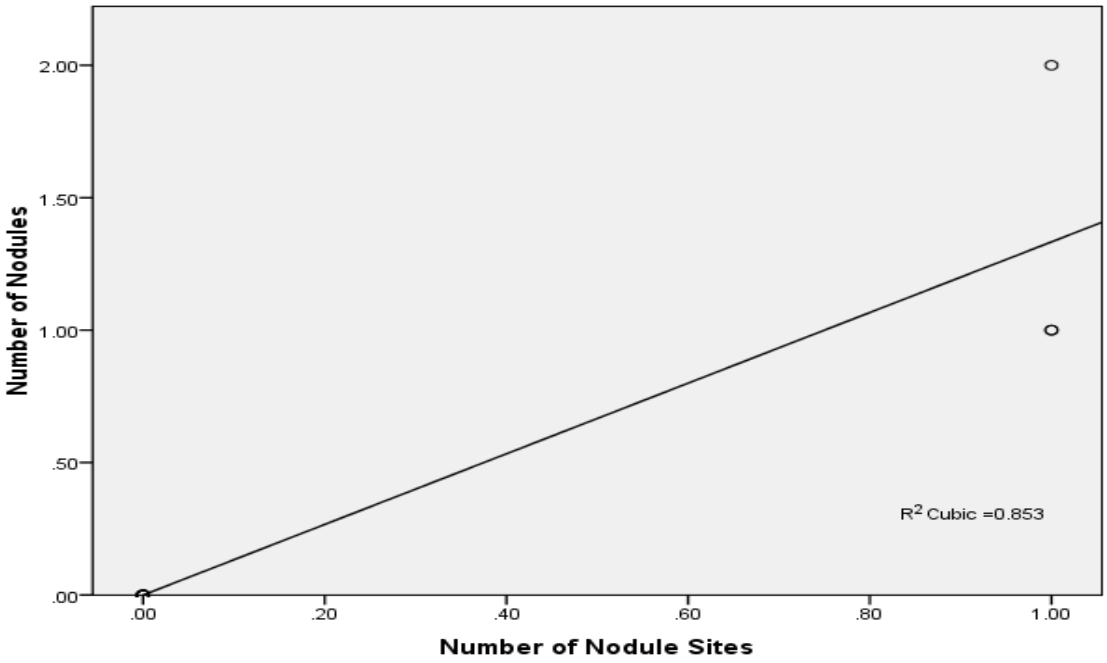

Figure 4.7: Correlation of number of nodules with nodule sites in Fawoman-Banda with cubic line fit

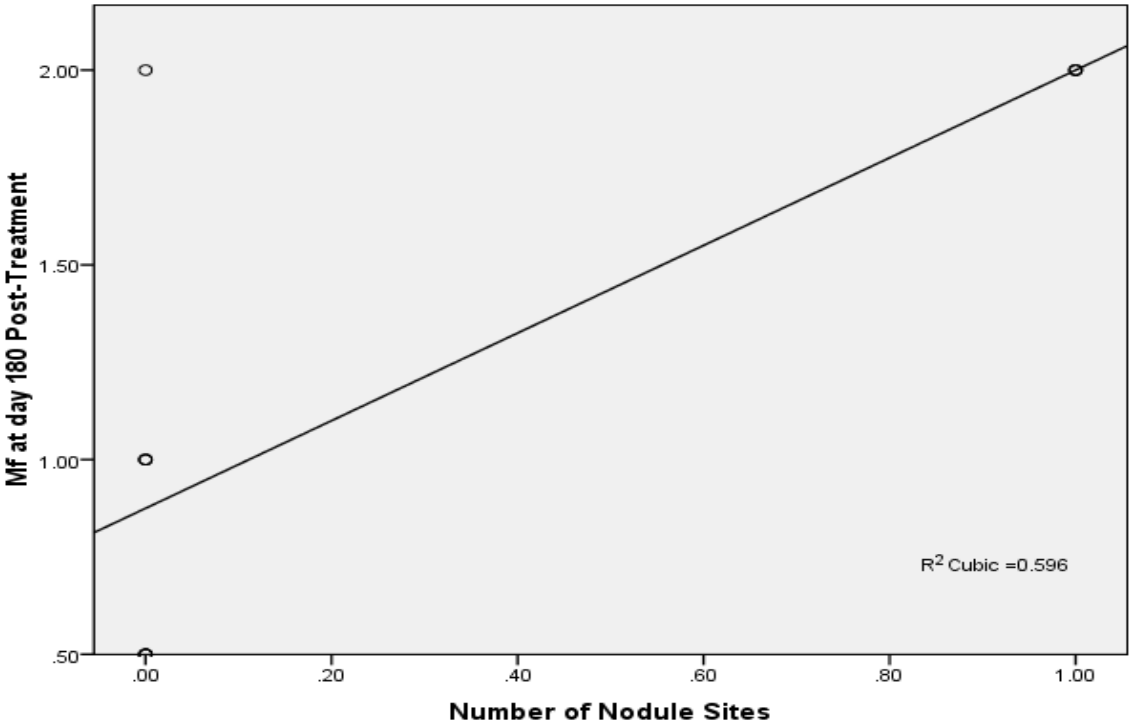

Figure 4.8: Correlation of number of Mf at day 180 post-treatment with number of nodule sites in Fawoman-Banda with cubic line fit

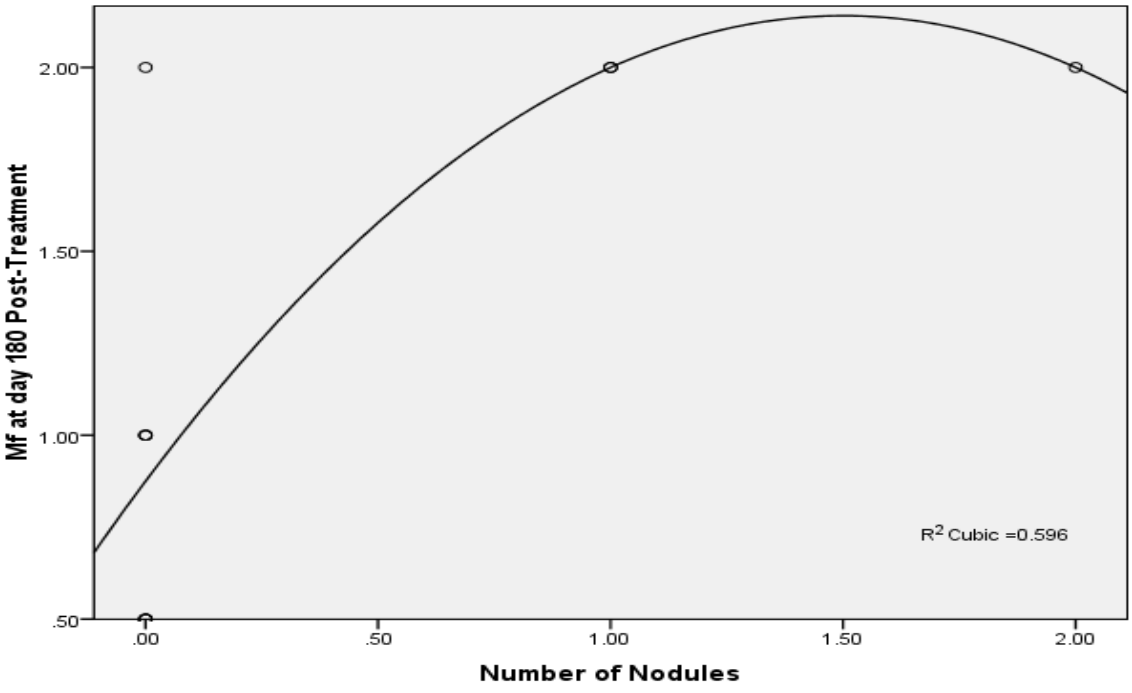

Figure 4.9: Correlation of number of Mf at day 180 post-treatment with number of nodules in Fawoman-Banda with cubic line fit

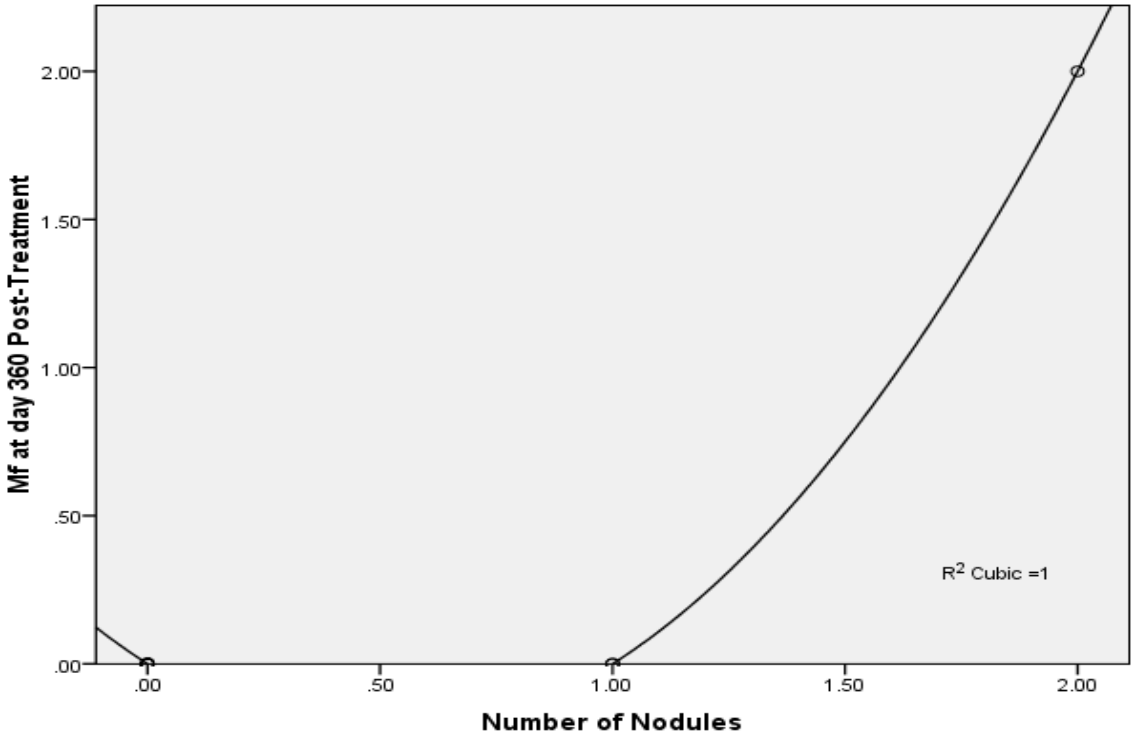

Figure 4.10: Correlation of number of Mf at day 360 post-treatment with number of nodules in Fawoman-Banda with cubic line fit

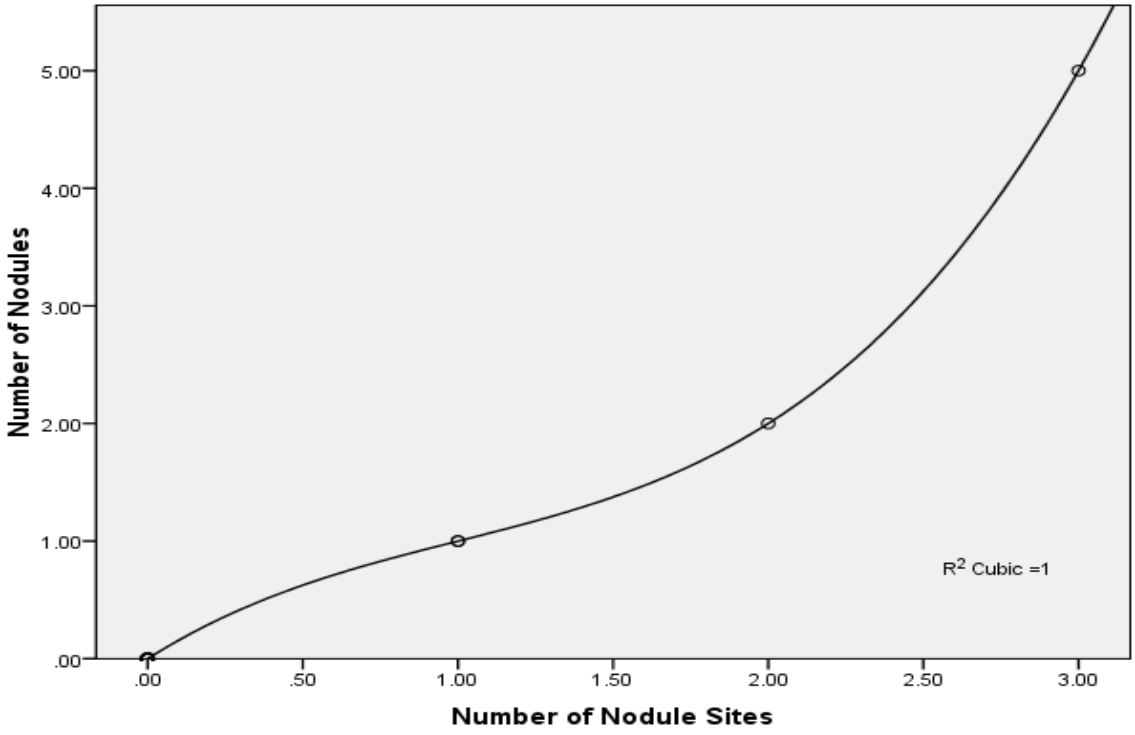

Figure 4.11: Correlation of number of nodules with nodule sites in Kojobone with cubic line fit

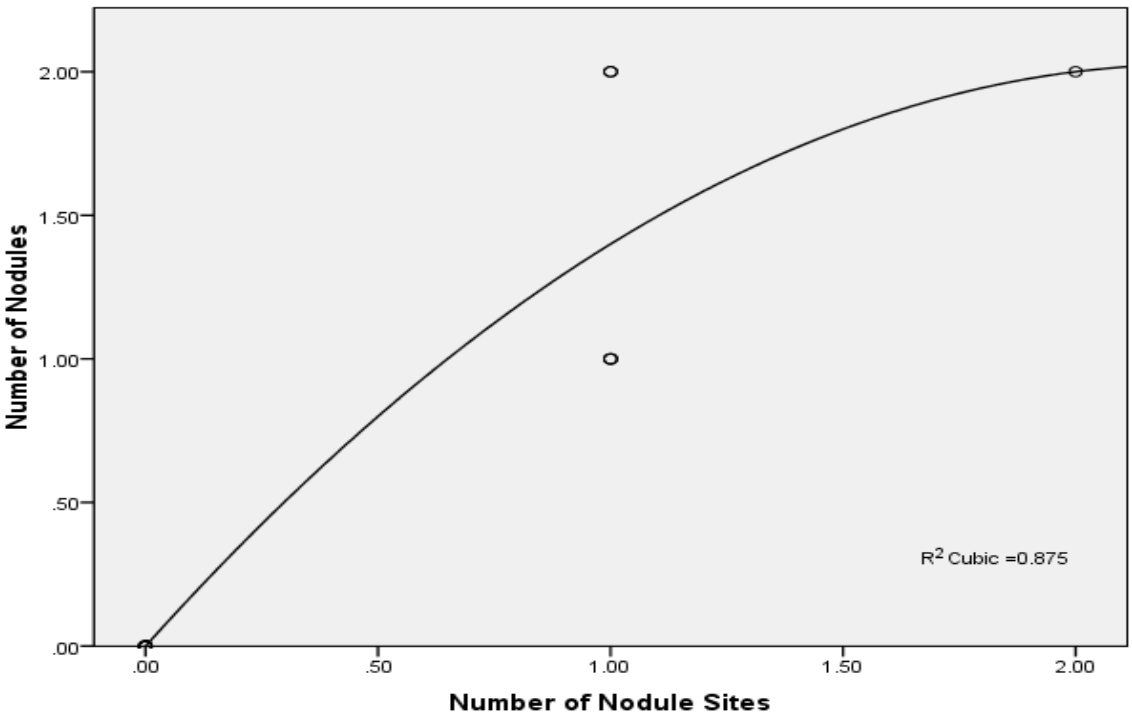

Figure 4.12: Correlation of number of nodules with nodule sites in Nyire with cubic line fit

Table 4.8: Descriptive Statistics of Mf at pre-treatment and at days 180-, 270- and 360 post-treatment in Chabon

Parameters	Mf at Pre-Treatment	Mf at day 180 Post-Treatment	Mf at day 270 Post-Treatment	Mf at day 360 Post-Treatment
Mean	5.77	2.27	0.08	0.60
Std. Error of Mean	1.53	0.46	0.05	0.20
Median	2.50	1.50	0.00	0.00
Range	39.00	10.50	1.50	4.00
Minimum	0.50	0.00	0.00	0.00
Maximum	39.50	10.50	1.50	4.00
Sum	173.00	68.00	2.50	18.00

Table 4.9: Descriptive Statistics of nodules and nodule sites in Chabon

	Mean	Std. Deviation	N
Number of Nodule Sites	0.63	0.93	30
Number of Nodules	1.00	1.91	30

Table 4.10: Wilcoxon Signed Ranks Test Statistics of Mf at pre-treatment and at days 180-, 270 and 360 post-treatment in Chabon

	Mf at day 180 Post-Treatment - Mf at Pre-Treatment	Mf at day 270 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at day 180 Post-Treatment
Z	-3.90[a]	-4.79[a]	-4.79[a]	-4.19[a]
Asymp. Sig. (2-tailed)	0.00	0.00	0.00	0.00

a. Based on positive ranks.

Table 4.11: Correlation of nodules, nodule sites and Mf at different treatment days in Chabon

Correlation between	**Pearson's R**	***p*-value**	**Spearman's rho R**	***p*-value**
No. Nodules & Nodule sites in Chabon	0.914	0.000	0.971	0.000
No. Nodule sites & Mf at pre-treatment in Chabon	0.546	0.002	0.223	0.236
No. Nodule sites & Mf at day 180 post-treatment in Chabon	0.532	0.002	0.353	0.056
No. Nodule sites & Mf at day 270 post-treatment in Chabon	0.178	0.347	0.011	0.954
No. Nodule sites & Mf at day 360 post-treatment Chabon	0.573	0.001	0.311	0.094
No. Nodules & Mf at pre-treatment in Chabon	0.705	0.000	0.216	0.251
No. Nodules & Mf at day 180 post-treatment in Chabon	0.607	0.000	0.322	0.083
No. Nodules & Mf at day 270 post-treatment in Chabon	0.030	0.873	-0.027	0.886
No. Nodules & Mf at day 360 post-treatment in Chabon	0.585	0.001	0.283	0.130

Table 4.12: Descriptive statistics of Mf at pre-treatment and at days 180-, 270 and 360 post-treatment in Fawoman-Banda

	Mf at Pre-Treatment	Mf at day 180 Post-Treatment	Mf at day 270 Post-Treatment	Mf at day 360 Post-Treatment
Mean	3.23	1.18	0.00	0.18
Std. Error of Mean	0.69	0.21	0.00	0.18
Median	2.50	1.00	0.00	0.00
Range	7.00	1.50	0.00	2.00
Minimum	0.50	0.50	0.00	0.00
Maximum	7.50	2.00	0.00	2.00
Sum	35.50	13.00	0.00	2.00

Table 4.13: Wilcoxon Signed Ranks Test statistics of Mf at pre-treatment and at days 180-, 270 and 360 post-treatment in Fawoman-Banda

	Mf at day 180 Post-Treatment - Mf at Pre-Treatment	Mf at day 270 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at day 180 Post-Treatment	Mf at day 360 Post-Treatment - Mf at day 270 Post-Treatment
Z	-2.812[a]	-2.946[a]	-2.943[a]	-2.836[a]	-1.000
Asymp. Sig. (2-tailed)	0.005	0.003	0.003	0.005	0.317

a. Based on positive ranks.

Table 4.14: Correlation of nodules, nodule sites and Mf at different treatment days in Fawoman

Correlation between	Pearson's R	*p*-value	Spearman's rho R	*p*-value
No. Nodules & Nodule sites in Fawoman-Banda	0.924	0.000	0.989	0.000
No. Nodule sites & Mf at pre-treatment in Fawoman-Banda	0.683	0.021	0.592	0.055
No. Nodule sites & Mf at day 180 post-treatment in Fawoman-Banda	0.772	0.005	0.718	0.013
No. Nodule sites & Mf at day 270 post-treatment in Fawoman-Banda	NA	NA	NA	NA
No. Nodule sites & Mf at day 360 post-treatment in Fawoman-Banda	0.516	0.104	0.516	0.104
No. Nodules & Mf at pre-treatment in Fawoman-Banda	0.426	0.192	0.532	0.092
No. Nodules & Mf at day 180 post-treatment in Fawoman-Banda	0.713	0.014	0.710	0.014
No. Nodules & Mf at day 270 post-treatment in Fawoman-Banda	NA	NA	NA	NA
No. Nodules & Mf at day 360 post-treatment in Fawoman-Banda	0.805	0.003	0.638	0.035

NA = Not Applicable. This is because all individual Mf loads were completely zero = Constant

Table 4.15: Descriptive Statistics of Mf at pre-treatment and at days 180-, 270- and 360 post-treatment in Kojobone

	Mf at Pre-Treatment	Mf at day 180 Post-Treatment	Mf at day 270 Post-Treatment	Mf at day 360 Post-Treatment
Mean	8.47	3.87	0.05	0.50
Std. Error of Mean	3.37	2.23	0.04	0.22
Median	3.00	1.00	0.00	0.00
Range	65.50	43.00	0.50	3.50
Minimum	0.50	0.50	0.00	0.00
Maximum	66.00	43.50	0.50	3.50
Sum	161.00	73.50	1.00	9.50

Table 4.16: Descriptive Statistics of number of nodules and nodule sites in Kojobone

	Mean	Std. Deviation	N
Number of Nodule Sites	0.37	0.83	19
Number of Nodules	0.47	1.22	19

Table 4.17: Wilcoxon Signed Ranks Test Statistics of Mf at pre-treatment and at days 180-, 270- and 360 post-treatment in Kojobone

	Mf at day 180 Post-Treatment - Mf at Pre-Treatment	Mf at day 270 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at day 180 Post-Treatment	Mf at day 360 Post-Treatment - Mf at day 270 Post-Treatment
Z	-3.662[a]	-3.825[a]	-3.725[a]	-3.551[a]	-2.410[b]
Asymp. Sig. (2-tailed)	.000	.000	.000	.000	.016

a. Based on positive ranks.
b. Based on negative ranks.

Table 4.18: Correlation of nodules, nodule sites and Mf at different treatment days in Kojobone

Correlation between	**Pearson's R**	***p*-value**	**Spearman's rho R**	***p*-value**
No. Nodules & Nodule sites in Kojobone	0.970	0.000	1.000	NA
No. Nodule sites & Mf at pre-treatment in Kojobone	-0.172	0.481	-0.147	0.549
No. Nodule sites & Mf at day 180 post-treatment in Kojobone	-0.145	0.553	-0.256	0.290
No. Nodule sites & Mf at day 270 post-treatment in Kojobone	0.056	0.820	0.198	0.417
No. Nodule sites & Mf at day 360 post-treatment in Kojobone	0.000	1.000	0.312	0.193
No. Nodules & Mf at pre-treatment in Kojobone	-0.157	0.520	-0.147	0.549
No. Nodules & Mf at day 180 post-treatment in Kojobone	-0.131	0.594	-0.256	0.290
No. Nodules & Mf at day 270 post-treatment in Kojobone	0.008	0.975	0.198	0.417
No. Nodules & Mf at day 360 post-treatment in Kojobone	0.000	1.000	0.312	0.193

Table 4.19: Descriptive Statistics of Mf at pre-treatment and at days 180-, 270- and 360 post-treatment in Nyire

	Mf at Pre-Treatment	Mf at day 180 Post-Treatment	Mf at day 270 Post-Treatment	Mf at day 360 Post-Treatment
Mean	1.93	0.80	0.07	0.10
Std. Error of Mean	0.52	0.19	0.07	0.05
Median	0.50	0.50	0.00	0.00
Range	5.50	2.50	1.00	0.50
Minimum	0.50	0.00	0.00	0.00
Maximum	6.00	2.50	1.00	0.50
Sum	29.00	12.00	1.00	1.50

Table 4.20: Wilcoxon Signed Ranks Test Statistics of Mf at pre-treatment and at days 180-, 270- and 360 post-treatment in Nyire

	Mf at day 180 Post-Treatment - Mf at Pre-Treatment	Mf at day 270 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at day 180 Post-Treatment
Z	-2.69[a]	-3.47[a]	-3.24[a]	-2.67[a]
Asymp. Sig. (2-tailed)	0.01	0.00	0.00	0.01

a. Based on positive ranks.

Table 4.21: Correlation of nodules, nodule sites and Mf at different treatment days in Nyire

Correlation between	**Pearson's R**	***p*-value**	**Spearman's rho R**	***p*-value**
No. Nodules & Nodule sites in Nyire	0.917	0.000		
No. Nodule sites & Mf at pre-treatment in Nyire	-0.002	0.995	-0.130	0.645
No. Nodule sites & Mf at day 180 post-treatment in Nyire	-0.015	0.957	-0.055	0.846
No. Nodule sites & Mf at day 270 post-treatment in Nyire	-0.202	0.471	-0.214	0.443
No. Nodule sites & Mf at day 360 post-treatment in Nyire	-0.108	0.702	-0.089	0.752
No. Nodules & Mf at pre-treatment in Nyire	-0.124	0.661	-0.192	0.493
No. Nodules & Mf at day 180 post-treatment in Nyire	-0.202	0.471	-0.175	0.532
No. Nodules & Mf at day 270 post-treatment in Nyire	-0.200	0.474	-0.211	0.450
No. Nodules & Mf at day 360 post-treatment in Nyirre	0.042	0.883	0.000	1.000

Table 4.22: Descriptive statistics of Mf at pre-treatment and at days 180-, 270 and 360 post-treatment in Agblekeme II

	Mf at Pre-Treatment	Mf at day 180 Post-Treatment	Mf at day 270 Post-Treatment	Mf at day 360 Post-Treatment
Mean	7.75	2.96	0.08	0.71
Std. Error of Mean	3.67	0.98	0.08	0.43
Median	2.25	1.75	0.00	0.00
Range	44.00	11.00	1.00	5.00
Minimum	0.50	0.50	0.00	0.00
Maximum	44.50	11.50	1.00	5.00
Sum	93.00	35.50	1.00	8.50

Table 4.23: Descriptive statistics of number of nodules and nodule sites in Agblekeme II

	Mean	Std. Deviation	N
Number of Nodule Sites	1.17	1.59	12
Number of Nodules	1.42	1.93	12

Table 4.24: Wilcoxon Signed Ranks Test Statistics of Mf at pre-treatment and at days 180-, 270 and 360 post-treatment in Agblekeme II

	Mf at day 180 Post-Treatment - Mf at Pre-Treatment	Mf at day 270 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at Pre-Treatment	Mf at day 360 Post-Treatment - Mf at day 180 Post-Treatment
Z	-2.61[a]	-3.06[a]	-3.07[a]	-2.81[a]
Asymp. Sig. (2-tailed)	0.01	0.00	0.00	0.01

a. Based on positive ranks.

Table 4.25: Correlation of nodules, nodule sites and Mf at different treatment days in Agblekeme II

Correlation between	Pearson's R	*p*-value	Spearman's rho R	*p*-value
No. Nodules & Nodule sites in Agblekeme II	0.986	0.000	0.988	0.000
No. Nodule sites & Mf at Pre-treatment in Agblekeme II	-0.259	0.416	-0.219	0.494
No. Nodule sites & Mf at day 180 post-treatment in Agblekeme II	-0.261	0.413	-0.083	0.797
No. Nodule sites & Mf at day 270 post-treatment in Agblekeme II	0.165	0.607	0.281	0.377
No. Nodule sites & Mf at day 360 post-treatment	-0.035	0.913	0.123	0.704
No. Nodules and Mf at Pre-treatment in Agblekeme II	-0.251	0.431	-0.227	0.477
No. Nodules & Mf at day 180 post-treatment in Agblekeme II	-0.248	0.438	-0.053	0.869
No. Nodules and Mf at day 270 post –treatment in Agblekeme II	0.095	0.768	0.235	0.462
No. Nodules and Mf at day 360 post-treatment in Agblekeme II	-0.065	0.841	0.094	0.771

An assessment of the relationship between worm load, Mf load and number of nodules indicated a moderate positive correlation of number of female worms with day 270 post-treatment Mf (R=0.477, p=0.009), and similar significant positive association realized in Total number of male & female worms with day 270 post-treatment worms (R=0.397, p=0.033). However, there was no significant correlation between the worm loads, Mf loads and number of nodules in the other treatment periods (Tables 4.26 - 4.28; Figures 4.13 and 4.14)

Table 4.26: Assessment of relationship between worm load and Mf load

Correlation between	**Pearson's R**	***p*-value**
Total Number of Male & Female Worms and Mf at Pre-Treatment	-0.039	0.843
Total Number of Male & Female Worms and Mf at day 180 Post-Treatment	-0.081	0.675
Total Number of Male & Female Worms and Mf at day 270 Post-Treatment	0.397	0.033

Table 4.27: Assessment of relationship between female worms load and Mf load

Correlation between	**Pearson's R**	***p*-value**
Number of Female Worms and Mf at Pre-Treatment	-0.146	0.450
Number of Female Worms and Mf at day 180 Post-Treatment	-0.102	0.598
Number of Female Worms and Mf at day 270 Post-Treatment	0.477	0.009

Table 4.28: Assessment of relationship between male worms load and Mf load

Correlation between	**Pearson's R**	***p*-value**
Number of Male Worms and Mf at Pre-Treatment	0.197	0.304
Number of Male Worms and Mf at day 180 Post-Treatment	0.000	0.996
Number of Male Worms and Mf at day 270 Post-Treatment	0.049	0.799

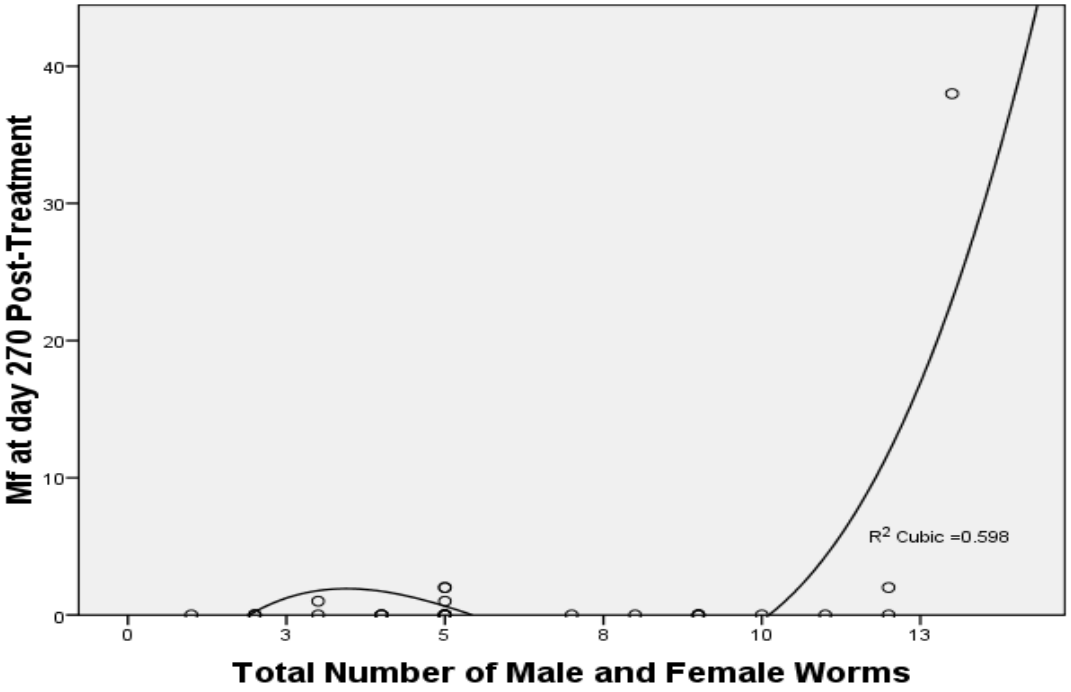

Figure 4.13: Correlation of Total number of male and female worms with Mf at Day 270 post-treatment

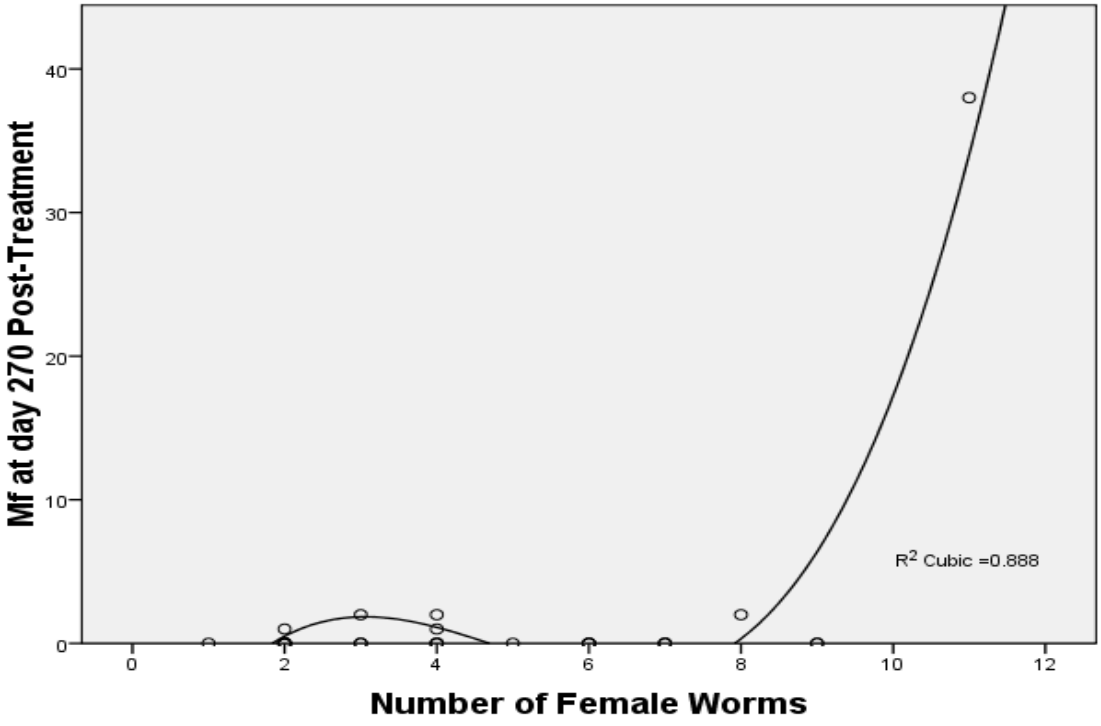

Figure 4.14: Correlation of number of female worms and Mf at day 270 Post-Treatment

Out of the 337 worms extracted from the 135 nodules (Plate 1), 247 (73.29%) were females whiles 90 (26.71%) were males (Plate 2), representing an average of 6.18 female worms and 2.25 male worms (Tables 4.29 and 4.30) and an average male to female ratio of approximately 1:3 when assessing total load per person but this value drops to 1:2 male to female ratio per nodule. This can be attributed to the fact that an average of one male becomes mobile in search of available female worms to mate with.

