Genetic selection by Ivermectin on *Onchocerca volvulus*

Jeffrey K. L. Eng

Institute of Parasitology
McGill University, Montreal

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Abstract

Onchocerca volvulus is a parasitic filarial nematode responsible for human onchocerciasis, a disease commonly known as "River Blindness". Although there are no well documented cases of ivermectin resistance in *O. volvulus*, reports of suboptimal responses to ivermectin have appeared. The purpose of this thesis was to examine genetic polymorphisms in *O. volvulus* and to determine whether there was genetic evidence of ivermectin selection on *O. volvulus* genes. Analysis of 17 genes from *O. volvulus* was undertaken in two populations of worms, either from ivermectin-naïve patients or from patients who had been repeatedly treated with ivermectin annually. In 14 of the genes no differences in genetic polymorphism were found (although polymorphisms were identified). However, chi square analysis (χ^2=0.05) indicated significant differences in allele frequencies for a P-glycoprotein, a β-tubulin and a putative dyf-8 gene. Analysis of the *O. volvulus* β-tubulin alleles identified three amino acid substitutions in the H3 region with ivermectin selection. Microtubules play a key structural role in the formation of neurons, and in ivermectin-resistant *Haemonchus contortus*, amphidial neurons show distorted microtubule bundles. Polymerization and depolymerization assays of the recombinant *O. volvulus* β-tubulin alleles showed interesting differences between the polymerized tubulin using the two different alleles. It is speculated that similar differences could cause the disorganization of the microtubules identified in the amphidial neurons in ivermectin resistant *H. contortus*. In addition to the coding mutations, a 24 bp deletion in the adjacent intron to the H3 was detected. A PCR diagnostic assay was developed to genotype individual macro- and microfilariae. Further analyses were conducted to investigate the possibility of a direct

relationship between ivermectin and β-tubulin. Data obtained from equilibrium dialysis experiments indicated that BODIPY FL ivermectin bound to purified *O. volvulus* α- and β-tubulins. More interesting, non-fluorescent ivermectin and taxol competed with the BODIPY FL ivermectin. The work presented in this thesis provides evidence of genetic selection by ivermectin on *O. volvulus* and suggests a putative binding site for ivermectin on tubulin. These data provide novel information on ivermectin selection in *O. volvulus* and on the possible involvement of tubulin in ivermectin resistance.

Abrégé

Onchocerca volvulus est un nématode parasite de type filaire qui provoque chez l'homme l'onchocercose, une maladie aussi connue sous le nom de « Cécité des Rivières ». Bien qu'il n'y ait aucun cas prouvé de résistance à l'ivermectine chez *O. volvulus*, des rapports ont fait leur apparition révélant une efficacité sous-optimale de l'ivermectine. L'objectif de cette thèse est d'examiner les polymorphismes génétiques chez *O. volvulus* et de déterminer s'il existe une sélection à l'ivermectine dans les gènes d'*O. volvulus*. L'analyse de 17 gènes d'*O. volvulus* a été effectuée dans deux populations de vers adultes en provenance de patients jamais exposés à l'ivermectine, et traités annuellement à maintes reprises à l'ivermectine. Le polymorphisme génétique n'a démontré aucune différence dans 14 des gènes examinés (bien que des polymorphismes aient été identifiés). Toutefois, un test khi-carré (χ^2=0.05) indique une différence significative dans la fréquence des allèles de la P-glycoprotéine, la tubuline β et le gène putatif dyf-8. L'étude de l'allèle tubuline β chez *O. volvulus* exposé à l'ivermectine a permis d'identifier trois substitutions d'acides aminés de la région H3. Les microtubules jouent un rôle structurel clé à la formation des neurones, et chez *Haemonchus contortus* résistant à l'ivermectine, les neurones amphibials démontrent des faisceaux de microtubules déformés. Des tests de polymérisation et de dépolymérisation des allèles recombinés de la tubuline β chez *O. volvulus* ont souligné des différences intéressantes entre la tubuline polymérisée de ces deux allèles. Il semblerait que des différences similaires pourraient causer la déstructuration des microtubules identifiés dans les neurones amphidials chez *H. contortus* résistant. En plus des mutations d'acides aminés, la suppression de 24 pb dans l'intron adjacent à H3

a été détectée. Une méthode diagnostique au PCR a été développée pour génotyper individuellement les macro- et microfilaires. Des analyses plus poussées ont démontré qu'une relation directe entre l'ivermectine et la tubuline β existerait. Les résultats obtenus par dialyse à l'équilibre indiquent que l'ivermectine BODIPY FL se lie aux tubulines purifiées α et β. De plus, l'ivermectine non fluorescente et le taxol ont fait concurrence à l'ivermectine BODIPY FL. Le travail de cette thèse indiquent l'existence d'une sélection génétique à l'ivermectine chez *O. volvulus* et suggèrent un site de liaison putatif à l'ivermectine de la tubuline. Ces résultats fournissent de nouvelles informations sur la sélection à l'ivermectine chez *O. volvulus* et une implication possible de la tubuline à la résistance à l'ivermectine.

Acknowledgments

I would first like to express gratitude towards my supervisor Dr. Roger K. Prichard (a.k.a. BOSS) for the opportunity to complete my PhD in his lab. His years of experience and knowledge in the field of veterinary and human parasites provided the proper environment not only for me to learn but to develop my own thought and confidence. I really appreciate his patience and helpful discussions through out my graduate studies.

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Finally, I would like to thank my parents Donald and Grace, and my wife to be, Phetsamone, for their continual support with the decisions I've made (good and bad) and who have never pestered me with scientific questions about PCR optimization or ivermectin resistance.

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List of Abbreviations

APOC,	African Program for Onchocerciasis Control
bp,	Base pair
DEC,	Diethylcarbamazine
DNA,	Deoxyribonucleic acid
GABA-Cl,	γ-aminobutyric acid Chloride Channel
Glu-Cl,	Glutamate-gated Chloride Channel
IVM,	Ivermectin
kDa,	kiloDalton
mf,	microfilariae
UN,	United Nations
OCP,	Onchocerciasis Control Program
PGP,	P-glycoprotein
PLP,	P-glycoprotein like protein
RFLP,	Restriction Fragment Length Polymorphism
SDS-PAGE,	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSCP,	Single Strand Conformational Polymorphism
WHO,	World Health Organization

Thesis Office Statement

As an alternative to the traditional thesis format, the thesis can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

1. a table of contents;
2. a brief abstract in both English and French;
3. an introduction which clearly states the rational and objectives of the research;
4. a comprehensive review of the literature (in addition to that covered in the introduction to each paper);
5. a final conclusion and summary;
6. a thorough bibliography;
7. Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.

Statement of Originality

The following aspects of this thesis are considered contributions of original knowledge.

Manuscript I

Eng J.K.L. and Prichard R.K. (2005). A Comparison of Genetic Polymorphism in Populations of Onchocerca volvulus from Untreated- and Ivermectin-Treated Patients. Molecular and Biochemical Parasitology. 142:193-202

In manuscript I, a comparison of 16 *O. volvulus* genes was conducted by single strand conformational polymorphism and/or restriction fragment length polymorphism to identify changes in the allele frequency in *O. volvulus* exposed to yearly doses of ivermectin. The analysis not only shows that the *O. volvulus* genome is genetically polymorphic, Chi-square analysis (x^2=0.05), indicated two of the 16 genes, P-glycoprotein and β-tubulin isotype 1, had significant changes in allelic frequencies in the population exposed to multiple yearly rounds of ivermectin. Subsequently a third gene, a putative dyf-8 gene, was also found to show genetic selection.

Manuscript II

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. and Prichard, R.K. (2006). Ivermectin selection on β-tubulin: Evidence in Onchocerca volvulus and Haemonchus contortus. Molecular and Biochemical Parasitology. 150: 229-235

In manuscript II, the focus was on the β-tubulin isotype I gene of *O. volvulus* and *H. contortus* and how it could be used as a potential genetic marker for ivermectin resistance. Sequence analysis of the *O. volvulus* β-tubulin isotype I gene revealed three amino acid substitutions and 24 bp of deletion in the intron of the helix 3 domain from the selected β-tubulin allele. The deletion was used as a target to design a PCR test which can be used to monitor populations of *O. volvulus* under pressure from

ivermectin. In addition, genetic selection was also identified on a β-tubulin gene in ivermectin selected strains of *H. contortus*. This is the first study which shows genetic selection in the β-tubulin gene due to ivermectin and suggests an involvement of β-tubulin with ivermectin resistance.

Manuscript III

Eng J.K.L., Osei-Atweneboana M.Y. and Prichard, R.K (2006). *Onchocerca volvulus* β-tubulin: functional role in ivermectin resistance? *In preparation.*

We have demonstrated that the three amino acid differences in the H3 domain found between the two β-tubulin alleles may alter the stability of the microtubules. Polymerization and depolymerization assays with recombinant *O. volvulus* β-tubulin alleles showed an interesting difference in the plateau region indicating a possible altered protein structure. Furthermore, ivermectin significantly reduced the rate of depolymerization of polymerized tubulin composed with the ivermectin selected β-tubulin allele. In addition, this is the first study that indicates that ivermectin, as BODIPY FL ivermectin, can directly bind to α- and β-tubulin. More interesting, the non-fluorescent ivermectin and taxol were able to compete with the BODIPY FL ivermectin binding to the purified tubulin. The data not only suggest direct binding to the purified tubulin protein but as well indicate a putative binding site for ivermectin.

Statement of Authorship

This thesis is comprised of three manuscripts which were written with scientific and editorial contributions from my PhD supervisor, Dr. Roger K. Prichard. All experimental data for manuscript I, II, III, and Appendix I were produced and analyzed by the author. In manuscript II co-author contributions include data in Figure 1 (a data set for the non-exposed 1989-90-West Africa samples) which was provided by Catherine Bourguinat. Figure 5 was based on data provided by Dr. William J. Blackhall, and Figure 6a and 6b were based on data provided by Daniel Galazzo. Mike Osei-Atweneboana collected and provided the *O. volvulus* microfilariae and patient information for the study on the microfilariae, including the day 90 skin microfilarial load assessments. Dr. Thomas Unnasch provided *O. volvulus* DNA samples from the 1989-90 West Africa samples, Dr. George W. Lubega provided *O. volvulus* DNA for the Uganda data set and Dr. Kwablah Awadzi provided the 1998 *O. volvulus* material. Dr. Roger K. Prichard provided financial support, a laboratory work environment, advice with regards to study objectives, experimental design, interpretation of the results and correction of the manuscripts and thesis.

