The Genetics of Potential Albendazole and Ivermectin Resistance in Lymphatic Filariae

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Your file Votre référence
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This thesis is dedicated to my parents, Rosemarie and Andreas.

Abstract

A current initiative to eliminate lymphatic filariasis (LF), headed by the World Health Organization, aims to interrupt transmission of the disease through yearly community-wide treatment with the broad spectrum anthelmintic albendazole (ABZ), in combination with ivermectin (IVM) or diethylcarbamazine (DEC). Over the years, the use of both ABZ and IVM in the treatment of veterinary parasites has led to widespread anthelmintic resistance against these drugs. In this study, we genotyped microfilaria of *Wuchereria bancrofti*, a causative agent of LF, in order to detect the presence of mutations which confer ABZ resistance in other parasites, and we identified such mutations in worms obtained from untreated patients in Ghana and Burkina Faso, West Africa. Microfilaria from patients who had been treated with ABZ + IVM, had a significantly higher frequency of the resistant genotype, and this frequency was even higher in worms from patients that had received two rounds of treatment. In addition, the untreated population of microfilaria had an excess of homozygotes in the population. This excess homozygosity was equivalent to a Wright's Inbreeding Statistic of $F_{IT} = 0.44$, and we found that the population was significantly subdivided between patients. In order to better understand the mechanisms and factors involved in the potential spread of ABZ resistance, caused by such mutations, through a population of *Culex*-transmitted *W. bancrofti*, we developed a deterministic model that incorporates genotype structure into the epidemiological model EPIFIL. This model predicts that the combination of ABZ + DEC leads to stronger selection for the resistant genotype than ABZ + IVM, and that drug efficacy assumptions are an important factor affecting the spread of drug resistance. Treatment coverage, non-random mating, initial allele frequency and number of treatments also had substantial impact on the speed and magnitude of the spread of ABZ resistance. When we expanded this model to include potential IVM-resistance alleles we found that, under ABZ + IVM treatment, selection for resistance to either drug is enhanced by the presence of resistance against the second drug. Similarly, excess homozygosity caused by parasite non-random mating may increase selection for a dominant IVM resistance allele through enhancing the spread of a recessive ABZ resistance allele. Resistence developed more slowly when it was inherited as a polygenic trait. Results from this study suggest that resistance monitoring is crucial, as resistance

may not be apparent until treatment is stopped, recrudescence occurs and treatment is reapplied.

Abrégé

Une initiative actuellement menée par l'Organisation mondiale de la santé a pour but d'éliminer la filariose lymphatique (LF) par l'interruption de la transmission de cette maladie, en traitant annuellement des communautés complètes avec l'antihelminthique à spectre large albendazole (ABZ), combiné avec l'ivermectin (IVM) ou la diethylcarbamazine (DEC). Avec les années, l'usage de l'ABZ et le l'IVM comme traitement contre les parasites vétérinaires a résulté en une pharmacorésistance répandue contre ces deux médicaments. Dans cette étude, nous avons déterminé le génotype de plusieurs microfilaires de *Wuchereria bancrofti*, l'agent causal de la LF, afin de détecter la présence de certaines mutations reconnues pour conférer une résistance à l'ABZ chez d'autres parasites du même type. Ces mutations ont été trouvées chez des vers obtenus de patients non traités provenant du Ghana et du Burkina Faso, en Afrique de l'Ouest. Les vers des patients du Burkina Faso qui avaient été traités avec de l'ABZ + IVM ont démontré une fréquence significativement accrue du génotype résistant, et cette fréquence était encore plus élevée chez les patients qui avaient été traités deux fois. De plus, la population non-traitée de microfilaires contenait un excès d'homozygotes, équivalent à une statistique de consanguinité de Wright $F_{IT} = 0.44$, et nous avons trouvé que cette population était subdivisée de façon significative entre les patients. Nous avons développé un modèle déterministe qui incorpore les structures de génotypes au modèle épidémiologique EPIFIL afin de mieux comprendre les mécanismes et les facteurs impliqués dans la dispersion potentielle de la résistance à l'ABZ dans une population de *W. bancrofti* transmise par le moustique *Culex*. Ce modèle prédit que la combinaison ABZ + DEC mène à une sélection pour le génotype résistant plus forte que l'ABZ + IVM, et que les suppositions sur les efficacités des médicaments sont des facteurs importants qui influencent la dispersion de la pharmacorésistance. La couverture des traitements, l'accouplement non-aléatoire des vers, les fréquences initiales des allèles ainsi que le nombre de traitements administrés influençaient aussi fortement la vitesse et l'ampleur de la dispersion de la résistance à l'ABZ. L'inclusion dans ce modèle d'allèles potentiels conférant une résistance à l'IVM a mené à la conclusion que, sous traitement avec l'ABZ + IVM, la sélection pour la pharmacorésistance à un des médicaments est accrue par la présence de pharmacorésistance à l'autre médicament. De même, un excès

d'homozygosité causé par un accouplement non-aléatoire des parasites peut augmenter la sélection pour l'allèle dominant de la pharmacorésistance à l'IVM, par l'accroissement de la dispersion de l'allèle récessif de la pharmacorésistance à l'ABZ. La résistance se développait plus lentement si elle était causée par plusieurs gènes. Les résultats de cette étude suggèrent que la surveillance pour la résistance est de grande importance, car celle ne sera pas apparente avant la fin du traitement, la recrudescence des parasites est le recommencement du traitement.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER1

CHAPTER2

CHAPTER 3

CHAPTER 4

CHAPTER 6

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX I

APPENDIX II

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CONTRIBUTION OF AUTHORS

Chapter 2: All experiments in this manuscript were designed and carried out by Anne Schwab under the supervision of Dr. R. Prichard. She obtained all data presented, and carried out all analyses. Dr. D. Kyelem and Dr. D. Boakye provided the microfilarial samples used in this analysis.

Chapter 3 &4: The extensions and alterations of the mathematical model, originally published as EPIFIL (Norman *et al*, 2000), in these manuscripts were conceived and programmed by Anne Schwab. This author also generated all the model outputs and prepared both manuscripts. Dr. Prichard acted as supervisor, and provided scientific and editorial advice. Dr. Basanez, Dr. Andreas Schwab and Thomas Churcher provided technical advice and expertise, as well as editorial advice in the manuscript preparation.

Appendix I: All the data used for this manuscript was collected by Anne Schwab. The statistical analyses and modeling was done by Thomas Churcher, who also prepared the manuscript with assistance from Anne Schwab, Dr. Prichard and Dr. Basáñez. Dr. Basáñez acted as primary supervisor to Mr. Churcher.

STATEMENT OF ORIGINALITY

The manuscripts contained in this thesis contributed original material, original scholarship and the advancement of knowledge in the field.

Chapter 2: A. E. Schwab, D. A. Boakye, D. Kyelem and R. K. Prichard (2005). Detection of Benzimidazole Resistance–associated Mutations in the Filarial Nematode Wuchereria Bancrofti and Evidence for Selection by Albendazole and Ivermectin Combination Treatment. Am J Trop Med Hyg 73, 234-238.

This manuscript reports cloning of the first full length β-tubulin coding sequence from *B. malayi* and the first partial genomic sequence of *W. bancrofti* β-tubulin containing both amino acid position 167 and 200, which have been implicated in benzimidazole resistance. These sequences have been deposited in GenBank. We also developed two novel diagnostic tests for both mutations in microfilariae of *W. bancrofti*. This work was also the first report of benzimidazole resistance associated mutations in filarial worms from both untreated and treated patients.

Chapter 3: Schwab A.E., ChurcherT.S., Schwab A.J., Basáñez, M-G., Prichard R.K. An Analysis of the Population Genetics of Potential Albendazole Resistance in Lymphatic Filariasis due to Combination Chemotherapy. Parasitology, *in press*.

In this manuscript we describe the first detailed model examining the spread of ABZ resistance alleles in filarial nematodes, based on the epidemiological model EPIFIL (Norman *et al,* 2000). We show here that drug-efficacy assumptions have an impact on the spread of resistance genes, and that increased treatment coverage, non-random mating and the number of treatments applied intensify selection for a recessive ABZ resistance allele in *W. bancrofti.* Such insights are essential in understanding if resistance can develop, in a population, and how it may spread.

Chapter 4: Schwab A.E., ChurcherT.S., Schwab A.J., Basáñez, M-G., Prichard R.K An Analysis of the Population Genetics of Potential Multi-drug Resistance in Lymphatic Filariasis due to Combination Chemotherapy

This is the first construction of a mathematical model examining multi-drug resistance in parasitic worms, based on the epidemiological model EPIFIL (Norman *et al,* 2000). Our model gives considerable new insight into the interaction of factors such as dominance and the number of genes involved. We show that selection with one drug may increase selection of other drugs and that if multiple resistance genes are associated with different pharmacodynamic properties then examining the changes to single loci may produce misleading results. These interactions have not been addressed before, to our knowledge.

Appendix I: T. S. Churcher, A. E. Schwab, R. K. Prichard, M.-G. Basáñez, Helminth inbreeding and the detection of drug resistance

This is the first in-depth analysis of population genetics to determine inbreeding in filarial parasites. We report here the first evidence of significant population subdivision between *W. bancrofti* in different human hosts. We also propose novel insight into sampling strategies for molecular detection of drug resistance.

THESIS OFFICE STATEMENT

The following statement is a regulation of the Faculty of Graduate Studies and Research, McGill University, concerning thesis preparation:

"Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted for publication, or the clearly duplicated text of a published paper(s). If this option is chosen, connecting texts, providing logical bridges between the different papers are mandatory.

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation", and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include: A table of contents, an abstract in English and French, an introduction which clearly states the rationale and objective of the study, a comprehensive review of the literature, a final overall conclusion, and/or summary, and a reference list.

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement as to who contributed to such work and to what extent. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers".

ABBREVIATIONS

ABZ-	Albendazole
ADLA-	Acute dermatolymphangioadenitis
AFL-	Acute filarial lymphangitis
BZ-	Benzimidazole
Bmax-	Maximum binding
CFA-	Circulating filarial antigen
COX-	Cyclooxygenase
DEC-	Diethylcarbamazine
DALY-	Disability adjusted life years
ELISA-	Enzyme-linked immunosorbent assay
F-	Phenylalanine
GABA	Gamma aminobutyric acid
GPELF-	Global Program to Eliminate Lymphatic Filariasis
HMZ-	Homozygote
HTZ-	Heterozygote
ICT-	Immunochromatographic test
IgE-	Immunoglobulin E
IVM-	Ivermectin
Kd-	Dissociation constant
LF-	Lymphatic filariasis
MF-	Microfilaria
ML-	Macrocyclic lactone
MAP-	Microtubule associated protein
PC-	Phosphorylcholine
PCR-	Polymerase chain reaction
PgP-	P-glycoprotein
RFLP-	Restriction fragment length polymorphism
SNP-	Single Nucleotide Polymorphism
WHO-	World Health Organization
Y-	Tyrosine

GENERAL INTRODUCTION

Many of the world's most debilitating diseases are caused by parasites. The filarial worms *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* are the causative agents of lymphatic filariasis, a disease which may lead to disability and has a severe impact on endemic countries. Recognizing this, the World Health Organization is currently implementing a global program to eliminate lymphatic filariasis (Maher and Ottesen, 2000). This program is designed to disrupt transmission of the disease using chemotherapy. The benzimidazole drug albendazole is used in combination with diethylcarbamazine or the macrocyclic lactone, ivermectin, in some geographical areas (WHO, 1999). However, like many of the current anthelmintics used to treat nematode parasites, resistance to both albendazole and ivermectin has occurred in some species, although in human populations, this has not been unequivocally confirmed despite some reports suggesting resistance (De Clercq *et al.*, 1997).

Resistance against these broad-spectrum anthelmintics has been a concern in veterinary science for many years (Prichard, 1990). Though resistance against anthelmintics has not yet impeded the treatment of human helminth infections (Coles, 1999), the development of drug resistance has had an adverse impact on the control of other parasites, such as *Plasmodium falciparum* (Krogstad *et al.*, 1987). Recently, there have been several reports of a poor response to ivermectin in a related filarial worm *Onchocerca volvulus*, the causative agent of River Blindness, after many years of exposure to the drug, possibly due to drug resistance (Awadzi *et al.*, 2004a; Awadzi *et al.*, 2004b). While there are no reports of resistant strains of *Wuchereria*, our current knowledge of the development of resistance suggests that it is likely that resistance to albendazole (ABZ) and ivermectin (IVM) will occur. Development of resistance against both ABZ and IVM would be a major threat to the elimination program, since chemotherapy is the primary strategy employed.

The overall objective of this thesis was to explore the risk of potential anthelmintic resistance developing in lymphatic filariasis. Specifically this thesis examines the genetics of potential drug resistance in *W. bancrofti*, from a practical, as well as a theoretical perspective. The first chapter reviews the current knowledge of

lymphatic filariasis and the parasites causing it, as well as the drugs used to treat the disease. A summary of advances made in the mathematical biology of LF and of drug resistance in parasites is also included. Chapter 2 presents evidence that ABZ resistance-associated mutations are present in populations of *W. bancrofti* from West Africa and explores the frequencies of these mutations in patients with different treatment histories. The third chapter of this thesis introduces a deterministic mathematical model, which incorporates population genetics with epidemiology, in order to better understand the mechanisms and key factors involved in the potential spread of ABZ resistance through a population of *W. bancrofti.* This mathematical model is extended in the fourth chapter, to include IVM-resistance genes, and thus explores important factors in the spread of polygenic multi-drug resistance in *W. bancrofti.* The fifth chapter of this thesis consists of a detailed discussion of the implications of the findings on our current thinking of potential drug resistance in filarial parasites. The manuscript in Appendix I explores the population genetics of the *W. bancrofti* population in Burkina Faso. Evidence of non-random mating in this population is presented and examined, in order to develop more appropriate sampling strategies. The second Appendix contains supplemental figures to Chapters 3 and 4.

References

Awadzi, K., Attah, S. K., Addy, E. T., Opoku, N. O., Quartey, B. T., Lazdins-Helds, J. K., Ahmed, K., Boatin, B. A., Boakye, D. A. and Edwards, G. (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, 359-370.

Awadzi, K., Boakye, D. A., Edwards, G., Opoku, N. O., Attah, S. K., Osei-Atweneboana, M. Y., Lazdins-Helds, J. K., Ardrey, A. E., Addy, E. T., Quartey, B. T., Ahmed, K., Boatin, B. A. and Soumbey-Alley, E. W. (2004b). An investigation of persistent microfilaridermias despite multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. Ann Trop Med Parasitol, 98, 231-249.

Coles, G. C. (1999). Anthelmintic resistance and the control of worms. J Med Microbiol, 48, 323-325.

De Clercq, D., Sacko, M., Behnke, J., Gilbert, F., Dorny, P. and Vercruysse, J. (1997). Failure of mebendazole in treatment of human hookworm infections in the southern region of Mali. Am J Trop Med Hyg, 57, 25-30.

Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M., Martin, S. K., Milhous, W. K. and Schlesinger, P. H. (1987). Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science, 238, 1283-1285.

Maher, D. and Ottesen, E. A. (2000). The Global Lymphatic Filariasis Initiative. Trop Doct, 30, 178-179.

Prichard, R. K. (1990). Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. Int J Parasitol, 20, 515-523.

WHO (1999). Building Partnerships for Lymphatic Filariasis: Strategic Plan WHO/FIL/99.198.

CHAPTER 1: LITERATURE REVIEW

1.1 Endemicity And Impact Of Lymphatic Filariasis

Lymphatic filariasis (LF) is endemic to over 90 countries (Fig. 1.1). It has been estimated that approximately 128 million people are currently infected or afflicted with the disease. The global prevalence of LF is thought to be 3.39% in the exposed population (Michael, 2000; Michael and Bundy, 1997; Michael *et al.*, 1996). *Wuchereria bancrofti* is associated with 91% of all cases, with *Brugia malayi* causing most of the remaining cases (WHO, 1992). A third species, *Brugia timori* , replaces *B. malayi* in the eastern islands of the lesser Sunda archipelago (Nusa Tenggara Timur) (Fischer *et al.*, 2004). Bancroftian filariasis is endemic throughout the tropics and subtropics, while brugian filariasis is confined to South and Southeast Asia (Michael, 2000). Though India accounts for the largest number of cases for a single country (Michael *et al.*, 1996), disease prevalences are higher in Africa and the Pacific islands where mean regional prevalences are estimated at 10.3% and 29% of positive countries respectively compared to 2.4% for positive countries in Asia and 0.2% in South America (Michael and Bundy, 1997).

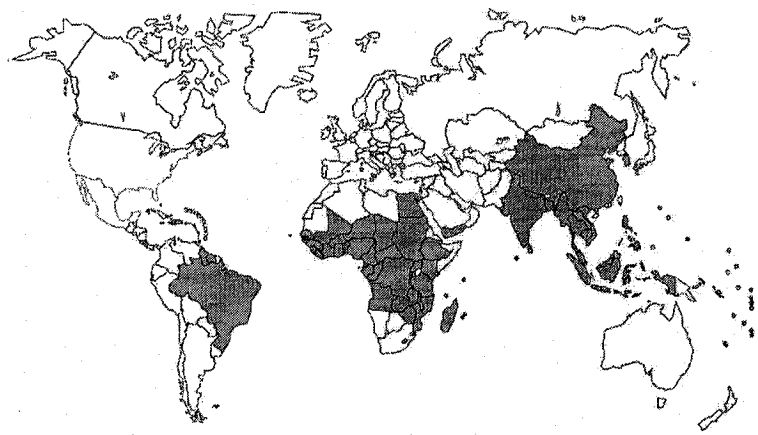

Figure 1.1: Geographical distribution of Lymphatic filariasis. Grey areas indicate countries endemic to LF. This map is reproduced from WHO (2002)

Lymphatic filariasis imposes a devastating socioeconomic burden on affected communities. It is estimated that in 1990, 560 000 Disability Adjusted Life Years (DALYs) and 290 000 DALYs were lost worldwide due to the disease for men and

women, respectively (World Bank, 1993). It is believed that in India, LF is responsible for a one billion US dollar loss in productivity (Ramaiah *et al.*, 2000). Studies in Ghana have also indicated that the disease greatly contributes to household poverty (Gyapong *et al.*, 1996). A recent study showed that LF is endemic in 89% of the 46 countries classified as the least developed (Durrheim *et al.*, 2004). In addition, there is a severe psychosocial impact on individuals infected with the disease. Severe cases lead to stigmatization and in turn social isolation (Krishna Kumari *et al.*, 2005).

1.2 Biology and Life Cycle of Lymphatic Filaria

A summary of the life-cycle of lymphatic filarial is shown in Fig. 1.2. Lymphatic filariae are nematodes, belonging to the order Spirurida and the family Onchocercidae. The Spirurida is most closely related to the Ascaridida and the Oxyurida (Scott 2000). Most nematode parasites pass through five life stages after hatching from the egg, separated by four moults. The first four stages are larval stages termed L1, L2, L3 and L4 and the fifth stage is the adult worm or macrofilaria. In parasitic nematodes of vertebrates, the infective stage is almost always at the beginning of the third stage, immediately or shortly after the second moult.

1.2.1 Development in The Human Host

In LF, the infective L3 stage enters the host upon a bite of an infected mosquito vector. Mature L3 are deposited onto the host's skin and enter the host via the puncture site (Scott, 2000). Studies in monkeys have shown that L3 develop into L4s in 14-38 days and into adults 42-103 days post infection (Cross *et al.*, 1979). The pre-patent period has been found to be 2-3 months for *B. malayi* and 7-8 months for *Wuchereria* (WHO, 1984; WHO, 1992), after which each female releases thousands of microfilariae every day. Calculations have shown that an adult female worm may produce as many as 1.32×10^7 microfilariae during her lifetime (Hairston and Jachowski Jr., 1968).

All species of lymphatic filaria are dioecious. Males are approximately 4 cm in length and 50 mm in diameter, while females are 200 μm in diameter and range from 8-10 cm in length (Scott, 2000). *Brugia* worms are slightly smaller than *Wuchereria*, ranging from 4-5.5 cm and 130-170 μm diameter in females and 1.5-2.3 cm in males

(Rao, 2005). Estimates for the lifespan of *W. bancrofti* adults vary greatly, and are based on blood microfilarial levels. Table1.1 (Dreyer *et al.*, 2005) summarizes some of these estimates. It has also been shown that the life-span of adult worms may be reduced in areas of high transmission, likely due to some form of density dependence (Dreyer *et al.*, 2005). Estimates for the reproductive lifespan of *B. malayi* females range from 3.4 years (Sabesan *et al.*, 1991) to 8-9 years (Wang *et al.*, 1994).

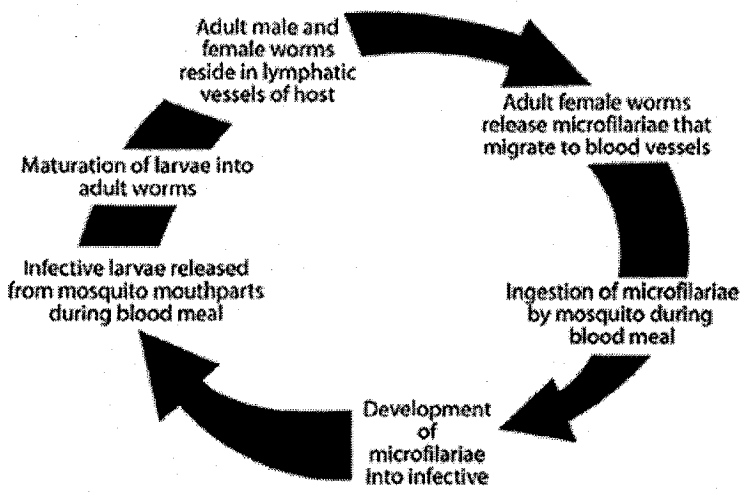

Figure 1.2: Life Cycle of lymphatic filaria
Reproduced from http://www.mectizan.org/lifecycle2.asp

Table 1.1: Estimates of *W. bancrofti* adult lifespan (Dreyer *et al.*, 2005).

Author	Location(s)	Estimated lifespan (years)	Sample size
Carme and Laigret, 1979	Tahiti/France	40	1
Leeuwin, 1962	Surname/Amsterdam	≤8	28
Jachowski *et al.*, 1951	American Samoa/Hawaii	≤6	11
WHO/WPRO, 2003	China	≤8	model
Vanamail *et al.*, 1990	Pondicherry, India	10.2	877
Vanamail *et al.*, 1996	Pondicherry, India	5	7525
Vanamail *et al.*, 1989	Pondicherry, India	5.4	633
Subramanian *et al.*, 2004	Pondicherry, India	10.2, 11.8	model
Guptavanij and Harinasuta, 1971	Thailand	5	1
Conn and Greenslit, 1952	Pacific/United States	7.5	1
Mahoney and Patrick, 1970	Samoa/Hawaii/California	≤8	25
Webber, 1977	Solomon Islands	8	model

Within the host, adult worms live in the lumen of dilated lymphatics (Amaral *et al.*, 1994). Worms are found in nests containing several individuals, and the location of nests remains stable over time in untreated patients (Dreyer *et al.*, 1994). In men, *W. bancrofti* worm nests are often found in the lymphatic vessels of the epididymis, spermatic cord and paratesticular region (Noroes *et al.*, 1996a; Reddy *et al.*, 2004). In women, worm nests have been found in lymphatic vessels in the uterus, the groin and between the muscular fibers of the thighs (Mand *et al.*, 2004). Lymphatic filaria are oviviparous (Scott 2000), or they lay motile vermiform eggs or very young larvae in diapause, known as microfilariae or L1 (Bain and Babayan, 2003). These sheathed microfilariae migrate to the peripheral circulation. The presence of microfilariae in the blood was first observed by Thomas Manson (1893-1958). Microfilaraemia is usually nocturnally periodic, and thus microfilariae appear in the blood between 10 PM and 2 AM, coinciding with the peak biting times for many night-biting *Culex* and *Anopheles* mosquito species. In some parts of Asia and the Pacific, lymphatic filaria display

subperiodic behaviour. Thus, they are present continuously, but peak at specific times of the day (McCarthy, 2000). This periodicity is a product of circadian migration of the microfilariae from the peripheral circulation to the lungs (Hawking and Thurston, 1951). Microfilariae react to physiological signals from the host, since reversal of the host's circadian cycle will also reverse the periodicity of microfilariae in the blood (Hawking, 1965). It has been shown that periodicity behaviour of microfilariae is related to changes in oxygen tension between venous and arterial blood. At night arterial O_2 tension may fall slightly, while venous O_2 tension may be slightly higher than during the day, thus providing a signal to microfilariae (Edeson *et al.*, 1957; Hawking *et al.*, 1966).

1.2.3 Development in the Vector

Microfilariae are picked up from the peripheral blood through the bite of a mosquito. Over ninety species of mosquitoes are known to be competent for the transmission of LF. Periodic *W. bancrofti* is transmitted primarily by *Anopheles* or *Culex* mosquitoes (except for two species of *Aedes* in South Asia), whereas the predominant vector species for subperiodic *W. bancrofti* belong to the genus *Aedes*. *Brugia malayi* is transmitted by both *Anopheles* and *Mansonia* species and *B. timori* is transmitted by *Anopheles barbirostis* (WHO, 1984). Inside the mosquito, the larvae penetrate the midgut and migrate to the flight muscles of the mosquito. They develop to a shorter form, known as the "sausage stage" in three days, and to the L2 stage in 7 days. L2 larvae molt to the L3 stage within 12-14 days and then migrate to the head of the mosquito (Samarawickrema *et al.*, 1985; Scott 2000). Though most infective L3 remain in the head of the mosquito, some migrate back and forth to the thorax or abdomen of the insect (Paily *et al.*, 1995). L3 are thought to be stimulated to move into the labium of the mosquito by the movement of the muscles of the pharyngeal pump (Zielke, 1977). *Wuchereria bancrofti* L3 were found to be alive and motile in *Culex* mosquitoes up to 46-50 days after ingestion (Paily *et al.*, 1995).

1.3 Pathogenesis of Lymphatic Filariasis

The pathology of LF is complex and still poorly understood. Its clinical manifestations are highly variable and depend on the endemic area and the filarial species involved (Kumaraswami, 2000; Partono, 1987). Pathology also varies with age and gender (Pani *et al.*, 1991). Traditionally, it has been assumed that the disease progresses from asymptomatic microfilaraemia to acute and finally chronic disease, depending on the patient's immunological state (Partono, 1987). This theory has been challenged recently, and secondary bacterial infections are now known to be strongly implicated in the development of chronic disease (Dreyer *et al.*, 2000). Acute disease is characterized by recurrent attacks of fever and adenolymphangitis (Helm *et al.*, 1989), often accompanied by retrograde lymphangitis known as acute filarial lymphangitis (AFL) (WHO, 1992). In bancroftian filariasis, the male genitalia may be affected, with symptoms such as funiculitis, epididymitis or orchitis (Partono, 1987). In Brugian filariasis, mostly lymph nodes in the inguinal region are affected. Repeated episodes of AFL have been implicated in the progression of the disease to a chronic state (Pani *et al.*, 1995). Chronic manifestations of lymphatic filariasis include lymphoedema, elephantiasis, hydrocoele and chyluria (WHO, 1992). Bancroftian disease causes primarily hydroceole and swelling of the testis, followed by elephantiasis of an entire lower limb, and less frequently of the scrotum, the arm, vulva and breasts (Kumaraswami, 2000). In Brugian filariasis, elephantiasis most commonly affects the leg below the knee and less frequently the arm below the elbow (Partono, 1987). Chyluria is the result of the rupture of a dilated lymphatic vessel, causing drainage of the lymph through the urinary tract (Dreyer *et al.*, 2000).

The use of ultrasound and lymphoscintigraphy has led to a re-evaluation of microfilaraemia that appears to be asymptomatic. It has recently been shown that virtually all patients bearing adult worms exhibit lymphangiectasia and lymphatic abnormalities, and thus have subclinical forms of the disease (Dissanayake *et al.*, 1995; Dreyer *et al.*, 2000; Dreyer *et al.*, 1996c; Freedman *et al.*, 1994; Freedman *et al.*, 1995; Kumaraswami, 2000). An inflammatory response was not evident in the lymphatics near

the adult worms in these asymptomatic patients. This is clearly different from patients with chronic pathology, who display a vigorous inflammatory reaction (Melrose, 2002). It is also known that AFLs are strongly associated with death of adult worms, which does lead to inflammation (Dreyer *et al.*, 2000). Acute hydrocoele and lymphoedema can be the result of temporary blockage of lymphatics due to this inflammation in response to damaged or dead worms. However, these conditions usually resolve quickly, and only sometimes lead to chronic hydrocoele and very rarely to chronic lymphoedema (Dreyer *et al.*, 2000). It is not yet clear what usually triggers the onset of chronic disease. Recent evidence suggests that immunological changes in response to filaria and lymphatic obstruction *per se* may not be as important in chronic lymphoedema as previously suspected (Melrose, 2002). It has recently been shown that acute attacks are often precipitated by secondary bacterial infections (Kumaraswami, 2000), and it was suggested that acute episodes of AFL caused by the death of adult worms can be clinically differentiated from acute attacks caused by bacterial infections, acute dermatolymphangioadenitis or ADLA. A recent hypothesis claims that ADLA is a common cause of elephantiasis (Dreyer *et al.*, 1999). In fact it was proposed that, though hydrocoele can sometimes be caused solely by the presence of worms, development of chronic elephantiasis always requires the presence of bacterial infections (Dreyer *et al.*, 2000).

A rare complication of LF is tropical pulmonary eosinophilia, whose symptoms are similar to those of bronchial asthma (Kumaraswami, 2000). It is characterized by marked elevations of immunoglobulin E (IgE) titers and circulating eosinophils in the serum, thought to be caused by immunologic hyperactivity to filarial parasites or their antigens (O'Bryan *et al.*, 2003). Patients do not have overt microfilaraemia, though dead or dying microfilariae have been found in the lung, liver and lymph nodes (Webb *et al.*, 1960) and adult worms have been detected (Dreyer *et al.*, 1996b). Lymphatic filariasis has also been associated with rheumatic symptoms (Adebajo, 1996).

Endemic normals are individuals that live in endemic areas, thus being continually exposed to filariasis, but remain amicrofilaraemic (Kumaraswami, 2000). Many so-called endemic normals show the presence of filarial antigens and anti-filarial antibodies in the intra-venous circulation, which suggests that these individuals are likely

not immune, but bear an amicrofilaremic and asymptomatic infection (Weil *et al.*, 1996). In other cases, ultrasound examination of endemic normals have revealed the presence of adult worms in their lymphatics (Dreyer *et al.*, 1996c; Faris *et al.*, 1998; Noroes *et al.*, 1996a; Noroes *et al.*, 1996b). Thus, true endemic normals may be rare in the population (Melrose, 2002).

1.4 Immunological Aspects of Filarial Infections

The host immune response to lymphatic filaria is complex and poorly understood. The host comes into contact with three distinct stages of the parasite's life-cycle, in immunologically distinct compartments, and each stage presumably elicits its own specific type of immune response (O'Connor *et al.*, 2003). In order to survive in the lymphatics as a multicellular organism, filarial worms must use a large number of adaptive strategies (Devaney and Yazdanbakhsh, 2001). It is well-documented that filarial infections with microfilaraemia lead to high levels of immunosuppression, as evidenced by a lack of proliferative T-cell responses (King *et al.*, 1992; Lawrence, 2001; Ottesen *et al.*, 1977). Interferon (IFN)-γ production has been shown to be absent (Sartono *et al.*, 1997). This lack of responsiveness appears to be primarily directed at the parasite (Nutman and Kumaraswami, 2001), though recent studies have shown that concurrent infection with *W. bancrofti* can diminish responses to unrelated antigens (Nookala *et al.*, 2004). In contrast, IgG4 levels and circulating eosinophils may be extremely high in microfilaraemic patients (Kwan-Lim *et al.*, 1990; Wong and Guest, 1969) . Surprisingly, there is also a lowered interleukin (IL)-5 production (Sartono *et al.*, 1997), which complicates any assumptions of a simple suppression of Th1 cytokines. It should be noted that in South India, induction of IL-5 was sometimes observed (Nutman and Kumaraswami, 2001). Interestingly, if given diethylcarbamazine (DEC) therapy, T-cell responsiveness in microfilaraemics may be restored (Sartono *et al.*, 1995). It has also been demonstrated that filarial antigens alter the function of antigen presenting cells to induce tolerance (Nutman and Kumaraswami, 2001; O'Connor *et al.*, 2003). Studies in mice have revealed that infection with only the microfilarial stage of the parasite induces IFN-γ production and causes elevated lymphocyte apoptosis (O'Connor *et al.*, 2003), suggesting the possibility of a different immunomodulary mechanism by this lifestage in an artificial host.

Protective immunity to LF in humans has been the subject of much debate. To date, extensive studies have not been able to produce conclusive evidence of its existence (Maizels and Yazdanbakhsh, 2000; Ravindran *et al.*, 2003). Mathematical modeling and combined data analysis have shown that the age prevalence pattern of infection can be

explained by exposure-driven herd immunity (Michael and Bundy, 1998). A mathematical model has also shown that transmission intensity has a profound effect on infection and disease, and that immunity against infection with new worms is likely modulated by adult worm experience. In addition, a separate type of immunity, which leads to pathology, appears to be mediated by cumulative experience of infective larvae (Michael *et al.*, 2001). It has also been shown that the age of the patient has an impact on the cytokine profile produced, thus the length of exposure clearly affects the immune response (Sartono *et al.*, 1997). Indirect evidence of the presence of density-dependent mechanisms such as acquired immunity is also provided in a study that showed a decrease in aggregation or overdispersion with age (Das *et al.*, 1990). In Papua-New Guinea, in an area with constant levels of microfilaraemia, antigenaemia tended to increase in patients younger than 20 years, but remained stable in older patients, suggesting some resistance to superinfection (Day *et al.*, 1991).

1.5 Diagnosis of Lymphatic Filariasis

Diagnosis of LF is inherently difficult, due to several factors, such as periodicity of microfilariae and incidence of amicrofilaraemic patients, as well as the difficulty of locating adult worms.

Traditionally, diagnosis has been achieved through the detection of microfilariae in the blood. This method requires a blood sample to be taken at a time of peak microfilaraemia. Thus in areas of nocturnal periodicity the sample must be taken at midnight, which may be unacceptable in many settings (McCarthy, 2000). A thick blood film stained with Giemsa stain is the simplest method (Melrose, 2002). However this test lacks sensitivity as it cannot detect microfilarial counts below 20 mf/ml (McCarthy, 2000). There may be an additional loss of microfilariae from the blood film during the steps of dehaemoglobinization, staining and rinsing in this procedure, further limiting the reliability of diagnosis (Denham *et al.*, 1971). An alternative is to use concentration techniques of larger blood samples. The method developed by Knott (1939) is still widely used today. One ml of blood is added to 9 ml of 2% formalin solution in normal saline. The sample is then centrifuged and the sediment analyzed. Formalin preserves the microfilariae and inactivates other infectious agents. However, since the sediment is often difficult to analyze (McCarthy, 2000; Melrose, 2002) other concentration techniques, such as filtration, are more common today. The filtration of blood through a polycarbonate membrane with a 5 μM pore-size to concentrate microfilariae was first described by Bell (1967). Membrane filtration has been shown to be more sensitive than using blood films (Moulia-Pelat *et al.*, 1992).

The recent development of tests that detect circulating filarial antigens in the blood has proven revolutionary, as they are capable of diagnosing amicrofilaraemics and can be performed during the daytime (Melrose, 2002). It has long been known that patients infected with lymphatic filaria present circulating antigen (Franks, 1946).

Early assays, which detected the *W. bancrofti* excretory/secretory product phosphorylcholine (PC) with a monoclonal antibody (Gib-13) were, however, not sensitive enough to recognize all patent infections (93% sensitivity) (Forsyth *et al.*, 1985;

Lal *et al.*, 1987). The first commercially available *W. bancrofti* diagnostic test for circulating filarial antigen (CFA) was a capture enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody Og4C3, which recognizes a major PC-containing epitope that was first characterized by Maore and Copeland (1990). It has proven to have high sensitivity and specificity (Chanteau *et al.*, 1994; Lammie *et al.*, 1994).

A rapid card format immunochromatographic test (ICT) using the antifilarial monoclonal antibody AD12.1, which recognizes CFA in the blood, is also available. This test is rapid and easy to use and shows specificity and sensitivity up to 100% (Simonsen and Dunyo, 1999; Weil *et al.*, 1997). No CFA tests are currently available for Brugian filariasis (McCarthy, 2000). Instead, diagnosis has been improved by immunodiagnostic tests for antifilarial IgG4 (Melrose *et al.*, 2004; Rahmah *et al.*, 1998a; Rahmah *et al.*, 2001a; Rahmah *et al.*, 2001b). This test is specific for filaria, but does not distinguish between *B. malayi, B. timori* and *W. bancrofti* (Rahmah *et al.*, 1998a).

DNA based tests for Brugian and bancroftian filariasis have also been developed. Such tests can detect parasites within mosquito vectors using polymerase chain reaction (PCR)-based assays (Dissanayake *et al.*, 1991; Goodman *et al.*, 2003; Hoti *et al.*, 2001; Vasuki *et al.*, 2001) or enzyme-labeled DNA probes (Dissanayake *et al.*, 1991). PCR-based tests which amplify repetitive DNA sequences capable of detecting *B. malayi* in blood samples (Lizotte *et al.*, 1994; Rahmah *et al.*, 1998b) and *W. bancrofti* in sputum (Abbasi *et al.*, 1999; Abbasi *et al.*, 1996; Lucena *et al.*, 1998; McCarthy *et al.*, 1996) and urin (Lucena *et al.*, 1998) of human patients, have also been useful. Recently, a PCR-test for combined detection of both species has been developed (Mishra *et al.*, 2005).

Molecular-based tests have shown high specificity and sensitivity (Abbasi *et al.*, 1996; Lizotte *et al.*, 1994; McCarthy *et al.*, 1996), though the latter has been questioned by other groups (Dissanayake *et al.*, 2000). PCR-based tests are more rapid than traditional methods, however they tend to be expensive and impractical in field settings (Melrose, 2002).

Recently ultrasonography has been added as a diagnostic tool. Live adult worms produce a characteristic signal in lymphatics known as 'filaria dance signs'(Amaral *et al.*, 1994), which can be used to detect infection with macrofilaria (Amaral *et al.*, 1994;

Dreyer *et al.*, 1996b; Dreyer *et al.*, 1996c; Faris *et al.*, 1998; Simonsen and Dunyo, 1999). Ultrasonography is widely available, portable, cost effective and non-invasive (Amaral *et al.*, 1994; Suresh *et al.*, 1997).

1.6 Treatment of Lymphatic Filariasis

Three drugs are currently being used for the treatment of lymphatic filariasis. Diethylcarbamazine (DEC), ivermectin (IVM) and albendazole (ABZ) are known to be effective against the parasite when administered on their own, or in combination (Tisch *et al.*, 2005). The efficacy of these drugs is currently not firmly established. DEC has been used to treat the disease for the last 60 years and has been shown to clear microfilariae for up to 12 months following a 12 day regimen of 6 mg DEC /kg body weight administered each day (Richards *et al.*, 1991), a dose regimen that was recommended for many years by WHO (WHO, 1992). Recent studies have shown that a single dose of 6 mg/kg can reduce microfilaraemia to levels and for a time-period comparable to the 12-day regimen (Cartel *et al.*, 1990; Cartel *et al.*, 1991; Kimura *et al.*, 1985; Kimura *et al.*, 1992; Weerasooriya *et al.*, 1998). A single dose of the drug also appears to exert an adulticidal effect (Dreyer *et al.*, 1995; Figueredo-Silva *et al.*, 1996). Ultrasonographic studies found that 41.5% of worm nests in an infected individual were inactivated by DEC treatment at doses ranging from 1-12 mg/kg (Noroes *et al.*, 1997) and studies measuring filarial antigen found a 34% macrofilaricidal effect with a dose rate of 72 mg/kg (Day *et al.*, 1991). The drug may also be consumed in the form of table-salt fortified with 0.25% DEC, which has proven highly effective, though with some minimal side-effects (Freeman *et al.*, 2001; Gelband, 1994; Panicker *et al.*, 1997; Tisch *et al.*, 2005).

A single dose of IVM at 200 μg has been shown to rapidly reduce blood microfilarial levels for up to 12 months (Brown *et al.*, 2000; Cao *et al.*, 1997; Kazura *et al.*, 1993; Kumaraswami *et al.*, 1988; Ottesen *et al.*, 1990). This drug does not appear to have a macrofilaricidal effect on *W. bancrofti* (Dreyer *et al.*, 1996a; Dreyer *et al.*, 1995), but does affect microfilarial production, presumably through an effect on reproduction (Plaisier *et al.*, 1999; Stolk *et al.*, 2005).

Several studies have explored the effect of a single dose of ABZ on lymphatic filariasis. However, there is not enough evidence to support the efficacy of this treatment. Some studies have shown a reduction in microfilaraemia due to ABZ (Ismail *et al.*, 1998;

Makunde *et al.*, 2003; Pani *et al.*, 2002), but others have found that single doses of ABZ have little or no impact on microfilariae (Addiss *et al.*, 1997; Dunyo *et al.*, 2000a). It has, however, been found that high and repeated doses of ABZ cause large decreases in microfilarial levels and are clearly macrofilaricidal for *W. bancrofti* in humans (Jachowski *et al.*, 1993) and *B. malayi* and *B. pahangi* in laboratory animals (Denham *et al.*, 1980; Mak *et al.*, 1984).

Many studies have shown that combination treatment with the various drugs is more efficacious than the use of any drug alone (Addiss *et al.*, 1997; Beach *et al.*, 1999; Bockarie *et al.*, 1998; Ismail *et al.*, 2001; Ismail *et al.*, 1998; Moulia-Pelat *et al.*, 1995; Simonsen *et al.*, 2004), though some studies reported no advantage to combination therapy (Dunyo *et al.*, 2000a; Dunyo *et al.*, 2000b). Recently, meta-analysis studies have questioned the efficacy of ABZ in lymphatic filariasis treatment, stating that there is insufficient evidence to conclude that there is any benefit to adding this drug to control programs and that further investigation is required (Critchley *et al.*, 2005a; Critchley *et al.*, 2005b; Tisch *et al.*, 2005). However, this has been refuted by other groups (Sunish *et al.*, 2006) and a recent ultrasonographic study found ABZ/DEC treatment to be strongly adulticidal (Hussein *et al.*, 2004).

Some studies have examined the potential of antibiotic treatment targeting the obligate filarial endosymbiont *Wolbachia* (Rao, 2005). Studies in animal models have shown that targeting *Wolbachia* with tetracycline has a strong effect on the development and reproduction of filarial nematodes (Taylor, 2002). It was found that treatment of *Litomosoides sigmodontis* with tetracycline eliminates the bacteria and leads to growth retardation and infertility of filaria (Hoerauf, 2000; Hoerauf *et al.*, 1999). In humans, treatment with doxycycline resulted in complete long-term blockage of embryogenesis of *Onchocerca volvulus* (Taylor and Hoerauf, 2001). It was then shown that doxycycline treatment led to a 96% loss of *Wolbachia* and a 99% reduction in microfilaraemia in *W. bancrofti* infections (Hoerauf *et al.*, 2003). It was recently found that doxycycline treatment displays some adulticidal activity in addition to high efficacy against microfilariae (Taylor *et al.*, 2005). However, the long-term multi-dose treatment regimes tested to date have proven to be impractical (Rao, 2005).

1.6.1 Albendazole

Albendazole belongs to the benzimidazole (BZ) class of drugs (Townsend, 1990). Benzimidazoles are broad-spectrum anthelmintics, with some additional activity against fungi and protozoans (Lacey and Gill, 1994). These drugs are composed of a bicyclic ring system, in which benzene is fused to the 4- and 5-position of the heterocycle (Townsend, 1990). Albendazole metabolism is characterized by oxidation of the nucleophilic heteroatom, with sulfoxide and sulfone metabolites dominating the plasma profile (Lacey, 1990).

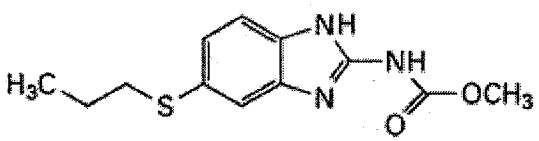

Figure 1.3: Albendazole

Many mechanisms of action have been proposed for benzimidazoles. An early report demonstrated an inhibition of the fumarase reductase reaction (Prichard, 1973), while another reported an inhibitory effect on protein secretion (Watts *et al.*, 1982), and disruption of the parasite's energy balance, due to dissipation of the transmembrane proton gradient (McCracken and Stillwell, 1991). In 1975, Borgers first proposed that the benzimidazole mebendazole had an effect on microtubules in *Ascaris suum* (Borgers and De Nollin, 1975). Microtubules are involved in many cellular functions, thus providing a link between the various biochemical reactions previously associated with benzimidazoles (Lacey and Prichard, 1986). Binding studies confirmed the mode of action of benzimidazoles to be interference with microtubule polymerization, via tubulin binding (Ireland *et al.*, 1979; Kohler and Bachmann, 1981; Lacey, 1988; Lacey, 1990; Lubega and Prichard, 1990). Benzimidazoles were found to compete for binding with the microtubule inhibitor colchicine (Friedman and Platzer, 1978; Kohler and Bachmann, 1981). Lubega and Prichard also found that the anthelmintic potency of BZs correlates with their affinities for tubulin binding (Lubega and Prichard, 1991). BZs bind specifically to helminth β-tubulin, though some binding to mammalian tubulin does occur

(Lacey, 1990). In *Haemonchus contortus*, BZs are lethal due to inhibition of secretory vesicle transport via depolymerization of microtubules (Jasmer *et al.*, 2000). There has been speculation on the specific binding site of benzimidazoles to tubulin. Photolabeling studies determined that the binding site of benzimidazoles on β-tubulin isotype-1 is between amino acids 63 and 103 (Nare *et al.*, 1996). In a review, Prichard proposed that the benzimidazole binding site includes both α and β-tubulin and that the planar benzimidazole ring binds between the phenyl rings of phenylalanines at position 167 and 200 of β-tubulin (Prichard, 2001). However, it was suggested that this model was not in accordance with the crystal structure of β-tubulin (Robinson *et al.*, 2002). Recently, it was proposed that residues implicated in binding lie deeply within the microtubule, and that a mechanism, such as an inter-domain movement between the N-terminal and the intermediate region of β-tubulin, may occur to allow binding (Robinson *et al.*, 2004), though it must be noted that this work was based on modeling with albendazole-sulphoxide, an albendazole metabolite which shows lower binding affinity to tubulin than does albendazole (Lubega & Prichard, 1991), while still being active as an anthelmintic.

1.6.1.1 B-tubulin

Microtubules are the principal structural components of meiotic and mitotic spindles of eukaryotic cilia and flagella, and of elongated processes such as those found in neuronal cells. They are also involved in overall cell structure, maintaining cell surface properties, and in intracellular transport (Cleveland and Sullivan, 1985). Most organelles, such as mitochondria, Golgi apparatus, ribosomes, lysozomes, cell membranes and the nucleus, associate with microtubules (Dustin, 1984). They are also directly associated with hormone and neurotransmitter secretion, as well as nutrient, enzyme and receptor action (Lacey, 1988).

Microtubules are hollow tubular organelles with a diameter of about 25 nm. Mammalian microtubules are made up of 13 parallel aligned protofilaments (Dustin, 1984), whereas helminth tubulin consists of 11 or 15 (Chalfie and Thomson, 1979). The protein, which makes up microtubules is the soluble dimeric protein tubulin, which is composed of an α and a β-subunit. Tubulin proteins are in dynamic equilibrium with microtubules, as they are added and subtracted on opposite ends of the insoluble

microtubule, in a 'treadmilling' action (Lacey, 1988). This equilibrium may be disturbed by changes in temperature or in levels of endogenous co-factors, such as GTP, Mg_2^+, Ca_2^+, and microtubule associated proteins (MAPs) (Lacey and Gill, 1994). Tubulin subunits are acidic, have a primary structure of about 450 amino acids, a molecular weight of about 50 KDa (Lacey and Gill, 1994), and the genes coding for the two subunits are 36-42% homologous to each other (Little and Seehaus, 1988). Different isotypes of both α and β-tubulin have been identified, and may be distinguished using isoelectric focusing on gels (Cleveland and Sullivan, 1985). It has been found that some specificity in function of different isotypes exists, yet generally, communal association in the microtubule matrix occurs (Lopata and Cleveland, 1987).

Using a monoclonal antibody shown to bind microfilariae, Helm (1989) localized β-tubulin in *B. malayi* and *B. pahangi* microfilariae. Screening of an adult *B. pahangi* cDNA library with this antibody allowed the identification of a small, 40 amino acid segment, which showed great similarity to the C-terminus of vertebrate β-tubulin. In *B. malayi,* β- tubulin was found to be abundant in somatic tissues (Helm *et al.*, 1989). Tang and Prichard (1989) found 4-5 β-tubulin isotypes in *B. malayi* and 5 in *B. pahangi* through Western blotting with anti-chicken β-tubulin monoclonal antibodies. The isoelectric point of these isoforms ranged from pH 4.7-5.4. Tubulin accounted for 2.8% and 2.9% of soluble protein in *B. malayi* and *B. pahangi,* respectively. A *B. pahangi* β-tubulin gene (b 1) was sequenced and found to span 3.8 kb, contained 9 exons, and 1.8 kb of coding region. It coded for a 448 aa protein, which showed over 85 % similarity with chicken and *Caenorhabditis elegans* β-tubulins (Guenette *et al.*, 1991). The b-1 tubulin also contains a 22 nucleotide trans spliced leader sequence, which is homologous to SL1 of *C. elegans* (Guenette *et al.*, 1992). A partial sequence for a second β-tubulin gene (b2), which is developmentally regulated to be expressed primarily in adult males, has also been identified (Guenette *et al.*, 1992). At least three different genes of β-tubulin have been cloned and sequenced from the sheep parasite *Haemonchus contortus*, including the highly similar b12-16 and b12-164 (thought to belong to the same isotype group), and b8-9 (Geary *et al.*, 1992). These genes were later determined to code for isotype 1 and isotype 2 β-tubulin (Kwa *et al.*, 1993b). The *H. contortus* genome project indicates that

there may be 3 β-tubulin genes in this nematode (R.K. Prichard, personal communication). B-tubulins have also been cloned from the filarial worms *Onchocerca volvulus* and *Dirofilaria immitis*, and have high similarity to the b-1 of *B. pahangi* (Geary *et al.*, 1998).

1.6.1.2 Resistance to Albendazole

Resistance to BZs in livestock was reported as early as the 1960s (Drudge *et al.*, 1964). Thereafter, there have been many reports of resistance (Le Jambre *et al.*, 1979; Wolstenholme *et al.*, 2004). Early on it was shown that differential expression of β-tubulin isotypes may be involved in benzimidazole resistance in protozoa. Of three known isotypes of β-tubulin expressed in *Physarium*, a mutation in only one gene, known as *benD*, was sufficient to cause resistance (Burland *et al.*, 1984). This mutant isotype was equally incorporated into microtubules and was expressed in constant proportion to wild-type benD with increasing drug concentrations (Foster *et al.*, 1987). Later it was found that the resistance mechanism to BZs in *Trichostrongylus colubriformis* involves a change in binding affinity of BZs to tubulin (Sangster *et al.*, 1985), which is dependent on the BZ structure (Lacey and Prichard, 1986). In addition, there is reduced stability in the BZ-tubulin complex in resistant strains (Lacey *et al.*, 1987; Russell and Lacey, 1992). This change in binding has been attributed to a reduction in high affinity receptors, and does not appear to be linked to low-affinity binding (Lubega and Prichard, 1990; Lubega and Prichard, 1991). Studies involving the free-living nematode *C. elegans* found that a mutation in, or complete deletion of, the *ben-1* β-tubulin gene conferred BZ resistance. This appeared to be the only locus susceptible to BZs in *C. elegans,* and other β-tubulin isotypes in this nematode, such as *mec-7* and *tub-1,* were not sensitive to BZs. Worms lacking the *ben-1* isotype were shown to be completely viable in the presence of BZs, supporting the hypothesis that β-tubulin isotypes may be homologous in function (Driscoll *et al.*, 1989). Benzimidazole-resistant populations of *H. contortus* show a decrease in polymorphism in at least two β-tubulin genes (Beech *et al.*, 1994; Lubega *et al.*, 1994; Roos *et al.*, 1990) and *T. colubriformis* (Grant and Mascord, 1996).

Kwa (1993 a,b) then showed that lower levels of BZ resistance in *H. contortus* were associated with selection of the isotype 1 β-tubulin gene (Kwa *et al.*, 1993a).

Resistance was due to a mutation at amino acid position 200, changing from a phenylalanine to a tyrosine (Kwa *et al.*, 1993a). It was also found that resistance could be enhanced by a deletion of the β-tubulin isotype 2 gene, although this occurred only at very high levels of resistance (Kwa *et al.*, 1993a; Roos, 1995). However, Beech *et al.* (1992) found selection associated with BZ resistance in both isotype 1 and isotype 2 genes, and hypothesized that resistant alleles are not novel, but were present in the population at low levels before selection pressure occurred, explaining the relatively rapid emergence of BZ resistance. Upon drug exposure, the above mentioned mutation at codon 200 in β-tubulin isotype 1 was also found in resistant strains of *Teleadorsagia circumcincta* (Elard *et al.*, 1996) and appeared to be recessive (Elard and Humbert, 1999). In addition, the Tyr 200 genotype has been observed in *Cooperia oncophera* (Njue and Prichard, 2003; Winterrowd *et al.*, 2003) as well as in equine cyathostomes (Drogemuller *et al.*, 2004a; Pape *et al.*, 2003). Vertebrate β-tubulin possesses a Tyr200, and this may be the reason why vertebrate cells are relatively insensitive to BZs (Li *et al.*, 1996b; Sullivan, 1988). This has led to the conclusion that the nature of the amino acid at position 200 is fundamental for the development of BZ resistance or susceptibility (Elard *et al.*, 1999). As a result, a simple diagnostic PCR test detecting BZ resistance, which may replace egg hatch or microlarval development assays, was developed (Elard and Humbert, 1996).

ABZ resistance in the protozoan *Giardia* was, however, not linked to the amino acid 200 mutation (Upcroft *et al.*, 1996). In the fungus *Rynchosporidium secalis*, a mutation at codon 198, changing from glutamic acid to glycine, was associated with BZ resistance (Wheeler *et al.*, 1995). The same mutation also caused decreased binding of BZs to a a fungal β-tubulin expressed as a fusion protein (Hollomon *et al.*, 1998). A study in *R. secalis* by Butters and Hollomon (1999) detected BZ resistance in β-tubulin isoforms that have wild-type amino acids between codons 196-202, thus involving neither the mutations at residue 198 or 200. A mutation at codon 241 from arginine to histidine in *Sacchromyces cerevisiae*, (Thomas *et al.*, 1985) and at position 240 from leucine to phenylalanine in *Tapesia,* appeared to confer BZ-resistance as well. Residue 200 was found to be associated with benomyl resistance in *Aspergillus nidulans* (Jung *et al.*, 1992) and *Botrytis cinerea* (Yarden and Katan, 1993).

An important mutation in β-tubulin at residue 167, from phenylalanine to tyrosine, conferred resistance in *S. cerevisiae* and was confirmed with studies involving site-directed mutagenesis (Li *et al.*, 1996a). Similar results were found in *Neospora crassa* (Orbach *et al.*, 1986), trichostrongylids (Silvestre and Cabaret, 2002) and in equine cyathostomes (Drogemuller *et al.*, 2004b).

Studies in the human nematode parasite *W. bancrofti* using sequence analysis of bulk samples (Hoti *et al.*, 2003) and in the human hookworms *Ancylostoma duodenale* and *Necator americanus* (Albonico *et al.*, 2004), have failed to find the position 200 mutation in worm populations exposed to BZ drugs.

Hall *et al.* (1982) found BZ resistance to persist for many generations after removal of anthelmintic selection pressure. Lack of reversion of BZ resistance after prolonged use of levamisole has also been observed in *H. contortus* and *Ostertagia* spp. (Borgsteede and Duyn, 1989; Martin *et al.*, 1988). Elard *et al.* (1998) examined the relative fitness of BZ-resistant and sensitive strains of *T. circumcincta* and found no significant difference. This suggests that there is no selection against the resistant phenotype, and thus supports the hypothesis that BZ-resistance is unlikely to revert back to the susceptible form, once the drug is removed.

In 1977, Coles and Simpkin proposed the use of a test based on the ovicidal action of BZs to detect BZ resistance (Hall *et al.*, 1978). Hubert and Kerboeuf (1992) proposed a microlarval development assay to detect BZ resistence. However, discrepancies between faecal egg count reduction and the lethal dose 50% in the egg-hatch assay and larval development assay have been found (Maingi *et al.*, 1998). Furthermore, these tests have low sensitivity, and it is unlikely that resistance can be detected before it has become a problem in the treatment of parasites (Prichard, 1990). PCR diagnostic tests for resistance, such as the one developed by Elard and Humbert (1999), are thus very useful and necessary in order to detect selection for resistance as soon as it appears.