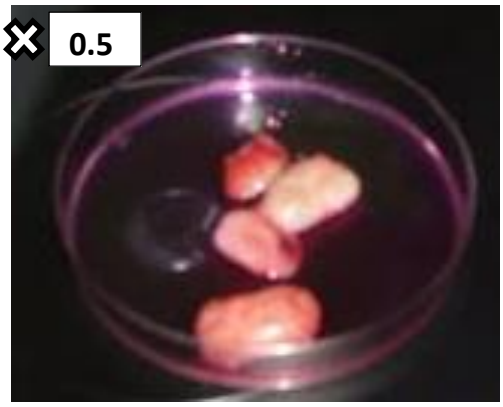

Plate 1: Nodules taken from *O. volvulus* infected subject

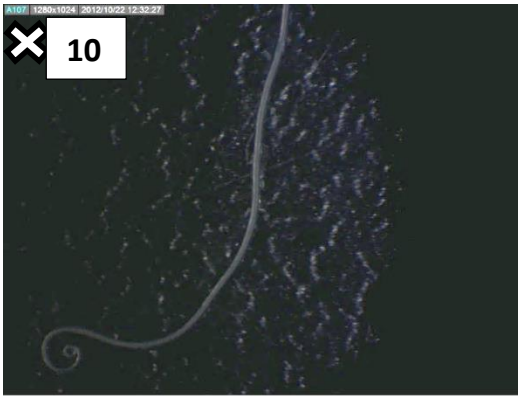

Plate 2: *O. volvulus* adult Male worm with coiled posterior end

Table 4.29: Descriptive Statistics of worms in nodules (alone or with opposite sex worm)

	Number of Female Worms	Number of Male Worms
Mean	6.18	2.25
Std. Error of Mean	0.36	.202
Median	6.00	2.50
Range	7	4
Minimum	2	0
Maximum	9	4
Sum	247	90

Table 4.30: Descriptive Statistics of worms in nodules (both male & female worms)

	Male Worms in Nodule	Female Worms in Nodule
Mean	1.05	2.45
Std. Error of Mean	.160	.251
Median	1.00	2.00
Range	3	5
Minimum	0	1
Maximum	3	6
Sum	42	98

A total of 285 worms were assessed for embryogram in Agblekeme II (45), Chabon (74), Jagbengbendo (77), Kojobone (27), Nyire (19) and Takumdo (43) (Figure 4.15 and Table 4.31; the rest in Appendix 1).

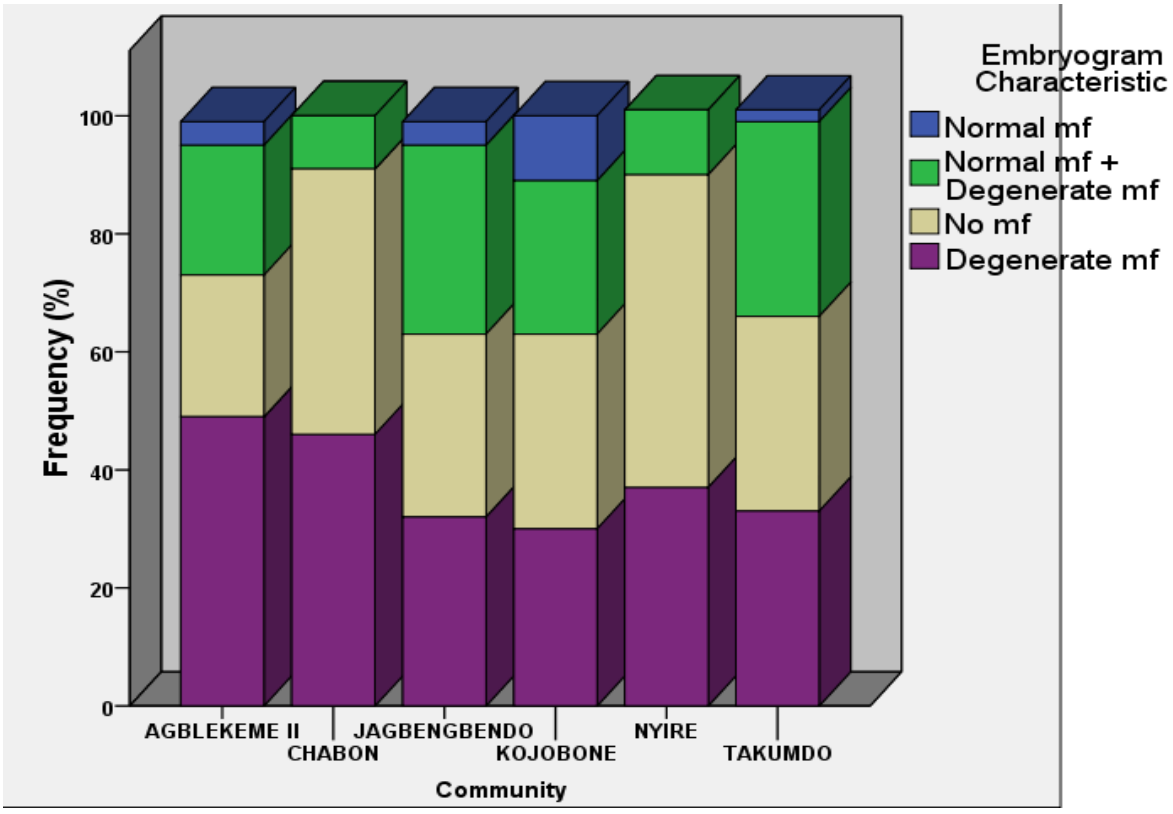

Figure 4.15: Embryogram characteristic for the six study communities

Table 4.31: Embryogram characteristic of the 285 worms in the six communities

Community	Frequency	Percent		Age	Frequency	Percent
AGBLEKEME II	45	15.80		M	144	50.50
CHABON	74	26.00				
JAGBENGBENDO	77	27.00		M+	38	13.30
KOJOBONE	27	9.50				
NYIRE	19	6.70		OLD	80	28.10
TAKUMDO	43	15.10		Y	23	8.10
TOTAL	285	100.00		TOTAL	285	100.00

In all of the 285 worms analysed from the six communities, 8.1% (23) were young, 50.5% (144) were middle aged, 13.3% (38) were matured than the middle aged but relatively younger than the old aged ones, and 28.1% (80) were of the old worm category (Table 4.31; the rest in appendix 1). Some of the worms associated with the poor response phenotype had lots of normal Mf (Plate 3) and a significant proportion of them were observed from all the embryonic stages (Plate 4). Of the total, 35.4% (101) had no Mf, 3.2% (9) had normal Mf (Plate 5), 22.8% (65) of the worms had both normal and degenerate Mf, and 38.6% (110) had Mf that were all degenerate (Plate 6).

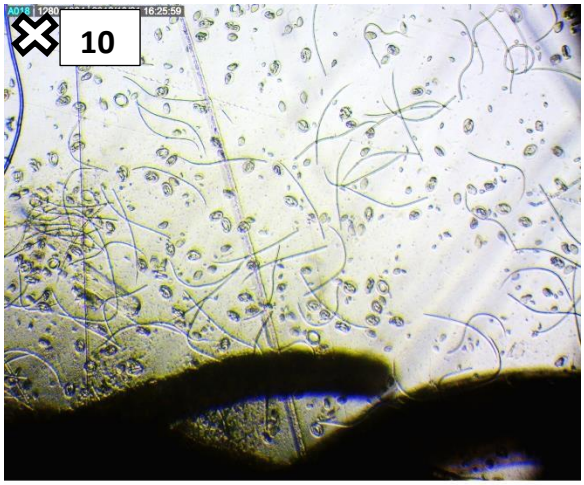

Plate 3: Microfilariae and embryonic stages of *O. volvulus* from a sub-optimal responding worm

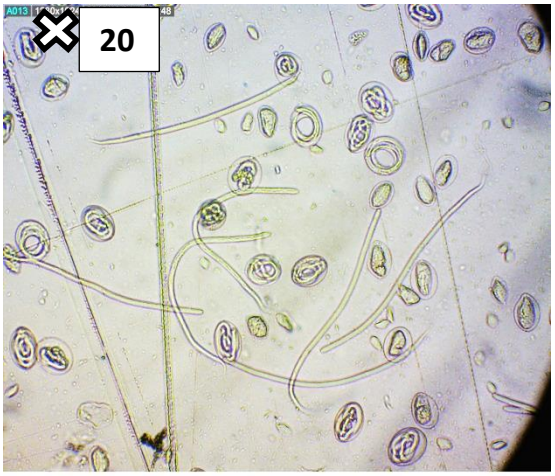

Plate 4: Stretched and coiled microfilariae with other embryonic stages of *O. volvulus*

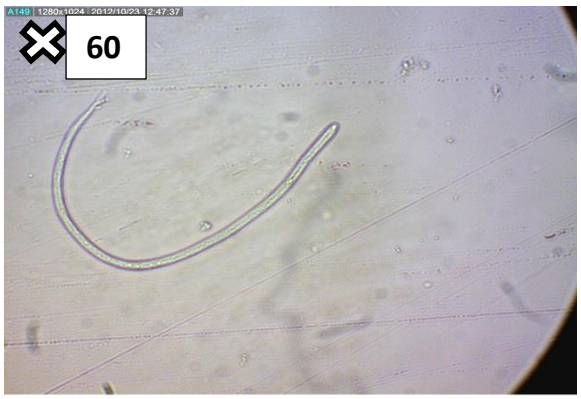

Plate 5: Normal Mf from a sub-optimal responding *O. volvulus* worm

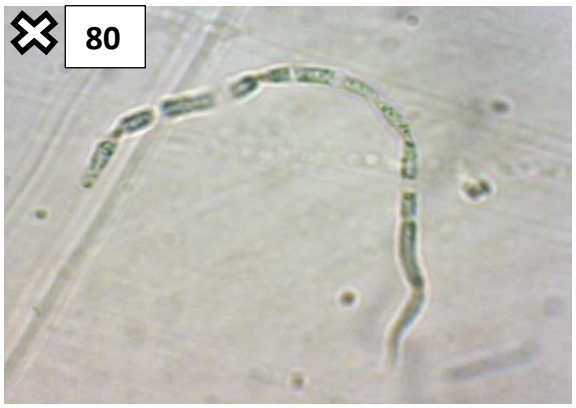

Plate 6: Degenerate Mf from a good responding *O. volvulus* worm

Generally, a comparison of the worms assessed for embryogram indicated that Chabon and Nyire showed better response to IVM treatment with no normal Mf present, higher proportion of degenerate Mf (46% and 37% respectively), lower proportion of those with combination of both normal and degenerate Mf (9% and 11% respectively), and higher percentage of those with no embryonic Mf present (45% and 53% respectively). The other communities had relatively higher proportion of normal Mf (2-11%), moderate to high proportion of degenerate Mf (30-49%), higher proportion of those with combination of both normal and degenerate Mf (22-33%) and moderate proportion of those with no embryonic Mf present (24-33%) (Figure 4.15).

A total of 59 adult female worms were selected from Jagbengbendo and Takumdo for PCR amplification and DNA sequencing. The DNA sequence region of 1012-169bp (684bp) of the full length genomic DNA sequence (3696bp) of beta tubulin gene of *O. volvulus* was successfully amplified (Plate 7) and sequenced. The optimization of reagents indicated that the higher the concentration of the 100bp DNA ladder, the more prominent and clearer the marker band became (Plate 7). However, the highest optimization concentration of 1.4 µg/µl (M1, Plate 7) was too much creating very prominent band and might be considered a waste of reagent. The moderate optimization concentration of 0.7 µg/µl (M2, Plate 7) was low and

created faint bands on the 1% agarose gel whiles the lowest optimization concentration of 0.4 µg/µl (M3, Plate 7) was too low that it could hardly be seen at all.

The 1 ng/µl DNA concentrations of samples used gave both good and poor amplification bands but the 20 ng/µl DNA sample (S2, Plate 7) gave band that was too prominent and can be considered waste of reagent in repeated works. The 0.5 ng/µl DNA sample did not produce observable amplification band on the 1% agarose gel and can be considered too low to be used for repeated works (S1, Plate 7). Nevertheless, the 10 ng/µl DNA sample gave consistently good amplification bands and can be considered good concentration to be used for future repeated works on the 1% agarose gel. The gel showed no amplification band for the negative template control indicating no contamination problem of the various samples (N2, Plate 7).

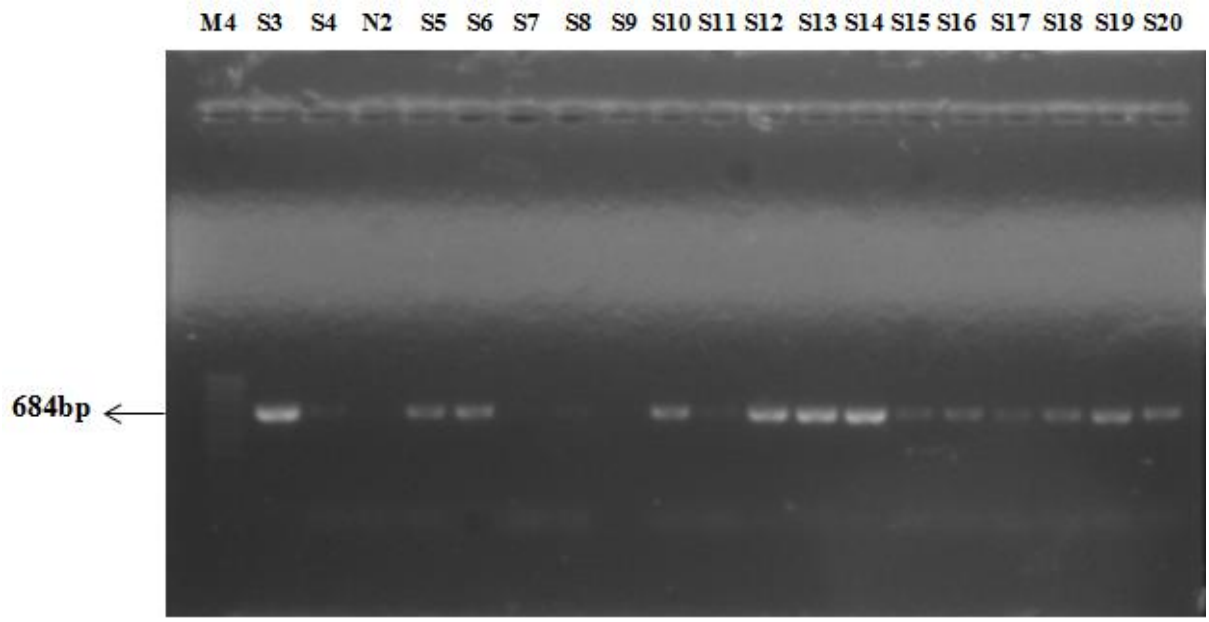

Plate 7: Agarose gel showing DNA bands of Beta tubulin gene amplicons

Key:

M4: 100bp DNA marker

S3, S4, S5, S6, S8, S10-S20: DNA samples with amplified gel bands

N2: Negative template control sample of gel

S7 and S9: DNA samples with no positive amplification gel bands

The sequence of nucleotides and chromatograms (Figure 4.16**)** were analysed by first looking at their quality. Of the 59 worms that were amplified and sequenced, 19 (32.2%) were of poor quality sequences, six (10.2%) were of good quality sequences but had confounding effects whiles the remaining 34 (57.6%) worms produced good quality sequences without any confounding effects (Table 4.32).

Table 4.32: Frequency of sequenced samples (of 79 fragments)

Sequenced Samples Characteristics	Frequency
Samples with Poor Sequences	19
Samples with Good Sequences (with Confounding effects)	6
Samples with Good Sequences (without Confounding effects)	34
Total	59

From all the samples sequenced and analysed, there were seven SNPs occurring at significant frequencies and some of them (Figure 4.16) were associated with IVM selection. The genomic DNA sequence positions where SNPs occurred are positions: 1268 (T to A), 1269 (A to T) and 1270 (T to A) 1183 (T to G), 1188 (T to C), 1308 (C to T) and 1545 (A to G). The chromatogram of Figure 4.16 shows all the expected sequences including the homozygote wild type sequence, the heterozygote sample sequence and the mutated homozygote sample sequence. The SNP at position 1545 occurs in an exon with the remaining six SNPs occurring in introns. The SNP at position 1545 caused a coding change from AAG to GAG but it did not translate into a functional amino acid.

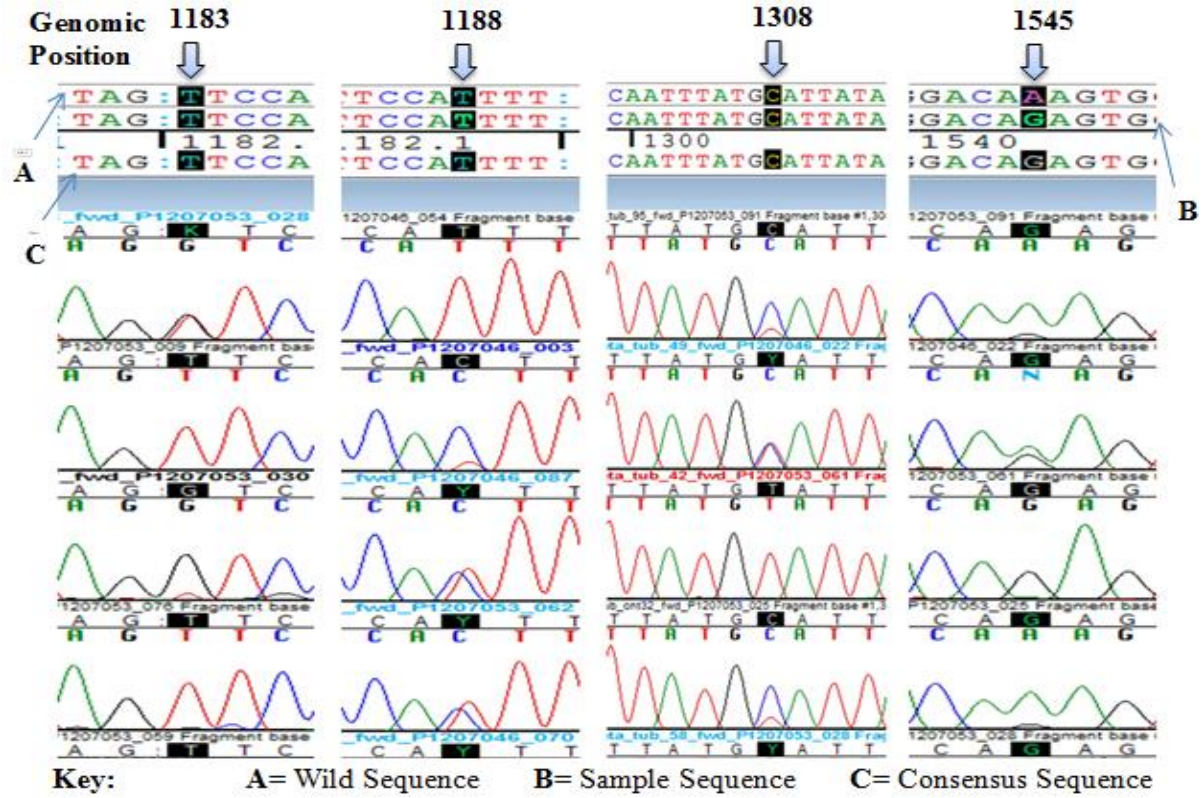

Figure 4.16: Chromatogram of sequenced samples showing SNP's associated with IVM selection

Out of the 34 worms, anterior and posterior sections, (Plates 8-10) used for the genotypic assessment in Jagbengbendo and Takumdo, approximately 62% (21) were of the poor response phenotype and can be said to be responding sub-optimally to IVM (Table 4.33). The embryos could clearly be seen by examining the adult female worm (Plate 11).

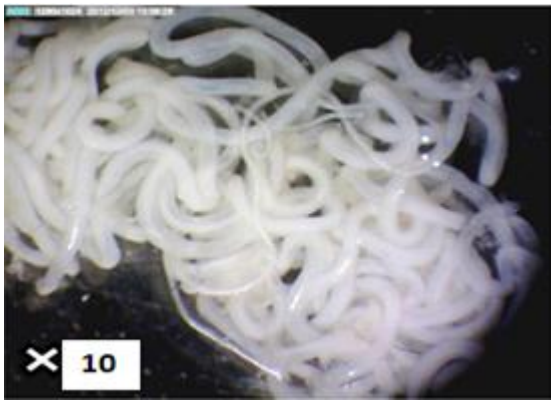

Plate 8: *O. volvulus* adult female worm freshly taken out of nodule

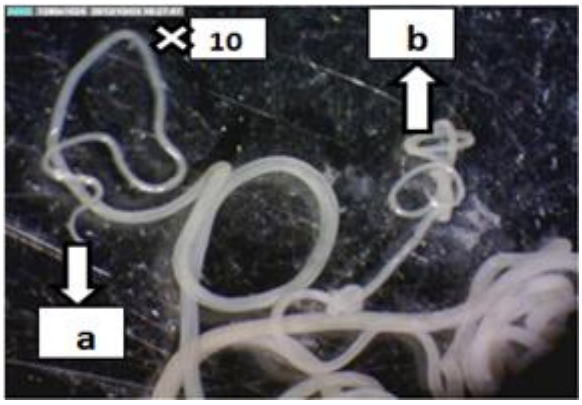

Plate 9: *O. volvulus* adult female worm stretched to show the (a) anterior and (b) posterior ends

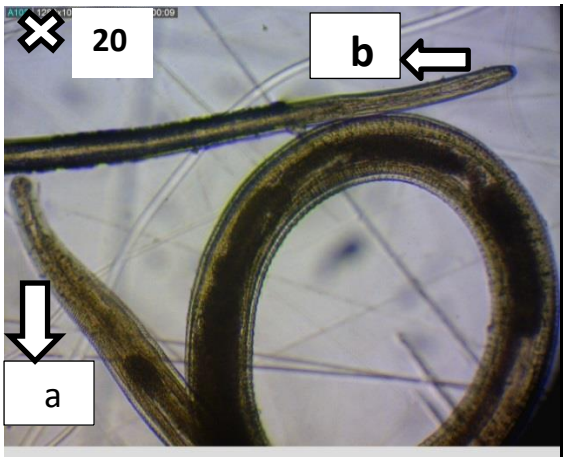

Plate 10: *O. volvulus* adult Female worm showing the (a) anterior and (b) posterior end at a higher magnification

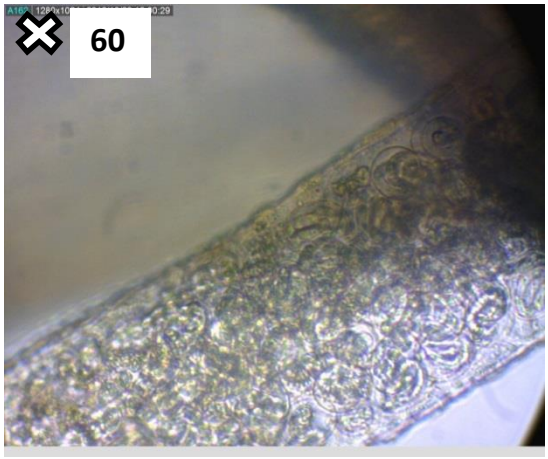

Plate 11: *O. volvulus* adult female worm showing intra-uterine embryos

Table 4.33: Worm response to IVM treatment by age category: used for molecular study (Jagbengbendo and Takumdo)

Worm Item	Number (%)
Number of worms responding sub-optimally to IVM	19 (55.9)
Total number of worms in study	34 (100)
Number of worms that are older or of middle age	32 (94.1)
Total number of worms in study	34 (100)

Categorization of the age of female worms was based among others on body structure, size, colour, cuticular ridges and the extent of inclusions (as detailed in the literature section). Following worm embryogram, the classification demonstrated that 94.1% of worms belonged to the older or middle age category (Plate 12 (a) and (b) respectively) and the majority of the Mf were produced by these matured worm age category previously exposed to multiple treatments with little contribution received from young worms (Plate 13) derived from the on-going transmission.

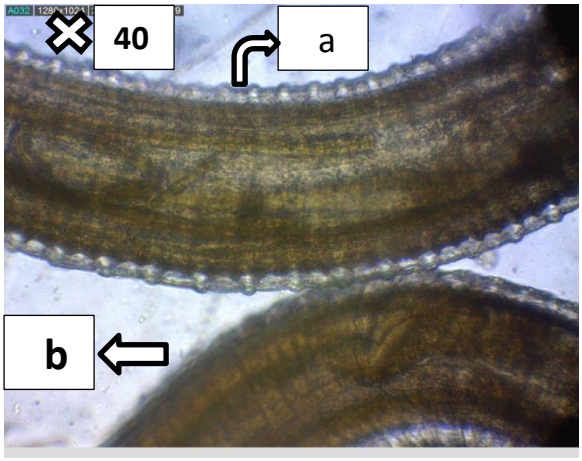

Plate 12: *O. volvulus* adult female worms that are (a) old aged and (b) middle aged

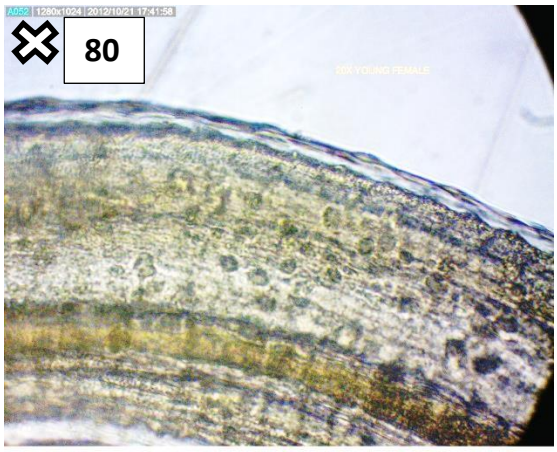

Plate 13: Young *O. volvulus* adult female worm

Table 4.34 shows the correlation test of total number of worms (male and female), worm phenotypes and worm genotypes at SNP positions 1183, 1188, 1308 and 1545. These showed positive significant (Pearson's) correlation between: total number of male and female worms (R = 0.317, p = 0.046); and worm phenotype with SNP genotype at position 1308 (R= 371, p=0.031) and 1545 (R = 0.384, p = 0.025). In addition, there was significant negative (Pearson's) correlation between worm phenotype and SNP genotype at position 1188 (R = -0.384, p = 0.025). There was no significant correlation between: number of male and female worms in the same nodules (R = 0.178, p = 0.273); and between worm phenotype and SNP genotype at position 1183 (R = -0.108, p = 0.542).

Table 4.34: Correlation of worms, worm phenotypes and SNP genotypes at different positions (1183, 1188, 1308 and 1545)

Correlation between	**Pearson's R**	***p*-value**	**Spearman's rho R**	***p*-value**
Total No. Male & Female worms	0.317	0.046	0.373	0.018
No. Male & Female worms in same nodules	0.178	0.273	0.225	0.164
Worm phenotype & SNP genotype at position 1183	-0.108	0.542	-0.087	0.626
Worm phenotype & SNP genotype at position 1188	-0.384	0.025	-0.444	0.009
Worm phenotype & SNP genotype at position 1308	0.371	0.031	0.442	0.009
Worm phenotype & SNP genotype at position 1545	0.384	0.025	0.444	0.009
Worm phenotype & SNP genotype sequence at position 1183, 1188, 1308 and 1545	0.273	0.119	0.354	0.040
Worm phenotype & SNP genotype sequence at position 1188, 1308 and 1545	0.370	0.031	0.423	0.013

Table 4.35 shows that five distinct SNP genotypes were identified and these are: TcttAg, TcCtAg, ccttgg, TcCtAg and TTCCAA.