Section I

Literature Review

Introduction

Filarial nematodes belong to the family Filariidae, and are an important class of parasites, infecting both animals and humans. The human filarial nematodes include *Onchocerca volvulus, Loa loa, Wuchereria bancrofti, Brugia malayi, Brugia timori, Mansonella perstans, Mansonella streptocerca, Mansonella ozzardi* and *Dracunculus medinensis.* The filarial nematodes are widely distributed. *O. volvulus* occurs in West Africa and the central belt of Africa, South and Central America, and Yemen (Arabian Peninsula). It has resulted in the infection of tens of million of individuals.

The disease caused by *O. volvulus* is commonly called "River Blindness" or onchocerciasis and is a major cause of preventable blindness. The name River Blindness originates from the fact that the vector, the *Similium* black fly, requires a source of fast flowing water to breed and for proper larval development. The disease is associated with blindness and severe skin pathology. In addition, a socioeconomic problem has arisen as a result of the black fly breeding occurring where there is water, with the adjacent land usually being the most fertile for agriculture. However, due to the incidence of onchocerciasis in these areas, they have had to be abandoned in favor of less fertile regions to avoid exposure from infected black flies.

Ivermectin was found to be effective at eliminating the microfilarial stage and reducing parasite transmission. Since 1988, ivermectin has been provided free of charge by Merck & Co. through the Mectizan Donation Program. It is currently the only safe and effective drug used to treat patients for onchocerciasis. Although there has yet to be unequivocal proof of ivermectin resistance in *O. volvulus*, resistance to ivermectin has been reported worldwide in nematode species of livestock such as *Haemonchus*

contortus, Trichostrongylus colubriformis, Teladorsagia circumcincta, Cooperia spp. (Feng et al. 2002; Kaplan 2004; Wolstenholme et al. 2004)

Since ivermectin is presently the sole safe drug available to treat onchocerciasis, the development of resistance could jeopardize the social and economic situation in these endemic countries. This would be a major setback to the onchocerciasis control programs initiated by the World Health Organization.

Nematode populations, like many other groups of diploid organisms which breed sexually, appear to be genetically quite heterogeneous (Blouin et al. 1995) and thus may be able to respond to selective pressures, such as from anthelmintic drugs. The development of ivermectin resistance involves a process called genetic selection which eventually develops a population of nematodes, which will have the genetic variants (alleles) for allow them to survive drug exposure. These alleles are heritable so that the genes for resistance phenotype can be passed on to following generations. The rate at which resistance spreads in the parasite population depends on many factors, such as drug pressure, gene flow, the frequency of resistance alleles in the population, number of genes involved, and the dominance or recessiveness of these genes (Grant 1994; Prichard 2001).

When genetic selection occurs, such as with the continual use of drugs to treat a disease, certain allele(s) within the gene pool are eliminated (susceptible allele(s)) whereas other genes are preserved (resistant allele(s)). Therefore, by comparing and/or monitoring the genetic profiles between populations under drug pressure verse drug naïve populations, we may be able to identify the genes directly involved or genetically linked to the resistance phenotype.

Chapter I

Literature Review

I.1 *Onchocerca volvulus*

The human filarial parasite *Onchocerca volvulus* is a major public health and socioeconomic problem in Africa and parts of Latin America. It is estimated that 18 million people around the world are infected of whom 500,000 are visually impaired, another 270,000 are blind with this parasite and 123 million people are at risk of infection (Brattig and Erttmann 1992; Luder et al. 1996; Dull and Meredith 1998).

Until recently, the disease caused by *O. volvulus* was not believed to be fatal, but rather debilitating. Recent analysis of endemic countries indicate that host mortality is directly associated with microfilariae burden and infection with *O. volvulus* reduces life expectancy (Pion et al. 2002; Little et al. 2004). The juvenile stage of *O. volvulus*, the microfilariae, is responsible for the majority of the pathology which occurs following microfilarial death (Hall and Pearlman 1999). Recently, several papers have been published which implicate the endosymbionent *Wolbachia* as the causative agent for pathology when the microfiliaria die rather than the microfilaria itself (Saint Andre et al. 2002; Taylor 2003). Pathology can include persistent and debilitating itching and loss of skin elasticity, skin depigmentation, severe visual impairment which eventually leads to blindness (hence the description "River Blindness"), nodule formation, epileptic seizures and growth retardation (Chandrashekar et al. 1995; Newell et al. 1997).

Filarial nematodes, which include *O. volvulus*, are transmitted by haematophagus

arthropod vectors (Figure 1). The black-fly (*Simulium sp.*) is the arthropod vector by which *O. volvulus* is transmitted from human to human. The black fly obtains a blood meal from an infected individual and acquires the L1 microfilariae (larval stage of the nematode) by ingesting tissue fluids and blood (1). The L1 microfilariae then migrate from the intestinal tract of the black fly to the thoracic muscle where two molts occur prior to the L3 larvae stage (2). The time frame between L1 and L3 takes approximately nine days to complete (Eichner et al. 1991). The L3 larvae then migrate to the labium of the black fly where they will be in position to be transmitted the next time the back fly feeds (Schmidt and Roberts 1996)(3). Interesting, for an unknown reasons not all L1 microfilariae develop into infective L3s.

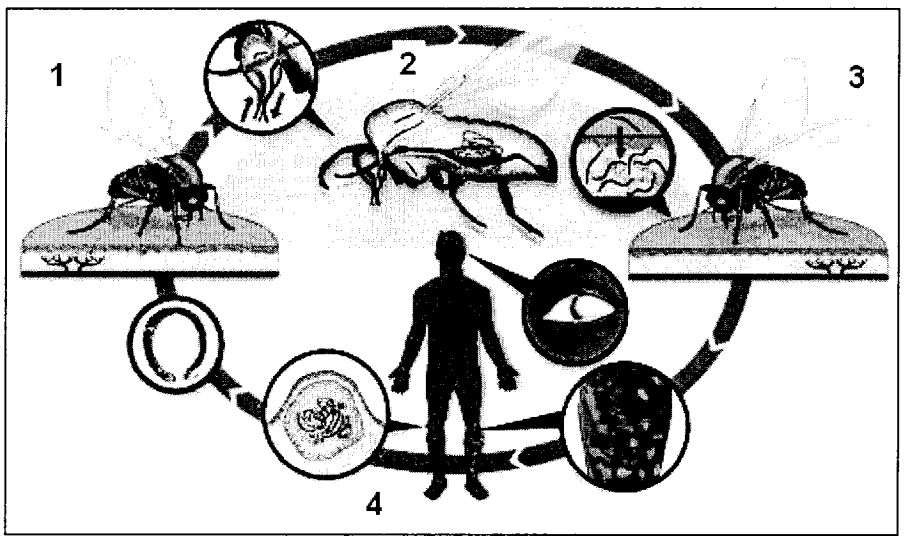

Figure 1. Life cycle of *Onchocerca volvulus*
(http://www.who.int/tdr/diseases/oncho/lifecycle.htm)

If the black fly feeds on a susceptible host, the lesion caused by the bite will allow infective L3 a passageway into the subcutaneous tissues. Within a year of entering the host, the microfilariae will develop into the L4 stage, the juvenile L5 stage and eventually into a sexually mature adult (Basanez and Boussinesq 1999)(4).

The much larger and less motile adult females are eventually encapsulated by fibrous tissue as a result of the host's response to the parasite and the inability of the human immune system to successfully remove the parasite. The smaller and more motile adult males are usually found within the nodule along with the female. The fibrous capsule often appears as a nodule under the skin but can also reside in deeper tissues. A single adult female worm can produce one thousand microfilariae a day, which migrate from the nodule and reside within the skin (Schmidt and Roberts 1996; Pearlman et al. 1999).

The large number of microfilariae released into the skin can potentially cause a harsh immune reaction, though the pathology of the immune response can vary from patient to patient. Some people can develop an intense skin disease due to the overreaction to the microfilariae or *Wolbachia* antigens whereas other infected people develop a very low level of skin disease though they possess a very high number of microfilariae residing in their skin (Abraham et al. 2004).

As with many parasitic infections, eosinophils appear to play a central role in killing the microfilariae. They can do so by releasing a cocktail of cytokines (i.e. IL-5), cationic protein, and eosinophil-derived neurotoxin which can be toxic to the microfilariae. Adhesion of eosinophils to the microfilariae may also allow complement to play a role (Cooper et al. 1999; Abraham et al. 2004).

The total size of the nuclear genome of *O. volvulus* is believed to be 1.5×10^8 bp, arranged in four pairs of chromosomes; one pair of dimorphic sex chromosomes and three pairs of autosomes (Unnasch and Williams 2000). Besides the nuclear and mitochondrial genome of *O. volvulus* (and many other tissue dwelling filariaes), a

bacterial endosymbiont genome is also present (Unnasch and Williams 2000). The bacterial endosymbiont, *Wolbachia*, has been recently implicated, thought still controversial, in some of the pathogenesis previously thought to be caused by *O. volvulus*. For example, visual impairment due to Onchocerciasis was once believed to be mediated via the inflammatory processes that occur due to the releases of antigenic material from dead or moribund microfilariae in the eye region. Current evidence suggests that the inflammatory responses are elicited not so much by the microfilarial antigens but rather from the endosymbiotic *Wolbachia* bacteria (Saint Andre et al. 2002).

Two strains of *O. volvulus* are believed to exist, forest and savannah. Morphologically, there is no difference between the two strains. However, individuals infected with the savannah strain appear to have a greater chance of developing eye pathology, whereas with the forest strain, infected individuals have an increased frequency of developing skin disorders such as dermatitis and depigmentation rather than eye pathology (Dadzie 1998). At the DNA level, a difference between these strains exists in the non-coding tandem repeat region called O-150. Therefore, a PCR for the strain specific O-150 sequence can be use to differentiate between them (Zimmerman et al. 1993). However, due to the fact that the O-150 assay probes for a noncoding repeated sequence, it is unlikely to be directly involved in determining pathogenicity (Higazi et al. 2005). Recently, reports have linked the burden population of the *Wolbachia* endosymbiont to the pathogenic differences seen in the two different strains of *O. volvulus* (Higazi et al. 2005). The authors concluded that the more severe strain of *O. volvulus* contained significantly greater number of endosymbionts

compared to the milder strain of *O. volvulus*. Although two different strains of *O. volvulus* have been reported, the strains appear to breed together, therefore differences in the extent of the pathology can be blurred.