1.6.2 Ivermectin

Ivermectin became commercially available in 1980 (Campbell *et al.*, 1983; Chabala *et al.*, 1980). This drug, which is an avermectin, belongs to the class of anthelmintics known as macrocyclic lactones, which were first developed during the

1970s (Burg *et al.*, 1979). Ivermectin is derived from the fermentation products of the soil organism *Streptomyces avermitilis,* which was first isolated in Japan in 1975 (Burg *et al.*, 1979). Avermectins are broad-spectrum compounds, which display activity against a wide variety of helminths and arthropods (Shoop, 1993).

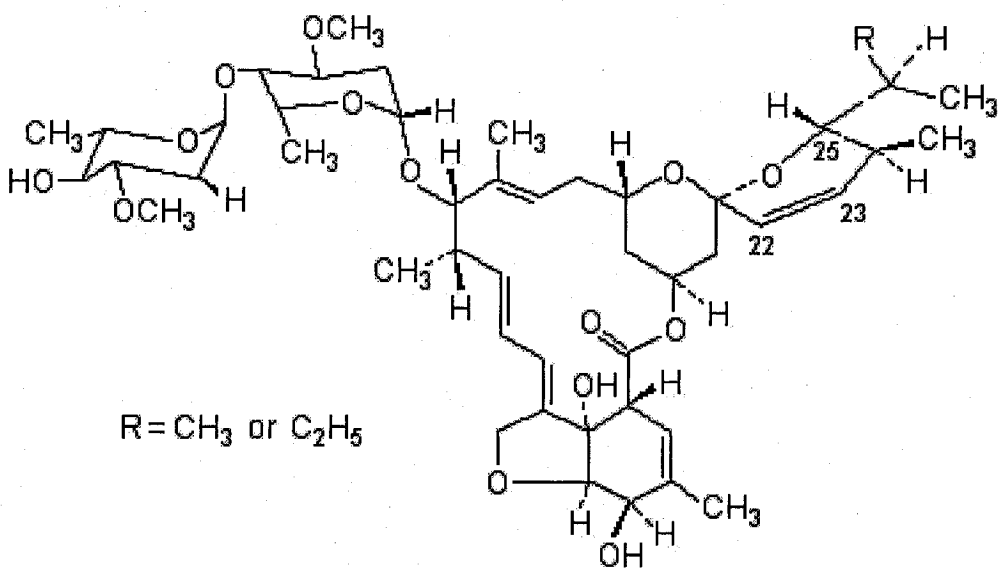

Figure 1.4: Ivermectin

The mode of action of avermectins was initially found to involve γ-aminobutyric acid (GABA) receptors (Holden-Dye *et al.*, 1989; Holden-Dye and Walker, 1990; Pong *et al.*, 1981; Pong and Wang, 1980; Pong and Wang, 1982; Wang and Pong, 1982). It was initially suggested that ivermectin binds to GABA-associated chloride channels (Fritz *et al.*, 1979), leading to flaccid paralysis (Kass *et al.*, 1984). However, studies by Martin and Pennington (1989) and Arena *et al.*, (1991) demonstrated that avermectins potently affect chloride channels not sensitive to GABA , and it was shown that there was interaction between ivermectin and glutamate-gated chloride channels (Scott and Duce, 1985; Zufall *et al.*, 1989). Two glutamate-gated chloride channel subunits (GluClα and GluClβ) were cloned from *C. elegans*. GluClα displayed sensitivity to ivermectin when expressed in *Xenopus* oocytes (Cully *et al.*, 1994). Two additional GluClα genes, Avr 15 (Dent *et al.*, 1997; Vassilatis *et al.*, 1997) and Avr 14 (Laughton *et al.*, 1997) were isolated from *C. elegans*, and were shown to bind glutamate reversibly, and ivermectin irreversibly, leading to channel activation (Dent *et al.*, 2000). Activation of GluCl by ivermectin was found to be correlated with its nematocidal activity (Arena *et al.*, 1995).

Glutamate binds GluCl reversibly, leading to an influx of Cl^- into the target neuromuscular cell, which inhibits muscle contraction. As ivermectin binds the α-subunit irreversibly, the chloride channel remains in the open conformation, leading to hyperpolarization of the cell, causing flaccid paralysis (Dent *et al.*, 2000). A similar GluClα was cloned from *Drosophila melanogaster*, though ivermectin binding led to an inhibition of glutamate binding in this channel at high concentrations of the drug (Cully *et al.*, 1996b). GluCl homologues have also been cloned from *H. contortus* and *Ascaris suum* (Delaney *et al.*, 1998; Jagannathan *et al.*, 1999; Hejmadi *et al.*, 2000). GluCls from *C. oncophera* have also been cloned (Njue and Prichard, 2004). Partial GluCl sequences have been obtained from *O. volvulus* and *D. immitis* (Cully *et al.*, 1996a), and recently an ivermectin-sensitive GluClα was cloned from *D. immitis* (Yates and Wolstenholme, 2004). Glutamate-gated chloride channels are not present in vertebrates, accounting for part of the relatively small effect of the avermectins on mammals (Laughton *et al.*, 1997; Vassilatis *et al.*, 1997). However, since avermectins are known to interact with mammalian GABA receptors, α7 nicotinic acetylcholine receptors and $P2X_4$ receptors (Yates *et al.*, 2003) this does not solely explain host selectivity (Geary, 2005). Recent studies have shown that disruption of the P-glycoprotein gene *mdr-1* in Collie dogs and mice leads to ivermectin hypersensitivity and toxicity (Mealey *et al.*, 2001; Schinkel *et al.*, 1994), implicating this protein in protection against IVM in mammals.

GluCls were found to be largely restricted to the pharynx in *C. elegans* (Laughton *et al.*, 1997; Vassilatis *et al.*, 1997) and in *A. suum* (Martin *et al.*, 1996). Ivermectin is thought to act on *C. elegans* by causing paralysis of the pharynx (Cleland, 1996; Cully *et al.*, 1996a; Dent *et al.*, 1997; Dent *et al.*, 2000; Pemberton *et al.*, 2001). Gill and Lacey (1998) found that avermectin affects the motility of *H. contortus* and *T. colubriformis*, and speculated that this may lead to their expulsion from the host. It has been shown that ivermectin acts by causing paralysis of the pharynx in *H. contortus* (Geary *et al.*, 1993). However the presence of GluCl in the pharynx of this parasite has not yet been confirmed. In *H. contortus*, GluCls were initially found only in the systemic musculature (Delany *et al.*, 1998; Jagannathan *et al.*, 1999), but micreoinjection of the *H. contortus* GluCl-α subunit gene *HcGluCla* into *C. elegans* led to expression of this gene in phanryngeal neurons (Liu *et al.*, 2004). A recent study did not find an effect of ivermectin on feeding

(Sheriff *et al.*, 2005). Dent *et al.*, (1997; 2000) found that Avr 15 is also expressed in the peripheral nervous system, and that Avr 14 is found in the somatic musculature of *C. elegans*. Glutamate binding sites are found in both α and β subunits of GluCl, whereas ivermectin appears to have a binding site solely on the α subunit (Dent *et al.*, 2000). Studies suggest that ivermectin and glutamate binding sites are separate on the α-subunit, and that ivermectin exerts an allosteric effect on glutamate binding (Forrester *et al.*, 2002; Paiement *et al.*, 1999a).

1.6.2.1 Resistance to Ivermectin in Parasites

The mechanism of resistance against macrocyclic lactones has not been completely elucidated. However, several genes have been shown to exhibit selection associated with resistance. Using single-stranded conformational polymorphism analysis, it was shown that there was selection for one allele of a glutamate-gated chloride channel α-subunit in ivermectin-resistant strains of *H. contortus* (Blackhall *et al.*, 1998b). Selection in such strains was also shown at a P-glycoprotein (P-gp) gene, which is usually highly polymorphic in nematodes (Blackhall *et al.*, 1998a; Xu *et al.*, 1998). Le Jambre *et al.* (1999) found selection at a second P-gp gene. The ivermectin-selected strains also showed an increase in mRNA levels of a P-gp in resistant strains (Xu *et al.*, 1998). Selection for an allele in a different P-glycoprotein gene was also found to be associated with ivermectin resistance in field strains (Sangster *et al.*, 1999). Ivermectin has been shown to bind these membrane transport proteins in humans (Didier and Loor, 1996), which may explain their involvement in ivermectin resistance. Kwa *et al.* (1998) in a limited restriction fragment length polymorphism (RFLP) analysis using only a single restriction enzyme and gene probes failed to detect an involvement of P-gp in ivermectin resistance. However, Le Jambre *et al.* (1999) conducted a more thorough genetic analysis and found that this same P-gp gene was associated with ivermectin resistance. There was also significant selection at a β-tubulin gene in ivermectin-resistant *H. contortus* strains (Blackhall *et al.*, 1999; Eng and Prichard, 2005a).

Resistance of *H. contortus* to macrocyclic lactones may be measured by the extent of pharyngeal pumping (Kotze, 1998). The effect of ivermectin on pharyngeal pumping was significantly different in susceptible and resistant strains. There was an 8-fold

decrease in the efficacy of ivermectin to inhibit pharyngeal pumping in resistant strains (Paiement *et al.*, 1999b). However, Gill *et al.* (1998) suggested that there may be more than one mechanism of resistance against ivermectin. Since avermectins may act either on pharyngeal pumping or through paralysis of the somatic musculature, different resistance mechanisms may develop depending on the mode of action (Gill and Lacey, 1998). Furthermore, the development of resistance may be affected by additional factors. Dent *et al.* (2000) found that a deleterious mutation in pharyngeal GluClα did not cause high levels of resistance in *C. elegans*. They speculated that ivermectin, acting on somatic GluClα, may cause a Cl influx, which is transferred to the pharyngeal musculature via gap junctions.

It was discovered that glutamate binding kinetics were altered in resistant *H. contortus* strains, with increased maximum binding (Bmax) and lowering dissociation constant (Kd) values, suggesting that changes in a glutamate binding site are directly related to ivermectin resistance (Paiement *et al.*, 1999b). Hejmadi *et al.* (2000) noted no difference in ivermectin binding between sensitive and resistant strains of *H. contortus*, though they did find an increase in low-affinity glutamate binding sites in resistant strains. Also, a study in *C. oncophera* showed that mutations in a GluClα subunit found in resistant strains led to a decrease in IVM sensitivity (Njue and Prichard, 2004).

Recently it has been documented that some females of *O. volvulus* in endemic foci in Ghana showed decreased sensitivity to ivermectin, possibly due to drug resistance (Awadzi *et al.*, 2004a; Awadzi *et al.*, 2004b). There is also some genetic evidence that selection by ivermectin is occurring in *O. volvulus*. Significant differences in allele frequencies between untreated and ivermectin treated worm populations were found for β-tubulin and P-glycoprotein (Eng and Prichard, 2005; Ardelli *et al.*, 2006 a,b ; Ardelli *et al.*, 2005; Ardelli and Prichard, 2004). This suggests that, at least in filarial nematodes, IVM is likely selecting on more than one gene (Prichard, 2005), contrary to an earlier claim that IVM resistance is a monogenic trait (Le Jambre *et al.*, 2000). Little is known about the inheritance of ivermectin resistance, however it has been speculated that it is a completely dominant trait (Barnes *et al.*, 2001; Le Jambre *et al.*, 2000). Other studies have shown that the mode of inheritance of macrocyclic lactone resistance in *T. circumcincta* may vary (Sutherland *et al.*, 2003; Sutherland *et al.*, 2002). Resistance to

abermectin in the Colorado potato beetle and the housefly is inherited as a polygenic, autosomal recessive trait (Clark *et al.*, 1995). The mode of inheritance of drug resistance will have a large impact on its selection, with dominant traits being selected more quickly (Barnes *et al.*, 1995; Dobson and Barnes, 1995).

1.6.3 Diethylcarbamazine

Despite its wide-spread use against lymphatic filariasis for over 50 years, the mode of action of DEC against filarial still remains poorly understood (Alves *et al.*, 2005). For many years, it was understood that the drug had no direct effect on the parasite *in vitro* at therapeutic concentrations (Hawking *et al.*, 1950; Johnson *et al.*, 1988; Jordan, 1958). This has led to the belief of an indirect mode of action of the drug. It was suggested that DEC acts as an anti-inflammatory drug, as it has an antagonistic effect on arachidonic acid metabolism. This could lead to vasoconstriction and enhanced microfilarial adherence to endothelial cells, which immobilizes the parasites as well as increased adherence and cytotoxic activity by host platelets and granulocytes (Maizels and Denham, 1992). Specifically, DEC has been shown to inhibit leukotriene synthases (Bach and Brashler, 1986) and to inhibit products of the cyclooxygenase (COX) pathway (Kanesa-thasan *et al.*, 1991; McGarry *et al.*, 2005). An augmentation of human neutrophil and eosinophil adherence due to DEC has been shown *in vitro* (King *et al.*, 1983).

Some recent studies have suggested that DEC may have a direct effect on *W. bancrofti in vitro* (Peixoto, 2005). It was found that diethylcarbamazine induced a loss of the microfilarial sheath of *W. bancrofti* (Florencio and Peixoto, 2003; Peixoto *et al.*, 2003). Thereafter, it was observed that there was cytolysis and damage to organelles and the formation of cellular vacuoles in microfilariae treated *in vitro* (Florencio and Peixoto, 2003) and *in vivo* (Peixoto *et al.*, 2004). Similar observations were made in infective L3 larvae of *W. bancrofti* (Alves *et al.*, 2005). Recently, it was also shown that adult females of *W. bancrofti* obtained from patients treated with DEC contained few or no embryos, clearly indicating an effect on reproduction (Peixoto, 2005).

1.6.3.1 Resistance to DEC

There have been some reports of lymphatic filarial worms showing tolerance to treatment with DEC (Eberhard *et al.*, 1991; Eberhard *et al.*, 1988) and that some adult worms appear not to be susceptible to DEC (Dreyer *et al.*, 1995). A recent study also found a variable response in treatment efficacy of DEC, with some patients showing a very poor response (Stolk *et al.*, 2005).

1.7 The Global Alliance to Eliminate Filariasis.

In 1993, an international task force on disease eradication named LF amongst one of six diseases that could be eradicated, or potentially eradicated (WHA, 1993). Several years later, a World Health resolution called for global efforts to eliminate LF as a public health problem (WHO, 1997). In January 1998, the pharmaceutical company Glaxo-SmithKline, then SmithKline and Beecham, announced that it would donate the antiparasitic drug ABZ to the World Health Organization (WHO, 1998b) for as long as necessary to eliminate the disease. Later that year, Merck & Co., Inc., which was already supplying IVM free of charge to the WHO for onchocerciasis control, extended this donation program to include the treatment of LF, for the purpose of its elimination (WHO, 1998a). Hence, the global program to eliminate LF (GPELF) was formed. This program resolves to eliminate LF as a public health threat by the year 2020 (WHO, 2000). It aims to interrupt disease transmission, primarily by chemotherapy, and thereby reduce morbidity due to the disease. Recommended strategies of GPELF are community directed mass treatment programs with drug combinations of ABZ (400 mg) and DEC (6 mg/kg), or ABZ (400 mg) and IVM (200 mg/kg) for 4-6 years. The GPELF also calls for the use of DEC-fortified table salt in some areas, vector control *via* insecticide treated bed nets and morbidity control through home-based self care of lymphoedema and elephantiasis as well as surgical interventions for hydrocoele (Gyapong *et al.*, 2005). Patients suffering from onchocerciasis or loiasis experience severe adverse reactions to DEC, thus this drug cannot be administered in areas endemic for these diseases (WHO, 1999; WHO, 2000).

By 2004, half of all recognized endemic countries had initiated treatment programs for LF, and approximately 435 million doses of drug had been administered to the at-risk population (Gyapong and Twum-Danso, 2006). Though results of the program seem very positive, with successful reductions in microfilaraemia (WHO, 2004), the estimate of a required 4-6 years to interrupt transmission may be overly optimistic, and longer treatment periods will likely be required in many areas (Gyapong and Twum-Danso, 2006; Michael *et al.*, 2004).

1.8 Epidemiology and Mathematical Modeling of Lymphatic Filariasis

Mathematical models can be invaluable in helping to understand the population dynamics of parasites and to predict the impact of chemotherapy on these populations (Anderson and May, 1982; Anderson and May, 1985). Models involving populations of animals must attempt to recreate the most important dynamic features of the populations and their interactions (May, 1976). Models aiding in the design and implementation of control programs have been successfully used for the filarial nematode *Onchocerca volvulus* (Alley *et al.*, 2001; Basanez and Ricardez-Esquinca, 2001; Habbema *et al.*, 1992; Hairston and Jachowski, 1968).

1.8.1 Analytical Models

Mathematical models to examine various aspects of the biology of lymphatic filariasis have been in existence since the 1960s (Hayashi, 1962). This model made use of the two stage catalytical model introduced by Muench (1959) which describes the rate of change in the infected (microfilaraemic) and uninfected (amicrifilaraemic) populations in a system that is at equilibrium (Michael, 2000). Hayashi's model assumed that individuals are initially susceptible and may become infected and recover from the infection. Once they have recovered, they become resistant and may no longer become infected. Hairston and Jalowski (1968) later found that this model did not accurately describe the data they had collected in American Samoa. Instead they applied the reversible catalytical model (Muench, 1959), which allows individuals that have cleared the infection to become microfilaraemic again. The reversible catalytic model is defined by the following differential equations (Vanamail *et al.*, 1989):

$$\frac{dU}{dt} = -aU + bI \qquad 1.1$$

$$\frac{dI}{dt} = aU - bI \qquad 1.2$$

Where U is the number of uninfected (amicrofilaraemic) and I of infected (microfilaraemic) individuals of age t, a is the *per capita* rate of infection and b the *per capita* loss of infection rate.

The solution to this equation, assuming that no one is initially infected and that a and b are constant with host age is:

$$I(t) = \frac{a}{a+b}\left(1 - e^{-(a+b)t}\right) \qquad 1.3$$

Hairston and Jalowski (1968) applied this model to cross-sectional data and thereby estimated average life-span of adult worms and worm burden. This same reversible catalytic model was later applied by Vanamail *et al.* (1989) to longitudinal data from Pondicherry, India. This work indicated that the loss of infection is independent of age. However, the gain of infection displays a convex age-profile, increasing in the early age classes until 16-20 years and then stabilizing and declining. This analysis provides some evidence of acquired immunity acting against worm establishment in LF.

Michael and Bundy (1998) applied the two stage catalytic model and found that observed infection patterns in India are consistent with the assumption that there is herd immunity, and that this immunity is stronger and occurs earlier in areas where transmission is higher.

Other theoretical models have examined the relationship between prevalence and microfilarial intensity of filarial infections. Grenfell *et al.* (1990) modelled the relationship between macrofilarial prevalence and microfilarial burdens and found that most amicrofilaraemics are true negatives that arise from the absence of microfilariae or from single sex infections. Das *et al* (1990) fitted a zero-truncated negative binomial distribution to *W. bancrofti* microfilarial counts of different age groups, and found that there was a significant decrease in overdispersion with age above age 10, providing indirect evidence for density-dependence acting on microfilarial intensity in the host. However a similar study in *B.malayi* did not observe such an effect (Srividya *et al.*, 1991a).

There have also been mathematical models that investigate the dynamics of disease in lymphatic filariasis, in order to determine the relationship between lymphoedema and microfilaraemia. These models indicate a progression from uninfected, microfilaraemia, amicrofilaraemic to irreversible lymphatic pathology (Bundy *et al.*, 1991; Srividya *et al.*, 1991b). Recently, a model examining transmission intensity, immunoepidemiology and disease indicated that the *per capita* rate of chronic disease

may increase with transmission intensity (Michael *et al.*, 2001).

Much attention has also been given to modeling the transmission dynamics of lymphatic filariasis. It has been shown through laboratory studies, that the uptake of microfilariae from the host by the mosquito vector depends on the density and distribution of microfilariae in the host (Subramanian *et al.*, 1998). The relationship between microfilaraemia and uptake has been shown to be non-linear and saturating in some studies (Bryan and Southgate, 1988; McGreevy *et al.*, 1982; Subramanian *et al.*, 1998), though linear in others (Jayasekera *et al.*, 1991; Lowrie *et al.*, 1989). A meta-analysis by Snow and Michael (2002) attributes these divergent findings to differences in study design. These authors found that, though all genera of vectors displayed density dependence in MF uptake, the point at which saturation was reached was significantly higher for *Culex.*

L3 development in the vector from an ingested blood meal may follow three possible relationships: proportionality (a constant ratio between MF and L3), limitation (a decrease in this ratio with increasing MF) or facilitation (an increase in this ratio with increasing MF) (Southgate and Bryan, 1992). Limitation has been observed in some *Aedes* spp. (Pichon, 2002) and in *Culex* that transmit *W. bancrofti* (Southgate, 1992; Southgate and Bryan, 1992; Subramanian *et al.*, 1998). Proportionality has been found to occur in *Mansonia* and *Aedes*, whereas facilitation was observed in *Anopheles* (Southgate and Bryan, 1992). Limitation may in part be due to parasite-induced vector mortality, which has been shown in a number of analyses (McGreevy *et al.*, 1982; Samarawickrema and Laurence, 1978; Zielke, 1977). This process leads to a more efficient transmission at low microfilarial densities and thus makes interruption of transmission through LF control inherently more difficult (Duerr *et al.*, 2005; Pichon, 2002). It has been suggested that facilitation processes lead to unstable transmission due to the presence of "breakpoints" below which the infection can no longer persist (Duerr *et al.*, 2005). However, this concept has been questioned by others (Wada *et al.*, 1995).

Recently, Stolk *et al.* (2004) argued that in mathematical models, the relationship between microfilarial intensity in the patient and the number of L3 developing from a blood meal is more accurately modeled by a hyperbolic saturating function than by a linearized power curve.

1.8.2 Simulation Models

In addition to the above analytical models, there has also been some effort to produce simulation models. These models go beyond the previously described prevalence models and explore adult and larval dynamics of *Culex* transmitted *W. bancrofti*.

Two simulation models have been developed. LYMFASYM is a stochastic micro simulation model which is individual based, and thus each individual is tracked through time (Plaisier *et al.*, 1998). This model is a result of a collaboration between the Vector Control Research Centre in Pondicherry and the Department of Public Health, Erasmus University in Rotterdam (Das and Subramanian, 2002). As it is a micro-simulation model, LYMFASIM may consider variations in the human population (such as age, sex, exposure to mosquitoes, immune responsiveness) and in the parasites (adult life-span, reproduction).

LYMFASIM has recently been quantified using data from Pondicherry, collected between 1981 and 1985. This work compared different assumptions about acquired immunity and found that the model fitted the data best when anti L3 or anti-fecundity immunity was assumed. The mean life-span of the parasite was estimated to be about 10 years using LYMFASIM (Subramanian *et al.*, 2004). This model has also been used to make predictions by simulating different control programs. When a community representative of Pondicherry, India was used, the simulation predicted that 8 yearly treatments at 65% coverage gave a 99% probability of elimination (Stolk *et al.*, 2003). It was also shown that the benefit of vector control in terms of a reduction in MF prevalence outlasts the cessation of the control method (Subramanian *et al.*, 2004). Another prediction, based on preliminary data, was that at least 90% coverage will be required in order to reach the goal of elimination within 5 years using DEC-based mass drug administration (Das and Subramanian, 2002).

EPIFIL is a deterministic macro-simulation model which incorporates some probabilistic elements to describe vector transmission dynamics and also incorporates host age structure (Chan *et al.*, 1999; Chan *et al.*, 1998; Norman *et al.*, 2000). This model is a result of a collaboration between the Vector Control Research Centre in

Pondicherry and the Department of Parasite Epidemiology, Oxford University, Oxford (Das and Subramanian, 2002). It consists of a set of differential equations and initially described changes by age in worm burden, intensity in microfilaraemia, immunity and prevalence of lymphatic damage, lymphoedema and hydrocoel. It was fitted to epidemiological data collected in Pondicherry. It predicts that both hydrocoel and lymphoedema are irreversible and develop due to lymphatic damage produced by adult worms (Chan *et al.*, 1998). The EPIFIL model was then extended to explicitly incorporate host age structure and vector transmission dynamics (Norman *et al.*, 2000). The type of framework used in this model was first proposed by Anderson and May (1985). It has previously been used to model schistosomiasis (Chan *et al.*, 1995). EPIFIL predicts that chemotherapy has a larger short-term effect than vector control, but that, like predictions made with LYMFASIM, benefits of vector control may last beyond the treatment period. It also predicts that DEC is the most effective drug in reducing community microfilarial loads (Norman *et al.*, 2000). In a recent review, Michael *et al* (2004) stated that predictions using EPIFIL suggested that the proposed 6 year time frame for disease elimination is unlikely to be achieved in areas of moderate and high transmission. They suggest that the achievement of such goals would require the incorporation of vector control methods. They also conclude that high coverage will have to be maintained in order for the elimination program to be successful. EPIFIL is now available as a free resource for health workers in operational settings (Chan *et al.*, 1999).

1.8.3 Mathematical Models of Drug Resistance

The use of computer or mathematical models to examine the evolution and behaviour of drug resistance is extremely useful in order to examine the interaction of many different factors (Barnes and Dobson, 1990). Often field experiments in drug resistance are expensive and technically difficult to carry out. Furthermore, a mathematical or computer model facilitates altering conditions and factors involved in producing different outcomes.

Models that examine the development of drug resistance have been developed for a number of parasites (Barnes *et al.*, 1995; Hastings and D'Alessandro, 2000). Antibiotic

resistance models are generally purely epidemiological models. Due to sexual reproduction of helminths, models examining anthelmintic resistance are based on population genetics and tend to follow individual genes (Hastings, 2001). In 1990, two models for anthelmintic resistance were developed. A computer model examining the spread of resistance, in the sheep parasite *Trichostrongylus colubriformis* considers factors such as sheep management and anthelmintic delivery in a simple model that allows for three anthelmintic resistance genes (Barnes and Dobson, 1990). This model has been used to explore factors such as treatment rotations, treatment combinations and treatment with persistent drugs (Barnes, 1995; Dobson *et al.*, 1996). From this model, it was derived that resistance developed more quickly when two genes were involved than when a single gene conferred resistance (Barnes, 1995). If this gene was dominant, resistance developed more quickly than if it was recessive. Interestingly, the model also predicted that a drug which remains in the host for a long time-period at low levels is more likely to lead to resistance (Dobson *et al.*, 1996). A second general model applicable to direct life-cycle parasites considers one resistance gene with two alleles and examines different anthelmintic treatment strategies (Smith, 1990). More complex deterministic and stochastic models for the evolution of anthelmintic resistance in trichostongylids were later developed (Smith *et al.*, 1999). These models predict that host immunity, fecundity and aggregation have an important impact on the development of resistance.

The impact of spatial heterogeneity and metapopulation dynamics of transmission on the spread of drug resistance has been examined more recently, using stochastic models. These works predict that rare recessive alleles may spread through overdispersed parasite populations more rapidly than initially anticipated (Cornell *et al.*, 2000; Cornell *et al.*, 2003).

References

Abbasi, I., Githure, J., Ochola, J. J., Agure, R., Koech, D. K., Ramzy, R. M., Williams, S. A. and Hamburger, J. (1999). Diagnosis of *Wuchereria bancrofti* infection by the polymerase chain reaction employing patients' sputum. Parasitol Res, 85, 844-849.

Abbasi, I., Hamburger, J., Githure, J., Ochola, J. J., Agure, R., Koech, D. K., Ramzy, R., Gad, A. and Williams, S. A. (1996). Detection of *Wuchereria bancrofti* DNA in patients' sputum by the polymerase chain reaction. Trans R Soc Trop Med Hyg, 90, 531-532.

Addiss, D. G., Beach, M. J., Streit, T. G., Lutwick, S., LeConte, F. H., Lafontant, J. G., Hightower, A. W. and Lammie, P. J. (1997). Randomised placebo-controlled comparison of ivermectin and albendazole alone and in combination for *Wuchereria bancrofti* microfilaraemia in Haitian children. Lancet, 350, 480-484.

Adebajo, A. O. (1996). Rheumatic manifestations of tropical diseases. Curr Opin Rheumatol, 8, 85-89.

Albertini, C., Gredt, M. and Leroux, P. (1999). Mutations of the beta-tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis*. Pest Biochem Phys. 64, 17-31.

Albonico, M., Wright, V. and Bickle, Q. (2004). Molecular analysis of the beta-tubulin gene of human hookworms as a basis for possible benzimidazole resistance on Pemba Island. Mol Biochem Parasitol, 134, 281-284.

Alley, W. S., van Oortmarssen, G. J., Boatin, B. A., Nagelkerke, N. J., Plaisier, A. P., Remme, J. H., Lazdins, J., Borsboom, G. J. and Habbema, J. D. (2001). Macrofilaricides and onchocerciasis control, mathematical modelling of the prospects for elimination. BMC Public Health, 1, 12.

Alves, L. C., Brayner, F. A., Silva, L. F. and Peixoto, C. A. (2005). The ultrastructure of infective larvae (L3) of *Wuchereria bancrofti* after treatment with diethylcarbamazine. Micron, 36, 67-72.

Amaral, F., Dreyer, G., Figueredo-Silva, J., Noroes, J., Cavalcanti, A., Samico, S. C., Santos, A. and Coutinho, A. (1994). Live adult worms detected by ultrasonography in human bancroftian filariasis. Am J Trop Med Hyg, 50, 753-757.

Anderson, R. M. and May, R. M. (1982). Population dynamics of human helminth infections: control by chemotherapy. Nature, 297, 557-563.

Anderson, R. M. and May, R. M. (1985). Helminth infections of humans: mathematical models, population dynamics, and control. Adv Parasitol, 24, 1-101.

Ardelli, B. F., Guerriero, S. B. and Prichard, R. K. (2005). Genomic organization and effects of ivermectin selection on Onchocerca volvulus P-glycoprotein. Mol Biochem Parasitol, 143, 58-66.

Ardelli, B. F., Guerriero, S. B. and Prichard, R. K. (2006a). Characterization of a half-size ATP-binding cassette transporter gene which may be a useful marker for ivermectin selection in Onchocerca volvulus. Mol Biochem Parasitol, 145, 94-100.

Ardelli, B. F., Guerriero, S. B. and Prichard, R. K. (2006b). Ivermectin imposes selection pressure on P-glycoprotein from Onchocerca volvulus: linkage disequilibrium and genotype diversity. Parasitology, 132, 375-386.

Ardelli, B. F. and Prichard, R. K. (2004). Identification of variant ABC-transporter genes among Onchocerca volvulus collected from ivermectin-treated and untreated patients in Ghana, West Africa. Ann Trop Med Parasitol, 98, 371-384.

Arena, J. P., Liu, K. K., Paress, P. S. and Cully, D. F. (1991). Avermectin-sensitive chloride currents induced by *Caenorhabditis elegans* RNA in *Xenopus* oocytes. Mol Pharmacol, 40, 368-374.

Arena, J. P., Liu, K. K., Paress, P. S., Frazier, E. G., Cully, D. F., Mrozik, H. and Schaeffer, J. M. (1995). The mechanism of action of avermectins in *Caenorhabditis elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. J Parasitol, 81, 286-294.

Awadzi, K., Attah, S. K., Addy, E. T., Opoku, N. O., Quartey, B. T., Lazdins-Helds, J. K., Ahmed, K., Boatin, B. A., Boakye, D. A. and Edwards, G. (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, 359-370.

Awadzi, K., Boakye, D. A., Edwards, G., Opoku, N. O., Attah, S. K., Osei-Atweneboana, M. Y., Lazdins-Helds, J. K., Ardrey, A. E., Addy, E. T., Quartey, B. T., Ahmed, K., Boatin, B. A. and Soumbey-Alley, E. W. (2004b). An investigation of persistent microfilaridermias despite multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. Ann Trop Med Parasitol, 98, 231-249.

Awasthi, S., Pande, V. K. and Fletcher, R. H. (2000 Jan). Effectiveness and cost-effectiveness of albendazole in improving nutritional status of pre-school children in urban slums. Indian J Med Res, 37, 19-29.

Bach, M. K. and Brashler, J. R. (1986). Inhibition of the leukotriene synthetase of rat basophil leukemia cells by diethylcarbamazine, and synergism between diethylcarbamazine and piriprost, a 5-lipoxygenase inhibitor. Biochem Pharmacol, 35, 425-433.

Bain, O. and Babayan, S. (2003). Behaviour of filariae: morphological and anatomical signatures of their life style within the arthropod and vertebrate hosts. Filaria J, 2, 16.

Barnes, E. H. and Dobson, R. J. (1990). Population dynamics of *Trichostrongylus colubriformis* in sheep: computer model to simulate grazing systems and the evolution of anthelmintic resistance. Int J Parasitol, 20, 823-831.

Barnes, E. H., Dobson, R. J. and Barger, I. A. (1995). Worm control and anthelmintic resistance: adventures with a model. Parasitol Today, 11, 56-63.

Barnes, E. H., Dobson, R. J., Stein, P. A., Le Jambre, L. F. and Lenane, I. J. (2001). Selection of different genotype larvae and adult worms for anthelmintic resistance by persistent and short-acting avermectin/milbemycins. Int J Parasitol, 31, 720-727.

Basanez, M. G. and Ricardez-Esquinca, J. (2001). Models for the population biology and control of human onchocerciasis. Trends Parasitol, 17, 430-438.

Beach, M. J., Streit, T. G., Addiss, D. G., Prospere, R., Roberts, J. M. and Lammie, P. J. (1999). Assessment of combined ivermectin and albendazole for treatment of intestinal helminth and *Wuchereria bancrofti* infections in Haitian schoolchildren. Am J Trop Med Hyg, 60, 479-486.

Beech, R. N., Prichard, R. K. and Scott, M. E. (1994). Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics, 138, 103-110.

Bell, D. (1967). Membrane filters and microfilariae: a new diagnostic technique. Ann Trop Med Parasitol, 61, 220-223.

Blackhall, W. J., Liu, H. Y., Xu, M., Prichard, R. K. and Beech, R. N. (1998a). Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of *Haemonchus contortus*. Mol Biochem Parasitol. 95, 193-201.

Blackhall, W. J., Pouliot, J. F., Prichard, R. K. and Beech, R. N. (1998b). *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin-and moxidectin-selected strains. Exp Parasitol. 90, 42-48.

Blackhall W. 1999. Genetic variation and multiple mechanisms of anthelmintic resistance in *Haemonchus contortus*. Ph.D. thesis. McGill University, Montreal, Quebec, Canada

Bockarie, M. J., Alexander, N. D., Hyun, P., Dimber, Z., Bockarie, F., Ibam, E., Alpers, M. P. and Kazura, J. W. (1998). Randomised community-based trial of annual single-dose diethylcarbamazine with or without ivermectin against *Wuchereria bancrofti* infection in human beings and mosquitoes. Lancet, 351, 162-168.

Borgers, M. and De Nollin, S. (1975). Ultrastructural changes in *Ascaris suum* intestine after mebendazole treatment in vivo. J Parasitol, 61, 110-122.

Borgsteede, F. H. and Duyn, S. P. (1989). Lack of reversion of a benzimidazole resistant strain of *Haemonchus contortus* after six years of levamisole usage. Res Vet Sci, 47, 270-272.

Brown, K. R., Ricci, F. M. and Ottesen, E. A. (2000). Ivermectin: effectiveness in lymphatic filariasis. Parasitol, 121 Suppl, S133-146.

Bryan, J. H. and Southgate, B. A. (1988). Factors affecting transmission of *Wuchereria bancrofti* by anopheline mosquitoes. 1. Uptake of microfilariae. Trans R Soc Trop Med Hyg, 82, 128-137.

Bundy, D. A., Grenfell, B. T. and Rajagopalan, P. K. (1991). Immunoepidemiology of lymphatic filariasis: the relationship between infection and disease. Immunol Today, 12, A71-75.

Burg, R. W., Miller, B. M., Barker, E. E., Birnbaum, J., Currie, J. A., Harman, R., Kong, V. L., Monaghan, R. L., Olson, G., Putter, I., Tunac, J. D., Wallick, H., Stapley, E. O., Oiwa, R. and Omura, S. (1979). Avermectins, a new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob Ag Chem, 15, 361-367.

Burglin, T. R., Lobos, E. and Blaxter, M. L. (1998). *Caenorhabditis elegans* as a model for parasitic nematodes. Intern J Parasitol, 28, 395-411.

Burland, T. G., Schedl, T., Gull, K. and Dove, W. F. (1984). Genetic analysis of resistance to benzimidazoles in *Physarum*: differential expression of beta-tubulin genes. Genetics, 108, 123-141.

Campbell, W. C., Fisher, M. H., Stapley, E. O., Albers-Schonberg, G. and Jacob, T. A. (1983). Ivermectin: a potent new antiparasitic agent. Science, 221, 823-828.

Cao, W. C., Van der Ploeg, C. P., Plaisier, A. P., van der Sluijs, I. J. and Habbema, J. D. (1997). Ivermectin for the chemotherapy of bancroftian filariasis: a meta-analysis of the effect of single treatment. Trop Med Int Health, 2, 393-403.

Carme B, Laigret J. (1979). Longevity of *Wuchereria bancrofti* var. pacifica and mosquito infection acquired from a patient with low level parasitemia. Am J Trop Med Hyg, 28, 53-55.

Cartel, J. L., Celerier, P., Spiegel, A., Burucoa, C. and Roux, J. F. (1990). A single diethylcarbamazine dose for treatment of *Wuchereria bancrofti* carriers in French Polynesia: efficacy and side effects. Southeast Asian J Trop Med Public Health, 21, 465-470.

Cartel, J. L., Spiegel, A., Nguyen Ngnoc, L., Cardines, R., Plichart, R., Martin, P. M. and Roux, J. F. (1991). Single versus repeated doses of ivermectin and diethylcarbamazine for the treatment of *Wuchereria bancrofti* var. pacifica microfilaremia. Results at 12 months of a double-blind study. Trop Med Parasitol, 42, 335-338.

Chabala, J. C., Mrozik, H., Tolman, R. L., Eskola, P., Lusi, A., Peterson, L. H., Woods, M. F., Fisher, M. H., Campbell, W. C., Egerton, J. R. and Ostlind, D. A. (1980). Ivermectin, a new broad-spectrum antiparasitic agent. J Med Chem, 23, 1134-1136.

Chalfie, M. and Thomson, J. N. (1979). Organization of neuronal microtubules in the nematode *Caenorhabditis elegans*. J Cell Biol, 82, 278-289.

Chan, M. S., Guyatt, H. L., Bundy, D. A., Booth, M., Fulford, A. J. and Medley, G. F. (1995). The development of an age structured model for schistosomiasis transmission dynamics and control and its validation for *Schistosoma mansoni*. Epidemiol Infect, 115, 325-344.

Chan, M. S., Norman, R. A., Michael, E., Bundy, D. A., Das, P. K., Pani, S. P. and Ramaiah, K. D. (1999). http://www.schoolsandhealth.org/epidynamics.htm.

Chan, M. S., Srividya, A., Norman, R. A., Pani, S. P., Ramaiah, K. D., Vanamail, P., Michael, E., Das, P. K. and Bundy, D. A. (1998). Epifil: a dynamic model of infection and disease in lymphatic filariasis. Am J Trop Med Hyg, 59, 606-614.

Chanteau, S., Moulia-Pelat, J. P., Glaziou, P., Nguyen, N. L., Luquiaud, P., Plichart, C., Martin, P. M. and Cartel, J. L. (1994). Og4C3 circulating antigen: a marker of infection and adult worm burden in *Wuchereria bancrofti* filariasis. J Infect Dis, 170, 247-250.

Cheeseman, C. L., Delany, N. S., Woods, D. J. and Wolstenholme, A. J. (2001 May). High-affinity ivermectin binding to recombinant subunits of the *Haemonchus contortus* glutamate-gated chloride channel. Molecular& Biochemical Parasitology, 114, 161-168.

Clark, J. M., Scott, J. G., Campos, F. and Bloomquist, J. R. (1995). Resistance to avermectins: extent, mechanisms, and management implications. Annu Rev Entomol, 40, 1-30.

Cleland, T. A. (1996). Inhibitory glutamate receptor channels. Mol Neurobiol, 13, 97-136.

Cleveland, D. W. and Sullivan, K. F. (1985). Molecular biology and genetics of tubulin. Annual Review of Biochemistry, 54, 331-365.

Coles, G. C. (1999). Anthelmintic resistance and the control of worms. J of Med Micro, 48, 323-325.

Conn HC, Greenslit FS. (1952). Filariasis residuals in veterans with report of a case of microfilaremia. Am J Trop Med Hyg 1, 474-476.

Cornell, S. J., Isham, V. S. and Grenfell, B. T. (2000). Drug-resistant parasites and aggregated infection--early-season dynamics. J Math Biol, 41, 341-360.

Cornell, S. J., Isham, V. S., Smith, G. and Grenfell, B. T. (2003). Spatial parasite transmission, drug resistance, and the spread of rare genes. Proc Natl Acad Sci U S A, 100, 7401-7405.

Critchley, J., Addiss, D., Ejere, H., Gamble, C., Garner, P. and Gelband, H. (2005a). Albendazole for the control and elimination of lymphatic filariasis: systematic review. Trop Med Int Health, 10, 818-825.

Critchley, J., Addiss, D., Gamble, C., Garner, P., Gelband, H. and Ejere, H. (2005b). Albendazole for lymphatic filariasis. Cochrane Database Syst Rev, CD003753.

Cross, J. H., Partono, F., Hsu, M. Y., Ash, L. R. and Oemijati, S. (1979). Experimental transmission of *Wuchereria bancrofti* to monkeys. Am J Trop Med Hyg, 28, 56-66.

Cully, D. F., Paress, P. S., Liu, K. K., Schaeffer, J. M. and Arena, J. P. (1996a). Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. J Bio Chem, 271, 20187-20191.

Cully, D. F., Vassilatis, D. K., Liu, K. K., Paress, P. S., Van der Ploeg, L. H., Schaeffer, J. M. and Arena, J. P. (1994). Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature, 371, 707-711.

Cully, D. F., Wilkinson, H., Vassilatis, D. K., Etter, A. and Arena, J. P. (1996b). Molecular biology and electrophysiology of glutamate-gated chloride channels of invertebrates. Parasitol, 113, S191-200.

Das, P. K., Manoharan, A., Srividya, A., Grenfell, B. T., Bundy, D. A. and Vanamail, P. (1990). Frequency distribution of *Wuchereria bancrofti* microfilariae in human populations and its relationships with age and sex. Parasitol, 101, 429-434.

Das, P. K. and Subramanian, S. (2002). Modelling the epidemiology, transmission and control of lymphatic filariasis. Ann Trop Med Parasitol, 96 Suppl 2, S153-164.

Davidse, L. C. and Flach, W. (1977). Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. J Cell Bio, 72, 174-193.

Davies, J. A. and Schwalbach, L. M. (2000 Sep). A study to evaluate the field efficacy of ivermectin, fenbendazole and pyrantel pamoate, with preliminary observations on the efficacy of doramectin, as anthelmintics in horses. South Afr Vet Ass, 71, 144-147.

Day, K. P., Spark, R., Garner, P., Raiko, A., Wenger, J. D., Weiss, N., Mitchell, G. F., Alpers, M. P. and Kazura, J. W. (1991). Serological evaluation of the macrofilaricidal effects of diethylcarbamazine treatment in bancroftian filariasis. Am J Trop Med Hyg, 44, 528-535.

Delany, N. S., Laughton, D. L. and Wolstenholme, A. J. (1998). Cloning and localisation of an avermectin receptor-related subunit from *Haemonchus contortus*. Mol Biochem Parasitol, 97, 177-187.

Denham, D. A., Dennis, D. T., Ponnudurai, T., Nelson, G. S. and Guy, F. (1971). Comparison of a counting chamber and thick smear methods of counting microfilariae. Trans R Soc Trop Med Hyg, 65, 521-526.

Denham, D. A., Liron, D. A. and Brandt, E. (1980). The anthelmintic effects of albendazole on *Brugia pahangi*. J Helminthol, 54, 199-200.

Dent, J. A., Davis, M. W. and Avery, L. (1997). avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. EMBO, 16, 5867-5879.

Dent, J. A., Smith, M. M., Vassilatis, D. K. and Avery, L. (2000). The genetics of ivermectin resistance in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A, 97, 2674-2679.

Devaney, E. and Yazdanbakhsh, M. (2001). Prospects and challenges in lymphatic filariasis. Parasite Immunol, 23, 323-325.

Didier, A. and Loor, F. (1996). The abamectin derivative ivermectin is a potent P-glycoprotein inhibitor. Anticancer Drugs, 7, 745-751.

Dissanayake, S., Min, X. and Piessens, W. F. (1991). Detection of amplified *Wuchereria bancrofti* DNA in mosquitoes with a nonradioactive probe. Mol Biochem Parasitol, 45, 49-56.

Dissanayake, S., Rocha, A., Noroes, J., Medeiros, Z., Dreyer, G. and Piessens, W. F. (2000). Evaluation of PCR-based methods for the diagnosis of infection in bancroftian filariasis. Trans R Soc Trop Med Hyg, 94, 526-530.

Dissanayake, S., Watawana, L. and Piessens, W. F. (1995). Lymphatic pathology in *Wuchereria bancrofti* microfilaraemic infections. Trans R Soc Trop Med Hyg, 89, 517-521.

Dobson, R. J. and Barnes, E. H. (1995). Interaction between *Ostertagia circumcincta* and *Haemonchus contortus* infection in young lambs. Int J Parasitol, 25, 495-501.

Dobson, R. J., LeJambre, L. and Gill, J. H. (1996). Management of anthelmintic resistance: inheritance of resistance and selection with persistent drugs. Int J for Parasitol, 26, 993-1000.

Dreyer, G., Addiss, D. and Noroes, J. (2005). Does longevity of adult *Wuchereria bancrofti* increase with decreasing intensity of parasite transmission? Insights from clinical observations. Trans R Soc Trop Med Hyg, 99, 883-892.

Dreyer, G., Addiss, D., Noroes, J., Amaral, F., Rocha, A. and Coutinho, A. (1996a). Ultrasonographic assessment of the adulticidal efficacy of repeat high-dose ivermectin in bancroftian filariasis. Trop Med Int Health, 1, 427-432.

Dreyer, G., Amaral, F., Noroes, J. and Medeiros, Z. (1994). Ultrasonographic evidence for stability of adult worm location in bancroftian filariasis. Trans R Soc Trop Med Hyg, 88, 558.

Dreyer, G., Medeiros, Z., Netto, M. J., Leal, N. C., de Castro, L. G. and Piessens, W. F. (1999). Acute attacks in the extremities of persons living in an area endemic for bancroftian filariasis: differentiation of two syndromes. Trans R Soc Trop Med Hyg, 93, 413-417.

Dreyer, G., Noroes, J., Amaral, F., Nen, A., Medeiros, Z., Coutinho, A. and Addiss, D. (1995). Direct assessment of the adulticidal efficacy of a single dose of ivermectin in bancroftian filariasis. Trans R Soc Trop Med Hyg, 89, 441-443.

Dreyer, G., Noroes, J., Figueredo-Silva, J. and Piessens, W. F. (2000). Pathogenesis of lymphatic disease in bancroftian filariasis: a clinical perspective. Parasitol Today, 16, 544-548.

Dreyer, G., Noroes, J., Rocha, A. and Addiss, D. (1996b). Detection of living adult *Wuchereria bancrofti* in a patient with tropical pulmonary eosinophilia. Braz J Med Biol Res, 29, 1005-1008.

Dreyer, G., Santos, A., Noroes, J., Rocha, A. and Addiss, D. (1996c). Amicrofilaraemic carriers of adult *Wuchereria bancrofti*. Trans R Soc Trop Med Hyg, 90, 288-289.

Driscoll, M., Dean, E., Reilly, E., Bergholz, E. and Chalfie, M. (1989). Genetic and molecular analysis of a *Caenorhabditis elegans* beta-tubulin that conveys benzimidazole sensitivity. J Cell Bio, 109, 2993-3003.

Drogemuller, M., Failing, K., Schnieder, T. and von Samson-Himmelstjerna, G. (2004a). Effect of repeated benzimidazole treatments with increasing dosages on the phenotype of resistance and the beta-tubulin codon 200 genotype distribution in a benzimidazole-resistant cyathostomin population. Vet Parasitol, 123, 201-213.

Drogemuller, M., Schnieder, T. and von Samson-Himmelstjerna, G. (2004b). Beta-tubulin complementary DNA sequence variations observed between cyathostomins from benzimidazole-susceptible and -resistant populations. J Parasitol, 90, 868-870.

Drudge, J. H., Szanto, J., Wyant, Z. N. and Elam, G. (1964). Field Studies on Parasite Control in Sheep: Comparison of Thiabensazole, Ruelene, and Phenothiazine. Am J Vet Res, 25, 1512-1518.

Duerr, H. P., Dietz, K. and Eichner, M. (2005). Determinants of the eradicability of filarial infections: a conceptual approach. Trends Parasitol, 21, 88-96.

Dunyo, S. K., Nkrumah, F. K. and Simonsen, P. E. (2000a). A randomized double-blind placebo-controlled field trial of ivermectin and albendazole alone and in combination for the treatment of lymphatic filariasis in Ghana. Trans R Soc Trop Med Hyg, 94, 205-211.

Dunyo, S. K., Nkrumah, F. K. and Simonsen, P. E. (2000b). Single-dose treatment of *Wuchereria bancrofti* infections with ivermectin and albendazole alone or in combination: evaluation of the potential for control at 12 months after treatment. Trans R Soc Trop Med Hyg, 94, 437-443.

Durrheim, D. N., Wynd, S., Liese, B. and Gyapong, J. O. (2004). Editorial: Lymphatic filariasis endemicity--an indicator of poverty? Trop Med Int Health, 9, 843-845.

Dustin, P. (1984). Microtubules, 2nd edn. Springer, Berlin.

Eberhard, M. L., Lammie, P. J., Dickinson, C. M. and Roberts, J. M. (1991). Evidence of nonsusceptibility to diethylcarbamazine in *Wuchereria bancrofti*. J Infect Dis, 163, 1157-1160.

Eberhard, M. L., Lowrie, R. C., Jr. and Lammie, P. J. (1988). Persistence of microfilaremia in bancroftian filariasis after diethylcarbamazine citrate therapy. Trop Med Parasitol, 39, 128-130.

Edeson, J. F., Hawking, F. and Symes, C. B. (1957). The periodicity of microfilariae. VI. The response of microfilariae of *Wuchereria malayi* and *W. bancrofti*, Pacific type, to various stimuli. Trans R Soc Trop Med Hyg, 51, 359-365.

Elard, L., Cabaret, J. and Humbert, J. F. (1999). PCR diagnosis of benzimidazole-susceptibility or -resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. Vet Parasitol, 80, 231-237.

Elard, L., Comes, A. M. and Humbert, J. F. (1996). Sequences of beta-tubulin cDNA from benzimidazole-susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Mol Biochem Parasitol, 79, 249-253.

Elard, L. and Humbert, J. F. (1999). Importance of the mutation of amino acid 200 of the isotype 1 beta-tubulin gene in the benzimidazole resistance of the small-ruminant parasite *Teladorsagia circumcincta*. Parasitolo Rese, 85, 452-456.

Elard, L., Sauve, C. and Humbert, J. F. (1998). Fitness of benzimidazole-resistant and -susceptible worms of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Parasitol, 117, 571-578.

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, 193-202.

Faris, R., Hussain, O., El Setouhy, M., Ramzy, R. M. and Weil, G. J. (1998). *bancroftian* filariasis in Egypt: visualization of adult worms and subclinical lymphatic pathology by scrotal ultrasound. Am J Trop Med Hyg, 59, 864-867.

Figueredo-Silva, J., Jungmann, P., Noroes, J., Piessens, W. F., Coutinho, A., Brito, C., Rocha, A. and Dreyer, G. (1996). Histological evidence for adulticidal effect of low doses of diethylcarbamazine in bancroftian filariasis. Trans R Soc Trop Med Hyg, 90, 192-194.

Fischer, P., Supali, T. and Maizels, R. M. (2004). Lymphatic filariasis and *Brugia timori*: prospects for elimination. Trends Parasitol, 20, 351-355.

Florencio, M. S. and Peixoto, C. A. (2003). The effects of diethylcarbamazine on the ultrastructure of microfilariae of *Wuchereria bancrofti*. Parasitol, 126, 551-554.

Forrester, S. G., Prichard, R. K. and Beech, R. N. (2002). A glutamate-gated chloride channel subunit from *Haemonchus contortus*: expression in a mammalian cell line, ligand binding, and modulation of anthelmintic binding by glutamate. Biochem Pharmacol, 63, 1061-1068.

Forsyth, K. P., Spark, R., Kazura, J., Brown, G. V., Peters, P., Heywood, P., Dissanayake, S. and Mitchell, G. F. (1985). A monoclonal antibody-based immunoradiometric assay for detection of circulating antigen in *bancroftian* filariasis. J Immunol, 134, 1172-1177.

Foster, K. E., Burland, T. G. and Gull, K. (1987). A mutant beta-tubulin confers resistance to the action of benzimidazole-carbamate microtubule inhibitors both in vivo and in vitro. Eur J Biochem, 163, 449-455.

Franks, M. B. (1946). Specific soluble antigen in the blood of filarial patients. J Parasitol, 32, 400-406.

Freedman, D. O., de Almeida Filho, P. J., Besh, S., Maia e Silva, M. C., Braga, C. and Maciel, A. (1994). Lymphoscintigraphic analysis of lymphatic abnormalities in symptomatic and asymptomatic human filariasis. J Infect Dis, 170, 927-933.

Freedman, D. O., de Almeido Filho, P. J., Besh, S., Maia e Silva, M. C., Braga, C., Maciel, A. and Furtado, A. F. (1995). Abnormal lymphatic function in presymptomatic bancroftian filariasis. J Infect Dis, 171, 997-1001.

Freeman, A. R., Lammie, P. J., Houston, R., LaPointe, M. D., Streit, T. G., Jooste, P. L., Brissau, J. M., Lafontant, J. G. and Addiss, D. G. (2001). A community-based trial for the control of lymphatic filariasis and iodine deficiency using salt fortified with diethylcarbamazine and iodine. Am J Trop Med Hyg, 65, 865-871.

Friedman, P. A. and Platzer, E. G. (1978). Interaction of anthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. Biochim Biophys Acta, 544, 605-614.

Fritz, L. C., Wang, C. C. and Gorio, A. (1979). Avermectin B1a irreversibly blocks postsynaptic potentials at the lobster neuromuscular junction by reducing muscle membrane resistance. Proc Natl Acad Sci U S A, 76, 2062-2066.

Geary, T. G. (2005). Ivermectin 20 years on: maturation of a wonder drug. Trends Parasitol, 21, 530-532.

Geary, T. G., Nulf, S. C., Alexander-Bowman, S. J., Mahmoud, B. M., Prichard, R. K. and Klein, R. D. (1998). Cloning and characterization of cDNAs encoding beta-tubulin from *Dirofilaria immitis* and *Onchocerca volvulus*. J Parasitol, 84, 356-360.

Geary, T. G., Nulf, S. C., Favreau, M. A., Tang, L., Prichard, R. K., Hatzenbuhler, N. T., Shea, M. H., Alexander, S. J. and Klein, R. D. (1992). Three beta-tubulin cDNAs from the parasitic nematode *Haemonchus contortus*. Mol Biochem Parasitol, 50, 295-306.

Geary, T. G., Sims, S. M., Thomas, E. M., Vanover, L., Davis, J. P., Winterrowd, C. A., Klein, R. D., Ho, N. F. and Thompson, D. P. (1993). *Haemonchus contortus*: ivermectin-induced paralysis of the pharynx. Exp Parasitol, 77, 88-96.

Gelband, H. (1994). Diethylcarbamazine salt in the control of lymphatic filariasis. Am J Trop Med Hyg, 50, 655-662.

Gill, J. H., Kerr, C. A., Shoop, W. L. and Lacey, E. (1998). Evidence of multiple mechanisms of avermectin resistance in *Haemonchus contortus*--comparison of selection protocols. Int J Parasitol, 28, 783-789.

Gill, J. H. and Lacey, E. (1998). Avermectin/milbemycin resistance in trichostrongyloid nematodes. Int J Parasitol, 28, 863-877.

Goodman, D. S., Orelus, J. N., Roberts, J. M., Lammie, P. J. and Streit, T. G. (2003). PCR and Mosquito dissection as tools to monitor filarial infection levels following mass treatment. Filaria J, 2, 11.

Grant, W. N. and Mascord, L. J. (1996). Beta-tubulin gene polymorphism and benzimidazole resistance in *Trichostrongylus colubriformis*. Int J Parasitolo, 26, 71-77.