Table 4.35: Composite Crosstabulation of worm phenotype status with SNP genotype (SNP positions 1188, 1308 & 1545)

		SNP Genotype Sequence					
		TcttAg	*TcCtAg	ccttgg	*TcCtAg	TTCCAA	Total
Worm	Poorer	0	1	0	1	0	2
Phenotype	Poor	0	0	1	0	1	2
Status	Moderate	1	3	1	11	2	18
	Good	0	2	0	3	7	12
Total		1	6	2	15	10	34
Percentage (%)		2.94	17.65	5.88	44.12	29.41	100

*= These genotypes appear to be the same but are distinctly different when the genotypes at SNP position 1183 are added

Tables 4.36-4.39 indicate the individual crosstabulation of worm phenotype with SNP genotype at positions 1188, 1308 and 1545.

Table 4.36: Crosstabulation of worm phenotype with SNP genotype at position 1183

		SNP Genotype at position 1183			
		gg	Tg	TT	Total
Worm Phenotype	Poorer	0	1	1	2
Status	Poor	0	0	2	2
	Moderate	1	3	14	18
	Good	0	2	10	12
Total		1	6	27	34
Percentage (%)		2.94	17.65	79.41	100

Table 4.37: Crosstabulation of worm phenotype with SNP Genotype at position 1188

		SNP Genotype at position 1188			
		cc	Tc	TT	Total
Worm Phenotype	Poorer	0	2	0	2
Status	Poor	1	0	1	2
	Moderate	1	15	2	18
	Good	0	5	7	12
Total		2	22	10	34
Percentage (%)		5.88	64.71	29.41	100

Table 4.38: Crosstabulation of worm phenotype with SNP genotype at position 1308

		SNP Genotype at position 1308			
		CC	Ct	tt	Total
Worm Phenotype	Poorer	0	2	0	2
Status	Poor	1	0	1	2
	Moderate	2	14	2	18
	Good	7	5	0	12
Total		10	21	3	34
Percentage (%)		29.41	61.76	8.82	100

Table 4.39: Crosstabulation of worm phenotype with SNP genotype at position 1545

		SNP Genotype at position 1545			
		AA	Ag	gg	Total
Worm Phenotype	Poorer	0	2	0	2
Status	Poor	1	0	1	2
	Moderate	2	15	1	18
	Good	7	5	0	12
Total		10	22	2	34
Percentage (%)		29.41	64.71	5.88	100

For all the three SNP positions, the proportion of genotyped worms associated with the Good, Moderate, Poor and Poorer response phenotypes were 35.3% (12), 52.9% (18), 5.9% (2) and 5.9% (2) respectively. The homozygote dominant and heterozygote genotypes were

dominated by good (0-7 frequency) and moderate (1-11) response phenotypes whiles the homozygote recessive genotype was dominated by moderate (1 frequency) and poor (1 frequency) response phenotypes. None of the selected homozygote recessive genotype (ccttgg) was associated to the Good response phenotype, and none of the homozygote dominant genotype (TTCCAA) was associated to the poorer response phenotype. A comparison of the frequencies of the three SNPs (1188, 1308 and 1545) between the four IVM response categories indicated significant differences ($p<0.05$) in the beta-tubulin gene. The sample genotype without mutation, TTCCAA, occurred at significantly higher frequency of 70% (7) ($p<0.05$) in the Good response phenotype than in the other poor response phenotypes (0-2). The SNP genotype mutation TcCtAg occurred at significantly higher frequency of 73.3% (11) in the Moderate response phenotype than in the rest (0-3). The SNP genotype with complete mutation ccttgg occurred at equal frequency of 50% (1) in the poor and moderate response phenotypes.

Individual genotypic and phenotypic assessment at SNP positions 1188, 1308 and 1545 (Tables 4.37-39 and Figures 4.17-19), indicate similar pattern of the frequencies of the heterozygote genotypes being generally higher (61.8-64.7%) than that of the homozygote dominant genotypes (29.4%) with the selected homozygote recessive genotypes showing the lowest frequencies (5.9-8.8%).

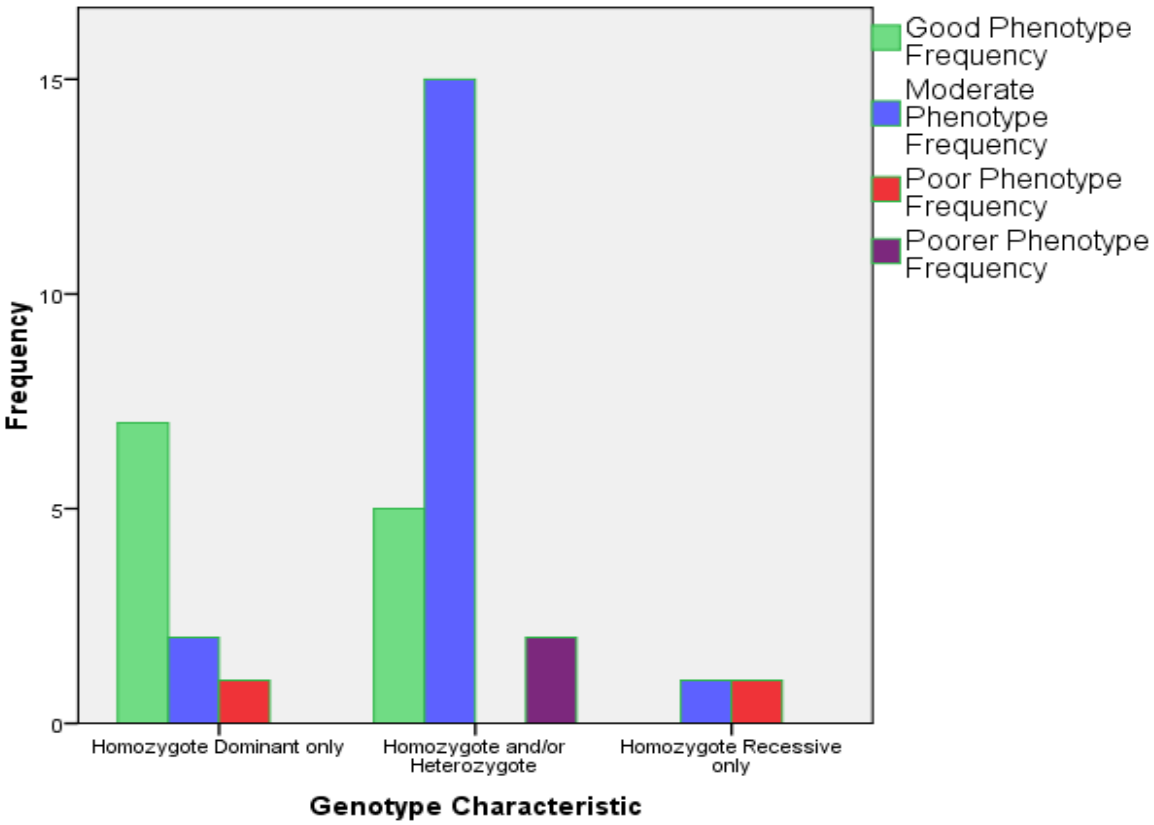

Figure 4.17: Worm phenotypes by their genotypes (All 4 SNPs) of the 34 worms sequenced

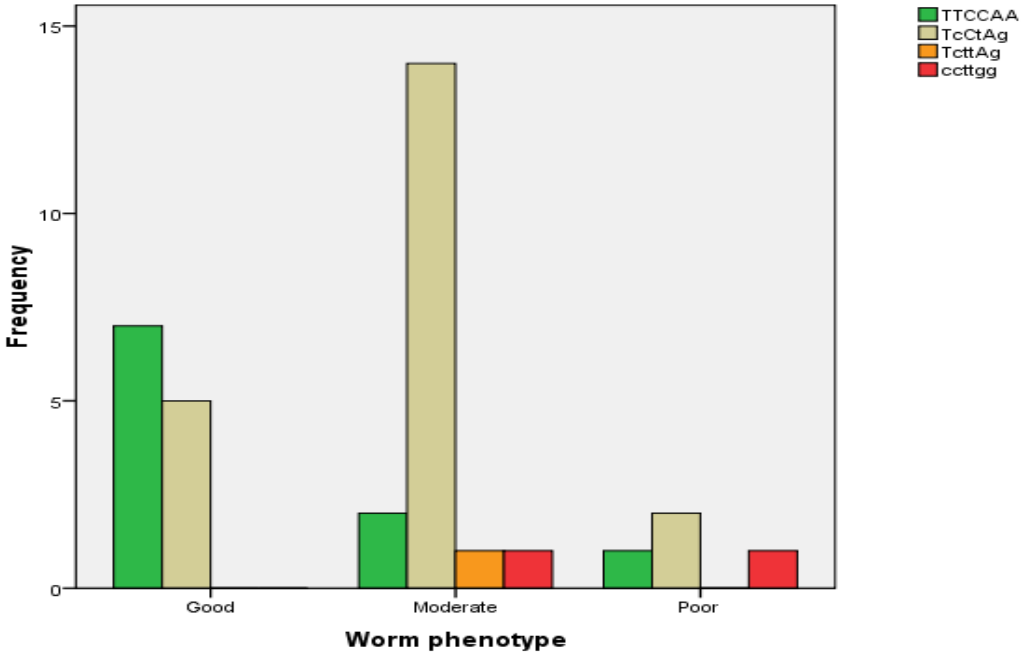

Figure 4.18: Worm phenotypes by their genotypes (last 3 SNPs) of the 34 worms sequenced

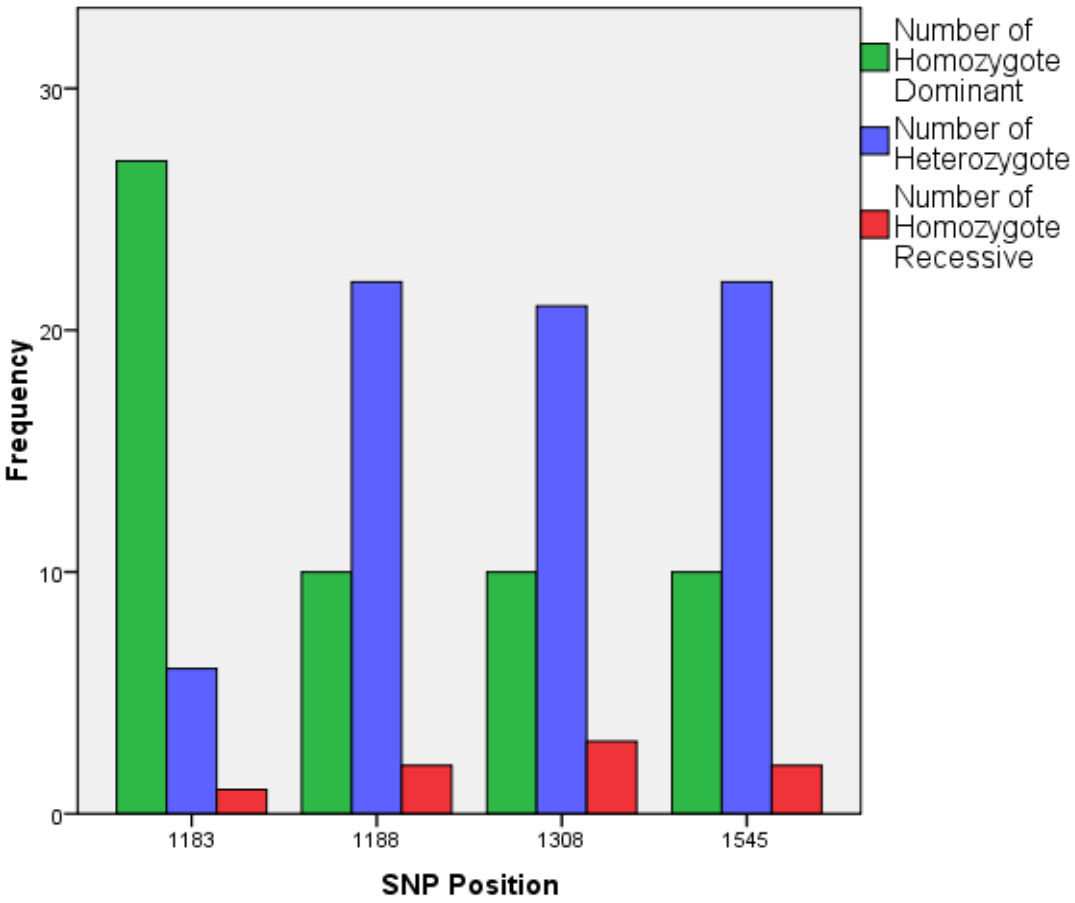

Figure 4.19: Comparison of genotype frequencies at SNP positions 1183, 1188, 1308 and 1545

However, SNP position 1183 (Table 4.36) differed from this trend whereby the homozygote dominant genotype occurred at significantly higher frequency (79.4%) than that of the heterozygote genotype (17.7%) with the selected homozygote recessive genotype showing the lowest frequency (2.9%). These indicate greater selection pressure at SNP positions 1188, 1308 and 1545 compared with SNP position 1183.

The Hardy-Weinberg expected frequency had a pattern of moderately high frequency (20.242 – 20.698%) of heterozygote genotypes at all the SNP positions except for position 1183 with low frequency of 8.026% (Tg); a relatively moderate expected frequency (13.171 – 14.869%) of homozygote dominant genotypes at all the SNP positions except for position 1183 with

high frequency of 33.493% (TT); and a relatively low expected frequency (6.889 – 8.131%) of homozygote recessive genotypes at all the SNP positions to such an extent that position 1183 is expected to have very low expected frequency of 0.481% (gg) (Figure 4.20).

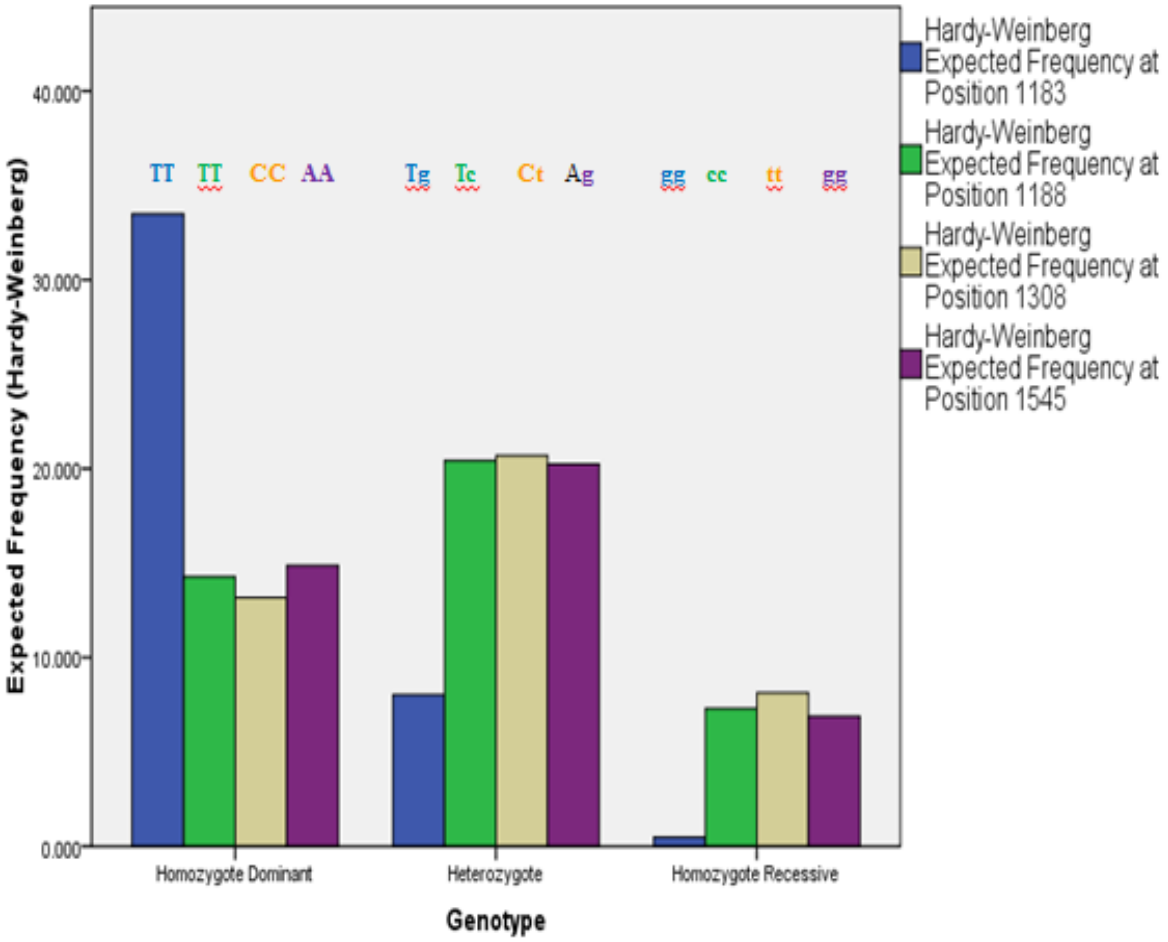

Figure 4.20: Hardy-Weinberg Expected Frequency at positions 1183, 1188, 1308 and 1545 in the 5 communities

Figures 4.21 and 4.22 respectively show the genotype per total sample and the genotype frequency at positions 1183, 1188, 1308 and 1545. All the chi square values of the observed genotype frequencies were less than the 5% significance level for the 1 degree of freedom value of 3.84. Thus, the study population is in Hardy-Weinberg equilibrium. Although there

is selection pressure, the pattern is not too different from that of the Hardy-Weinberg expected frequency and the alleles under selection have not been fixed in the population.

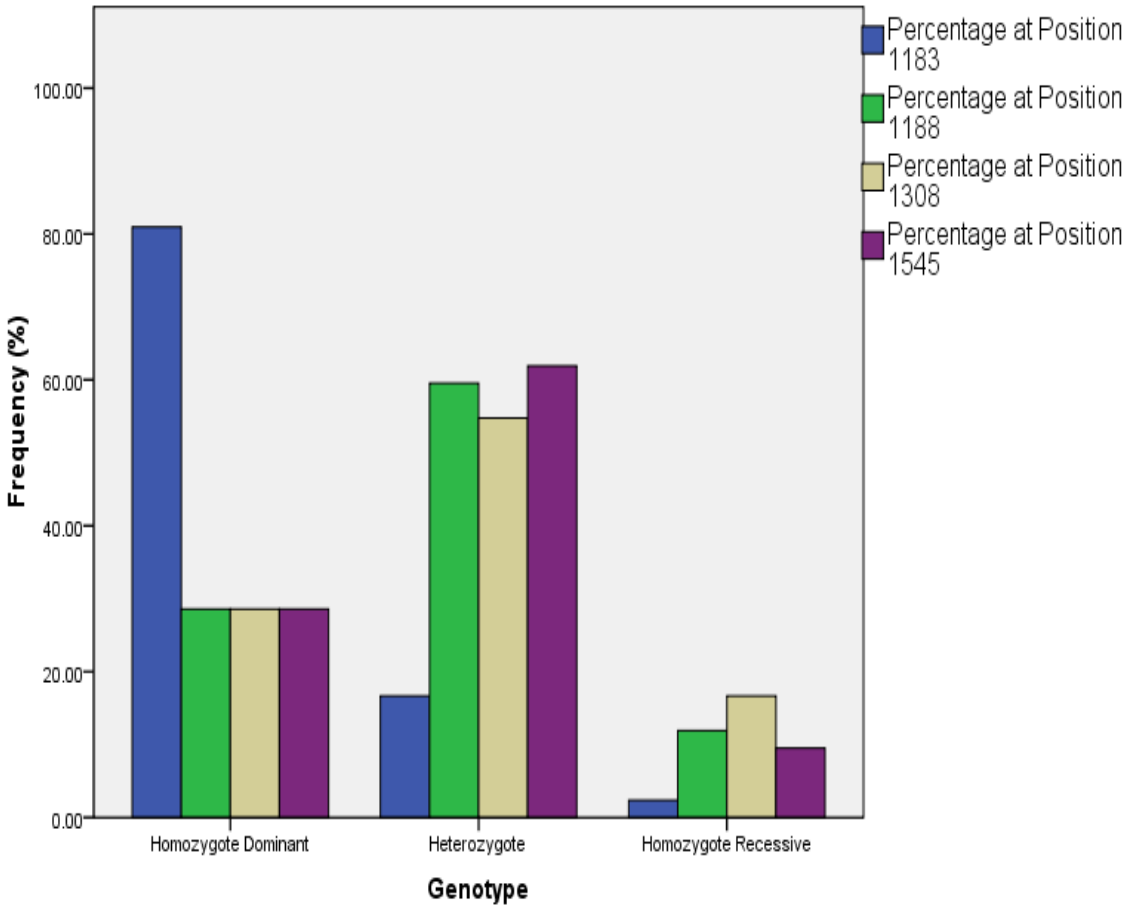

Figure 4.21: Genotype per total sample at positions 1183, 1188, 1308 and 1545 in the 5 communities

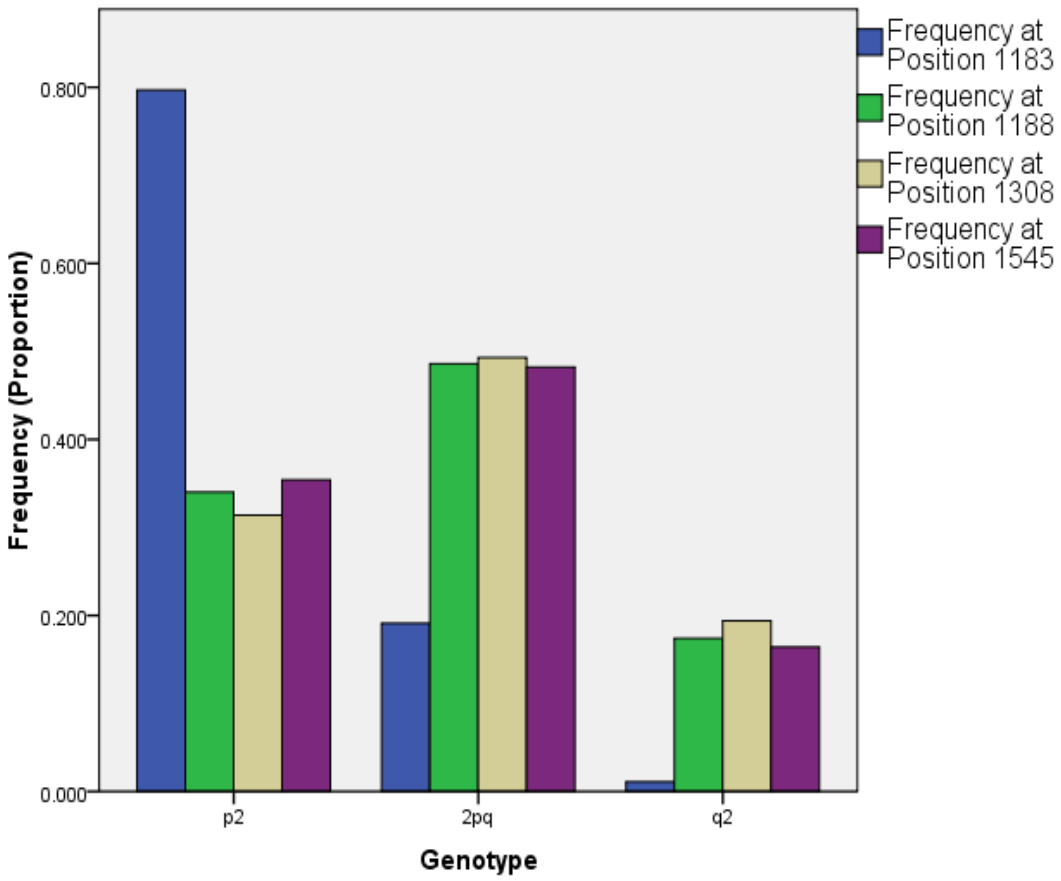

Figure 4.22: Genotype Frequency at positions 1183, 1188, 1308 and 1545 in the 5 communities

According to Hardy-Weinberg Law (Hardy's Law and Principle of Wilhelm Weinberg), both allele and genotype frequencies remain constant or stay in equilibrium in a population from generation to generation unless specific disturbing influences are acting and seven assumptions need to be fulfilled to be valid: organisms are expected to be diploid; only sexual reproduction should occur; generations should not overlap; there should be random mating; the size of a population is expected to be significantly large; allele frequencies should be equal in the sexes; and there should be no migration, mutation or selection (Stern, 1962; Crow, 1999; Hartl and Clarke, 2007). Hartl and Clarke (2007) further point out that a violation of assumptions like the random mating (as can be caused by inbreeding to increase

homozygosity for all genes) will make the population not have Hardy-Weinberg proportions but a violation of the following assumptions will make the population continue to be in equilibrium with the tendency for allele frequencies to change over time: selection, mutation and small population size. Hence, it is logical for this present study to have mutation and selection at SNP postions 1188, 1308 and 1545 whiles the population stays in Hardy-Weinberg equilibrium seen in Figures 4.17-23.

From Figure 4.23, the allele frequencies were generally relatively higher (0.560 – 0.595) in the nucleotides determining the dominant genotypes when compared with the relatively lower frequencies (0.405 – 0.440) in the nucleotides determining the recessive genotypes for the three SNP positions (1188, 1308 and 1545). It appears that the alleles determining the dominant genotypes in the wild types are decreasing, being selected against, whiles the alleles determining the recessive genotypes in the mutagenic forms are increasing, being selected for. Thus, the allele frequencies of the mutated genotypes have almost caught up with that of the wild type non-mutated forms. If this trend continues, the homozygote recessive alleles can become fixed in the population.

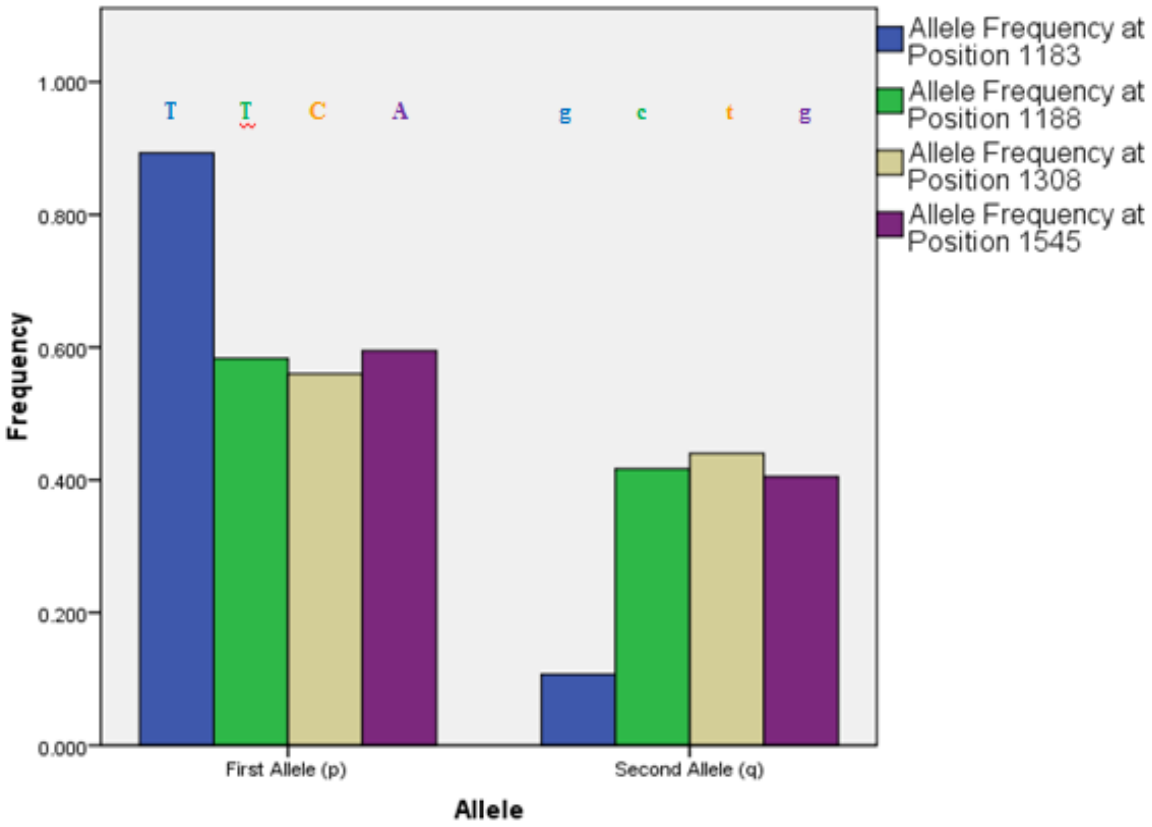

Figure 4.23: Allele Frequency at positions 1183, 1188, 1308 and 1545 in the 5 communities

The highest inbreeding coefficient occurred at position 1545 (0.284), followed by the one at position 1188 (0.224) shown in Figure 4.24. It was however lower at position 1183 (0.128) and lowest at position 1308 (0.111). This indicates a decrease in the extent of heterozygosity in the order of position 1545, 1188, 1183 and 1308. Furthermore, this result shows that there is an increase in the extent of being identical by descent in the order of position 1308, 1183, 1188 and 1545.