The current treatment for onchocerciasis is ivermectin. The use of ivermectin to treat human onchocerciasis began when the drug became available in 1987, following a generous donation from Merck & Co. (Molyneux 1995). The beneficial effects of ivermectin are numerous and include: (1) a reduction in the number of circulating microfilariae in the host; (2) decrease in the transmission of the parasite to the black fly vector; (3) alleviation of intense itching and dermatitis; and (4) a significant reduction in developing blindness.

Research on ivermectin resistance is currently being conducted with nematode species such as *H. contortus*, *Caenorhabditis elegans*, *Te. circumcincta*, *Cooperia* spp., *Ostertagia ostertagi* and *O. volvulus*. With nematodes like *H. contortus*, drug resistant strains can be obtained by a process of repeatedly treating the wild type or susceptible strains with sub-lethal doses of drug, such as ivermectin, until the desired level of resistance is obtained (Ranjan et al. 2002). With this technique, any genetic selection detected by techniques such as Restriction Fragment Length Polymorphism (RFLP) or Single Strand Conformational Polymorphism (SSCP) can be correlated with the susceptible strains since the resistant strain is derived from the susceptible strain.

Although ivermectin is currently the drug of choice to treat onchocerciasis, the lack of significant macrofilaricidal activity is a major weakness. Recently, the identification of a symbiotic bacterial species, *Wolbachia*, within *O. volvulus* has been identified as a possible novel target for therapy. In an animal model, the African bovine

parasite *Onchocerca ochengi* has been successfully used as a model to test the effectiveness of the antibiotic doxycycline as a macrofiliaricide . In filarids, Taylor et al. (2005), used doxycycline daily for eight weeks to eliminate the *Wolbachia* endosymbiont in *Wuchereria bancrofti* adult worms. After the drug regime, microfilarial counts were performed 5, 8, 11 and 14 months post treatment. The results indicated that a sustained reduction in the number of microfilariae was maintained for 8-12 months after treatment. However, the use of doxycycline as a macrofilicaide does not appear to be promising due to the prolonged drug regime (daily for two to three months), the contraindications for pregnant women and children younger than eight years old, and the lack of a donation programme for the antibiotic (Langworthy et al. 2000; Taylor et al. 2005).

I.2 *Onchocerca volvulus* Control Programmes

In the last two decades, the control of onchocerciasis has been the responsibility of three main organizations: (1) the Onchocerciasis Control Program (OCP) in West Africa; (2) the Onchocerciasis Elimination Program of the Americas; and (3) the African Program for Onchocerciasis Control (Dadzie 1998). The first control programme started in 1974, with the initiation of the OCP in West Africa. The OCP was a result of the combined efforts of the World Health Organization, the World Bank, the United Nations Development Programme and the UN Food and Agriculture Organization.

The goal of the OCP was to eliminate the threat of River Blindness from seven West African countries (Benin, Burkina Faso, Côte d'Ivoire, Ghana, Mali, Niger and

Togo) with vector control consisting of the larviciding of breeding sites. With the promising success of the OCP, four additional countries, Guinea Bissau, Guinea, Senegal, and Sierra Leone were added to the control programme in 1986 with a total operational area of 1.23 million sq. km (Molyneux 1995). Elimination of the parasite was thought to be possible by the intensive use of vector control consisting of aerial larviciding over vector breeding areas. This strategy aimed to kill the black-fly larvae and interrupt the transmission of disease. Vector control has led to the virtual elimination of the parasite from many formerly endemic areas. With the donation of Mectizan® (ivermectin) by Merck & Co., Inc. in 1987, the control strategy changed from exclusive vector control to vector control combined with ivermectin treatment and subsequently, in most areas, to ivermectin treatment alone.

In 2002, 28 years after the initiation of the OCP, the mandate of the OCP ended without the complete elimination of River Blindness. Although the complete elimination of *O. volvulus* was not achieved, the work of the OCP had accomplished the following:

- Prevention of over 30 million people in 11 OCP countries from developing ocular lesions due to *O. volvulus* infections. (Molyneux 1995)
- A new generation of children had been spared the risk of infection and developing blindness from *O. volvulus*. (Molyneux 1995)
- 1.5 million people originally infected were relieved of symptoms. (Molyneux 1995)
- 25 million hectares of riverine valley became safe for resettlement. (Molyneux 1995)
- The disease (as a public health problem) has been eliminated from Burkina Faso, Niger, large areas of southeast Mali, and northern areas of Côte d'Ivoire, Ghana, Togo and Benin. (Molyneux 1995).

The African Program for Onchocerciasis Control (APOC), which included 19 affected countries in Africa: Angola, Burundi, Cameroon, Central African Republic, Chad, Congo, Democratic Republic of Congo, Ethiopia, Equatorial Guinea, Gabon, Kenya, Liberia, Malawi, Mozambique, Nigeria, Rwanda, Sudan, Tanzania and Uganda, was established in December, 1995 (Remme 1995). The APOC was built on the success of the Onchocerciasis Control Program for West Africa, with the objective, to be achieved by 2007, to control the disease by establishing community-directed treatment with ivermectin. Unfortunately, APOC solely aims to treat infected individuals within the communities but is not designed to prevent transmission of the disease, since newly infected individuals may enter the treatment area and be a source for transmission (Richards et al. 2000). The program is also not monitoring the impact of the mass treatment.

Despite the benefits of these programs and the closing of the OCP in 2002, a conference on the "Eradicability of Onchocerciasis" concluded that onchocerciasis in Africa is not eradicable using current tools (Dadzie et al. 2003), particularly since a major concern is the possibility of re-entry of infected black flies into the OCP areas from bordering (APOC) countries which can reinitiate infections.

I.3 Macrocyclic Lactones: Ivermectin

Drugs belonging to the class of macrocyclic lactones are effective against a wide range of ecto- and endoparasites of animals and man. Ivermectin was the first of the macrocyclic lactone endectocides to be developed. It was very effective at low

concentrations and had a wide margin of safety (Campbell et al. 1983). The macrocyclic lactones are grouped into two major chemical families: the avermectins (including ivermectin and doramectin) and the milbemycins (including moxidectin) (Campbell et al. 1983). Ivermectin is a large molecule composed of a 16-member macrocyclic lactone ring derived from soil bacteria of the species *Steptomyces avermitilis* (Burg et al. 1979). Ivermectin is a mixture of its two isomeric forms and therefore has the chemical formula $C_{48}H_{74}O_{14}$ (22,23-dihydroavermectin B_{1a}) + $C_{47}H_{72}O_{14}$ (22,23-dihydroavermectin B_{1b}).

Ivermectin is the sole safe and effective drug given to individuals infected with *O. volvulus*. The standard dose given to patients is 150 μg/Kg bodyweight annually (Dull and Meredith 1998). Many millions of people worldwide have taken ivermectin for various parasitic infections. With the vast majority of cases, no serious drug-related adverse events have been reported although side effects can include one or more of the following: fever, headache, chills, arthralgia, rash, eosinophilia, and anorexia. Many of these symptoms are thought to result from the death of parasites rather than as a reaction to the drug (Kar et al. 1994). Ivermectin has been used extensively in the past against onchocerciasis with very low incidence of serious adverse effects. Unfortunately, ivermectin has been linked to an increased death rate in humans if the individuals are also co-infected with *Loa loa* (Boussinesq et al. 1998). In addition, there have been reports that elderly individuals treated with ivermectin for scabies infection had an increased death rate compared to a control group (Barkwell and Shields 1997).

In humans, oral ivermectin is absorbed rapidly into the blood stream where it reaches a serum concentration peaking at 30–46 ng/mL by 2.7 – 4.3 hours and slowly

decreases thereafter. Ivermectin has a half-life of 28 ± 10 hours (Edwards et al. 1988; Ette et al. 1990; Baraka et al. 1996). The drug is metabolized in the liver and the metabolites are mainly excreted in the feces (98%) and a small amount in the urine (1%) (Edwards et al. 1988). In *O. volvulus*, the mechanism of entry and exposure level to ivermectin is unknown. *In vivo* experiments were conducted with *Onchocerca ochengi*, a cattle filiarid related to *O. volvulus* by Cross et al. (1998), in which [^{3}H]ivermectin was used to determine if ivermectin uptake can occur via the pharyngeal-oral and/or transcuticular routes. Their results indicate a minority of the [^{3}H]ivermectin entered via the pharyngeal oral route and suggested that the majority of the uptake was by a transcuticular mechanism. They did not investigate other possible mechanisms of entry.

Although ivermectin has helped eliminate the majority of the skin pathology and prevented the development of blindness in infected patients since its introduction, it lacks significant macrofilaricide activity. Although ivermectin is poorly macrofilaricidal, it is effective in preventing microfilaria production in female worms for a prolonged period of time (Awadzi et al. 1999). Adwazi *et al.* (1999) have shown that increasing the dosage of ivermectin to five times, and even ten times that of the standard dose, had no significant effect on the survival of the adult worms. Due to the lack of significant macrofilaricidal activity, infected individual must take ivermectin for at least the life span of the parasite, which can be up to 13 years (Calamari et al. 1998).

Experiments in *H. contortus* determined that concentrations of ivermectin ≥0.1 nM paralysed the pharynx of adult worms . At higher concentrations, ≥10 nM, systemic paralysis occurs (Geary et al. 1993; Yates et al. 2003) but paralysis is limited to the

mid-body region of the worm. Interestingly, the head and tail regions of the worm were still able to move normally . Ivermectin exerts its effect by selectively binding and opening inhibitory chloride channels. Two chloride channels have been associated with resistance to ivermectin in nematodes; the glutamate - gated chloride channels and γ-aminobutyric acid (GABA) – gated chloride channels. The opening of the channels by the natural ligands, glutamate or GABA or by a drug such as ivermectin, allows an influx of extracellular chloride ions causing hyperpolarization of the cellular membrane. The end result is an elevation of the resting potential, hence no action potential can occur and the target neruo muscular system is paralyzed (Geary et al. 1993; Courtney and Robertson 1995; Omura and Crump 2004).