Grenfell, B. T., Das, P. K., Rajagopalan, P. K. and Bundy, D. A. (1990). Frequency distribution of lymphatic filariasis microfilariae in human populations: population processes and statistical estimation. Parasitol, 101, 417-427.

Guenette, S., Prichard, R. K., Klein, R. D. and Matlashewski, G. (1991). Characterization of a beta-tubulin gene and a beta-tubulin gene products of *Brugia pahangi*. Mol Biochem Parasitol, 44, 153-164.

Guenette, S., Prichard, R. K. and Matlashewski, G. (1992). Identification of a novel *Brugia pahangi* beta-tubulin gene (beta 2) and a 22-nucleotide spliced leader sequence on beta 1-tubulin mRNA. Mol Biochem Parasitol, 50, 275-284.

Guptavanij PH, C. (1971). Spontaneous disappearance of microfilaria *Brugia malayi* and *Wuchereria bancrofti* in infected patients living in a non-endemic area. Southeast Asian J Trop Med Pub Health, 2, 578-584.

Gyapong, J. O., Gyapong, M., Evans, D. B., Aikins, M. K. and Adjei, S. (1996). The economic burden of lymphatic filariasis in northern Ghana. Ann Trop Med Parasitol, 90, 39-48.

Gyapong, J. O., Kumaraswami, V., Biswas, G. and Ottesen, E. A. (2005). Treatment strategies underpinning the global programme to eliminate lymphatic filariasis. Expert Opin Pharmacother, 6, 179-200.

Gyapong, J. O. and Twum-Danso, N. A. (2006). Editorial: Global elimination of lymphatic filariasis: fact or fantasy? Trop Med Int Health, 11, 125-128.

Habbema, J. D., Alley, E. S., Plaisier, A. P., van Oortmarssen, G. J. and Remme, J. H. (1992). Epidemiological modelling for onchocerciasis control. Parasitol Today, 8, 99-103.

Hairston, N. G. and Jachowski Jr., L. A. (1968). Analysis of the *Wuchereria bancrofti* population of the people of American Samoa. Bull World Health Organ, 33, 29-59.

Hall, C. A., Campbell, N. J. and Richardson, N. J. (1978). Levels of benzimidazole resistance in *Haemonchus contortus* and *Trichostrongylus colubriformis* recorded from an egg hatch test procedure. ResVet Sci, 25, 360-363.

Hall CA, Ritchie L, Kelly JD. (1982). Effect of removing anthelmintic selection pressure on the benzimidazole resistance status of *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep. Res Vet Sci, 33, 54-57.

Hastings, I. M. (2001). Modelling parasite drug resistance: lessons for management and control strategies. Trop Med Int Health, 6, 883-890.

Hastings, I. M. and D'Alessandro, U. (2000). Modelling a predictable disaster: the rise and spread of drug-resistantmalaria. Parasitol Today, 16, 340-347.

Hawking, F. (1965). Advances in Filariasis Especially Concerning Periodicity of Microfilariae. Trans R Soc Trop Med Hyg, 59, 9-25.

Hawking, F., Pattanayak, S. and Sharma, H. L. (1966). The periodicity of microfilariae. XI. The effect of body temperature and other stimuli upon the cycles of *Wuchereria bancrofti*, *Brugia malayi*, *B. ceylonensis* and *Dirofilaria repens*. Trans R Soc Trop Med Hyg, 60, 497-513.

Hawking, F., Sewell, P. and Thurston, J. P. (1950). The mode of action of hetrazan on filarial worms. Br J Pharmacol Chemother, 5, 217-238.

Hawking, F. and Thurston, J. P. (1951). The periodicity of microfilariae. I. The distribution of microfilaria in the body. Trans R Soc Trop Med Hyg, 45, 307-328.

Hayashi, S. (1962). A mathematical analysis on the epidemiology of *bancroftian* and *malayian* filariasis in Japan. Jpn J Exp Med, 32, 13-43.

Hejmadi, M. V., Jagannathan, S., Delany, N. S., Coles, G. C. and Wolstenholme, A. J. (2000). L-glutamate binding sites of parasitic nematodes: an association with ivermectin resistance? Parasitol, 120. 535-545.

Helm, R., Selkirk, M. E., Bradley, J. E., Burns, R. G., Hamilton, A. J., Croft, S. and Maizels, R. M. (1989). Localization and immunogenicity of tubulin in the filarial nematodes *Brugia malayi* and *B. pahangi*. Parasite Immunology, 11, 479-502.

Hoerauf, A. (2000). Targeting of *Wolbachia* endobacteria in litomosoides sigmodontis: comparison of tetracyclines with chloramphenicol, macrolides and ciprofloxacin. Trop Med Int Health, 5, 275-279.

Hoerauf, A., Mand, S., Fischer, K., Kruppa, T., Marfo-Debrekyei, Y., Debrah, A. Y., Pfarr, K. M., Adjei, O. and Buttner, D. W. (2003). Doxycycline as a novel strategy against bancroftian filariasis-depletion of *Wolbachia* endosymbionts from *Wuchereria bancrofti* and stop of microfilaria production. Med Microbiol Immunol, 192, 211-216.

Hoerauf, A., Nissen-Pahle, K., Schmetz, C., Henkle-Duhrsen, K., Blaxter, M. L., Buttner, D. W., Gallin, M. Y., Al-Qaoud, K. M., Lucius, R. and Fleischer, B. (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. J Clin Invest, 103, 11-18.

Holden-Dye, L., Krogsgaard-Larsen, P., Nielsen, L. and Walker, R. J. (1989). GABA receptors on the somatic muscle cells of the parasitic nematode, *Ascaris suum*: stereoselectivity indicates similarity to a GABAA-type agonist recognition site. Br J Pharmacol, 98, 841-850.

Holden-Dye, L. and Walker, R. J. (1990). Avermectin and avermectin derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic muscle cells of *Ascaris*; is this the site of anthelmintic action? Parasitol, 101 Pt 2, 265-271.

Hollomon, D. W., Butters, J. A., Barker, H. and Hall, L. (1998). Fungal beta-tubulin, expressed as a fusion protein, binds benzimidazole and phenylcarbamate fungicides. Antimicrob Ag Chemother, 42, 2171-2173.

Hoti, S. L., Subramaniyan, K. and Das, P. K. (2003). Detection of codon for amino acid 200 in isotype 1 beta-tubulin gene of *Wuchereria bancrofti* isolates, implicated in resistance to benzimidazoles in other nematodes. Acta Trop, 88, 77-81.

Hoti, S. L., Vasuki, V., Lizotte, M. W., Patra, K. P., Ravi, G., Vanamail, P., Manonmani, A., Sabesan, S., Krishnamoorthy, K. and Williams, S. A. (2001). Detection of *Brugia malayi* in laboratory and wild-caught Mansonioides mosquitoes (Diptera: Culicidae) using Hha I PCR assay. Bull Entomol Res, 91, 87-92.

Hubert, J. and Kerboeuf, D. (1992). A microlarval development assay for the detection of anthelmintic resistance in sheep nematodes. Vet Rec, 130, 442-446.

Hussein, O., Setouhy, M. E., Ahmed, E. S., Kandil, A. M., Ramzy, R. M., Helmy, H. and Weil, G. J. (2004). Duplex Doppler sonographic assessment of the effects of diethylcarbamazine and albendazole therapy on adult filarial worms and adjacent host tissues in *bancroftian* filariasis. Am J Trop Med Hyg, 71, 471-477.

Ireland, C. M., Gull, K., Gutteridge, W. E. and Pogson, C. I. (1979). The interaction of benzimidazole carbamates with mammalian microtobule protein. Biochem Pharmacol, 28, 2680-2682.

Ismail, M. M., Jayakody, R. L., Weil, G. J., Fernando, D., De Silva, M. S., De Silva, G. A. and Balasooriya, W. K. (2001). Long-term efficacy of single-dose combinations of albendazole, ivermectin and diethylcarbamazine for the treatment of bancroftian filariasis. Trans R Soc Trop Med Hyg, 95, 332-335.

Ismail, M. M., Jayakody, R. L., Weil, G. J., Nirmalan, N., Jayasinghe, K. S., Abeyewickrema, W., Rezvi Sheriff, M. H., Rajaratnam, H. N., Amarasekera, N., de Silva, D. C., Michalski, M. L. and Dissanaike, A. S. (1998). Efficacy of single dose combinations of albendazole, ivermectin and diethylcarbamazine for the treatment of bancroftian filariasis. Trans R Soc Trop Med Hyg, 92, 94-97.

Jachowski, L. A., de Silva, D. C. and Weerasooriya, M. V. (1993). Treatment of bancroftian filariasis with albendazole: evaluation of efficacy and adverse reactions. Trop Biomed, 10, 19-24.

Jachowski Jr. LAO, G.F. ; Wharton,J.D. (1951). Filariasis in American Samoa I. Loss of microfilaria in the absence of continued reinfectionFilariasis in American Samoa I. Loss of microfilaria in the absence of continued reinfection. Proceedings of the Helminth Soc Wash, 18, 26-28.

Jagannathan, S., Laughton, D. L., Critten, C. L., Skinner, T. M., Horoszok, L. and Wolstenholme, A. J. (1999). Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and Ascaris suum orthologues of the *Caenorhabditis elegans* gbr-2 (avr-14) gene. Mol Biochem Parasitol, 103, 129-140.

Jasmer, D. P., Yao, C., Rehman, A. and Johnson, S. (2000 Jan 5). Multiple lethal effects induced by a benzimidazole anthelmintic in the anterior intestine of the nematode *Haemonchus contortus*. Mol Biochem Parasitol, 105, 81-90.

Jayasekera, N., Kalpage, K. S. and De Silva, C. S. (1991). The significance of low density microfilaraemia in the transmission of *Wuchereria bancrofti* by *Culex (Culex) quinquefasciatus* Say in Sri Lanka. Trans R Soc Trop Med Hyg, 85, 250-254.

Johnson, P., Mackenzie, C. D., Denham, D. A. and Suswillo, R. R. (1988). The effect of diethylcarbamazine on the in vitro serum-mediated adherence of feline granulocytes to microfilariae of *Brugia pahangi*. Trop Med Parasitol, 39, 291-294.

Jordan, P. (1958). Action of diethylcarbamazine in vitro on infective larvae of *Wuchereria bancrofti*. Br J Pharmacol Chemother, 13, 318-320.

Jung MK, Wilder IB, Oakley BR. (1992). Amino acid alterations in the benA (beta-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. Cell Motil Cytoskeleton, 22, 170-174

Kanesa-thasan, N., Douglas, J. G. and Kazura, J. W. (1991). Diethylcarbamazine inhibits endothelial and microfilarial prostanoid metabolism in vitro. Mol Biochem Parasitol, 49, 11-19.

Kass, I. S., Stretton, A. O. and Wang, C. C. (1984). The effects of avermectin and drugs related to acetylcholine and 4-aminobutyric acid on neurotransmission in *Ascaris suum*. Mol Biochem Parasitol, 13, 213-225.

Kazura, J., Greenberg, J., Perry, R., Weil, G., Day, K. and Alpers, M. (1993). Comparison of single-dose diethylcarbamazine and ivermectin for treatment of bancroftian filariasis in Papua New Guinea. Am J Trop Med Hyg, 49, 804-811.

Kimura, E., Penaia, L. and Spears, G. F. (1985). The efficacy of annual single-dose treatment with diethylcarbamazine citrate against diurnally subperiodic bancroftian filariasis in Samoa. Bull World Health Organ, 63, 1097-1106.

Kimura, E., Spears, G. F., Singh, K. I., Samarawickrema, W. A., Penaia, L., Sone, P. F., Pelenatu, S., Faaiuaso, S. T., Self, L. S. and Dazo, B. C. (1992). Long-term efficacy of single-dose mass treatment with diethylcarbamazine citrate against diurnally subperiodic *Wuchereria bancrofti*: eight years' experience in Samoa. Bull World Health Organ, 70, 769-776.

King, C. H., Greene, B. M. and Spagnuolo, P. J. (1983). Diethylcarbamazine citrate, an antifilarial drug, stimulates human granulocyte adherence. Antimicrob Agents Chemother, 24, 453-456.

King, C. L., Kumaraswami, V., Poindexter, R. W., Kumari, S., Jayaraman, K., Alling, D. W., Ottesen, E. A. and Nutman, T. B. (1992). Immunologic tolerance in lymphatic filariasis. Diminished parasite-specific T and B lymphocyte precursor frequency in the microfilaremic state. J Clin Invest, 89, 1403-1410.

Knott, J. (1939). A method for making microfilarial surveys on night blood. Trans R Soc Trop Med Hyg, 33, 191.

Kohler, P. and Bachmann, R. (1981). Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes. Mol Biochem Parasitol, 4, 325-336.

Kotze, A. C. (1998). Effects of macrocyclic lactones on ingestion in susceptible and resistant *Haemonchus contortus* larvae. J Parasitol, 84, 631-635.

Krishna Kumari, A., Harichandrakumar, K. T., Das, L. K. and Krishnamoorthy, K. (2005). Physical and psychosocial burden due to lymphatic filariasis as perceived by patients and medical experts. Trop Med Int Health, 10, 567-573.

Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M., Martin, S. K., Milhous, W. K. and Schlesinger, P. H. (1987). Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science, 238, 1283-1285.

Kumaraswami, V. (2000). Clinical Manifestations of Lymphatic Filariasis. In Lymphatic Filariasis, Vol. 1 (ed. Nutman, T. B.), pp. 103-125. Imperial College Press, London.

Kumaraswami, V., Ottesen, E. A., Vijayasekaran, V., Devi, U., Swaminathan, M., Aziz, M. A., Sarma, G. R., Prabhakar, R. and Tripathy, S. P. (1988). Ivermectin for the treatment of *Wuchereria bancrofti* filariasis. Efficacy and adverse reactions. JAMA, 259, 3150-3153.

Kwa, M. S., Okoli, M. N., Schulz-Key, H., Okongkwo, P. O. and Roos, M. H. (1998). Use of P-glycoprotein gene probes to investigate anthelmintic resistance in *Haemonchus contortus* and comparison with *Onchocerca volvulus*. Int J Parasitol, 28, 1235-1240.

Kwa, M. S. G., Kooyman, F. N. J., Boersema, J. H. and Roos, M. H. (1993a). Effect of selection for benzimidazole resistance in *Haemonchus contortus* on beta-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res, 191, 413-419.

Kwa, M. S. G., Veenstra, J. G. and Roos, M. H. (1993b). Molecular characterisation of beta-tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol, 60, 133-144.

Kwan-Lim, G. E., Forsyth, K. P. and Maizels, R. M. (1990). Filarial-specific IgG4 response correlates with active *Wuchereria bancrofti* infection. J Immunol, 145, 4298-4305.

Lacey, E. (1988). The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. Int J Parasitol, 18, 885-936.

Lacey, E. (1990). Mode of action of Benzimidazoles. Parasitol Today, 6, 112-124.

Lacey, E. and Gill, J. H. (1994). Biochemistry of benzimidazole resistance. Acta Tropica, 56, 245-262.

Lacey, E. and Prichard, R. K. (1986). Interactions of benzimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. Mol Biochem Parasitol, 19, 171-181.

Lacey, E., Snowdon, K. L., Eagleson, G. K. and Smith, E. F. (1987). Further investigation of the primary mechanism of benzimidazole resistance in *Haemonchus contortus*. Int J Parasitolo, 17, 1421-1429.

Lal, R. B., Paranjape, R. S., Briles, D. E., Nutman, T. B. and Ottesen, E. A. (1987). Circulating parasite antigen(s) in lymphatic filariasis: use of monoclonal antibodies to phosphocholine for immunodiagnosis. J Immunol, 138, 3454-3460.

Lammie, P. J., Hightower, A. W. and Eberhard, M. L. (1994). Age-specific prevalence of antigenemia in a *Wuchereria bancrofti*-exposed population. Am J Trop Med Hyg, 51, 348-355.

Laughton, D. L., Lunt, G. G. and Wolstenholme, A. J. (1997). Reporter gene constructs suggest that the *Caenorhabditis elegans* avermectin receptor beta-subunit is expressed solely in the pharynx. J Exper Bio, 200, 1509-1514.

Lawrence, R. A. (2001). Immunity to filarial nematodes. Vet Parasitol, 100, 33-44.

Le Jambre, L. F., Dobson, R. J., Lenane, I. J. and Barnes, E. H. (1999). Selection for anthelmintic resistance by macrocyclic lactones in *Haemonchus contortus*. Int J Parasitol, 29, 1101-1111.

Le Jambre, L. F., Gill, J. H., Lenane, I. J. and Baker, P. (2000). Inheritance of avermectin resistance in *Haemonchus contortus*. Int J Parasitol, 30, 105-111.

Le Jambre, L. F., Royal, W. M. and Martin, P. J. (1979). The inheritance of thiabendazole resistance in *Haemonchus contortus*. Parasitol, 78, 107-119.

Leeuwin RS. (1962). Microfilaraemia in Surinamese living in Amsterdam. Trop Geogr Med, 14, 355-360.

Li, J., Katiyar, S. K. and Edlind, T. D. (1996a). Site-directed mutagenesis of *Saccharomyces cerevisiae* beta-tubulin: interaction between residue 167 and benzimidazole compounds. FEBS Letters, 385, 7-10.

Li, J., Katiyar, S. K., Hamelin, A., Visvesvara, G. S. and Edlind, T. D. (1996b). Tubulin genes from AIDS-associated microsporidia and implications for phylogeny and benzimidazole sensitivity. Mol Biochem Parasitol, 78, 289-295.

Little, M. and Seehaus, T. (1988). Comparative analysis of tubulin sequences. Comp Biochem, 90, 655-670.

Liu, J., Dent, J. A., Beech, R. N. and Prichard, R. K. (2004). Genomic organization of an avermectin receptor subunit from *Haemonchus contortus* and expression of its putative promoter region in *Caenorhabditis elegans*. Mol Biochem Parasitol, 134, 267-274.

Lizotte, M. R., Supali, T., Partono, F. and Williams, S. A. (1994). A polymerase chain reaction assay for the detection of *Brugia malayi* in blood. Am J Trop Med Hyg, 51, 314-321.

Lopata, M. A. and Cleveland, D. W. (1987). In vivo microtubules are copolymers of available beta-tubulin isotypes: localization of each of six vertebrate beta-tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens. J Cell Biol, 105, 1707-1720.

Lowrie, R. C., Jr., Eberhard, M. L., Lammie, P. J., Raccurt, C. P., Katz, S. P. and Duverseau, Y. T. (1989). Uptake and development of *Wuchereria bancrofti* in *Culex quinquefasciatus* that fed on Haitian carriers with different microfilaria densities. Am J Trop Med Hyg, 41, 429-435.

Lubega, G. W., Klein, R. D., Geary, T. G. and Prichard, R. K. (1994). *Haemonchus contortus*: the role of two beta-tubulin gene subfamilies in the resistance to benzimidazole anthelmintics. Biochem Pharmacol, 47, 1705-1715.

Lubega, G. W. and Prichard, R. K. (1990). Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. Mol Biochem Parasitol, 38, 221-232.

Lubega, G. W. and Prichard, R. K. (1991). Interaction of benzimidazole anthelmintics with *Haemonchus contortus* tubulin: binding affinity and anthelmintic efficacy. Exp Parasitol, 73, 203-213.

Lucena, W. A., Dhalia, R., Abath, F. G., Nicolas, L., Regis, L. N. and Furtado, A. F. (1998). Diagnosis of *Wuchereria bancrofti* infection by the polymerase chain reaction using urine and day blood samples from amicrofilaraemic patients. Trans R Soc Trop Med Hyg, 92, 290-293.

Maher, D. and Ottesen, E. A. (2000). The Global Lymphatic Filariasis Initiative. Trop Doct, 30, 178-179.

Mahoney LE, Patrick I.A.U. (1970). Filariasis in Samoan immigrants to the United States Patrick, Filariasis in Samoan immigrants to the United States. Am J Trop Med Hyg, 19, 629-636.

Maingi, N., Bjorn, H. and Dangolla, A. (1998). The relationship between faecal egg count reduction and the lethal dose 50% in the egg hatch assay and larval development assay. Vet Parasitol, 77, 133-145.

Maizels, R. M. and Denham, D. A. (1992). Diethylcarbamazine (DEC): immunopharmacological interactions of an anti-filarial drug. Parasitol, 105 Suppl, S49-60.

Maizels, R. M. A., J.E.; Yazdanbakhsh, M. (2000). Immunology of Lymphatic Filariasis: Current controversies. In Lymphatic Filariasis, Vol. 1 (ed. Nutman, T. B.), pp. 217-244. Imperial College Press, London.

Mak, J. W., Sim, B. K. L. and Yen, P. F. K. (1984). Filaricidal effect of albendazole against subperiodic *Brugia malayi* infection in the leaf-monkey. Presbytis melalophos. Trop. Biomed., 1, 121-123.

Makunde, W. H., Kamugisha, L. M., Massaga, J. J., Makunde, R. W., Savael, Z. X., Akida, J., Salum, F. M. and Taylor, M. J. (2003). Treatment of co-infection with bancroftian filariasis and onchocerciasis: a safety and efficacy study of albendazole with ivermectin compared to treatment of single infection with bancroftian filariasis. Filaria J, 2, 15.

Mand, S., Debrah, A., Batsa, L., Adjei, O. and Hoerauf, A. (2004). Reliable and frequent detection of adult *Wuchereria bancrofti* in Ghanaian women by ultrasonography. Trop Med Int Health, 9, 1111-1114.

Martin, P. J., Anderson, N., Brown, T. H. and Miller, D. W. (1988). Changes in resistance of *Ostertagia* spp. to thiabendazole following natural selection or treatment with levamisole. Int J Parasitol, 18, 333-340.

Martin, R. J. and Pennington, A. J. (1989). A patch-clamp study of effects of dihydroavermectin on *Ascaris* muscle. Br J Pharmacol, 98, 747-756.

Martin, R. J., Valkanov, M. A., Dale, V. M., Robertson, A. P. and Murray, I. (1996). Electrophysiology of *Ascaris* muscle and anti-nematodal drug action. Parasitol, 113, S137-156.

May, R. M. (1976). Models for Single Populations, Saunders Company, England.

McCarthy, J. (2000). Diagnosis of Lymphatic Filarial Infections. In Lymphatic Filariasis, Vol. 1 (ed. Nutman, T. B.), pp. 127-150. Imperial College Press, London.

McCarthy, J. S., Zhong, M., Gopinath, R., Ottesen, E. A., Williams, S. A. and Nutman, T. B. (1996). Evaluation of a polymerase chain reaction-based assay for diagnosis of *Wuchereria bancrofti* infection. J Infect Dis, 173, 1510-1514.

McCracken, R. O. and Stillwell, W. H. (1991). A possible biochemical mode of action for benzimidazole anthelmintics. Int J Parasitol, 21, 99-104.

McGarry, H. F., Plant, L. D. and Taylor, M. J. (2005). Diethylcarbamazine activity against *Brugia malayi* microfilariae is dependent on inducible nitric-oxide synthase and the cyclooxygenase pathway. Filaria J, 4, 4.

McGreevy, P. B., Kolstrup, N., Tao, J., McGreevy, M. M. and Marshall, T. F. (1982). Ingestion and development of *Wuchereria bancrofti* in *Culex quinquefasciatus*, *Anopheles gambiae* and *Aedes aegypti* after feeding on humans with varying densities of microfilariae in Tanzania. Trans R Soc Trop Med Hyg, 76, 288-296.

Mealey, K. L., Bentjen, S. A., Gay, J. M. and Cantor, G. H. (2001). Ivermectin sensitivity in collies is associated with a deletion mutation of the mdr1 gene. Pharmacogen, 11, 727-733.

Melrose, W. D. (2002). Lymphatic filariasis: new insights into an old disease. Int J Parasitol, 32, 947-960.

Melrose, W. D., Durrheim, D. D. and Burgess, G. W. (2004). Update on immunological tests for lymphatic filariasis. Trends Parasitol, 20, 255-257.

Michael, E. (2000). The Population Dynamics and Epidemiology of Lymphatic Filariasis. In Lymphatic Filariasis, Vol. 1 (ed. Nutman, T. B.), pp. 41-81. Imperial College Press, London.

Michael, E. and Bundy, D. A. (1997). Global mapping of lymphatic filariasis. Parasitol Today, 13, 472-476.

Michael, E. and Bundy, D. A. (1998). Herd immunity to filarial infection is a function of vector biting rate. Proc Biol Sci, 265, 855-860.

Michael, E., Bundy, D. A. and Grenfell, B. T. (1996). Re-assessing the global prevalence and distribution of lymphatic filariasis. Parasitol, 112 (Pt 4), 409-428.

Michael, E., Malecela-Lazaro, M. N., Simonsen, P. E., Pedersen, E. M., Barker, G., Kumar, A. and Kazura, J. W. (2004). Mathematical modelling and the control of lymphatic filariasis. Lancet Infect Dis, 4, 223-234.

Michael, E., Simonsen, P. E., Malecela, M., Jaoko, W. G., Pedersen, E. M., Mukoko, D., Rwegoshora, R. T. and Meyrowitsch, D. W. (2001). Transmission intensity and the immunoepidemiology of bancroftian filariasis in East Africa. Parasite Immunol, 23, 373-388.

Mishra, K., Raj, D. K., Dash, A. P. and Hazra, R. K. (2005). Combined detection of *Brugia malayi* and *Wuchereria bancrofti* using single PCR. Acta Trop, 93, 233-237.

More, S. J. and Copeman, D. B. (1990). A highly specific and sensitive monoclonal antibody-based ELISA for the detection of circulating antigen in bancroftian filariasis. Trop Med Parasitol, 41, 403-406.

Moulia-Pelat, J. P., Glaziou, P., Nguyen-Ngoc, L., Cardines, D., Spiegel, A. and Cartel, J. L. (1992). A comparative study of detection methods for evaluation of microfilaremia in lymphatic filariasis control programmes. Trop Med Parasitol, 43, 146-148.

Moulia-Pelat, J. P., Glaziou, P., Weil, G. J., Nguyen, L. N., Gaxotte, P. and Nicolas, L. (1995). Combination ivermectin plus diethylcarbamazine, a new effective tool for control of lymphatic filariasis. Trop Med Parasitol, 46, 9-12.

Muench, H. (1959). Catalytic Models in Epidemiology, Harvard University Press, Cambridge, MA.

Nare, B., Lubega, G., Prichard, R. K. and Georges, E. (1996). p-Azidosalicyl-5-amino-6-phenoxybenzimidazole photolabels the N-terminal 63-103 amino acids of *Haemonchus contortus* beta-tubulin 1. J Biol Chem, 271, 8575-8581.

Njue, A. I. and Prichard, R. K. (2003). Cloning two full-length beta-tubulin isotype cDNAs from *Cooperia oncophora*, and screening for benzimidazole resistance-associated mutations in two isolates. Parasitol, 127, 579-588.

Njue, A. I. and Prichard, R. K. (2004). Genetic variability of glutamate-gated chloride channel genes in ivermectin-susceptible and -resistant strains of *Cooperia oncophora*. Parasitol, 129, 741-751.

Nookala, S., Srinivasan, S., Kaliraj, P., Narayanan, R. B. and Nutman, T. B. (2004). Impairment of tetanus-specific cellular and humoral responses following tetanus vaccination in human lymphatic filariasis. Infect Immun, 72, 2598-2604.

Norman, R. A., Chan, M. S., Srividya, A., Pani, S. P., Ramaiah, K. D., Vanamail, P., Michael, E., Das, P. K. and Bundy, D. A. (2000). EPIFIL: the development of an age-structured model for describing the transmission dynamics and control of lymphatic filariasis. Epidemiol Infect, 124, 529-541.

Noroes, J., Addiss, D., Amaral, F., Coutinho, A., Medeiros, Z. and Dreyer, G. (1996a). Occurrence of living adult *Wuchereria bancrofti* in the scrotal area of men with microfilaraemia. Trans R Soc Trop Med Hyg, 90, 55-56.

Noroes, J., Addiss, D., Santos, A., Medeiros, Z., Coutinho, A. and Dreyer, G. (1996b). Ultrasonographic evidence of abnormal lymphatic vessels in young men with adult *Wuchereria bancrofti* infection in the scrotal area. J Urol, 156, 409-412.

Noroes, J., Dreyer, G., Santos, A., Mendes, V. G., Medeiros, Z. and Addiss, D. (1997). Assessment of the efficacy of diethylcarbamazine on adult *Wuchereria bancrofti* in vivo. Trans R Soc Trop Med Hyg, 91, 78-81.

Nutman, T. B. and Kumaraswami, V. (2001). Regulation of the immune response in lymphatic filariasis: perspectives on acute and chronic infection with *Wuchereria bancrofti* in South India. Parasite Immunol, 23, 389-399.

O'Bryan, L., Pinkston, P., Kumaraswami, V., Vijayan, V., Yenokida, G., Rosenberg, H. F., Crystal, R., Ottesen, E. A. and Nutman, T. B. (2003). Localized eosinophil degranulation mediates disease in tropical pulmonary eosinophilia. Infect Immun, 71, 1337-1342.

O'Connor, R. A., Jenson, J. S., Osborne, J. and Devaney, E. (2003). An enduring association? Microfilariae and immunosuppression in lymphatic filariasis. Trends Parasitol, 19, 565-570.

Orbach MJ, Porro EB, Yanofsky C. (1986). Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol Cell Biol, 6, 2452-2461.

Ottesen, E. A., Vijayasekaran, V., Kumaraswami, V., Perumal Pillai, S. V., Sadanandam, A., Frederick, S., Prabhakar, R. and Tripathy, S. P. (1990). A controlled trial of ivermectin and diethylcarbamazine in lymphatic filariasis. N Engl J Med, 322, 1113-1117.

Ottesen, E. A., Weller, P. F. and Heck, L. (1977). Specific cellular immune unresponsiveness in human filariasis. Immunol, 33, 413-421.

Paiement, J. P., Leger, C., Ribeiro, P. and Prichard, R. K. (1999a). *Haemonchus contortus*: effects of glutamate, ivermectin, and moxidectin on inulin uptake activity in unselected and ivermectin-selected adults. Exp Parasitol, 92, 193-198.

Paiement J. P., Prichard R. K., Ribeiro P. (1999b). *Haemonchus contortus*: characterization of a glutamate binding site in unselected and ivermectin-selected larvae and adults. Exp Parasitol , 92, 32-39.

Paily, K. P., Hoti, S. L., Manonmani, A. M. and Balaraman, K. (1995). Longevity and migration of *Wuchereria bancrofti* infective larvae and their distribution pattern in relation to the resting and feeding behaviour of the vector mosquito, *Culex quinquefasciatus*. Ann Trop Med Parasitol, 89, 39-47.

Pani, S., Subramanyam Reddy, G., Das, L., Vanamail, P., Hoti, S., Ramesh, J. and Das, P. (2002). Tolerability and efficacy of single dose albendazole, diethylcarbamazine citrate (DEC) or co-administration of albendazole with DEC in the clearance of *Wuchereria bancrofti* in asymptomatic microfilaraemic volunteers in Pondicherry, South India: a hospital-based study. Filaria J, 1, 1.

Pani, S. P., Balakrishnan, N., Srividya, A., Bundy, D. A. and Grenfell, B. T. (1991). Clinical epidemiology of bancroftian filariasis: effect of age and gender. Trans R Soc Trop Med Hyg, 85, 260-264.

Pani, S. P., Yuvaraj, J., Vanamail, P., Dhanda, V., Michael, E., Grenfell, B. T. and Bundy, D. A. (1995). Episodic adenolymphangitis and lymphoedema in patients with bancroftian filariasis. Trans R Soc Trop Med Hyg, 89, 72-74.

Panicker, K. N., Arunachalam, N., Kumar, N. P., Prathibha, J. and Sabesan, S. (1997). Efficacy of diethylcarbamazine-medicated salt for microfilaraemia of *Brugia malayi*. Natl Med J India, 10, 275-276.

Pape, M., Posedi, J., Failing, K., Schnieder, T. and von Samson-Himmelstjerna, G. (2003). Analysis of the beta-tubulin codon 200 genotype distribution in a benzimidazole-susceptible and -resistant cyathostome population. Parasitol, 127, 53-59.

Partono, F. (1987). The spectrum of disease in lymphatic filariasis. Ciba Found Symp, 127, 15-31.

Peixoto, C. A. (2005). Some morphological aspects of *Wuchereria bancrofti* uterus after treatment with diethylcarbamazine. Micron, 36, 17-22.

Peixoto, C. A., Alves, L. C., Brayner, F. A. and Florencio, M. S. (2003). Diethylcarbamazine induces loss of microfilarial sheath of *Wuchereria bancrofti*. Micron, 34, 381-385.

Peixoto, C. A., Rocha, A., Aguiar-Santos, A. and Florencio, M. S. (2004). The effects of diethylcarbamazine on the ultrastructure of microfilariae of *Wuchereria bancrofti* in vivo and in vitro. Parasitol Res, 92, 513-517.

Pemberton, D. J., Franks, C. J., Walker, R. J. and Holden-Dye, L. (2001). Characterization of glutamate-gated chloride channels in the pharynx of wild-type and mutant *Caenorhabditis elegans* delineates the role of the subunit GluCl-alpha2 in the function of the native receptor. Mol Pharmacol, 59, 1037-1043.

Pichon, G. (2002). Limitation and facilitation in the vectors and other aspects of the dynamics of filarial transmission: the need for vector control against *Anopheles*-transmitted filariasis. Ann Trop Med Parasitol, 96 Suppl 2, S143-152.

Plaisier, A. P., Cao, W. C., van Oortmarssen, G. J. and Habbema, J. D. (1999). Efficacy of ivermectin in the treatment of *Wuchereria bancrofti* infection: a model-based analysis of trial results. Parasitol, 119, 385-394.

Plaisier, A. P., Subramanian, S., Das, P. K., Souza, W., Lapa, T., Furtado, A. F., Van der Ploeg, C. P., Habbema, J. D. and van Oortmarssen, G. J. (1998). The LYMFASIM simulation program for modeling lymphatic filariasis and its control. Methods Inf Med, 37, 97-108.

Pong, S. S., Dehaven, R. and Wang, C. C. (1981). Stimulation of benzodiazepine binding to rat brain membranes and solubilized receptor complex by avermectin B1a and gamma-aminobutyric acid. Biochim Biophys Acta, 646, 143-150.

Pong, S. S. and Wang, C. C. (1980). The specificity of high affinity binding of avermectin B1a to mammalian brain. Neuropharmacol, 19, 311-317.

Pong, S. S. and Wang, C. C. (1982). Avermectin B1a modulation of gamma-aminobutyric acid receptors in rat brain membranes. J Neurochem, 38, 375-379.

Prichard, R. (2001). Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends Parasitol, 17, 445-453.

Prichard, R. K. (1973). The fumarate reductase reaction of *Haemonchus contortus* and the mode of action of some anthelmintics. Int J Parasitol, 3, 409-417.

Prichard, R. K. (1990). Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. Int J Parasitol, 20, 515-523.

Prichard, R. K. (2005). Is anthelmintic resistance a concern for heartworm control? What can we learn from the human filariasis control programs? Vet Parasitol, 133, 243-253.

Rahmah, N., Anuar, A. K., Ariff, R. H., Zurainee, M. N., A'Shikin A, N., Fadzillah, A., Maimunah, A. and Haq, J. A. (1998a). Use of antifilarial IgG4-ELISA to detect *Brugia malayi* infection in an endemic area of Malaysia. Trop Med Int Health, 3, 184-188.

Rahmah, N., Ashikin, A. N., Anuar, A. K., Ariff, R. H., Abdullah, B., Chan, G. T. and Williams, S. A. (1998b). PCR-ELISA for the detection of *Brugia malayi* infection using finger-prick blood. Trans R Soc Trop Med Hyg, 92, 404-406.

Rahmah, N., Lim, B. H., Khairul Anuar, A., Shenoy, R. K., Kumaraswami, V., Lokman Hakim, S., Chotechuang, P., Kanjanopas, K. and Ramachandran, C. P. (2001a). A recombinant antigen-based IgG4 ELISA for the specific and sensitive detection of *Brugia malayi* infection. Trans R Soc Trop Med Hyg, 95, 280-284.

Rahmah, N., Taniawati, S., Shenoy, R. K., Lim, B. H., Kumaraswami, V., Anuar, A. K., Hakim, S. L., Hayati, M. I., Chan, B. T., Suharni, M. and Ramachandran, C. P. (2001b). Specificity and sensitivity of a rapid dipstick test (Brugia Rapid) in the detection of *Brugia malayi* infection. Trans R Soc Trop Med Hyg, 95, 601-604.

Ramaiah, K. D., Das, P. K., Michael, E. and Guyatt, H. (2000). The economic burden of lymphatic filariasis in India. Parasitol Today, 16, 251-253.

Rao, R. U. (2005). Endosymbiotic *Wolbachia* of parasitic filarial nematodes as drug targets. Indian J Med Res, 122, 199-204.

Ravindran, B., Satapathy, A. K., Sahoo, P. K. and Mohanty, M. C. (2003). Protective immunity in human lymphatic filariasis: problems and prospects. Med Microbiol Immunol, 192, 41-46.

Reddy, G. S., Das, L. K. and Pani, S. P. (2004). The preferential site of adult *Wuchereria bancrofti*: an ultrasound study of male asymptomatic microfilaria carriers in Pondicherry, India. Natl Med J India, 17, 195-196.

Richards, F. O., Jr., Eberhard, M. L., Bryan, R. T., McNeeley, D. F., Lammie, P. J., McNeeley, M. B., Bernard, Y., Hightower, A. W. and Spencer, H. C. (1991). Comparison of high dose ivermectin and diethylcarbamazine for activity against bancroftian filariasis in Haiti. Am J Trop Med Hyg, 44, 3-10.

Robinson, M., Trudgett, A., Fairweather, I. and McFerran, N. (2002). Benzimidazole binding to *Haemonchus contortus* tubulin: a question of structure. Trends Parasitol, 18, 153-154.

Robinson, M. W., McFerran, N., Trudgett, A., Hoey, L. and Fairweather, I. (2004). A possible model of benzimidazole binding to beta-tubulin disclosed by invoking an inter-domain movement. J Mol Graph Model, 23, 275-284.

Roos, M. H., Boersema, J. H., Borgsteede, F. H., Cornelissen, J., Taylor, M. and Ruitenberg, E. J. (1990). Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. Mol Biochem Parasitol, 43, 77-88.

Roos, M. H. K., M.S.G.; Grant, W.N. (1995). New genetic and practical implications of selection for anthelmintic resistance in parasitic nematodes. Parasitol Today, 11, 148-150.

Russell, G. J. and Lacey, E. (1992). Differential stability of the benzimidazole (BZ)-tubulin complex in BZ-resistant and BZ-susceptible isolates of *Haemonchus contortus* and *Trichostrongylus colubriformis*. Int J Parasitol, 22, 399-402.

Sabesan, S., Krishnamoorthy, K., Panicker, K. N. and Vanamail, P. (1991). The dynamics of microfilaraemia and its relation with development of disease in periodic *Brugia malayi* infection in south India. Epidemiol Infect, 107, 453-463.

Samarawickrema, W. A. and Laurence, B. R. (1978). Loss of filarial larvae in a natural mosquito population. Ann Trop Med Parasitol, 72, 561-565.

Samarawickrema, W. A., Spears, G. F., Sone, F., Ichimori, K. and Cummings, R. F. (1985). Filariasis transmission in Samoa. II. Some factors related to the development of microfilariae in the intermediate host. Ann Trop Med Parasitol, 79, 101-107.

Sangster, N. C., Bannan, S. C., Weiss, A. S., Nulf, S. C., Klein, R. D. and Geary, T. G. (1999). *Haemonchus contortus*: sequence heterogeneity of internucleotide binding domains from P-glycoproteins. Expl Parasitol, 91, 250-257.

Sangster, N. C., Prichard, R. K. and Lacey, E. (1985). Tubulin and benzimidazole-resistance in *Trichostrongylus colubriformis* (Nematoda). J Parasitol, 71, 645-651.

Sartono, E., Kruize, Y. C., Kurniawan, A., Maizels, R. M. and Yazdanbakhsh, M. (1997). Depression of antigen-specific interleukin-5 and interferon-gamma responses in human lymphatic filariasis as a function of clinical status and age. J Infect Dis, 175, 1276-1280.

Sartono, E., Kruize, Y. C., Kurniawan, A., van der Meide, P. H., Partono, F., Maizels, R. M. and Yazdanbakhsh, M. (1995). Elevated cellular immune responses and interferon-gamma release after long-term diethylcarbamazine treatment of patients with human lymphatic filariasis. J Infect Dis, 171, 1683-1687.

Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P. and *et al.* (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell, 77, 491-502.

Scott , A. L. (2000). Lymphatic-Dwelling Filaria. In Lymphatic Filariasis, Vol. 1 (ed. Nutman, T. B.), Imperial College Press, London.

Scott, R. H. and Duce, I. R. (1985). Effects of 22,23 dihydroavermectin B1a on locust (*Schistocerca gregaria*) muscles may involve several sites of action. Pest Sci, 16, 599-604.

Sheriff, J. C., Kotze, A. C., Sangster, N. C. and Hennessy, D. R. (2005). Effect of ivermectin on feeding by *Haemonchus contortus* in vivo. Vet Parasitol, 128, 341-346.

Shoop (1993). Ivermectin Resistance. Parasitol Today, 9, 154-159.

Silvestre, A. and Cabaret, J. (2002). Mutation in position 167 of isotype 1 beta-tubulin gene of *Trichostrongylid* nematodes: role in benzimidazole resistance? Mol Biochem Parasitol, 120, 297-300.

Simonsen, P. E. and Dunyo, S. K. (1999). Comparative evaluation of three new tools for diagnosis of bancroftian filariasis based on detection of specific circulating antigens. Trans R Soc Trop Med Hyg, 93, 278-282.

Simonsen, P. E., Magesa, S. M., Dunyo, S. K., Malecela-Lazaro, M. N. and Michael, E. (2004). The effect of single dose ivermectin alone or in combination with albendazole on *Wuchereria bancrofti* infection in primary school children in Tanzania. Trans R Soc Trop Med Hyg, 98, 462-472.

Smith, G. (1990). A mathematical model for the evolutions of anthelmintic resistance in a direct life cycle nematode parasite. Int J Parasitol, 20, 913-921.

Smith, G., Grenfell, B. T., Isham, V. and Cornell, S. (1999). Anthelmintic resistance revisited: under-dosing, chemoprophylactic strategies, and mating probabilities. Int J Parasitol, 29, 77-91; discussion 93-74.

Snow, L. C. and Michael, E. (2002). Transmission dynamics of lymphatic filariasis: density-dependence in the uptake of *Wuchereria bancrofti* microfilariae by vector mosquitoes. Med Vet Entomol, 16, 409-423.

Southgate, B. A. (1992). The significance of low density microfilaraemia in the transmission of lymphatic filarial parasites. J Trop Med Hyg, 95, 79-86.

Southgate, B. A. and Bryan, J. H. (1992). Factors affecting transmission of *Wuchereria bancrofti* by anopheline mosquitoes. 4. Facilitation, limitation, proportionality and their epidemiological significance. Trans R Soc Trop Med Hyg, 86, 523-530.

Srividya, A., Krishnamoorthy, K., Sabesan, S., Panicker, K. N., Grenfell, B. T. and Bundy, D. A. (1991a). Frequency distribution of *Brugia malayi* microfilariae in human populations. Parasitol, 102 Pt 2, 207-212.

Srividya, A., Pani, S. P., Rajagopalan, P. K., Bundy, D. A. and Grenfell, B. T. (1991b). The dynamics of infection and disease in bancroftian filariasis. Trans R Soc Trop Med Hyg, 85, 255-259.

Stolk, W. A., van.Oortmarssen, G.J., Pani, S. P., de Vlas, S.J, Subramanian, S., Das, P. K. and Habbema, J. D. (2005). Effects of ivermectin and diethylcarbamazine on microfilariae and overall microfilaria production in bancroftian filariasis. Am J Trop Med Hyg, 73, 881-887.

Stolk, W. A., Swaminathan, S., van Oortmarssen, G. J., Das, P. K. and Habbema, J. D. (2003). Prospects for elimination of bancroftian filariasis by mass drug treatment in Pondicherry, India: a simulation study. J Infect Dis, 188, 1371-1381.

Stolk, W. A., Van Oortmarssen, G. J., Subramanian, S., Das, P. K., Borsboom, G. J., Habbema, J. D. and de Vlas, S. J. (2004). Assessing density dependence in the transmission of lymphatic filariasis: uptake and development of *Wuchereria bancrofti* microfilariae in the vector mosquitoes. Med Vet Entomol, 18, 57-60.

Subramanian, S., Krishnamoorthy, K., Ramaiah, K. D., Habbema, J. D., Das, P. K. and Plaisier, A. P. (1998). The relationship between microfilarial load in the human host and uptake and development of *Wuchereria bancrofti* microfilariae by *Culex quinquefasciatus*: a study under natural conditions. Parasitol, 116 (Pt 3), 243-255.

Subramanian, S., Stolk, W. A., Ramaiah, K. D., Plaisier, A. P., Krishnamoorthy, K., Van Oortmarssen, G. J., Dominic Amalraj, D., Habbema, J. D. and Das, P. K. (2004). The dynamics of *Wuchereria bancrofti* infection: a model-based analysis of longitudinal data from Pondicherry, India. Parasitol, 128, 467-482.

Sullivan, K. F. (1988). Structure and utilization of tubulin isotypes. Annu Rev Cell Biol, 4, 687-716.

Sunish, I., Rajendran, R., Mani, T., Dash, A. and Tyagi, B. (2006). Evidence for the use of albendazole for the elimination of lymphatic filariasis. Lancet Infect Dis, 6, 125-126.

Suresh, S., Kumaraswami, V., Suresh, I., Rajesh, K., Suguna, G., Vijayasekaran, V., Ruckmani, A. and Rajamanickam, M. G. (1997). Ultrasonographic diagnosis of subclinical filariasis. J Ultrasound Med, 16, 45-49.

Sutherland, I. A., Brown, A. E. and Leathwick, D. M. (2003). The effect of anthelmintic capsules on the egg output and larval viability of drug-resistant parasites. Vet Res Commun, 27, 149-157.

Sutherland, I. A., Leathwick, D. M., Moen, I. C. and Bisset, S. A. (2002). Resistance to therapeutic treatment with macrocyclic lactone anthelmintics in *Ostertagia circumcincta.* Vet Parasitol, 109, 91-99.

Tang, L. and Prichard, R. K. (1989). Characterization of tubulin from *Brugia malayi* and *Brugia pahangi.* Mol Biochem Parasitol, 32, 145-152.

Taylor, M. J. (2002). *Wolbachia* endosymbiotic bacteria of filarial nematodes. A new insight into disease pathogenesis and control. Arch Med Res, 33, 422-424.

Taylor, M. J. and Hoerauf, A. (2001). A new approach to the treatment of filariasis. Curr Opin Infect Dis, 14, 727-731.

Taylor, M. J., Makunde, W. H., McGarry, H. F., Turner, J. D., Mand, S. and Hoerauf, A. (2005). Macrofilaricidal activity after doxycycline treatment of *Wuchereria bancrofti*: a double-blind, randomised placebo-controlled trial. Lancet, 365, 2116-2121.

Thomas, J. H., Neff, N. F. and Botstein, D. (1985). Isolation and characterization of mutations in the beta-tubulin gene of *Saccharomyces cerevisiae.* Genetics, 111, 715-734.

Tisch, D. J., Michael, E. and Kazura, J. W. (2005). Mass chemotherapy options to control lymphatic filariasis: a systematic review. Lancet Infect Dis, 5, 514-523.

Townsend, L. B. W., D.S. (1990). The synthesis and chemistry of certain anthelmintic benzimidazoles. Parasitol Today, 6, 107-112.

Upcroft, J., Mitchell, R., Chen, N. and Upcroft, P. (1996). Albendazole resistance in Giardia is correlated with cytoskeletal changes but not with a mutation at amino acid 200 in beta-tubulin. Microbial Drug Resistance, 2, 303-308.

Vanamail, P., Subramanian, S., Das, P. K., Pani, S. P., Rajagopalan, P. K., Bundy, D. A. and Grenfell, B. T. (1989). Estimation of age-specific rates of acquisition and loss of *Wuchereria bancrofti* infection. Trans R Soc Trop Med Hyg, 83, 689-693.

Vanamail P, Ramaiah KD, Pani SP, Das PK, Grenfell BT, Bundy DA. (1996). Estimation of the fecund life span of *Wuchereria bancrofti* in an endemic area. Trans R Soc Trop Med Hyg ,90, 119-121.

Vanamail P, Subramanian S, Das PK, Pani SP, Rajagopalan PK. (1990). Estimation of fecundic life span of *Wuchereria bancrofti* from longitudinal study of human infection in an endemic area of Pondicherry (south India). Indian J Med Res, 91, 293-297.

Vassilatis, D. K., Elliston, K. O., Paress, P. S., Hamelin, M., Arena, J. P., Schaeffer, J. M., Van der Ploeg, L. H. and Cully, D. F. (1997). Evolutionary relationship of the ligand-gated ion channels and the avermectin-sensitive, glutamate-gated chloride channels. J Mol Evol, 44, 501-508.

Vasuki, V., Patra, K. P. and Hoti, S. L. (2001). A rapid and simplified method of DNA extraction for the detection of *Brugia malayi* infection in mosquitoes by PCR assay. Acta Trop, 79, 245-248.

Wada, Y., Kimura, E., Takagi, M. and Tsuda, Y. (1995). Facilitation in *Anopheles* and spontaneous disappearance of filariasis: has the concept been verified with sufficient evidence? Trop Med Parasitol, 46, 27-30.

Wang, C. C. and Pong, S. S. (1982). Actions of avermectin B1a on GABA nerves. Prog Clin Biol Res, 97, 373-395.

Wang, P. Y., Zhen, T. M., Wang, Z. Z., Gu, Z. F., Ren, S. P., Liu, L. H., Hou, L. W. and Liu, J. L. (1994). A ten-year observation on experimental infection of periodic *Brugia malayi* in man. J Trop Med Hyg, 97, 269-276.

Watts, S. D., Rapson, E. B., Atkins, A. M. and Lee, D. L. (1982). Inhibition of acetylcholinesterase secretion from *Nippostrongylus brasiliensis* by benzimidazole anthelmintics. Biochem Pharmacol, 31, 3035-3040.

Webb, J. K., Job, C. K. and Gault, E. W. (1960). Tropical eosinophilia: demonstration of microfilariae in lung, liver, and lymphnodes. Lancet, 1, 835-842.

Webber RH. (1977). The natural decline of *Wuchereria bancrofti* infection in a vector control situation in the Solomon Islands. Trans R Soc Trop Med Hyg, 71, 396-400.

Weerasooriya, M. V., Kimura, E., Dayaratna, D. A., Weerasooriya, T. R. and Samarawickrema, W. A. (1998). Efficacy of a single dose treatment of *Wuchereria bancrofti* microfilaria carriers with diethylcarbamazine in Matara, Sri Lanka. Ceylon Med J, 43, 151-155.

Weil, G. J., Lammie, P. J. and Weiss, N. (1997). The ICT Filariasis Test: A rapid-format antigen test for diagnosis of bancroftian filariasis. Parasitol Today, 13, 401-404.

Weil, G. J., Ramzy, R. M., Chandrashekar, R., Gad, A. M., Lowrie, R. C., Jr. and Faris, R. (1996). Parasite antigenemia without microfilaremia in bancroftian filariasis. Am J Trop Med Hyg, 55, 333-337.

WHA (1993). Recommendations of the International Task Force for Disease Eradication. MMWR Recomm Rep., 42, 1-38.

Wheeler, I. E., Kendall, S. J., Butters, J., Hollomon, D. W. and Hall, L. (1995). Using Allele-Specific Oligonucleotide probes to characterize benzimidazole resistance in *Rhynchosporium secalis*. Pest Sci, 43, 201-209.

WHO (1984). Lymphatic Filariasis. In WHO Technical Report Series, Vol. 702 pp. 1-110. World Health Organization, Geneva.

WHO (1992). Lymphatic Filariasis: The Disease and its Control: the fifth report of the WHO Expert Commitee on Filariasis. In WHO Technical Report Series, Vol. 821 pp. 1-80. World Health Organization, Geneva.

WHO (1997). Elimination of lymphatic filariasisas a public health problem: a world health resolution, 50th World Health Assembly, Agenda item 20, WHA 50.29.

WHO (1998a). Major Private Sector Partner, Merck, welcomed to lymphatic filariasis control effort, Press Release WHO/76

WHO (1998b). WHO, SmithKline & Beecham to cooperate on elephantiasis elimination, Press Release WHO/l 2.

WHO (1999). Building Partnerships for Lymphatic Filariasis: Strategic Plan WHO/FIL/99.198.

WHO (2000). Eliminate Filariasis: attack poverty. WHO booklet.

WHO. (2001). Lymphatic Filariasis. Wkly Epidemiol Rec, 76, 149-176.

WHO. (2002). Lymphatic filariasis, progress report on mass drug administration, 2001. Wkly Epidemiol Rec, 77, 125.

WHO (2004). Report on the mid-term assessment of microfilaraemia reduction in sentinel sites of 13 countries of the Global Programme to Eliminate Lymphatic Filariasis. Wkly Epidemiol Rec, 79, 358-365.

Wolstenholme, A. J.,Fairweather, I., Prichard, R. K., von Samson-Himmelstjerna, G. and Sangster, N. C. (2004). Drug resistance in veterinary helminths. Trends Parasitol, 20, 469-476.

Winterrowd, C. A., Pomroy, W. E., Sangster, N. C., Johnson, S. S. and Geary, T. G. (2003). Benzimidazole-resistant beta-tubulin alleles in a population of parasitic nematodes (*Cooperia oncophora*) of cattle. Vet Parasitol, 117, 161-172.

Wong, M. M. and Guest, M. F. (1969). Filarial antibodies and eosinophilia in human subjects in an endemic area. Trans R Soc Trop Med Hyg, 63, 796-800.

World Bank (1993). Investing in Health. In World Development Report pp. 344. Oxford University Press, New York.

Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R. and Prichard, R. (1998). Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol, 327-335.

Yarden O, Katan T. (1993). Mutations leading to substitutions at amino-acid 198 and 200 of beta-tubulin that correlate with benomyl-resistant phenotypes of field strains of *Botrytis cinerea*. Mol Plant Pathol, 83, 1478-1483.

Yates, D. M., Portillo, V. and Wolstenholme, A. J. (2003). The avermectin receptors of *Haemonchus contortus* and *Caenorhabditis elegans*. Int J Parasitol, 33, 1183-1193.

Yates, D. M. and Wolstenholme, A. J. (2004). An ivermectin-sensitive glutamate-gated chloride channel subunit from *Dirofilaria immitis*. Int J Parasitol, 34, 1075-1081.

Zielke, E. (1977). On the escape of infective filarial larvae from the mosquitoes. Tropenmed, 28, 461-466.

Zufall, F., Franke, C. H. and Hatt, H. (1989). The insecticide avermectin B1a activates a chloride channel in crayfish muscle membranes, J Exp Bio, 142, 191-205.

CHAPTER 2:

DETECTION OF BENZIMIDAZOLE RESISTANCE-ASSOCIATED MUTATIONS IN THE FILARIAL NEMATODE *WUCHERERIA BANCROFTI* AND EVIDENCE FOR SELECTION BY ALBENDAZOLE AND IVERMECTIN COMBINATION TREATMENT

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Abstract

The Global Program to Eliminate Lymphatic Filariasis has been implemented to reduce human microfilaraemia to levels low enough to break the transmission of the disease, using single annual doses of albendazole in combination with diethylcarbamazine or ivermectin. Many veterinary helminth parasites have developed resistance against both albendazole and ivermectin. Resistance to albendazole in veterinary nematodes is known to be caused by either of two single amino acid substitutions from phenylalanine to tyrosine in parasite β-tubulin at position 167 or 200. We have developed assays capable of detecting these single nucleotide polymorphisms (SNPs) in *Wuchereria bancrofti*, and have applied them to microfilariae obtained from patients in Ghana and Burkina Faso. One of the SNPs was found in untreated populations of worms from both locations. Worms from treated patients had significantly higher frequencies of these mutations. These findings indicate that a β-tubulin allele associated with benzimidazole resistance is being selected for in these populations.

2.1. Introduction

The Global Alliance to Eliminate Lymphatic Filariasis relies on chemotherapy with the anthelmintic albendazole in combination with ivermectin or diethylcarbamazine to block transmission of the main causative agent of lymphatic filariasis, *Wuchereria Bancrofti* (WHO, 1999). Avermectins and benzimidazoles have been used extensively in veterinary medicine, and this has led to high levels of drug resistance (Prichard, 1990). Though resistance against anthelmintics has not yet impeded the treatment of human helminth infections (Coles, 1999), the development of drug resistance has had an adverse impact on the control of other parasites, such as *Plasmodium falciparum* (Krogstad *et al.*, 1987). The development of drug resistance in the lymphatic filariae could severely compromise the international control program, which will involve an estimated 350 million people.