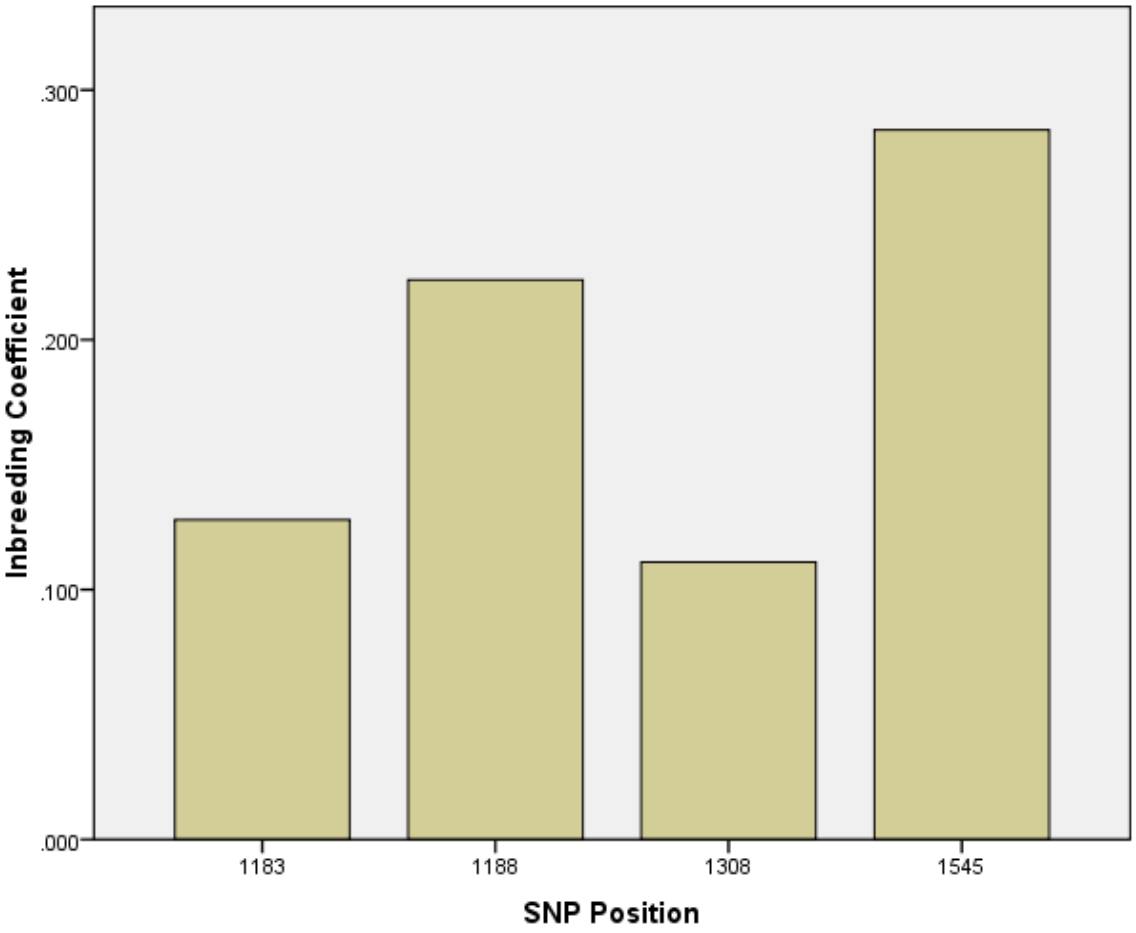

Figure 4.24: Inbreeding Coefficient at positions 1183, 1188, 1308 and 1545

Generally, selected form of the mutated female homozygotes and heterozygotes were less fertile than wild-type non-mutated homozygotes at SNP position 1183 (Figure 4.25). However, variations exist at the other SNP positions with the situation appearing to be gradually reversing, characterized by significant proportions of the wild-type homozygotes increasing in the frequency of non-fertile female worms whiles both the selected mutated form of the heterozygotes and homozygotes increasing in the frequency of fully fertile females at SNP positions 1188, 1308 and 1545 (Figures 4.26-4.28).

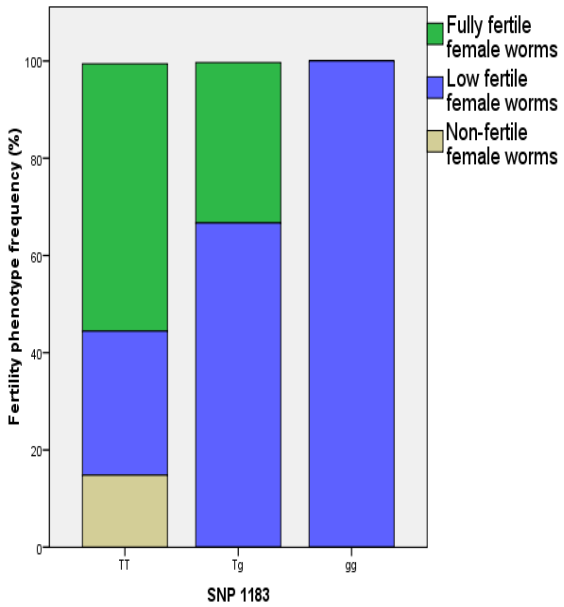

Figure 4.25: Frequency of worm fertility phenotype at SNP position 1183

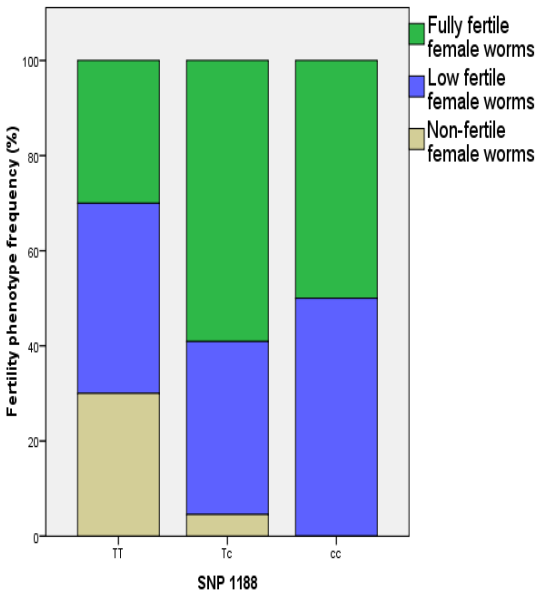

Figure: 4.26: Frequency of worm fertility phenotype at SNP position 1188

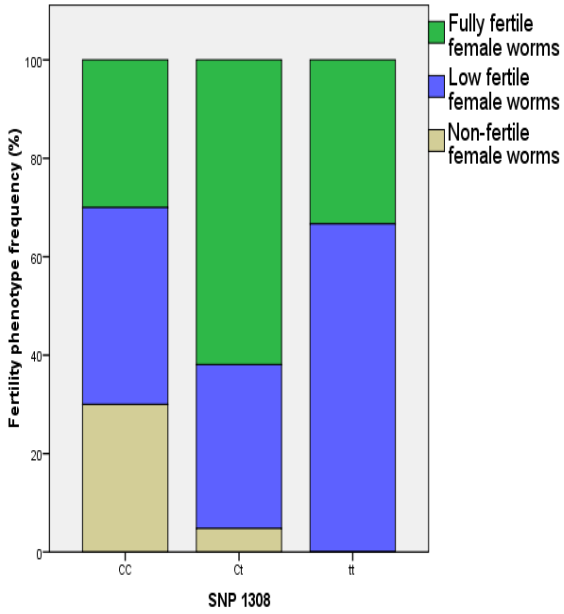

Figure 4.27: Frequency of worm fertility phenotype at SNP position 1308

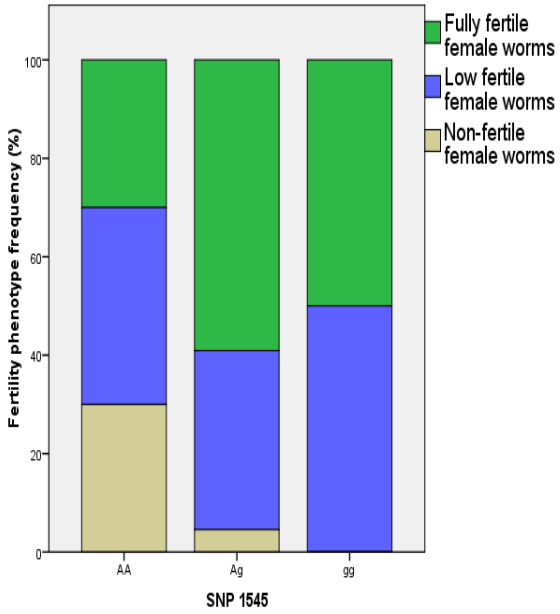

Figure 4.28: Frequency of worm fertility phenotype at SNP position 1545

This presently indicates that the worms without mutations are being selected against because IVM is effective in decreasing their reproductive potential in the long term, whiles the mutant

worms associated with poor response are being selected for because they have relatively higher reproductive potentials in view of a reduced IVM effect on them.

A nucleotide sequences has been proposed consisting of 364bp (encompassing SNPs occurring between sequence position 1185 and 1548) for developing molecular marker to monitor IVM resistance.

Candidate nucleotide sequence (364bp) from position 1185 to 1548 of beta tubulin gene for developing marker for the monitoring of IVM resistance is proposed as:

CCA**C**TTTCTAATCTCTTCTCTTCTTAATGGAATTAAAATTGAACTAAACATTTGTT
TAGAATATGATCTTTTCTGACGACCAATATTTTTATTTATTAAATTCTAAATAGCT
GTCAATTTATG**T**ATTATATTGTAATACATCTCTTTACCCCTTTATATCATTCGCTCT
TTGCTGTTTCGTAATATTTCTTTTTTCTTTAATTCAAGCAATATTATAGTGAGAAA
TTTCTTTTTTTTATCTTTAGGTGGCAAATATGTACCACGAGCAATCCTTGTCGATC
TGGAACCGGGTACTATGGATTCCATTCGAGGAGGTGGATTTGGCCAACTGTTCCG
ACCGGACAATTTTGTATTTGGACA**G**AGT

(Colour Code: **Red**=Nucleotide at position 1188; **Blue** = Nucleotide at position 1308; and **Green** = Nucleotide at position 1545)

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

There was no significant correlation ($p>0.05$) between the Mf loads observed from skin snips taken from both iliac crest in individuals across the study communities. One of the main challenges in onchocerciasis studies is the determination of intensity of *O. volvulus* infection in humans following natural exposure to the disease. The use of Mf counts from skin snips and worms from excised palpable nodule are regarded as relatively rough estimates, especially when infection levels are low, and the number of infected individuals can only be roughly estimated (Thylefors, 1978; Duke, 1993). The number of palpable nodules is rough estimation of the total number of nodule counts, because majority of nodules are not palpable and are usually found in deeper tissues (Duke, 1993). Although microfilarial density is generally accepted as the standard measure of the infection intensity of *O. volvulus*, it has been indicated as being of little accuracy because of its variation even at adjacent skin snip locations (Schulz-Key, 1978; Toe *et al*., 1998).

Studies have demonstrated that irrespective of the initial levels of microfilarial load at pre-treatment, repeated annual IVM treatments for five years leads to the reduction of Mf density to a mean value less than 10 Mf/s just about a year after the fifth treatment (Alley *et al*., 1994; Plaisier *et al*., 1995; Kennedy *et al*., 2002). Sub-optimal responders were defined by Awadzi *et al*. (2004) as individuals who still have 10 Mf/snip or greater despite receiving nine or more ivermectin treatments. The results of this study (Table 4.7) show that most of the individuals under study had Mf densities less than 10 Mf/snip to such extent that Fawoman-Banda and Nyire recorded no subject with more than 10 Mf/snip at both pre-treatment and day 180 post-treatment. Besides, an average of at least 80% of subjects in

Agblekeme II, Chabon and Kojobone had both pre-treatment and day 180 Mf densities less than 10 Mf/snip. These are good responding subjects consistent with the findings of Plaisier *et al*. (1995), Alley *et al*. (1994), Osei-Atweneboana *et al*. (2011) and Kennedy *et al*. (2002) who all followed up on subjects receiving treatments and observed reduction in the mean Mf counts of over 90 Mf/s to counts less than 8 Mf/s. However, some subjects in three of the five study communities (Agblekeme II, Chabon and Kojobone) had mean Mf counts greater than the 10 Mf/s (16.7-21.1%) prior to the first study ivermectin treatment. Just six months after the first ivermectin treatment of this study, the mean Mf counts showed that 3.3% (Chabon), 5.3% (Kojobone) and 8.3% (Agblekeme II) of the treated subjects had mean microfilarial densities greater than 10 Mf/s to as high as 43.5 Mf/s in Kojobone despite over a decade of ivermectin treatment. Our findings agree with those of Osei-Atweneboana *et al*. (2011) whereby they observed that communities responding poorly had average Mf counts of more than 10 Mf/s to as high as 16.54 Mf/s. Our results further indicate that *O. volvulus* in some individuals in the study communities are responding sub-optimally to ivermectin.

Our study shows that six years after the work of Osei-Atweneboana *et al*. (2007), nodule prevalence in Chabon had almost doubled from 21.8% to 36.2% despite IVM treatment, however, microfilaria prevalence has decreased from 51.8% to 31.9%. The tremendous decrease in microfilaria prevalence in Chabon may suggest that the semi-ivermectin treatment introduced in Ghana since 2010 has been effective in clearing skin Mf. This is evident in our study as we observed drastic reduction in Mf densities over the one year study period involving two rounds of IVM treatment as shown in fig 4.3.1. However, the observed increase in nodule prevalence suggests that transmission is still on-going resulting in reinfections in human populations. This has been confirmed in current entomological studies in these areas (Osei-Atweneboana, unpublished data). The increased transmission is also as a result of early release of Mf in the skin (skin Mf repopulation) by adult female worms

showing SOR. This makes Mf available in the skin soon after IVM treatment to sustain transmission. Our embryogramme and worm aging analysis revealed that about 8.1% of the adult worms were young indicating recent infections and may explain the increase in nodule prevalence over the years (Table 4.31). This result agrees with the proportion of young worms of 5.1% and 8.1% in the poor and intermediate response categories respectively found in a similar study (Osei-Atweneboana *et al*., 2011).

The findings of our investigation across the five study communities show that the average nodule prevalence and microfilaria prevalence value of 25.9% and 17.4% respectively are lower than the ones (35% and 70% respectively) found by Timmann *et al*. (2008). However, variations exist among individual communities. Our observed average community microfilarial load value of 3.1 across the five study communities was also lower than theirs which ranged from 5 to 40.3 Mf per skin snip. The relatively lower nodules and Mf loads observed in our study communities could be attributed to the fact that our communities have had longer ivermectin treatment periods and there is cumulative action of the microfilaricidal effect of long time usage of ivermectin.

Generally, the ratio of male to female worms per nodule is approximately 1:3. There is a significant positive correlation between the total number of male and female worms either in the same or different nodules in individuals across the various study communities. Classifying the female worms in Jagbengbendo and Takumdo according to age and intra-uterine content, showed that approximately 94% of worms belonged to the older or middle age category, of which 56% were still reproductively active, when compared with the young ones contributing less Mf to the on-going transmission even at three months after the previous IVM treatment and these matured female worms are showing as responding sub-optimally to ivermectin (Awadzi *et al*., 2004a; Osei-Atweneboana *et al*., 2011).

This is similar but relatively higher than observation by Osei-Atweneboana *et al*. (2011) who demonstrated that about 25% of the individual subjects in the study had adult female worms responding sub-optimally and that approximately 90% of the worms were older or middle aged with most of the Mf being produced by the older or middle aged worms. Thus, the young worms in their study also contributed very little to on-going transmission.

Nineteen of the 59 samples had poor sequences and were excluded from further analysis. Thus, the remaining 40 samples were considered as good sequences. Six (15%) of the remaining 40 samples produced good sequences that had confounding effects. These included sequences from worms that were not reproductively active (absence of intra-uterine Mf), however, the individuals from whom these worms were obtained were found to be microfilaridermic. The absence of Mf in the uteri implies that the Mf in the skin could be coming from worms in other nodules that may not be palpable or deeply located in tissues. These worms could not be linked to the Mf in the skin to accurately characterize the phenotypes without leading to confounding results. Hence, they were excluded from subsequent analysis. The remaining 34 (57.6%) samples yielded good sequences without any confounding effects and were used for the subsequent analysis.

Although the observed genotype frequencies (61.8-64.7% heterozygote; 29.4% homozygote dominant; and 5.9-8.8% homozygote recessive) for SNP positions 1188, 1308 and 1545 were not significantly different ($p>0.05$) from the Hardy-Weinberg expected frequencies (20.2-20.7% heterozygote; 13.2-14.9% homozygote dominant; and 6.9-8.1% homozygote recessive) (Figure 4.6.2.4-6) with an indication of the genotypes being constant from generation to generation, the allele frequencies (0.405 – 0.440) of the nucleotides determining the mutated recessive genotypes showed greater selection almost reaching the same frequency magnitude as that of the nucleotides determining the wild-type dominant genotypes (0.560 – 0.595) (Figure 4.6.2.7). Thus, phenotypic and genotypic results of our

work indicate that ivermectin SOR has been selected for (Hartl and Clarke, 2007) with the genotypes at SNP positions 1183, 1188, 1308 and 1545 showing different levels of selection consistent with the findings of Eng and Prichard (2005), Nana-Djeunga *et al*. (2012), and Osei-Atweneboana *et al*. (2012). Hence, there is selection against worms carrying susceptible genotypes by IVM, whiles worms carrying the sub-optimally responding genotypes are being selected for (favoured) to continue to reproduce and contribute their gene pool to those of the next generation (Prichard *et al*., 1980). Whiles these workers found stronger selection at all four SNP positions, our study indicated that the genotype at the SNP position 1183 showed lower selection. Moreover, their work showed stronger association of the genotypes at all four SNP positions with the resistance phenotypes. However, our results showed that the sub-optimal phenotype worms were strongly associated with genotypes at SNP positions 1188, 1308 and 1545. Four distinct genotype configurations were formed from these three SNP sites that were associated with SOR phenotype worms. The worm samples with DNA sequences having no nucleotide change, are considered as the same as the wild-type, TTCCAA, and this genotype showed strong association ($p<0.05$) with the good response phenotype. The genotypes with heterozygote change at each of the three SNP positions, TcCtAg, were found to show strong association ($p<0.05$) with the moderate response phenotype worms. Also, the genotype with complete change (homozygote) at each of the three SNP positions (1188, 1308 and 1545), ccttgg, showed strong association ($p<0.05$) with the poor response phenotype. A mix proportion of TcCtAg and TcttAg were either found at the good, moderate, poor or poorer response phenotype but none of the genotypes ccttgg was found to be associated with the good response phenotype (Figure 4.18 and Table 4.36). Nevertheless, the greatest inbreeding coefficient, indicating the probability of two alleles at a SNP locus being identical by descent and the occurrence of selection pressure from heterozygote towards homozygote, was found higher at position 1545 (0.284), followed by 1188 (0.224) and then quite lower at 1183 (0.128) and 1308 (0.111) (Figure 4.24).

At the SNP position 1183, selected female homozygotes and heterozygotes were less fertile than wild-type homozygotes (Figure 4.25). However, slight variations exist at the different SNP positions (Figures 4.26-4.28). The highest frequency of the wild-type homozygotes of the fully fertile female worms (55.56%) was realized at SNP position 1183 compared with the 30% lower frequency in each of the remaining three SNP positions. The frequencies of the heterozygotes of the fully fertile female worms were generally higher at SNP positions 1188 (59.09%), 1308 (62%) and 1545 (59.09%) compared with the lower frequencies of 30% of the wild-type homozygotes at the same SNP positions, as well as being higher than the relatively lower frequencies of the selected homozygotes at SNP positions 1188 (50%), 1308 (33%) and 1545 (50%). Moreover, there was a relatively high frequency of the non-fertile female worms of the wild-type homozygotes at SNP positions 1183 (14.81%), 1188 (30%), 1308 (30%) and 1545 (30%) compared with the low frequency of the selected heterozygotes at SNP positions 1183 (0%), 1188 (4.55%), 1308 (5%) and 1545 (4.55%) as well as that of the selected homozygotes with complete absence of non-fertile female worms at all the four SNP positions. These indicate variation in genotypic and phenotypic traits.

Usually, traits resulting in greater reproductive success of organisms may be selected for whiles those with reduced reproductive success will be selected against. In some cases, the selection for traits can result in the selection of other correlated traits that do not by themselves directly have effect on reproductive advantages as a result of gene linkage or pleiotropy (Sober, 1984; 1993). It appears that the evolution of the nucleotides at SNP positions 1183, 1188, 1308 and 1545 originally came from genes located proximal to each other on a chromosome before being inherited together during meiosis. Thus the pleiotropic-like action of the beta tubulin gene seems to limit the rate of multivariate evolution in the presence of on-going natural selection and sexual selection on the poor response phenotype trait and favours the recessive allele at SNP positions 1188, 1308 and 1545, while selection on the good response phenotype trait favours the dominant allele at SNP position 1183. The

ability of pleiotropy to cause genetic correlations influenced the nucleotides at some SNP positions into exhibiting correlated responses to such extent that the fertility phenotype frequency at SNP positions 1188 and 1545 were exactly the same characterized by a relatively higher proportions of the selected female homozygotes (50%) falling in the low fertile category compared with the 40% frequency of the wild-type homozygotes, but with a 36.36% of the heterozygotes falling in this low fertile category (Figures 4.26 and 4.28). At these SNP positions (1188 and 1545), the frequencies of the selected female homozygotes (50%) and heterozygotes (59.09%) were higher than that of the wild-type homozygotes (30%). Nevertheless, a higher frequency (30%) of the wild-type homozygotes in the non-fertile female worm category was observed compared with the low frequency (4.55%) in the selected female heterozygotes and none of the selected female homozygotes falling in this category of non-fertile female worms. These findings indicate that the formerly observed trend of selected homozygotes and heterozygotes that were relatively infertile is reversing and gradually changing into a trend of relatively higher fertile worms with homozygotes and heterozygotes selection associated with SOR. If this trend continues without any form of monitoring and subsequent changes in control programs being implemented, there could be repercussions on the successes gained through decades of ivermectin usage.

Our findings agree with those of Eng *et al*. (2006) who observed that ivermectin selection changes the frequency of beta tubulin alleles in both *Haemonchus contortus* and *O. volvulus*. Similarly, our findings were consistent with Bourguinat *et al* (2007b) who also found significant selection for beta tubulin heterozygotes in female worms. Their findings however differed at the point whereby quarterly ivermectin treatment within a period of three years reduced the frequency of the beta tubulin homozygotes whiles increasing the heterozygotes. Again, our findings agreed with theirs at the section whereby the female worms that were

homozygous selected became more fertile than the heterozygous female worms before and after the annual treatments.

Our results were consistent with the findings of Nana-Djeunga *et al*. (2012), who investigated four SNPs occurring in the beta tubulin gene of *O. volvulus*, at the same SNP positions that we investigated (SNPs 1183, 1188, 1308 and 1545). Our studies and theirs determined changes in genotype frequencies associated with ivermectin treatments and observed significant increase in the frequency of the selected homozygote in the female worms following ivermectin treatment. Their results further showed that the selected homozygotes and heterozygotes were less fertile than the wild-type homozygotes. Our result was also consistent with this latter part of their findings for SNP position 1183. However, our results differed from theirs at the other SNP positions (1188, 1308 and 1545) because, comparatively, the selected homozygotes and heterozygotes found in our investigation became relatively more fertile whiles the wild-type homozygote became relatively less fertile. These results offer evidence of genetic selection and augment the warning that selection for ivermectin resistance in *O. volvulus* is occurring gradually and it is currently in its initial stages. There is urgency for control programmes to take this serious and incorporate mitigation measures in their activities as the gradual change in the fertility status of the selected worms that are associated with SOR can have dire consequences on the successes of ivermectin control.

Pion *et al*. (2013) studied the dynamics of *O. volvulus* microfilarial densities after treatment in an ivermectin-naïve and a multiply treated population from Cameroon. They observed that worms from the multi-treated area had early Mf productivity recovery. Our findings agree with theirs as some of the individuals in our study communities, whose responses having not been up to expectations, had early Mf repopulation of the skin. In addition, their findings in a way agree with that of Nana-Djeunga *et al*. (2012), as well as our findings for few of the

worms with phenotypic association to genotypes at SNP position 1183, since the worms from the multi-treated area were less productive than those from the ivermectin-naïve area.

There are uncertainties in future outcomes so far as mutations, selection and evolution are concerned partly due to a changing environment that living organism must either adapt to and survive long enough to reproduce their kind or cease to exist. What is certain now is that there is selection on beta tubulin gene and the reproductive successes of the sub-optimally responding worms may change in the course of time due to changes in their immediate environment. Presently, only one SNP at position 1545 occurs in an exon but does not translate into a functional protein. The other two SNPs occur in introns. Formerly, effects of mutations were considered neutral or close to neutral because they occurred in noncoding region of the DNA sequence or resulted in synonymous substitution. Recent findings however suggest that lots of mutations in introns do have slight deleterious effects (Bejerano *et al*., 2004; Kryukov, Schmidt and Sunyaev, 2005). Some workers argue that both mutation rates and average fitness effects of mutations are dependent on living organisms and estimates from human data suggest that majority of mutations are slightly deleterious (Eyre-Walker, Woolfit and Phelps, 2006). In view of these, control programmes must take a second look at the factors influencing ivermectin efficacy and changes in the response of *O. volvulus* to ivermectin treatment.

The region in the beta tubulin gene with the three SNPs associated with response phenotypes is within 364bp and the nucleotide sequence in this region with the SNP changes has been proposed for developing genetic marker for the early detection and monitoring of ivermectin resistance.

5.2 CONCLUSION

The findings of this study show that following the two rounds of semi-annual IVM treatment, Mf intensities of *O. volvulus* at pre-treatment had reduced drastically across the entire study communities and this confirms the findings of other workers that ivermectin still remains a potent microfilaricide. However, a few individuals had high Mf intensities and also carried worms that were reproductively active even at 90 days after treatment. This suggest that a population of adult worms are emerging that are responding poorly to the anti-fecundity effects of ivermectin. There was variation in the average nodule and Mf prevalence across the five study communities with Agblekeme II and Chabon being mesoendermic, whiles Fawoman-Banda, Kojobone and Nyire are Hypoendermic. Genetic differences in the beta tubulin gene of *O. volvulus* were found at four SNP positions between worms from individuals responding normally and those responding sub-optimally to ivermectin treatment. The sub-optimal responding worms were found to be strongly associated with genotype with nucleotide changes at all three SNP positions, (1188CC/1308TT/1545GG). From the genetic analysis, a DNA sequence consisting of 364bp from the region of 1012-1695bp of the genomic DNA sequence of beta tubulin gene has been proposed for developing a molecular marker to monitor ivermectin resistance.

5.3 RECOMMENDATIONS

The following are recommended:

1. Similar investigation should be carried out on other genes in *O. volvulus* known to be associated with ivermectin resistance in other nematodes like *Haemonchus contortus* since IVM resistance has been suggested to be polygenic.
2. A molecular marker should be developed for monitoring of ivermectin resistance development and this should be incorporated into control programmes for continuous assessment of the efficacy of IVM.
3. This study should be carried out in all areas alongside control programmes to assess changes in the efficacy of ivermectin and response profile of *O. volvulus*. There should be a modification in control strategy of the programmes to include vector control in communities where poor ivermectin responses have been documented. Modelling studies should be carried out on the rate of mutation of the nucleotides associated with the phenotypic responses and their rate of transmission.

REFERENCES

Abiose, A. (1998). Onchocercal eye disease and the impact of Mectizan treatment. *Ann Trop Med Parasitol.* **92**: S1–S22.

African Programme for Onchocerciasis Control (APOC). (2004). Year 2004 Progress Report of WHO/APOC. Available at: http://www.apoc.bf/en/download.htm. Accessed: March 4, 2013

African Programme for Onchocerciasis Control (APOC). (2005). Final communiqué of the 11th session of the Joint Action Forum (JAF) of APOC, Paris, France, 6–9 December 2005. Ouagadougou (Burkina Faso).

African Programme for Onchocerciasis Control (APOC). (2008). Report of the twenty sixth session of the Technical Consultative Committee. Ouagadougou: APOC. Available at: http://www.who.int/apoc/about/structure/tcc/ 10-15 Mars 2008, Final_Report_TCC_26_English_23_05_08.pdf. **Accessed**: March 18, 2013.

Alley, E. S., Plaisier, A. P., Boatin, B. A., *et al*. (1994). The impact of five years of annual ivermectin treatment on skin microfilarial loads in the onchocerciasis focus of Asubende, Ghana. *Trans R Soc Trop Med Hyg*. **88**: 581–84.

Ali, M. M., Mukhtar, M. M., Baraka, O. Z., *et al*. (2002). Immunocompetence may be important in the effectiveness of Mectizan (ivermectin) in the treatment of human onchocerciasis. *Acta Trop*. **84:** 49-53.

Ali, M. M., Baraka, O. Z., AbdelRahman, S. I., *et al*. (2003). Immune responses directed against microfilariae correlate with severity of clinical onchodermatitis and treatment history. *J Infect Dis*. **187**: 714–717.

Amazigo, U. V., Brieger, W. R., Katabarwa, M., *et al*. (2002a). The challenges of community-directed treatment with ivermectin (CDTI) within the African Programme for Onchocerciasis Control (APOC). *Ann Trop Med Parasitol*. **96** (Suppl. 1): S41 S58.

Amazigo, U. V., Obono, M., Dadzie, K. Y., *et al*. (2002b). Monitoring Community-Directed Treatment Programs for Sustainability: Lessons from the African Programme for Onchocerciasis Control (APOC). *Ann Trop Med Parasitol*. **96** (Suppl. 1): S75–92.

Ault, S. K. (2007). Pan American Health Organization's Regional Strategic Framework for addressing neglected diseases in neglected populations in Latin America and the Caribbean. *MemÓrias do Instituto Oswaldo Cruz*. **102** (Suppl. 1): 99-107.

Australian Shepherd Health & Genetics Institute (ASHGI). (2013). MDR1 FAQs. Home Page. Available at: http://www.ashgi.org/articles/mdr1.htm. **Accessed**: May 3, 2013.

Awadzi, K., Dadzie, K. Y., Schulz-Key, H., *et al*. (1985). The chemotherapy of onchocerciasis X: An assessment of four single doses treatment regimens of MK-933 (ivermectin) in human onchocerciasis. *Ann Trop Med Parasitol*. **79**: 63–67.

Awadzi, K., Dadzie, K. Y., Kläger, S., *et al*. (1989). The chemotherapy of onchocerciasis. XIII. Studies with ivermectin in onchocerciasis patients in northern Ghana, a region with long lasting vector control. *Ann Trop Med Parasitol*. **40**: 361–66.

Awadzi, K. (1993). Drug surveillance: international cooperation past, present and future. In *Proceedings of the XXVIIth CIOMS Conference*, eds Bankowski, Z. & Dunne, J. F. Geneva: Council for International Organizations of Medical Sciences. pp. 136–140.

Awadzi, K., Attah, S. K., Addy, E. T., *et al*. (2004a). Thirty-month follow-up of sub-optimal responders to multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. *Ann Trop Med Parasitol*. 98(4):359–370.

Awadzi, K., Boakye, D. A., Edwards, G., *et al*. (2004b). An investigation of persistent microfilaridermias despite multiple treatments with ivermectin, in two onchocerciasis endemic foci in Ghana. *Ann Trop Med Parasitol*. **98**(3): 231–249.

Basáñez, M. G. and Boussinesq, M. (1999). Population biology of human Onchocerciasis. *Philos Trans R SocLond B Bio Sci*. **354** (1384): 808-26.

Basáñez, M. G., Collins, R. C., Porter, C. H., *et al*. (2002). Transmission intensity and the patterns of *Onchocerca volvulus* infection in human communities. *Am J Trop Med Hyg*. **67** (6): 669–679.

Basáñez, M. G., Pion, S. D. S., Churcher, T. S., *et al*. (2006). River Blindness: A Success Story under Threat? *PLoS Med* **3** (9): e371.

Basáñez, M. G., Pion, S. D. S., Boakes, E., *et al*. (2008). Effect of single-dose ivermectin on

Onchocerca volvulus: a systematic review and meta-analysis. *Lancet Infect Dis*. **8**: 310-22.

Bejerano, G., Pheasant, M., Makunin, I., *et al*. (2004). Ultraconserved elements in the human genome. *Science* **304**: 1321-5

Benton, B. (1998). Economic impact of onchocerciasis control through the African Programme for Onchocerciasis Control: an overview. *Ann Trop Med Parasitol*. **92** (Suppl. 1): S33–S39.

Blackhall, W. J., Prichard, R. K. and Beech, R. N., *et al*., (2003). Selection at a ϒ-aminobutyric acid receptor gene in *Haemonchus contortus* resistant to avermectins/milbemycins. *Mol Biochem Parasitol*. **131**: 137–145.