The lack of a strong macrofilaricidal activity is unfortunate, but studies suggest that the therapeutic dose of ivermectin is directed towards the larval stages of *O. volvulus* and the reproductive system in adult females. In the case of the microfilaria, the exact mechanism of killing remains essentially unknown. With respect to the action toward the adult females, ivermectin has been shown to prevent the release of microfilariae and/or increase the number of degraded microfilaria present in the uteri. This was visible in the embryograms of worms from treated people studied by Awadzi et al. (1999). A possible mode of action, perhaps, is by muscle paralysis via hyperpolarization of the cellular membrane of the muscles in the reproductive organs and inhibition of release of the microfilariae from the female worms.

I.4 Ivermectin Resistance

Drug resistance to ivermectin has developed in many parts of the world where the drug has been used to treat parasitic infections in livestock, such as *H. contortus* in sheep and goats (Wolstenholme et al., 2004), *Oesophagostomum spp.* in pigs (Varady et al. 1997), and sheep (Waller et al. 1996). Fortunately, there is as yet no unequivocal evidence that resistance to ivermectin in *O. volvulus* populations exist (Prichard 2005). However, due to the large number of individuals taking ivermectin for long periods, (between 1987-1997, 96 million doses of ivermectin had been taken) it is possible that ivermectin resistance will develop in *O. volvulus* and that there could be severe consequences for public health (Hall and Pearlman 1999; Prichard 2005).

Currently, ivermectin resistance has only been reported in nematodes of livestock. However, ivermectin is presently used to treat onchocerciasis in humans. The concern for ivermectin resistance is great, due to the speed with which ivermectin resistance can develop. For example, ivermectin was introduced to South Africa in the middle 1980's; within 33 months, ivermectin resistant organisms had been detected (Shoop 1993).

Unfortunately, ivermectin resistance in *O. volvulus* may have already emerged. Awadzi et al. (2004), compared two populations of patients in Ghana, West Africa. One population of patients had received annual doses of ivermectin, whereas the second population did not. Their analysis of microfilariae counts, 90 days after treatment with ivermectin, indicated the existence of patients with a sub-optimal response phenotype,

defined as greater than 10 microfilariae per skin snip after nine or more treatments of ivermectin.

Despite multiple treatments with ivermectin, female parasites in sub-responding patients almost continuously released microfilariae and tested positive for embryos in the embryogrammes. The article concluded that the lack of response to ivermectin was likely not due to the placebo effect, host factors or new infection, but rather from the lack of response in adult female worms.

Besides ivermectin, other filarial drugs such as diethylcarbamazine (DEC) and suramin, which have macrofilaricidal activities, have been developed and used. DEC is currently used extensively for lymphatic filariasis, except in sub-Saharan Africa (Prichard 2005). Both drugs have some macrofilaricidal effects. Unfortunately, both can cause severe side effects, suramin being more severe, in individual infected with onchocerciasis (Thylefors and Rolland 1979; Francis et al. 1985). Since ivermectin is currently the only safe drug available to treat onchocerciasis, the possible development of resistance may threaten the people living in endemic areas as well as undo all the years of work that have gone into controlling this parasite (Gardon et al. 1997).

I.5 Multifactoral Resistance - Possible Mechanism of Ivermectin Resistance

Multifactoral resistance is a term used to describe how more than one mechanism of drug resistance operates simultaneously in organisms that are drug resistant (Gottesman et al. 2002). This phenomenon develops when a nematode population is composed of genetically heterogeneous individuals. Individual responses to an external

stimulus, such as a drug, can range from toxic to tolerance. Repeated exposures of the population to the drug will continually select individuals for their ability to survive and grow in the presence of the drug. Because the initial population is genetically heterogeneous, the drug selected population may have more than one mechanism of drug resistance. The following sections will describe some nematodes genes which have been linked to ivermectin selection.

I.5.a. Glutamate-gated Chloride Channels (Glu-Cl)

Glutamate-gated chloride channels (Glu-Cl) are members of a ligand-gated ion channel superfamily. They are members of the 'cys-loop' class of ligand-gated ion channels that are found only in invertebrates (Cleland 1996). Structurally, they are composed of multiple subunits, each of which contains an N-terminal extracellular domain and four transmembrane domains (m1-m4) with a cysteine loop between m3 and m4 which contains a protein kinase C phosphorylation site (Unwin 1995). The proposed channel consists of five subunits, which form a ring structure that spans the cell membrane (Unwin 1989). Other members of the ligand-gated ion channel superfamily include glycine receptors, serotonin type-3 receptors, γ-aminobutyric acid chloride receptor and nicotinic acetylcholine receptors (Unwin 1989; Hosie et al. 1997).

Glutamate-gated chloride channels were first discovered in arthropods and have also been found in nematodes like *Caenorhabditis elegans*, *Haemonchus. contortus* (Cully et al. 1996; Blackhall et al. 1998), *Cooperia oncophora* (Njue and Prichard

2004) and *O. volvulus* (Cully et al. 1996). Interestingly, Glu-Cl not been identified in any vertebrate species, which makes the Glu-Cl receptors an ideal target for drug development.

In the invertebrate nervous system, the Glu-Cl channels are responsible for the majority of inhibitory transmission signals. These channels are located extra-junctionally on the muscle and on neuronal cell bodies. The release of glutamate targets the glutamate-gated chloride channels and activates the channel (opens the channel) in a reversible manner. The opening of the channel allows the influx of chloride ions which results in the hyperpolarization of the cell (Cully et al. 1996; Dent et al. 2000). Analogs of glutamate such as ibotenic acid can compete directly for the ligand binding site or bind elsewhere on the channel to cause it to open, as is the case with the avermectins (Cully et al. 1996). In nematodes, the α-subunit of the Glu-Cl channel has been shown to be the target for ivermectin (Cully et al. 1994; Dent et al. 1997; Forrester et al. 2002; Forrester et al. 2004). Ivermectin binds to the α-subunit in an essentially irreversible manner leading to the opening of the Glu-Cl channel. The prolonged opening of the channel alters the membrane potential of the cell membrane by allowing excess chloride ions to enter the cell from the extra-cellular matrix resulting in a state of hyperpolarization of the cell and flaccid paralysis of the worm (Blackhall et al. 1998; Prichard 2005).

The mechanism of ivermectin resistance in relation to Glu-Cl was initially investigated by Rohrer et al. (1994) who concluded that the resistance to ivermectin was not the result of an alteration in the binding of ivermectin to the Glu-Cl. However, a single amino acid mutation in the α-subunit may be responsible for an alteration in

the conformational change that occurs when ivermectin binds to the Glu-Cl channels, limiting the action of ivermectin on the Glu-Cl channels (Etter et al. 1996; Blackhall et al. 1998). Dent et al. (2000), generated null mutations in the three α-subunit type genes, which encode the glutamate-gated chloride channel subunits, *avr-14, avr-15*, and *glc-1*. The null mutations resulted in high-level ivermectin resistance, confirming the role of these gene products in the action of ivermectin. Interestingly, they noted that mutating any two, instead of all three, α-subunit genes confers only a modest or no resistance. Genetic selection for the Glu-Cl α-subunits was also identified in ivermectin selected strains of *H. contortus* (Blackhall et al. 1998; Forrester et al. 2002) and ivermectin resistant *C. oncophora* (Njue and Prichard 2004). There was no significant selection in the β-subunit in either *H. contortus* (Blackhall et al. 1998) or *C. oncophora* (Njue and Prichard 2004).

Cook et al. (2006), has provided evidence that genes that encode the α-subunits, *avr-14b, avr-15* and *glc-1* are potentially involved in locomotion patterns in the non-parasitic nematode *C. elegans.* Nematodes with mutations in any of these three α-subunits exhibit a decrease in the duration that *C. elegans* moves in a particular direction (Cook et al. 2006). Interestingly, these three α-subunits are also the three α-subunits which have been shown to be involved in ivermectin susceptibility by Dent et al. (2000).

The relationship between glutamate-gated chloride channels and ivermectin has been studied in laboratory selected strains of *H. contortus* with results pointing toward Glu-Cl channels as a contributing factor to ivermectin resistance (Dent et al. 2000; Forrester et al. 2004). The only evidence of a functional relationship between coding

changes in a Glu-Cl subunit and ivermectin resistance in a field isolate of a parasitic nematode remains the data of (Njue et al. 2004) with *C. oncophora*. Unfortunately the physiological role of the Glu-Cl channels in relation to ivermectin resistance in other field isolates of parasitic nematodes remains uncertain.

The lack of Glu-Cl in vertebrates suggests that Glu-Cl is an ideal target for drug design. However, it may also be a good target to focus on for development of a marker for ivermectin resistance in *O. volvulus*. Currently, only a limited amount of information and experimental work have been performed on the family of glutamate-gated chloride channels in *O. volvulus*. Eng and Prichard (2005) performed single strand conformational polymorphism (SSCP) and restriction fragment length polymorphism analysis of a Glu-Cl channel subunit and identified polymorphisms, but did not find a significant change in allele frequency between ivermectin exposed and unexposed *O. volvulus*.

I.5.b γ-aminobutyric acid Chloride Channels (GABA-Cl)

In addition to the glutamate-gated chloride channels, an additional member of the ligand-gated ion channel superfamily, the γ-aminobutyric acid chloride channels (GABA-Cl) is believed to be linked to ivermectin action and resistance in nematodes. Similar to other members of the ligand-gated ion channels, the purposed three-dimensional structure consists of a pentameric assembly of five different protein subunits. Each of the GABA subunits contains a cysteine loop on the N-terminus and four transmembrane domains. The second transmembrane domain (M2) of each subunit forms the pore lining of the ion channel (Unwin 1989; Bloomquist 2003).