The mode of action of benzimidazoles was found to be their interference with tubulin polymerization into microtubules, by binding to β-tubulin (Friedman and Platzer, 1978; Kohler and Bachmann, 1981; Lubega and Prichard, 1990). Resistance against benzimidazoles in a number of nematode species has been shown to be caused by a phenylalanine (TTT/TTC) to tyrosine (TAT/TAC) substitution at either position 167 or 200 of nematode β-tubulin (Driscoll *et al.*, 1989; Elard *et al.*, 1996, Elard and Humbert, 1999; Kwa *et al.*, 1993a; Kwa *et al.*, 1993b; Pape *et al.*, 1999; Prichard, 2001; Silvestre and Cabaret, 2002; von Samson-Himmelstjerna *et al.*, 2001). The position 200-tyrosine mutation appears to be more common in parasitic nematodes and was found to be recessive (Elard and Humbert, 1999; Silvestre and Cabaret, 2002). Resistance to benzimidazoles was also found to be associated with 200Tyr or 167Tyr substitutions in normally benzimidazole susceptible fungi (Jung *et al.*, 1992; Koenraadt *et al.*, 1992; Orbach *et al.*, 1986; Yarden and Katan, 1993) and the 200Tyr mutation in β-tubulin was also found in benzimidazole-resistant strains of protozoa (Edlind *et al.*, 1994). However, the position 200 mutation was not found in *Necator americanus* hookworms from Pembla Island in which possible benzimidazole-resistance was suspected (Albonico *et al.*, 2004). Eukaryotes which normally code for tyrosine at codon 200 appear to be uniformly benzimidazole tolerant. Phenylalanine at codons 167 and 200 appears to be critical for

the high affinity binding of benzimidazole anthelmintics to nematode tubulin and for sensitivity to these anthelmintics (Prichard, 1990).

In a study carried out in India (Hoti *et al.*, 2003), pooled *W. bancrofti* microfilariae from 14 patients from 5 geographical regions were sequenced in order to detect the codon 200 resistance mutation. The consensus sequences of the 14 pooled samples failed to show the codon 200 resistance mutation. However, sequencing the pooled samples would not have shown the mutation unless it was abundant, since sequencing pooled DNA samples is not a sensitive tool for the detection of rare SNPs. The sequencing result of a pooled sample will show the most abundant sequence.

The objectives of this study were to assess whether mutations associated with benzimidazole-resistance, in other nematodes, are present in *W. bancrofti*, to examine their frequency in untreated populations from Ghana and Burkina Faso and to determine their frequency in microfilariae obtained from patients treated with albendazole and ivermectin.

2.2. Materials and Methods

The study and microfilarial collections were approved by the McGill University, Faculty of Medicine Institutional Review Board, the Noguchi Memorial Institute for Medical Research Insitutional Review Board, Ghana and the Ministry of Health, Burkina Faso. Informed consent was obtained from all human participants.

2.2.1 Amplification of *B. malayi* and *W. bancrofti* β-tubulin.

Primers were designed, based on the *Brugia pahang*i β-tubulin sequence - Accession No. M36380, in order to amplify the full length ß-tubulin cDNA from *B. malayi*. Total RNA was extracted from bulk adult worms (obtained from NIAID, NIH, Bethesda Maryland), using TRIzol reagent and methods described by the manufacturer (Invitrogen, Ontario, Canada). Total RNA was reverse transcribed with the adaptor primer (5'-3')GGC CAC GCG TCG ACT AGT $AC(T)_{17}$ and 200 U of murine moloney leukemia virus reverse transciptase (M-MLV, Invitrogen) according to the manufacturers instructions. cDNA was used to amplify a fragment of ß -tubulin, using a reaction containing 2.5 µl 10× PCR Buffer (200 mMTris-HCl pH 8.4, 500 mM KCl), 1 µl of $MgCl_2$ [50 µM], 0.5 µl each of primers , sense (5'-3') GGT ACC ATG GAT TCT ATT CG (AY705382 position 211-230) and antisense (5'-3') GAT CGG CGT TCA ACT GTC CA (position 729-748) at a concentration of 12.5 µM, 1U Taq polymerase, 1 µl dNTP (10 µM), and sterile H_2O up to 25 µl. The amplification conditions were 2 min at 94°C, followed by 30 cycles at 94°C for 55 sec, 55°C for 55 sec and 72°C for 55 sec and a final extension at 72°C for 10 min. The full length sequence was obtained using a 3' RACE (rapid amplification of cDNA ends) procedure. Briefly, 5 µl of cDNA was amplified in a nested reaction containing 2.5 µl 10× PCR Buffer (200 mMTris-HCl pH 8.4, 500 mM KCl), 1.5 µl of $MgCl_2$ [50 µM], 0.5 µl each of the sense primers (5'-3') GGC AAA TAT GTG CCA CGA GC (position 169-188) and (5'-3') GGT ACC ATG GAT TCT ATT CG (position 211-230) at a concentration of 20 µM, 1U Taq polymerase, 1 µl dNTP [10 µM], and sterile H_2O up to 25 µl. The amplification conditions for both steps were 2 min at 94°C, followed by 40 cycles at 94°C for 30 sec, 56°C for 30 sec and

72°C for 1 min 15 sec and a final extension at 72°C for 10 min. The 5'end of the gene was amplified using the same conditions as the 3'RACE, however with different primers: the sense primer, which corresponded to the nematode splice leader sequence SL1 (5'-3') GGT TTA ATT ACC AAG TTT GAG, and the antisense primers (5'-3') AGG GCT CGA TAA GCA GCA GC (position 832-851) and (5'-3') GAT CGG CGT TCA ACT GTC CA (position 729-748). The resulting fragments were cloned into a pCR 2.1 vector using a TA-cloning kit (Invitrogen), as per the manufacturer's instructions and sequenced with a Beckman CEQ DNA Sequencer.

Primers to amplify genomic DNA from *W. bancrofti* were designed based on the full length *B. malayi* β- tubulin cDNA, in order to PCR amplify a region surrounding the mutations to be diagnosed from *W. bancrofti* microfilariae, in a reaction containing 2.5 µl 10× PCR Buffer, 1 µl of $MgCl_2$ [50 µM], 0.5 µl each of the primers, sense (5'-3') GGC AAA TAT GTG CCA CGA GC (AY705382 position 169-188) and (5'-3') AGG GCT CGA TAA GCA GCA GC (AY705382 position 832-851) at a concentration of 12.5 µM, 1U Taq polymerase, 1 µl dNTP [10µM]), and sterile H_2O to make 25 µl. The amplification conditions were 2 min at 94°C, followed by 30 cycles at 94°C for 55s, 55°C for 55s and 72°C for 55s and a final extension at 72°C for 10 min. All reactions were carried out on an MJ-Research Inc. PTC-200 Thermal Cycler. Resulting fragments were cloned and sequenced as above.

2.2.2 Diagnosis of Resistance Associated Mutations in *W. bancrofti.*

Blood samples (10 ml) containing microfilariae were transferred to centrifuge tubes and centrifuged at 4°C and 1100 rpm (300 x g) for 10 minutes. The pellet was re-suspended in 25 ml red blood cell lysis solution, incubated on ice for 10 minutes, and centrifuged again as above. The final pellet was re-suspended in 1.5 ml 70% isopropyl alcohol. To separate individual worms, a small amount of the sample was transferred to a small Petri dish. Single microfilariae were washed in 70% ethanol, separated into individual PCR-microcentrifuge tubes using a micropipette, under an inverted microscope and identified visually as *W. bancrofti*. DNA extraction was carried out directly in the PCR tube using the Qiagen® DNeasy tissue kit at one quarter the recommended volume and following the manufacturer's instructions.

In order to detect mutations using a pyrosequencer, a smaller fragment surrounding the SNPs was amplified using microfilarial DNA or, when DNA content was small, the product of the PCR-amplification above was used as a template. The reaction contained 5 µl 10× PCR Buffer, 2 µl of $MgCl_2$ [50 µM], 0.5 µl each of primers, sense (5'-3') GGG AAC ATT GCT GAT CTC GAA (AY705383 position 308-328) and antisense (5'-3') GGA AGC AGA TGT CAT ACA AAG CC (AY705383 position 672-694) at a concentration of 20 µM, 1U *Taq* polymerase, 2 µl dNTP [10µM], and sterile H_2O to make 50 µl. The second primer had biotin attached to its 3' end, in order to generate single stranded template using streptavidin beads. The amplification conditions were 2 min at 94°C, followed by 40 cycles at 94°C for 15 s, 55°C for 30s and 72°C for 30s and a final extension at 72°C for 10 min. The sequencing primers used for SNP analysis in the Pyrosequencer® were (5'-3') CGG ATC GAA TTA TGA GCT CT (position 346-365) for position 167 and (5'-3') GAA AAC ACT GAC GAA ACT T (position 637-655) for position 200.

Real time diagnosis of mutations at position 200 and 167 was done in a single reaction in a Roche Light Cycler®. The reaction contained 1.6 µl $MgCl_2$ [25 µM], 0.4µl each of the primers , sense (5'-3') GGT ACC ATG GAT TCT ATT CG (AY705383 position 1-20) and antisense (5'-3') GAT CGG CGT TCA ACT GTC CA (position 907-926) at a concentration of 12.5 µM, 0.2µl each of the probes 5'-CCGTGAGGAGTATCC GGATCGAATTATG (fluorescine)-3' (position 332-359) and 5'-(LC Red 640)CTC TTT TTC GGT TGT GCC GTC G (phosphate)-3'(position 362-382) for position 167 and 5'-ACT TTC TGC ATT GAT AAC GAG GC (fluorescine)-3' (position 652-674) and 5'-(LC Red 705) TGT ATG ACA TCT GCT TCC GAA CGT (phosphate)-3' (position 677-700) for position 200 at a concentration of 20 µM. The conditions were 20 s at 95°C and 20 °C/ s, followed by 40 cycles at 95°C for 10 s and 20 °C/ s, 55°C for 10 s and 20 °C/ s and 72°C for 40 s and 20 °C/ s, a melting step from 50°C to 80°C at 20 °C/ s and a final cooling step to 40°C. Melting temperatures for the position 200 probes are 67 °C for the wild type gene and 63°C for the mutant. Melting temperatures for the position 167 probes are 63°C for the wild type gene and 58.5°C for the mutant.

Both diagnostic tests were optimized with a plasmid containing the wild type gene

and plasmids containing the mutation(s), generated by site-directed mutagenesis, using the QuickChange kit (Stratagene, LaJolla, USA) and the following mutagenesis primers: sense (5'-3') ATG AGC TCT TAT TCG GTT GTG CCG TCG (AY705383 position 357-383 and antisense (5'-3') ACA ACC GAA TAA GAG CTC ATA ATT CG (position 351-377) for the mutation at codon 167, and sense (5'-3') CGA AAC TTA CTG CATT GAT AAC G (position 648-670) and antisense (5'-3) CGT TAT CAA TGC AGT AAG TTT CG (position 648-670) for the mutation at codon 200, following the manufacturer's instructions.

2.2.3 Experimental Design and Statistical Analyses.

Microfilariae from patients infected with *W. bancrofti* were collected from Burkina Faso and Ghana, West Africa. Treatment programs for lymphatic filariasis have been instituted in communities in Ghana and Burkina Faso and records and patient questionnaires were available to establish the treatment histories in the individuals sampled. We analyzed microfilariae from patients before treatment, after one round of treatment and after two rounds of treatment with 400 mg albendazole and 200 μg/kg ivermectin. In the samples obtained from Burkina Faso, five to fifteen microfilariae per patient were genotyped. Microfilariae from Ghana were obtained as pooled samples from several patients. Two pooled samples were obtained that had been collected at different times in 2001. These two samples were treated separately, as they were sampled from different populations. A Chi-square test was used to detect significant differences in genotype frequencies and in allele frequencies between the three treatment groups within each country. It was determined whether genotype frequencies of the whole population and within each patient were in Hardy-Weinberg equilibrium using a Chi-square analysis to compare observed and expected values.

2.3. Results

We had access to fresh *Brugia malayi* which permitted us to extract high quality RNA and generate a full length β-tubulin cDNA (Accession No. AY705382). The high sequence homology between *B. malayi* and *W. bancrofti* allowed us to design primers, based on the *B. malayi* sequence, in order to clone and sequence a partial genomic sequence of *W. bancrofti* β-tubulin containing the regions coding for both amino acid 167 and 200 (Accession No. AY705383). Only one isotype of β-tubulin was detected. Based on these sequences, two diagnostic tests for mutations at positions 167 and 200 of β-tubulin of *W. bancrofti* were developed. The first assay was developed on a Pyrosequencer. This technique is able to sequence short fragments of DNA very rapidly; the Pyrosequencer can process 96 samples in under 1 hour and the results are reliable and easy to interpret. A second test made use of FRET (fluorescence resonance energy transfer) probes and melting point analysis for mutation detection on a Roche Light-Cycler. This test is able to identify both mutations in a single Light Cycler reaction. Both assays are capable of genotyping single microfilariae. We evaluated these tests with the help of several plasmids. These plasmids contained either the wild-type β-tubulin gene, or the β-tubulin gene containing the position 167 or the position 200 mutations introduced by site-directed mutagenesis. Absence/presence of the mutations was verified by sequencing. Though the FRET assay is not as quick and easy to perform as the Pyroquencer assay, it is more accessible for large-scale use, since real-time PCR technology is now available in many research facilities.

We obtained several hundred microfilariae from Ghana and Burkina Faso. The Ghanaian samples consisted of LF microfilariae from patients that had not been treated with albendazole and ivermectin as part of the Global Alliance to Eliminate Lymphatic Filariasis. The blood samples containing *Wuchereria bancrofti* collected in Burkina Faso, came from either untreated patients or patients treated once or twice with albendazole and ivermectin. Samples were taken at least seven days after the last anthelmintic treatment. Microfilaraemia was low in the treated patients, although the patients were still microfilaria positive despite recent treatment. The first two batches of microfilariae from Ghana were analyzed using the FRET assay. We were able to identify several

microfilariae that had the position 200 tyrosine genotype. The frequency of the mutant allele in the first group of 130 microfilariae was 2.7%. The frequency of the mutant allele in the second group of 300 microfilariae was 0.33% (Table 2.1). Because of a lack of information regarding the size of the human population from which the microfilariae were pooled, we cannot make a reliable estimate of the frequency of these resistance associated mutations in the entire parasite population. However, these findings indicate that the allele, which potentially causes albendazole resistance, is present in the population prior to treatment with albendazole. This suggests that with the advent of the mass treatment program in Ghana, selection for this allele in the population could occur.

Table 2.1: Frequencies of Codon 200 genotypes in β-tubulin in two populations of *W. bancrofti* from Ghana. F = phenylalanine, Y = tyrosine. Homozygous FF and heterozygous YF have been associated with a benzimidazole-susceptibility phenotype and homozygous YY has been associated with a benzimidazole-resistance phenotype. Total Y is the allele frequency of the resistance-associated allele in the population. Group1, n= 130, Group 2, n = 300.

	FF (%)	FY (%)	YY (%)	Total Y (%)
Group 1	96.15	1.54	2.31	2.69
Group 2	99.67	0	0.33	0.33

We have also analyzed a total of 400 microfilariae from 48 patients residing in the villages of Gora, Perigban, Bandongo and Tangonko, Burkina Faso. Thirty of these patients had not received any treatment for lymphatic filariasis, 14 had received one round of treatment with 400 mg of albendazole in combination with 200 μg/kg ivermectin. Four patients had received two yearly doses of the same treatment combination. Microfilarial counts obtained from these villages are found in Table 2.2. The microfilarial counts from the patients treated twice were taken 7 days after treatment.

Table 2.2: Microfilarial levels in patients from four villages in Burkina Faso.
"n" is the sample size, MF is microfilariae , and range is the difference between the highest and lowest counts.

Village	Treatment history	n	Geometric Mean MF/ml	Range
Tangonko	Untreated	16	15091	27050
Bandongo	Untreated	14	7674	39250
Gora	Treated once	1	9300	0
	Treated twice	4	964	350
Perigban	Treated once	13	1608	262

Using the Pyrosequencer, we found the resistance associated mutation at position 200 at an allele frequency of 26.2% in the untreated population. In the microfilariae from patients treated once, the allele frequency of this mutation was 60.2%; and in worms from patients treated twice, the allele frequency was 86.2 %. These allele frequencies were significantly different using Chi-square analysis ($Chi^2 = 139.8$). The genotype frequencies for all three groups from Burkina Faso were as follows (Fig. 2.1). Worms from untreated patients were 63% homozygous wild type (200phe/200phe), 21.5% heterozygous (200phe/200tyr) and 15.5% homozygous for the resistance mutation (200tyr/200tyr). Microfilariae from patients treated once were 26.3% homozygous wild type, 27.1% heterozygous and 46.6% homozygous for the resistance mutation. Microfilariae from patients treated twice were 0% homozygous wild type, 13.8% heterozygous and 86.2% homozygous for the resistance mutation. All genotype frequencies were significantly different using Chi-square analysis (Fig. 2.1). A resistance mutation was not detected at codon 167.

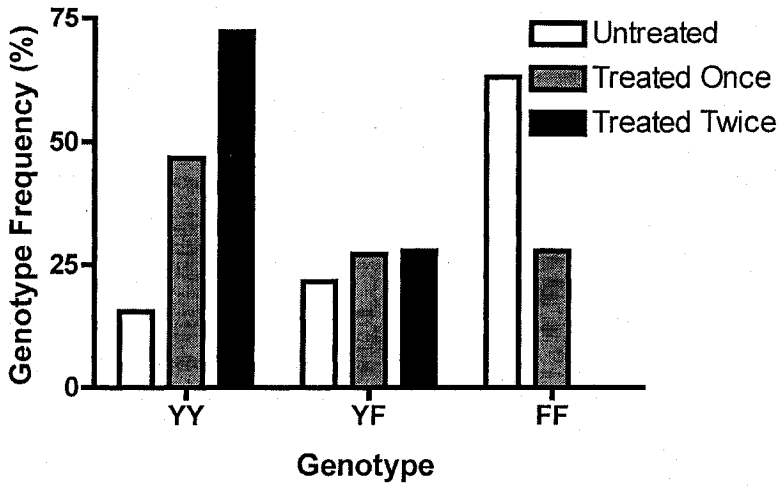

Figure 2.1: Genotype frequencies of β-tubulin codon 200 of *W. bancrofti* Burkina Faso, before and after treatment with albendazole/ivermectin.
Genotype frequencies (YY = homozygous 200tyr; YF = heterozygous 200tyr/200phe; FF = homozygous 200phe) for different treatment groups were significantly different from each other at p = 0.001 (Chi2 = 106.5). YY is associated with a resistance phenotype, YF and FF code for the susceptibility phenotype. Untreated, n = 246, treated once, n = 118, treated twice, n = 36.

2.4. Discussion

These results of this study indicate that the resistance associated mutation at codon 200 was present in the populations of *W. bancrofti* sampled, especially those from Burkina Faso, at a reasonably high frequency even before the mass treatment program had been initiated. It has been found that alleles associated with benzimidazole resistance in *Haemonchus contortus* were found in an unselected population at an allele frequency of 46% (Beech *et al.*, 1994), indicating that such resistance alleles may commonly be in the population prior to drug treatment at relatively high frequencies. However, it must be taken into consideration that benzimidazole resistance is recessive and that albendazole or mebendazole are common treatments for intestinal helminths, and yearly deworming of schoolchildren is common in many parts of West Africa (Hall *et al.*, 1996). Thus, parasites may have been exposed to benzimidazoles in the past, and some selection for the 200 tyrosine mutation may have occurred prior to the onset of the mass treatment program.

We show here that there was a significantly higher allele frequency of the 200 tyrosine genotype in worms collected from patients that had been treated with albendazole in combination with ivermectin. This indicates that this mutation may be selected for with drug treatment. The total allele frequency was 31.6% higher in the treated versus the non-treated worm population. In worms collected from patients that had been exposed to two rounds of albendazole/ivermectin treatment, the allele frequency was an additional 25.9% higher. Such selection is a strong indication of the development of drug resistance. Because pre-treatment microfilarial counts were not taken, we cannot make conclusions about the success of treatment. However, it was only those microfilariae that were still present in the treated patients after treatment which were genotyped. In the case of the patients treated twice, the microfilariae were harvested seven days after their last treatment and could therefore represent a population of microfilarial survivors of treatment or progeny of adult worms which were able to maintain microfilarial production despite treatment.

In some parasitic nematodes a second β-tubulin gene appears to be present (Geary *et al.*, 1992; Guenette *et al.*, 1992), which may contribute to benzimidazole

sensitivity and resistance (Beech *et al.*, 1994). We did not detect a second β-tubulin isotype in *W. bancrofti*. However, if present, it could contribute to maintaining susceptibility to albendazole.

It must also be noted that this was not a prospective study, but that parasites from different treatment groups were collected from different patients residing in separate villages. We can therefore not rule out natural genetic variation, which may occur between populations, being responsible for the differences in polymorphism between the treated and untreated populations.

We tested whether alleles of this gene were in Hardy-Weinberg equilibrium. When considering all worms in a treatment group (including untreated patients) as part of one population, the frequencies were not in balance, but showed an excess of homozygotes. When considering all worms sampled from an individual patient as being one population, the sum of the resulting allele frequencies from all patients was in Hardy-Weinberg equilibrium. The resistant-associated allele appeared to be aggregated within certain patients, rather than evenly distributed throughout the population. This result may be due to the fact that random mating does not occur between worms in different hosts. An excess of homozygotes may strongly accelerate selection for resistance, since it is believed to be a completely recessive trait (Elard *et al.*, 1996), thus a larger proportion of the 200tyr as homozygotes could lead to a greater proportion of treatment failures.

Combination treatment with ivermectin and albendazole could be expected to reduce the rate of selection for resistance to either of these anthelmintics, provided that the resistance mechanisms involve different genes. However, it has recently been found that ivermectin selects on β-tubulin in the filarial nematode *Onchocerca volvulus* (Eng and Prichard, 2005). Although ivermectin selection is not associated with the phe200tyr SNP examined here, selection by the two anthelmintics can involve the same gene, thus there could be linkage between the region implicated in ivermectin selection and possible albendazole selection for the phe200tyr SNP which could modify the rate of selection for albendazole resistance. The implications of combination treatment on genetic selection need to be examined in future studies.

Based on these findings, it is imperative to continue monitoring for the presence of this mutation, in other treatment zones, in order to detect early evidence of possible resistance selection and to correlate this with responses to treatment.

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References

Albonico, M., Wright, V. and Bickle, Q. (2004). Molecular analysis of the beta-tubulin gene of human hookworms as a basis for possible benzimidazole resistance on Pemba Island. *Mol Biochem Parasitol,* 134, 281-284.

Beech, R. N., Prichard, R. K. and Scott, M. E. (2004). Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. *Genetics*, 138, 103-110.

Coles, G. C. (1999). Anthelmintic resistance and the control of worms [editorial]. *Journal of Medical Microbiology,* 48, 323-325.

Driscoll, M., Dean, E., Reilly, E., Bergholz, E. and Chalfie, M. (1989). Genetic and molecular analysis of a Caenorhabditis elegans beta-tubulin that conveys benzimidazole sensitivity. *Journal of Cell Biology,* 109, 2993-3003.

Edlind, T., Visvesvara, G., Li, J. and Katiyar, S. (1994). Cryptosporidium and microsporidial beta-tubulin sequences: predictions of benzimidazole sensitivity and phylogeny. *J Eukaryot Microbiol,* 41, 38S.

Elard, L., Comes, A. M. and Humbert, J. F. (1996). Sequences of beta-tubulin cDNA from benzimidazole-susceptible and -resistant strains of Teladorsagia circumcincta, a nematode parasite of small ruminants. *Molecular & Biochemical Parasitology,* 79, 249-253.

Elard, L. and Humbert, J. F. (1999). Importance of the mutation of amino acid 200 of the isotype 1 beta-tubulin gene in the benzimidazole resistance of the small-ruminant parasite Teladorsagia circumcincta. *Parasitology Research,* 85, 452-456.

Eng, J. K. and Prichard, R. K. (2005). A comparison of genetic polymorphism in populations of *Onchocerca volvulus* from untreated- and ivermectin-treated patients. *Molecular Biochemistry and Parasitology* 142, 193-202.

Friedman, P. A. and Platzer, E. G. (1978). Interaction of anthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. *Biochimica et Biophysica Acta,* 544, 605-614.

Guenette, S., Prichard, R. K. and Matlashewski, G. (1992). Identification of a novel *Brugia pahangi* beta-tubulin gene (beta 2) and a 22-nucleotide spliced leader sequence on beta 1-tubulin mRNA. *Mol Biochem Parasitol*, 50, 275-284.

Geary, T. G., Nulf, S. C., Favreau, M. A., Tang, L., Prichard, R. K., Hatzenbuhler,

N. T., Shea, M. H., Alexander, S. J. and Klein, R. D. (1992). Three beta-tubulin cDNAs from the parasitic nematode *Haemonchus* contortus. *Mol Biochem Parasitol,* 50, 295-306.

Hall A, Adjei S, C. K. (1996). School Health Programs. *Afr Health Sci* 18 22-23.

Hoti, S. L., Subramaniyan, K. and Das, P. K. (2003). Detection of codon for amino acid 200 in isotype 1 beta-tubulin gene of Wuchereria bancrofti isolates, implicated in resistance to benzimidazoles in other nematodes. *Acta Trop,* 88, 77-81.

Jung, M. K., Wilder, I. B. and Oakley, B. R. (1992). Amino acid alterations in the benA (beta-tubulin) gene of Aspergillus nidulans that confer benomyl resistance. *Cell Motil Cytoskeleton,* 22, 170-174.

Koenraadt, H., Sommerville, S. C. and Jones, A. L. (1992). Characterisation of mutations on the beta-tubulin gene of benomyl-resistant field strains of Venturia inaequalis and other pathogenic fungi. *Mol Plant Pathol,* 82, 1348-1354.

Kohler, P. and Bachmann, R. (1981). Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes. *Molecular & Biochemical Parasitology,* 4, 325-336.

Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M., Martin, S. K., Milhous, W. K. and Schlesinger, P. H. (1987). Efflux of chloroquine from Plasmodium falciparum: mechanism of chloroquine resistance. *Science,* 238, 1283-1285.

Kwa, M. S. G., Veenstra, J. G. and Roos, M. H. (1993). Molecular characterisation of beta-tubulin genes present in benzimidazole-resistant populations of Haemonchus contortus. *Molecular & Biochemical Parasitology*, 60, 133-144.

Kwa, M. S. G., Veenstra, J. G. and Roos, M. H. (1994). Benzimidazole resistance in Haemonchus contortus is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. *Molecular & Biochemical Parasitology,* 63, 299-303.

Lubega, G. W. and Prichard, R. K. (1990). Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in Haemonchus contortus. *Molecular & Biochemical Parasitology,* 38, 221-232.

Orbach, M. J., Porro, E. B. and Yanofsky, C. (1986). Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of Neurospora crassa and its use as a dominant selectable marker. *Mol Cell Biol,* 6, 2452-2461.

Pape, M., von Samson-Himmelstjerna, G. and Schnieder, T. (1999). Characterisation of the beta-tubulin gene of Cylicocyclus nassatus? *Int J Parasitol,* 29, 1941-1947.

Prichard, R. (2001). Genetic variability following selection of Haemonchus contortus with anthelmintics. *Trends Parasitol,* 17, 445-453.

Prichard, R. K. (1990). Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. *Int J Parasitol,* 20, 515-523.

Silvestre, A. and Cabaret, J. (2002). Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? *Mol Biochem Parasitol,* 120, 297-300.

von Samson-Himmelstjerna, G., Harder, A., Pape, M. and Schnieder, T. (2001 Feb). Novel small strongyle (Cyathostominae) beta-tubulin sequences. *Parasitology Research,* 87, 122-125.

WHO (1999). Building Partnerships for Lymphatic Filariasis: Strategic Plan WHO/FIL/99.198.

Yarden, O. and Katan, T. (1993). Mutations leading to substitutions at amino-acid 198 and 200 of beta-tubulin that correlate with benomyl-resistant phenotypes of field strains of Botrytis cinerea. *Mol Plant Pathol,* 83, 1478-1483

CONNECTING STATEMENT 1:

In Chapter 2 of this thesis we present evidence that mutations known to cause benzimidazole resistance were present in microfilariae of *W. bancrofti*, and that the frequency of these mutations was higher in worms obtained from patients that had been treated with ABZ and IVM. This suggests that resistance may be developing and could pose a threat to the current international control program. The GPELF is implementing large-scale treatment programs that have the potential to exert strong selection pressure for resistance alleles. However it is not known how resistance alleles may spread through populations of *W. bancrofti* and how epidemiological factors, as well as current treatment strategies, will impact selection for resistance. In the following chapter we present a mathematical model that incorporates population genetics into an epidemiological model of *W. bancrofti* in order to further analyze the mechanisms and relative importance of factors involved in the spread of drug-resistance through populations of lymphatic filaria, due to the current combination treatment strategies applied by the GPELF. Due to the differences in initial allele frequencies for the putative ABZ resistance mutation, between populations sampled in Burkina Faso (26%) and Ghana (2.6%, 0.33%), for the purpose of the model, we have assumed that initial resistance-allele frequencies to be 5%.

CHAPTER 3:

AN ANALYSIS OF THE POPULATION GENETICS OF POTENTIAL ALBENDAZOLE RESISTANCE IN LYMPHATIC FILARIASIS DUE TO COMBINATION CHEMOTHERAPY

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Abstract

The Global Program for the Elimination of Lymphatic Filariasis (GPELF) intends to achieve its aims through yearly mass treatments with albendazole (ABZ) combined with ivermectin (IVM) or diethylcarbamazine (DEC). The use of ABZ and IVM separately to combat parasites of veterinary importance has, on many occasions, resulted in widespread drug resistance. In order to help predict the spread of potential ABZ resistance alleles through a population of *Wuchereria bancrofti*, we have developed a mathematical model that incorporates population genetics into EPIFIL, a model which examines the transmission dynamics of the parasite. Our model considers the effect of the combined treatments on the frequency of a recessive allele, which confers ABZ resistance. The model predicts that after 10 yearly treatments with ABZ and DEC, 85% coverage and an initial resistance allele frequency of 5%, the frequency of the resistance genotype will increase from 0.25 to 12.7%. If non-random mating is assumed, the initial genotype frequency will be 2.34% and will increase to 62.7 %. ABZ and IVM combination treatment may lead to weaker selection for this genotype. Treatment coverage, initial allele frequencies and number of treatments also affect the rate of selection.

3.1. Introduction

Lymphatic filariasis (LF) is a disease caused by a group of lymphatics-dwelling filarial nematodes transmitted by mosquito vectors which infect approximately 120 million people in over 90 countries, and whose disease sequelae impose a severe economic and social burden on affected communities and individuals (Michael & Bundy, 1997; Ramaiah *et al.*, 1999; Zagaria & Savioli, 2002). Currently, a global public-private partnership, under the auspices of the World Health Organization (the Global Alliance for the Elimination of Lymphatic Filariasis), aims to eliminate this disease as a public health problem within the next twenty years. It is hoped that this will be achieved by community-wide yearly mass treatment with the broad spectrum anthelmintic albendazole (ABZ) in combination with the well-known microfilaricide diethylcarbamazine (DEC) or ivermectin (IVM) (Dean, 2002; Maher & Ottesen, 2000; Ottesen, 2000, 2002; Zagaria & Savioli, 2002). Benzimidazoles and avermectins have been used extensively in veterinary medicine for over two decades, and this has led to the development of drug resistance to both types of compounds by many helminth parasites affecting livestock (Prichard, 1990; Prichard *et al.*, 1980; Wolstenholme *et al*, 2004). Consequently, there is concern that lymphatic filarial nematodes in humans may also develop such drug resistance, as this could severely hamper the control programmes. There have already been some reports of tolerance to DEC by filarial parasites (Eberhard *et al.*, 1988, 1991). Recently, we have shown that a mutation at position 200 of the β-tubulin gene, from phenylalanine to tyrosine (TYR200), known to cause benzimidazole resistance in veterinary parasites is present in populations of *Wuchereria bancrofti* from West Africa, and is significantly higher in treated than in non-treated populations (Schwab *et al.*, 2005). Thus, selection for albendazole resistance may already be occurring.

Mathematical models can be invaluable in helping to understand parasite population dynamics and predict the impact of chemotherapy on these dynamics (Anderson & May, 1982, 1985). Models aiding in the design and implementation of control programmes have been successfully used for the filarial nematode *Onchocerca volvulus* (Alley *et al.*, 2001; Basáñez & Ricárdez-Esquinca, 2001; Habbema *et al.*, 1992).

Simple analytical models have examined various aspects of LF transmission and disease dynamics (Grenfell *et al.*, 1990; Grenfell & Michael, 1992; Hayashi, 1962; Michael & Bundy, 1998; Subramanian *et al.*, 1989) by assuming equilibrium with respect to age or time and therefore exploring temporal dynamics or age infection profiles. EPIFIL is an age-structured, deterministic model which incorporates some probabilistic elements (the frequency distribution of the number of microfilariae that will be transmitted to vectors) and describes changes in parasite population abundance with both age and time (Chan *et al.*, 1998, 1999; Norman *et al.*, 2000). Stochastic microsimulation models have also been developed [e.g. LYMFASIM (Plaisier *et al.*, 1998)]. Both EPIFIL and LYMFASIM models have been used to examine the effect of various control strategies based on different antiparasitic and antivectorial measures (Das & Subramanian, 2002; Michael *et al.*, 2004; Stolk *et al.*, 2003; Subramanian *et al.*, 2004). In addition, EPIFIL is now available as a free resource for health workers in operational settings (Chan *et al.*, 1999). However, neither of these models has so far been used to explore the spread of drug resistance, which requires incorporation of genetic structure into the parasite population (Anderson *et al.*, 1989).

One such model has been presented for the investigation of the spread of anthelmintic resistance in the sheep parasite *Trichostrongylus colubriformes*, considering various sheep management and anthelmintic delivery practices in a simple framework which includes up to three anthelmintic resistance genes, each with two alleles, associated with resistance to a single drug or to each of three drugs (Barnes & Dobson, 1990). A second, more general model applicable to direct life-cycle parasites, based on the simpler one locus-two allele system examines different anthelmintic treatment strategies (Smith, 1990). More complex, deterministic and stochastic models for the evolution of anthelmintic resistance in trichostrongylids (Smith *et al.*, 1999) predict that host immunity, parasite fecundity and aggregation will have an important impact on the spread of resistance. More recently, the impact of spatial heterogeneity and metapopulation transmission dynamics on the spread of drug resistance has been examined using stochastic models (Cornell *et al.*, 2000, 2003). These models predict that rare recessive alleles may spread through overdispersed parasite populations more rapidly than initially anticipated.

An excess of homozygotes for the TYR200 mutation was observed in a population of *W. bancrofti* microfilariae from West Africa (Schwab *et al.*, 2005). A preliminary analysis of these data indicates that excess homozygosity is equivalent to Wright's hierarchical (inbreeding) *F*-statistic or $F_{IT} = 0.44$ (measuring the degree of parasite inbreeding within individuals in the total host population; Churcher, 2006). Although the mechanisms generating this are unknown, an increased homozygosity will likely lead to a faster spread of recessive drug resistance. Here we describe a model that incorporates simple parasite population genetics into the transmission dynamics model EPIFIL in order to explore the consequences of both random and non-random parasite mating upon the evolution and spread of drug resistance in filarial parasites under the current combination therapy treatment regimes.

3.2. Model Development

3.2.1 Population Dynamics.

The model described in this paper is based on EPIFIL, whose code is now publicly available (Chan *et al.*, 1999; Norman *et al.*, 2000). EPIFIL consists of a system of partial differential equations which describe, with respect to time and host age, the rates of change of mean adult worm burden (W), mean microfilarial count per 20 μl of blood (M), mean numbers of L3 larvae per mosquito (L), and mean strength of acquired protective immunity by the human host (I). In EPIFIL, acquired immunity is assumed to depend solely on past experience to adult worms and to affect the establishment of L3 larvae within the definitive host. Density dependence within the mosquito vector is represented by a saturating function of L3 output with increasing microfilarial input, and is influenced by an assumed negative binomial distribution of microfilariae in the human population (Norman *et al.*, 2000). The equations of the EPIFIL model are reproduced in Appendix A. Parameters for EPIFIL are summarized in Table 3.1.

3.2.1 Population Genetics.

In order to model the spread of anthelmintic resistance through the parasite population, we have assumed that resistance to ABZ is associated with a single autosomal locus with two alleles S, r, with r, the recessive allele conferring resistance. Each of the EPIFIL equations, originally for homogeneous macrofilarial, microfilarial, and infective larvae populations, were structured into worms homozygotes and drug sensitive (with genotype SS), heterozygous drug-sensitive (Sr), and homozygous resistant (rr) (see Appendix B). We have assumed that there are no costs or trade-offs associated with resistance (e.g., resistant parasites may have a lower fecundity rate). Under chemotherapeutic pressure, the fitness of the resistant parasites is higher than that of the susceptible worms.

Table 3.1. Definition and values of parameters used in the model.

Parameter	Value† and units	Definition
λ	10 month^{-1}	biting rate per mosquito on human hosts
$\lambda(V/H)$	5,760 month^{-1}	monthly biting rate per person
g	0.37	proportion of mosquito bites made on microfilaraemic hosts that result in the mosquito acquiring infection
ρ	0.047	initial proportion of microfilariae per 20 µl of blood that once ingested become infective larvae in the mosquito
κ	6	the maximum number of infective larvae produced per mosquito as microfilaraemia increases
φ_1	0.414	proportion of L3 leaving the mosquito during bite
φ_2	0.32	proportion of L3 entering host
s_2	1.13 x 10^{-4}	proportion of L3 entering host that become adult worms
β	0.112	the severity of constraints on larval establishment effected by protective acquired immunity
α	2 month^{-1}	the rate of microfilarial production per worm per 20 µl of blood
μ	0.0104 month^{-1}	per capita death rate of adult worms
γ	0.1 month^{-1}	per capita death rate of microfilaria
σ	5 month^{-1}	per capita death rate of L3 larvae
Δ	0.005 month^{-1}	rate of decay of protective immunity (value from Man-Suen Chan, pers. comm.)
k_0	0.0029	overdispersion parameters of the negative binomial distribution, with $k_b = k_0 + k_1 M_b$ as a function of treated (b=T) or untreated (b=U) microfilariae
k_1	0.0236 microfilaria^{-1}	

†Parameter values are taken from Norman *et al.* (2000) for *Culex*-transmitted filariasis. We use these parameters in order to remain as close as possible to the EPIFIL model. However, our evidence for increased parasite homozygosity comes from West African locations, where *Anopheles*- rather than *Culex*-transmitted filariasis prevails.

3.2.1.1 Random Mating.

Initially, random mating was assumed and thus genotype distributions of microfilariae were calculated from those of adult worm populations by means of simple Mendelian genetics. In order to reflect treatment coverage, treated and untreated parasite populations were modelled separately. At the point of transmission, the respective contributions of each infective larval population (depending on coverage level) were introduced into the equations for adult worms. The model thus assumes that the same individuals are treated at each round. Total mean parasite loads are a combination of the three genotypes for the treated and untreated sections of the parasite populations, the latter in proportions depending on coverage.

3.2.1.2 Non-random Mating.

In order to examine the effect of non-random mating, we have included a parameter F in the equations to indicate deviation from Hardy-Weinberg equilibrium, as a crude way to incorporate increased homozygosity (see eqns. (B3.6) to (B3.8) of Appendix B). Wright's inbreeding F_{IT}-statistic is a phenomenological rather than a mechanistic measure of homozygosity, and therefore does not explicitly describe the biological processes involved in producing an excess of homozygotes. By setting a fixed value for this parameter, the same adjustment factor for increased homozygosity is applied from generation to generation. The amount of excess homozygosity was calculated from a preliminary analysis of data obtained in Burkina Faso (Schwab *et al.*, 2005); a detailed description of this calculation and of the possible mechanisms generating homozygosity will be presented elsewhere.

3.2.2 Drug Efficacy

Assumptions on drug efficacy, measured as percentage of worms (macro- and microfilariae) killed and female worms sterilized, were based on those made by Michael *et al.* (2004) and other published drug trials (Addiss *et al.*, 1997; Dunyo *et al.*, 2000; Ismail *et al.*, 1998) and are summarized in Table 3.2. Treatment was modelled as instantaneous reductions in adult worms (macrofilaricidal effect), microfilarial populations (microfilaricidal effect), or female worm reproduction rate (sterilizing effect)

by the percentage efficacy following treatment. Sterilized females did not resume microfilarial production immediately but after a period of time specified for each drug (Table 3.2). (In Table 3.2 it is assumed that microfilaricidal efficacy for the ABZ + IVM combination is higher than that of combinations containing DEC, but see Tisch *et al.*, 2005.) All model outcomes in this paper examine the effect of ten yearly treatments on the genotype distribution of the microfilariae (the stages more feasibly sampled for genetic analyses).

Table 3.2. Drug efficacy assumptions (adapted from Michael *et al.*, 2004)

	DEC alone	IVM alone	DEC and ABZ	IVM and ABZ
Months without reproduction	3	9	9	9
% Adults killed	30	10	55	35
% Microfilariae killed	90	99	95	99

Model code was written using the JSIM numerical integration software from http://nsr.bioeng.washington.edu/PLN/Members/butterw/JSIMDOC1.6/JSim_Home.stx/view. Differential equations were solved using the Euler method. Prior to the initiation of control perturbations, parasite populations were assumed to be at endemic equilibrium.

3.3. Results

We have examined different factors affecting the spread of anthelmintic resistance in *W. bancrofti*. Two different treatment regimes are currently used in the LF control programme. ABZ and DEC are used in most areas of the world though in parts of Africa, where onchocerciasis is co-endemic, DEC cannot be used and patients are treated with ABZ and IVM. We have examined the effect of both of these treatment regimes on the spread of ABZ resistance. Results of this analysis can be seen in Fig. 3.1. Fig. 3.1a shows the genotype frequency of the recessive, resistant homozygote in the microfilarial population, after ten yearly treatments with either drug combination. Coverage was assumed to be 85% of the total population, and the initial resistance allele frequency was taken as 5%. Fig. 3.1b shows the mean microfilaraemia (all microfilariae genotypes combined) for each treatment regime. Model outcomes without the presence of ABZ resistance are virtually indistinguishable from those portrayed in Fig. 3.1b, and are therefore not shown. This indicates that the spread of ABZ resistance is unlikely to impair the impact of the control programme on microfilarial levels. Treatment with ABZ and IVM leads to an increase of the resistant genotype by a factor of 1.74. However since the initial genotype frequency is 0.25%, it will only reach 0.44%, and thus will still be uncommon in the population. Treatment with ABZ and DEC has a much larger impact on the frequency of the ABZ-resistant genotype. Our model indicates that the genotype frequency will rise to 12.7% and thus increase by a factor of 4.8 (Fig. 3.1 a).

In order to further examine these results, we have carried out a sensitivity analysis on different parameters of the model. The results of these analyses are displayed in Figs. 3.2 to 3.6. It can be seen that when treating with the ABZ + DEC combination, selection for ABZ resistance is strongly dependent on increasing the differential microfilaricidal efficacy of the ABZ + DEC combination over DEC alone (Fig. 3.2a), and the assumed macrofilaricidal efficacy of DEC (Fig. 3.2b) (Table 3.2). When treating with the ABZ + IVM combination, the spread of resistance is less affected by small changes in drug efficacies (see Appendix II, Fig. 7.1).

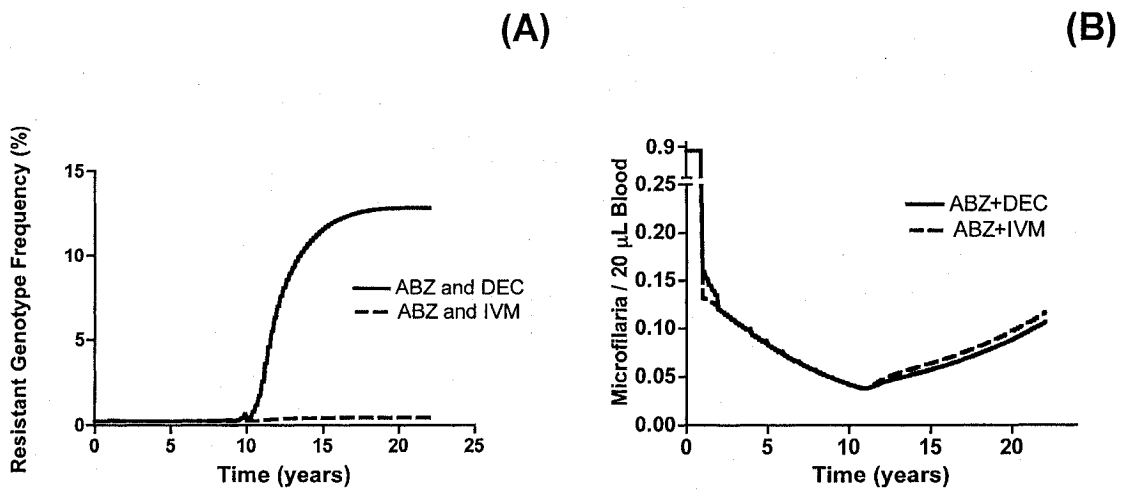

Figure 3.1: The effect of 10 yearly treatments with albendazole (ABZ) + diethylcarbamazine (DEC), or ABZ + ivermectin (IVM), with 85% coverage of the total population, on the spread of ABZ resistance in *Wuchereria bancrofti*. A) the average frequency (in percentage) of resistant genotype microfilariae; B) the mean microfilaraemia per 20 µl of blood in the human host population. Solid line: ABZ + DEC, dashed line: ABZ + IVM.

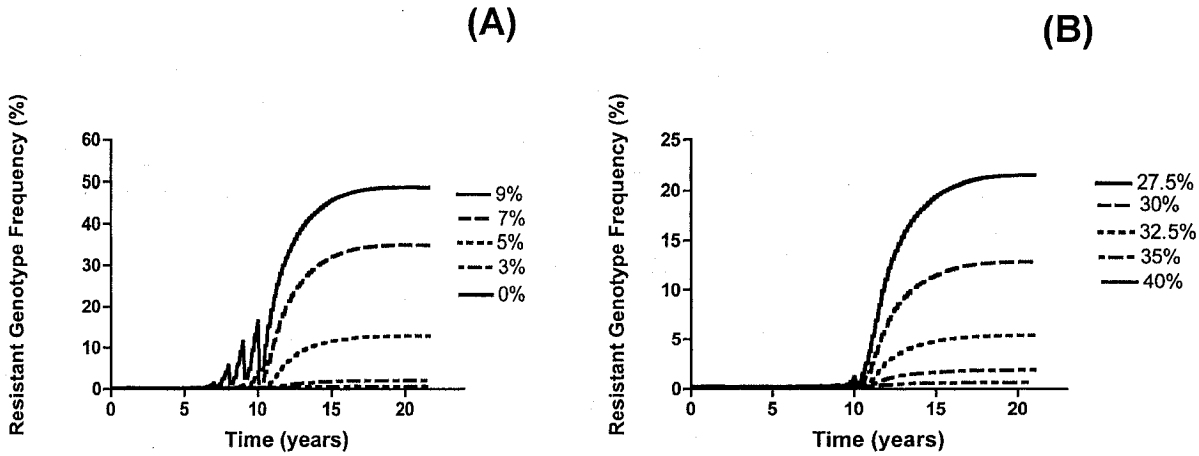

Figure 3.2: The effect, on the ABZ resistant genotype frequency (%) of *W. bancrofti* microfilariae, of increasing drug efficacy parameters.
A) the differential microfilaricidal efficacy (% of microfilariae killed) due to adding ABZ to DEC, and B) adulticidal efficacy (% of macrofilariae killed) of DEC, when administering 10 annual treatments with ABZ + DEC to 85% of the human host population.

Treatment coverage affects the speed at which ABZ resistance spreads, with increasing coverage considerably increasing selection for the resistant genotype (Fig.

3.3). When coverage reaches 95% with ABZ + DEC, the ABZ resistant genotype frequency in microfilariae will reach almost 40% 10 years after cessation of the 10 yearly treatments (Fig. 3.3a). Coverage will also affect the frequency of the ABZ resistant genotype after treatment with ABZ + IVM. However, though the resistant genotype will increase by a factor of 2.5 when changing treatment coverage to 95%, this frequency will reach only 0.64% 10 years after halting the annual treatments, and thus will only have a marginal effect (Fig. 3.3c). As expected, mean microfilaraemia, during the period of treatment, depends on treatment coverage with either ABZ + DEC or ABZ + IVM (respectively Figs. 3.3b and 3.3d), with lowest microfilaraemia corresponding to highest coverage. Therefore, very low microfilaraemia levels during control, mask strong selection and increased frequency of resistant genotypes once control is halted.

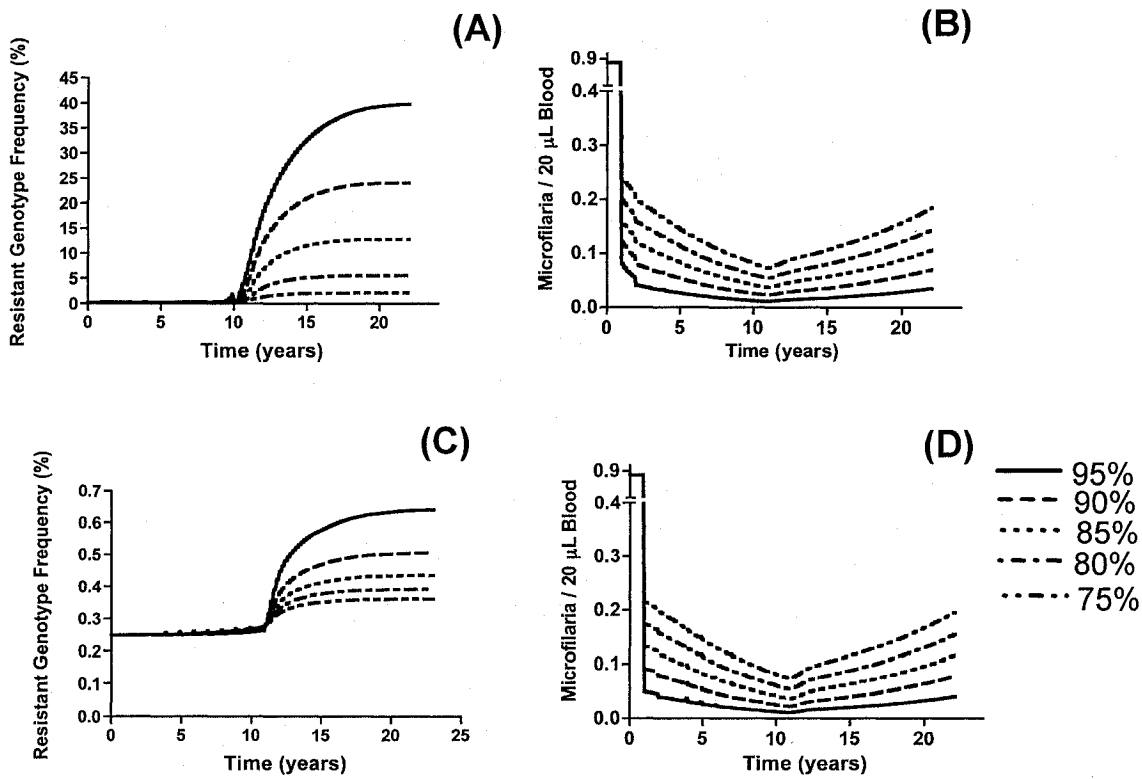

Figure 3.3: The effect on the ABZ resistant genotype frequency (%) of increasing therapeutic coverage of 10 yearly treatments. ABZ-resistant genotype microfilariae (A, C), and average microfilaraemia (B, D), for combination therapy with ABZ + DEC (A, B), and ABZ + IVM (C, D).

We have also examined the effect of non-random mating. Inbreeding changes the

distribution of genotypes, increasing homozygosity and hence the number of resistant genotypes at a given allele frequency. Non-random mating can have a dramatic impact on the outcomes of selection, as shown in Fig.3.4. With a Wright's F_{IT}-statistic equivalent to parameter F= 0.44 in the model, the ABZ resistant genotype would reach a frequency of over 60% after ceasing 10 yearly treatments with ABZ + DEC. It may also be noted that even a much lower F value of 0.11 will lead to a resistant genotype frequency of over 50% (Fig. 3.4a). In the case of 10 yearly treatments with ABZ + IVM, an F value of 0.44 will lead the resistant genotype frequency to increase from 0.25% to just over 20%, under the assumptions of 85% coverage. The corresponding microfilarial intensities are shown in Figs 3.4b (ALB + DEC) and 4c (ALB + IVM).

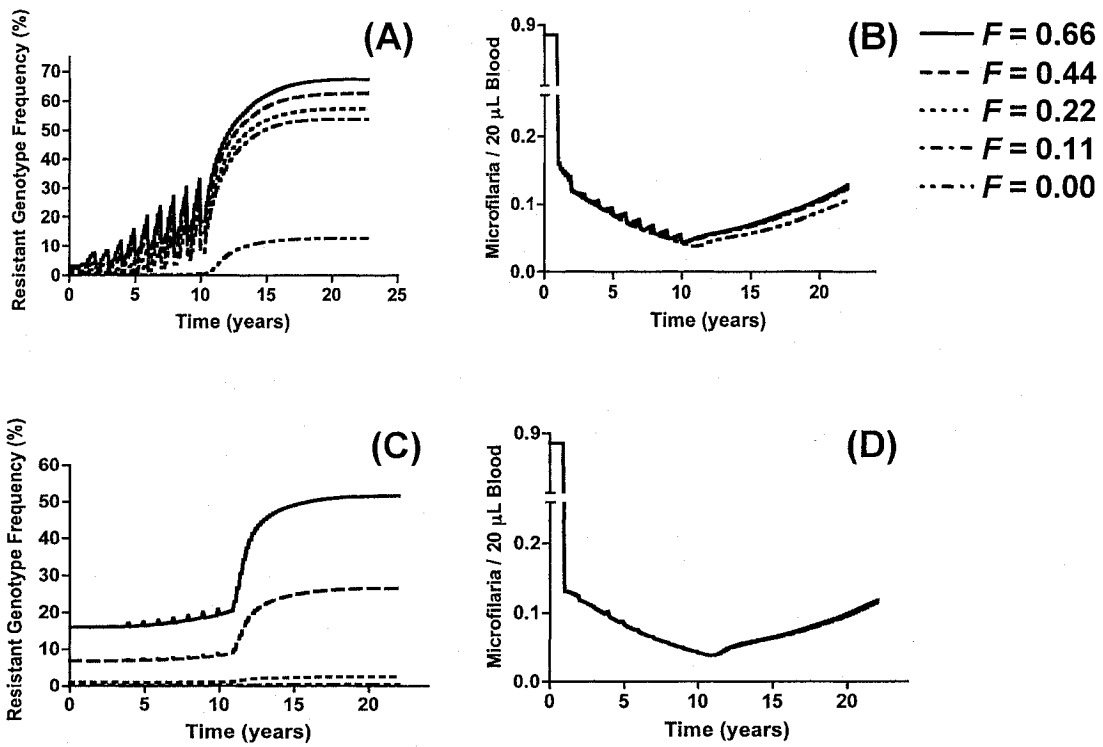

Figure 3.4: The effect, on the spread of ABZ resistance in *W. bancrofti*, of increasing non-random parasite mating (i.e., increased homozygosity as measured by the Fisher F_{IT} statistic), following 10 yearly treatments at 85% coverage ABZ + DEC (A, B), and ABZ + IVM (C, D). Panels (A) and (C) represent the frequency (%) of resistant genotype microfilariae, and panels (B) and (D) the mean microfilaraemia.

Schwab *et al* (2005) found initial frequencies of the ABZ resistant genotype of up

to 26%. Thus we examined how quickly resistance will rise if initial frequencies are higher than the 5% assumed in the other models. In Fig. 3.5, it may be observed how changing the initial ABZ resistance frequencies before treatment will dramatically change the spread of resistance once mass drug administration is stopped (Fig. 3.5). An initial resistant genotype frequency of 10% will lead to an increase in the resistant genotype frequency to over 50% after 10 treatments with ABZ + DEC. However, if the initial frequency is further increased to 40%, the proportion of resistant microfilariae will rise to reach 70% (Figs. 3.5a, b). When treating with ABZ + IVM (Figs. 3.5c, d), the effect of increasing initial frequency of ABZ resistance is similar, but less dramatic than with ABZ + DEC. These simulations assume no resistance to DEC or IVM.