Blanks, J., Richards, F., Beltrán, F., *et al*. (1998). The Onchocerciasis Elimination Program for the Americas: a history of partnership [Programa de Eliminación de la Oncocercosis para las Américas: historia de solidaridad]. *Rev Panam Salud Publica*. **1998** (3): 367–374.

Borsboom, G. J. J. M., Boatin, B. A., Nagelkerke, N. J. D., *et al*. (2003). Impact of Ivermectin on Onchocerciasis Transmission: Assessing the Empirical Evidence That Repeated Ivermectin Mass Treatments May Lead to Elimination/Eradication in West-Africa. *Filaria J*. **2** (1): 8.

Borst, P. and Schinkel, A. H. (1996). What have we learnt thus far from mice with disrupted P-glycoprotein genes? *Europe J Canc.* **32** (6): 985–990.

Bourguinat, C., Ardelli, B. F., Pion, S. D. S., *et al.* (2007a). P-glycoprotein-like protein, a possible genetic marker for ivermection resistance selection in *Onchocerca volvulus*. *Mol Biochem Parasitol*. 158 (2008) 101-111; doi:10.1016/j.molbiopara.2007.11.017

Bourguinat, C., Pion, S. D., Kamgno, J., *et al*. (2007b). Genetic Selection of Low Fertile *Onchocerca volvulus* by Ivermectin Treatment, *PloS Negl Trop Dis*. **1:** e72.

Boussinesq, M. **and** Hougard, J. M. **(**1998**)**. La lutte contre l'onchocercose en Afrique: aspects actuels. *Médecine Tropicale*. **58**: 285–296.

Boussinesq, M. and Gardon, J. (1999). La re´sistance de *Onchocerca volvulus* a`

l'ivermectine: une e´ventualite´ a` conside´rer. *Annales de l'Institut Pasteur*. **10:** 81–91.

Boussinesq, M., Pion, S. D., Demanga-Ngangue, *et al*. (2002). Relationship between onchocerciasis and epilepsy: A matched case-control study in the Mbam Valley, Republic of Cameroon. *Trans R Soc Trop Med Hyg*. **96**: 537–541.

Brattig, N. W. (2004). Pathogenesis and host responses in human onchocerciasis: Impact of *Onchocerca* filariae and *Wolbachia* endobacteria. *Microbes Infect*. **6**: 113–128.

Brieger, W. R., Awedoba, A.K., Eneanya, C.I., *et al*. (1998). The effect of ivermectin on Onchocerca skin disease and severe itching: results of a multicenter trial. *Trop Med Int Health*. **3**: 951–961.

Brown, K. R. and Neu, D. C. (1990). Ivermectin-clinical trials and treatment schedules in onchocerciasis. *Acta Leidensia*. **59**: 169–175.

Brunton, L., Lazo, J. and Parker, K. (2005). Goodman & Gilman's The Pharmacological Basis of Therapeutics, Eleventh Edition, *McGraw Hill Professional*, *McGraw-Hill Medical*. pp. 11-1984.

Carmichael, I., Visser, R., Schneider, D., *et al*. (1987). *Haemonchus contortus* resistance to ivermectin. *J South Afric Vet Ass*. **58**: 93.

Centers for Disease Control and Prevention (CDC). (2013a). Onchocerciasis (also known as River Blindness): Onchocerciasis, DPDx. Available at http://www.dpd.cdc.gov/pdx, Accessed: March 2, 2013.

Centers for Disease Control and Prevention (CDC). (2013b). Parasites-Onchocerciasis (also known as River Blindness): Epidemiology & Risk Factors**.** *CDC Home Page*. Available at: http://www.cdc.gov/parasites/onchocerciasis/epi.html. Accessed: June 9, 2013

Chabner, B. A. and Longo, D. L. (1996). Cancer Chemotherapy and Biotherapy: Principles and Practice. Lippincot Williams and Wilkins, Philadelphia, PA. Edition **4**: 12-879.

Chavasse, D. C., Post, R. J., Lemoh, P. A., *et al*. (1992). The effect of repeated doses of ivermectin on adult female *Onchocerca volvulus* in Sierra Leone. *Trop Med Parasitol*. **43:**256-262.

Cheke, R. A. and Garms, R. (1983). Re-infestation of the southern flank of the Onchocerciasis Control Programme area by windborne vectors. *Phil Trans Royal Soc London* series B. **302**: 471-482.

Crosskey, R. W. and Chichester, W. (1990). The natural history of blackflies. John Wiley & Sons, Ltd. pp. 2-711. Available at: http://onlinelibrary.wiley.com/doi/10.1002/rrr.3450080313/abstract. Accessed: May 9, 2013.

Crosskey, R. W. and Howard, T. M. (2004). A revised taxonomic and geographical inventory of world blackflies (Diptera : Simuliidae). London: The Natural History Museum. Available at: http://www.nhm.ac.uk/research-curation/projects/blackflies. Accessed May 9, 2013.

Crow, J. F. (1999). Hardy, Weinberg and language impediments. *Genetics*. **152** (3): 821-825.

Crump, A., Morel, C.M. and Õmura, S. (2012). The onchocerciasis chronicle: from the beginning to the end? *Trends Parasitol*. **28**: 208-88.

Cupp, E. W., Ochoa, J. O., Collins, R. C., *et al*. (1992). The effects of repetitive community-wide ivermectin treatment on transmission of *Onchocerca volvulus* in Guatemala. *Am J Trop Med Hyg*. **47**: 170–180.

Cupp, E. W., Richards, F., Lammie, P., *et al*. (2007). Efficacy of ivermectin against *Onchocerca volvulus* in Ghana. *Lancet*. **370** (9593): 1123.

Cupp, E. W., Sauerbrey, M., Richards, F., *et al*. (2011). Elimination of human onchocerciasis: History of progress and current feasibility using ivermectin (Mectizan®) monotherapy. *Acta Trop*. **120** (Suppl. 1): S100-8.

Dadzie, K. Y., Remme, J., Rolland, A., *et al*. (1989). Ocular onchocerciasis and intensity of infection in the community. II. West African rainforest foci of the vector *Simulium yahense*. *Trop Med Parasitol*. **40**: 348 – 354.

Dadzie, K. Y. **(**1997**)**. Onchocerciasis control: the APOC strategy. *Africa Health*. **3**: 13–15.

Dadzie, K. Y., Neira, M. and Hopkins, D. (2003). Final report of the Conference on the Eradicability of Onchocerciasis. *Filaria Journal*. **2:** 2.

David, M., Pariser, M. D., Meinking, T. L., *et al*. (2012). Topical 0.5% Ivermectin

Lotion for Treatment of Head Lice. *New Eng J Med*. **367**: 1687–1693.

Debrah, A. Y., Mand, S., Marfo-Debrekyei, Y., *et al*. (2006). Assessment of microfilarial loads in the skin of onchocerciasis patients after treatment with different regimens of doxycycline plus ivermectin. *Filaria*. *J.* **5**:1.

Donald, G. and McNeil, J. (2012). Pill Could Join Arsenal Against Bedbugs. The New York Times. Availbale at: http://well.blogs.nytimes.com/2012/12/31/pill-could-join-arsenal-against-bedbugs. Accessed: March 10, 2013.

Dourmishey, A. L., Dourmishey, L. A. and Schwartz, R. A. (2005). Ivermectin: pharmacology and application in dermatology. *Int J Dermatol.* **44** (12): 981–8.

DPCU. (2012). Strategic Environmental Assessment on District Medium Term Development Plan (2010-2013) Report, Tain District Assembly. pp. 1-101

Duerr, H. P., Dietz, K., Schulz-Key, H., *et al*. (2004). The relationships between the burden of adult parasites, host age and the microfilarial density in human onchocerciasis. *Int J Parasitol*. **34**: 463–73.

Duke, B. O. and Moore, P. J. (1968). The contributions of different age groups to the transmission of onchocerciasis in a Cameroon forest village. *Trans R Soc Trop Med Hyg*. **62**:22– 8.

Duke, B. O. **(**1990**)**. Human onchocerciasis – an overview of the disease. *Acta Leidensia.* **59**: 9-24.

Duke, B. O., Zea-Flores, G., Castro, J., *et al*. (1991). Comparison of the effects of a single dose and of four six monthly doses of ivermectin on adult *Onchocerca volvulus*. *Am J Trop Med Hyg.* **45**: 132–137.

Duke, B. O. L., Zeafl, O. G., Castro, J., *et al*. (1992). Effects of 3-month doses of ivermectin on adult *Onchocerca volvulus*. *Am J Trop Med Hyg*. **46**: 189–94.

Duke, B. O. (1993). The population dynamics of *Onchocerca volvulus* in the human host. *Trop Med Parasitol*. **44**: 61– 8.

Eng, J.K. and Prichard, R.K. (2005). A comparison of genetic polymorphism in populations of *Onchocerca volvulus* from untreated- and ivermectin-treated patients. *Mol Biochem Parasitol*. **142**(2): 193–202.

Eng, J. K. L., Blackhall, W. J., Osei-Atweneboana, M. Y., *et al.* (2006). Ivermectin selection on ß-tubulin: Evidence in *Onchocerca volvulus* and *Haemonchus contortus*. *Mol Biochem Parasitol*. **150**: 229–235.

Epidemiological profiles of neglected diseases and other infections related to poverty in Latin America and the Caribbean (EPND-LAC). (2009). Washington DC, Pan American Health Organization. Available at: http://new.paho.org/hq/index.php?option=com_content&task=view&id=1247&Itemid=211, Accessed: March 10, 2013.

Evans, T. G. (1995). Socioeconomic consequences of blinding onchocerciasis in West. *Bull World Health Organ.* **73**: 495–506.

Eyre-Walker, A., Woolfit, M. and Phelps, T. (2006). The distribution of fitness effects of new deleterious amino acid mutations in humans. *Genetics* **173** (2): 891–900.

Gardon, J., Boussinesq, M., Kamgno J., *et al*. (2002). Effects of standard and high doses of ivermectin on adult worms of *Onchocerca volvulus*: a randomised controlled trial. *Lancet* 2002 , **360:**203-210.

Gasparini, G. (1964). Problems of Onchocerciasis IN New Suspected Areas: Sowda and Onchocerciasis. *Arch ItalSci Med Trop Parasitol*. **45**: 242-60.

Ghanadistricts.com (GHA-COM). (2012). Tain District, Brong Ahafo: Tain. Available at: http://www.ghanadistricts.com/districts/?news&r=10&_=49. Accessed: May 28, 2013.

Grant, W. (2000). What is the real target for ivermectin resistance in *Onchocerca volvulus*. *Parasitol Today*. **16**: 458–459.

Greene, B. M., Taylor, H. R., Cupp, E. W., *et al*. (1985). Comparism of ivermectin and diethylcarbamazine in the treatment of onchocerciasis. *N Engl J Med.* **313**: 133–38.

Green, B. M., Brown, K. R. and Taylor, H. R. (1989). Use of ivermectin in humans. In Ivermectin and Abamectin (ed. Campbell, W. C.). pp. 311–323. Springer-Verlag, New York.

Hall, L. R. and Pearlman, E. (1999). Pathogenesis of onchocercal keratitis (river blindness).

Clin Microbiol Rev. **12**: 445–453. Available at: http://www.plosmedicine.org/article/findcited/710078. Accessed: February 4, 2013.

Harlem, B. G. (2002). Onchocerciasis Control Programme closure ceremony [speech]. Available at: http://www.who.int/dg/speeches/2002/Ouagadougou/en. Accessed March 10, 2013.

Hartl, D. L. and Clarke, A. G. (2007). Principles of population genetics. Ed. 4. Sunderland, MA: Sinauer Associates, Incorporated. pp. 2-652.

Hawking, F. (1979). Diethylcarbamazine and new compounds for the treatment of filariasis. *Adv Pharmacol Chemother*. **16**: 129-94.

Heald, R. and Nogales, E. (2002). Microtubule dynamics. *J Cell Sci*. **115** (Pt 1): 3–4.

Higazi, T. B., Filiano, A. and Katholi, C. R. (2005). *Wolbachia* endosymbiont levels in severe and mild strains of *Onchocerca volvulus*. *Mol Biochem Parasitol.* **141**: 109.

Hoerauf, A. and Brattig, N. (2002). Resistance and susceptibility in human onchocerciasis Beyond Th1 vs. Th2. *Trends Parasitol*. **18**: 25–31.

Homeida, M., Braide, E., Elhassan, E., *et al*. (2002). APOC's Strategy of CDTI and Its Potential for Providing Additional Health Services to the Poorest Populations: African Programme for Onchocerciasis Control. *Ann Trop Med Parasitol*. **96** (Suppl. 1): S93–104.

Hopkins, A. D. (2005). Ivermectin and onchocerciasis: Is it all solved? *Eye*. **19**: 1057–1066.

Hotez, P., Raff, S., Fenwick, A., *et al*. (2007). Recent progress in integrated neglected tropical disease control. *Trends Parasitol*. **23** (11): 511–514.

Howard, J. and Hyman, A. A. (2003). Dynamics and mechanics of the microtubule plus end. *Nature*. **422** (6933): 753–8.

Jackson, F. (1993). Anthelmintic resistance – the state of play. *Brit Vet J*. 149: 123–138.

Kelly, J.D., Whitlock, H.V., Thompson, H.G., *et al*. (1978). Physiological characteristics of

free-living and parasitic stages of strains of *Haemonchus contortus*, susceptible or resistant to benzimidazole anthelmintics. *Res Vet Sci*. **25**: 376–385.

Kennedy, M. H., Bertocchi, I., Hopkins, A. D., *et al*. (2002). The effect of 5 years of annual treatment with ivermectin (Mectizan) on the prevalence and morbidity of onchocerciasis in the village of Gami in the Central African Republic. *Ann Trop Med Parasitol*. **96**: 297–307.

Kim, A. and Benton, B. (1995). Cost-Benefit Analysis of the Onchocerciasis Control Programme (OCP). Bruce Benton Series on river blindness control in West Africa. Washington, DC: World Bank. *World Bank Technical/Discussion Paper*. 282: 23-282.

Kläger, S., Whitworth, J. A., Post, R. J., *et al*. (1993). How long do the effects of ivermectin on adult *Onchocerca volvulus* **persist?** *Trop Med Parasitol*. **44:** 305-310.

Kläger, S. L., Whitworth, J. A. G. and Downham, M. D. (1996). Viability and fertility of adult *Onchocerca volvulus* after 6 years of treatment with ivermectin. *Trop Med Int Health*. **1**: 581–589.

Kryukov, G. V., Schmidt, S. and Sunyaev, S. (2005). Small fitness effect of mutations in highly conserved non-coding regions. *Human Mol Gen*. **14** (15): 2221–9.

LAT. (2012). (2012). Study shows Ivermectin ending lice problem in one treatment, *Los Angeles Times* for Nov 5, 2012. In: Wikimedia Foundation Inc., Ivermectin, Available at: https://en.wikipedia.org/wiki/Ivermectin#cite_ref-15. Accessed: March 3, 2013.

Lindblade, K. A., Arana, B., Zea-Flores, G., *et al*. (2007). Elimination of *Onchocerca volvulus* transmission in the Santa Rosa focus of Guatemala. *Am J Trop Med Hyg*. **77**: 334-41.

Little, M. P., Breitling, L. P., Basáñez M-G., *et al*. (2004a). Association between microfilarial load and excess mortality in human onchocerciasis: An epidemiological study. *Lancet*. **363**: 1514–1521.

Little, M. P., Basáñez, M-G., Breitling, L. P., *et al*. (2004b). Incidence of blindness during the entire duration of the Onchocerciasis Control Programme in western Africa, 1971–2002. *J Infect Dis*. **189**: 1932–1941.

McLean, C. M. (1959). Ocular Onchocerciasis in Northern Ghana: A treatment survey. *Brit J Ophth*. **43**: 477-485.

Mackenzie, C. D. (2007). Efficacy of ivermectin against *Onchocerca volvulus* in Ghana. *Lancet*. **370** (9593): 1123.

Mazzotti, L. (1948). Possibilidad de utilizarcomomedio diagnostic auxiliar en la oncocercosis, lasreaccionesalergicasconsecutivas a la administracion de 'Hetrazan'. *Rev InstSalubrEnferm Trop (Mex)*. **9**: 235-7.

McKechnie, N. M., Gürr, W., Yamada, H., *et al*. (2002). Antigenic mimicry: *Onchocerca volvulus* antigen-specific T cells and ocular inflammation. *Invest Ophthalmol Vis Sci*. **43**: 411–418.

Metropolitan, Municipal and District Assembly (MMDA). (2012). The Composite Budget of the Kpandai District Assembly for the 2012 Fiscal Year. MMDA's Composite Budget, The Coordinating Director, Kpandai District Assembly, Northern Region. pp: 4-75. Available at: http://www.mofep.gov.gh/sites/default/files/budget/Kpandai.pdf. Accessed: March 3, 2013.

Meyer, C. G., Gallin, M., Erttmann, K. D., *et al*. (1994). HLA-D alleles associated with generalized disease, localized disease, and putative immunity in *Onchocerca volvulus* infection. *Proc Natl Acad Sci*. U S A. **91**: 7515–7519.

Morbidity and Mortality Weekly Report (MMWR). (2013). Progress Toward Elimination of Onchocerciasis in the Americas-1993-2012. *CDC Home Page*. Weekly May 24, 2013 / **62**(20): 405-408. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6220a4.htm. Accessed: March 9, 2013.

Mottier, M. L. and Prichard, R.K. (2008). Genetic analysis of a relationship between macrocyclic lactone and benzimidazole anthelmintic selection on *Haemonchus contortus*. *Pharmacogen Genomics*. **18:** 129–140.

Murdoch, M. E., Asuzu, M. C, Hagan, M., *et al*. (2002). Onchocerciasis: The Clinical and Epidemiological Burden of Skin Disease in Africa. *Ann Trop Med Parasitol*. **96** (3): 283–96.

Murdoch, M. E. (2010). Onchodermatitis. *Current Opinion Infect Dis*. **23** (2): 124-449.

Murdoch, M. E. (2012). Onchocerciasis. Available at: http://www.uptodate.com/contents/onchocerciasis /contributors. Accessed: March 8, 2013.

Murray, C. J. L. **and** Lopez, A. D. **(**1996**)**. The Global Burden of Disease. A Comprehensive Assessment of Mortality and Disability from Diseases, Injuries, and Risk Factors in 1990 and Projected to 2020. Havard University Press, Cambridge.

Nana-Djeunga, H., Bourguinat, C., Pion, S. D., *et al*. (2012). Single nucleotide polymorphisms in β-tubulin selected in *Onchocerca volvulus* following repeated ivermectin treatment: possible indication of resistance selection. *Mol Biochem Parasitol*. **185** (1): 10-8.

Njue, A., Hayashi, J., Kinne, L., *et al*. (2004). Mutations in the extracellular domains of glutamate-gated chloride channel a3 and b subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity. *J Neurochem*. **89:** 1137–1147.

Nogales, E., Wolf, S. G. and Downing, K. H. (1998). Structure of the alpha beta tubulin dimer by electron crystallography. *Nature.* **391**: 199–203.

Noma, M., Nwoke, B. E., Nutall, I., *et al*. (2002). Rapid Epidemiological Mapping of Onchocerciasis (REMO): Its Application by the African Programme for Onchocerciasis Control (APOC). *Ann Trop Med Parasitol*. **96** (Suppl. 1): S29–39.

Oladepo, O., Brieger, W. R., Otusanya, S., *et al*. (1997). Farm land size and onchocerciasis status of peasant farmers in south-western Nigeria. *Trop Med Int Health*. **2**: 334–340.

Õmura, S. and Crump, A. (2004). The Life and Times of Ivermectin- A Success Story. *Nature Rev*. **2**: 984-989. Available at: http://courses.umass.edu/mic590s/2009/Reading/Omura2004.pdf. Accessed: May 3, 2013.

Onchocerciasis. (2005). Parasitology, Topley and Wilson's microbiology and microbial infections. 10th edition. In: Cox FEG, Wakelin, D., Gillespie, S. H., Despommier, D. D., editors. London: Hodder Arnold. pp. 781–801.

Osei-Atweneboana, M. Y., Eng, J. K., Boakye, D. A., *et al*. (2007). Prevalence and intensity of *Onchocerca volvulus* infection and efficacy of ivermectin in endemic communities in Ghana: A two phase epidemiological study. *Lancet*. **369**: 2021-2029.

Osei-Atweneboana, M. Y., Awadzi, K., Attah, S. K., *et al*. (2011). Phenotypic Evidence of Emerging Ivermectin Resistance in *Onchocerca volvulu*s. *PLoS Negl Trop Dis*. **5** (3): e998.

Osei-Atweneboana, M. Y., Boakye, D. A., Awadzi, K., *et al*. (2012). Genotypic analysis of ß-tubulin in *Onchocerca volvulus* from communities and individuals showing poor parasitological response to ivermectin treatment. *Int J Parasitol: Drugs and Drug Res*. **2**: 20-28.

Keskin, O., Durell, S. R., Bahar, I., *et al*. (2002). Relating Molecular Flexibility to Function: A Case Study of Tubulin. *Biophysical J*. **83**: 663-680.

Pampiglione, S., Majori, G., Petrangeli, G., *et al*. (1985). Avermectins, MK-933 and MK-936, for mosquito control. *Trans R Soc Trop Med Hyg*. **79** (6): 797–9.

Pearlman, E., Garhart, C. A., Grand, D. J., *et al*. (1999). Temporal recruitment of neutrophils and eosinophils to the skin in a murine model for onchocercal dermatitis. *Am J Trop Med Hyg.* **61**: 14–18.

Pion, S. D., Kamgno, J., Demanga-Ngangue , *et al*. (2002). Excess mortality associated with blindness in the onchocerciasis focus of the Mbam Valley, Cameroon. *Ann Trop Med Parasitol*. **96**: 181–189.

Pion, S. D., Nana-Djeunga, H. C., Kamgno, J., *et al*. (2013). Dynamics of *Onchocerca volvulus* Microfilarial Densities after Ivermectin Treatment in an Ivermectin-naïve and a Multiply Treated Population from Cameroon. *PLoS Negl Trop Dis*. **7** (2): e2084

Plaisier, A. P., Alley, E. S., Boatin BA, *et al*. (1995). Irreversible effects of ivermectin on adult parasites in onchocerciasis patients in the onchocerciasis control program in west-Africa. *J Inf Dis*. **172**: 204–210.

Prichard, R. K. (2007). Ivermectin resistance and overview of the Consortium for Anthelmintic Resistance SNPs. *Expert Opin Drug Discov.* **2:** S41-S52.

Prichard, R. K., Hall, C. A., Kelly, J. D., *et al*. (1980). The problem of anthelmintic resistance in nematodes. *Aust Vet J*. **56**: 239–251.

Prost, A. (1986). The burden of blindness in adult males in the savanna villages of West Africa exposed to onchocerciasis. *Trans R Soc Trop Med Hyg*. **80**: 525–527.

Program Coordinating Committee and OEPA staff (PCC-OEPA). (2012). Guide to detecting a potential recrudescence of onchocerciasis during the posttreatment surveillance period: the American paradigm. *Res Rep Trop Med*. **3**:21-33.

Remme, J., Ba, O., Dadzie, K. Y., *et al*. (1986). A force-of-infection model for onchocerciasis and its applications in the epidemiological evaluation of the Onchocerciasis Control Programme in the Volta River Basin area. *Bull World Health Organ*. **64**: 667– 81.

Remme, J., Baker, R. H., De-Sole, G., *et al*. (1989). A community trial of ivermectin in the onchocerciasis focus of Asubende, Ghana. I. Effect on the microfilarial reservoir and the transmission of *Onchocerca volvulus*. *Trop Med Parasitol*. **40** (3): 367–374.

Remme, J. H. F. **(**1995**)**. The African programme for onchocerciasis control: preparing to launch. *Parasitol Today*. **11**: 403–406.

Remme, J. H. F., Alley, E. S. and Plaisier, A. P. (1995). Estimation and Prediction in Tropical Disease Control: The Example of Onchocerciasis. In: Epidemic Models: Their Structure and Relation to Data, ed. Mollison, D. Cambridge University Press. Cambridge, U.K. pp. 372–92.

Remme, J. H. F. (2004a). The Global Burden of Onchocerciasis in 1990. In *Global Burden of Disease 1990*. Geneva: World Health Organization. Available at: http://www3.who.int/whosis/burden/gbd2000docs/Onchocerciasis%201990.pdf. Accessed: January 9, 2013.

Remme, J. H. F. (2004b). Research for control: The onchocerciasis experience. *Trop Med Int Health*. **9** (2): 243–254.

Remme, J. H. F., Feenstra, P., Lever, P. R., *et al*. (2006). Tropical diseases targeted for elimination: Chagas disease, lymphatic Filariasis, onchocerciasis, and leprosy. In: Jamison, D. T., Breman, J. G. and Measham, A. R., eds. Disease control priorities in developing countries, 2nd ed. New York, Oxford University Press. pp.: 433-449.

Remme, J. H., Amazigo, U., Engels, D., *et al*. (2007). Efficacy of ivermectin against *Onchocerca volvulus* in Ghana. *Lancet*. **370** (9593): 1123–1124.

Richards, F. O., Boatin, B., Saurebrey, M., *et al*. (2004). Control of Onchocerciasis today: status and challenges. *Trends Parasitol*. **17** (12): 558-563.

Saint-André, A., Blackwell, N. M., Hall, L. R., *et al*. (2002). The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. Science 295: 1892–1895. Find this article online

Sauerbrey, M. (2008). The Onchocerciasis Elimination Program for the Americas (OEPA). Ann Trop Med Parasitol. **102** (Suppl 1): S25-9.

Seketeli, A., Adeoye, G., Eyamba, A., *et al*. (2002). The Achievements and Challenges of the African Programme for Onchocerciasis Control (APOC). *Ann Trop Med Parasitol*. **96** (Suppl. 1): 15–28.

Schulz-Key, H. (1978). A simple technique to assess the total number of *Onchocerca volvulus* microfilariae in skin snips. *Tropenmed Parasitol*. **29**: 51– 4.

Schulz-Key, H. (1988). The collagenase technique: how to isolate and examine adult *Onchocerca volvulus* for the evaluation of drug trials. Trop Med Parasitol. **39**: 423–440.

Schulz-Key, H., Albiez, E. J. and Buttner, D. W. (1977). Isolation of living adult *Onchocerca volvulus* from nodules. *Tropenmed Parasitol*. **28**: 428–430.

Schulz-Key, H., Jean, B., Albiez, E. J. (1980). Investigations on female *Onchocerca volvulus* for the evaluation of drug trials. *Tropenmed Parasitol*. **31**: 34–40.

Schulz-Key, H. and Karam, M. (1986). Periodic reproduction of *Onchocerca volvulus*. *Parasitol Today*. **2**: 284–86.

Shoop, W. L. (1993). Ivermectin resistance. *Parasitol Today*. **9:** 154–159.

Sober, E. (1984; 1993). The Nature of Selection: Evolutionary Theory in Philosophical Focus. *Univ Chicago Press*. ISBN 0-226-76748-5.

Specht, S., Brattig, N., Buttner, M., *et al*. (2009). Criteria for the differentiation between young and old *Onchocerca volvulus* filariae. *Parasitol Res*. **105**: 1531–1538.

Stern, C. (1962). Wilhelm Weinberg. *Genetics*. **47**: 1–5.

Strong, M. and Johnstone, P. W. (2007). Interventions for treating scabies. In: Strong, M. Cochrane Database of Systematic Reviews (Online). **3**: CD000320.

Strycharz, J. P., Yoon, K. S. and Clark, J. M. (2008). A new ivermectin formulation topically kills permethrin-resistant human head lice (Anoplura: Pediculidae). *J Med Entomol.* **45** (1): 75–81.

Sturchio, J. L. (2001). The case of ivermectin: Lessons and implications for improving access to care and treatment in developing countries. *Comm Eye Health*. **14** (38): 22–23.

Taylor, H. R., Greene, B. M. and Langham M. E. (1980). Controlled clinical trial of oral and topical diethylcarbamazine in treatment of onchocerciasis. *Lancet.* **i**: 943-6.

Taylor, M. J., Awadzi, K., Basáñez, M. G., *et al*. (2009). Meeting Report on Onchocerciasis Control: Vision for the Future from a Ghanaian perspective. *Parasites & Vectors*, **2**:7.

Thylefors, B. (1978). Ocular onchocerciasis. *Bull World Health Organ.*56 (1): 63-73.

Thylefors, B. and Alleman, M. (2006). Towards the elimination of onchocerciasis. *Ann Trop Med Parasitol*. **100**:733–746.

The Carter Center (TCC). (2013a). River Blindness Program: Health Programmes. *Home Page*. Available at: http://www.cartercenter.org/health/river_blindness/index.html. Accessed: May 3, 2013.

The Carter Center (TCC). (2013b). Lymphatic Filariasis Elimination Program: Health Programmes. *Home Page*. Available at: http://www.cartercenter.org/health/lf/index.html. Accessed: May 3, 2013.

Timmann, C., Kamp, E., Kleensang, A., *et al*. (2008). Human Genetic Resistance to *Onchocerca volvulus*: Evidence for Linkage to Chromosome 2p from an Autosome-Wide Scan. *JID*. **198** (427): 1-7

Toe, L., Boatin, B. A., Adjami, A., *et al*. (1998). Detection of *Onchocerca volvulus* infection by O-150 polymerase chain reaction analysis of skin scratches. *J Infect Dis.* **178**: 282–5.

Udall, D. N. (2007). Recent updates on onchocerciasis: diagnosis and treatment. *Clin Infect Dis*. **44**: 53–60.

Victoria, J. and Trujillo, R. (2001). Topical ivermectin: a new successful treatment for

scabies. *Pediatr Dermatol.* **18** (1): 63–5.

Vlassoff, C., Weiss, M., Ovuga, E. B., *et al*. (2000). Gender and the stigma of onchocercal skin disease in Africa. *Soc Sci Med*. **50** (10): 1353–1368.

Williams, R. C. J., Shah, C. and Sackett, D. (1999). Separation of tubulin isoforms by isoelectric focusing in immobilized pH gradient gels. *Anal Biochem.* **275** (2): 265–7

Wolstenholme, A. J., Fairweather, I., Prichard, R., *et al*. (2004). Drug resistance in veterinary helminths. *Trends Parasitol.* **20**: 469–476.

Workneh, W**.,** Fletcher, M. **and** Olwit, G. **(**1993**)**. Onchocerciasis in fields workers at Baya Farm, Teppi Coffee Plantation Project, Southwestern Ethiopia: Prevalence and Impact on Production. *Acta Tropica.* **54:** 89–97.

World Health Organization (WHO). (1995). Onchocerciasis and its control. Report of a WHO expert committee on onchocerciasis control. WHO Technical Report Series, number **852**. Geneva: World Health Organization.

World Health Organization (WHO). (1997**)**. Twenty years of onchocerciasis control in West Africa. Review of the Work of the Onchocerciasis Control Programme in West Africa. Geneva: World Health Organization.

World Health Organization (WHO). (2001). Certification of elimination of human onchocerciasis: criteria and procedures. Geneva, Switzerland: World Health Organization. Available at: http://whqlibdoc.who.int/hq/2001/WHO_CDS_CPE_CEE_2001.18b.pdf. Accessed: June 9, 2013.