γ-aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in vertebrate and invertebrate nervous systems and is an important constituent in the nematode nervous system which controls locomotor function . The release of GABA molecules specifically targets the GABA receptor type A located on the neuromuscular cells and causes the opening of chloride ion channels. The opening of the ion channel allows the entry of negatively-charged chloride ions into the cell and results in a hyperpolarizing inhibition of the cell (Casida 1993; McIntire et al. 1993). GABA type A receptors have been targeted for pesticide development in which many compounds such as picrotoxin can act as antagonists of the GABA receptors. In insects and mammals, the result of antagonist exposure is hyperexcitability, spontaneous nerve activity and muscle convulsions due to the lack of coordinated inhibition of nerves and muscle excitation (Bloomquist 2003).

Experimental data with the parasitic nematode *Trichinella spiralis* showed that [^{3}H]ivermectin can bind and directly compete with GABA at the GABA receptor (Ros-Moreno et al. 1999). Therefore, ivermectin can be used to target and disrupt the GABA-mediated interneuron-motorneuron transmission in nematodes. Experimental results have shown clear evidence that ivermectin is an agonist of glutamate-gated chloride channels. However, with the GABA-Cl channels, the ligand receptor interaction is not fully agreed upon, due to contrary experimental results. In experiments conducted with somatic muscle cells of *Ascaris* (Kass et al. 1980; Holden-Dye and Walker 1990), the results indicated that the interaction between ivermectin and GABA receptors appears to be an antagonistic mechanism whereas experimental results in *H. contortus* lean

towards an agonist effect (Feng et al. 2002). It is therefore possible that the function of the GABA receptor diverges in function in different species or tissues.

Blackhall et al. (2003), investigated the M2 region of the *H. contortus* (HG1) GABA-Cl channel by SSCP and compared the allele frequency profiles between an unselected and two anthelmintic-selected strains (moxidectin and ivermectin selected). Seven alleles (A-G) were identified, and in both anthelmintic-selected strains the frequency of allele E increased significantly (55%) compared to the parental strain (15 %). The identification of selection for the HG1 allele E (HG1E) was evidence that the GABA receptors in *H. contortus* were subject to genetic selection during the development of resistance and could be implicated in the modes of action and of resistance. All nucleotide differences identified between the two alleles, in the M2 region investigated, were silent mutations.

At the same time, Feng et al. (2002) cloned and sequenced the complete cDNA of the resistance-associated HG1E allele and the susceptible associated HG1A allele of the GABA-Cl receptor and identified single nucleotide polymorphisms that translated into four amino acid differences between the two GABA-Cl alleles. Two amino acid changes were identified in the N-terminal extracellular cysteine loop and two were identified in M4.

Electrophysiological characterization of two HG1 alleles was conducted in a *Xenopus oocyte* expression system. To form a functional channel, a *C. elegans* GABA β-subunit was co-expressed along with the HG1 constructs. Co-expression of the HG1A along with a *C. elegans* GABA β-subunit formed a channel which was able to potentiate a current when ivermectin and GABA were applied. Interestingly, when the

HG1E allele was coexpressed with *C. elegans* GABA β-subunit to form the hetero-oligomeric receptors, the current produced was much attenuated in the presence of ivermectin. This results suggest that the reduced activity of the GABA receptor from the resistance associate allele, HG1E, may limit the effects of ivermectin and hence result in a possible mechanism to resist the toxic effects of ivermectin (Feng et al. 2002).

In filarids, putative GABA sequences have been identified in *Dirofilaria immitis* (Yates and Wolstenholme 2004) and *O. volvulus* (T. Egwang Pers. Comm.). Eng and Prichard (2005) analyzed a 539 bp sequence of the putative *O. volvulus* GABA cDNA sequence by SSCP and, contrary to the results obtain from *H. contortus* M2 region, no significant changes were identified in allele frequency in the *O. volvulus* population exposed to multiple yearly treatments of ivermectin compared to the worms from IVM naïve subjects.

A strong emphasis has been placed on the ligand gated ion channels as the target for ivermectin resistance, particularly in *H. contortus*. The effects of targeting the glutamate-gated chloride and GABA-chloride channels results in the paralysis and death of the worm. In *O. volvulus*, genetic analysis by Eng and Prichard (2005) showed that neither the glutamate-gated chloride, nor the GABA-chloride channels investigated, had a significant change in allele frequency after multiple yearly doses of ivermectin. One important consideration is that motility may not play a large factor in the survival of the filarids compared to ruminant or terrestrial nematodes. Therefore, the selective pressures upon the filarids may differ, which may lead to an alternative mechanism of drug resistance. Hence, the ligand-gated ion channels may not play a

strong contributing role in ivermectin resistance in *O. volvulus*. However, further work may show their possible involvement in developing mechanisms of IVM resistance in filarial worms.

I.5.c. P-glycoprotein (PGP) and P-glycoprotein like proteins

P-glycoproteins (PGP) are a group of ATP-binding cassette transporter proteins that are part of a superfamily of integral plasma membrane proteins that function as ATP-dependent transport pumps (Aouali et al. 2005). The transmembrane regions of the PGP can bind to hydrophobic drugs that are either neutral or positively charged. In order for the pump to transport a molecule of the drug, two molecules of ATP are hydrolyzed. The hydrolysis of the first ATP is required to efflux the drug, whereas the hydrolysis of the second ATP, on the second ATP binding domain, is required to 'reset' the transporter (Gottesman et al. 2002).

These protein pumps are located in various areas of the organism such as the gut, gonads, kidneys, biliary system, and nervous system. The family of PGPs function by non-specifically transporting a diverse range of molecules from the interior of the cell and the cell membrane to the exterior milieu via the hydrolysis of ATP. PGPs are not only found in many normal cells but have been reported to be involved in multi-drug resistance in human cancer cells (Aouali et al. 2005). Substrates for the P-glycoprotein are quite diverse but do share some similarities, such as being amphiphilic, lipophilic, planar in shape and usually possess a ring structure (Ford and Hait 1990). Ivermectin has many of the characteristics described and has been shown to be a candidate ligand for P-glycoprotein (Didier and Loor 1996; Pouliot et al. 1997)

The function of these protein pumps have been conserved during evolution and they are present in a diverse range of organisms ranging from insects (*Drosophilia melanogaster*) (Wu et al. 1991), protozoa (*Plasmodium falciparum*) (Foote et al. 1989; Broeks et al. 1995; Sharom et al. 1995), and nematodes such as *C. elegans* (Lincke et al. 1992), *H. contortus* (Xu et al. 1998) and *O. volvulus* (Huang and Prichard 1999; Eng and Prichard 2005). In *C. elegans*, 15 PGPs have been identified and countless more ABC transporter genes with similar activity have been described and/or identified, i.e. *abt, mdr, coq, mrp*, and *haf.* (http://www.sanger.ac.uk).

The current accepted (but still controversial) PGP structure consists of a 170 kDa membrane-bound protein with two similar halves each containing six transmembrane domains, which are linked by a hydrophilic region and an ATP binding domain on the cytoplasmic side. The final structure is a single aqueous pore (Sangster et al. 1999; Jones and George 2000). ABC transporters can exist in two forms, either as a single whole transporter ("full-size") or as the result of two subunits which may be the same or different ("half size") (Schneider and Hunke 1998).

Over expression of P-glycoprotein has been implicated in multi-drug resistance in human cancer cells (Scala et al. 1997) and in the nematode *H. contortus* (Xu et al. 1998). In the mouse model, the *mdr1a* gene codes for P-glycoprotein, which when knocked out, results in a dramatic increase in ivermectin sensitivity (Schinkel et al. 1994). In nematodes, Xu et al. (1998) performed a Northern blot experiment that provided supporting evidence of P-glycoprotein over expression in ivermectin selected strains of *H. contortus*. Blackhall et al. (1998) then supported the notion that the *H.*

contortus P-glycoproteins were involved in ivermectin resistance by showing allele selection in a P-glycoprotein gene in macrocyclic lactone selected *H. contortus*.

In another study of genetic polymorphism in *H. contortus*, Sangster et al. (1999) also found selection on P-glycoprotein genes in ivermectin resistant strains of the parasite, and Le Jambre et al. (1999) suggested that a particular P-glycoprotein gene was involved in macrocyclic lactone resistance *H. contortus*. However, Le Jambre et al. suggested that *H. contortus* P-glycoprotein may not be the main determinant of ivermectin resistance but rather linked to genes which are directly involved. This conclusion was based on results that showed that not all the individual worms of the *H. contortus* strain investigated, which survived ivermectin treatment, contained a P-glycoprotein allele thought to be linked to resistance. In *O. volvulus*, Eng and Prichard (2005) used single strand conformational polymorphism and restriction fragment length polymorphism and identified a statistically significant change in allele frequencies between worms from ivermectin exposed and ivermectin naïve people. Ardelli et al. (2006), further concluded that the annual doses of IVM for onchocerciasis control reduce gene diversity in P-glycoprotein from *O. volvulus*.

The P-glycoprotein like protein (PLP) belongs to a class of "half-size" transporters and contains six transmembrane domains and one ATP-binding domain. Individual half size transporters can either form a hetero- or homodimer to become a functional "full-size" transporter (Gottesman et al. 2002). Currently, very little information is available on PLPs of nematodes and their role in drug resistance, although we can postulate that PLPs may be involved in multidrug resistance since a

particular human PLP (ABCG2) has been linked to mitoxantrone resistance in acute myelogenous leukemia (Gottesman et al. 2002).

Currently, only one putative P-glycoprotein like protein (PLP) has been identified in *O. volvulus* which was initially described by Huang and Prichard (1999). Genetic analysis with SSCP by Eng and Prichard (2005) on a 356 bp fragment of the *O. volvulus* PLP gene identified genetic polymorphism but did not reveal any genetic selection in worms exposed to multiple treatments of ivermectin. Subsequently, a more detailed analysis of the complete *O. volvulus* PLP gene by Ardelli and Prichard (2006) did reveal a loss of genetic polymorphism in the PLP allele profiles in *O. volvulus* exposed to multiple treatments of ivermectin compared to naïve worms.