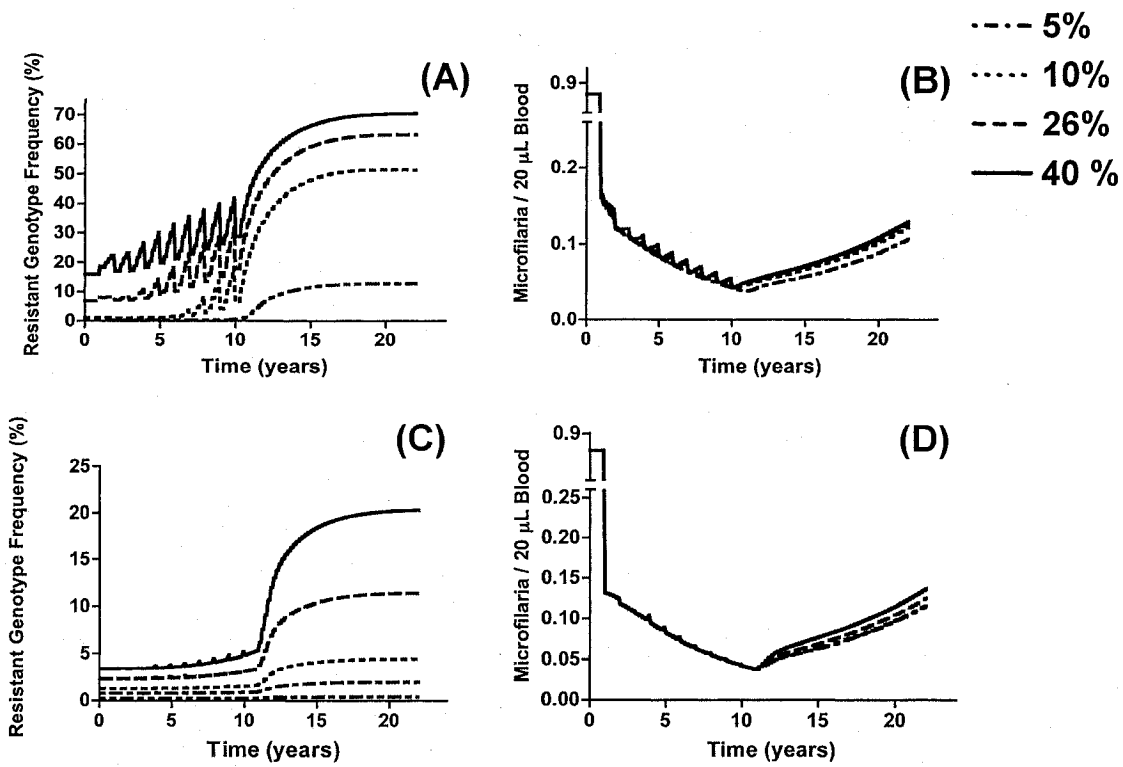

Figure 3.5: The effect, on the spread of ABZ resistance, of varying the initial resistance allele frequency (in %) following 10 yearly treatments with 85% coverage. A) the frequency (%) of resistant genotype microfilariae, and B) the mean microfilaraemia for ABZ + DEC; panels (C) and (D) present the corresponding results for ABZ + IVM.

Increasing the duration of the treatment programme also considerably affects the spread of ABZ resistance. Here we consider the effect of up to 15 yearly treatments with ABZ + DEC. It was estimated that it will take 15 years of treatment for the ABZ resistant genotype frequency to reach over 50% (Fig. 3.6a). When treating with ABZ + IVM, 15 yearly treatments are estimated to lead to an increase in the resistant genotype frequency from 0.25% to just under 0.6% (Fig. 3.6c). The effects of these treatment schedules on microfilarial intensity are shown in Figs. 3.6b and 3.6d.

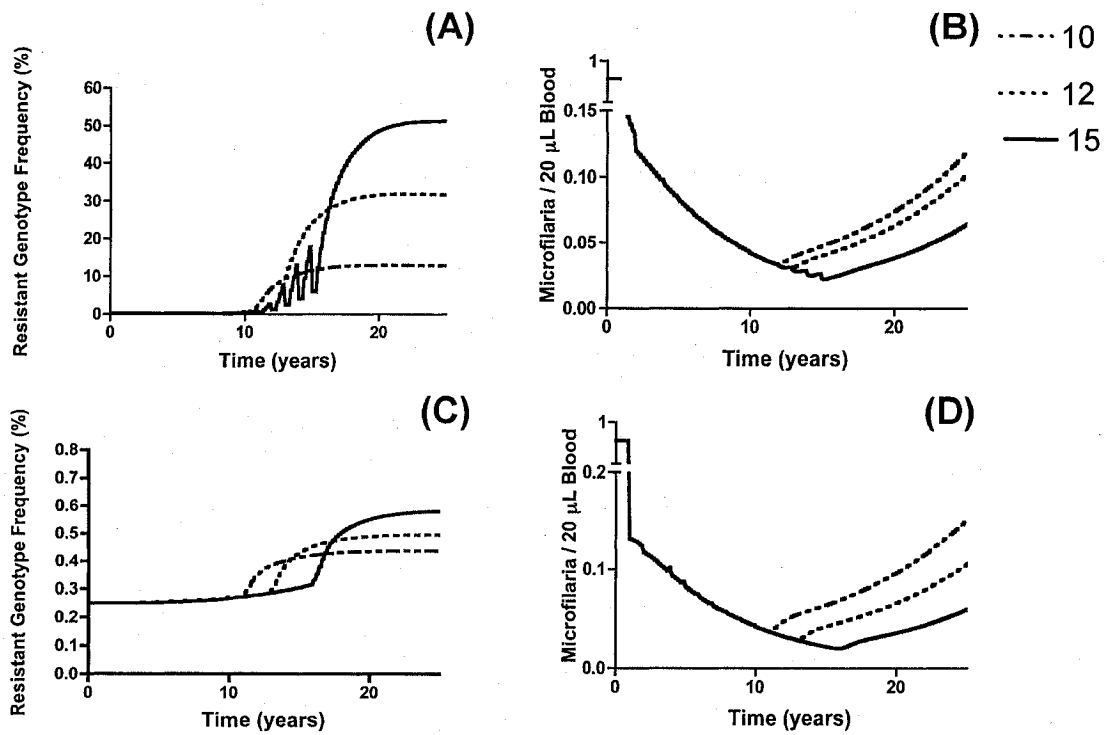

Figure 3.6:. The effect of increasing the number of annual treatments with 85% coverage on the spread of ABZ resistance in *W. bancrofti*:
A) the frequency (%) of resistant genotype microfilariae, and B) the mean microfilaraemia for ABZ + DEC; panels (C) and (D) correspond to treatment with ABZ + IVM.

We have used parameters for homozygosity and initial frequency obtained from villages in Burkina Faso to indicate the spread of ABZ resistance in these communities

(Fig. 3.7). Both treatment regimes are estimated to result in the frequency of the ABZ resistant homozygote reaching 50% after ten yearly treatments at 85% coverage, though little difference will be seen in microfilarial burdens.

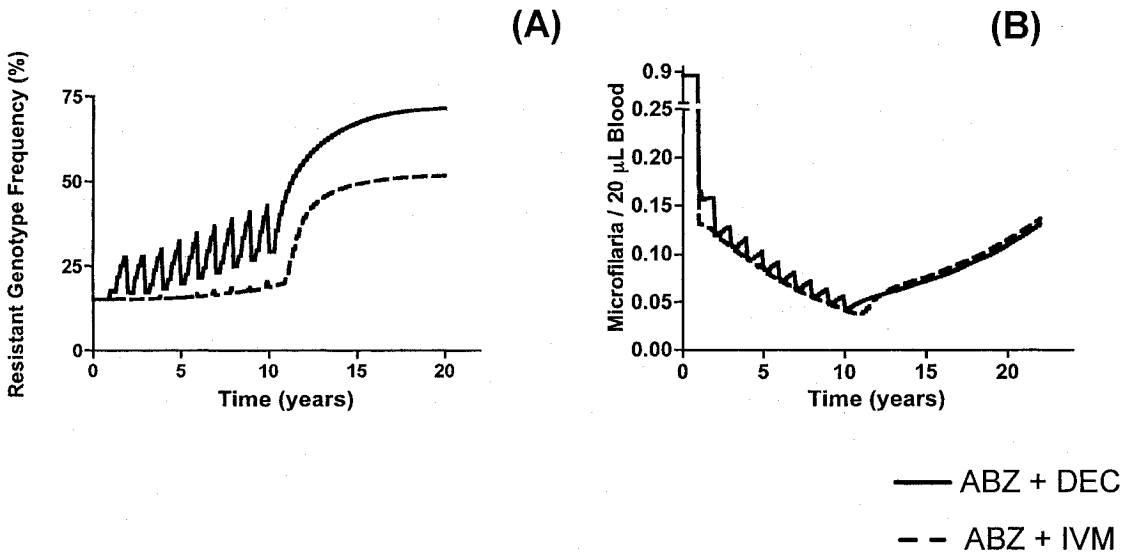

Figure 3.7: The effect, on the spread of ABZ resistance in *W. bancrofti*, of 10 annual treatments at 85% coverage with ABZ + DEC (solid line), or ABZ + IVM (dashed line), using the initial allele frequencies and F_{IT} values recorded in the village of Tangonko, Burkina Faso (Schwab *et al.*, 2005): A) the frequency (%) of resistant genotype microfilariae; B) the average microfilaraemia

3.4. Discussion

The development of drug resistance in lymphatic filarial parasites could represent a serious threat to the GPELF, which currently aims to eliminate the disease by blocking transmission. The model presented in this paper examines factors affecting the spread of ABZ resistance under current treatment strategies for *W. bancrofti*, under a series of structural and parameter assumptions. Although the model provides quantitative results, given the uncertainties still remaining about such assumptions, the qualitative insights gained are more important than the actual predictions. Based on the assumptions of drug efficacies made here, treatment with ABZ + DEC would lead to a much quicker spread of ABZ resistance than that with ABZ + IVM. The model indicates that treatment with ABZ + DEC for ten years, at 85% coverage and an initial ABZ resistant genotype frequency of 0.25%, would increase the resistant microfilarial genotype frequency to approximately 13%. Treatment with ABZ + IVM would lead to a negligible increase in the resistant genotype.

Sensitivity analysis of the of ABZ + DEC treatment regime shows that the spread of ABZ resistance would be highly dependent on the additional microfilaricidal activity gained when adding ABZ to DEC (as compared to DEC alone), and the degree to which DEC alone can kill adult parasites. Some additional microfilaricidal activity, caused by ABZ, is required in order to give resistant worms enough of a selective advantage for the allele frequency to change after the introduction of treatment. Increasing DEC adulticidal activity dramatically reduces the spread of ABZ resistance. Therefore, care must be taken when assumptions about drug efficacy are made, as small parameter changes can lead to larger changes in the outcomes predicted by the model. The ABZ + IVM combination treatment is much less affected by small changes in the microfilaricide and macrofilaricide efficacy parameters. An important factor in the behaviour of different treatment regimes is the fact that DEC sterilizes adult worms for a shorter period than IVM. Thus, following each IVM treatment, very little reproduction occurs for 9 months and selection for the ABZ resistant allele is minimal (as we have assumed no resistance to IVM).

The spread of ABZ resistance is strongly dependent on treatment coverage.

Higher levels of therapeutic coverage would lead to faster microfilarial reductions, but also to quicker spread of ABZ resistance. Our model indicates that increasing the treatment coverage by 10% (from 85% to 95%) would lead to an almost four-fold increase in the frequency of ABZ-resistant microfilariae in the population 10 years after cessation of the 10 yearly treatments with ABZ + DEC. This is due to the fact that untreated hosts act as refugia of susceptible parasites. Similar results have been observed for animal parasites (Coles, 2002; van Wyk, 2001). In this model it is assumed that the same individuals in the population are being treated at each round, as is the case for systematic compliers. Without using individual-based, more realistic models, our conclusions remain tentative. During the treatment period, little increase in the resistant homozygote frequency in the microfilarial population is apparent. During this time, the treated hosts are being continually infected by L3 larvae derived from untreated hosts. These larvae have the genotype distribution of the untreated group, which is similar to the initial overall genotype distribution. Within the treated hosts, susceptible worms are dying at a faster rate than resistant worms (the macrofilaricidal efficacy of DEC + ABZ is assumed to be 55% whereas that of DEC alone is 30%). Therefore, although the resistant genotype frequency of the adult worm population is increasing continually over time during treatment, this is not reflected in substantial changes in microfilarial genotype, partly because adult worms in treated hosts are reproducing only to a very small degree. Consequently, the overall genotype distribution in the microfilarial population is predominantly influenced by that of the microfilariae in untreated hosts, where the adult genotype frequency remains fairly constant. Thus, there is no considerable change in the genotype distribution of the microfilarial population during the course of the treatment. Following the end of treatment, the adult worms in the treated individuals can reproduce once again. These adult worms have a high ABZ resistance allele frequency so the microfilariae resistant genotype frequency starts to rise until the adult worm allele frequency in the treated and untreated hosts reaches equilibrium.

Though the genotype structure of the microfilarial population is clearly changing, there is only a small impact on microfilaraemia. This is a consequence of using combination treatment. While ABZ resistance may be developing, it has been assumed that DEC or IVM still remain effective in clearing microfilariae from the blood of

patients. Thus, ABZ resistance may not become immediately apparent, but it may have a considerable impact should DEC or IVM become no longer effective for treatment (e.g. if DEC or IVM resistance were to develop).

Based on the results obtained from the models, and with default parameters, it would take 15 years of treatment with ABZ + DEC in order for half of the microfilaria population to be homozygous resistant to ABZ. This exceeds the time frame intended by the GPELF, which proposes that duration of chemotherapy should last the time equivalent to the lifespan of an adult worm, estimated to vary between 4 and 6 years (Michael *et al.*, 2004; Vanamail *et al.*, 1996). However, there is considerable uncertainty around this estimate, and values ranging from 5 years (Vanamail *et al.*, 1989) to ~40 (Carme & Laigret, 1979), with a mean of ~10 years (Subramanian *et al.*, 2004) for the LYMFASIM model have been presented. The adult worm life span assumed by EPIFIL is approximately eight years (Norman *et al.*, 2000). Michael *et al* (2004) believe that it may not be feasible to reach the goals of the GPELF in areas of high endemicity within the proposed time-span of 4 – 6 years.

Some areas may have high levels of initial benzimidazole resistance allele frequencies, as demonstrated by Schwab *et al* (2005). Similar observations were also made in trichostrongylid parasites (Beech *et al.*, 1994). Our model indicates that when ABZ + DEC are used together, an increase of the initial resistant allele frequency from 5% to 10%, would result in half of all microfilariae in the population becoming resistant after halting the 10 years of annual treatment. If the resistance allele frequency is initially 26%, this proportion would increase to 63%. Initial allele frequencies may vary in different geographical locations and were found to be around 26% in some areas of Burkina Faso, and less than 1% in some microfilarial samples from Ghana (Schwab *et al.*, 2005). It is therefore advisable to develop resistance markers for monitoring purposes in areas where treatment is being introduced. Given that the sampling protocol will greatly influence the estimated values of ABZ resistance frequencies, the results above need to be taken with caution. We are developing population genetics models to address these issues and the results will be presented elsewhere (Churcher *et al.*, unpublished-see).

Results from this model suggest that excess homozygosity, caused by non-random parasite mating, would lead to a rapid increase in selection of recessive resistance alleles.

Inbreeding is incorporated very crudely within the modelling framework, and it had the aim to illustrate the serious implications that excess homozygosity would have on the spread of drug resistance. Our current model makes no assumptions about the mechanisms driving non-random mating. It has been demonstrated in the past that recessive alleles may be selected far more quickly than initially anticipated, due to metapopulation structure and resulting inbreeding (Cornell *et al.*, 2000, 2003). Filarial worm populations are subdivided in the host population, and thus random mating is unlikely to occur. In addition, vectors may not evenly ingest microfilariae and distribute infective larvae through the host populations and are more likely to take consecutive blood meals from people within the same household (Michael *et al.*, 1998; 2001), which may lead to inbreeding.

We show here, using parameters obtained from villages in Burkina Faso, that the effects investigated previously (i.e. inbreeding, high initial allele frequency), when combined together will have an additive affect on the final resistance allele frequency. This is because the relationship between allele frequency and homozygosity is non-linear.

Though mathematical models are not necessarily accurate quantitative predictors of population dynamics in the field, because assumptions made in the models may turn out to be not entirely correct, they do help highlight influential factors (Cornell *et al.*, 2005). EPIFIL was optimized in India, particularly in the locality of Pondicherry, where LF prevalence is relatively low, and integrated vector management has taken place for a long time (Subramanian *et al.*, 2004). Also, the operation of acquired immunity, which was thought to be essential for reproducing observed infection patterns in Pondicherry, has not been substantiated in other geographical regions (Stolk *et al.*, 2004). Transmission intensity may be different in other areas, such as Africa, where there are different vectors (EPIFIL is parameterised for *Culex*-transmitted LF). In addition, this is a deterministic model that does not consider transmission breakpoints (e.g., no explicit mating probabilities for adult worms are incorporated), and is unable to take into account variability among individual hosts and parasites. Incorporating greater biological complexity into the model will be necessary for obtaining accurate quantitative conclusions, though we feel this is not likely to affect in great measure the qualitative conclusions drawn from our results.

3. 5. Concluding Remarks.

We show that it is important to take into account the efficacies and mechanisms of drug effects when applying control strategies in order to predict the spread of drug resistance. Furthermore, it is important to be aware of initial resistance allele frequencies, as these will affect the speed at which resistance spreads, and thus monitoring field situations with appropriate resistance markers is advisable. The requirements of high coverage and compliance, and the lengthy duration of chemotherapy-based control programmes risks anthelmintic resistance or at best, a decrease in the efficacy of the drugs of choice. Incorporation of vector control into control programmes can reduce the time required to meet control goals (Basáñez *et al.*, 2002; Michael *et al.*, 2004) and may thus prevent drug resistance from undermining the achievement of these goals. In addition, if aspects of the parasite's biology increase homozygosity in the population, the spread of resistance will be faster than if the parasite population is mating randomly. The results of the model also indicate that if monitoring of allele frequencies is not undertaken during the control programme, and low *W. bancrofti* infection levels persist by the time control is stopped, drug resistance may only become apparent once regular treatment has been interrupted, recrudescence occurs, and treatment is reapplied.

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References

Addiss, D. G., Beach, M. J., Streit, T. G., Lutwick, S., LeConte, F. H., Lafontant, J. G., Hightower, A. W. and Lammie, P. J. (1997). Randomised placebo-controlled comparison of ivermectin and albendazole alone and in combination for *Wuchereria bancrofti* microfilaraemia in Haitian children. *Lancet* 350, 480-484.

Alley, W. S., van Oortmarssen, G. J., Boatin, B. A., Nagelkerke, N. J., Plaisier, A. P., Remme, J. H., Lazdins, J., Borsboom, G. J. and Habbema, J. D. (2001). Macrofilaricides and onchocerciasis control, mathematical modelling of the prospects for elimination. *BMC Public Health* 1, 12.

Anderson, R. M. and May, R. M. (1982). Population dynamics of human helminth infections: control by chemotherapy. *Nature* 297, 557-563.

Anderson, R. M. and May, R. M. (1985). Helminth infections of humans: mathematical models, population dynamics, and control. *Advances in Parasitology* 24, 1-101.

Anderson, R. M., May, R. M. and Gupta, S. (1989). Non-linear phenomena in host-parasite interactions. *Parasitology* 99 Suppl, S59-79.

Barnes, E. H. and Dobson, R. J. (1990). Population dynamics of *Trichostrongylus colubriformis* in sheep: computer model to simulate grazing systems and the evolution of anthelmintic resistance. *International Journal for Parasitology* 20, 823-831.

Basáñez, M.-G. and Ricárdez-Esquinca, J. (2001). Models for the population biology and control of human onchocerciasis. *Trends in Parasitology* 17, 430-438.

Basáñez. M.-G., Collins, R. C., Porter, C. H., Little, M. P. and Brandling-Bennett D. (2002). Transmission intensity and the patterns of *Onchocerca volvulus* infection in human communities. *American Journal of Tropical Medicine and Hygiene* 67, 669-679.

Beech, R. N., Prichard, R. K. and Scott, M. E. (1994). Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. *Genetics* 138, 103-110.

Carme, B. and Laigret, J. (1979). Longevity of *Wuchereria bancrofti* var. *pacifica* and mosquito infection acquired from a patient with low level parasitemia. *American Journal of Tropical Medicine and Hygiene* 28, 53-55.

Chan, M. S., Norman, R. A., Michael, E., Bundy, D. A., Das, P. K., Pani, S. P. and Ramaiah, K. D. (1999). http://www.schoolsandhealth.org/epidynamics.htm.

Chan, M. S., Srividya, A., Norman, R. A., Pani, S. P., Ramaiah, K. D., Vanamail, P., Michael, E., Das, P. K. and Bundy, D. A. (1998). EPIFIL: a dynamic model of infection and disease in lymphatic filariasis. *American Journal of Tropical Medicine and Hygiene* 59, 606-614.

Churcher, T.S. (2006). Modelling the spread of anthelmintic resistance. *PhD Thesis* Imperial College London.

Coles, G. C. (2002). Sustainable use of anthelmintics in grazing animals. *Veterinary Record* 151, 165-169.

Cornell, S. (2005). Modelling nematode populations: 20 years of progress. *Trends in Parasitology* 21, 542-545.

Cornell, S. J., Isham, V. S. and Grenfell, B. T. (2000). Drug-resistant parasites and aggregated infection--early-season dynamics. *Journal of Mathematical Biology* 41, 341-360.

Cornell, S. J., Isham, V. S., Smith, G. and Grenfell, B. T. (2003). Spatial parasite transmission, drug resistance, and the spread of rare genes. *Proceedings of the National Academy of Science U S A* 100, 7401-7405.

Das, P. K. and Subramanian, S. (2002). Modelling the epidemiology, transmission and control of lymphatic filariasis. *Annals of Tropical Medicine and Parasitology* 96 Suppl 2, S153-164.

Dean, M. (2002). Towards the elimination of lymphatic filariasis. *Lancet* 359, 1677.

Dunyo, S. K., Nkrumah, F. K. and Simonsen, P. E. (2000). A randomized double-blind placebo-controlled field trial of ivermectin and albendazole alone and in combination for the treatment of lymphatic filariasis in Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94, 205-211.

Eberhard, M. L., Lammie, P. J., Dickinson, C. M. and Roberts, J. M. (1991). Evidence of nonsusceptibility to diethylcarbamazine in *Wuchereria bancrofti*. *Journal of Infectious Diseases* 163, 1157-1160.

Eberhard, M. L., Lowrie, R. C., Jr. and Lammie, P. J. (1988). Persistence of microfilaremia in bancroftian filariasis after diethylcarbamazine citrate therapy. *Tropical Medicine and Parasitology* 39, 128-130.

Grenfell, B. T., Das, P. K., Rajagopalan, P. K. and Bundy, D. A. (1990). Frequency distribution of lymphatic filariasis microfilariae in human populations: population processes and statistical estimation. *Parasitology* 101 Pt 3, 417-427.

Grenfell, B. T. and Michael, E. (1992). Infection and disease in lymphatic filariasis: an epidemiological approach. *Parasitology* 104 Suppl, S81-90.

Habbema, J. D., Alley, E. S., Plaisier, A. P., van Oortmarssen, G. J. and Remme, J. H. (1992). Epidemiological modelling for onchocerciasis control. *Parasitology Today* 8, 99-103.

Hayashi, S. (1962). A mathematical analysis on the epidemiology of Bancroftian and Malayan filariasis in Japan. *Japanese Journal of Experimantal Medicine* 32, 13-43.

Ismail, M. M., Jayakody, R. L., Weil, G. J., Nirmalan, N., Jayasinghe, K. S., Abeyewickrema, W., Rezvi Sheriff, M. H., Rajaratnam, H. N., Amarasekera, N., de Silva, D. C., Michalski, M. L. and Dissanaike, A. S. (1998). Efficacy of single dose combinations of albendazole, ivermectin and diethylcarbamazine for the treatment of bancroftian filariasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92, 94-97.

Maher, D. and Ottesen, E. A. (2000). The Global Lymphatic Filariasis Initiative. *Tropical Doctor* 30, 178-179.

Michael, E. and Bundy, D. A. (1997). Global mapping of lymphatic filariasis. *Parasitology Today* 13, 472-476.

Michael, E. and Bundy, D. A. (1998). Herd immunity to filarial infection is a function of vector biting rate. *Proceedings of the Royal Society: Biological Science* 265, 855-860.

Michael, E., Grenfell, B. T., Isham, V. S., Denham, D. A. and Bundy, D. A. (1998). Modelling variability in lymphatic filariasis: macrofilarial dynamics in the *Brugia pahangi*--cat model. *Proceedings of the Royal Society: Biological Science* 265, 155-165.

Michael, E., Malecela-Lazaro, M. N., Simonsen, P. E., Pedersen, E. M., Barker, G., Kumar, A. and Kazura, J. W. (2004). Mathematical modelling and the control of lymphatic filariasis. *Lancet Infectious Diseases* 4, 223-234.

Michael, E., Simonsen, P. E., Malecela, M., Jaoko, W. G., Pedersen, E. M., Mukoko, D., Rwegoshora, R. T. and Meyrowitsch, D. W. (2001). Transmission intensity and the immunoepidemiology of bancroftian filariasis in East Africa. *Parasite Immunology* 23, 373-388.

Norman, R. A., Chan, M. S., Srividya, A., Pani, S. P., Ramaiah, K. D., Vanamail, P., Michael, E., Das, P. K. and Bundy, D. A. (2000). EPIFIL: the development of an age-structured model for describing the transmission dynamics and control of lymphatic filariasis. *Epidemiology and Infection* 124, 529-541.

Ottesen, E. A. (2000). The global programme to eliminate lymphatic filariasis. *Tropical Medicine and International Health* 5, 591-594.

Ottesen, E. A. (2002). Major progress toward eliminating lymphatic filariasis. *New England Journal of Medicine* 347, 1885-1886.

Plaisier, A. P., Subramanian, S., Das, P. K., Souza, W., Lapa, T., Furtado, A. F., Van der Ploeg, C. P., Habbema, J. D. and van Oortmarssen, G. J. (1998). The LYMFASIM simulation program for modeling lymphatic filariasis and its control. *Methods of Information in Medicine* 37, 97-108.

Prichard, R. K. (1990). Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. *International Journal for Parasitology* 20, 515-523.

Prichard, R. K., Hall, C. A., Kelly, J. D., Martin, I. C. and Donald, A. D. (1980). The problem of anthelmintic resistance in nematodes. *Austrian Veterinary Journal* 56, 239-251.

Ramaiah, K. D., Guyatt, H., Ramu, K., Vanamail, P., Pani, S. P. and Das, P. K. (1999). Treatment costs and loss of work time to individuals with chronic lymphatic filariasis in rural communities in south India. *Tropical Medicine and International Health* 4, 19-25.

Schwab, A. E., Boakye, D., Kyelem, D. and Prichard, R. K. (2005). Detection of benzimidazole-resistance associated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection with albendazole and ivermectin treatment. *American Journal of Tropical Medicine and Hygiene* 73, 234-238.

Smith, G. (1990). A mathematical model for the evolutions of anthelmintic resistance in a direct life cycle nematode parasite. *International Journal for Parasitology* 20, 913-921.

Smith, G., Grenfell, B. T., Isham, V. and Cornell, S. (1999). Anthelmintic resistance revisited: under-dosing, chemoprophylactic strategies, and mating probabilities. *International Journal for Parasitology* 29, 77-91; discussion 93-74.

Stolk, W. A., Swaminathan, S., van Oortmarssen, G. J., Das, P. K. and Habbema, J. D. (2003). Prospects for elimination of bancroftian filariasis by mass drug treatment in Pondicherry, India: a simulation study. *Journal of Infectious Diseases* 188, 1371-1381.

Stolk, W. A., Ramaiah, K. D., Van Oortmarssen, G. J., Das, P. K., Habbema, J. D. and De Vlas, S. J. (2004). Meta-analysis of age-prevalence patterns in lymphatic

filariasis: no decline in microfilaraemia prevalence in older age groups as predicted by models with acquired immunity. *Parasitology* 129, 605-612.

Subramanian, S., Stolk, W. A., Ramaiah, K. D., Plaisier, A. P., Krishnamoorthy, K., Van Oortmarssen, G. J., Dominic Amalraj, D., Habbema, J. D. and Das, P. K. (2004). The dynamics of *Wuchereria bancrofti* infection: a model-based analysis of longitudinal data from Pondicherry, India. *Parasitology* 128, 467-482.

Subramanian, S., Vanamail, P., Ramaiah, K. D., Pani, S. P., Das, P. K. and Rajagopalan, P. K. (1989). A simple deterministic model for host-parasite relationship in *Wuchereria bancrofti* infection & its relevance to parasite regulation in human host. *Indian Journal of Medical Research* 89, 411-417.

Tisch, D.J., Michael, E., and Kazura, J.W. (2005). Mass chemotherapy options to control lymphatic filariasis: a systematic review. Lancet Infectious Diseases 5, 514-523.

van Wyk, J. A. (2001). Refugia--overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. *Onderstepoort Journal of Veterinary Research* 68, 55-67.

Vanamail, P, Subramanian, S., Das, P. K., Pani, S. P., Rajagopalan, P. K., Bundy, D. A. P. and Grenfell, B. T. (1989). Estimation of age-specific rates of acquisition and loss of Wuchereria bancrofti infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 83, 689-693.

Vanamail, P., Ramaiah, K. D., Pani, S. P., Das, P. K., Grenfell, B. T. and Bundy, D. A. (1996). Estimation of the fecund life span of *Wuchereria bancrofti* in an endemic area. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90, 119-121.

Wolstenholme, A. J.,Fairweather, I., Prichard, R. K., von Samson-Himmelstjerna, G. and Sangster, N. C. (2004). Drug resistance in veterinary helminths. *Trends in Parasitology* 20, 469-476.

Zagaria, N. and Savioli, L. (2002). Elimination of lymphatic filariasis: a public-health challenge. *Annals of Tropical Medicine and Parasitology* 96 Suppl 2, S3-13.

Appendix 3A. Brief Description of the EPIFIL Model

Our model was based on the epidemiological model EPIFIL (Norman *et al.* 2000), which describes, with respect to time, t, and host age, a, the rate of change of the mean number of adult worms per host, W; the mean microfilarial count per 20μl of blood, M, and the average magnitude of the protective immune response, I, acquired due to infection experience (and assumed to be elicited by the adult worm stage, but targeted against establishment of incoming larvae) as follows,

$$\frac{\partial W(a,t)}{\partial t}+\frac{\partial W(a,t)}{\partial a}=\lambda\frac{V}{H}\varphi_1\varphi_2 s_2 h(a)L^* e^{-\beta I}-\mu W(a,t) \qquad \text{(A3.1)}$$

$$\frac{\partial M(a,t)}{\partial t}+\frac{\partial M(a,t)}{\partial a}=\alpha W(a,t)-\gamma M(a,t) \qquad \text{(A3.2)}$$

$$\frac{\partial I(a,t)}{\partial t}+\frac{\partial I(a,t)}{\partial a}=W(a,t)-\Delta I(a,t) \qquad \text{(A3.3)}$$

$$L^*=\frac{\lambda\kappa g\int\pi(a)f[M(a,t)]da}{\sigma+\lambda\varphi_1} \qquad \text{(A3.4)}$$

L^* is the average number of L3 larvae per mosquito, with the asterisk denoting equilibrium as the temporal changes in the infective larval population take place at a much faster rate than those in the adult worm and microfilarial stages, and therefore it has been assumed that $\frac{\partial L(a,t)}{\partial t}+\frac{\partial L(a,t)}{\partial a}=0$. The function $f[M(a,t)]$ combines the density-dependent relationship between microfilaraemia in the host and development of L3 larvae in the vector with the assumed negative binomial distribution of microfilariae among hosts,

$$f[M(a,t)]=1-\left\{1+\frac{M(a,t)}{k}\left[1-\exp(-\rho/\kappa)\right]\right\}^{-k} \qquad \text{(A3.5)}$$

with ρ being the initial, linear rate of increase in L3 larvae per microfilariae in 20μl of blood, ρ/κ the severity of density-dependent constraints upon larval uptake and/or development; κ the level at which the function saturates (the maximum number of L3

produced per mosquito as microfilaraemia increases), and k the overdispersion parameter of the negative binomial; g is the proportion of bites which result in infection of mosquitoes, and $\pi(a)$ is the proportion of the host population surviving to age a, based on a population in Pondicherry, India, with equation,

$$\pi(a) = \frac{\exp(-a/m)}{m}\left[1 - \exp(-n/m)\right] \qquad \text{(A3.6)}$$

with $m = 29$ and $n = 65$.

Since λ is the biting rate per mosquito on humans, and V/H is the vector to human ratio, $\lambda(V/H)$ is the mosquito biting rate as measured in the field by human landing catches; φ_1 is the proportion of L3 larvae that leave the mosquito's proboscis at the time of biting and are deposited onto human's skin, φ_2 is the proportion of the latter which enter the host through the wound caused by the mosquto bite, s_2 is the proportion of larvae that, having entered the host, will develop into adult worms; $h(a)$ is the proportion of hosts of age a that are bitten, thereby making the biting rate on humans age-dependent (it is assumed to increase linearly until the age of 9 years and then to become unity); β measures the severity by which larval establishment is decreased by acquired immunity, I (which is assumed to increase with accumulated worm burden or worm experience); μ, γ and σ are, respectively, the per capita death rates of adult worms, microfilariae and L3 larvae; Δ is the rate of decay of the immune response, and α is the per capita reproductive rate of adult worms (the rate at which fertilized females produce microfilariae per 20 µl of blood).

Prevalence of microfilaraemia is estimated from microfilarial density using the negative binomial distribution and is given by

$$pM(a,t) = 1 - \left(1 + \frac{M(a,t)}{k}\right)^{-k} \qquad \text{(A3.7)}$$

Appendix 3B. Incorporation of Parasite Population Genetics into EPIFIL

We now extend the EPIFIL model by incorporating genetic structure into the worm population in order to examine the spread of benzimidazole resistance. We track the number of worms of each of three genotypes (homozygote susceptible (*SS*), heterozygote susceptible (*Sr*) and homozygote resistant (*rr*) (Durrheim *et al.*), 2004) in both the treated and untreated sections of the human population. The mean number of adult worms per person of each genotype is denoted, after dropping the age and time dependency, by W_b^q, with subscript b indicating the treatment category ($b=T$ or $b=U$ for worms within, respectively, hosts treated or untreated with benzimidazoles), and superscript q specifying the worm genotype ($q=$ *SS*, *Sr*, or *rr*). For example, W_T^{SS} denotes the mean number of adult homozygote susceptible worms (of genotype *SS*) in the treated population. Similar notation is used to represent the mean number of microfilariae per 20 µl blood, M_b^q, and the mean number of L3 larvae per mosquito, L_b^q. The level of acquired immunity in the treated and untreated human population is specified by, respectively, I_T and I_U. The modified EPIFIL equations are as follows,

$$\frac{\partial W_b^q}{\partial t}+\frac{\partial W_b^q}{\partial a}=\lambda\frac{V}{H}\varphi_1\varphi_2 s_2 h(a)L^{*q}e^{-\beta I}-\mu W_b^q \tag{B3.1}$$

$$\frac{\partial M_b^q}{\partial t}+\frac{\partial M_b^q}{\partial a}=\alpha W_b\,\omega_b^q-\gamma M_b^q \tag{B3.2}$$

$$\frac{\partial I_b}{\partial t}+\frac{\partial I_b}{\partial a}=W_b-\Delta I_b \tag{B3.3}$$

$$L^{*q}=\left[C\frac{L_T^{*q}}{L_T^*}+(1-C)\frac{L_U^{*q}}{L_U^*}\right]\left[CL_T^*+(1-C)L_U^*\right]^- \tag{B3.4}$$

$$L_b^{*q}=\frac{\lambda\kappa g\int\pi(a)\dfrac{M_b^q}{M_b}f(M_b)da}{\sigma+\lambda\varphi_1} \tag{B3.5}$$

where C is the proportion of the population treated (coverage), $M_b = M_b^{SS} + M_b^{Sr} + M_b^{rr}$ and $L_b = L_b^{SS} + L_b^{Sr} + L_b^{rr}$. The expression for L^{*q} determines the average number of L3 larvae per mosquito for each genotype that infect the treated and untreated sections of the population. As with EPIFIL, the L3 population is assumed to be at equilibrium and is calculated using equation (B3.5).

In order to obtain microfilariae of a particular genotype, we use Mendelian genetics to calculate the probability ω_b^q that new microfilariae will belong to a particular genotype given the genotype frequencies of their parent adult worms,

$$\omega_b^{SS} = \frac{1}{4}\left(2\frac{W_b^{SS}}{W_b} + \frac{W_b^{Sr}}{W_b}\right)^2 (1-F) + \left(\frac{W_b^{SS}}{W_b} + \frac{1}{2}\frac{W_b^{Sr}}{W_b}\right)F \qquad \text{(B3.6)}$$

$$\omega_b^{rr} = \frac{1}{4}\left(2\frac{W_b^{rr}}{W_b} + \frac{W_b^{Sr}}{W_b}\right)^2 (1-F) + \left(\frac{W_b^{rr}}{W_b} + \frac{1}{2}\frac{W_b^{Sr}}{W_b}\right)F \qquad \text{(B3.7)}$$

$$\omega_b^{Sr} = \frac{1}{2}\left(2\frac{W_b^{rr}}{W_b} + \frac{W_b^{Sr}}{W_b}\right)\left(2\frac{W_b^{SS}}{W_b} + \frac{W_b^{Sr}}{W_b}\right)(1-F) \qquad \text{(B3.8)}$$

Parameter F adjusts for excess homozygosity. In the case of random mating, F is set to 0. In the case of non-random mating, F is set to 0.44 (see main text).

Functions that model density dependence operating within the mosquito are calculated separately for treated and untreated populations,

$$f(M_b) = 1 - \left\{1 + \frac{M_b}{k_b}\left[1 - \exp(-\rho/\kappa)\right]\right\}^{-k_b} \qquad \text{(B3.9)}$$

CONNECTING STATEMENT 2

In the previous chapter, the potential spread of ABZ resistance through populations of *W. bancrofti* due to combination treatments, currently applied by the GPELF, were explored. We chose to initially examine ABZ resistance, as the mechanisms involved in its development are well known. We found that factors such as drug efficacy, treatment coverage, mating structure and initial allele frequency of the resistance mutation have a considerable impact on the development of resistance. It is well known that the use of IVM in veterinary medicine has led to many cases of drug resistance, and we suspect that such IVM resistance could also develop in lymphatic filaria, in addition to ABZ resistance. The interation between selection of resistance against these two separate drugs will likely affect how resistance develops, and how many individual parasites ultimately survive treatment.. In Chapter 4, we expand the mathematical model presented in Chapter 3, by incorporating additional genes responsible for potential IVM resistance. As the inheritance of such resistance has not been fully elucidated, we consider several possibilities of dominance and polygenicity of IVM resistance. We use this mathematical model to explore mechanisms and factors involved in the possible spread of multidrug resistance through populations of *W. bancrofti*, due to combination chemotherapy.

CHAPTER 4:

ANALYSIS OF THE POPULATION GENETICS OF POTENTIAL MULTI-DRUG RESISTANCE IN LYMPHATIC FILARIASIS DUE TO COMBINATION CHEMOTHERAPY

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Abstract

Currently, yearly mass treatments with albendazole (ABZ) plus ivermectin (IVM) or diethylcarbamazine (DEC) are administered under the Global Programme to Eliminate Lymphatic Filariasis (GPELF). Drug resistance against both ABZ and IVM is prevalent in veterinary nematodes, raising awareness that if anthelmintic resistance were to develop among *Wuchereria bancrofti* populations, this would jeopardise GPELF's goals. Genetic structure was incorporated into an existing LF transmission dynamics model, EPIFIL, to investigate the potential development of concurrent resistance to ABZ and IVM. The resulting models explore the impact of different inheritance modes of resistance to ABZ and IVM on the likely risk of treatment failure under our model assumptions. Results indicate that under ABZ + IVM combination, selection for resistance to one drug is enhanced if resistance to the other drug is already present. Excess parasite homozygosity may increase selection for dominant IVM resistance via enhancing the frequency of recessive ABZ resistance. The model suggests that if multiple resistance genes are associated with different efficacy properties of a drug combination, then examining changes at single loci may be misleading. Sampling schemes of genetic epidemiological surveys that investigate the frequency of an allele under selection should consider host age, as individuals of different ages may acquire parasites at different rates.

4.1 Introduction

Lymphatic filariasis (LF) is a parasitic disease caused by the infection with one or several of a group of filarial nematodes dwelling in the lymph system and being transmitted by mosquito vectors. Recently, revised figures estimate that LF is endemic in 83 countries and that 119 million people are infected worldwide. LF is a major cause of morbidity, with the loss of 4.6 million DALYs (disability-adjusted life years) (Remme *et al.* 2006), severely affecting socioeconomic development in endemic areas (Ramaiah *et al.* 1999; Zagaria and Savioli, 2002). The Global Programme to Eliminate Lymphatic Filariasis (GPELF), launched in 2000, involves community-wide annual treatment with the broad spectrum anthelmintic albendazole (ABZ) in combination with ivermectin (IVM) or diethylcarbamazine (DEC). The combination chosen depends on whether or not human onchocerciasis is co-endemic (Maher and Ottesen, 2000; Ottesen, 2000, 2002; Dean, 2002; Zagaria and Savioli, 2002).

Resistance to both benzimidazoles (including ABZ) and macrocyclic lactones (MLs, to which IVM belongs), has been documented in many parasitic nematodes affecting livestock (Prichard *et al.* 1980; Prichard, 1990, Wolstenholme *et al.* 2004), suggesting that the possibility of resistance developing in lymphatic filariae of humans should not be dismissed. There have been some reports of tolerance to DEC by filarial parasites (Eberhard *et al.* 1988; Eberhard *et al.* 1991), and we have shown that a polymorphism, known to be associated with benzimidazole resistance in veterinary parasites, is present in West African populations of *Wuchereria bancrofti* (see Schwab *et al.* 2005). ABZ resistance is known to be associated with selection on β-tubulin and is thought to be recessive in most helminths of veterinary importance (Elard and Humbert, 1999; Prichard, 2001; Silvestre and Cabaret, 2002).

Schwab *et al.* (2006) incorporated population genetics into the deterministic mathematical model EPIFIL, which examines the population dynamics of *W. bancrofti* (see Chan *et al.* 1998, 1999; Norman *et al.* 2000), with the aim of investigating the impact of mass drug administration (MDA) on the development of ABZ resistance in the absence of resistance to either DEC or IVM. The model suggested that if ten annual

treatments with ABZ + DEC were applied, this would lead to a five-fold increase in a (homozygous) ABZ resistant genotype, whereas, in the case of the ABZ + IVM combination, the increase in ABZ resistance would be less pronounced.

Recently, it has been documented that in areas of Ghana endemic for the related filarial nematode *Onchocerca volvulus*, in which humans have been treated more than 9 times with IVM, some infected individuals exhibit a sub-optimal response phenotype and experience a faster recovery of microfilaridermia after treatment than normal responders (Awadzi *et al.* 2004*a,b*). Whether this phenotype is associated with genetic changes indicative of selection under IVM pressure has not yet been ascertained. However, there is genetic evidence that selection by IVM treatment may indeed be occurring in *O. volvulus* populations. Significant differences in allele frequencies between worms obtained from untreated and IVM-treated human populations were found for β-tubulin, P-glycoprotein and other ABC transporter genes (Eng and Prichard, 2005; Ardelli and Prichard, 2004; Ardelli *et al.* 2005, 2006*a,b*; Eng *et al.* 2006).

In *Haemonchus contortus*, the barber's pole worm of small ruminants, more than one gene appears to be under selection by repeated treatments with IVM or other MLs (Blackhall *et al.* 1998*a,b*; Prichard, 2005). Although previous reports had concluded that IVM resistance in *H. contortus* was a monogenic trait (Le Jambre *et al.* 2000), more recent evidence, based on the responses of field isolates of resistant *H. contortus* and *Trichostrongylus colubriformis* to MLs, suggests that ML resistance is likely to be polygenic (Le Jambre *et al.* 2005). The results of a mathematical model investigating anthelmintic resistance in sheep parasites examined concurrent selection by more than one drug upon multiple resistance genes, and concluded that under the assumption of linkage equilibrium, resistance is selected more slowly when it is polygenic rather than monogenic (Barnes *et al.* 1995).

Little is known about the relative dominance of alleles conferring ML resistance in parasitic nematodes. Mathematical models have demonstrated how the mode of inheritance of drug resistance has a large impact on its selection, with dominant traits being selected more rapidly (Barnes *et al.* 1995). In *H. contortus*, in particular, it has been speculated that IVM resistance is a completely dominant trait (Le Jambre *et al.* 2000; Barnes *et al.* 2001), whereas Le Jambre *et al.* (2005) have suggested that resistance

to moxidectin and abamectin, also members of the ML family, are semirecessive and semidominant, respectively. Another study has shown that the mode of inheritance of ML resistance to moxidectin in *Teladorsagia circumcincta* may be semi-dominant or recessive (Sutherland *et al.* 2002).

In this paper, we expand our previous work (Schwab *et al.* 2006) to include the possibility of concurrent selection for resistance to ABZ and IVM in *W. bancrofti*. We have chosen to consider resistance to IVM and not to DEC, because, unlike the many cases of IVM resistance in veterinary nematodes, resistance against DEC has not been unequivocally demonstrated to date.

4.2 Model Development

4.2.1 Population Dynamics

The model described in this paper is based on EPIFIL (Chan *et al.* 1999; Norman *et al.* 2000), into which genetic structure was incorporated by Schwab *et al.* (2006), where all equations and parameters for EPIFIL may be found. Since EPIFIL was initially parameterized for the locality of Pondicherry, India, where age-specific microfilarial infection profiles peak in young humans, the model assumes some degree of protective immunity against incoming infective larvae. This conjecture has been maintained in the work presented here. Parameters determining the intensity of transmission and the particular epidemiology in the model are such that the annual biting rate (by *Culex quinquefasciatus*) is ~ 70,000 bites per person per year, the annual transmission potential is 940 third-stage larvae per person per year, the mean microfilaraemia is 0.85 per 20 μl of blood, and the prevalence of microfilarial infection is 8% prior to the introduction of chemotherapy, based on the EPIFIL model (Norman *et al.* 2000).

4.2.2 Population Genetics.

Analysis of patients from villages in Burkina Faso, prior to the introduction of anti-LF chemotherapy, indicated that the β-tubulin genotype distribution of *W. bancrofti* microfilariae deviates from the Hardy-Weinberg equilibrium (Schwab *et al.* 2005). The non-random mating of *W. bancrofti* causes a deficiency in the proportion of heterozygote offspring, which can be described using Wright's inbreeding *F*-statistic (Wright, 1951). *W. bancrofti* from the untreated population in Burkina Faso was shown to have an overall inbreeding coefficient of $F_{IT} = 0.44$ (Churcher, 2006). A detailed description of this calculation and a discussion of the possible mechanisms generating homozygosity in *W. bancrofti* will be presented elsewhere. Throughout this paper, and unless otherwise stated, the genotype distribution for microfilariae shall be altered to account for this degree of non-random mating (see Tables A4.1. and A4.2 of Appendix 4A), as was done in the previous study (Schwab *et al.* 2006).

We have assumed that there are no costs or trade-offs associated with the

resistance alleles (*i.e.*, resistant and susceptible *W. bancrofti* have identical fitness prior to the introduction of chemotherapy). We have also assumed that resistance to ABZ is associated with a single autosomal locus with two alleles, with the resistance allele being recessive. IVM resistance was assumed to be associated with either one autosomal locus with two alleles (i.e., to be monogenic), or with two autosomal loci with two alleles each (*i.e.*, to be polygenic). In the default setting, IVM resistance was assumed to be dominant, but this assumption was later relaxed in order to explore the influence of different modes of inheritance, ranging from a fully dominant to a fully recessive trait. Unless otherwise stated, the initial frequencies of the ABZ and IVM resistance alleles were (respectively) set to 0.2 (based on Schwab *et al.* 2005) and 0.05 (loosely based on polymorph/allele frequencies observed in *O. volvulus*; Eng and Prichard, 2005; Eng *et al.* 2006). In order to test the sensitivity of model outcomes to lower initial values, we also conducted simulations using 0.02 for both putative ABZ and IVM resistance allele frequencies.

4.2.2.1 Monogenic IVM Resistance

When ABZ and IVM resistances were both considered and each was assumed to be monogenic, each of the EPIFIL equations for adult worms, microfilariae and infective larvae populations, was written to represent parasites which were either: i) drug sensitive for both drugs; ii) ABZ-sensitive and IVM-resistant; iii) ABZ-resistant and IVM-sensitive, or iv) resistant to both drugs. It is thought that ABZ and IVM resistance may both involve the β-tubulin gene (Prichard, 2001; Eng and Prichard, 2005; Eng *et al.* 2006), such that there is a chance that the loci conferring ABZ and IVM resistance may be completely or partially linked. Various degrees of dominance of IVM resistance and linkage, with recessive ABZ resistance, were considered by altering assumptions regarding drug efficacy and the degree of recombination between ABZ and IVM resistance loci (full equations are given in Appendix 4A).

4.2.2.2 Polygenic IVM Resistance

For the situation in which IVM resistance would be conferred by two loci, we assumed that one of these loci is completely linked to ABZ resistance (*i.e.*, no

recombination between loci), as would be the case if both ABZ and IVM resistance were to involve β-tubulin. The other IVM resistance locus was assumed to be unlinked (*e.g.*, involving a P-glycoprotein or another gene), resulting in worm populations with 30 possible genotype combinations. We considered three different scenarios: (1) one IVM resistance locus was responsible for IVM resistance in adult nematodes and a second locus for IVM resistance in microfilariae, as may be the case if adults and microfilariae have different resistance mechanisms, a scenario which may be consistent with the differences in the responses of adult worms and microfilariae to IVM; (2) neither locus on its own led to IVM resistance, but selection on both loci together was required for resistance, which will occur if genetic change at both loci is required for the resistance mechanism to be functional; and (3) one locus conferred resistance in all microfilariae and 70% of adults, and a second locus conferred resistance in the remaining 30% of adults, suggesting that one mechanism of resistance protects all microfilariae and most adults, and that a second mechanism has developed in the adult worms that survive treatment. This scenario may again, in part, reflect differences in the responses of macrofilariae and microfilariae to IVM. Linkage equilibrium was assumed between the two IVM loci.

4.2.2 Treatment Related Parameters

Once the model is run to equilibrium, and unless otherwise stated, MDA with the ABZ + IVM combination starts in year 1 and proceeds annually for 10 years uninterruptedly. (Although the main strategy of GPELF is based on a programme duration of 5 years in each endemic area, doubts have been raised as to whether this would be enough to attain intended goals in all regions (Michael *et al.* 2004).) In order to reflect therapeutic coverage (set at a default level of 85% of the total population), treated and untreated parasite populations were modelled separately. At the point of transmission, the contributions of each population of infective larvae, from treated and untreated people, respectively (depending on coverage level), were introduced into the equations for adult worms. Due to the constrains imposed by a deterministic model which considers population averages, and to reflect that often the same individuals fail to

comply with treatment in successive years, the model assumes that the same section of the host population is treated at each round. After cessation of chemotherapy, no further treatments take place, but the model is run for ten more years. Model outputs regarding microfilarial genotype frequencies (expressed as percentages) and host microfilaraemia (expressed as microfilariae per 20 μl of blood) are shown up to years 15 and 20, respectively.

Assumptions on drug efficacy were based on those made by Michael *et al.* (2004) and other published drug trials (Addiss *et al.* 1997; Ismail *et al.* 1998; Dunyo, Nkrumah and Simonsen, 2000) and have been summarized in Table 4.1. Treatment was modelled as instantaneous reductions in adult worms (macrofilaricidal effect), microfilarial populations (microfilaricidal effect), or female worm reproduction rate (sterilizing effect) by the percentage efficacy given in Table 4.1. Following chemotherapy, sterilized females did not resume microfilarial production for a period of time specified for each drug. Model outcomes in this paper examine the effect, during and up to 5 years after the cessation of MDA, of ten annual treatments on the genotype distribution of the microfilariae (the stage more feasibly sampled for genetic analyses), and up to 10 years after the cessation of MDA on the mean microfilaraemia.

Table 4.1. Drug efficacy assumptions (adapted from Michael *et al.* 2004)

Effect	ABZ-susceptible IVM-susceptible	ABZ-resistant IVM-susceptible	ABZ-susceptible IVM-resistant	ABZ-resistant IVM-resistant
Months without reproduction	9	9	9	0
% Adults killed	35	10	25	0
% Microfilariae killed	99	99	10	0

All equations for the models explored are given in Appendix 4A and Tables A4.1 and A4.2. Model code was written using the JSim numerical integration software,

available from

http://nsr.bioeng.washington.edu/PLN/Members/butterw/JSIMDOC1.6/JSim_Home.stx/view.

Differential equations were solved using the Euler method (Edwards and Penney, 1989). Prior to the initiation of control perturbations, *W. bancrofti* populations were assumed to be at endemic equilibrium, as it has been ascertained that prevalences of infection in areas of Burkina Faso have remained relatively stable for at least 30 years (Gyapong *et al.* 2002).

4.3. Results

4.3.1 Influence of parasite inbreeding

Model outputs indicate that parasite non-random mating (which results in increased offspring homozygosity) increases the projected rate at which ABZ and IVM resistant genotypes would spread throughout the genome of *W. bancrofti*. Increased microfilarial homozygosity (as reflected by the value of F_{IT}) increases the frequency of the dominant IVM resistant genotypes during MDA. For $F_{IT} = 0.44$ (the value of homozygosity observed in Burkina Faso), the frequency of IVM resistant genotypes in year 10 (63%) is 8 times higher than that prior to the initiation of control (8%), whereas for $F_{IT} = 0$ (no excess homozygosity), the frequency in year 10 is 6 times as high (Fig. 4.1A). If initial allele frequencies are assumed to be lower (0.02 for both ABZ and IVM), the frequency of IVM resistant microfilariae would increase 17 fold (from 3% to 51%) when $F_{IT} = 0.44$, but only about 6 fold (from 4% to 24%) if random mating is assumed. It is important to note that this increase only occurs in the presence of ABZ resistance. At the cessation of treatment, the percentage of microfilarial genotypes resistant to both ABZ and IVM would have increased to 52% in the case of $F_{IT} = 0.44$ as opposed to 8% when $F_{IT} = 0$ and parasite mating is random (Fig. 4.1B). The higher frequency of resistant genotypes caused by parasite non-random mating is reflected in higher levels of microfilaraemia, particularly after 5 years of MDA (Fig. 4.1C).

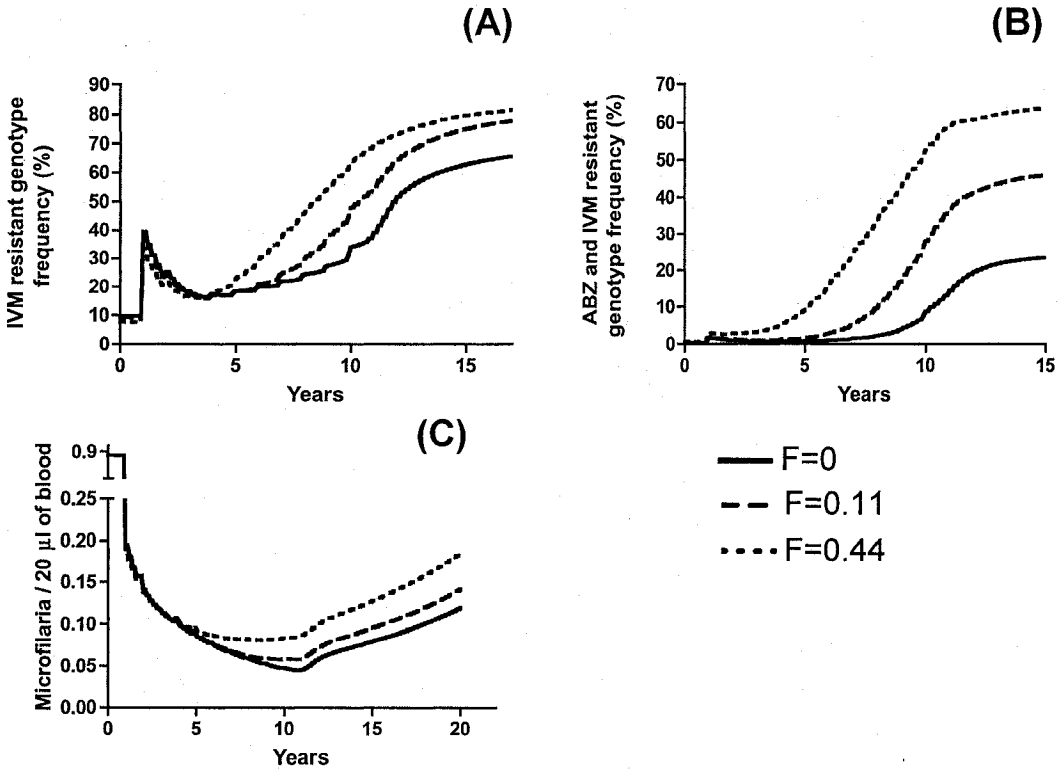

Figure 4.1: The effect of increasing levels of parasite inbreeding (i.e., increased homozygosity) as measured by Wright's *F*-statistic, F_{IT}, on the development of anthelmintic resistant *Wuchereria bancrofti*, during 10 annual treatments at 85% therapeutic coverage with the ABZ + IVM combination, and after 5 (genotype frequency) or 10 years (microfilaraemia) following cessation of mass drug administration (MDA). The initial ABZ and IVM resistance allele frequencies were set, respectively, at 0.2 and 0.05. IVM resistance is assumed to be a fully dominant trait. (A) The average frequency (expressed as percentage) of IVM resistant microfilarial genotypes; (B) the average frequency (%) of microfilarial genotypes resistant to both ABZ and IVM, and (C) the mean microfilaraemia per 20 µl of blood in the human host population, with chemotherapy starting in year 1.