World Health Organization/African Programme for Onchocerciasis Control (WHO). (2002). Onchocerciasis control in special intervention zones including Sierra Leone in the OCP area. Joint Programme Committee Report, September 2002. Ouagadougou (Burkina Faso): World Health Organization/African Programme for Onchocerciasis Control.

World Health Organization (WHO). (2005). Onchocerciasis (river blindness). *Wkly Epidemiol Rec*. **80**: 257–260. Available:http://www.who.int/wer/2005/wer8030.pdf. Accessed 26 July 2006.

World Health Organization (WHO). (2008). Global programme to eliminate lymphatic

Filariasis. *Wkly Epidemiol Rec*. **83**:333-341.

World Health Organization (WHO). (2012a). InterAmerican Conference on Onchocerciasis, 2011: interruption of transmission in Guatemala and Mexico. *Wkly Epidemiol Rec*. **87**: 309-15.

World Health Organization (WHO). (2012b). Research Priorities for Helminth Infections. Technical Report of the TDR Disease Reference Group on Helminth Inections. WHO Technical Report Series; number **972**. Geneva: World Health Organization.

World Health Organization (WHO). (2013a). Onchocerciasis. Media Centre; Fact sheet. Fact sheet N°374 (February 2013). Available at: http://www.who.int/mediacentre/factsheets/fs374/en/index.html. Accessed: July 6, 2013

World Health Organization (WHO). (2013b). African Programme for Onchocerciasis Control. Available at: http://www.who.int/blindness/partnerships/APOC/en/. Accessed May 3, 2013.

Xu, M., Molento, M., Blackhall, W., *et al*. (1998). Ivermectin resistance in nematodes may be caused by alteration of Pglycoprotein homolog. *Mol Biochem Parasitol*. **91:** 327–335.

Yates, D. M. and Wolstenholme, A. J. (2004). An ivermectin-sensitive glutamate-gated chloride channel subunit from Dirofilaria immitis. *Int J Parasitol.* **34** (9): 1075–81.

APPENDICES

Appendix 1

Additional Tables of Results

Table A3: Nodule infection in the 5 communities

Community	Total Patients Assessed for Nodules	No. Nodule Positive Patients	Nodule Prevalence (%)
Agblekeme II	53	19	35.8490566
Chabon	94	34	36.17021277
Fawoman	92	19	20.65217391
Kojobone	219	28	12.78538813
Nyire	126	22	17.46031746
Total	**584**	**128**	20.89041096

Table A4: Microfilaria infection in the 5 communities

Community	Tot Patients Assessed for Mf at Pre Trt	No. Mf Positive patients at Pre Trt	Microfilariae Prevalence (%)
Agblekeme II	53	12	22.64150943
Chabon	94	30	31.91489362
Fawoman	92	11	11.95652174
Kojobone	219	19	8.675799087
Nyire	126	15	11.9047619
Total	**584**	**87**	14.89726027

Table A5: Cross-tabulation of Embryogram characteristics by age in the six communities

			Embryogram Characteristic				
Age			Degenerate Mf	No Mf	Normal Mf	Normal Mf + Degenerate Mf	Total
M	Community	AGBLEKEME II	9	4	1	6	20
		CHABON	19	20	0	4	43
		JAGBENGBENDO	13	10	2	15	40
		KOJOBONE	3	1	1	5	10
		NYIRE	5	5	0	1	11
		TAKUMDO	5	6	1	8	20
	Total		54	46	5	39	144
M+	Community	AGBLEKEME II	5	1	0	2	8
		CHABON	4	2	0	2	8
		JAGBENGBENDO	3	2	0	6	11
		KOJOBONE	1	2	1	1	5
		NYIRE	0	1	0	0	1
		TAKUMDO	3	0	0	2	5
	Total		16	8	1	13	38
old	Community	AGBLEKEME II	8	4	1	1	14
		CHABON	9	6	0	1	16
		JAGBENGBENDO	7	7	1	4	19
		KOJOBONE	4	6	1	1	12
		NYIRE	1	2	0	0	3
		TAKUMDO	6	6	0	4	16
	Total		35	31	3	11	80
Y	Community	AGBLEKEME II	0	2		1	3
		CHABON	2	5		0	7
		JAGBENGBENDO	2	5		0	7
		NYIRE	1	2		1	4
		TAKUMDO	0	2		0	2
	Total		5	16		2	23

Table A6: Embryogram characteristic of the 285 worms in the six communities

Embryogram Characteristic	Frequency	Percentage
Degenerate mf	110	38.6
No mf	101	35.4
Normal mf	9	3.2
Normal mf + Degenerate mf	65	22.8
Total	285	100.0

Table A7: Proportion of subjects with Mf repopulation at day 90 after second study IVM treatment

Community	Number of subjects with Mf repopulation at day 270 Post Treatment	Total Subjects Assessed at day 270 Post-Treatment	Proportion of subjects with Mf repopulation at day 270 Post-Treatment (%)
Agblekeme II	1	12	8.3
Chabon-Wiae	3	30	10
Fawoman	0	11	0
Kojobone	2	19	10.5
Nyire	1	15	6.7
Total	**7**	**87**	**8**

Appendix 2

Additional Graphs of Results

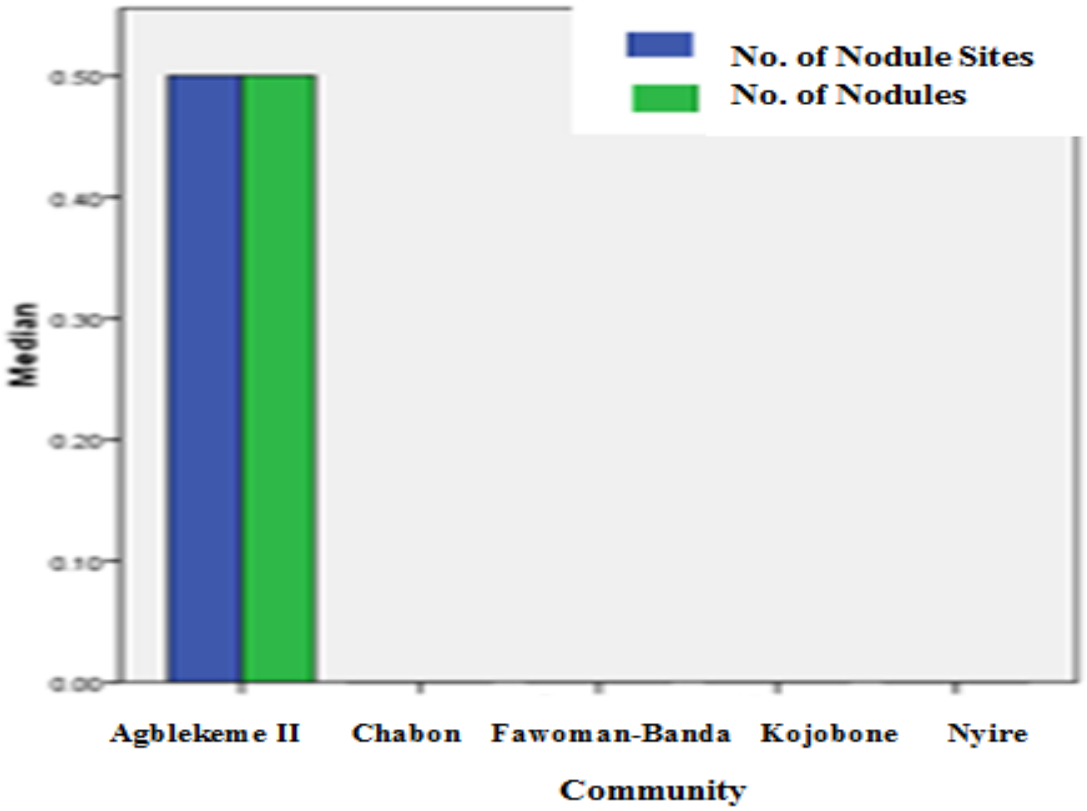

Figure A1: Average (Median) number of nodules and nodule sites

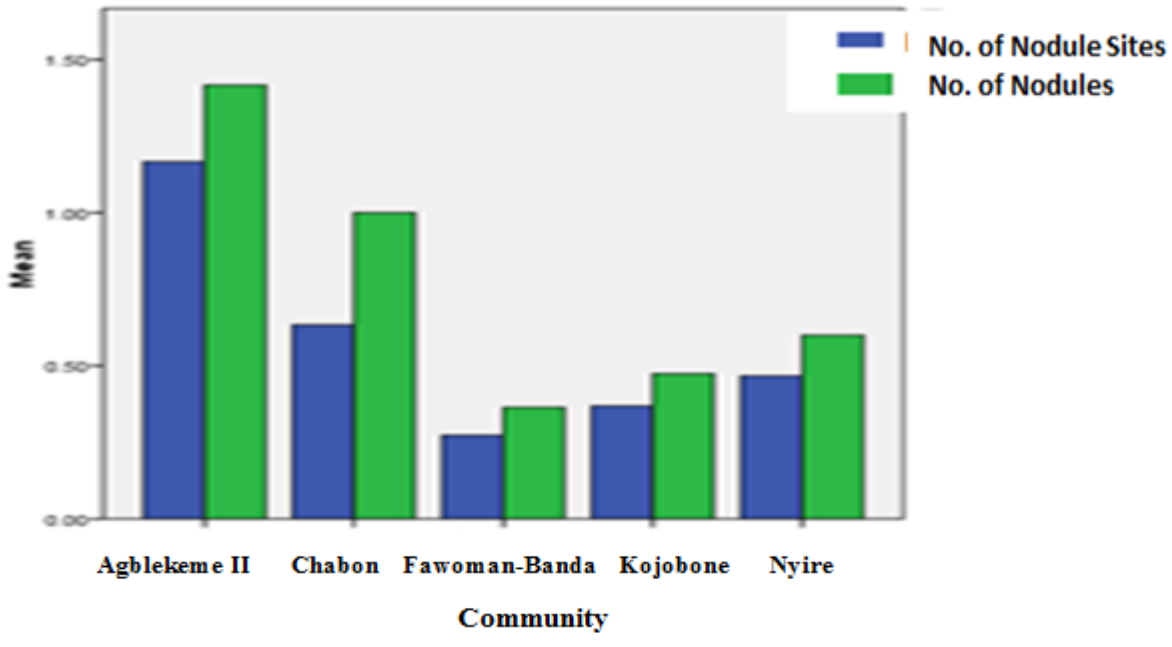

Figure A2: Average (Mean) number of nodules and nodule sites

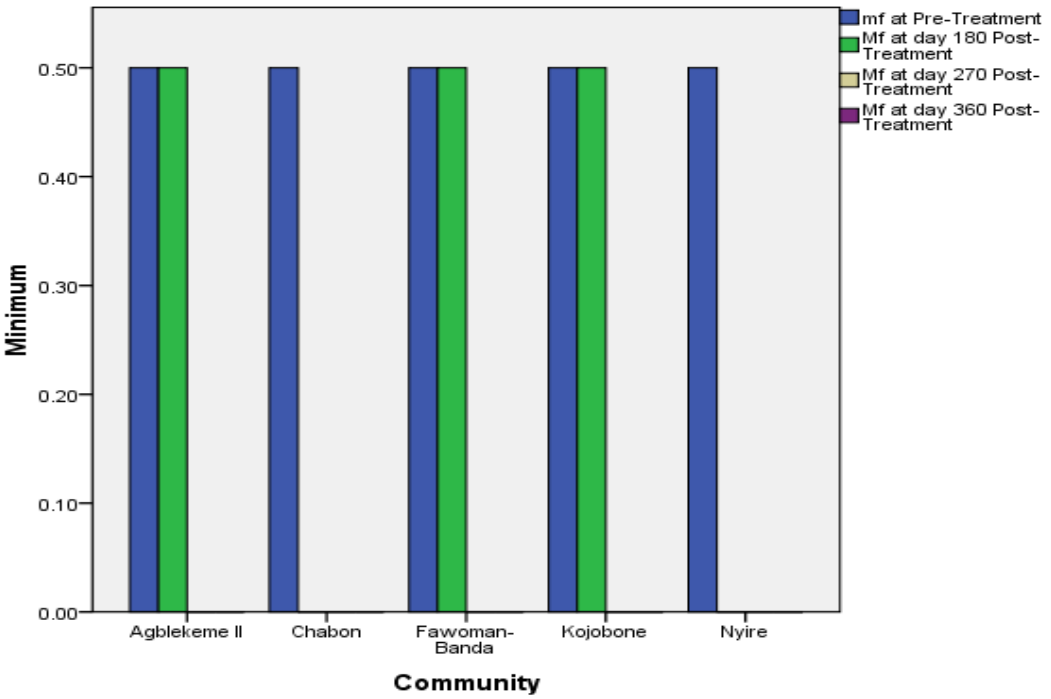

Figure A3: Minimum microfilaria at pre-treatment and at days 180-, 270- and 360 post-treatment

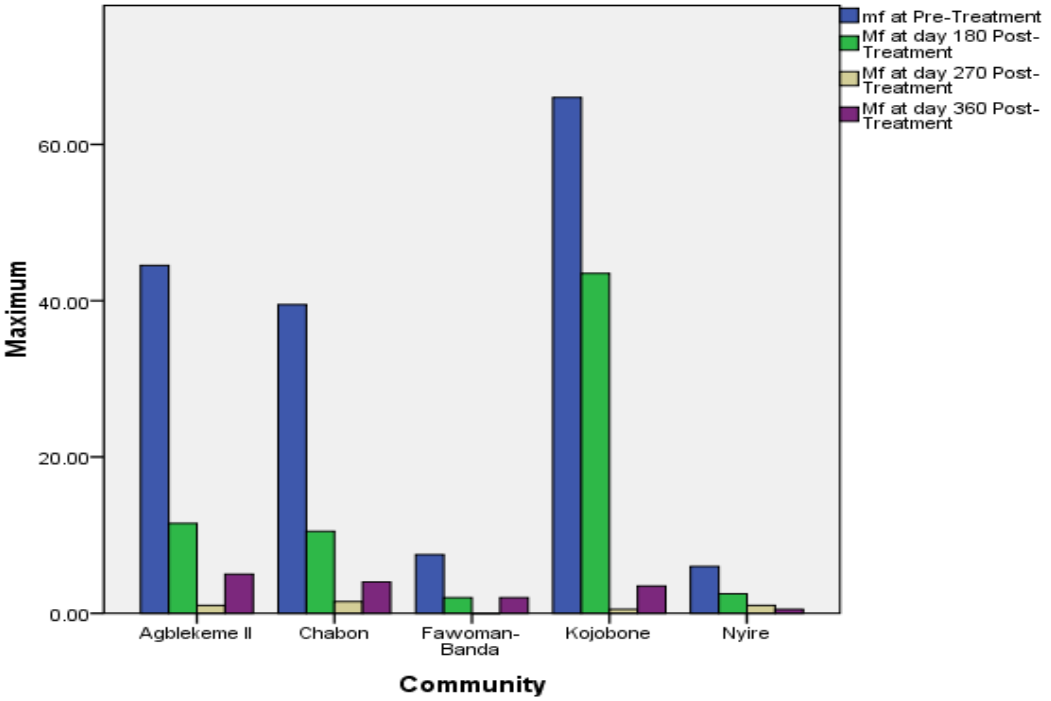

Figure A4: Maximum microfilaria at pre-treatment and at days 180-, 270- and 360 post-treatment

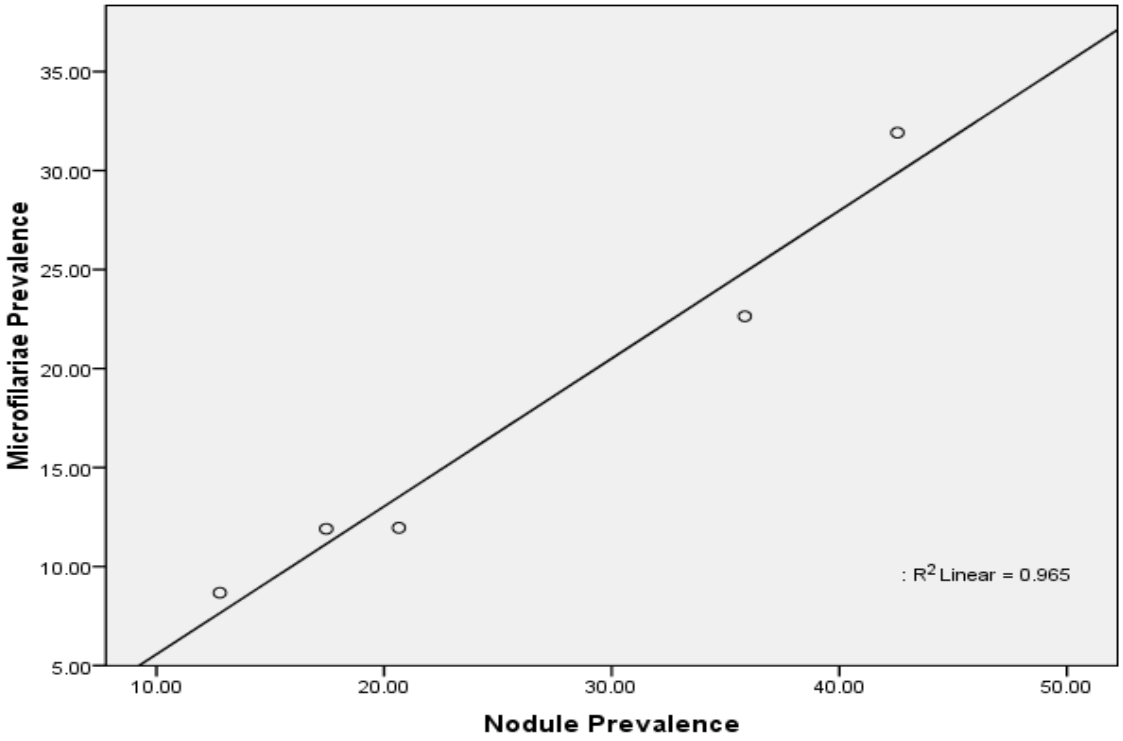

Figure A5: A plot of microfilariae prevalence against nodule prevalence in the 5 communities with linear line fit

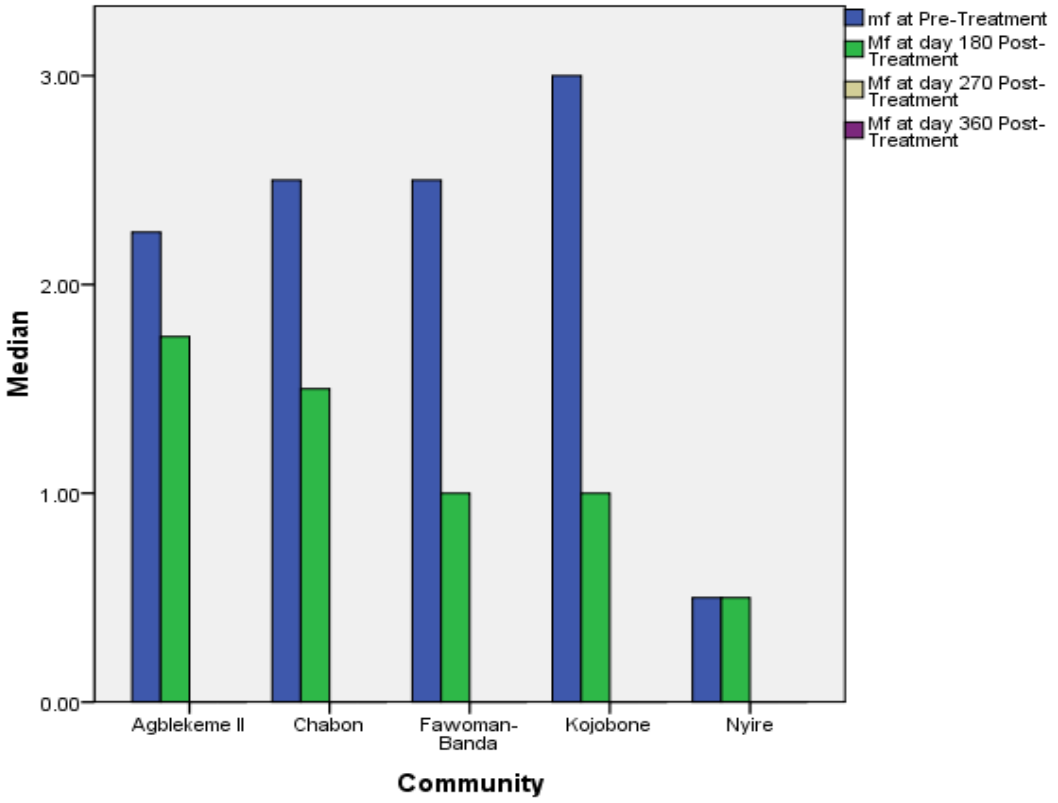

Figure A6: Average (median) microfilaria at pre-treatment and at days 180-, 270- and 360 post-treatment

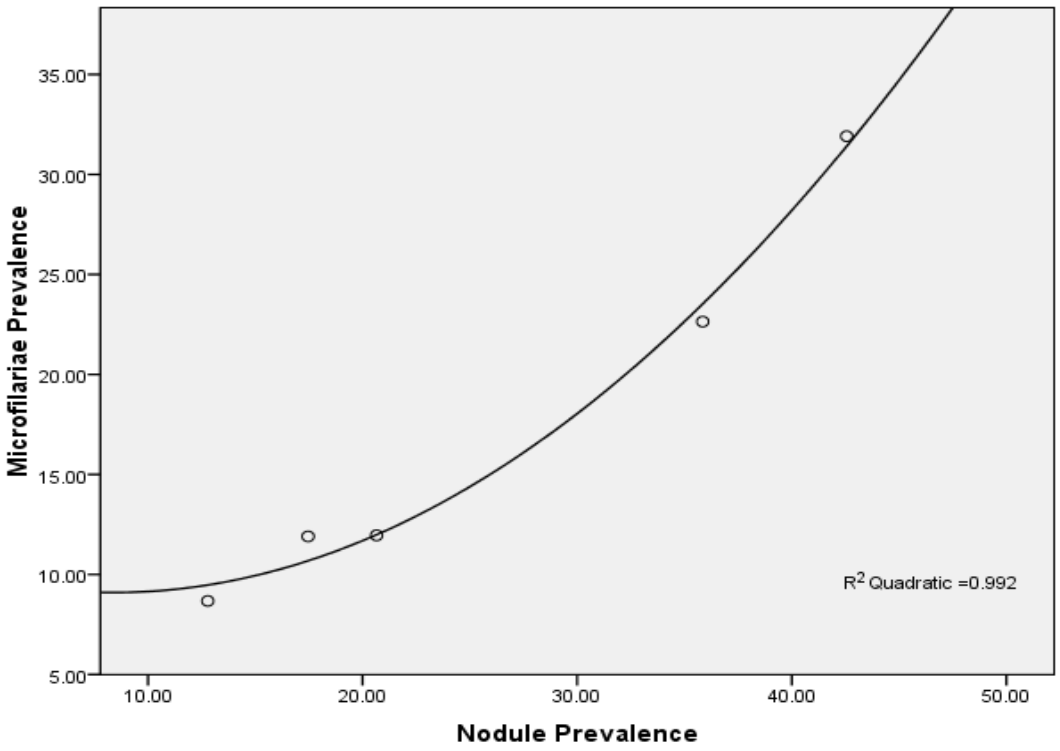

Figure A7: A plot of microfilariae prevalence against nodule prevalence in the 5 communities with quadratic line fit

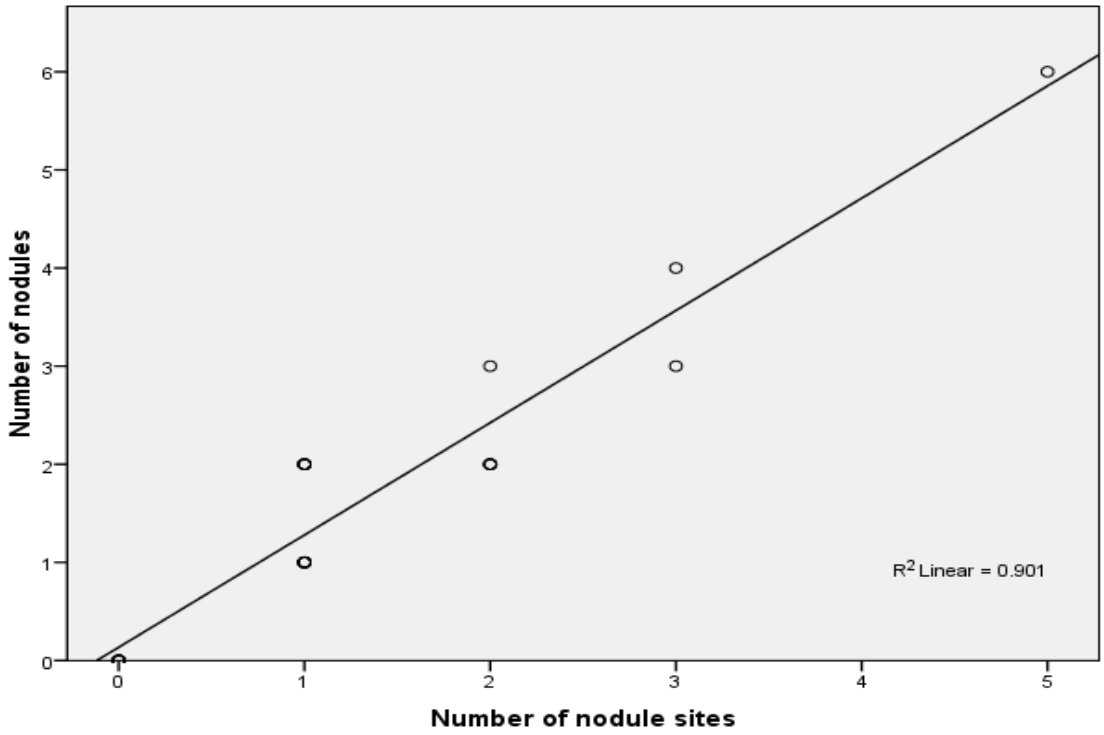

Figure A8: Correlation of number of nodules and nodule sites in Agblekeme II with linear line fit

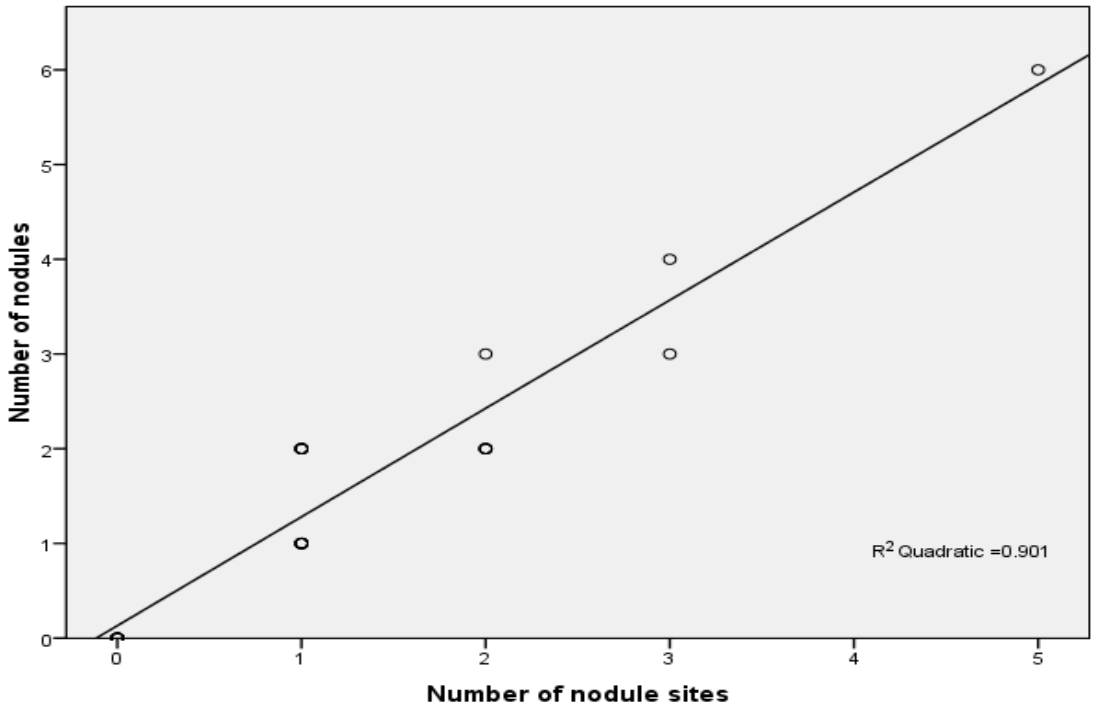

Figure A9: Correlation of number of nodules and nodule sites in Agblekeme II with quadratic line fit

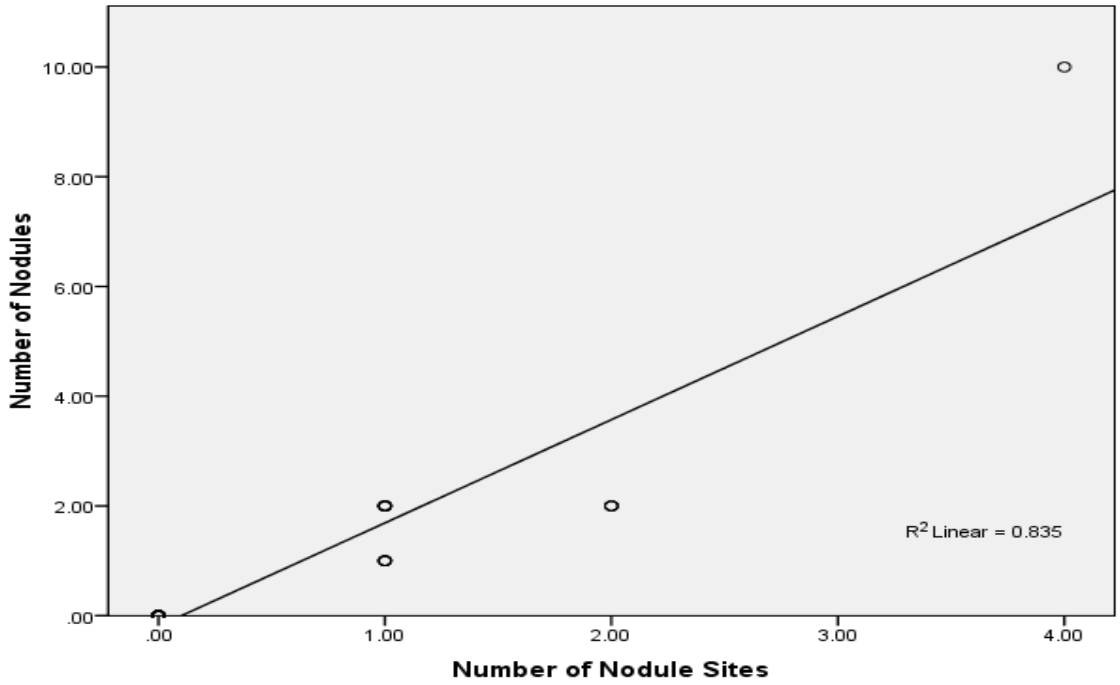

Figure A10: Correlation of number of nodules and nodule sites in Chabon with linear line fit