I.5.d. β-tubulin

Tubulin is a ubiquitous protein molecule which makes up microtubules. Microtubules are composed of two distinct polypeptides, α- and β-tubulin. The two subunits are similar but have distinct activities, for example, both the α-subunit and β-subunit can bind GTP but only the β-subunit E-site (exchange) possesses GTPase activity (Dustin 1984). In some eukaryotes cells, an additional five members of the tubulin superfamily have been identified, some of which have been identified in parasitic organisms; γ-tubulin (*Trypanosoma brucei)*, δ-tubulin (trypanosomes and *Plasmodium*), ε-tubulin (*T. brucei* and *Leishmania major*), ζ-tubulin and η-tubulin, having been identified to date (McKean et al. 2001). Currently only the β-tubulin isotype 1 and α-tubulin have been identified in *O. volvulus*; none of the other tubulin family members has been identified in *O. volvulus*.

In vertebrate species, *in vitro* experiments have identified seven β-tubulin isotypes

with tissue-specific distributions and differences in stability and function. Tissue specific localization of individual β-tubulin isotypes may provide the cell with a means of regulating the dynamic behaviour of the microtubules (Schwarz et al. 1998). It is still uncertain if differential localization truly exists *in vivo* since some studies have indicated that microtubules can be composed of all available isotypes and are functionally interchangeable within the same cell (Lopata and Cleveland 1987), whereas another report suggested that the high degree of homology present in the different β-isotypes results in redundancy of gene products (Joshi and Cleveland 1990). In lower eukaryotes, such as with *Drosophila spp.*, a single β-tubulin isotype gene product is sufficient for constructing multiple classes of microtubules (Joshi and Cleveland 1990).

Within the cell, the dimerization of α- and β-tubulin occurs in a head-to-tail arrangement onto the (+) ends of microtubules. The process of dimerization is a complex mechanism involving several steps with co-factors (A-E) and a small monomeric G protein called ADP ribosylation factor-like protein 2 (Bhamidipati et al. 2000). The newly synthesized tubulin protein is captured by chaperon proteins such as chaperonin immediately after translation. The chaperonin then begins to fold the linear protein into its native three dimensional conformation. After release from the chaperonin, the α-tubulin gets bound by cofactor B, while β-tubulin is bound to cofactor A. In the following chain of reactions, cofactors A and B are replaced by cofactors D and E, respectively. In the final step of dimerization, a super-complex is formed consisting of cofactor C and the ADP ribosylation factor-like protein 2 interacting with cofactor D. After this, a super complex is formed, GTP catalysis is

required to release the tubulin heterodimers from the dimerization complex (Bhamidipati et al. 2000; Grynberg et al. 2003).

Polymerization follows dimerization if favorable conditions are present, such as the presence of GTP, cofactors like Mg^{+2} and assistance from microtubule-associated proteins. Microtubule-associated proteins such as MAP2 and Tau, are believed to promote the stabilization of the microtubule by binding to the surface of the microtubule which can bridge several tubulin subunits together and may also play a role in neutralizing any repulsive charge on the microtubule surface (Heald and Nogales 2002). The assembling and disassembling of tubulin subunits requires binding, hydrolysis and exchange of GTP. The polymerization of the subunits forms a hollow tube shape structure called a protofilament. The protofilaments are further assembled into microtubules which are involved with cellular processes such as cell division, intracellular transport, extracellular secretion, cell signaling, cell motility, chromosomal segregation and cell morphology (Dustin 1984; Zabala et al. 1996; Correia and Lobert 2001).

Microtubules are dynamic molecular structures which are in a state of continuous polymerization and depolymerization. Interestingly, the half life of mammalian β-tubulin protein varies from hours to as long as 24 days (Sedman et al. 1986). Since tubulin is interconnected to virtually every cellular organelle, a normal functional tubulin protein is essential to the nematode's survival, since the proper organization of each structure depends not only on the α- and β-tubulin interactions as heterodimers but also on the interactions between tubulin and other proteins (Raff et al. 1997). Aboobaker and Blaxter (2003) showed the importance of a functional tubulin protein

by RNA interference (RNAi) experiments in *B. malayi*. It was possible to suppress production of β-tubulin by soaking the nematodes in a solution containing RNAi for β-tubulin. The effect of the RNAi was not immediate, but all the experimental worms eventually died, due to loss of β-tubulin protein synthesis.

The three-dimensional structure of the β-tubulin protein was a mystery until Nogales et al. (1998) generated a 3.7 Å resolution of the protein using electron crystallography. The three-dimensional images demonstrated that α- and β-tubulin monomers have nearly identical structures. The difficulty of generating a proper crystal structure was due to the dynamic nature of the β-tubulin protein. In order to crystallize the β-tubulin protein, Nogales et al. (1998) added Zn^{+2} molecules to form stabilized tubulin sheets in which a crystal was generated. This model provided the first detailed description of the β-tubulin gene product, which included the predicated site where the taxol molecule binds (Nogales et al. 1998).

The dynamic nature of tubulin also makes it a target for drug development. Antimitotic drugs such as taxol, colchicines, podophyllotoxin and vinblastine/vincristine have been used to suppress the function of tubulin following binding of the drug, either to free tubulin or to tubulin heterodimers. The drug-tubulin complex is recruited to the growing (+) ends of microtubule, preventing further polymerization (Correia and Lobert 2001).

Besides being a target for antimitotic drugs, tubulin is also the target for a group of broad-spectrum anthelmintics called the benzimidazoles. The benzimidazole family of drugs also possess antimitotic properties. *In vitro* studies using purified recombinant α- and two β-tubulin protein from *H. contortus* indicated that mebendazole was able to

bind to all three proteins with α-tubulin having the highest affinity (Oxberry et al. 2001). The benzimidazole drug is believed to bind on or near the colchicine binding site on the tubulin molecule, near the GTP binding domain and therefore exerts its effect by interfering with either the nucleotide exchange process and/or preventing the elongation of the polymers, resulting in the depolymerization of the microtubules into individual subunits (Borgers and De Nollin 1975; Lacey 1988; Roos et al. 1990).

Bioinformatic analyses of different β-tubulin sequences from various groups of nematodes, including *Dirofilaria immitis*, *O. volvulus, B. pahangi* and *Onchocerca gibsoni*, were aligned for comparison and it was determined that there were a number of β-tubulin isotypes, each with a different degree of benzimidazole sensitivity (Geary et al. 1998). For example, in *H. contortus,* two β-tubulin loci, isotype 1 and 2, have been identified and it is believed that benzimidazoles exert selection pressure on both β-tubulin isotypes (Kwa et al. 1993). With the isotype 1 locus, it was determined that there was a single amino acid substitution (mutation) from a phenylalanine to a tyrosine, at position 200, which confers resistance to benzimidazoles (Kwa et al. 1993). This specific point mutation in β-tubulin was present in benzimidazole resistant strains of *H. contortus* (Kwa et al. 1993; Roos et al. 1995). Experimental results published by Kwa et al. (1995) showed the functional importance of that single amino acid substitution at position 200 by heterologous expression of a benzimidazole-susceptible *H. contortus* β-tubulin isotype 1 in benzimidazole-resistant *C. elegans*. Expression of the BZ-susceptible *H. contortus* gene resulted in a susceptible phenotype. In addition to selection for particular alleles of isotype 1 and isotype 2 β-tubulins (Beech et al. 1994),

intense selection with benzimidazoles may result in deletion of the isotype 2 β-tubulin (Kwa et al. 1993).

Currently, there is little evidence linking β-tubulin directly with ivermectin resistance. Preliminary results from Blackhall (1999), using single strand conformational polymorphism, identified a significant change in β-tubulin allele frequencies from ivermectin selected strains of *H. contortus* compared with the parental strain. Genetic selection in the β-tubulin gene was hypothesized as a result of a genetic hitch hiking event in which β-tubulin selection was due to linkage to another gene on the same chromosome which was involved in the resistance mechanism. Subsequently, (Kneussel and Betz 2000) provided possible evidence that microtubules play a role in anchoring and concentrating glycine and GABA ion channels in the membrane of synaptic junctions. It is, therefore, possible that microtubules play a similar role in localizing surface receptors involved in ivermectin binding, such as the glutamate and GABA-gated chloride channels in *H. contortus* and *C. elegans*. Hence, it is conceivable to imagine that selection for a particular allele of a gene such as a GABA-gated chloride channel subunit and/or a glutamate gated chloride channel subunit, in ivermectin selected nematode, may indirectly cause selection for a particular β-tubulin allele.

Recently, Eng and Prichard (2005) identified a significant change in allele frequency in the β-tubulin isotype 1 gene in *O. volvulus* exposed to multiple yearly doses of ivermectin. Sequencing the two β-tubulin alleles identified three amino acid changes in the third helix (H3). The third helix of the β-tubulin protein is believed to interact with the M-loop of the adjacent β-tubulin in the formation of the microtubule

and is also believed to be involved in the binding of the γ-phosphate of the E-site (Lowe et al. 2001). Therefore, the three amino acid changes in the H3 may alter the overall stability of the microtubule. Although strong evidence which links β-tubulin to a direct mechanism of ivermectin resistance is lacking, β-tubulin may still be of value in the development of a genetic marker to monitor the development of resistance to ivermectin.

I.5.e. Amphid-dye filling protein

Three hundred and two neurons have been identified in the soil nematode *C. elegans*, of which 12 are located in the amphids for chemosensing. Chemicals can enter the amphid neurons via the amphid pores on the anterior head of the nematode. Within the pore lie dendritic processes. It is by these processes that the nematode can "taste" the environment. The amphids aid in seeking out and reacting to food, avoiding toxic substances, such as drugs, and in finding suitable mates (Ashton et al. 1999; Wicks et al. 2001; Fujii et al. 2004).

In *C. elegans*, 13 dye-filling (dyf) defective mutants have been identified (www.wormbase.org). Dyf defective nematodes are so-named because of their inability to take up lipophillic dye, i.e. fluoroisothiocyanate (FITC) into the amphids and phasmid neurons, from the environment via the amphid sensory endings (Starich et al. 1995). A phenotypical consequence of a dyf mutant is that the amphid becomes highly disorganized; there is a profound defect on behavior. The worm requires an intact amphid for chemosensation, such as toxin avoidance and mating (Starich et al. 1995; Dent et al. 2000).