4.3.2 Concurrent development of ABZ and IVM resistance

Model outputs indicate that when the initial frequencies of the ABZ and IVM resistance alleles is 0.2 and 0.05, respectively, MDA with ABZ + IVM would increase the frequency of microfilarial genotypes resistant to both drugs from under 1% prior to control to 52% at its cessation (Fig. 4.2A), or to 40% when initial resistance allele frequency to both drugs is 0.02. The presence of IVM resistance increases the rate of ABZ resistance development. In the absence of IVM resistance, the frequency of ABZ resistant homozygotes only increases from 12% at year 0 to 14% at year 10, and to 40% 5 years after halting MDA (Schwab *et al.* 2006). If the IVM resistance gene is present at an initial allele frequency of 0.05, the frequency of ABZ resistant homozygotes would reach 57% and 70%, following the same treatment schedule at years 10 and 15, respectively (Fig. 4.2A). If the initial resistance allele frequency, in relation to both drugs, is 0.02, the frequency of the ABZ resistant homozygotes would reach 39% and 54% at years 10 and 15, respectively. The rate of IVM resistance spread is similarly affected by the concurrent selection for the ABZ resistant genotype (see Appendix II). The presence of IVM resistance would also increase the average microfilaraemia relative to that with no resistance, and this is particularly marked after 5 rounds of MDA (Fig. 4.2B). The frequency of resistant genotypes, following the cessation of treatment, increased with the number of years of annual MDA treatment. Fifteen years after the commencement of treatment, the frequencies of microfilariae resistant to both ABZ and IVM were estimated to be 20%, 40% and 65%, after 5, 7 and 10 years of MDA (combination treatment), respectively. The same trend was observed with the lower initial allele frequencies (see Appendix II).

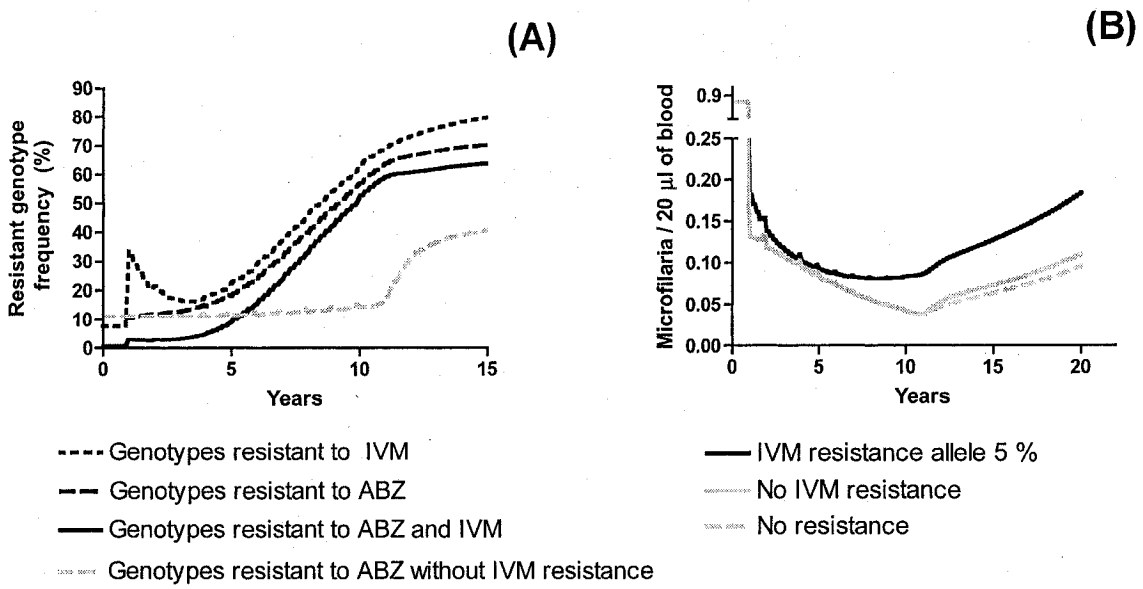

Figure 4.2: Estimated development of anthelmintic resistance in *W. bancrofti* during 10 annual treatments with ABZ + IVM at 85% coverage and after 5 (genotype frequency) or 10 years (microfilaraemia) following cessation of MDA. (A) The average frequency (%) of microfilarial genotypes resistant to ABZ, IVM, or to both ABZ and IVM, where resistance to both ABZ and IVM can develop, or if only ABZ resistance occurs, and (B) the mean microfilaraemia per 20 µl of blood in the human host population, with chemotherapy starting in year 1, if there is no drug resistance, resistance to ABZ, but not to IVM or when resistance can develop to both ABZ and IVM

4.3.3 Age-specific profiles of ABZ and IVM resistant genotypes

The proportion of resistant microfilariae that a host harbours will depend on the age of the host and the time since the start of chemotherapy. Fig. 4.3 indicates that, in a treatment programme of 10 years duration and following the onset of MDA, the proportion of resistant genotypes will be highest in the youngest hosts, and this is particularly marked 5 and 10 years into the programme. The peak resistant genotype frequency shifts towards older ages (teenagers and young adults) 5 and 10 years after the cessation of treatment, whilst the frequency in children starts returning to that in the overall population. Hosts which were young at the start of chemotherapy continue to have a higher than average number of resistant parasites for a number of years after treatment ceases, and this result reflects the assumption of acquired protective immunity as a function of established worm burden embedded in EPIFIL. The same effect was

observed with the lower initial allele frequencies.

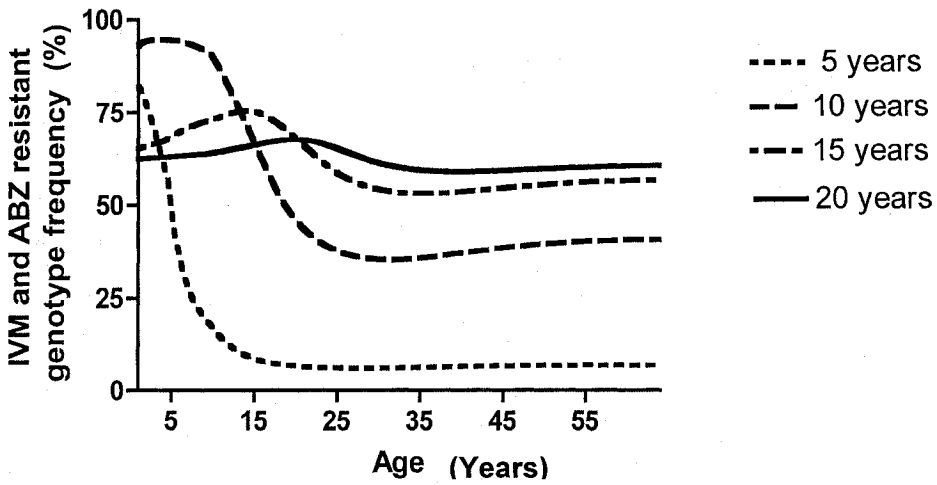

Figure 4.3. Host age-specific profiles of the proportion of microfilariae which are resistant to ABZ and IVM, 5, 10, 15 and 20 years after commencement of 10 annual rounds of MDA with ABZ + IVM with 85% therapeutic coverage. Initial ABZ and IVM resistance allele frequencies, were 0.2 and 0.05, respectively, with $F_{IT} = 0.44$.

4.3.4 Variable ivermectin resistance dominance

The default parameters of the model assume that the IVM resistance allele is fully dominant. However, although it is generally believed that the ABZ resistance allele will be recessive (benzimidazole resistance has been found to be recessive in nematodes of non-human animals), it is still unclear how IVM resistance would be inherited. We examined the effect of varying the degree of dominance of the IVM resistance allele. Results (not shown) indicate that varying the dominance of IVM resistance has a small impact on ABZ resistance. The frequency of the ABZ resistant genotype is marginally higher if the IVM resistance allele is recessive (74.5 % 5 years after the cessation of treatment) than if it is dominant (71.2 %). As may be expected, the frequency of the IVM resistant genotype increases with the dominance of IVM resistance. Projected host microfilaraemia, after 10 years of chemotherapy, would be slightly higher if the IVM resistance gene is recessive. However, this increase is relatively minor (0.198 microfilariae per 20 μl of blood after treatment if IVM resistance is recessive vs. 0.184 microfilariae per 20 μl of blood if it is dominant). Dominance of IVM resistance has the same impact at the lower initial allele frequencies of 0.02 for both drugs.

4.3.5 Dependence of Selection on Linkage

In the monogenic IVM resistance model, the rate of increase in the frequency of resistant genotypes was relatively insensitive to whether the loci conferring ABZ and IVM resistance were unlinked, partially linked, or fully linked, as long as prior to the introduction of chemotherapy, the resistance loci were in random association within the parasite genome (i.e., genotypes were at linkage equilibrium, outputs not shown). However, if both loci were 100% linked (*i.e.*, no recombination occurs between the two loci at meiosis), the result of selection would be affected by the number of chromosomes in the *W. bancrofti* population bearing only IVM resistance or both ABZ and IVM resistance before the start of treatment (initial linkage disequilibrium). If the (linked) alleles conferring ABZ and IVM resistance were assumed to be always on the opposite strands of a chromosome (high initial linkage disequilibrium with discordant alleles), the ABZ and IVM resistant genotypes would be less frequent in the population after treatment than would be the case if they were initially at linkage equilibrium. The highest frequency of resistant genotypes would result from initially high linkage disequilibrium when both ABZ and IVM resistance alleles are located on the same chromosome (concordant alleles). These results are illustrated in Fig. 4.4. The effect of linkage was the same when the lower initial frequencies were assumed (see Appendix II, Fig.7.6).

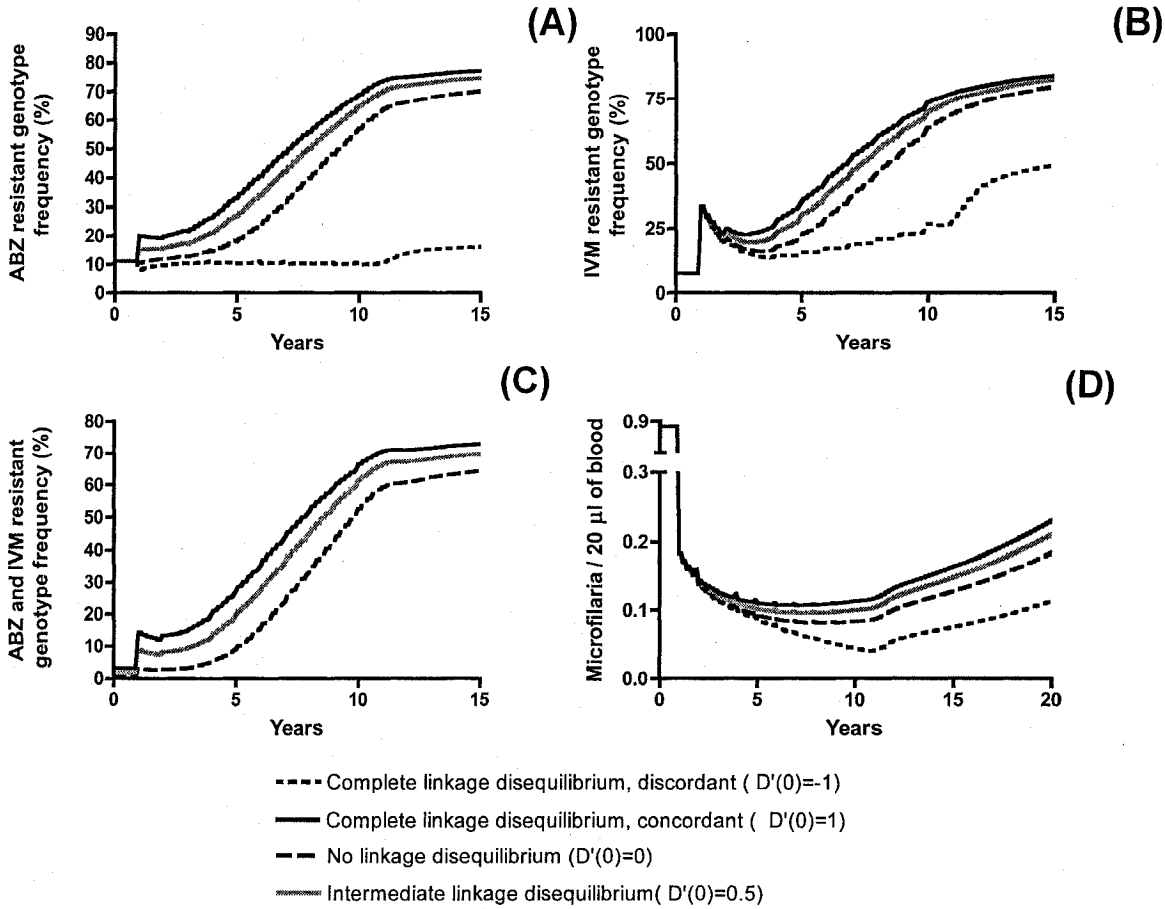

Figure 4.4: . The estimated effect, on the development of ABZ and IVM resistance, of 100% linkage between the alleles conferring these traits during 10 annual treatments with ABZ + IVM at 85% coverage. The initial ABZ and IVM resistance allele frequencies, and the value of F_{IT} are as in Fig. 3. (A) The average frequency (%) of ABZ resistant microfilarial genotypes; (B) the average frequency (%) of IVM resistant microfilarial genotypes; (C) the average frequency (%) of ABZ and IVM resistant microfilarial genotypes; and (D) the mean microfilaraemia per 20 µl of blood in the human host, with chemotherapy starting in year 1. Linkage disequilibrium was calculated after Hartl and Clark (1997); standardized linkage disequilibrium (*D'*) was calculated after Lewontin (1988).

4.3.6 Polygenic Ivermectin Resistance

The rate of increase of IVM resistance will be higher if it is inherited as a single gene rather than as a two-gene system, although the difference will depend on the initial allele frequencies and the selective advantage that the different IVM resistance genes confer individually. If two genes are both required for complete IVM resistance and individually they do not confer any resistance, the development of resistance will be delayed if the resistance alleles are at a low initial frequency. However, the development

of monogenic and polygenic IVM resistance is relatively similar at higher initial allele frequencies, as a high proportion of worms will already have both IVM resistance genes (Fig. 4.5). Results also indicate that the development of polygenic IVM resistance will be influenced by the life-cycle stage of the parasite at which resistance is manifested. If one gene conferred IVM resistance to microfilariae (impeding microfilaricidal effects) and the other gene conferred resistance to all adult worms (preventing macrofilaricidal or anti-fecundity effects in 100% of the worms), treatment failure, under our model assumptions, would develop more rapidly than would be the case if the first gene was, for instance, responsible for both microfilarial resistance and resistance in 70% of adults, and the second gene conferred resistance to the remaining 30% of the macrofilariae. These conclusions were the same when the lower initial allele frequencies were assumed.

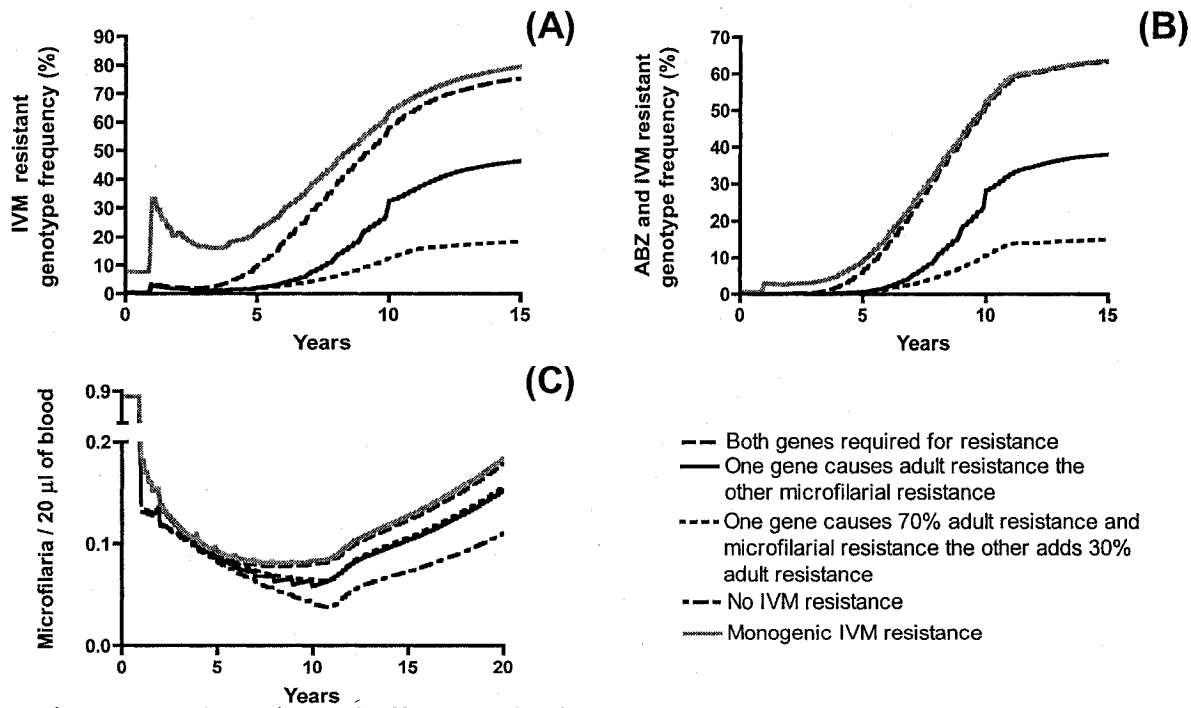

Figure 4.5: The estimated effect of selection on one ABZ resistance gene and two IVM resistance genes on the development of ABZ and IVM resistance during 10 annual treatments with ABZ + IVM at 85% coverage. The initial ABZ and IVM resistance allele frequencies, and the value of F_{IT} are as in Fig. 3. (A) The average frequency (%) of IVM resistant microfilarial genotypes; (B) the average frequency (%) of microfilarial genotypes resistant to both ABZ and IVM, and (C) the mean microfilaraemia per 20 µl of blood in the human host, with chemotherapy starting in year 1. the ABZ resistance genotypes, and (C) the mean microfilaraemia per 20 µl of blood in the human host.

4.4. **Discussion**

Combination therapy with two anthelmintics will influence the rates of development of single and multi-drug resistant parasites according to the efficacies of the drugs (in combination and individually) and the underlying genetics conferring resistance. Due to the many uncertainties regarding the population dynamics of the parasite (*e.g.*, operation of acquired protective immunity) and the inheritance of IVM resistance (*e.g.*, whether it is dominant), among others, the results of this study provide qualitative insight (rather than quantitative predictions) into how assumptions regarding the genetics of IVM resistance may influence the development of drug resistance in *W. bancrofti* under a combination treatment with ABZ + IVM.

The models highlight a number of results which may be of interest to those investigating the possibility of resistance developing, following prolonged co-administration of two anthelmintics. One salient prediction was the peak and subsequent temporary decline in the proportion of microfilariae resistant to IVM following the first round of chemotherapy. Results suggest that molecular epidemiological surveys conducted in the first three years of treatment might conclude that the genes conferring drug resistance had negative pleiotropic effects, therefore reducing the chance that IVM resistance will impede parasite elimination. However, the model indicates that the decline in microfilariae resistant to IVM may mask an increase in the number of adult worms resistant to both ABZ and IVM. The initial peak in IVM resistance is caused by the relative dominance of the alleles conferring ABZ and IVM resistance, and the assumed efficacies of the two drugs against the different life cycle stages of the parasite. Microfilariae resistant to IVM prior to the start of chemotherapy survive the first round of treatment, but subsequently start to decrease in number as ABZ would have sterilized the majority of IVM resistant adult worms. The rate of decline observed in the proportion of microfilariae resistant to IVM is highly sensitive to the assumed life-span of microfilariae, a parameter particularly difficult to quantify, with estimates ranging from 6 months to 2 years (Plaisier *et al.* 1999). It should be noted that if multiple genes are associated with different pharmacodynamic properties of a drug or drug combination, then examining the changes at single loci may produce misleading results.

Previous work had indicated that the change in proportion of ABZ resistant microfilariae may only become apparent after the end of the treatment period, due to the small amount of reproduction that occurs in the treated population while chemotherapy is in progress and the fact that the microfilariae population is, at that time, composed primarily of parasites from untreated patients (Schwab *et al.* 2006). However, the present work suggests that concurrent selection of both ABZ and IVM resistance may cause observable changes in the proportion of ABZ, IVM, and ABZ and IVM resistant microfilariae during the treatment period, as some parasites can overcome the combined action of both drugs and are able to reproduce. According to model outcomes, the presence of IVM resistance would enhance the rate of increase of any ABZ resistance, and *vice versa*, as a parasite resistant to both drugs has a higher probability of completing its life-cycle.

There is considerable debate as to the number of years that combination chemotherapy should be provided under the current GPELF programmes for achieving the goals of elimination. The number of years of annual combination treatment influences the proportion of the microfilarial population with ABZ and IVM resistant genotypes following the cessation of treatment. The higher frequency of resistant genotypes that follows a longer period of treatment indicates that for a given location, if transmission has not been interrupted by the programme and the parasite population has not been eliminated, a return to chemotherapy would be considerably less effective if the initial phase of treatment had lasted for 10 years rather than for 5 years. However, the present (deterministic) framework is not appropriate to explore the probability of parasite elimination with duration of control. In most of the scenarios explored here, levels of microfilaraemia which might cause concern would not be evident until after 5 years into the programme. Also, although the levels of parasite inbreeding used here are those estimated for *W. bancrofti* populations in West Africa, vector parameters derived from *Culex* (a vector for which the per microfilariae probability of completing its development within the mosquito increases with decreasing microfilaraemia) were used. LF in West Africa is transmitted by *Anopheles* species (for which the per-microfilaria probability of development may decrease markedly at low levels of microfilaraemia due to the increased proportion of ingested microfilariae damaged by the cibarial armatures of these

vectors) (Bryan and Southgate, 1988; Bryan *et al.* 1990).

An interesting result is that hosts of different ages may harbour different proportions of resistant microfilariae. Following the start of chemotherapy, the model indicates that children will, on average, acquire a higher proportion of resistant *W. bancrofti* than adult hosts, because they are becoming infected with new parasites at a faster rate. The exact age-profile of resistance will depend on the age-dependent heterogeneity in exposure, the acquisition of protective immune responses, and the time since the start of chemotherapy at which genetic surveys are undertaken. The model outputs in this study depend on the ability of EPIFIL to represent adequately the processes regulating *W. bancrofti* population abundance. EPIFIL assumes that the acquisition of new infections may be reduced in adult hosts due to acquired immunological responses protecting against incoming infection (Norman *et al.* 2000), but this has not yet been confirmed in different geographical locations (Stolk *et al.* 2004). Regardless of the mechanisms underlying infection age-profiles, molecular epidemiological surveys should take into consideration that, after the start of chemotherapy, hosts which acquire new infections at the fastest rate will have the highest resistance allele frequency. This also has implications if vector control is added to annual chemotherapy, because vector control would reduce the acquisition of new parasites and thus assist in reducing the proportion of resistant genotypes in the parasite population.

An analysis of the microfilarial data collected by Schwab *et al.* (2005) indicates that genetic differentiation in *W. bancrofti* between hosts, and assortative parasite mating may cause excess homozygosity to a degree equivalent to Wright's inbreeding *F*-statistic of $F_{IT} = 0.44$ (Churcher, 2006). Helminth inbreeding typically enhances the spread of recessive alleles but is not expected to increase selection for alleles which are dominant, as excess homozygosity will reduce the number of offspring with the resistance allele. Model outputs suggest that, under the assumed modes of inheritance of IVM and ABZ resistances, and in the presence of ABZ resistance, helminth inbreeding would increase markedly the spread of IVM and of ABZ and IVM resistant parasites (Fig. 4.1). This is because the selective advantage conferred by the more rapid increase in ABZ resistance (which is increased by parasite inbreeding) outweighs the loss of microfilariae which are heterozygous at the IVM resistance locus (thus being resistant to IVM). How the degree

of parasite inbreeding will influence the frequency of parasites resistant to both ABZ and IVM will depend on the different efficacies of the two drugs and the relative dominance of the resistance alleles under selection.

Based on the assumptions made here regarding inheritance, linkage between the genes conferring ABZ and IVM resistance is unlikely to influence the incidence of combination treatment failure, unless the genes are completely linked and are at linkage disequilibrium. The model was not extended to investigate how linkage would influence the development of polygenic IVM resistance, so we cannot speculate on more complicated genetic scenarios. If the genes conferring resistance to both drugs were always present in the same gamete, selection for one type of resistance would also lead to selection for resistance against the second drug, reducing the time that it takes for widespread treatment failure. A recent study indicates that this may be the case for the two genes under consideration here, as an increase in the frequency of the Phe200Tyr polymorphism in the β-tubulin gene was observed after selection of *H. contortus* with IVM and in IVM resistant worms (Eng *et al.* 2006).

Model outcomes suggest, under our assumptions and parameterisation, that microfilarial intensity would decrease to as low as 0.04 microfilariae per 20 μl of blood by the end of MDA in our simulations. Using the calculations proposed by Norman *et al.* (2000), this would translate into a microfilarial prevalence of 1%. There has been some debate about the threshold prevalence below which it is deemed that transmission can no longer occur, with the 1% cut-off microfilaraemia stemming from the Chinese experience in eliminating (bancroftian and brugian) LF transmitted by *Culex quinquefasciatus* and *Anopheles sinensis* (see WHO, 2003). Michael *et al.* (2004) assumed the threshold prevalence to be 0.5%. In Western Samoa, transmission was not interrupted even after the microfilarial prevalence fell to 0.2% due to mass DEC administration (Esterre *et al.* 2001). It is thus likely that transmission would continue in the scenarios explored by our model and that resistance could develop. However, we stress that stochastic frameworks (Plaisier *et al.* 1998) are more suitable than deterministic models (Norman *et al.* 2000) to investigate the probability of parasite elimination or recrudescence ensuing the cessation of MDA.

Notwithstanding modelling approaches, the presence of multi-drug

resistance would hinder the success of combination chemotherapy control programmes and may prevent parasite elimination. However, it is important to note that even though our models have relatively high resistance allele frequencies prior to the introduction of chemotherapy, as default values, the presence of resistance will only become phenotypically manifested in the temporal trends of host microfilaraemia a number of years after the start of treatment (in the present model, typically after 5 years). It should also be noted that the frequency of a potentially ABZ resistant genotype was found to be already high in West African populations of *W. bancrofti* microfilariae before the introduction of LF chemotherapy (Schwab *et al.* 2005). It is also important to recall that none of the anthelmintics used for LF control is very effective at eliminating the parasite from the host, because *W. bancrofti* is relatively tolerant to all of the anthelmintics used by the GPELF (Michael *et al.* 2004). If anthelmintic resistance genes were present in the population prior to the onset of MDA, and assuming that they are randomly distributed among the worm population initially, relatively few parasites will be resistant to both drugs at the first round of treatment. Successive rounds of MDA are likely to promote assortative mating within the surviving (fertile) worm population, thus increasing the number of parasites resistant to both drugs. Only after the genes have re-assorted in the parasite population in this way will treatment failure start to become apparent. With this in mind, it is advisable to implement promptly the monitoring and evaluation of possible changes in the parasite genome, by means of appropriate molecular markers, and to link this to the monitoring of responses to treatment in the host population. These actions may identify, before anthelmintic resistance becomes a major public health concern, treatment factors that could enhance the spread of drug resistance. Although it is typically thought that combination therapy should delay the emergence of treatment failure, factors such as moderately high initial resistance allele frequencies to both drugs, dominance, genetic linkage, parasite inbreeding and high treatment coverage in MDA programmes may cause multi-drug resistance to hinder the achievement of GPELF's goals. Continued updating and improvement of transmission models, particularly those which merge the population dynamics and population genetics of the parasite, should be essential components of any epidemiological surveillance system implemented to protect the investments made by the Global Alliance to Eliminate Lymphatic Filariasis and its

donors (Dadzie *et al.* 2004; Michael *et al.* 2006; Schwab *et al.* 2006).

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References

Addiss, D. G., Beach, M. J., Streit, T. G., Lutwick, S., LeConte, F. H., Lafontant, J. G., Hightower, A. W. and Lammie, P. J. (1997). Randomised placebo-controlled comparison of ivermectin and albendazole alone and in combination for *Wuchereria bancrofti* microfilaraemia in Haitian children. *Lancet* 350, 480-484.

Ardelli, B. F., Guerriero, S. B. and Prichard, R. K. (2005). Genomic organization and effects of ivermectin selection on Onchocerca volvulus P-glycoprotein. *Molecular Biochemistry and Parasitology,* 143, 58-66.

Ardelli, B. F., Guerriero, S. B. and Prichard, R. K. (2006a). Characterization of a half-size ATP-binding cassette transporter gene which may be a useful marker for ivermectin selection in Onchocerca volvulus. *Molecular Biochemistry and Parasitology,* 145, 94-100.

Ardelli, B. F., Guerriero, S. B. and Prichard, R. K. (2006b). Ivermectin imposes selection pressure on P-glycoprotein from Onchocerca volvulus: linkage disequilibrium and genotype diversity. *Parasitology*, 132, 375-386.

Ardelli, B. F. and Prichard, R. K. (2004). Identification of variant ABC-transporter genes among Onchocerca volvulus collected from ivermectin-treated and untreated patients in Ghana, West Africa. Ann Trop Med Parasitol, 98, 371-384.

Awadzi, K., Attah, S. K., Addy, E. T., Opoku, N. O., Quartey, B. T., Lazdins-Helds, J. K., Ahmed, K., Boatin, B. A., Boakye, D. A. and Edwards, G. (2004a). Thirty-month follow-up of sub-optimal responders to multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. *Annals of Tropical Medicine and Parasitology* 98, 359-370.

Awadzi, K., Boakye, D. A., Edwards, G., Opoku, N. O., Attah, S. K., Osei-Atweneboana, M. Y., Lazdins-Helds, J. K., Ardrey, A. E., Addy, E. T., Quartey, B. T., Ahmed, K., Boatin, B. A. and Soumbey-Alley, E. W. (2004b). An investigation of persistent microfilaridermias despite multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. *Annals of Tropical Medicine and Parasitology* 98, 231-249.

Barnes, E. H., Dobson, R. J. and Barger, I. A. (1995). Worm control and anthelmintic resistance: adventures with a model. *Parasitology Today* 11, 56-63.

Barnes, E. H., Dobson, R. J., Stein, P. A., Le Jambre, L. F. and Lenane, I. J. (2001). Selection of different genotype larvae and adult worms for anthelmintic resistance by persistent and short-acting avermectin/milbemycins. *International Journal for Parasitology* 31, 720-727.

Blackhall, W.J., Pouliot, J.-F., Prichard, R.K. and Beech, R.N. (1998a). *Haemonchus contortus*: Selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. *Experimental. Parasitology* 90, 42-48.

Blackhall, W., Liu, H.Y., Xu, M., Prichard, R.K. and Beech, R.N. (1998b). Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of *Haemonchus contortus. Molecular and Biochemical Parasitology* 95, 193-201.

Bryan, J. H. and Southgate, B. A. (1988). Factors affecting transmission of Wuchereria bancrofti by anopheline mosquitoes. 2. Damage to ingested microfilariae by mosquito foregut armatures and development of filarial larvae in mosquitoes. Transactions of the Royal Society of Tropical Medicine and Hygiene 82, 138-145.

Bryan, J. H., McMahon, P. and Barnes, A. (1990). Factors affecting transmission of Wuchereria bancrofti by anopheline mosquitoes. 3. Uptake and damage to ingested microfilariae by Anopheles gambiae, An. arabiensis, An. merus and An. funestus in East

Chan, M. S., Norman, R. A., Michael, E., Bundy, D. A., Das, P. K., Pani, S. P. and Ramaiah, K. D. (1999). http://www.schoolsandhealth.org/epidynamics.htm.

Chan, M. S., Srividya, A., Norman, R. A., Pani, S. P., Ramaiah, K. D., Vanamail, P., Michael, E., Das, P. K. and Bundy, D. A. (1998). Epifil: a dynamic model of infection and disease in lymphatic filariasis. *American Journal of Tropical Medicine and Hygiene* 59, 606-614.

Churcher, T.S. (2006). Modelling the spread of anthelmintic resistance. *PhD Thesis* Imperial College London.

Dean, M. (2002). Towards the elimination of lymphatic filariasis. *Lancet* 359, 1677.
Dobson, R. J. and Barnes, E. H. (1995). Interaction between *Ostertagia circumcincta* and *Haemonchus contortus* infection in young lambs. *International Journal for Parasitology* 25, 495-501.

Dunyo, S. K., Nkrumah, F. K. and Simonsen, P. E. (2000). A randomized double-blind placebo-controlled field trial of ivermectin and albendazole alone and in combination for the treatment of lymphatic filariasis in Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94, 205-211.

Eberhard, M. L., Lammie, P. J., Dickinson, C. M. and Roberts, J. M. (1991). Evidence of nonsusceptibility to diethylcarbamazine in *Wuchereria bancrofti*. *Journal of Infecious Diseases* 163, 1157-1160.

Eberhard, M. L., Lowrie, R. C., Jr. and Lammie, P. J. (1988). Persistence of microfilaremia in bancroftian filariasis after diethylcarbamazine citrate therapy. *Tropical Medicine and Parasitology* 39, 128-130.

Edwards Jr., C.H. and Penney, D.E. (1989). Elementary differential equations with boundary value problems, 2nd edn. Prentice-Hall, New Jersey.

Elard, L. and Humbert, J. F. (1999). Importance of the mutation of amino acid 200 of the isotype 1 beta-tubulin gene in the benzimidazole resistance of the small-ruminant parasite *Teladorsagia circumcincta*. *Parasitology Research* 85, 452-456.

Eng, J. K. and Prichard, R. K. (2005). A comparison of genetic polymorphism in populations of *Onchocerca volvulus* from untreated- and ivermectin-treated patients. *Molecular Biochemistry and Parasitology* 142, 193-202.

Eng, J. K., Blackhall W.J., Osei-Atweneboana M.Y., Bourguinat C., Galazzo D., Beech R.N., Unnasch, T.R., Awadzi K., Lubega G.W. and Prichard, R.K. (2006). Ivermectin selection on β-tubulin: Evidence in *Onchocerca volvulus* and *Haemonchus contortus*. *Molecular and Biochemical Parasitology* 150, 229-235.

Esterre, P., Plichart, C., Séchan, Y. and Nguyen, N. L. (2001). The impact of 34 years of massive DEC chemotherapy on Wuchereria bancrofti infection and transmission: the Maupiti cohort. Tropical Medicine and International Health 6, 190-195.

Gyapong, J. O., Kyelem, D., Kleinschmidt, I, Agbo, K., Ahouandogbo, F., Gaba, J., Owusu-Banahene, G., Sanou, S., Sodahlon, Y, K., Biswas, G., Kale, O. O, Molyneux, D. H., Roungou, J. B., Thomson, M. C. and Remme, J. (2002). The use of spatial analysis in mapping the distribution of bancroftian filariasis in four West African countries. Annals of Tropical Medicine and Parasitology 96, 695-705

Hartl, D.L. and Clark, A.G. (1997). Principles of Population Genetics. 3rd edn. Sinauer Associates, Sunderland, Massachusetts, USA.

Ismail, M. M., Jayakody, R. L., Weil, G. J., Nirmalan, N., Jayasinghe, K. S., Abeyewickrema, W., Rezvi Sheriff, M. H., Rajaratnam, H. N., Amarasekera, N., de Silva, D. C., Michalski, M. L. and Dissanaike, A. S. (1998). Efficacy of single dose combinations of albendazole, ivermectin and diethylcarbamazine for the treatment of bancroftian filariasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92, 94-97.

Le Jambre, L.F., Geoghegan, M. and Lyndal-Murphy, M. (2005) Characterization of moxidectin resistant *Trichostrongylus colubriformis* and *Haemonchus contortus*. *Veterinary Parasitology* 128, 83-90.

Le Jambre, L. F., Gill, J. H., Lenane, I. J. and Baker, P. (2000). Inheritance of avermectin resistance in *Haemonchus contortus*. *International Journal for Parasitology* 30, 105-111.

Lewontin, R. C. (1988) On measures of gametic disequilibrium. *Genetics* 120, 849-52

Maher, D. and Ottesen, E. A. (2000). The Global Lymphatic Filariasis Initiative. *Tropical Doctor* 30, 178-179.

Michael, E. and Bundy, D. A. (1997). Global mapping of lymphatic filariasis. *Parasitology Today* 13, 472-476.

Michael, E., Malecela-Lazaro, M. N., Simonsen, P. E., Pedersen, E. M., Barker, G., Kumar, A. and Kazura, J. W. (2004). Mathematical modelling and the control of lymphatic filariasis. *Lancet Infectious Diseases* 4, 223-234.

Norman, R. A., Chan, M. S., Srividya, A., Pani, S. P., Ramaiah, K. D., Vanamail, P., Michael, E., Das, P. K. and Bundy, D. A. (2000). EPIFIL: the development of an age-structured model for describing the transmission dynamics and control of lymphatic filariasis. *Epidemiology and Infection* 124, 529-541.

Ottesen, E. A. (2000). The global programme to eliminate lymphatic filariasis. *Tropical Medicine and International Health* 5, 591-594.

Ottesen, E. A. (2002). Major progress toward eliminating lymphatic filariasis. *New England Journal of Medicine* 347, 1885-1886.

Plaisier, A. P., Cao, W. C., Van Oortmarssen, G. J. and Habbema, J. D.F. (1999) Efficacy of ivermectin in the treatment of Wuchereria bancrofti infection: a model-based analysis of trial results. Parasitology: 119, 385-394

Plaisier, A. P., Subramanian, S., Das, P. K., Souza, W., Lapa, T., Furtado, A. F., Van der Ploeg, C. P., Habbema, J. D. F. and van Oortmarssen, G. J. (1998). The LYMFASIM simulation program for modeling lymphatic filariasis and its control. Methods of Information in Medicine 37, 97-108.

Prichard, R. K. (1990). Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research. *International Journal for Parasitology* 20, 515-523.

Prichard, R. K. (2001). Genetic variability following selection of *Haemonchus contortus* with anthelmintics. *Trends in Parasitology* 17, 445-453.

Prichard, R. K. (2005). Is anthelmintic resistance a concern for heartworm control? What can we learn from the human filariasis control programs? *Veterinary Parasitology* 133, 243-253.

Prichard, R. K., Hall, C. A., Kelly, J. D., Martin, I. C. and Donald, A. D. (1980). The problem of anthelmintic resistance in nematodes. *Australian Veterinary Journal* 56, 239-251.

Ramaiah, K. D., Guyatt, H., Ramu, K., Vanamail, P., Pani, S. P. and Das, P. K. (1999). Treatment costs and loss of work time to individuals with chronic lymphatic filariasis in rural communities in south India. *Tropical Medicine and International Health* 4, 19-25.

Remme, J. H. F., Feenstra, P., Lever, P. R., Medici, A. C., Morel, C. M., Noma, M., Ramaiah, K.D., Richards, F., Sékétéli, A., Schmunis, G., van Brakel, W. H. and Vassall, A. (2006). Tropical diseases targeted for elimination: Chagas disease, lymphatic filariasis, onchocerciasis, and leprosy. In: *Disease Control Priorities in Developing Countries* (Ed. Jamison, D. T., Breman, J. G., Measham, A. R., Alleyne, G., Claeson, M., Evans, D. B., Jha, P., Mills, A. and Musgrove, P.), pp. 433-449. The World Bank and Oxford University Press, USA.

Schwab, A. E., Boakye, D., Kyelem, D. and Prichard, R. K. (2005). Detection of benzimidazole-resistance associated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection with albendazole and ivermectin treatment. *American Journal of Tropical Medicine and Hygiene* 73, 234-238.

Schwab, A. E., Churcher, T.S., Schwab, A.J., Basáñez, M-G. and Prichard, R. K. (2006). Population genetics of concurrent selection with albendazole and ivermectin or diethylcarbamazine on the possible spread of albendazole resistance in *Wuchereria bancrofti*. *Parasitology* 133, 589-601.

Silvestre, A. and Cabaret, J. (2002). Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? *Mol Biochem Parasitol,* 120, 297-300.

Stolk, W. A., Ramaiah, K. D., Van Oortmarssen, G. J., Das, P. K., Habbema, J. D. and De Vlas, S. J. (2004). Meta-analysis of age-prevalence patterns in lymphatic

filariasis: no decline in microfilaraemia prevalence in older age groups as predicted by models with acquired immunity. *Parasitology* 129, 605-612.

Sutherland, I. A., Leathwick, D. M., Moen, I. C. and Bisset, S. A. (2002). Resistance to therapeutic treatment with macrocyclic lactone anthelmintics in *Ostertagia circumcincta*. *Veterinary Parasitology* 109, 91-99.

WHO (2003). Control of lymphatic filariasis in China. World Health Organization, Western Pacific Region.

Wolstenholme, A. J.,Fairweather, I., Prichard, R. K., von Samson-Himmelstjerna, G. and Sangster, N. C. (2004). Drug resistance in veterinary helminths. Trends in Parasitology 20, 469-476.

Wright, S. (1951). The genetical structure of populations. Annals of Eugenics 15, 323-354.

Zagaria, N. and Savioli, L. (2002). Elimination of lymphatic filariasis: a public-health challenge. *Annals of Tropical Medicine and Parasitology* 96 Suppl 2, S3-13.

Appendix 4.A. Incorporation Of Parasite Population Genetics Into Epifil

The EPIFIL model is extended by incorporating genetic structure into the worm population in order to examine the spread of ABZ, IVM, and ABZ and IVM resistances. The mathematical models track the number of worms, in both the treated and untreated sections of the human host population, of each of ten genotype combinations in the case of the ABZ monogenic-IVM monogenic model (2 loci with 2 alleles each), and thirty genotype combinations in the case of the ABZ monogenic-IVM polygenic model (3 loci with 2 alleles each). ABZ resistance is assumed to be recessive throughout, whilst the model investigates the effects of variable IVM resistance dominance, with the default setting assuming that IVM resistance is fully dominant. For each of these loci the different genotypes are identified using the following system: 0 corresponds to the susceptible homozygote, 1 to the heterozygote, and 2 to the resistant homozygote. The mean number of adult worms per person of each genotype is denoted, after dropping the age and time dependency, by W_b^{cde}, with subscript b indicating the treatment category ($b=T$ or $b=U$ for worms within, respectively, hosts treated or untreated with ABZ + IVM), and the superscripts c, d and e specifying the worm genotype at each of the different loci (c = the ABZ resistance locus, d = the first, linked IVM resistance locus, and e = the second unlinked IVM resistance locus). If IVM resistance is conferred by a single locus (the IVM monogenic model) the superscript e is dropped and superscript d indicates resistance to macrofilaricidal/inhibition of reproduction and embryostatic effects of IVM. For example, W_b^{00} denotes the mean number of adult worms (in the IVM monogenic model) which have the homozygote susceptible genotype at both the ABZ and IVM resistance loci; W_b^{201} indicates the mean number of worms (in the IVM polygenic model) which are homozygous resistant at the ABZ resistance locus, homozygous susceptible at the linked, IVM resistance locus, and heterozygous at the second, unlinked IVM resistance locus. Similar notation is used to represent the mean number of microfilariae per 20 µl blood, M_b^{cde}, and the mean number of L3 larvae per mosquito, L_b^{cde}. The level of acquired immunity in the treated and untreated human

population is specified by, respectively, I_T and I_U. The modified EPIFIL equations are as follows,

$$\frac{\partial W_b^{cde}}{\partial t}+\frac{\partial W_b^{cde}}{\partial a}=\lambda\frac{V}{H}\varphi_1\varphi_2 s_2 h(a) L^{*cde} e^{-\beta I}-\mu W_b^{cde} \tag{A4.1}$$

$$\frac{\partial M_b^{cde}}{\partial t}+\frac{\partial M_b^{cde}}{\partial a}=\alpha W_b \omega_b^{cde}-\gamma M_b^{cde} \tag{A4.2}$$

$$\frac{\partial I_b}{\partial t}+\frac{\partial I_b}{\partial a}=W_b-\Delta I_b \tag{A4.3}$$

$$L^{*cde}=\left[C\frac{L_T^{*cde}}{L_T^*}+(1-C)\frac{L_U^{*cde}}{L_U^*}\right]\left[CL_T^*+(1-C)L_U^*\right] \tag{A4.4}$$

$$L_b^{*cde}=\frac{\lambda\kappa g\int_{a=0}^{a=\infty}\pi(a)\frac{M_b^{cde}}{M_b}f(M_b)da}{\sigma+\lambda\varphi_1} \tag{A4.5}$$

where λ is the biting rate per mosquito on humans; V/H is the vector to human ratio; φ_1 is the proportion of L3 larvae that leave the mosquito's proboscis at the time of biting and are deposited onto human's skin; φ_2 is the proportion of the latter which enter the host through the wound caused by the mosquito bite; s_2 is the proportion of larvae that having entered the host will develop into adult worms; $h(a)$ is the proportion of hosts of age a that are bitten, thereby making the biting rate on humans age-dependent (it is assumed that this proportion increases linearly until the age of 9 years and becomes unity thereafter); β is the severity by which larval establishment is decreased by acquired immunity I (which is assumed to increase with cumulative worm burden or worm experience and decay at a rate Δ); μ, γ and σ are, respectively, the per capita death rates of adult worms, microfilariae and L3 larvae; α is the per capita reproductive rate of adult

worms (the rate at which fertilized females produce microfilariae per 20 μl of blood), and. ω_b^{cde} is the proportion of microfilariae with the *cde* genotype (calculated as described in Appendix B). The proportion of the total host population treated (therapeutic coverage) is denoted by C; $M_b = \sum_{c=0}^{2}\sum_{d=0}^{2}\sum_{e=0}^{2} M_b^{cde}$ and $L_b^* = \sum_{c=0}^{2}\sum_{d=0}^{2}\sum_{e=0}^{2} L_b^{*cde}$. The expression for L^{*cde} determines the average number of L3 larvae per mosquito for each genotype that infect the treated and untreated sections of the population, As with EPIFIL, the L3 population is assumed to be at equilibrium (denoted by the asterisk) and is calculated using equation (A5). In this equation, the function that models larval development within the (*Culex*-type) mosquito vector as dependent on the level of microfilaraemia, $f(M_b)$, is calculated separately for treated and untreated sections of the host population with expression,

$$f(M_b) = 1 - \left\{1 + \frac{M_b}{k_b}\left[1 - \exp(-\rho/\kappa)\right]\right\}^{-k_b} \qquad \text{(A4.6)}$$

where ρ is the initial, linear rate of increase in L3 larvae per microfilaria in 20 μl of blood, ρ/κ the severity of density-dependent constraints upon larval uptake and/or development; κ the level at which the function saturates (the maximum number of L3 produced per mosquito as microfilaraemia increases), and k_b the overdispersion parameter of the negative binomial. In the remainder of equation (A5), g is the proportion of bites which result in infection of mosquitoes, and $\pi(a)$ is the proportion of the host population surviving to age a, based on a population in Pondicherry, India (for which EPIFIL was first parameterised). The equation for $\pi(a)$ is given by,

$$\pi(a) = \frac{\exp(-a/m)}{m}\left[1 - \exp(-n/m)\right] \qquad \text{(A7)}$$

with $m = 29$ and $n = 65$. Finally, it is assumed (motivated by data), that the

overdispersion parameter of the negative binomial distribution of microfilariae in the host population is itself a density-dependent function with expression $k_b = k_0 + k_1 M_b$, representing a degree of overdispersion which depends linearly on microfilaraemia and will therefore increase in the treated sections of the population under microfilaricidal therapy. All the parameter values of the basic EPIFIL model have been listed in Schwab *et al.* (*in press*).

IVM Monogenic Model

In the IVM monogenic model we allow the IVM resistance locus to be either linked or unlinked to the ABZ resistance locus. Linkage increases the number of possible genotype combinations a parasite can have as helminths which are heterozygous for both ABZ and IVM resistance can either have both resistance alleles on the same chromosome (alleles are concordant, denoted by superscript *C*), or have resistance alleles on the opposite, partner chromosome (alleles are discordant, denoted by superscript *D*). The different genotype combinations for the two loci model and their associated abbreviations are listed in Table A.4.1.

Table A.4.1 : The abbreviations used within the IVM monogenic (2 loci) model. For the linked loci it is specified on which chromosome (denoted Ch.) the allele occurs.

Abbreviation	ABZ locus		IVM locus		Equation used to calculate the genotype frequency of the next generation
	Ch. 1	Ch. 2	Ch. 1	Ch. 2	
00	S	S	S	S	$\omega_b^{00} = (G_b^{SS})^2(1-F) + (G_b^{SS})F$
01	S	S	R	S	$\omega_b^{01} = 2(G_b^{SR})(G_b^{SS})(1-F)$
02	S	S	R	R	$\omega_b^{02} = (G_b^{SR})^2(1-F) + (G_b^{SR})F$
10	R	S	S	S	$\omega_b^{10} = 2(G_b^{RS})(G_b^{SS})(1-F)$
11^C	R	S	R	S	$\omega_b^{11^C} = 2(G_b^{RR})(G_b^{SS})(1-F)$
11^D	R	S	S	R	$\omega_b^{11^D} = 2(G_b^{RS})(G_b^{SR})(1-F)$
12	S	R	R	R	$\omega_b^{12} = 2(G_b^{SR})(G_b^{RR})(1-F)$
20	R	R	S	S	$\omega_b^{20} = (G_b^{RS})^2(1-F) + (G_b^{RS})F$
21	R	R	R	S	$\omega_b^{21} = 2(G_b^{RR})(G_b^{RS})(1-F)$
22	R	R	R	R	$\omega_b^{22} = (G_b^{RR})^2(1-F) + (G_b^{RR})F$

The genotype distribution of microfilariae in the next generation is determined by the allele frequency of the adult worm population using simple Mendelian genetics. The frequency of gametes produced by the adult worm population is denoted by G_b^{hi}. Gametes can have either the resistance or susceptibility allele at each of the loci under investigation (where *h* denotes the ABZ resistance locus and *i* denotes the IVM resistance locus, with resistance in both cases referring to macrofilarial resistance as described above). For the 2 loci model the gamete frequencies are calculated as follows,

$$G_b^{SS} = w_b^{00} + 0.5\left[w_b^{01} + (1-r) w_b^{11^C} + r\, w_b^{11^D} + w_b^{10} \right] \quad \text{(A4.7)}$$

$$G_b^{SR} = w_b^{02} + 0.5\left[w_b^{01} + r\, w_b^{11^C} + (1-r) w_b^{11^D} + w_b^{12} \right] \quad \text{(A4.8)}$$

$$G_b^{RS} = w_b^{20} + 0.5\left[w_b^{10} + r\, w_b^{11^C} + (1-r) w_b^{11^D} + w_b^{21} \right] \quad \text{(A4.9)}$$

$$G_b^{RR} = w_b^{22} + 0.5\left[w_b^{12} + (1-r) w_b^{11^C} + r\, w_b^{11^D} + w_b^{21} \right] \quad \text{(A4.10)}$$

where w_b^{cd} is the proportion of the adult worm population with the *cd* genotype (given by $w_b^{cd} = \frac{W_b^{cd}}{W_b}$). The proportion of new microfilariae with the *cd* genotype, ω_b^{cd}, is calculated from the different gamete frequencies; for individual equations see Table A4.1. Parameter *r* is the recombination fraction between the two loci or the proportion of recombinant gametes that are produced by a double heterozygote (Hartl and Clark 1997). Genes that are not linked have a recombination fraction $r = 0.5$. Genes which are linked have a value of $r < 0.5$. When genes are 100% linked, $r = 0$ (i.e., no recombination occurs between the two loci). Unless otherwise stated, the model assumes non-random parasite mating, with *F* (see Tables A4.1 and A4.2) set at 0.44 (the value of F_{IT} estimated for untreated populations in Burkina Faso, see main text).

IVM Polygenic Model

Here we model the parasite genotype frequency when there are three loci associated with resistance; one which determines ABZ resistance and another two loci that determine IVM resistance. The 3 loci model assumes that one of the IVM resistance loci is completely linked to the ABZ locus (with no recombination occurring between the loci), with the other remaining loci being unlinked to either of the first two loci. The frequencies of parental gametes can be derived from the 2 loci case as follows,

$$G_b^{SSS} = w_b^{000} + 0.5\left(w_b^{010} + w_b^{11^C0} + w_b^{100} + w_b^{001}\right) + 0.25\left(w_b^{011} + w_b^{11^C1} + w_b^{101}\right) \quad \text{(A4.11)}$$

$$G_b^{SSR} = w_b^{002} + 0.5\left(w_b^{012} + w_b^{11^C2} + w_b^{102} + w_b^{001}\right) + 0.25\left(w_b^{011} + w_b^{11^C1} + w_b^{101}\right) \quad \text{(A4.12)}$$

$$G_b^{SRS} = w_b^{020} + 0.5\left(w_b^{010} + w_b^{11^D0} + w_b^{120} + w_b^{021}\right) + 0.25\left(w_b^{011} + w_b^{11^D1} + w_b^{121}\right) \quad \text{(A4.13)}$$

$$G_b^{SRR} = w_b^{022} + 0.5\left(w_b^{012} + w_b^{11^D2} + w_b^{122} + w_b^{021}\right) + 0.25\left(w_b^{011} + w_b^{11^D1} + w_b^{121}\right) \quad \text{(A4.14)}$$

$$G_b^{RSS} = w_b^{200} + 0.5\left(w_b^{210} + w_b^{11^D0} + w_b^{100} + w_b^{201}\right) + 0.25\left(w_b^{11^D1} + w_b^{211} + w_b^{101}\right) \quad \text{(A4.15)}$$

$$G_b^{RSR} = w_b^{202} + 0.5\left(w_b^{212} + w_b^{11^D2} + w_b^{102} + w_b^{201}\right) + 0.25\left(w_b^{11^D1} + w_b^{211} + w_b^{101}\right) \quad \text{(A4.16)}$$

$$G_b^{RRS} = w_b^{220} + 0.5\left(w_b^{210} + w_b^{11^C0} + w_b^{120} + w_b^{221}\right) + 0.25\left(w_b^{11^C1} + w_b^{211} + w_b^{121}\right) \quad \text{(A4.17)}$$

$$G_b^{RRR} = w_b^{222} + 0.5\left(w_b^{212} + w_b^{11^C2} + w_b^{122} + w_b^{221}\right) + 0.25\left(w_b^{11^C1} + w_b^{211} + w_b^{121}\right) \quad \text{(A4.18)}$$

where w_b^{cde} is the frequency of the adult worm population with the *cde* genotype (given by $w_b^{cde} = \frac{W_b^{cde}}{W_b}$). G_b^{ghS} denotes the frequency of the gamete G_b^{gh} with the susceptibility allele at the third, unlinked locus, and G_b^{ghR} denotes the frequency of the gamete G_b^{gh} bearing the resistance allele at the third unlinked locus. For individual equations calculating the proportion of new microfilariae with the *cde* genotype see Table A4.2.

Table A.4.2 : The abbreviations used within the IVM polygenic (3 loci) model. For the linked loci it is specified on which chromosome (denoted Ch.) the allele occurs.