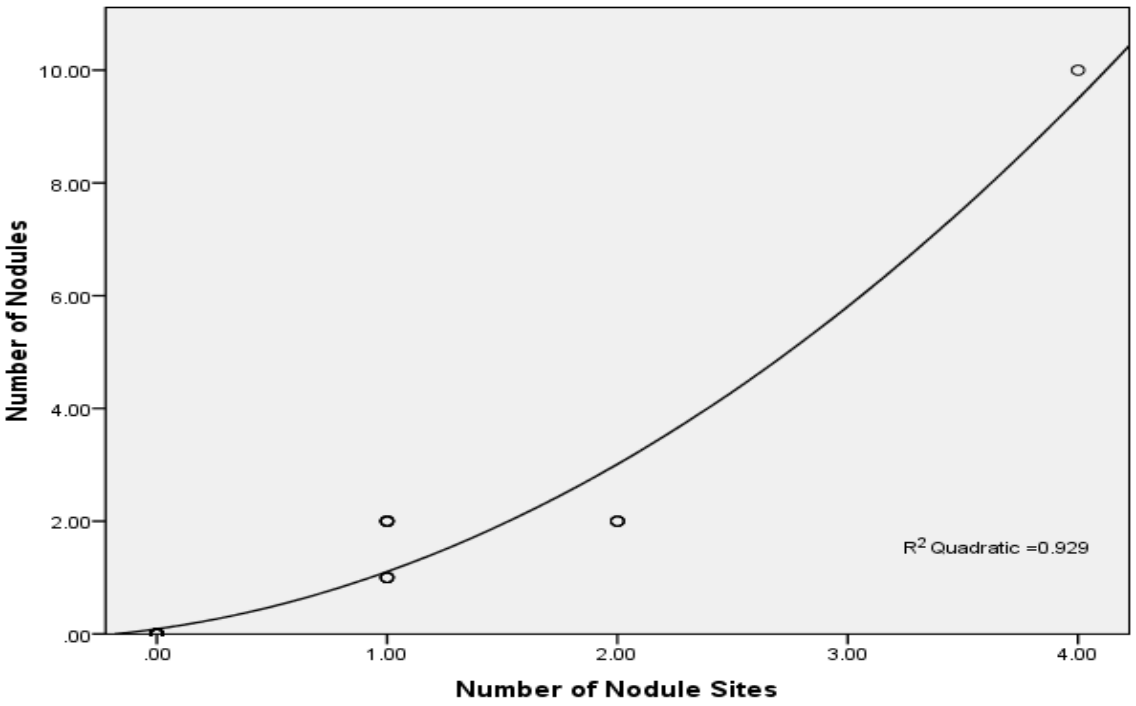

Figure A11: Correlation of number of nodules and nodule sites in Chabon with quadratic line fit

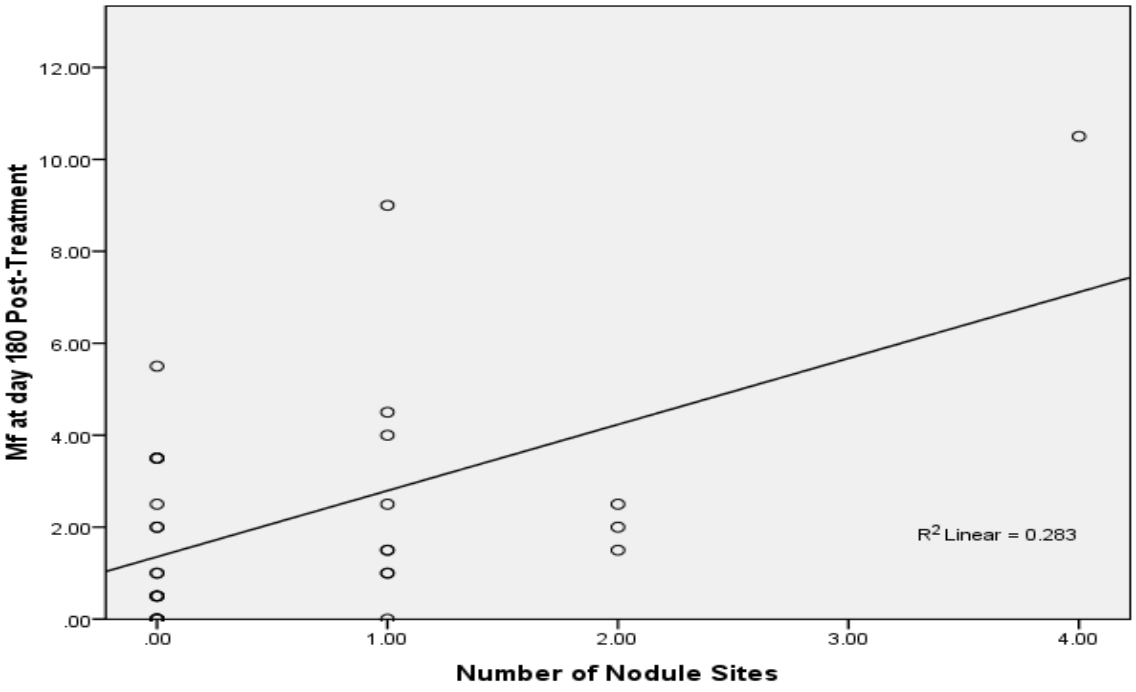

Figure A12: Correlation of Mf at day 180 post-treatment and number of nodule sites in Chabon with linear line fit

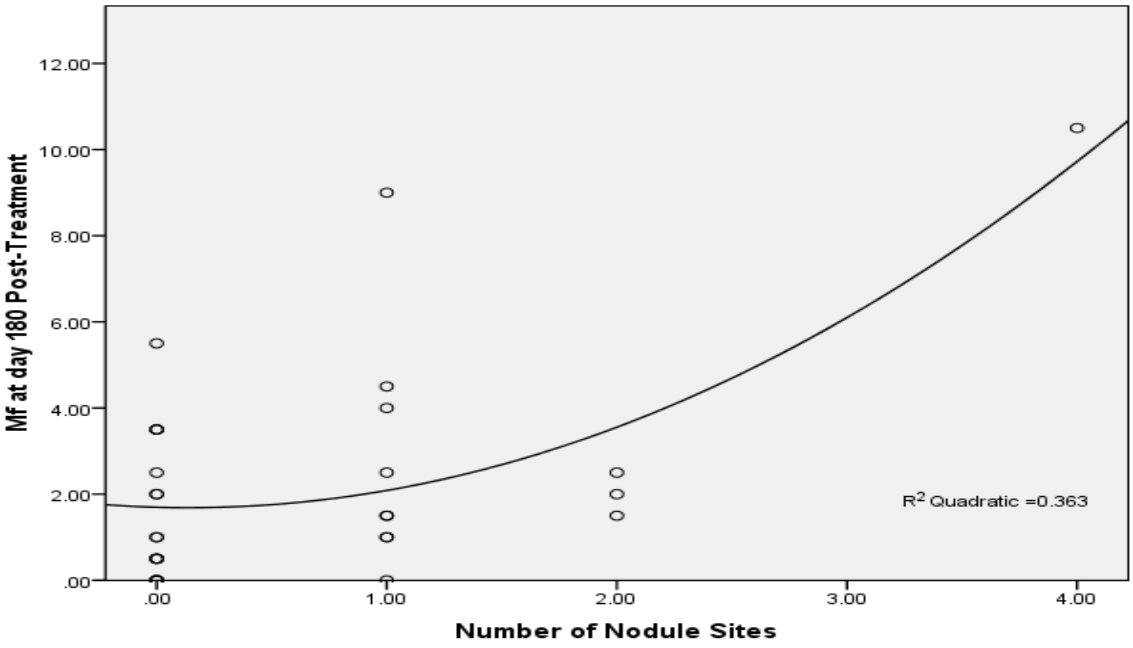

Figure A13: Correlation of Mf at day 180 post-treatment and number of nodule sites in Chabon with quadratic line fit

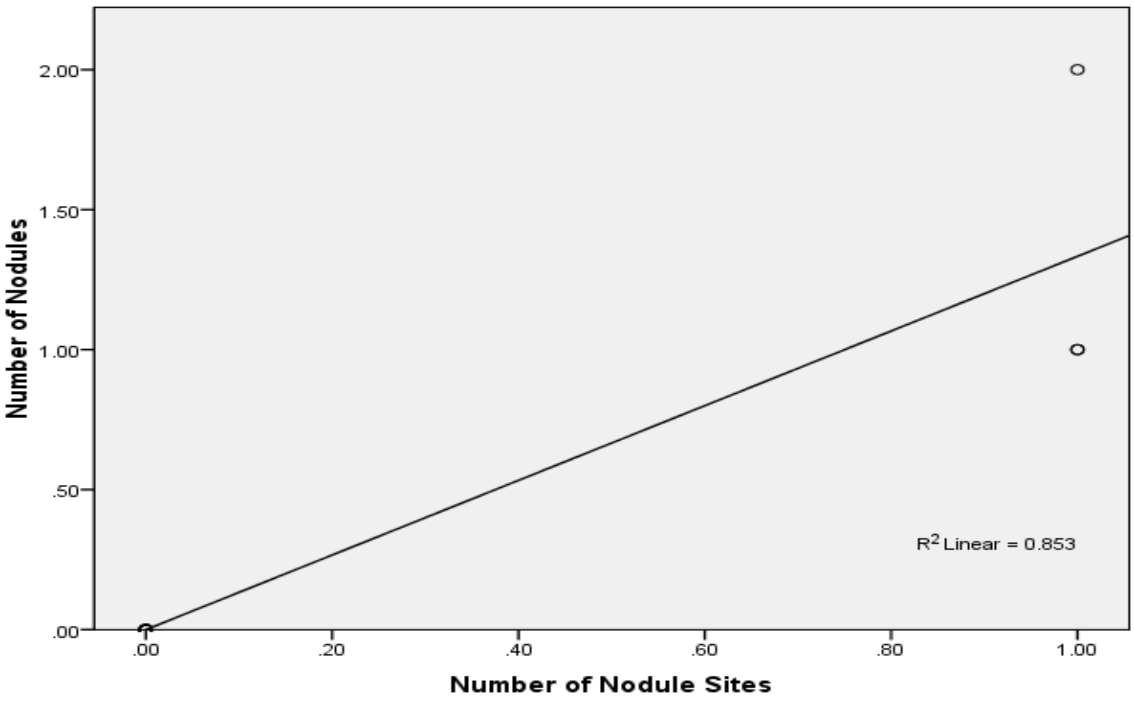

Figure A14: Correlation of number of nodules with nodule sites in Fawoman-Banda with linear line fit

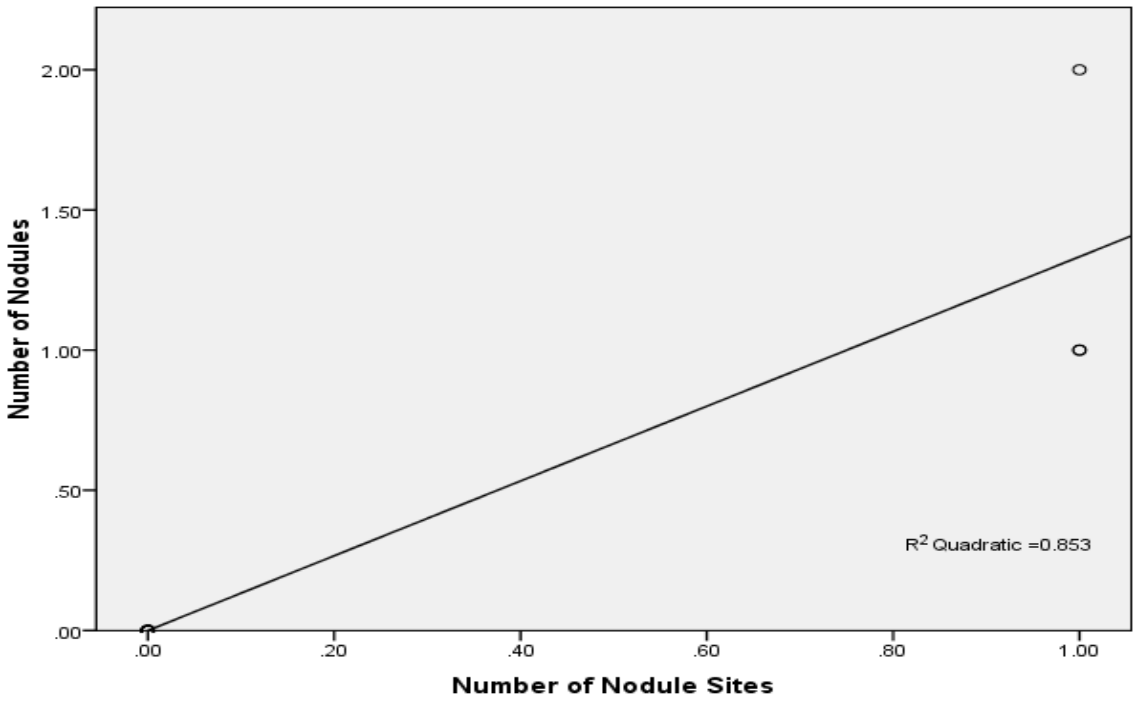

Figure A15: Correlation of number of nodules with nodule sites in Fawoman-Banda with quadratic line fit

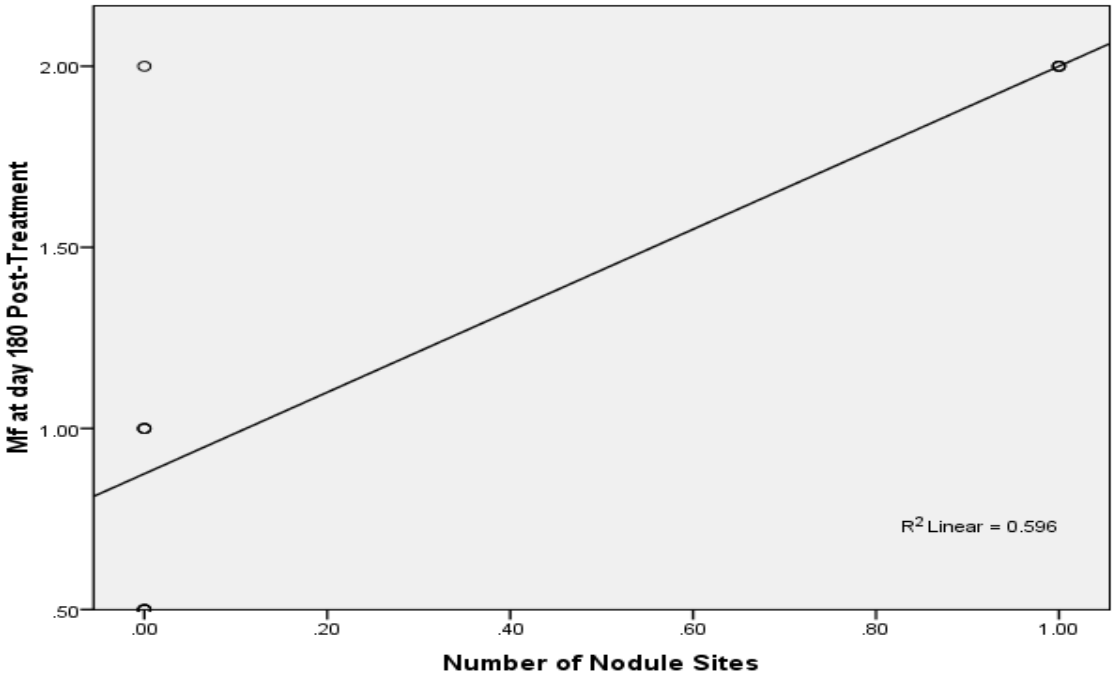

Figure A16: Correlation of number of Mf at day 180 post-treatment with number of nodule sites in Fawoman-Banda with linear line fit

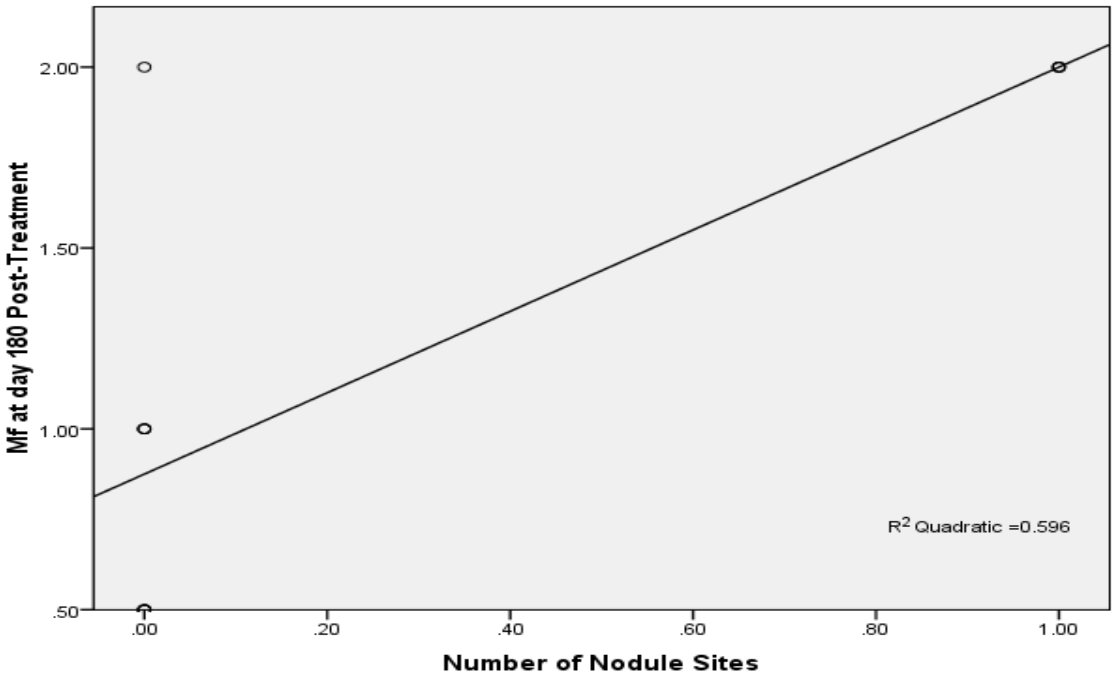

Figure A17: Correlation of number of Mf at day 180 post-treatment with number of nodule sites in Fawoman-Banda with quadratic line fit

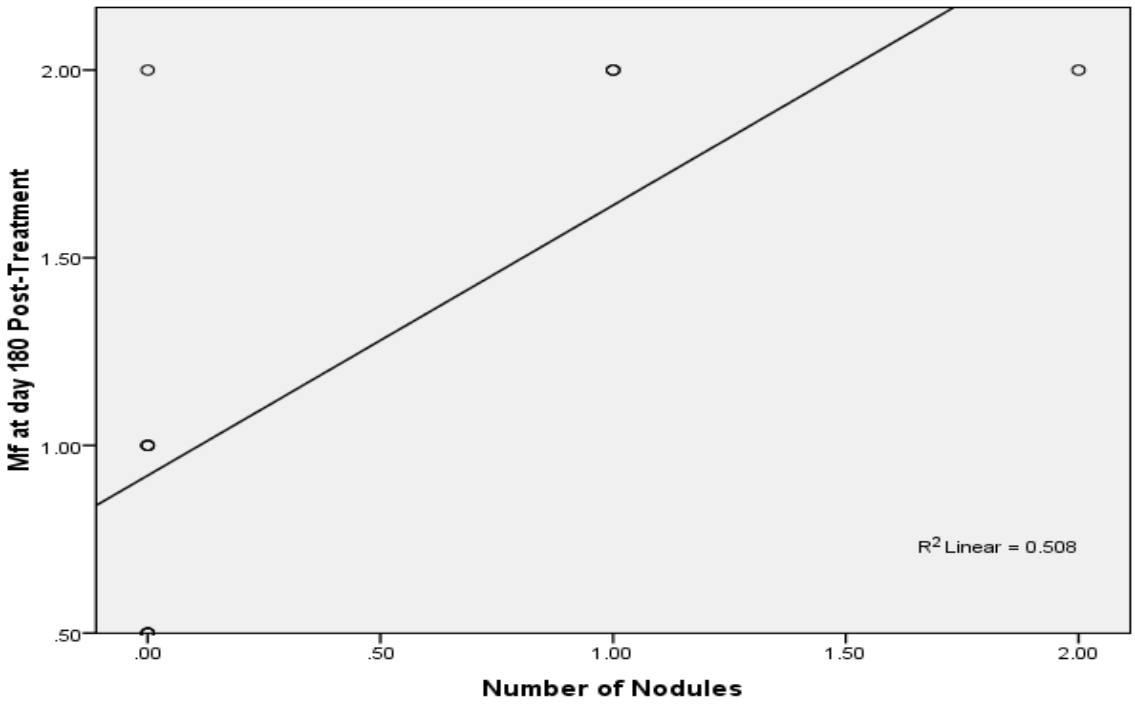

Figure A18: Correlation of number of Mf at day 180 post-treatment with number of nodules in Fawoman-Banda with linear line fit

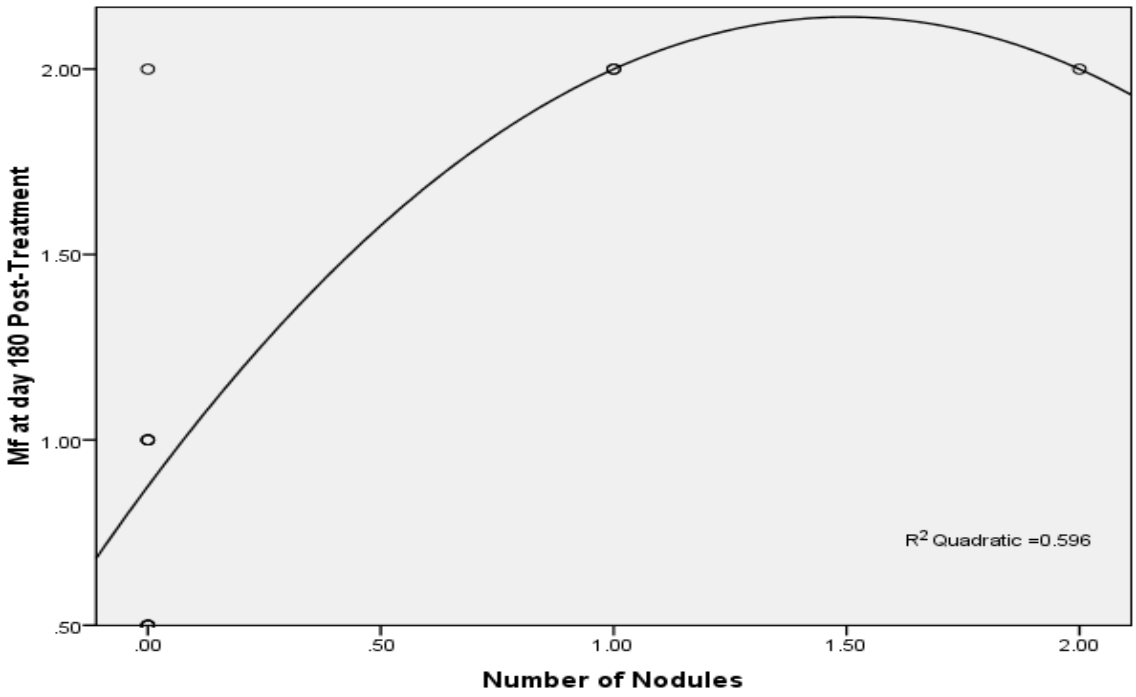

Figure A19: Correlation of number of Mf at day 180 post-treatment with number of nodules in Fawoman-Banda quadratic line fit

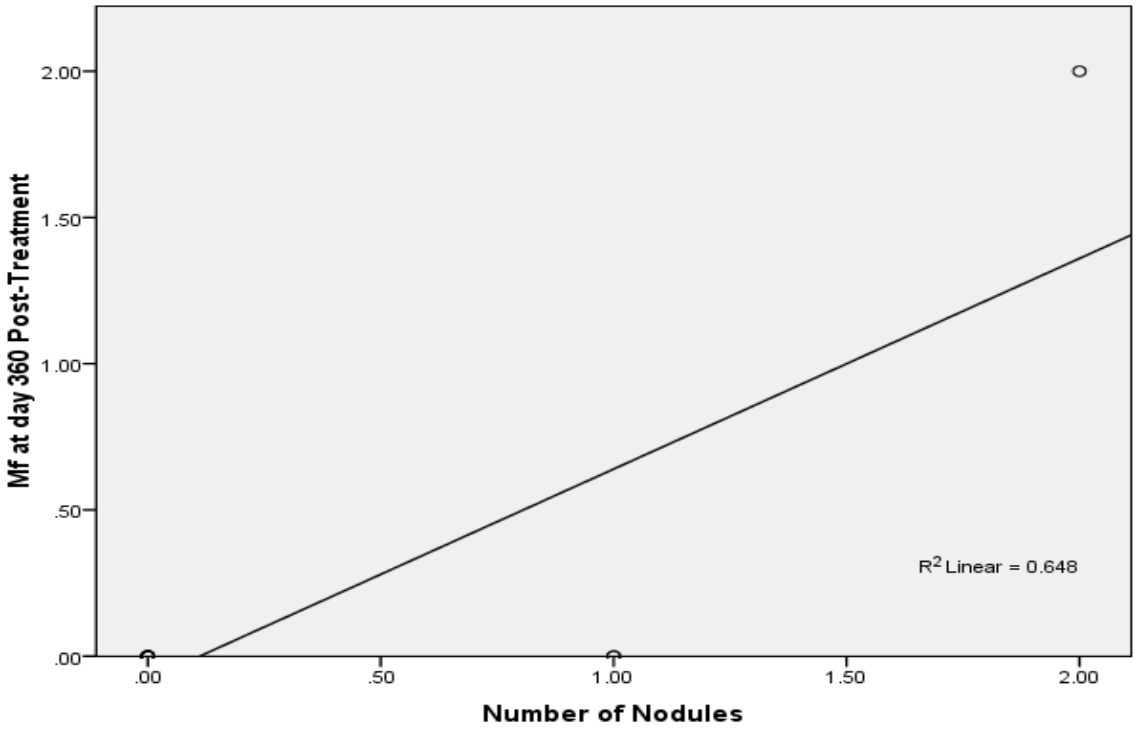

Figure A20: Correlation of number of Mf at day 360 post-treatment with number of nodules in Fawoman-Banda with linear line fit

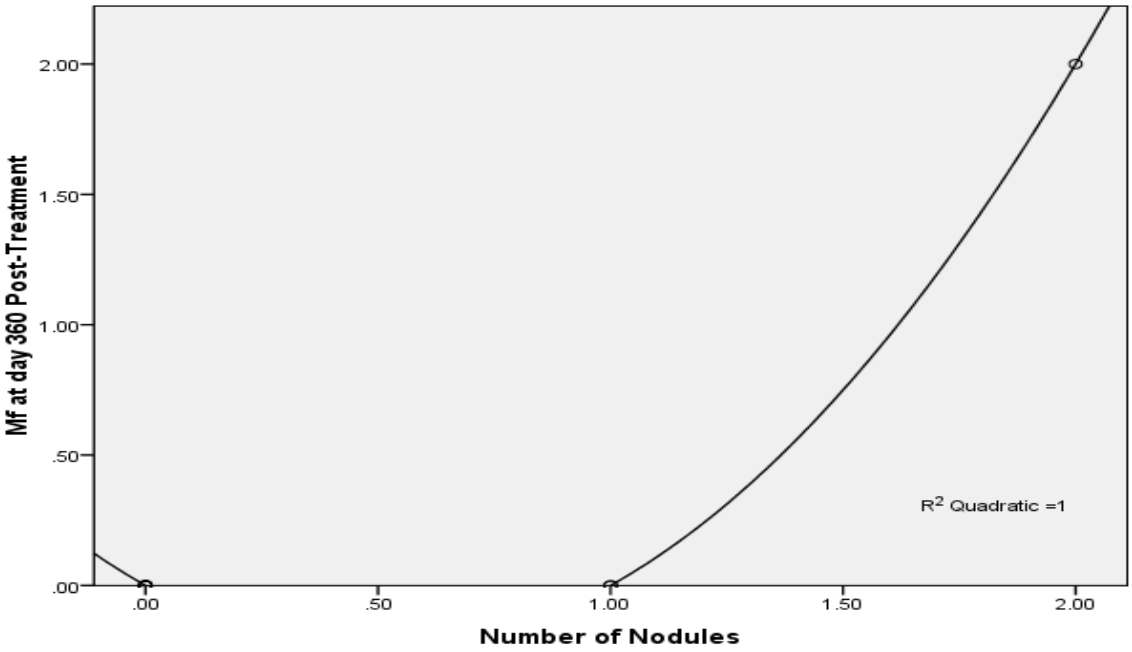

Figure A21: Correlation of number of Mf at day 360 post-treatment with number of nodules in Fawoman-Banda with quadratic line fit

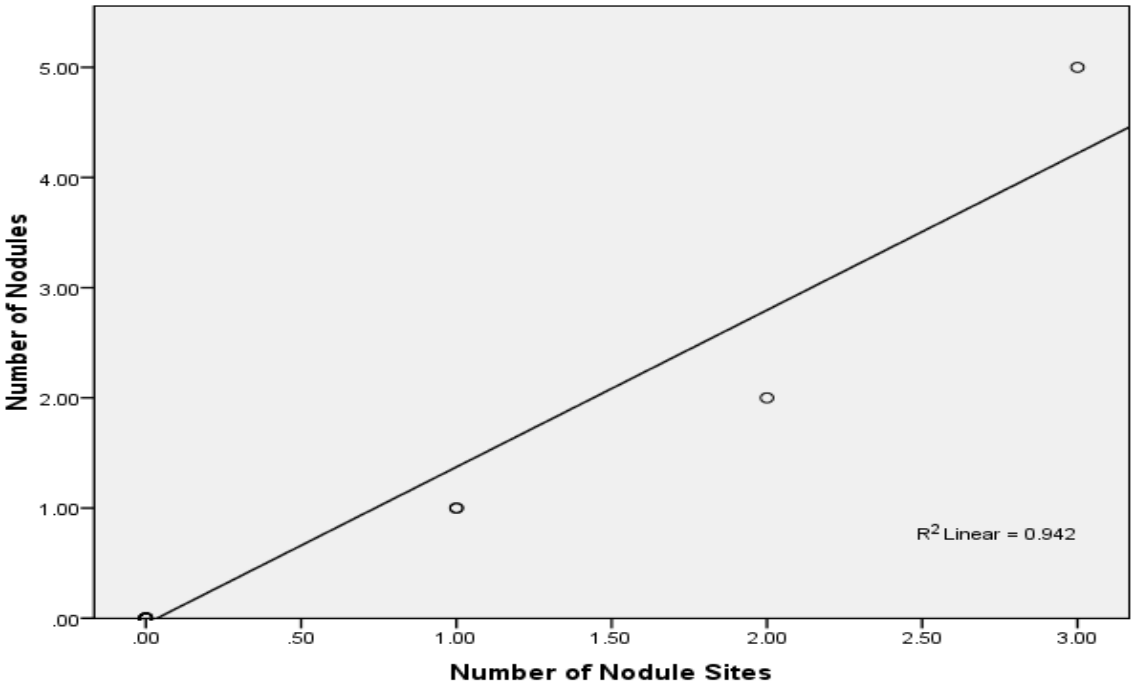

Figure A22: Correlation of number of nodules with nodule sites in Kojobone with linear line fit