Experimental uptake experiments with gastrointestinal nematodes suggest that one mode of entry for ivermectin is by ingestion via the oral pharyngeal route. The drug then targets the glutamate-gated channels and GABA-gated chloride channels causing flaccid paralysis of the nematode (Cully et al. 1994). But experiments, performed by Smith and Campbell (1996.), demonstrated that temporarily inducing paralysis of *C. elegans*, by soaking the worms in 0.15% 1-phenoxy-2-propanol, caused suppression of pharyngeal pumping. If the worms were then placed in a solution of ivermectin, the worms remain paralyzed compared to the control worms in water, in which motility returned. The results suggest an alternative route of entry, in addition to the oral/pharyngeal route.

Possible alternative routes could be a cuticular uptake mechanism (Ho et al. 1990; Cross et al. 1998) and/or uptake via the anterior amphidial neurons. The nematode amphid sensory neurons are used for chemical and thermal signaling in *C. elegans* (Dent et al. 2000), *H. contortus* (Ashton et al. 1999) and *O. volvulus* (Strote et al. 1996). Since there is evidence that the amphid are used for "tasting" the environment, it is therefore reasonable to think that amphid mutants, such as dyf-8 mutants, may result in ivermectin resistance, because they can render the worm less permeable to the drug.

A limited amount of information is available on the phenotype of dyf-8 mutants. Wicks et al. (2001) predicted that the dyf-8 gene encoded a protein with three characteristic features. First, it was suggested that the protein was secreted due to the presence of an N-terminus signal sequence. Second, the protein is membrane bound due to a single predicted transmembrane domain found near the C-terminus. Thirdly, on

the extracellular domain of this putative membrane-bound protein, there is a domain called the *zona pellucida*. The *zona pellucida*, which has been shown in other proteins, mediates protein-protein interactions on the extracellular surface of cells. Therefore, the dyf-8 gene product may be required to form the connection between sheath and socket cells during the brief period in late embryogenesis when the amphid is being formed (Wicks et al. 2001).

Grant and Hunt (pers. commun. cited in Starich et al. 1995) observed that all ivermectin-resistant *C. elegans* larvae were dyf mutants and determined that the amphidial neurons played a significant role in determining ivermectin sensitivity. Johnson et al. (pers. commun. cited in Starich et al. 1995) also proposed the same hypothesis and added that amphidial defective *C. elegans* mutants were able to grow in culture medium containing 5 ng/ml of ivermectin.

In 2003, Freeman et al. compared the amphid structure between ivermectin laboratory naïve and resistant strains of *H. contortus*. Their findings showed a significant shorting and diffused morphology of the amphidial dendrites in ivermectin resistant strains, compared with wild-type strains. This is a similar phenotype to that identified in dyf mutants in *C. elegans* (De Riso et al. 1994). The authors suggested that the shortening and possible dysfunction of the amphid dendrites may prevent access of the drug into the nematode.

Evidence consistent with the findings of Freeman et al. (2003) was provided by experimental paraquat resistance in *C. elegans* mutants. The paraquat mutants, *mev-5-7*, were not only resistant to paraquat but were also identified with the dye-filling defective phenotype . The authors suggested that the chemosensory neurons in *C.*

elegans were most likely the primary target for paraquat . Therefore the presence of the dyf phenotype resulted in a defect in the uptake of the drug, hence leading to the resistance phenotype.

Experiments of (Fujii et al. 2004; Fujii et al. 2005; Kotze and Bagnall 2006) also suggest the possibility of drugs entering the parasite via a non-oral route based on their results of RNAi experiments against the β-tubulin gene *H. contortus*. The experiments were conducted with various stages of the parasite, but it was interesting that results obtained for the L3 larvae stage, which contains no functional feeding mouth, still showed an ability of the RNAi to decrease the expression level of the targeted β-tubulin gene.

Recently, Eng and Prichard (unpublished) amplified and cloned a small fragment of a putative *O. volvulus* dyf-8 gene in which genetic analysis by SSCP identified a significant difference in the allele frequency from nematodes exposed to multiple yearly treatments of ivermectin compared to an ivermectin naïve population. Since the oral-pharyngeal route in *O. volvulus* is believed to be non-functional (Strote et al. 1996), a loss of function in the amphid neurons may allow *O. volvulus* to limit the amount of ivermectin taken up.

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Connecting Statement I

Limited genetic analysis by Keddie *et al.* (1999) and Unnasch and Williams (2000) revealed a low level of genomic heterogeneity in *O. volvulus* which they hypothesized as the reason why ivermectin resistance had not yet emerged in *O. volvulus* even after nearly than two decades of ivermectin treatment. Due to the limited number of genes previously analyzed, we undertook to analyze a greater number of *O. volvulus* genes. In contrast to previous conclusions, we have shown that there is genetic heterozygosity in some *O. volvulus* genes. Furthermore, we have shown that a few genes may be linked to ivermectin selection. These results are presented in Chapter II.

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Chapter II: Manuscript I

A Comparison of Genetic Polymorphism in Populations of *Onchocerca volvulus* from Untreated- and Ivermectin-Treated Patients

J.K.L. Eng and R.K. Prichard

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Abstract

An analysis of the polymorphism of 16 genes from *Onchocerca volvulus* was undertaken, in two populations of worms from either ivermectin-naïve patients or patients who had been repeatedly treated with ivermectin, in Ghana. Six genes were selected for analysis because studies in other nematodes had suggested a possible association with ivermectin resistance. The other 10 genes were included as control genes and have not been associated with ivermectin resistance. Twelve of the 16 genes were polymorphic, including five of the candidate genes and seven of the control genes. In all of the control genes and four of the candidate genes, there were no differences in genetic polymorphism between the untreated and ivermectin treatment worms. However, there were statically significant differences (χ^2 = 0.05) in allelic frequencies between the untreated and treatment derived worms for P-glycoprotein and β-tubulin genes; both genes which have been previously associated with ivermectin resistance in other nematodes. These genes were in Hardy-Weinberg equilibrium in the untreated population. However, the P-glycoprotein alleles, in the worms from the patients under treatment were not in Hardy-Weinberg equilibrium, and analysis of the allele frequencies of β-tubulin suggested that this gene may have also been under selection in the worms from the ivermectin treated patients. This data provides evidence of genetic selection by ivermectin on *O. volvulus* and indicates that investigations should be made to determine whether ivermectin resistance is developing. The β-tubulin and P-glycoprotein genes may prove useful for monitoring for possible development of ivermectin resistance.

Keywords: *Onchocerca volvulus*, Ivermectin, Genetic Polymorphism, β-tubulin, P-glycoprotein

Introduction

Onchocerca volvulus is a human filarial nematode which causes onchocerciasis or river blindness. It is a major public health and socioeconomic problem in Africa and parts of the Americas. It is estimated that 18 million people worldwide are infected with this parasite with a further 120 million people at risk (Luder et al. 1996; Meredith and Dull 1998). The only treatment approved for onchocerciasis is ivermectin (*Mectizan*®). In 1987, WHO commenced using ivermectin (IVM) to treat human onchocerciasis, following a generous donation from Merck & Co. (Molyneux 1995). Ivermectin is microfilaricidal and hence reduces transmission. It alleviates the intense itching of onchocerciasis and significantly reduces the incidence of blindness (Molyneux 1995). Ivermectin is not significantly macrofilaricidal. However, of critical importance to the effectiveness of IVM, as a single annual treatment, is its ability to markedly impair reproduction in adult worms and to maintain this inhibition of microfilarial production for several months (Awadzi et al. 1999). Annual IVM treatment has drastically improved the social and economical well being of people in areas affected by this parasite.

Widespread resistance to IVM has been reported in other nematode species, such as *Haemonchus contortus* (Prichard 2001). Ivermectin has been used for onchocerciasis for over 16 years of annual treatment in some parts of West Africa, including Ghana. Nevertheless, IVM resistance in *O. volvulus* has not been unequivocally documented. However, recently the finding of patients with surprisingly high microfilarial counts, despite many rounds of IVM treatment (Ali 2002; Awadzi et al. 2004; Awadzi and Edwards 2004) indicates that the parasite may not be responding to treatment as

expected. A possible cause of sub-optimal response to treatment could be a developing drug resistance. Because IVM does not kill most of the adult worms, but causes a temporary reduction in microfilarial production, and because there are no animal hosts for *O. volvulus* which would allow experimental investigation of resistance, parasitological definition of resistance in onchocerciasis is difficult. However, drug resistance is a genetic process and so selection for anthelmintic resistance would be seen as a selection for particular alleles of genes which would improve the reproductive fitness of *O. volvulus* in the presence of an IVM-based control program. Changes in the genetic polymorphism of genes involved in a resistance mechanism, or other genes which are linked to genes involved in a resistance mechanism, would be seen. Provided the appropriate genes are investigated, changes in genetic polymorphism are likely to be apparent in advance of a widespread failure of control due to IVM resistance. Furthermore, changes in genetic polymorphism could be used to monitor for resistance development.

Studies in other nematodes have suggested glutamate-gated and GABA-gated chloride channel subunit genes (Blackhall et al. 1998; Ashton et al. 1999; Dent et al. 2000; Blackhall et al. 2003; Njue and Prichard 2004), P-glycoprotein genes (Blackhall et al. 1998; Xu et al. 1998; Le Jambre et al. 1999; Kerboeuf et al. 2003), β-tubulin (Blackhall 1999) and *che-3* (Starich et al. 1995) may be associated with ivermectin resistance. Based on these and other findings and we have selected six *O. volvulus* candidate genes for which we were able to obtain sequence information and 10 other *O. volvulus* genes with no known association with ivermectin resistance in any nematodes, as control genes, to investigate genetic polymorphism in the untreated and ivermectin

exposed populations of *O. volvulus* using Restriction Fragment Length Polymorphism (RFLP) and Single Strand Conformational Polymorphism (SSCP).

Material and Methods

Informed consent was obtained from each patient following review and approval by the McGill University Faculty of Medicine Institutional Review Board, the Ghanaian Ministry of Health and the WHO Secretariat Committee for Research Involving Human Subjects. Nodules were excised from patients, naturally infected with *Onchocerca volvulus*, in a number of villages in Ghana (Table 1). The infected patients could be divided into two treatment groups, a group who had never received IVM and another group who had taken at least six annual doses of IVM as part of the Onchocerciasis Control Program in West Africa (OCP). Samples were obtained from many villages in different regions in Ghana, to reduce the influence of founder effects which might affect the comparison of genetic polymorphism. The sample population of patients comprised both males and females and the age group ranged between 13-76 years (Table 1).