Abbreviation	ABZ locus		IVM locus		IVM 2nd locus	Equation used to calculate the genotype frequency of the next generation
	Ch. 1	Ch. 2	Ch. 1	Ch. 2	Linkage equilibrium	
000	S	S	S	S	SS	$\omega_b^{000} = (G_b^{SSS})^2(1-F) + (G_b^{SSS})F$
001	S	S	S	S	RS	$\omega_b^{001} = 2(G_b^{SSR})(G_b^{SSS})(1-F)$
002	S	S	S	S	RR	$\omega_b^{002} = (G_b^{SSR})^2(1-F) + (G_b^{SSR})F$
010	S	S	R	S	SS	$\omega_b^{010} = 2(G_b^{SRS})(G_b^{SSS})(1-F)$
011	S	S	R	S	RS	$\omega_b^{011} = 2[(G_b^{SRR})(G_b^{SSS}) + (G_b^{SRS})(G_b^{SSR})](1-F)$
012	S	S	R	S	RR	$\omega_b^{012} = 2(G_b^{SRR})(G_b^{SSR})(1-F)$
020	S	S	R	R	SS	$\omega_b^{020} = (G_b^{SRS})^2(1-F) + (G_b^{SRS})F$
021	S	S	R	R	RS	$\omega_b^{021} = 2(G_b^{SRS})(G_b^{SRR})(1-F)$
022	S	S	R	R	RR	$\omega_b^{022} = (G_b^{SRR})^2(1-F) + (G_b^{SRR})F$
100	R	S	S	S	SS	$\omega_b^{100} = 2(G_b^{RSS})(G_b^{SSS})(1-F)$
101	R	S	S	S	RS	$\omega_b^{101} = 2[(G_b^{RSR})(G_b^{SSS}) + (G_b^{RSS})(G_b^{SSR})](1-F)$
102	R	S	S	S	RR	$\omega_b^{102} = 2(G_b^{RSR})(G_b^{SSR})(1-F)$
110^C	R	S	R	S	SS	$\omega_b^{110^C} = 2(G_b^{SSS})(G_b^{RRS})(1-F)$
110^D	R	S	S	R	SS	$\omega_b^{110^D} = 2(G_b^{SRS})(G_b^{RSS})(1-F)$
111^C	R	S	R	S	RS	$\omega_b^{111^C} = 2[(G_b^{SSR})(G_b^{RRS}) + (G_b^{SSS})(G_b^{RRR})](1-F)$
111^D	R	S	S	R	RS	$\omega_b^{111^D} = 2[(G_b^{SRR})(G_b^{RSS}) + (G_b^{SRS})(G_b^{RSR})](1-F)$
112^C	R	S	R	S	RR	$\omega_b^{112^C} = 2(G_b^{SSR})(G_b^{RRR})(1-F)$
112^D	R	S	S	R	RR	$\omega_b^{112^D} = 2(G_b^{SRR})(G_b^{RSR})(1-F)$
120	S	R	R	R	SS	$\omega_b^{120} = 2(G_b^{SRS})(G_b^{RRS})(1-F)$
121	S	R	R	R	RS	$\omega_b^{121} = 2[(G_b^{SRR})(G_b^{RRS}) + (G_b^{SRS})(G_b^{RRR})](1-F)$
122	S	R	R	R	RR	$\omega_b^{122} = 2(G_b^{SRR})(G_b^{RRR})(1-F)$
200	R	R	S	S	SS	$\omega_b^{200} = (G_b^{RSS})^2(1-F) + (G_b^{RSS})F$
201	R	R	S	S	RS	$\omega_b^{201} = 2(G_b^{RSR})(G_b^{RSS})(1-F)$
202	R	R	S	S	RR	$\omega_b^{202} = (G_b^{RSR})^2(1-F) + (G_b^{RSR})F$
210	R	R	R	S	SS	$\omega_b^{012} = 2(G_b^{SSR})(G_b^{SRR})(1-F)$
211	R	R	R	S	RS	$\omega_b^{211} = 2[(G_b^{RRR})(G_b^{RSS}) + (G_b^{RRS})(G_b^{RSR})](1-F)$
212	R	R	R	S	RR	$\omega_b^{212} = 2(G_b^{RRR})(G_b^{RSR})(1-F)$
220	R	R	R	R	SS	$\omega_b^{220} = (G_b^{RRS})^2(1-F) + (G_b^{RRS})F$
221	R	R	R	R	RS	$\omega_b^{221} = 2(G_b^{RRR})(G_b^{RRS})(1-F)$
222	R	R	R	R	RR	$\omega_b^{222} = (G_b^{RRR})^2(1-F) + (G_b^{RRR})F$

CHAPTER 5: GENERAL DISCUSSION

The current initiative to control lymphatic filariasis, implemented under the auspices of the World Health Organization, promises, if successful, to prevent the suffering of millions of people. In order to reach its goal of eliminating the disease as a public health problem by the year 2020, it is proposed to repetitively administer anthelmintic drugs to people in endemic areas. By the year 2004, approximately 435 million doses of drug had been administered to the at-risk population in half of all recognized LF endemic countries (Gyapong and Twum-Danso, 2006) and in 2005 a total of 610 million people were targeted with Mass Drug Administration for LF (www.who.int/lymphatic_filariasis/disease/en/, accessed June, 8, 2006). Experience, from the field of veterinary parasitology, indicates that large scale, repetitive distribution of anthelmintic drugs will inevitably lead to the emergence of drug resistance (Kaplan, 2004). Further examination of the potential for similar drug-resistance development is imperative in order to predict the success of the control program (Michael *et al.*, 2004).

Drug resistance to benzimidazoles (BZ) has been reported in many species of helminths of veterinary importance, as well as in protozoa and fungi and has been shown to be caused by SNPs in the β-tubulin gene (Driscoll *et al.*, 1989; Edlind *et al.*, 1994; Elard *et al.*, 1996; Jung *et al.*, 1992; Koenraadt *et al.*, 1992; Kwa *et al.*, 1993; Kwa *et al.*, 1994; Orbach *et al.*, 1986; Pape *et al.*, 1999; Prichard, 2001; Silvestre and Cabaret, 2002; von Samson-Himmelstjerna *et al.*, 2001; Yarden and Katan, 1993).

In the second chapter of this thesis we present the first evidence that such BZ resistance SNPs exist in *Wuchereria bancrofti* lymphatic filaria. Observed frequencies, of the β-tubulin 200TYR resistance SNP, were variable in untreated populations, ranging from less than 1% in Ghana to 26 % in Burkina Faso. High initial allele frequencies of BZ resistance alleles have been observed in unselected populations of other helminths (Beech *et al.*, 1994). However, in the case of the parasites obtained from Burkina Faso, prior exposure to ABZ cannot be ruled out, as regular de-worming of school children using ABZ is common in many communities in West Africa (Hall *et al.*, 1996) and a large number of doses of ABZ have been administered, over many years, in numerous countries.

The mathematical model that is presented in the third chapter of this thesis

examined the effect of differing initial allele frequencies on selection for the ABZ resistance genotype. The output of these simulations indicates that a higher initial allele frequency will lead to a higher proportion of the population that will be resistant to ABZ after the treatment period (assumed to be ten yearly treatments at 85% coverage). A simplistic model proposed in a review by Michael *et al* (2004) draws the same conclusion. Thus communities with higher initial frequencies may develop high levels of resistance more quickly. This highlights the importance of measuring how common resistant alleles are in the population prior to the onset of treatment programs. Diagnostic tests such as those described in Chapter 2 will be useful in setting up such monitoring systems.

The data collected in Burkina Faso indicates that the frequency of the ABZ resistance allele, as well as the resistance genotype are significantly higher in populations treated with a combination of IVM and ABZ. The frequency was again significantly higher in Mf sampled from people that had been treated twice, though there was a considerably smaller sample size for this group. The statistical analysis in this study was done using a Chi-square test. These results suggest that selection for the ABZ resistance allele may be occurring in *W. bancrofti* from Burkina Faso.

In this study, we only investigated one isotype of tubulin. None of the partial sequences obtained suggested that they originated from different isotypes. However, only three clones spanning the entire coding sequence were sequenced, thus the presence of a second isotype can not be excluded. A second isotype of tubulin could contribute to ABZ sensitivity in *W. bancrofti.*

The Mf from doubly treated patients in Burkina Faso, that were genotyped in Chapter 2, were collected only seven days after the last treatment had occurred. Thus adult worms affected by the anthelmintics should not have resumed reproduction, and the population genotyped is probably a surviving population after drug treatment. In addition, this time period is far too short for worms acquired since treatment to have reached sexual maturity, as the pre-patent period of *W. bancrofti* is estimated to be 7-8 months (WHO, 1984; WHO, 1992).

It should be noted that the genotype frequencies of Mf sampled from treated people exclusively, considers the proportion of Mf in treated people and not the entire Mf population of the community in question. The simulations carried out in the third chapter,

which assume that the same people are treated each year and the untreated people are also the same from year to year, show that, according to the mathematical model proposed, resistance would only become apparent in the Mf population after the cessation of the treatment program. This is explained by the fact that during the treatment period, very little parasite reproduction would occur in the treated people, and most of the Mf population would be from people that have not been treated. Presumably, some people in the treated villages in Burkina Faso did not undergo treatment, and, as they likely had higher Mf counts, their parasite load would contribute greatly to the overall population of Mf in the community. The predicted increase in resistant genotypes after the end of the treatment period raises the concern that, should the control program end before elimination of the parasite, a newly established population of lymphatic filaria will have a higher proportion of resistant individuals and will thus be more difficult to control.

The Mf samples from Burkina Faso were not in Hardy-Weinberg equilibrium at the β-tubulin locus. There was a significantly higher frequency of homozygotes in the population than expected. Parasite populations are known to be strongly subdivided, as they are distributed amongst hosts, and cannot directly mate with individuals that are not within the same host. Population subdivision leads to a deficit in heterozygotes, a phenomenon termed the Wahlund effect (Hartl and Clark, 1997). It has been previously noted that genetic differentiation between hosts exists in parasites (Fisher and Viney, 1998; Lymbery *et al.*, 1990; Vilas *et al.*, 2003).

A detailed analysis of genotyping data from the study carried out in Burkina Faso and presented in Chapter 2 (details are included as Appendix I to this thesis) reveals that the excess homozygosity observed is unlikely to be generated simply by stochastic processes or sampling strategies. It also indicates that there is significant genetic differentiation between human hosts, though no geographical subdivision between villages was observed. In addition, we found some indication of assortative mating within patients, though this was not statistically significant.

Since ABZ resistance is known to be recessive in nematode species in which this has been examined, increased homozygosity will, if ABZ resistance is also recessive in *W. bancrofti*, increase the number of worms surviving chemotherapy. This will lead to a more rapid spread of the resistance allele. The impact of increased homozygosity on the

development of drug resistance was examined using the mathematical model presented in Chapter 3. Simulations that vary homozygosity through the use of Wright's inbreeding statistic F_{IT} suggest that even very low levels of increased homozygosity greatly increase the spread of an ABZ resistance allele after selection with combination chemotherapy. A similar analysis was also carried out in Chapter 4, using a mathematical model which incorporates a recessive ABZ resistance allele as well as a dominant IVM resistance allele. In the case of such multi-drug resistance, our model predicts that the spread ABZ resistance is increased by a positive F_{IT} value to a similar degree to that calculated for ABZ resistance alone. Simultaneous selection for dominant IVM resistance is also increased by excess homozygosity. As increased homozygosity and decreased heterozygosity is expected to reduce the number of genotypes surviving treatment in the case of dominant resistance. The increase in IVM resistance genotypes, in the case of the non-random mating observed here, is likely to be due to the fact that fewer individuals would be sterilized by ABZ in this case, and more selection may occur.

Genetic differentiation between hosts may also increase selection for resistance, since there is a greater chance that resistant worms co-habit the same host and mate to produce homozygous resistant offspring. However, density dependent constraints on resistant populations within a single host may slow the spread of resistance.

In light of the findings in Appendix I, a re-analysis of the data presented in Chapter 2 indicates that a proportional sampling scheme may have been more appropriate in order to conclusively detect a difference in the underlying microfilarial allele frequencies from Burkina Faso. The number of Mf sampled per patient should, more appropriately, be proportional to the microfilarial load of each host.

It must however be stressed that the results obtained in Appendix I are based on the analysis of only one gene. It will be necessary to further analyze a number of different genes from *W. bancrofti* microfilarial populations in order to conclude with certainty the cause of such excess homozygosity. Work in our laboratory on the related filarial parasite *O. volvulus* indicates a similar level of increased homozygosity as that found in appendix I in β-tubulin. However such genotype distributions were not found in control genes, suggesting the possibility of assortative mating (Catherine Bourguinat, personal communication).

In Chapter 3, there is further analysis of the potential spread of drug resistance in *W. bancrofti*, using a mathematical model. The simulations carried out in this model indicate that the assumptions made about drug efficacies greatly impact the speed and magnitude of the spread of an ABZ resistance allele due to combination chemotherapy. The importance of correctly estimating the effect of drugs on lymphatic filariae, in mathematical models designed to predict the impact of control programs, was previously highlighted by Michael *et al* (2004). Studies examining drug effects against LF show great variability, and it has been traditionally very problematic to predict such drug effects, mainly due to the difficulty in the observation of adult worms (Stolk *et al.*, 2005; Tisch *et al.*, 2005). Increasing our knowledge of the precise impact that each of the drugs, used in the GPELF, alone or in combination, has on the parasite is imperative in order to more accurately predict how quickly ABZ resistance is likely to become a problem for the control program. Based on the efficacy assumptions made in Chapter 3, the combination of DEC and ABZ will allow a more rapid spread of ABZ resistance alleles than the combination of IVM and ABZ. This can be explained by the stronger microfilaricidal activity of, as well as a longer suppression of reproduction, caused by IVM in comparison with DEC.

In order to achieve its goal, the GPELF will have to maintain high levels of therapeutic coverage (Michael *et al.*, 2004). We examined how different levels of coverage affect selection for resistance in the case of an ABZ resistance allele (Chapter 3) or in the case of a dominant IVM resistance allele, in addition to ABZ recessive resistance (Appendix II). Our model predicts that higher levels of coverage will decrease the overall population of Mf after treatment, but that a greater proportion of the remaining Mf will be resistant to ABZ. Similar observations have been made when examining the case of *refugia* in the development of anthelmintic resistance in helminths of veterinary importance (Coles, 2002; van Wyk, 2001). However the impact of increasing coverage in the development of dominant IVM resistance is considerably more complicated. Our model predicts that increasing coverage up to 85% will increase the spread of the resistance allele. However, above this point, higher levels of coverage may actually decrease the spread of the resistance alleles (see Appendix II). This may be related to the high microfilaricidal effect of IVM, as well as adult worm longevity, in combination with

selection for ABZ resistance. Estimates of adult longevity are variable, ranging from 5-40 years (Carme and Laigret, 1979; Vanamail *et al.*, 1990). Thus, a better understanding of the length of the adult *W. bancrofti* lifespan, as well as drug efficacy data, should be given considerable attention in the future in order to improve the predictive value of mathematical models of lymphatic filariasis.

In Chapter 3 we also examine how the length of the treatment period affects the spread of selection. As in the case of increased treatment coverage, more years of yearly treatment reduces the average microfilarial load per patient. However, more of the remaining microfilariae will be resistant to ABZ. A rapid progress towards the goal of the GPELF with a minimum number of treatments is thus desirable in order to prevent the spread of problematic ABZ resistance.

The simulations in this thesis show a considerable increase in anthelmintic allele frequencies, despite a continued reduction in total Mf load throughout the treatment period. This is a consequence of combination treatment. Thus if two drugs are used concurrently, resistance to one drug may still be selected, although there may be few cases of treatment failure during the initial treatment period. Thus, should IVM and DEC become unavailable for the treatment of LF, there may already be a significant amount of drug resistance against ABZ that had been selected during the period of combination therapy.

In Chapter 4, we examined the impact of concurrent selection for IVM resistance on the development of ABZ resistance. Our simulations show that the presence of a dominant IVM resistance gene may increase selection for ABZ resistance and vice versa. This is due to a larger proportion of the parasite population surviving combination treatment when IVM resistance is present. The model presented in Chapter 4 also highlights the importance of dominance in the spread of resistance alleles. Our simulations show that higher levels of dominance of an IVM resistance gene will lead to a quicker and more pronounced spread of IVM resistance alleles through the parasite population. Dominance was shown to have a similar effect on the development of anthelmintic resistance in other helminths with a direct life-cyle (Barnes, 1995; Dobson *et al.*, 1996).

Based on the model used in Chapter 4, linkage only affects selection of resistance

alleles if genes are completely linked and not in linkage equilibrium. If resistance loci to both drugs lie on the same allele more often than expected at linkage equilibrium, selection for ABZ will increase selection for IVM and vice versa. We also found that if IVM is inherited as a polygenic trait, resistance against IVM will develop more slowly than in the case of a single gene, a finding which corroborates what is known about the development of anthelmintic resistance in sheep nematodes (Dobson *et al.*, 1996). More detailed information on the mechanism of IVM resistance will be of great value in order to improve the accuracy of model predictions.

The models presented in this thesis are deterministic models, and thus are limited in the accuracy of their quantitative outputs. However, such models are highly useful in providing qualitative insight into the mechanisms and relative importance of the factors involved in biological systems. Stochastic simulation models provide more realistic outputs, however the high levels of complexity involved in such models can make it difficult to draw clear conclusions. Nonetheless, a stochastic model examining single and multidrug resistance in filarial worms could be extremely beneficial and provide further insight into this complex issue. We are presently developing such a stochastic model, as with it, we will be able to further examine some of the questions examined in the preceding chapters.

In addition, our models were based on EPIFIL, which was parameterized for *Culex*-transmitted filariasis. We use these parameters in order to remain as close as possible to the EPIFIL model. However, our evidence for increased parasite homozygosity comes from West African locations, where *Anopheles*- rather than *Culex*-transmitted filariasis prevails. Sensitivity analysis (data not shown) reveals that worm populations in EPIFIL are controlled primarily by immunity in the definitive host, and differences in density dependence in the vector will likely have been small, and would not affect the qualitative conclusions of this work.

Our models assume no trade-offs for resistance. It has been shown that BZ resistance persists for many generations after removal of anthelmintic selection pressure (Hall *et al.,* 1982). In addition, lack of reversion of BZ resistance after prolonged use of levamisole has been observed (Borgsteede and Duyn, 1989; Martin *et al.*, 1988) and Elard *et al.* (1998) examined the relative fitness of BZ-resistant and sensitive strains of *T.*

circumcincta and found no significant difference, which supports our assumption. We made similar assumptions for IVM resistance, though there is no experimental evidence supporting this assumption. We can therefore not comment on whether IVM resistant phenotypes will affect fitness, in the absence of the drugs.

In summary, this thesis provides a detailed analysis of the genetics of the potential spread of drug resistance in the lymphatic filarial worm, *W. bancrofti.* We have shown that mutations, which cause ABZ resistance in other nematodes, exist in *W. bancrofti* in West Africa, and our models predict that such resistance mutations will likely spread if the current control strategy of the GPELF is continued without monitoring and the addition of vector control, in the event that significant frequencies of resistance alleles are determined. We find that the mating structure of filarial worms may lead to a quicker spread of resistance than expected and highlight the fact that factors such as drug-efficacy assumptions, treatment coverage, number of treatments and concurrent selection for IVM resistance may impact how quickly resistance spreads. We also find that dominance and polygenicity of IVM resistance impacts the spread of ABZ and IVM resistance and that these factors must be examined in more detail in the field.

References

Barnes, E. H. Dobson, R. .J.; Barger, I. A. (1995). Worm control and anthelminthic resistance: adventures with a model. Parasitol Today, 11, 56-63.

Beech, R. N., Prichard, R. K. and Scott, M. E. (1994). Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics, 138, 103-110.

Borgsteede, F. H. and Duyn, S. P. (1989). Lack of reversion of a benzimidazole resistant strain of *Haemonchus contortus* after six years of levamisole usage. Res Vet Sci, 47, 270-272.

Carme, B. and Laigret, J. (1979). Longevity of *Wuchereria bancrofti* var. pacifica and mosquito infection acquired from a patient with low level parasitemia. Am J Trop Med Hyg, 28, 53-55.

Coles, G. C. (2002). Sustainable use of anthelmintics in grazing animals. Vet Rec, 151, 165-169.

Dobson, R. J., LeJambre, L. and Gill, J. H. (1996). Management of anthelmintic resistance: inheritance of resistance and selection with persistent drugs. Int J Parasitol, 26, 993-1000.

Driscoll M., Dean E., Reilly E, Bergholz E.. and Chalfie M. (1989). Genetic and molecular analysis of a *Caenorh.abditis elegans* beta-tubulin that conveys benzimidazole sensitivity. Cell Biol, 109, 2993-3003.

Edlind T., Visvesvara G., Li J., Katiyar S. (1994). Cryptosporidium and microsporidial beta-tubulin sequences: predictions of benzimidazole sensitivity and phylogeny. J Eukaryot Microbiol, 41, 38S.

Elard L., Comes A. M, Humbert J. F. (1996). Sequences of beta-tubulin cDNA from benzimidazole-susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Mol Biochem Parasitol, 79, 249-253.

Elard, L., Sauve, C. and Humbert, J. F. (1998). Fitness of benzimidazole-resistant and -susceptible worms of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Parasitology, 117, 571-578.

Fisher, M. C. and Viney, M. E. (1998). The population genetic structure of the facultatively sexual parasitic nematode *Strongyloides ratti* in wild rats. Proc Biol Sci, 265, 703-709.

Gyapong, J. O. and Twum-Danso, N. A. (2006). Global elimination of lymphatic filariasis: fact or fantasy? Trop Med Int Health, 11, 125-128.

Hall, A., Adjei, S. and C., K. (1996). School Health Programs. Afr Health Sci, 18 22-23.

Hall C. A., Ritchie L., Kelly J. D. (1982). Effect of removing anthelmintic selection pressure on the benzimidazole resistance status of *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep. Res Vet Sci, 33, 54-57.

Hartl D.L. and Clark, A. G. (1997). Principles of Population Genetics, third edition edn. Sinauer Associates, Inc., Sunderland, Massachusetts.

Jung, M. K., Wilder, I. B. and Oakley, B. R. (1992). Amino acid alterations in the benA (beta-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. Cell Motil Cytoskeleton, 22, 170-174.

Kaplan R. M. (2004). Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol, 20, 477-481.

Koenraadt, H., Sommerville, S. C. and Jones, A. L. (1992). Characterisation of mutations on the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequal*is and other pathogenic fungi. Mol Plant Pathol, 82, 1348-1354.

Kwa M. S. G., Veenstra J. G., Roos M. H. (1993). Molecular characterisation of beta-tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol, 60, 133-144.

Kwa M.S.G, Veenstra J.G., Roos M.H. (1994). Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol Biochem Parasitol, 63, 299-303.

Lymbery, A. J., Thompson, R. C. and Hobbs, R. P. (1990). Genetic diversity and genetic differentiation in *Echinococcus granulo*sus (Batsch, 1786) from domestic and sylvatic hosts on the mainland of Australia. Parasitology, 101, 283-289.

Martin, P. J., Anderson, N., Brown, T. H. and Miller, D. W. (1988). Changes in resistance of *Ostertagia* spp. to thiabendazole following natural selection or treatment with levamisole. Int J Parasitol, 18, 333-340.

Michael, E., Malecela-Lazaro, M. N., Simonsen, P. E., Pedersen, E. M., Barker, G., Kumar, A. and Kazura, J. W. (2004). Mathematical modelling and the control of lymphatic filariasis. Lancet Infect Dis, 4, 223-234.

Orbach, M. J., Porro, E. B. and Yanofsky, C. (1986). Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol Cell Biol, 6, 2452-2461.

Pape M., von Samson-Himmelstjerna G., Schnieder T. (1999). Characterisation of the beta-tubulin gene of *Cylicocyclus nassatus*. Int J Parasitol, 29. 1941-1947.

Prichard R. K. (2001). Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends Parasitol, 17, 445-453.

Silvestre A., Cabaret J. (2002). Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? Mol Biochem Parasitol, 120, 297-300.

Stolk, W. A., GJ, Van Oortmarssen G.J.., Pani, S. P., de Vlas, S. J.., Subramanian, S., Das, P. K. and Habbema, J. D. (2005). Effects of ivermectin and diethylcarbamazine on microfilariae and overall microfilaria production in bancroftian filariasis. Am J Trop Med Hyg, 73, 881-887.

Tisch, D. J., Michael, E. and Kazura, J. W. (2005). Mass chemotherapy options to control lymphatic filariasis: a systematic review. Lancet Infect Dis, 5, 514-523.

van Wyk, J. A. (2001). Refugia--overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort J Vet Res, 68, 55-67.

Vanamail, P., Subramanian, S., Das, P. K., Pani, S. P. and Rajagopalan, P. K. (1990). Estimation of fecundic life span of *Wuchereria bancrofti* from longitudinal study of human infection in an endemic area of Pondicherry (south India). Indian J Med Res, 91, 293-297.

Vilas, R., Paniagua, E. and Sanmartin, M. L. (2003). Genetic variation within and among infrapopulations of the marine digenetic trematode *Lecithochirium fusiforme*. Parasitology, 126, 465-472.

von Samson-Himmelstjerna G., Harder A., Pape M., Schnieder T. (2001) Feb. Novel small strongyle (Cyathostominae) beta-tubulin sequences. Parasitol Res, 87, 122-125.

WHO (1984). Lymphatic Filariasis. In WHO Technical Report Series, Vol. 702 pp. 1-110. World Health Organization, Geneva.

WHO (1992). Lymphatic Filariasis: The Disease and its Control: the fifth report of the WHO Expert Commitee on Filariasis. In WHO Technical Report Series, Vol. 821 pp. 1-80. World Health Organization, Geneva.

WHO, www.who.int/lymphatic_filariasis/disease/en/, accessed June, 8, 2006.

Yarden, O. and Katan, T. (1993). Mutations leading to substitutions at amino-acid 198 and 200 of beta-tubulin that correlate with benomyl-resistant phenotypes of field strains of *Botrytis cinerea*. Mol Plant Pathol, 83, 1478-1483.

INTRODUCTION TO APPENDIX I

In Chapter 2, we identified mutations associated with ABZ resistance in *W. bancrofti.* We genotyped microfilariae from untreated and treated patients in order to determine the presence of this mutation. Upon close examination of the data, we observed that the total population of microfilariae was not in Hardy-Weinberg equilibrium. This indicated that lymphatic filaria may not have been mating randomly, and that the population may be subdivided. Such a finding could have serious implications. Selection for recessive resistance alleles may occur faster if the population has elevated frequencies of homozygotes, and treatment failures may occur at lower overall levels of resistance allele frequencies. In addition, the sampling strategies employed in order to detect the frequencies of resistance alleles may have to take into account the levels of microfilaraemia of patients sampled in the case of non-random mating in the parasite population. The following appendix is a detailed analysis of the microfilarial population, from Burkina Faso, genotyped in Chapter 2, taking into account the number of microfilariae sampled per host, as well as the hosts' microfilarial counts. Inbreeding coefficients are calculated and implications for sampling strategies are discussed.

APPENDIX I:
HELMINTH INBREEDING AND THE DETECTION OF DRUG RESISTANCE

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To be submitted

Abstract

Genetic epidemiological studies of helminth infections of humans typically sample the worm population indirectly, through the transmission stages that gain access to the environment. The results of these studies should be interpreted with caution, as the allele frequency and genotype distribution estimates obtained may differ greatly from those of the underlying adult worm population. Analysis of data collected for the lymphatic filarial parasite *Wuchereria bancrofti*, investigating a single polymorphic locus (a genetic change at position 200 on β-tubulin, known to cause benzimidazole resistance in nematodes of farmed ruminants), was conducted in microfilariae obtained from West African patients before and after the introduction of mass drug administration with albendazole plus ivermectin combination therapy. An individual-based stochastic model was developed to show that a wide range of microfilarial allele and genotype frequencies can be sampled from a single adult worm population, suggesting that appropriate theoretical null models are required in order to interpret adequately the results of genotyping solely transmission stages. Wright's hierarchical *F*-statistic was used to investigate the population structure in *W. bancrofti* microfilariae and showed significant deficiency of heterozygotes compared to the Hardy-Weinberg equilibrium. Results indicate strong parasite genetic differentiation between hosts, and a high degree of helminth assortative mating, which are unlikely to have occurred from population subdivision, helminth overdispersion, parasite mating behaviour, or the sampling scheme employed. If the allele conferring drug resistance were recessive, parasite inbreeding would contribute to substantial loss of anthelmintic efficacy, and eventually to treatment failure. Genetic epidemiological surveys of helminth populations should consider the parasite's biology when designing sampling schemes, in order to maximise the accuracy of allele frequency estimates and identify genetic changes indicative of selection under chemotherapeutic pressure.

6.1. Introduction

In recent years there has been a dramatic increase in the use of mass drug administration (MDA) to reduce the morbidity associated with helminth infections of humans (Lammie *et al.*, 2006), increasing the probability that anthelmintic resistance may become a public health concern in the future. One such annual MDA programme is the Global Programme to Eliminate Lymphatic Filariasis (GPELF) which, in 2005, treated over 145 million people with albendazole (a broad spectrum benzimidazole) in combination with either ivermectin or diethylcarbamazine (WHO, 2006). GPELF targets mainly *Wuchereria bancrofti*, the most widely distributed of the human filariae.

Evidence from the spread of anthelmintic resistance in veterinary nematodes indicates that once resistance reaches phenotypically detectable levels it may be too late to prevent widespread treatment failure (Roos *et al.*, 1995). Sensitive molecular assays are required to detect the presence of anthelmintic resistance before it becomes a major public health concern. Surveys of helminth parasites of humans are being conducted to establish whether genetic changes at certain polymorphic loci, which have been associated with resistance to the same or similar anthelmintic drugs in veterinary helminths, are present and subject to detectable selection under chemotherapeutic pressure (Albonico *et al.*, 2004; Ardelli and Prichard, 2004; Ardelli *et al.*, 2005; Eng and Prichard, 2005; Schwab *et al.*, 2005; Ardelli *et al.*, 2006a, 2006b; Eng *et al.*, 2006). A phenylalanine to tyrosine substitution at position 200 (TYR 200) on the β-tubulin isotype 1 molecule is thought to be associated with benzimidazole (BZ) resistance, and has been identified in a number of helminth parasites of farmed ruminants including *Haemonchus contortus* (Kwa *et al.*, 1993; Kwa *et al.*, 1994), *Cooperia oncophora* (Njue and Prichard, 2004), and *Teladorsagia circumcincta* (Elard and Humbert, 1999; Silvestre and Cabaret, 2002), as well as, worryingly, in *W. bancrofti* (Schwab *et al.*, 2005).

Parasite allele frequency can differ between infrapopulations (the populations of parasites within individual hosts) due to the ecology of the infection or through the random nature of infection events (all groups may have an equal probability of having a rare allele, but actual numbers may vary between groups by chance). Helminth parasites

have a particularly subdivided population structure as adult worms are confined to live within their definitive host, and are only able to mate with other worms that belong to the same infrapopulation. Parasite genetic differentiation between hosts has been observed in a number of helminth species (Criscione *et al.*, 2005). Infrapopulation genetic differentiation will cause a reduction in the frequency of heterozygote offspring, a principle known as the Wahlund effect (Hartl and Clarke, 1989). Other facets of a species' biology may also influence parasite genotype distribution, such as parasite assortative mating (when mate choice is determined by phenotype).

Alleles conferring drug resistance, including the TYR200 genetic change, which confers BZ resistance, are thought to be recessive in a number of nematode species (Elard and Humbert, 1999; Le Jambre *et al.*, 1999; Prichard, 2001), making heterozygote worms susceptible to treatment and the resistant phenotype unstable from generation to generation. If an allele conferring drug resistance is partially recessive, excess parasite homozygosity will increase the probability that a resistance allele will survive treatment. The degree of parasite genetic differentiation among hosts can be quantified using F_{ST} (or related analogues; see Criscione *et al.* (2005) and references therein).

The adult stages of most parasitic helminths of humans cannot be obtained routinely for direct examination or investigation, so genetic epidemiological surveys resort to sampling transmission stages, i.e. those life-stages that gain access to the environment to be transmitted to and from hosts or through vectors (Fisher and Viney 1998; Paterson *et al.*, 2000; Brouwer *et al.*, 2001; Curtis *et al.*, 2002; Schwab *et al.*, 2005; Shrivastava *et al.*, 2005). The majority of studies investigating the spread of anthelmintic resistance in helminth infections of humans will have to rely on sampling transmission stages as adult worms are often difficult to obtain. The results of these surveys should be interpreted with caution, as the underlying allele frequency of the adult worm population may in fact differ substantially from the allele frequency of the sampled transmission stages. Variations in transmission stage allele frequency and genotype distribution could be generated randomly or be a product of the parasite's spatial structure and life-history traits. For example, population subdivision will randomly cause variation in adult worm

allele frequencies between hosts at low parasite densities. Filarial parasites have separate sexes and are thought to be highly polygamous, which may accentuate the variability in microfilarial allele frequency, e.g. a rare allele may be highly over-represented in the subsequent generation if a male worm with this allele inhabits a host harbouring females but no other males. In addition, the inherent random sampling of gametes during sexual reproduction, and the overdispersed distribution of parasite numbers among hosts (Anderson and May, 1992) may cause variations in allele frequency and genotype distribution from generation to generation.

This paper analyses population genetic data collected for a study by Schwab *et al.* (2005) who identified the presence of the TYR200 change of the β-tubulin gene in populations of *W. bancrofti*. Firstly, the extent of parasite inbreeding is estimated from *W. bancrofti* microfilarial samples taken from patients in Burkina Faso, West Africa. Samples were obtained from different villages, some of which had received a single round of MDA with ivermectin and albendazole, under the auspices of the GPELF. Secondly, an individual-based stochastic model is presented which simulates microfilarial genetic diversity from adult worm allele frequencies. The model generates sample allele and genotype frequencies using the same number of hosts, and the same number of microfilariae per host as in Schwab *et al.* (2005). Finally, this model is used to assess the likely range of adult worm allele frequencies which would give rise to the observed microfilarial data and to identify whether observed genotype distributions are likely to be generated by chance. We discuss the implications of our results in terms of the development and detection of anthelmintic resistance.

6.2. Materials and Methods

6.2.1 Sampled Data

Table 1 summarises the data collected for the study by Schwab *et al.* (2005) and indicates the number of microfilariae and hosts sampled. In some hosts it was possible to genotype only a few microfilariae; thus their underlying infrapopulation allele frequencies are highly uncertain. Results are grouped according to parasite treatment history. The average resistance allele frequency of microfilariae from untreated and treated hosts was 0.26 and 0.60, respectively (Schwab *et al.*, 2005). Parasite heterozygosity in each of the villages indicates a deviation from the Hardy-Weinberg Equilibrium (HWE), revealing a strong deficit of heterozygotes.

Table 6.1: Summary of the genetic survey conducted on *Wuchereria bancrofti* microfilariae from Burkina Faso of genetic changes at the β-tubulin locus associated with benzimidazole resistance (in nematodes of ruminants). Results were presented by Schwab *et al.* (2005). The range of microfilarial samples obtained per host is given in brackets. The expected microfilariae heterozygosity according to the Hardy-Weinberg equilibrium is given in square brackets

Village	No. hosts sampled	Mean no. of microfilariae genotyped per host	Mean microfilaraemia (per 20μl blood)	Sample resistance allele frequency, $\hat{q}^M$	Sample and [expected] heterozygosity
Untreated villages					
Tangonko	16	9.6 (1, 15)	323 (162, 703)	0.28	0.20 [0.40]
Badongo	14	6.6 (1, 10)	212 (60, 845)	0.23	0.24 [0.35]
Villages that had received one round of chemotherapy (albendazole + ivermectin)					
Perigban	13	8.5 (3, 12)	35 (18, 86)	0.62	0.27 [0.47]
Gora	1	7	186	0.29	0.29 [0.41]

This paper refers to two different types of allele frequency: (1) underlying allele

frequency, q^l, the allele frequency of the entire parasite population of a given locality, and (2) the parasite allele frequency within the host population that is sampled, ${}^H q^l$. The superscript l denotes the parasite life-stage under investigation, be it microfilariae ($l = M$) or adult worms ($l = W$). The sampled (observed) allele frequency, $\ddot{\varphi}^M$, may not correspond to the true underlying allele frequency, q^l, (that needs to be estimated) either because the hosts sampled are not representative of the whole host population, or because the parasites genotyped do not represent adequately the allele frequency in the host.

6.2.1 Estimating Parasite Inbreeding

By genotyping transmission stages before they leave the definitive host prior to the introduction of mass chemotherapy, insight can be gained into the different causes of microfilarial excess homozygosity. For a single locus trait, the genotype frequency of an infrapopulation's offspring is dependent on the allele frequencies of the male and female worms reproducing within the host, and not on their genotype distribution (assuming male and female adult worms have the same underlying allele frequency). If it is assumed that the number of microfilariae produced, their survival, and their probability of being sampled is independent of their genotype, it can be concluded that deviation from the HWE is a result of non-random mating. If the locus being investigated is not under selection, the excess microfilarial homozygosity will most likely be the result of infrapopulation genetic differentiation or assortative parasite mating within hosts. Genotyping transmission stages would allow the relative contributions of each of these two sources of inbreeding to be estimated, as excess microfilarial homozygosity within each host will result from assortative mating. Filarial parasites are unable to self-fertilise or reproduce asexually, both life-history traits that are thought to influence parasite genotype distribution (Prugnolle *et al.*, 2005). Instead, they exhibit separate sexes (dioecious) and are thought to be highly polygamous (Schulz-Key and Karam, 1986).

The Wright's hierarchical *F*-statistic is used to investigate the correlation of parasite genes within and between human hosts (Fisher and Viney 1998; Paterson *et al.*,

1998; Brouwer *et al.*, 2001; Curtis *et al.*, 2002). It is assumed that the infrapopulation is the first hierarchical group in the parasite population, and F_{IS} is defined as the correlation of genes between microfilariae within the infrapopulation; F_{ST}^{H}, as the correlation of microfilarial genes between different hosts living in the same village; F_{ST}^{V}, as the correlation of microfilarial genes between different villages within the overall microfilarial population; and F_{IT}, as the correlation of genes between individual microfilariae relative to the overall microfilarial population of the region. The different inbreeding terms introduced are summarised in Table 2. A value of $F_{IS} > 0$ points towards adult worm assortative mating, $F_{ST}^{H} > 0$ indicates variation in worm allele frequency between hosts, and $F_{ST}^{V} > 0$ suggests differences in the worm allele frequency between villages. The same statistical frameworks used to estimate Wright's *F*-statistic may be employed, taking into account variable sample sizes (Weir, 1996). Estimates of the 95% confidence intervals for F_{IS}, F_{ST}^{H}, F_{ST}^{V} and F_{IT}, were generated by bootstrapping simultaneously worms within each host and bootstrapping over hosts within each village (Efron and Tibshirani, 1993). The village of Gora (Table 6.1) was removed from the *F*-statistic analysis since only one host was sampled in this village, making the underlying allele frequency highly uncertain.

Table 6.2: The extension of Wright's *F*-statistic to represent the hierarchical population structure of obligatory parasites of humans, exemplified in this paper with *Wuchereria bancrofti* (see text)

Symbol	Definition
F_{IT}	Correlation of parasite genes between individual worms relative to the overall worm population (total inbreeding coefficient)
F_{IS}	Correlation of genes between individual worms within the host infrapopulation (assortative mating)
F_{ST}^{H}	Correlation of parasite genes between different infrapopulations within the same host population (village) (parasite genetic differentiation between hosts within villages)
F_{ST}^{V}	Correlation of parasite genes between different host populations within the overall parasite population (parasite genetic differentiation between villages)

6.2.2 Modelling the allele frequency and genotype distribution of microfilariae

A dioecious adult worm helminth population with a 1:1 male to female ratio was randomly generated for a given mean number of worms per host and degree of parasite overdispersion (as determined by the k parameter of the negative binomial distribution). Each adult worm infrapopulation was randomly allocated an allele frequency, as regression analysis of pre-treatment data did not detect any significant relationship between the host's putative resistance allele frequency and microfilarial burden. The variance in adult worm infrapopulation allele frequency can be generated using the probability function of the beta distribution φ, using the equation,

$$\varphi\left({}^{H}q_i^W \mid F_{ST}^H, q^W\right) = \frac{\Gamma\left[\left(F_{ST}^H\right)^{-1} - 1\right] {}^{H}q_i^{W\left[\left(F_{ST}^H\right)^{-1}-1\right]q^W - 1} \; {}_{H}p_i^{W\left[\left(F_{ST}^H\right)^{-1}-1\right]p^W - 1}}{\Gamma\left\{\left[\left(F_{ST}^H\right)^{-1} - 1\right]q^W\right\} \Gamma\left\{\left[\left(F_{ST}^H\right)^{-1} - 1\right]p^W\right\}} \qquad 6.1$$

where ${}^{H}q_i^W$ is the resistance allele frequency of adult worms in a single host infrapopulation (i.e. in host *i*); ${}^{H}p_i^W = \left(1 - {}^{H}q_i^W\right)$; F_{ST}^H quantifies the variability in allele frequency between hosts estimated from data (Table 6.2); q^W is the true underlying resistance allele frequency; $p^W = \left(1 - q^W\right)$, and Γ denotes the gamma distribution (Wright, 1969; Porter, 2003).

A microfilarial population was generated for each host *i* according to the size and allele frequency of the adult worm infrapopulation. Worms were assumed to be highly polygamous; implying that if only one male parasite were present within a host, all fertile females within that infrapopulation would be mated. The number of microfilariae produced by each parasite infrapopulation was assumed to be proportional to the number of fertilised females within that host. It was also assumed that gametes separate independently and re-assort according to the degree of assortative mating (F_{IS}). The probability with which a microfilaria within host *i*, will be of genotype *j* is denoted ϑ_i^j, and is given by the equations,

$$\vartheta_i^{SS} = \left({}_{male}{}^{H}p_i^W \; {}_{fem}{}^{H}p_i^W\right)\frac{\left[\left(p^W\right)^2\left(1 - F_{IS}\right) + p^W F_{IS}\right]}{\left(p^W\right)^2} \qquad 6.2$$

$$\vartheta_i^{SR} = \left({}_{male}{}^{H}q_i^W \; {}_{fem}{}^{H}p_i^W + {}_{male}{}^{H}p_i^W \; {}_{fem}{}^{H}q_i^W\right)\frac{\left[2p^W q^W\left(1 - F_{IS}\right)\right]}{2p^W q^W} \qquad 6.3$$

$$\vartheta_i^{RR} = \left({}_{male}{}^{H}q_i^W \; {}_{fem}{}^{H}q_i^W\right)\frac{\left[\left(q^W\right)^2\left(1 - F_{IS}\right) + q^W F_{IS}\right]}{\left(q^W\right)^2} \qquad 6.4$$

where ${}^{H}_{male}q_i^W$ and ${}^{H}_{fem}q_i^W$ are, respectively, the resistance allele frequency in the male and female adult worms within host *i*, and ${}^{H}_{male}p_i^W = \left(1 - {}^{H}_{male}q_i^W\right)$ and ${}^{H}_{fem}p_i^W = \left(1 - {}^{H}_{fem}q_i^W\right)$ are the susceptible allele frequencies generated from equation (6.1). To allow random stochastic fluctuations in genotype distribution, we use the fact that the actual number of microfilariae in host *i* with genotype *j* follows a binomial distribution, with the number of trials being equal to the number of microfilariae produced by host *i*, and with genotype probability equal to ϑ_i^j.

Microfilarial allele frequencies and genotype distributions were generated by sampling a specific number of microfilariae from the generated hypothetical population according to the sampling scheme used in Schwab *et al.* (2005). The exact numbers of samples taken from each of the 30 hosts were: 11, 10, 15, 9, 11, 9, 13, 10, 10, 7, 10, 10, 7, 1, 11, 9, 1, 7, 4, 1, 10, 9, 8, 6, 4, 6, 9, 10, 10, 8, for a total of 246 microfilariae. Regression analysis of pre-treatment data had indicated that the number of samples taken from each host by Schwab *et al.* (2005) was independent of host microfilaraemia and host allele frequency, allowing the number of microfilariae sampled per host to be randomly allocated. The program code for the simulations implemented was written in C++ and run 100,000 times, with each run generating a new helminth population and genotype distribution.

The model was parameterised for the village of Tangonko, Burkina Faso, which had an initial prevalence of microfilaraemia of 25%. The deterministic model EPIFIL (see original formulation in Norman *et al.* (2000)) was used to estimate mean adult worm burden from observed microfilarial counts, giving a mean adult worm burden of 3.5 host^{-1}. The degree of adult worm overdispersion was estimated from the recorded microfilarial prevalence (taken here as a proxy for the prevalence of adult worms producing microfilariae) and the mean adult worm burden, using the prevalence vs. intensity relationship that derives from assuming a negative binomial distribution of worms among hosts (Anderson and May, 1992), yielding a *k* value of 0.07. The model

outlined above will only be valid for comparisons against the pre-treatment data, since chemotherapy is known to impede microfilarial production and / or survival (Tisch *et al.*, 2005).

The null model assumes that mating is random between male and female worms within each infrapopulation and that resistance alleles are randomly distributed across hosts, i.e. $F_{IS} = F_{ST}^{H} = F_{ST}^{V} = F_{IT} = 0$. Results of the inbreeding analysis can be incorporated into the individual-based model described in equations (6.1) to (6.4) to explore the range of adult worm resistance allele frequencies which can give rise to the observed microfilarial data.

6.3. Results

The generated microfilarial genotype distribution was found to deviate from the HWE. The results of the Wright's hierarchical *F*-statistic analysis are shown in Figure 6.1. Villages with no history of mass anthelmintic chemotherapy have an overall inbreeding coefficient of $F_{IT} = 0.44$ (indicating strong inbreeding). Fifteen percent of the microfilariae were found to be homozygous for the putative resistance-associated genetic (TYR 200) change, an estimate 2.3 times higher than would be expected in a random mating parasite population. Results indicate a significant amount of genetic differentiation in worm allele frequency among the host population. Infrapopulation allele frequency, ${}^{H}q_i^W$, varied from 0 to 0.77 in the villages with no history of treatment, increasing microfilarial homozygosity by 60%. The results suggest a degree of assortative mating within hosts measured by $F_{IS} = 0.28$, although the study had insufficient power for this to become statistically significant (95% confidence intervals for this estimate included zero). No difference was observed in the microfilarial allele frequency between the two villages at baseline ($F_{ST}^V \approx 0$). A similar degree of parasite inbreeding was observed in those villages that had received one round of MDA.

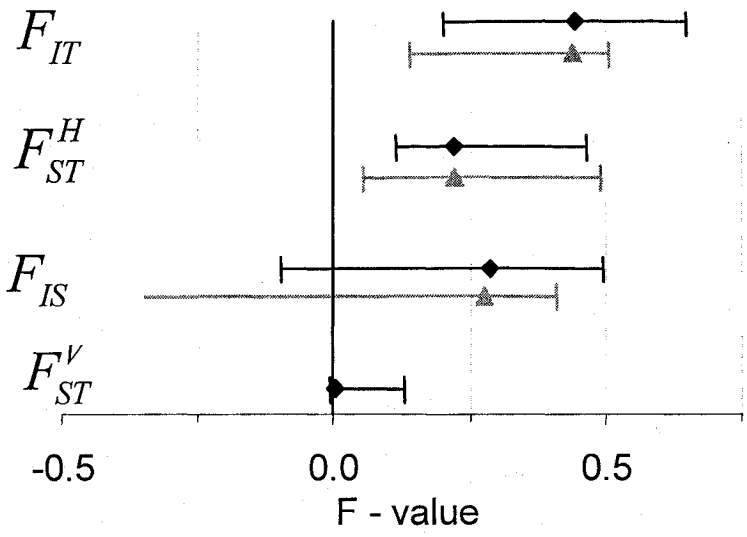

Figure 6.1. Estimates of Wright's *F*-statistics for the pre-treatment villages (black diamonds) and for the village of Perigban (grey triangles), which received one round of chemotherapy (albendazole + ivermectin). The error bars are the 95% confidence intervals. F_{IT} estimates the total degree of parasite inbreeding; F_{IS} describes the level of assortative mating within the infrapopulation; F_{ST}^{H} shows the variation in microfilarial allele frequency within the host subpopulation (village), and F_{ST}^{V} highlights the difference in allele frequencies between the villages under investigation (only one post-treatment village was investigated, so no F_{ST}^{V} value is given).

Parasite inbreeding increases the range of underlying adult worm allele frequencies, q^{W}, which can give rise to the observed microfilarial allele frequency of 0.26 (Figure 6.2). Results from the null model, where mating was random and resistance alleles were randomly distributed amongst hosts, indicate that q^{W} in the untreated villages of Tangonko and Badongo could range from 0.21 to 0.32. If we use the excess inbreeding estimate reported in Figure 6.1, then model simulations suggest that q^{W} could range from 0.18 to 0.37. The larger the infrapopulation size (i.e. the larger the parasite population size or the stronger the degree of parasite overdispersion), the more accurately the underlying microfilarial allele frequency will mirror q^{W}.

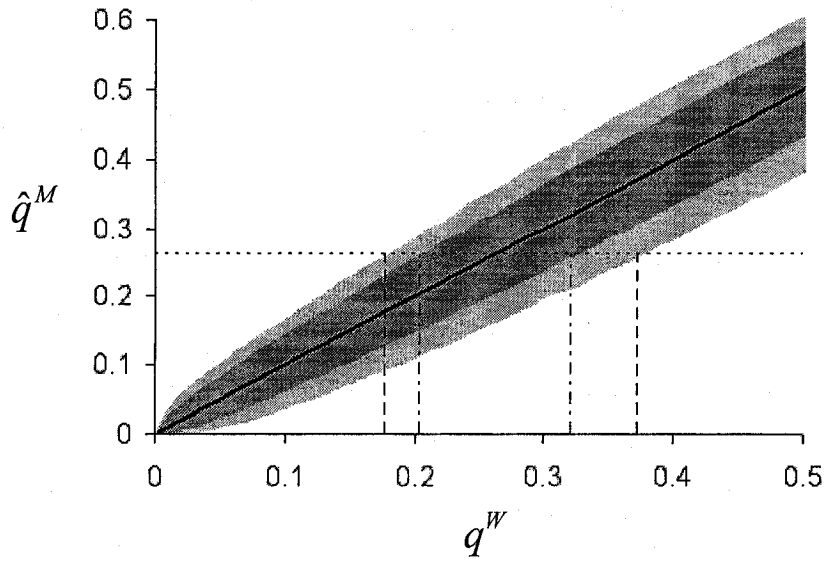

Figure 6.2. The impact of inbreeding on the relationship between the sample microfilarial allele frequencies $\hat{q}^M$ and the (inferred) underlying adult worm allele frequency, q^W. The figure shows 95% confidence intervals for a population with no excess inbreeding (the null model, dark grey shaded area), and a population with the observed levels of inbreeding ($F_{IS} = 0.28$, $F_{ST}^H = 0.22$, light grey shaded area). Simulations are based on the same sampling scheme used by Schwab *et al.* (2005). The thick black solid line indicates the mean result for both models. The observed pre-treatment microfilarial allele frequency (black thin, horizontal dotted line) was compared to simulation results to indicate the possible range of adult worm allele frequencies which could have given rise to the West African data. The null model (black vertical dotted-dashed lines) indicated values of q^W ranging from 0.21 to 0.32 compared to the inbred model ($F_{IS} = 0.28$, $F_{ST}^H = 0.22$, black vertical dashed lines), which gave values of q^W between 0.18 and 0.37.

The microfilarial genotype diversity model indicates that the observed homozygosity is unlikely to be solely a result of genetic sampling, demographic stochasticity, population subdivision, or the sampling scheme employed. Figure 6.2 indicates the range of likely microfilarial genotype distributions that can be generated from a given q^W value using the null (random) model. The observed excess homozygosity in the untreated villages was greater than the 95% confidence interval estimates generated by the null model. It is interesting to note the wide range of microfilarial genotype distributions that can be generated by the null model.

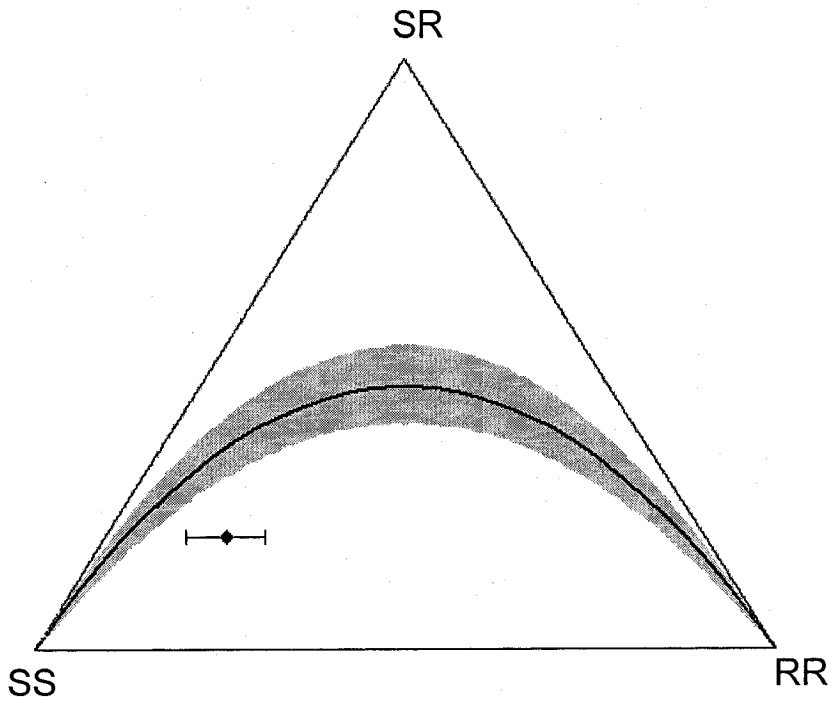

Figure 6.3. De Finetti diagram (Hartl and Clark, 1989) showing the genotype distribution of *W. bancrofti* microfilariae generating from a given underlying adult worm allele frequency, q^W, taken from villages prior to the introduction of chemotherapy. The black diamond represents the value originating from the observed data (with q^W =0.26, and $F_{IT} = 0.44$), and the error bars indicate the uncertainty in genotype distribution stemming from the values of q^W that were estimated from the null (random) model in Figure 6.2. *R* indicates the allele coding for tyrosine in the position 200 of β-tubulin that is associated with (recessive) benzimidazole (BZ) resistance in nematodes of livestock, and *S* denotes the allele (coding for phenylalanine) indicative of BZ susceptibility. The solid-black curve represents the Hardy-Weinberg equilibrium (HWE). The null model generating microfilarial populations was used to investigate the range of sample microfilarial genotype distributions that could be obtained from a population exhibiting no excess inbreeding (i.e. assuming that the underlying adult parasite population would have values of $F_{IS} = F_{ST}^H = F_{ST}^V = F_{IT} = 0$). Simulations mimic the same sampling scheme described in Schwab *et al.* (2005). The observed microfilarial genotype distribution falls outside the 95% confidence interval range (grey shaded area surrounding the HWE curve) generated by the null model, despite the uncertainty in the q^W estimates, indicating strong parasite

inbreeding even before introduction of antifilarial combination therapy.

High genetic differentiation between hosts reduces the prevalence of parasites homozygous for the putative resistance-associated genetic change (Figure 6.4). Despite the large increase in microfilarial homozygosity attributable to parasite inbreeding, there is only a modest increase in the prevalence of resistance homozygotes. High parasite overdispersion reduces the number of hosts who are microfilaria-positive and concentrates the resistance genotypes into a smaller proportion of hosts. High parasite assortative mating and infrapopulation genetic differentiation increases the number of hosts (and the number of samples per host) that needs to be sampled in order to quantify accurately the resistance allele frequency in the microfilarial population. To illustrate this, the model is used to investigate the minimum number of hosts, and the overall number of samples necessary to detect a given resistance allele frequency (Figure 6.5). Results indicate that the observed level of parasite inbreeding dramatically increases the number of hosts that need to be sampled to be 95% confident of detecting the rare allele. The sampling scheme used within Figure 6.5 assumes that the number of parasites genotyped per host is weighted by the host's microfilariae load.

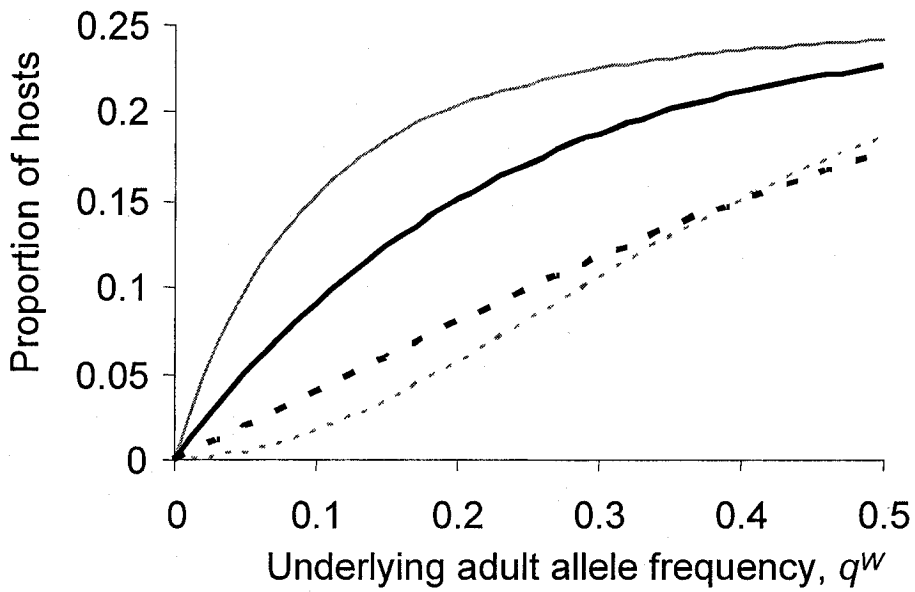

Figure 6.4. The impact of inbreeding on the relationship between the mean proportion of hosts harbouring microfilariae with one or two copies of the resistance allele and the (assumed) underlying adult worm allele frequency, q^W. The figure shows the proportion of hosts who have microfilariae with the resistance allele (solid lines), as well as the proportion who have microfilariae which are homozygous for resistance (broken lines). Model outcomes are compared for two hypothetical parasite populations; the first (thin grey lines) without excess inbreeding (generated by the null model), and the second (thick black lines) with the levels of inbreeding ($F_{IS} = 0.28$, $F_{ST}^H = 0.22$) observed in the Burkina Faso data. Simulations used the same sampling scheme described in Schwab *et al.* (2005) and assume an overall microfilarial prevalence of ~25% (see text).