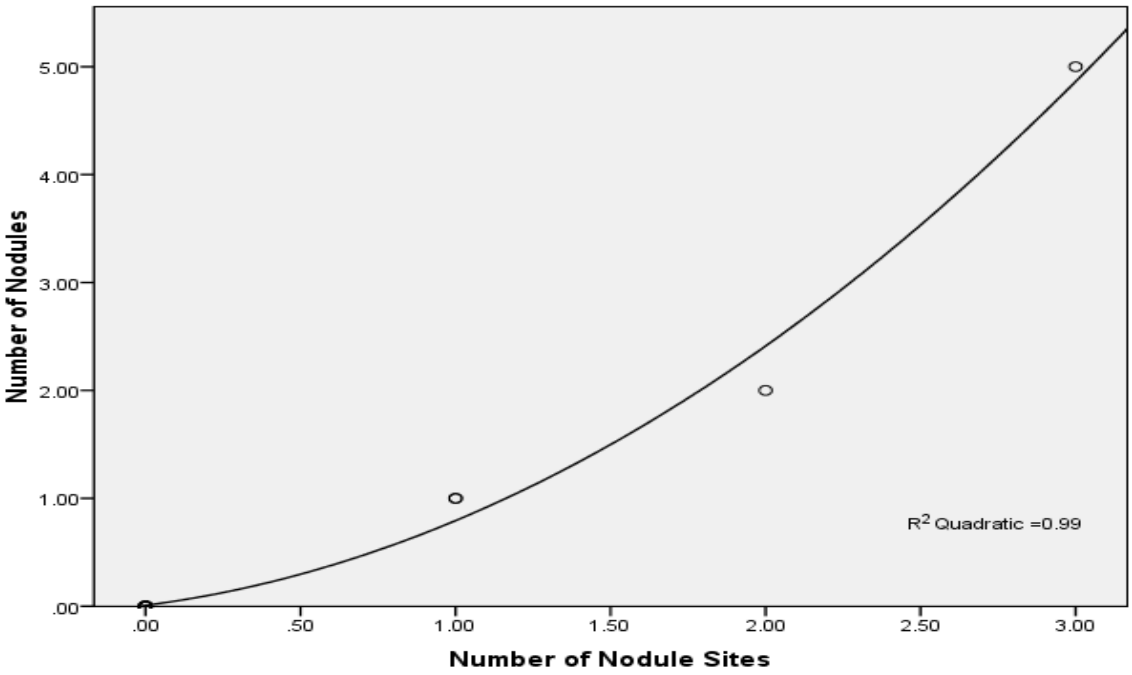

Figure A23: Correlation of number of nodules with nodule sites in Kojobone with quadratic line fit

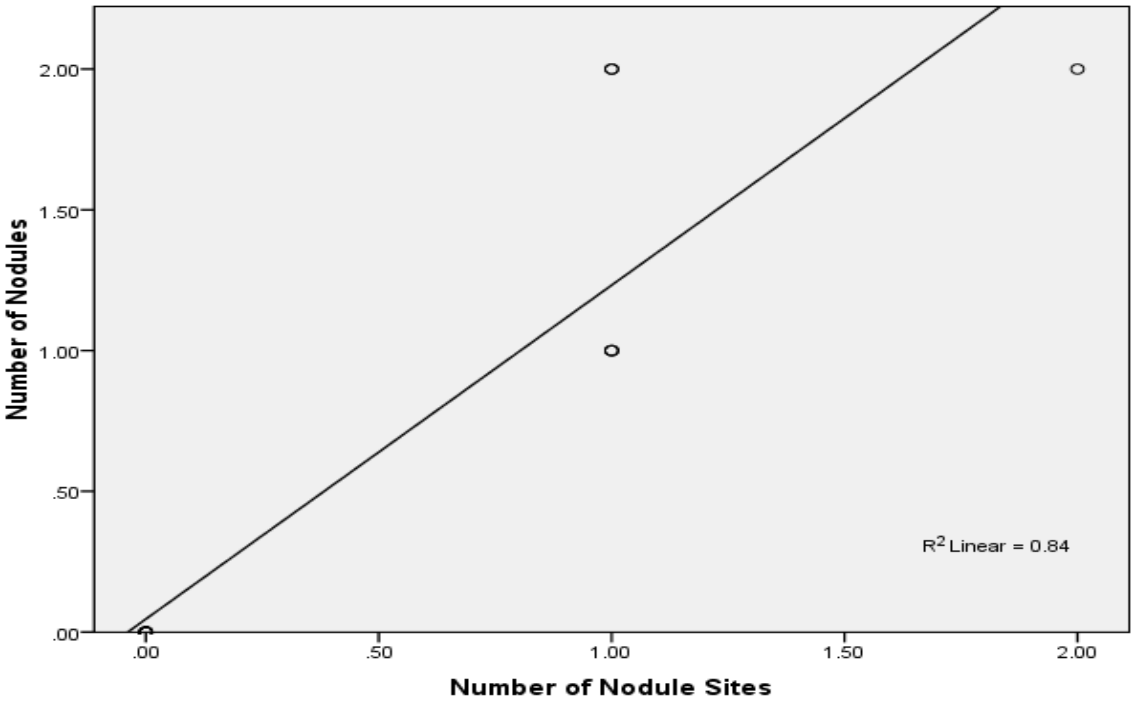

Figure A24: Correlation of number of nodules with nodule sites in Nyire with linear line fit

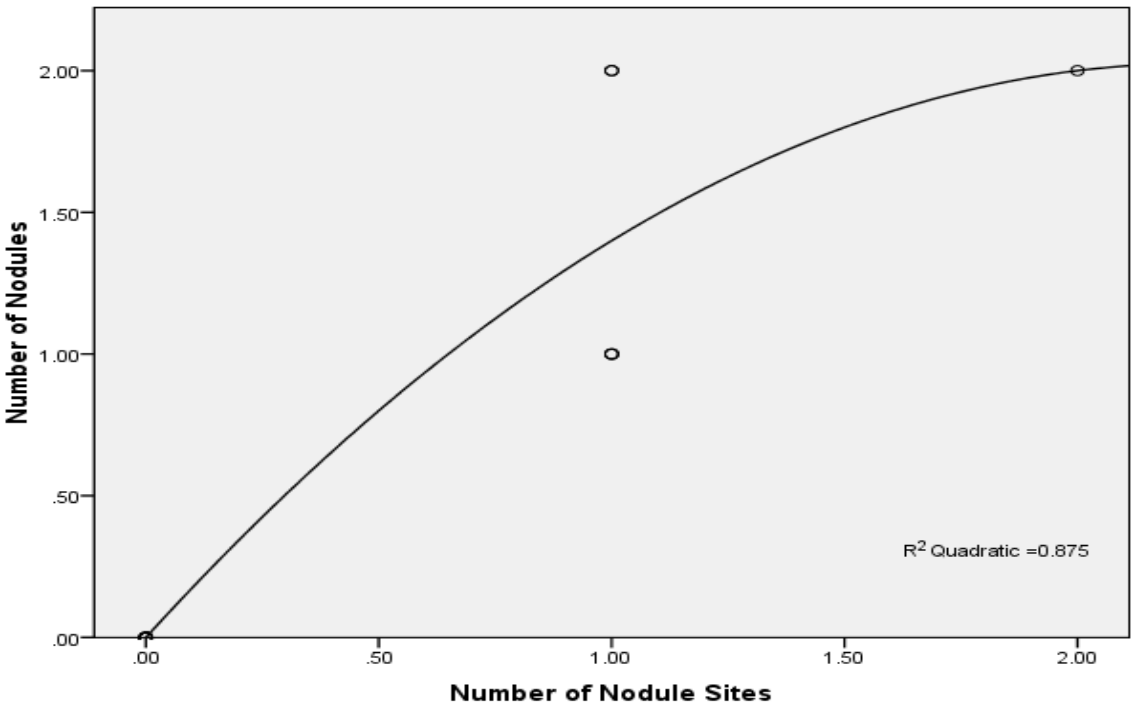

Figure A25: Correlation of number of nodules with nodule sites in Nyire with quadratic line fit

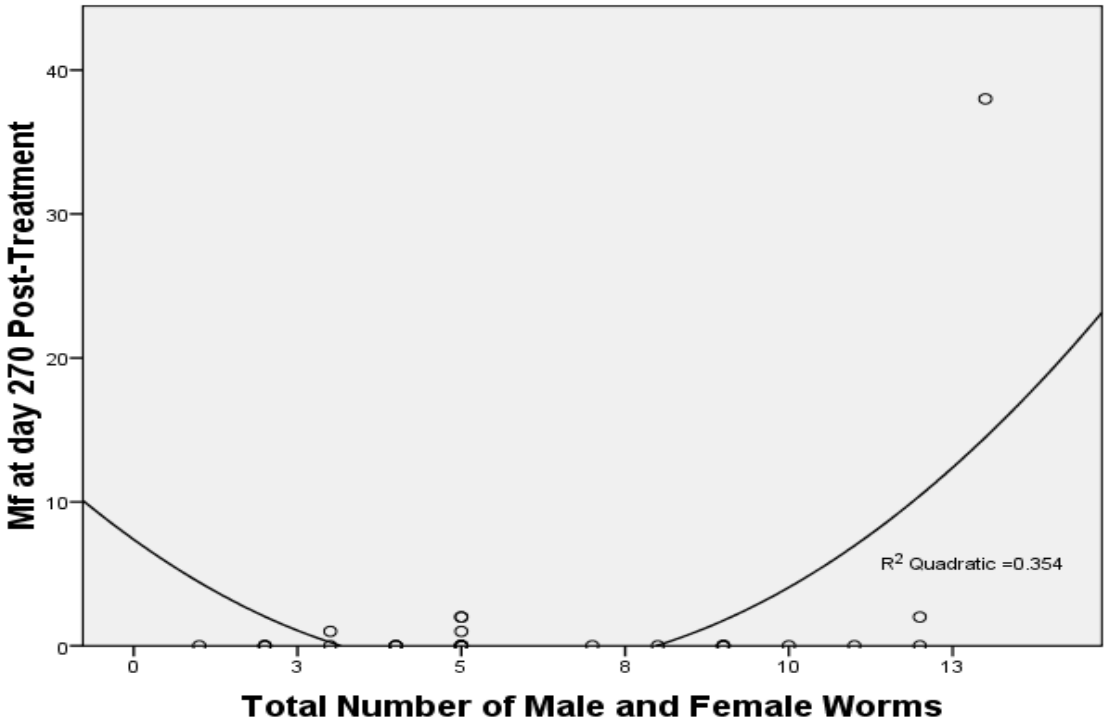

Figure A26: Correlation of Total number of male and female worms with Mf at day 270 Post-treatment with Quadratic line fit

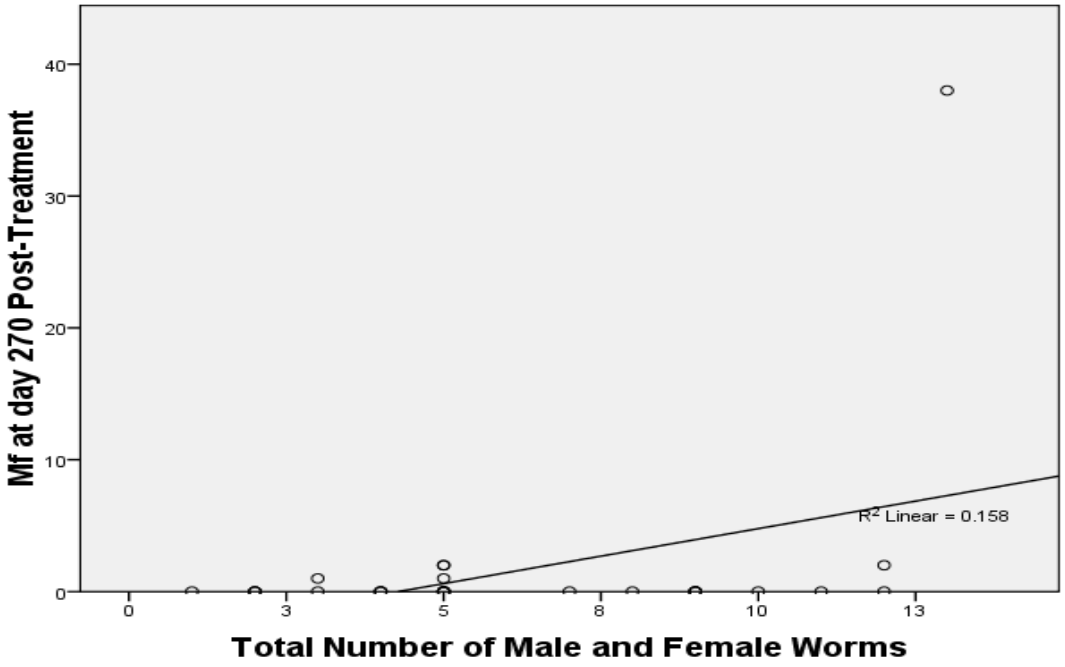

Figure A27: Correlation of Total number of male and female worms with Mf at day 270 Post-treatment with Linear line fit

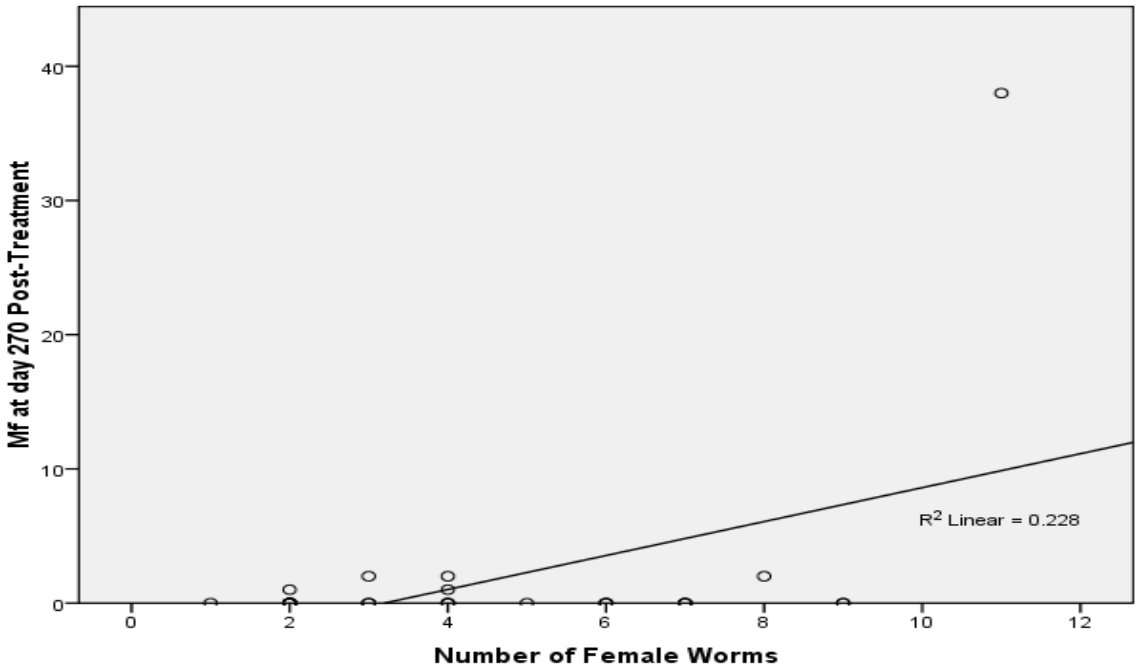

Figure A28: Correlation of number of female worms with Mf at day 270 Post-treatment with Linear line fit

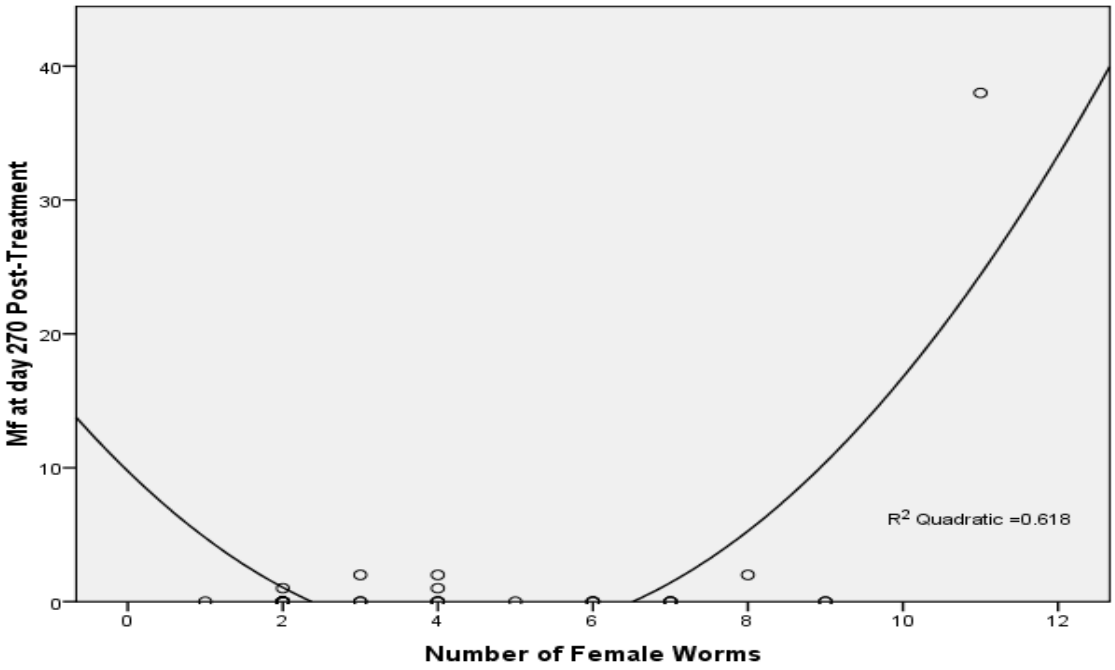

Figure A29: Correlation of number of female worms with Mf at day 270 Post-treatment with Quadratic line fit

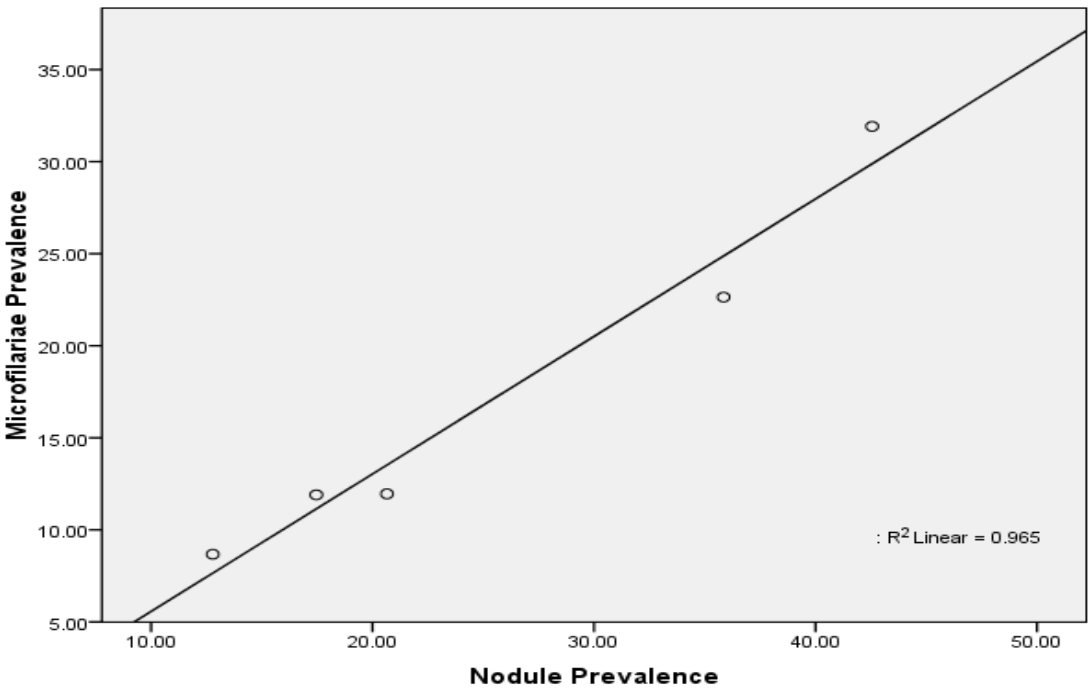

Figure A30: A plot of microfilariae prevalence against nodule prevalence in the 5 communities with linear line fit

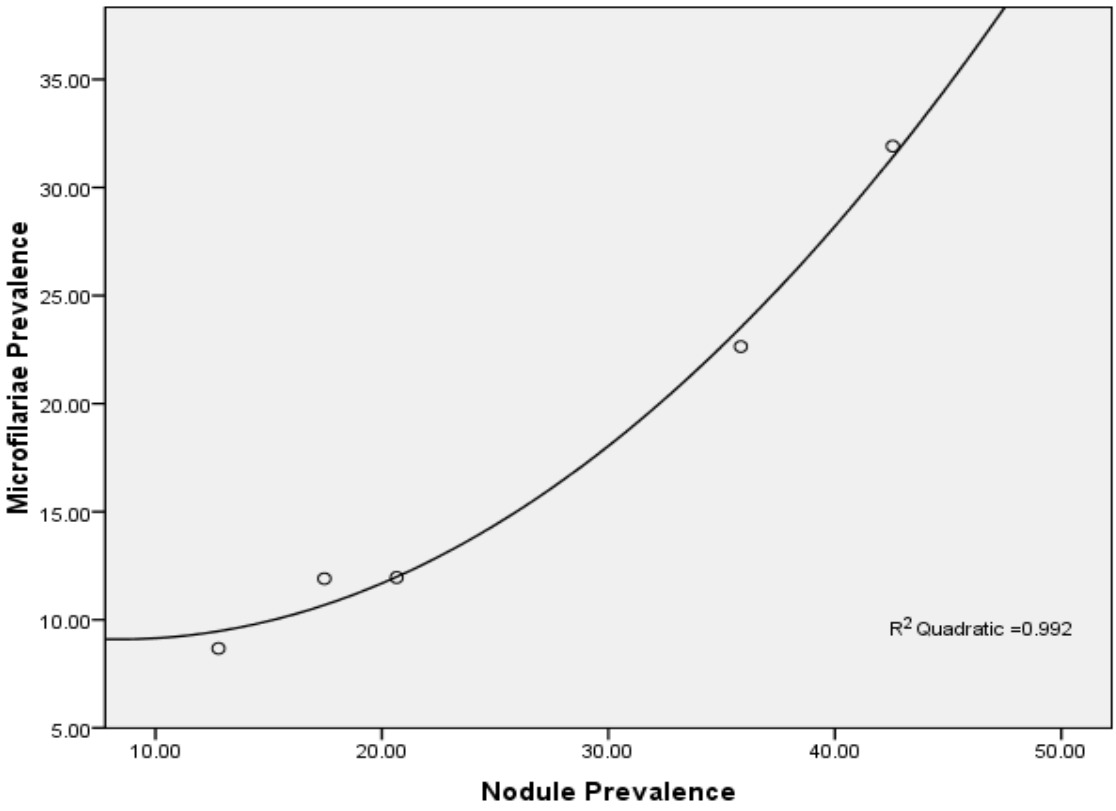

Figure A31: A plot of microfilariae prevalence against nodule prevalence in the 5 communities with quadratic line fit

Appendix 3

COMPLETE GENE SEQUENCE OF BETA TUBULIN GENE WITH SNPs (3696bp)

```
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 241 AAGATATTTC GGTAAATATG TTTGATTGTT TACATTCATA TTTCGAGCTG TCCATCGCTT
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                                                          G       C
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          ATA                                                   T
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3601 TCAGGAAGGT GAATCGGAAT ACATTGAACA GGAAGAGTAA AAGCCGAATT CCAGCACACT
3661 GGCGGCCGTT ACTAGTGGAT CCGAGCTCGG TACCAA

APPENDIX 4

Informed Consent Form

COUNCIL FOR SCIENTIFIC AND INDUSTRIAL RESEARCH-INSTITUTIONAL REVIEW BOARD

PARTICIPANTS INFORMATION AND CONSENT FORM FOR ONCHOCERCIASIS EPIDEMIOLOGICAL STUDY AND NODULECTOMIES

Title: Genetic monitoring for ivermectin resistance in *Onchocerca volvulus*

Principal Investigators:

1. Dr Mike Yaw Osei-Atweneboana (CSIR, Accra, Ghana)

Address: Council for Scientific and Industrial Research Accra, Ghana.

2. Prof. Grant Warwick

Address: Latrobe University, Australia

3. Prof. Roger Prichard

Address: Institute of Parasitology, McGill University, Montreal, Canada

Students Investigators:

1. Ernest Tawiah Gyan

Address: MPhil student, Department of Animal Biology & Conservation Science, University of Ghana, Legon

2. Samuel Armoo:

Address: PhD Student, Address: Latrobe University, Australia

General Information about Research

Human onchocerciasis is a disease that causes disability usually from troublesome itching, skin disease, and loss of vision. In the worst cases, people may become partly or totally blind as a result of getting this disease. The disease is caused by a worm that lives inside people (a parasite) and is carried among people by small biting flies known as "blackflies" Onchocerciasis is still a problem here in Ghana, and we need to find ways to stop this happening. Currently the World Health Organization (WHO) through the African Programme for Onchocerciasis Control (APOC) is finding ways of controlling the disease and prevents people from having skin and eye problems.

The strategy involves the administration of ivermectin to every person living in areas where the disease presents a problem. The treatment is available through the community distributors of ivermectin and it has to be taken once a year. In Ghana, many communities with onchocerciasis have received more than 10 annual rounds of ivermectin, yet the disease is still present. This probably means that in addition to the distribution of ivermectin we need to find other ways to help control the disease or the treatment has to be taken more often.

The purpose of the study is to find ways to monitor if the drugs are still working as well as they were 10 years ago and therefore we need people, like you, to volunteer to have you examined for the worms in your skin and to surgically remove them. The worm samples will

also be used for genetic analysis to help us find a way of monitoring the usefulness of the drug for onchocerciasis.

Possible Risks and Discomforts

Surgically removing the nodules will be painful, but you will be given medicine to reduce the pains. Also taking the skin snips will feel like a pinch, so you may experience a little discomfort during the procedure. The sites from where we take the snips should, be kept clean and dry for as long as possible. The examination of your body for the presence and number of nodules is not at all painful. There will be no risk of infection with onchocerciasis to yourself, and in any case you will receive ivermectin treatment after the study during community-directed treatment as scheduled for your village.

Possible Benefits

This study will directly help you to get better by removing all the adult worms we can see from your body. It will also benefit future generations of children and adults in Ghana by helping us to understand how well the treatment is working within individuals and the community and how best to combine control methods against the parasite that causes river blindness. Also, you will be treated with ivermectin which will help against onchocerciasis and other worms in your body.

Alternatives to Participation

If you decide not to participate in this study, you will still receive the regular annual ivermectin treatment which is being offered by the Ministry of Health (MOH). Moreover, medical care available to you in your respective community or village will not be affected or compromised.

Confidentiality

Once you agree to participate, we will assign you a code number. We will be very careful about the information that we have collected from you and we will make absolutely sure that when we tell people about our findings, no-one will be able to discover your personal identity.

Compensation

Since the surgical operation will not allow you to carry out your normal daily activities, we will compensate you for the time loss by giving you a daily farm replacement allowance of five Ghana cedis (Gh¢5.00) a day for four days.

Voluntary Participation and Right to Leave the Research

Before giving your consent, by signing this document, the methods, inconveniences, risks and benefits, and alternatives will be explained and your questions answered to your satisfaction.

Your participation in this study may be ended by the investigators for reasons that will be explained to you. New information that develops during the study will be given to you, especially if it may affect your willingness to continue with the study. We may also publish any interesting results that come from this study in journals.

You do not give up any legal rights by signing this document. You can obtain further information from Council for Scientific and Industrial Research (CSIR) –Water Research Institute (+233-302-775351) or Dr. Mike Osei-Atweneboana (at 0203176771).

The Institutional Review Board of the Council for Scientific and Industrial Research, Accra, Ghana, have reviewed this study, evaluated the potential risks and benefits and have granted approval to allow us to solicit participants in this study.

Notification of Significant New Findings

After this study, we will continue our studies in the area collecting data (flies collected from humans and from rivers) and we will analyze the data in Ghana. We will communicate these results as we go along in the project. We will let you know of our results when we revisit the communities later or through the Council for Scientific and Industrial Research, or through the community ivermectin distributors.

Contacts for Additional Information

If you have any further questions please contact Dr Mike Yaw Osei-Atweneboana of the Council for Scientific and Industrial Research, Water Research Institute, Accra, and he will be able to explain further to you what our study is about. You should also contact this person if you would like to withdraw from the study, or if you have any worries regarding your participation in this study. The contact address is as follows:

Dr Mike Y. Osei-Atweneboana

Council for Scientific and Industrial Research

Water Research Institute.

Division of Environmental Biology and Health

P.O. Box M 32, Accra

Ghana

Mobile: 0203176771

A copy of this written information sheet and of the signed Informed Consent form will be given to you to keep.

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of the Council for scientific and Industrial research (CSIR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302777651 (ext 1002) or email addresses: csirirb_iacuc@csir.org.gh You may also contact the Chairman of the IRB, through mobile number 0204362635 when necessary.

Volunteer Agreement

I confirm that I have read / been read the Participant Information Sheet explaining the research Project on "**Development of genetic markers for early detection and monitoring of ivermectin resistance in *Onchocerca volvulus* and its implication for onchocerciasis control**". I understand that the researchers are asking me to participate in their study by having onchocerciasis nodules that contain the adult worms removed from my body and skin snips taken from me. I have been given a chance to ask questions and feel that all of my questions have been answered. I know that my participation is entirely voluntary and that this will have no consequences for my usual participation in community-based ivermectin treatment. I also know that I can withdraw my consent at any time in the future.

_____	_____	_____
Name	Date	Signature or mark of volunteer
_____	_____	_____
Name	Date	Signature of Witness
_____	_____	_____
Name	Date	Signature of Person taking Consent

Appendix 5

Ethical Clearance

COUNCIL FOR SCIENTIFIC AND INDUSTRIAL RESEARCH
HEAD OFFICE

P. O. BOX M.32
ACCRA.
GHANA
WEST - AFRICA

TEL: 233-30-2777651-4 (4 Lines)
FAX: 233-30-2777655
E-MAIL: headoffice@csir.org.gh
WEBSITE: www.csir.org.gh

Our Ref: .....

Date: 25th November, 2011

ETHICAL CLEARANCE

RPN 003/CSIR-IRB/2011

On 12th October, 2011, the Council for Scientific and Industrial Research (CSIR) Institutional Review Board (IRB), at a full Board meeting reviewed and approved your protocol.

TITLE OF PROTOCOL : **Development of genetic markers for early detection and Monitoring of ivermectin resistance in *Onchocerca Volvulus* and its implementation for onchociasis control**

PRINCIPAL INVESTIGATOR : Dr Mike Yaw Osei-Atweneboana

COLLABORATOR : Prof. Mark Taylor

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation. Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to CSIR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 11th October, 2012. You are to submit annual reports for continuing review.

Okyere Boateng
(CSIR-IRB, Chairman)

Cc: Dr. Abdulai Baba Salifu
(Director General, CSIR)