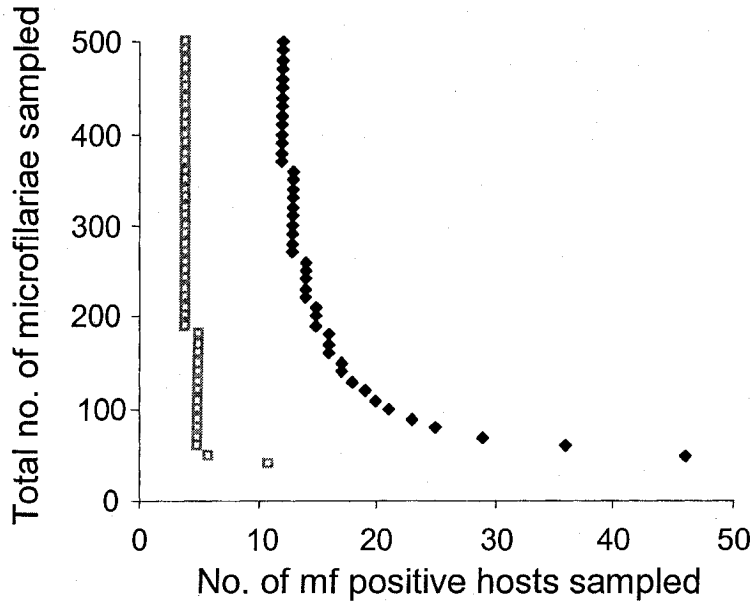

Figure 6.5. The impact of helminth inbreeding on the minimum number of microfilaria-positive hosts who should be sampled and the minimum number of microfilariae that should be genotyped to be 95% confident of detecting at least one rare allele. A randomly mating population ($F_{IS} = F_{ST}^{H} = 0$, grey open squares) is compared to an inbred population ($F_{IS} =$ 0.28 and $F_{ST}^{H} =$0.22, closed black diamonds). The underlying adult worm allele frequency of both populations is set at $q^{W} =$0.05. Each data point represents 100,000 runs of the stochastic model generating microfilarial populations. The number of microfilariae analysed per host is proportional to host microfilaraemia.

The consequences of parasite assortative mating and genetic differentiation between hosts will depend on the allele frequency and the relative dominance of the resistance allele. If the resistance allele is recessive, helminth inbreeding will dramatically increase the probability that a parasite will survive anthelmintic treatment. This is evident from Figure 6.6 which shows the influence of parasite inbreeding on the relative proportion of resistant genotypes for a given allele frequency. With a completely recessive resistance allele at a frequency of 0.05, the degree of inbreeding within the *W. bancrofti* population reported here, would on average increase the number of worms with the homozygote resistance genotype nine-fold. Conversely, if the resistance allele were dominant, helminth inbreeding would reduce the probability that a parasite will survive

chemotherapy, as fewer worms will have the resistant allele (the deficiency of heterozygous parasites caused by parasite inbreeding will be greater than the increase in resistant homozygous worms).

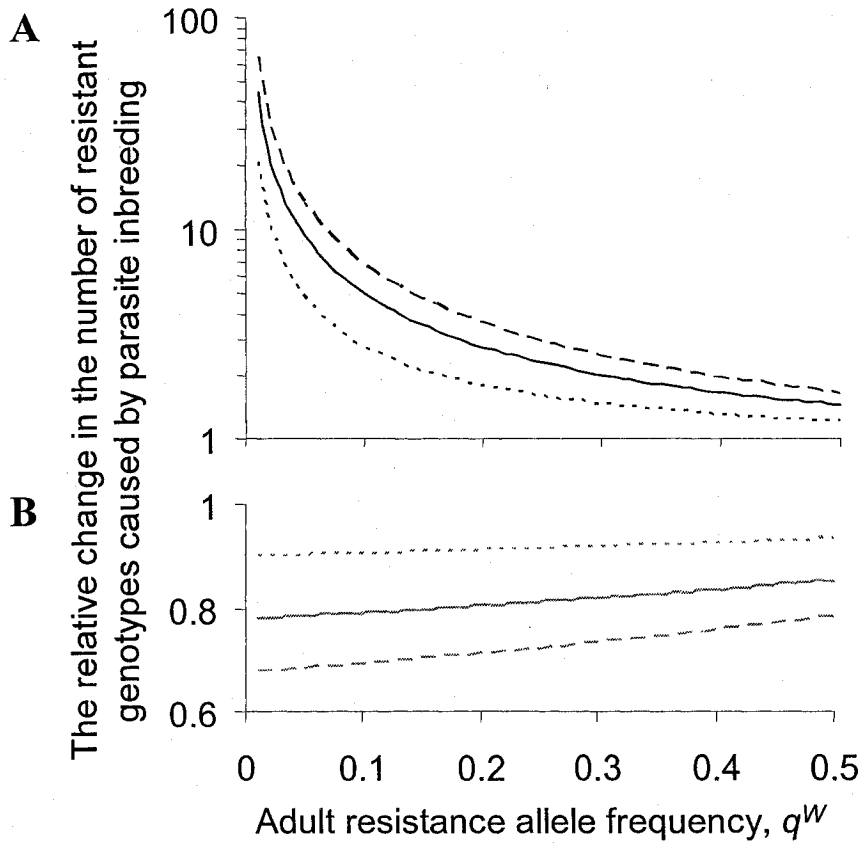

Figure 6.6. The number of resistant genotypes in an inbred population relative to that in a population at HWE. Results are shown for different resistance allele frequencies. The resistance allele is either recessive (**A**), black lines, or dominant (**B**), grey lines. The inbreeding coefficients are those estimated in the *Wuchereria bancrofti* microfilarial population reported in Figure 6.1: mean result ($F_{IT} = 0.44$, solid line); upper 95% confidence limit ($F_{IT} = 0.65$, dashed line); lower 95% confidence limit ($F_{IT} = 0.20$, dotted line). The relative change in the number of resistant genotypes caused by parasite inbreeding is estimated as $\frac{\left(q^2 F_{IT} + qF_{IT}\right)}{q^2}$ in (**A**) and $\frac{\left[1-\left(p^2 F_{IT} + qF_{IT}\right)\right]}{\left(1-q^2\right)}$ in (**B**).

6.4. Discussion

The degree of *W. bancrofti* excess homozygosity reported in this paper falls outside the range of values generated by the null model, indicating a significant degree of parasite inbreeding, present even before any MDA was implemented. This result highlights the crucial importance of developing sound theoretical null models that enable helminth population genetics data to be interpreted adequately (Prugnolle *et al.*, 2005). These models should take into account the uncertainty in result outcomes, given the sampling scheme employed and the life-history traits of the parasite. The null model described in this paper generates a wide range of microfilarial allele frequencies and genotype distributions indicating that caution should be exercised when interpreting results obtained by sampling transmission stages. The null model indicates that sampling transmission stages would cause estimates of the underlying adult worm allele frequency to be highly variable. This would make it harder to detect significant changes in the parasite genome with time after introduction of chemotherapeutic pressure.

Producing a null model to assess the range of adult populations that could give rise to the microfilarial population within the treated villages is complex and beyond the scope of this paper. A dynamic, full transmission model would be required that takes into account the pharmacodynamic properties of ivermectin and that of the albendazole plus ivermectin drug combination, as the effects of chemotherapy will influence microfilarial genetic diversity for a number of years after chemotherapy. Large changes in microfilarial resistance allele frequency may not reflect such substantial changes in the adult worm genome.

The results presented within this paper regarding the metapopulation dynamics of bancroftian filariasis stem from the analysis of a single nucleotide polymorphism in one gene. Further surveys, using multiple polymorphic loci, are required to confirm our findings of a high degree of parasite genetic differentiation between hosts (Anderson, 2001). The accuracy of the model developed to derive microfilarial genetic diversity is limited by uncertainties regarding the biology of *W. bancrofti*. Results are dependent on our current ability to mimic adult worm burden and its distribution among hosts.

Limitations inherent in the EPIFIL model, the presence of amicrofilaraemic yet circulating filarial antigen positive infections, and possible heterogeneity in host immune responses will make adult worm burden estimates highly uncertain from microfilarial prevalence and intensity data. The relationship between the number of adult filariae and the rate of microfilarial production is likely to be complex and may depend on the immune responses elicited during the infection. Our conclusions are based on the adequacy of the null model, which may be improved by the inclusion of further biological detail. For example, recent evidence suggests a possible association between β-tubulin genotype in the related filarial parasite, *Onchocerca volvulus*, and female worm fertility (Bourguinat *et al.* 2006), suggesting a cost of resistance. Whilst the same gene has been analyzed in the current study, it is not known whether a similar relationship between genotype and fertility applies to *W. bancrofti*.. Although no differences were seen in genotype frequency between the two pre-treatment villages studied, additional baseline surveys would be required before firm conclusions regarding the true underlying frequency of the TYR200 genetic change in pre-treatment *W. bancrofti* populations can be drawn. This emphasizes the importance of conducting genetic studies of appropriate molecular markers of selection under treatment pressure before (and during) implementation of large-scale MDA.

Notwithstanding the fact that the *F*-statistic provides a phenomenological tool rather than a mechanistic measure of inbreeding (and therefore does not describe the biological processes generating excess homozygosity), we proceed to propose some likely causes for the strong degree of inbreeding identified in *W. bancrofti*, as well as the implications that this may have for the development and detection of anthelmintic resistance.

6.4.1. Assortative mating

Our results suggest that adult *W. bancrofti* do not mate randomly within the infrapopulation. This is in agreement with ultrasonography studies that show adult parasites congregating in 'worm nests' along lymphatic vessels, which remain stable over time (Dreyer *et al.*, 1994). Spatial heterogeneity within the host may produce multiple

reproducing populations within each infrapopulation, which would increase host microfilarial homozygosity. Prior anthelmintic treatment may also increase assortative mating, depending on the selective advantage the TYR200 genetic change may have on the adult worm following drug treatment.

6.4.2. Parasite genetic differentiation between hosts

The degree of genetic differentiation in the parasite infrapopulation can shed insight into the microepidemiology of parasite transmission (Nadler, 1995; Anderson, 2001; Théron *et al.*, 2004; Criscione and Blouin, 2005). The metapopulation transmission dynamics of *W. bancrofti* will depend on the transmission efficiency and biting behaviour of the mosquito vector. *Anopheles gambiae sensu stricto* and *An funestus* are thought to be the main vectors of *W. bancrofti* in Burkina Faso (Gyapong *et al.*, 2002). Hosts can acquire multiple L3 larvae during the same bite. Although density-dependent processes are known to operate on the uptake and development of *W. bancrofti* in *An. gambiae*, infected vectors will regularly transmit multiple related L3 larvae simultaneously (Snow and Michael, 2002). Other mosquito vectors of *W. bancrofti* have even greater vector competence. For example, up to 32 L3 larvae were recovered from an experimental host after it was bitten by a single *Culex quinquefasciatus* (Gasarasi, 2000), the vector in East Africa. Mark-recapture studies and bloodmeal analysis indicate that various mosquito species appear to have high site fidelity, regularly biting multiple members of the same household (McCall *et al.*, 2001; Michael *et al.*, 2001). These aspects of *W. bancrofti* transmission increase the likelihood that a host will be infected with closely related parasites and will contribute to the observed genetic differentiation. Genetic metapopulation models have highlighted how the spread of rare recessive genes are promoted by hosts accumulating multiple related infections simultaneously (Cornell *et al.*, 2003).

If anthelmintic treatment kills adult worms, then MDA campaigns will increase infrapopulation genetic homogeneity, as those parasites within treated hosts which survive treatment may have a higher resistance allele frequency than those harboured within untreated hosts. In Burkina Faso, lymphatic filariasis is treated with albendazole

and ivermectin. Evidence indicates that the albendazole plus ivermectin combination has some macrofilaricidal effect, mainly due to the addition of albendazole (Tisch *et al.*, 2005), suggesting that possible albendazole resistance may act to accentuate parasite inbreeding in villages that have been repeatedly treated. It is possible that some of the excess homozygosity observed in the untreated villages may have resulted from individual members of the community seeking, for instance, treatment for geohelminth infection prior to the introduction of GPELF.

6.4.3. The spread of anthelmintic resistance

Population subdivision and assortative mating will influence the outcomes of selection under chemotherapeutic pressure in different ways, depending on the initial frequency of the allele under selection and the ecology of the infection. Before the rate of spread of drug resistant parasites can be reliably estimated, greater knowledge regarding the number, the linkage, the dominance, and the possible negative pleiotropic effects of putative resistance allele(s), is required together with accurate information on the pharmacodynamic properties of the drugs administered singly and / or in combination (Schwab *et al.*, in press).

If the resistance allele is recessive and it has a low initial frequency, inbreeding will increase parasite homozygosity and as a result, the spread of drug resistant worms across the parasite population (Schwab *et al.*, 2006). Parasite genetic differentiation between hosts will also increase the spread of resistance even when the resistance allele is initially present at a very low frequency, as it increases the probability that male and female resistant worms will inhabit the same infrapopulation.

6.4.4. The detection of anthelmintic resistance

The operation of a strong degree of parasite genetic differentiation between hosts reduces the prevalence of infection with drug resistant parasites and would therefore increase the number of hosts that should be sampled to detect and quantify anthelmintic

resistance reliably. Even at high resistance allele frequencies some hosts will have no phenotypic signs of resistance, particularly if the resistance allele is recessive. With this in mind, genetic epidemiological surveys of helminth parasites should carefully consider the sampling scheme they employ in order to maximise the accuracy of allele frequency estimates. The mathematical model developed in this paper can be used to derive sample size calculations, taking into consideration the approximate degree of parasite inbreeding, the resistance allele frequency, the intensity of infection, and the degree of parasite overdispersion.

6.4.5. Parasite elimination

For human helminth infections, the importance of parasite genetic differentiation between hosts stretches beyond population genetics and will influence the outcomes of parasite elimination campaigns such as the GPELF. The ability of a parasite species to persist in a host population following prolonged MDA will depend in part on the metapopulation dynamics of helminth transmission and the patterns of host compliance with treatment regimes. The aggregated nature of the passage of transmission stages between hosts will make parasite elimination harder to achieve by lowering the breakpoint density (the unstable equilibrium below which the parasite population will tend naturally to local extinction (Macdonald, 1965)), as parasites are less likely to inhabit a host with a single-sexed infection.

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References

Albonico, M., Wright, V., Bickle, Q., 2004. Molecular analysis of the beta-tubulin gene of human hookworms as a basis for possible benzimidazole resistance on Pemba Island. Mol. Biochem. Parasitol. 134, 281-284.

Anderson, R.M., May, R.M., 1992. Infectious diseases of humans: dynamics and control. Oxford University Press, Oxford.

Anderson, T.J.C., 2001. The dangers of using single locus markers in parasite epidemiology: *Ascaris* as a case study. Trends Parasitol. 17, 183-188.

Ardelli, B.F., Prichard, R.K., 2004. Identification of variant ABC-transporter genes among *Onchocerca volvulus* collected from ivermectin-treated and untreated patients in Ghana, West Africa. Ann. Trop. Med. Parasitol. 98, 371-384.

Ardelli, B.F., Guerriero, S.B., Prichard, R.K., 2005. Genomic organization and effects of ivermectin selection on *Onchocerca volvulus* P-glycoprotein. Mol. Biochem. Parasitol. 143, 58-66.

Ardelli, B.F., Guerriero, S.B., Prichard, R.K., 2006a. Characterization of a half-size ATP-binding cassette transporter gene which may be a useful marker for ivermectin selection in *Onchocerca volvulus*. Mol. Biochem. Parasitol. 145, 94-100.

Ardelli, B.F., Guerriero, S.B., Prichard, R.K., 2006b. Ivermectin imposes selection pressure on P-glycoprotein from *Onchocerca volvulus*: linkage disequilibrium and genotype diversity. Parasitology 132, 375-386.

Bourguinat, C., Pion, S.D.S., Kamgno, J., Gardon, J., Gardon-Wendel, N., Duke, B.O.L., Prichard, R.K., Boussinesq, M. 2006. Genetic polymorphism of the β-tubulin gene of *Onchocerca volvulus* in ivermectin naïve patients from Cameroon, and its relationship with fertility of the worms. Parasitology 132, 255-262.

Brouwer, K.C., Ndhlovu, P., Munatsi, A., Shiff, C.J., 2001. Genetic diversity of a population of *Schistosoma haematobium* derived from schoolchildren in east central Zimbabwe. J. Parasitol. 87, 762-769.

Churcher, T.S., 2006. Modelling the spread of anthelmintic resistance. PhD Thesis, Imperial College London, UK.

Cornell, S.J., Isham, V.S., Smith, G., Grenfell, B.T., 2003. Spatial parasite transmission, drug resistance, and the spread of rare genes. Proc. Natl. Acad. Sci. USA 100, 7401-7405.

Cotreau, M.M., Warren, S., Ryan, J.L., Fleckenstein, L., Vanapalli, S.R., Brown, K.R., Rock, D., Chen, C.Y., Schwertschlag, U.S., 2003. The antiparasitic moxidectin: safety, tolerability, and pharmacokinetics in humans. J. Clin. Pharmacol. 43, 1108-1115.

Criscione, C.D., Poulin, R., Blouin, M.S., 2005. Molecular ecology of parasites: elucidating ecological and microevolutionary processes. Mol. Ecol. 14, 2247-2257.

Criscione, C.D., Blouin, M.S., 2006. Minimal selfing, few clones, and no among-host genetic structure in a hermaphroditic parasite with asexual larval propagation. Evolution Int. J. Org. Evolution 60, 553-562.

Curtis, J., Sorensen, R.E., Minchella, D.J., 2002. Schistosome genetic diversity: the implications of population structure as detected with microsatellite markers. Parasitology 125 Suppl, S51-S59.

Dreyer, G., Amaral, F., Noroes, J., Medeiros, Z., 1994. Ultrasonographic evidence for stability of adult worm location in bancroftian filariasis. Trans. R. Soc. Trop. Med. Hyg. 88, 558-558.

Efron, B., Tibshirani, R. J., 1993. An introduction to the bootstrap. Chapman & Hall/CRC Press, London.

Elard, L., Humbert, J.F., 1999. Importance of the mutation of amino acid 200 of the isotype 1 beta-tubulin gene in the benzimidazole resistance of the small-ruminant parasite *Teladorsagia circumcincta*. Parasitol. Res. 85, 452-456.

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

Eng, J.K.L., Blackhall, W.J., Osei-Atweneboana, M.Y., Bourguinat, C., Galazzo, D., Beech, R.N., Unnasch, T.R., Awadzi, K., Lubega, G.W., Prichard, R.K., 2006. Ivermectin selection on β-tubulin: Evidence in *Onchocerca volvulus* and *Haemonchus contortus*. Mol. Biochem. Parasitol. 150, 229-235.

Fisher, M.C., Viney, M.E., 1998. The population genetic structure of the facultatively sexual parasitic nematode *Strongyloides ratti* in wild rats. Proc. Biol. Sci. 265, 703-709.

Gasarasi, D.B., 2000. The transmission dynamics of bancroftian filariasis: the distribution of the infective larvae of *Wuchereria bancrofti* in *Culex quinquefasciatus* and *Anopheles gambiae* and its effect on parasite escape from the vector. Trans. R. Soc. Trop. Med. Hyg. 94, 341-347.

Geary, T.G., Conder, G.A., Bishop, B., 2004. The changing landscape of antiparasitic drug discovery for veterinary medicine. Trends Parasitol. 20, 449-455.

Gyapong, J.O., Kyelem, D., Kleinschmidt, I., Agbo, K., Ahouandogbo, F., Gaba, J., Owusu-Banahene, G., Sanou, S., Sodahlon, Y.K., Biswas, G., Kale, O.O., Molyneux, D.H., Roungou, J.B., Thomson, M.C., Remme, J., 2002. The use of spatial analysis in mapping the distribution of bancroftian filariasis in four West African countries. Ann. Trop. Med. Parasitol. 96, 695-705.

Hartl, D.L., Clark, A.G., 1989. Principles of population genetics. Sinauer Associates Inc., Sunderland, Massachusetts.

Kwa, M.S., Veenstra, J.G., Roos, M.H., 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol. Biochem. Parasitol. 63, 299-303.

Kwa, M.S.G., Veenstra, J.G., Roos, M.H., 1993. Molecular characterization of beta-tubulin genes present in benzimidazole-resistant populations of *Haemonchus-contortus*. Mol. Biochem. Parasitol. 60, 133-144.

Lammie, P.J., Fenwick, A., Utzinger, J., 2006. A blueprint for success: integration of neglected tropical disease control programmes. Trends Parasitol. 22, 313-321.

Le Jambre, L.F., Dobson, R.J., Lenane, I.J., Barnes, E.H., 1999. Selection for anthelmintic resistance by macrocyclic lactones in *Haemonchus contortus*. Int. J. Parasitol. 29, 1101-1111.

Macdonald, G., 1965. The dynamics of helminth infections, with special reference to schistosomes. Trans. Roy. Soc. Trop. Med. Hyg. 59, 489-506.

McCall, P.J., Mosha, F.W., Njunwa, K.J., Sherlock, K., 2001. Evidence for memorized site-fidelity in *Anopheles arabiensis*. Trans. R. Soc. Trop. Med. Hyg. 95, 587-590.

Michael, E., Ramaiah, K.D., Hoti, S.L., Barker, G., Paul, M.R., Yuvaraj, J., Das, P.K., Grenfell, B.T., Bundy, D.A.P., 2001. Quantifying mosquito biting patterns on humans by DNA fingerprinting of bloodmeals. Am. J. Trop. Med. Hyg. 65, 722-728.

Nadler, S.A., Lindquist, R.L., Near, T.J., 1995. Genetic structure of midwestern *Ascaris suum* populations: a comparison of isoenzyme and RAPD markers. J. Parasitol. 81, 385-394.

Njue, A.I., Prichard, R.K., 2004. Genetic variability of glutamate-gated chloride channel genes in ivermectin-susceptible and -resistant strains of *Cooperia oncophora*. Parasitology 129, 741-751.

Norman, R.A., Chan, M.S., Srividya, A., Pani, S.P., Ramaiah, K.D., Vanamail, P., Michael, E., Das, P.K., Bundy, D.A., 2000. EPIFIL: the development of an age-structured model for describing the transmission dynamics and control of lymphatic filariasis. Epidemiol. Infect. 124, 529-541.

Paterson, S., Fisher, M.C., Viney, M.E., 2000. Inferring infection processes of a parasitic nematode using population genetics. Parasitology 120, 185-194.

Porter, A.H., 2003. A test for deviation from island-model population structure. Mol. Ecol. 12, 903-915.

Prichard, R.K., 2001. Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends Parasitol. 17, 445-453.

Prugnolle, F., Liu, H., de Meeus, T., Balloux, F., 2005. Population genetics of complex life-cycle parasites: an illustration with trematodes. Int. J. Parasitol. 35, 255-263.

Roos, M.H., Kwa, M.S.G., Grant, W.N., 1995. New genetic and practical implications of selection for anthelmintic resistance in parasitic nematodes. Parasitol. Today 11, 148-150.

Schwab, A.E., Boakye, D.A., Kyelem, D., Prichard, R.K., 2005. Detection of benzimidazole resistance-associated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection by albendazole and ivermectin combination treatment. Am. J. Trop. Med. Hyg. 73, 234-238.

Schwab, A.E., Churcher, T.S., Schwab, A.J., Basáñez, M.-G., Prichard, R.K., 2006. Population genetics of concurrent selection with albendazole and ivermectin or diethylcarbamazine on the possible spread of albendazole resistance in *Wuchereria bancrofti*. Parasitology 133, 589-601.

Schwab, A.E., Churcher, T.S., Schwab, A.J., Basáñez, M.-G., Prichard, R.K. An analysis of the population genetics of potential multi-drug resistance in lymphatic filariasis due to combination chemotherapy. *Parasitology* (in press).

Schulz Key, H., Karam, M., 1986. Periodic reproduction of *Onchocerca volvulus*. Parasitol. Today 2, 284-286.

Shrivastava, J., Qian, B.Z., McVean, G., Webster, J.P., 2005. An insight into the genetic variation of *Schistosoma japonicum* in mainland China using DNA microsatellite markers. Mol. Ecol. 14, 839-849.

Silvestre, A., Cabaret, J., 2002. Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? Mol. Biochem. Parasitol. 120, 297-300.

Snow, L.C., Michael, E., 2002. Transmission dynamics of lymphatic filariasis: density-dependence in the uptake of *Wuchereria bancrofti* microfilariae by vector mosquitoes. Med. Vet. Entomol. 16, 409-423.

Théron, A., Sire, C., Rognon, A., Prugnolle, F., Durand, P., 2004. Molecular ecology of *Schistosoma mansoni* transmission inferred from the genetic composition of larval and adult infrapopulations within intermediate and definitive hosts. Parasitology 129, 571-585.

Tisch, D.J., Michael, E., Kazura, J.W., 2005. Mass chemotherapy options to control lymphatic filariasis: a systematic review. Lancet Infect. Dis. 5, 514-523.

Weir, B.S., 1996. Genetic data analysis II. Sinauer Associates Inc., Sunderland, Massachusetts.

WHO, 2006. Weekly Epidemiological Review, 2 June 22, 221-232.

Wright, S., 1969. Evolution and the genetics of populations. Volume 2: The theory of gene frequencies. University of Chicago Press, Chicago.

APPENDIX II

Supplemental Figures

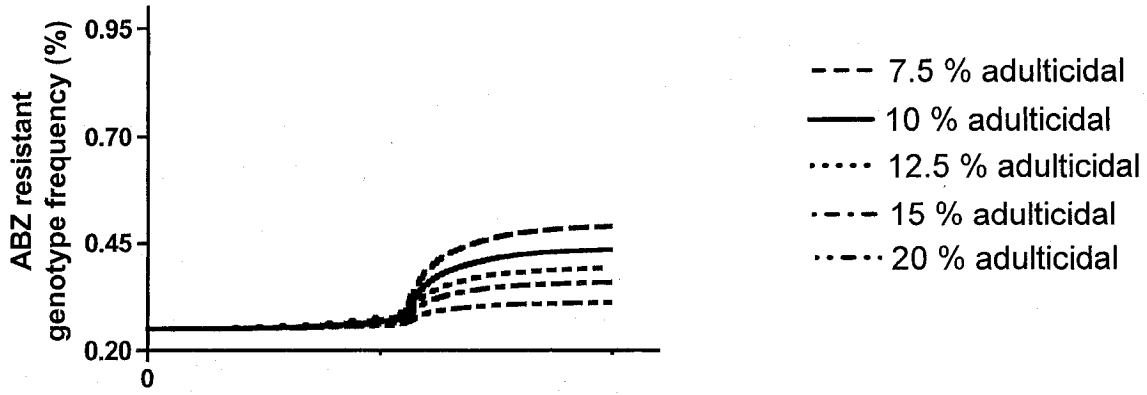

Figure 7.1: The effect, on the ABZ resistance genotype frequency (%) in *W. bancrofti* microfilariae, of increasing adulticidal efficacy (% of macrofilariae killed) of IVM, when administering 10 annual treatments of ABZ + IVM, with 85% coverage of the human host population.

This figure shows that altering the adulticidal activity of IVM from 7.5 % to 20 % changes the ABZ resistant genoptype frequency by less than 1 %. This is a smaller difference than that observed in a similar sensitivity analysis of the ABZ + DEC treatment combination. It was not possible to examine changes in additional microfilaricidal activity caused by adding ABZ to IVM, as was done for ABZ + DEC, since IVM alone is reported to kill 99% of microfilariae.

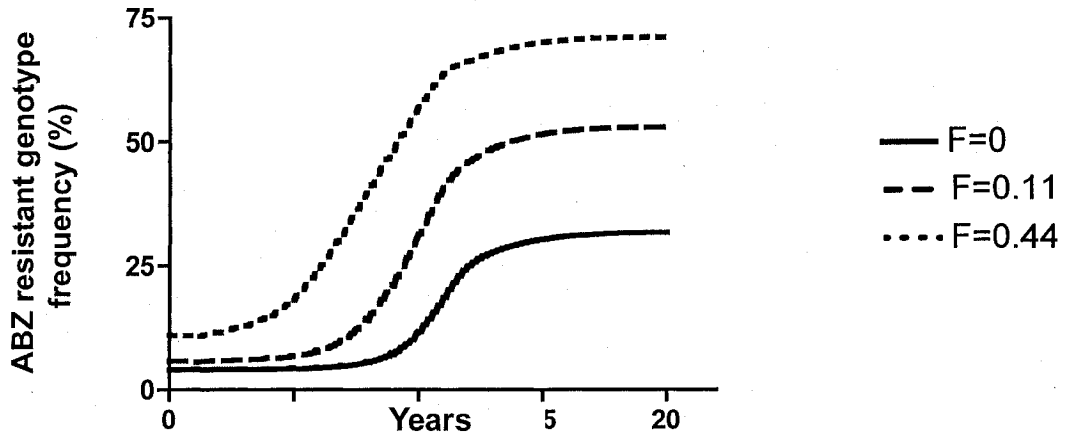

Figure 7.2: The effect of increasing the levels of non-random parasite mating (i.e., increased homozygosity, as measured by the Fisher F_{IT} statistic), on the frequency of the ABZ resistant genotype, assuming 20 % initial frequency of ABZ resistance and dominant IVM resistance (with 5 % initial frequency) in *W. bancrofti*, following 10 yearly treatments, at 85% coverage, with ABZ + IVM.

This figure shows that the spread of the ABZ resistance genotype is enhanced by increasing values of F_{IT}, or increased homozygosity, in the presence of IVM resistance. This increase is similar to that observed in Chapter 3 when no resistance to IVM was considered.

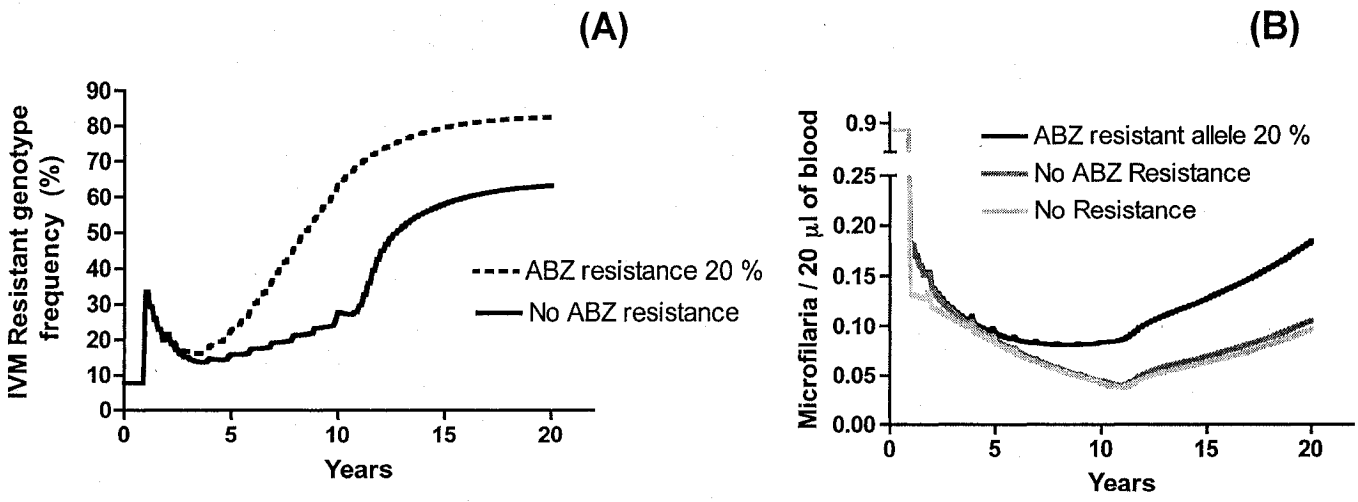

Figure 7.3: The effect of 10 yearly treatments with ABZ + IVM, with 85% coverage of the total population, on the spread of resistance in *W. bancrofti* populations, assuming dominant IVM resistance (5 % initial frequency) and ABZ resistance (0 or 20% initial frequency), with F_{IT}= 0.44. (A) The average frequency (%) of IVM resistance genotypes in microfilariae. (B) The mean microfilaraemia per 20 µl of blood in the human host population.

This figure shows that the presence of an ABZ resistance genotype increases selection for the IVM resistance genotype. This increase in resistance leads to higher overall microfilaraemia after the end of the treatment period.

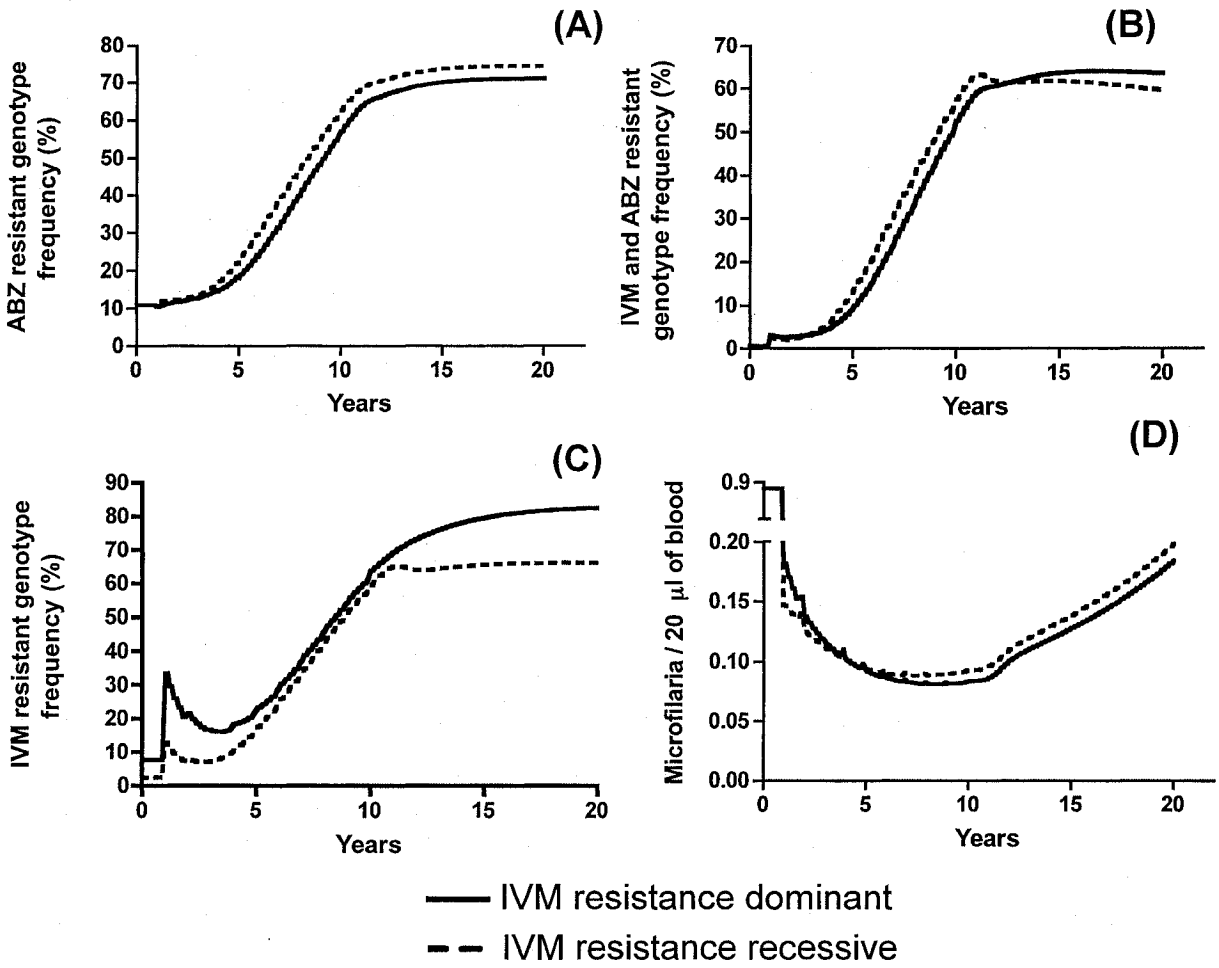

Figure 7.4: The effect of dominance of the IVM resistance gene following 10 yearly treatments with ABZ + IVM, and F_{IT}= 0.44, on the spread of resistance genotypes, assuming 20 % initial frequency of ABZ resistance genotype and 5 % initial frequency of IVM resistance. (A) the average frequency (%) of ABZ resistance genotypes in microfilariae, (B) the average frequency (%) of ABZ and IVM resistance genotypes in microfilariae, (C) the average frequency (%) of IVM resistance genotypes in microfilariae, and (D) the mean microfilaraemia per 20 µl of blood in the human host.

This figure shows that the IVM resistance genotype is higher after treatment if IVM resistance is dominant. The ABZ resistance genotype is higher if IVM resistance is reeessive, as is the total average Mf intensity, indicating a stronger impact on intensity of ABZ resistance.

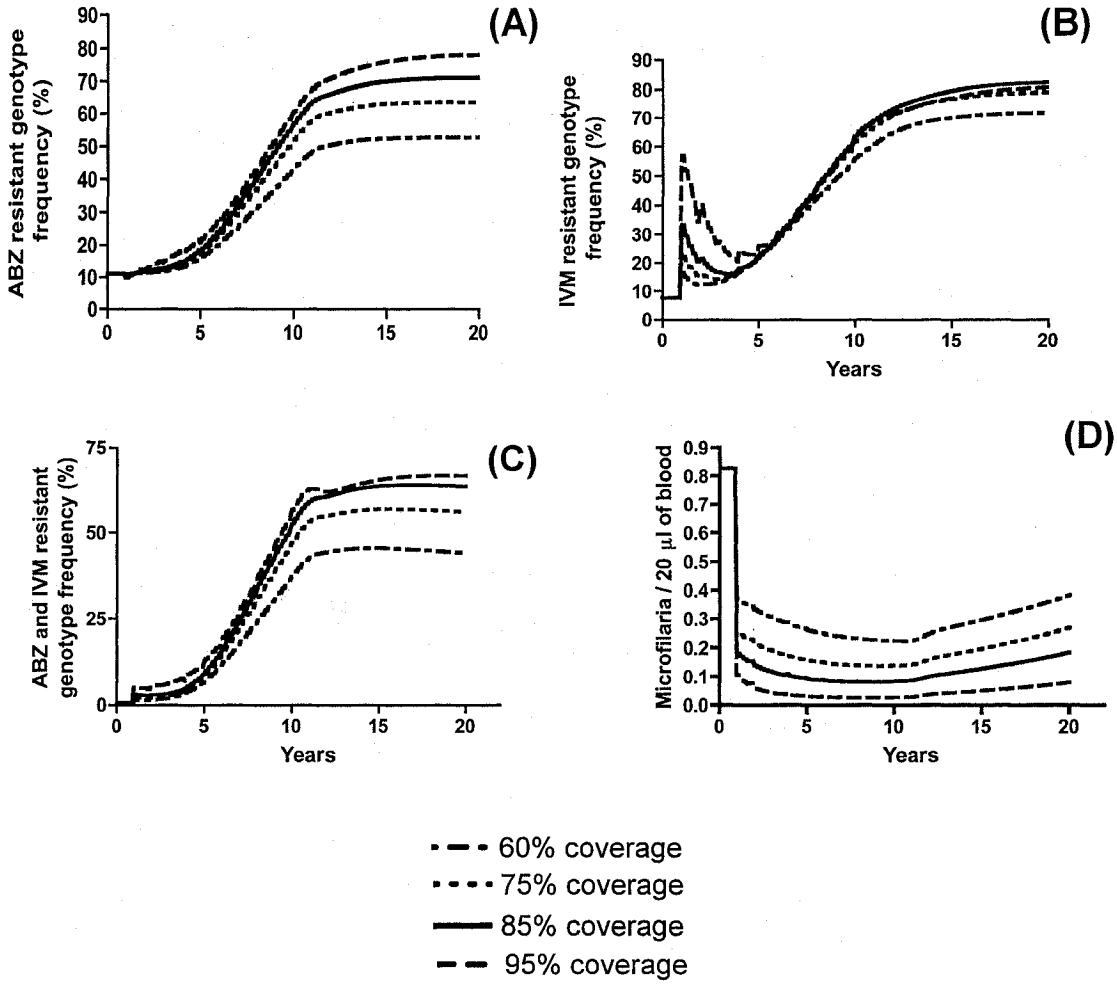

Figure 7.5: The effect of increasing therapeutic coverage (the percentage of the total host population treated) of 10 yearly treatments with ABZ + IVM, and FIT= 0.44, on the spread of resistance, assuming 20 % initial frequency of ABZ resistance genotype and dominant IVM resistance (with 5 % initial frequency) in microfilariae. (A) the average frequency (%) of ABZ resistance genotypes in microfilariae, (B) the average frequency (%) of IVM resistance genotypes in microfilariae, (C) the average frequency (%) of ABZ and IVM resistance genotypes in microfilariae, and (D) the mean microfilaraemia per 20 µl of blood in the human host.

This figure shows that the spread of the ABZ resistance genotype is enhanced, in the presence of IVM resistance, by increasing treatment coverage. This increase is similar to that observed in Chapter 3 when no IVM resistance was considered. Treatment coverage affected the ABZ and IVM resistance genes differently. When initial resistance

frequencies of ABZ and dominant IVM resistance were 20% and 5%, respectively, and F_{IT}=0.44, an increase in treatment coverage led to an increased spread of the ABZ resistance genotype after 10 yearly treatments (see Appendix II, Fig. 7.5), as was shown in the case of ABZ resistance alone (Schwab *et al*, *in press*). This increase was also observed when spread of genotypes resistant to both ABZ and IVM were considered (Fig. 4.3A). However, in the case of IVM resistance, the dominant resistance genotypes were only 10% more frequent after treatment with 85% coverage than with 60% coverage. If coverage was increased another 10% to 95%, the resistance genotypes were slightly less frequent than at 85% coverage and 10 yearly treatments (Fig. 4.3B). Varying treatment coverage from 60 to 95% led to a 4.75 fold reduction in the total average MF intensity after application of the treatment regime.

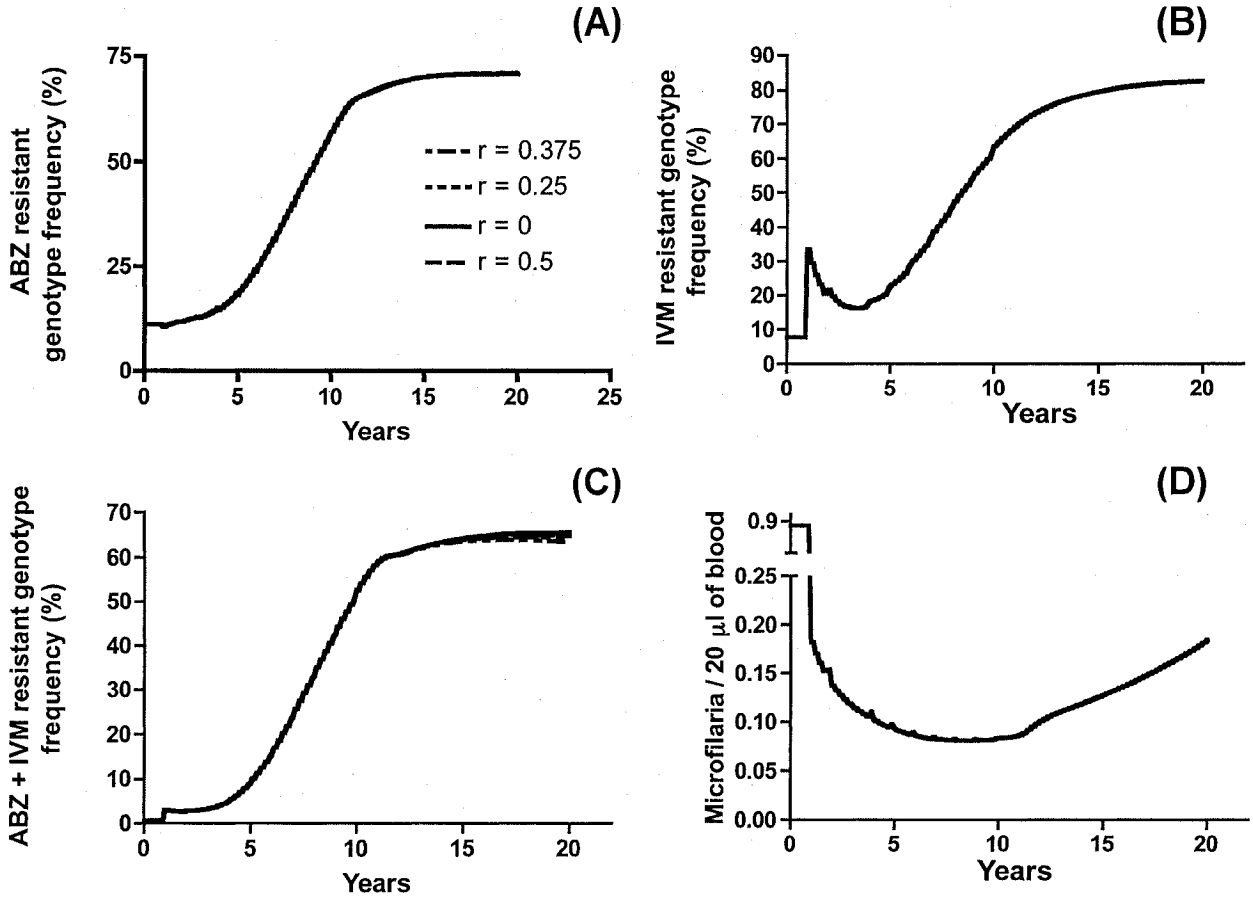

Figure 7.6 : The effect of varying amounts of recombination between ABZ and IVM resistance alleles on the spread of resistance, assuming 20 % initial frequency of ABZ resistance genotype and dominant IVM resistance (with a 5 % initial frequency), after 10 yearly treatments with ABZ + IVM at 85% coverage and F_{IT}= 0.44 on the spread of ABZ- and IVM-resistance. (A) the average frequency (%) of ABZ resistance genotypes in microfilariae, (B) the average frequency (%) of ABZ and IVM resistance genotypes in microfilariae, (C) the average frequency (%) of IVM resistance genotypes in microfilariae, and (D) the mean microfilaraemia per 20 µl of blood in the human host.

These panels demonstrate that linkage (decreased amount of recombination) between the ABZ and IVM resistance allele does not impact selection, if the population is at linkage equilibrium. This is due to the fact that alleles resistant to both drugs or to either drug, both exist in the population.

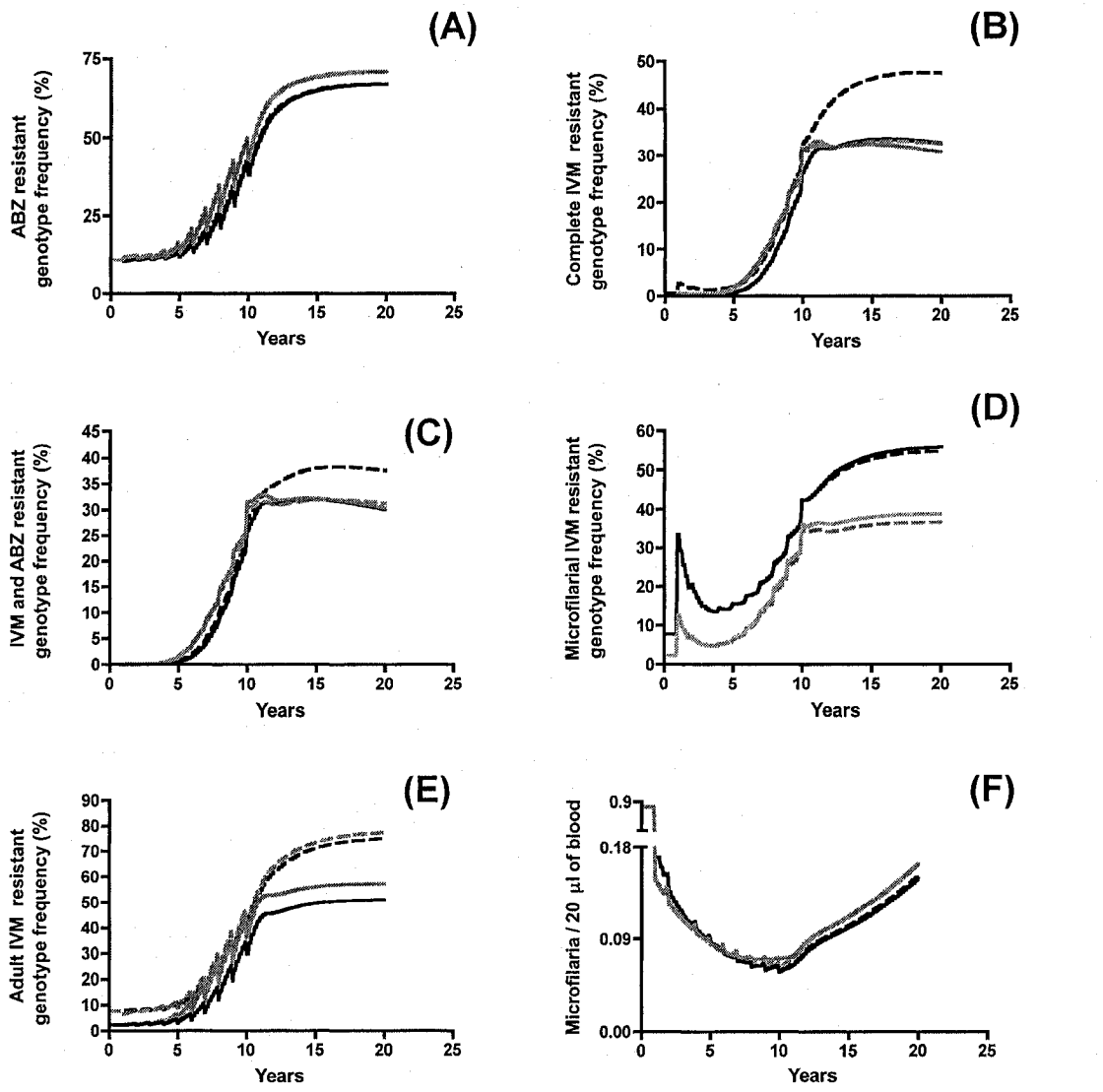

Figure 7.7: The effect of selection on one ABZ resistance gene and two IVM resistance genes on the spread of ABZ- and IVM- resistance at initial resistance allele frequencies of 20% for ABZ resistance, 5% for each IVM resistance allele and F_{IT}= 0.44. (A) The average frequency (%) of ABZ resistance genotypes in microfilariae, (B) the average frequency (%) of microfilariae with both IVM resistance genotypes, (C) the average frequency (%) of microfilariae with both IVM and the ABZ resistance genotypes, (D) the average frequency (%) of microfilariae with microfilarial IVM resistance genotypes, (E) the average frequency (%) of microfilariae with adult IVM resistance genotypes and (C) the mean microfilaraemia per 20 µl of blood in the human host.

In panel A of Figure 7.7 it may be noted that selection for the ABZ resistance genotype is stronger if microfilarial IVM resistance is recessive than if it is dominant. This is discussed in Chapter 4. Panels B and C indicate that complete IVM resistance and resistance against both drugs is only noticeably higher if both IVM resistance genes are inherited as dominant traits. In panel D and E, it is apparent that selection for each IVM resistance gene is enhanced by its own dominance and diminished by the dominance of the other IVM resistance gene, indicating the possibility of counter selection. Finally, panel F shows that the total average microfilarial intensity is slightly higher after treatment if the microfilarial IVM resistance gene is recessive, although this may be due to the consequent increase in the ABZ resistance genotype frequency (see panel A). ABZ has a larger impact on the adult population, which may contribute to this result.

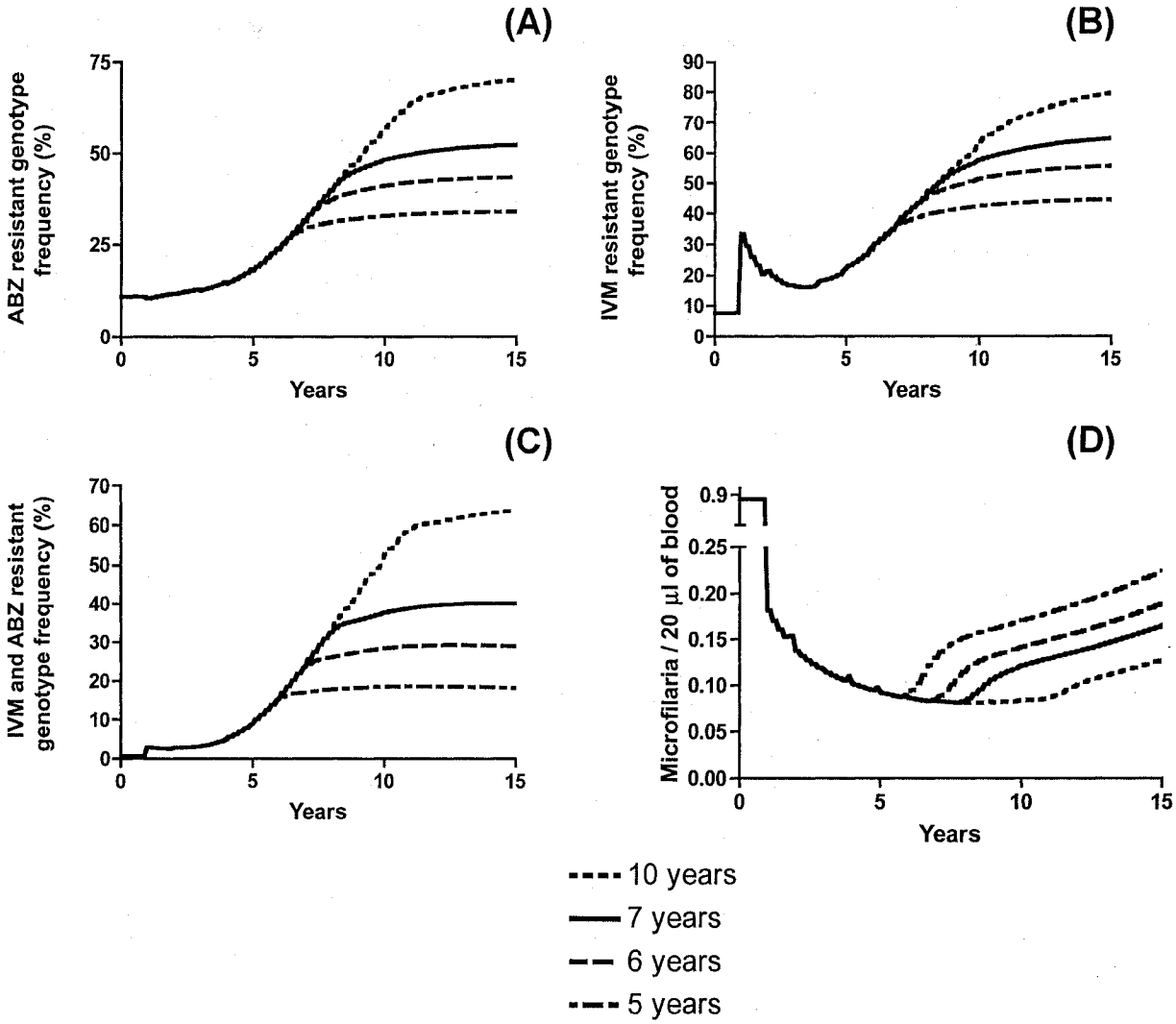

Figure 7.8: The effect of increasing the number of annual treatments with 85% coverage on the spread of ABZ resistance in *W. bancrofti*, (A) The average frequency (%) of ABZ resistance genotypes in microfilariae, (B) the average frequency (%) of microfilariae with both IVM resistance genotypes, (C) the average frequency (%) of microfilariae with both IVM and the ABZ resistance genotypes, (D) the mean microfilaraemia per 20 µl of blood in the human host.

This graph shows that fifteen years after the commencement of treatment, the frequencies of ABZ and IVM resistance genotypes were estimated to be 18%, 40% and 63%, after respectively 5, 7 and 10 years of annual combination treatment It may be noted that the higher frequency of resistance genotypes following a longer period of treatment indicates that a return to chemotherapy would be initially much less effective, assuming continued transmission, if the initial phase of treatment had been for 10 years compared with 5 years.