Worm Isolation

Nodules were excised from patients under aseptic conditions and frozen until ready for nodule digestion. Following thawing, each nodule was placed in a 50 ml disposable conical tube with 10 ml of Medium 199 supplemented with Earle's salt, L-glutamine, and sodium bicarbonate adjusted to pH 7.0 (Gibco BRL). The digestion medium was also supplemented with 0.2 mg/ml of gentamicin sulfate (Sigma) and collagenase (Type 1) purified from *Clostridium histolyticum* (Sigma) at a final concentration of 1.25 mg/ml. The nodules were incubated at 37 °C with constant gentle shaking for a minimum of 4 hours before removing the individual worms from the

digested nodule (Schulz-Key et al. 1977). The individual worms were washed and separated by sex and placed in a 1.5 ml Eppendorf tube with appropriate labeling, quickly frozen in dry ice and stored at -80 °C.

DNA extraction

Female worms were not used due to the possibility of DNA "contamination" from sperm, fertilized eggs or microfilaria. Male worms were washed twice with cold PBS (pH 7.0) and transferred to a clean Eppendorf tube containing 100 µl STE (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0)), 1 mM EDTA (pH 8.0), 0.6 M β-mercaptoethanol and 200 µg/ml Proteinase K. The worms were incubated for two hours at 65 °C. DNA was extracted using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) then precipitated with isopropanol:ammonium acetate (2:1). The DNA pellet was air dried, resuspended in 20 µl of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and stored at –20 °C.

PCR amplification of genomic DNA fragments

PCR products were amplified from the purified genomic DNA samples from the individual male worms on a PTC200 DNA Engine Thermalcycler (MJ Research). The standard PCR conditions follow: 95 °C for 1 min, 50 °C – 55 °C (depending on the gene) for 30 sec, and 72 °C for 1 min. The primer sequence for each of the genes can be found in Table 2. The PCR product was then immediately used for digestion with restriction enzymes and/or SSCP analysis.

Restriction Fragment Length Polymorphism

Based on similar work (Blackhall et al. 1998), restriction enzymes *Alu* I, *Rsa* I, *Dde* I, and *Hinf* I (Invitrogen Life Technologies) were used for digestion. The restriction digestion protocol was provided by Invitrogen. The digestion reactions were incubated at 37 °C for two hours and then analyzed on an 8% 39:1 polyacrylamide gel (acrylamide: N,N'-methylenebisacrylamide), 1 X TBE , electrophoresis at 115 V for 2.5 hours at 23 °C . The gels were stained with ethidium bromide (0.5 µg/ml), and visualized by a Molecular Imager FX Pro Fluorescent Imager (Bio-Rad).

Single Strand Conformation Polymorphism

Two µl of the PCR product were diluted with 10 µl of SSCP buffer (95% formamide, 10 mM sodium hydroxide, 0.25% bromophenol blue, and 0.25% xylene cyanole), denatured for 5 minutes at 95 °C and quickly cooled on ice for two minutes. The denatured samples were analyzed on a 12%-18% 49:1 polyacrylamide gel (depending on the gene analyzed) and subjected to an electric field for 20 - 24 hours. The gels were stained with ethidium bromide (0.5 µg/ml), and a scanned image was obtained from a Molecular Imager FX Pro Fluorescent Imager (Bio-Rad). The SSCP polymorphs for P-glycoprotein and ß-tubulin were cloned from worms that had been genotyped by SSCP.

Cloning and Sequencing Identified Polymorphs

Each SSCP polymorph for P-glycoprotein and β-tubulin was cloned into PCR 2.1 cloning vector (Invitrogen Life Technologies) and transformed into One Shot® INVαF′

chemically competent *E. coli* (Invitrogen Life Technologies). Positive colonies were selected on LB agar plates containing 40 mg/ml 5-bromo-4-chloro-3indolyl-β-D-galactoside (Invitrogen Life Technologies) and 100 μg/ml ampicillin (Sigma). Three positive colonies from each cloned polymorph were selected and sequenced to ensure accuracy of the sequence.

Results

Table 2 summarizes the results obtained by RFLP and SSCP analysis for each of the genes investigated. Chi Square analysis indicated that two of the genes, β-tubulin and P-glycoprotein, showed a significant difference ($\alpha^2 = 0.05$) in polymorph frequency between the IVM treated population and the untreated population. The frequency of each polymorph, observed by RFLP or SSCP, for P-glycoprotein and β-tubulin is shown in Table 3.

P-glycoprotein

The frequencies of polymorphs, obtained from the RFLP analysis of P-glycoprotein with the restriction enzyme *Dde* I is presented in Figure 1A. Figure 1B shows the polymorphs identified on the polyacrylamide gel. Three polymorphs were detected. Polymorph A-Dde more than doubled in frequency in the IVM exposed group in comparison to the unexposed population. At the same time, there was a decrease in the frequency of polymorph A/B Dde and a small decrease in the frequency of polymorph B-Dde in the IVM exposed group compared with the worms from the IVM naïve patients.

Using SSCP (Figures 1C and 1D), we were able to confirm that repeated IVM treatment resulted in a different frequency of PGP polymorphs. We identified seven polymorphs in comparison to the three obtained using RFLP. Two polymorphs were of interest, as there were large decreases in the frequencies of polymorphs B, A and B/E in the IVM exposed group in comparison to the naïve group and a large increase in the frequencies of polymorphs A/B and C and F in the ivermectin exposed worms compared to the IVM naïve group.

The PCR amplified products used for the P-glycoprotein SSCP analyses were sequenced and five alleles (Figure 1E) were identified with A/B corresponding to the heterozygote of allele A and allele B and B/E corresponding to the heterozygote of allele B and allele E. Hardy-Weinberg analysis was conducted on the RFLP and SSCP PGP data sets. PGP polymorphs in the worms from IVM naïve subjects were in Hardy-Weinberg equilibrium ($\chi^2 = 1.17$ for the RFLP and $\chi^2 = 5.82$ for the SSCP). However, the PGP polymorphs from the worms obtained from subjects under treatment were not in Hardy-Weinberg equilibrium. The calculated chi-squared value for the IVM-treated data set was $\chi^2 = 12.75$ for the RFLP data, and $\chi^2 = 8.18$ for the SSCP data set which were greater than the tabulated χ^2 value ($\chi^2_{0.05} = 3.84$, df = 1 for the RFLP and $\chi^2_{0.05} = 7.82$, df = 3 for the SSCP) indicating that the IVM exposed sample set was not in Hardy-Weinberg equilibrium.

β-Tubulin (isotype 1)

Data obtained by RFLP analysis of β-tubulin, using the *Alu* I restriction enzyme, is shown in Figure 2A. The polymorphs identified on the polyacrylamide gel are shown

in Figure 2B. We identified two polymorphs. This gene is particularly interesting as there was a large change in polymorph frequency. With the A-Alu polymorph, there was a dramatic decrease of approximately 81% in the worms from the IVM exposed group compared to the worms from unexposed patients. On the other hand we observed an 81% increase in the B-Alu polymorph in the worms from the IVM exposed compared to the naïve patients.

Similar results were obtained when SSCP was used for analysis (Figures 2C and 2D). Of particular interest was B polymorph. In the worms from naïve patients, SSCP polymorph pattern B accounted for only 2.5% of the population. The frequency of polymorph B increased to approximately 27.5% of the population from the IVM exposed subjects. The RFLP and SSCP analyses both clearly showed that there was a statistically significant difference in polymorph frequencies between the two populations of *O. volvulus* (Figures 2A and 2C).

The PCR amplified products used for the β-tubulin SSCP analyses were sequenced and two alleles were identified (Figure 2E), with A/B corresponding to the heterozygote. Hardy-Weinberg analysis was performed on the β-tubulin SSCP allele frequencies. The analysis of the SSCP data indicated that the non-exposed population of *O. volvulus* was in Hardy-Weinberg equilibrium (χ^2 = 1.01). Hardy-Weinberg analysis of the SSCP polymorph frequencies for the IVM exposed group produced a χ^2 = 3.49 which is close to the expected values for significance for disequilibrium of $\chi^2_{0.05}$ = 3.84 (df = 1).

Discussion

Ivermectin has drastically improved the health, social and economical well being of people in areas affected by onchocerciasis (Boatin 2003). However, in parts of the former OCP area in West Africa, 14 or more rounds of IVM treatment have been distributed. There have recently been a few reports of sub-optimal responses to IVM in terms of microfilarial loads after many rounds of treatment (Ali 2002; Awadzi et al. 2004). Blood levels of IVM were determined and the investigators were able to eliminate possible host effects such poor absorption, altered metabolism due to alcohol or other drug consumption as the cause of the sub-optimal responses and concluded that they were due to a parasite effect (Awadzi et al. 2004). However, unequivocal evidence of a resistance phenotype is still lacking. As indicated previously, it is difficult to unequivocally demonstrate a resistance phenotype in *O. volvulus*.

Resistance is a genetic phenomenon resulting from changes in the genetic profile of the parasite population which subsequently leads to a phenotype of a reduced response to treatment. Once the resistance phenotype has been unequivocally identified in the population, the genetic profile of the population has already been altered. This genetic alteration can be expected not only in genes whose products are involved in the resistance mechanism, but may also be seen in genes not themselves involved in the resistance mechanism, but genetically linked to other genes that are involved in a resistance mechanism. Identifying genes which may be linked to drug selection is a critical step in the development of possible markers for drug resistance. This then allows genetic surveillance to be coupled with parasitological and clinical surveillance of the control programs. All of these steps are important to ensure that the programs

achieve their objectives of long term control of onchocerciasis.

In this study, we have analyzed 16 genes, including six genes which, based on studies in other nematodes, could be candidate markers of genetic selection due to exposure to ivermectin and 10 genes, as control genes, for which there is no evidence of a link to ivermectin selection. *O. volvulus* samples were obtained from a number of communities in Ghana in an attempt to eliminate founder effects between different communities (Table 1). Many of the naïve and treated communities were located in the same region of Ghana, so that the likelihood of genetic differences between worms, derived from naïve and treated subjects, being due to founder effects is small.

Twelve of the 16 genes analyzed, including five of the candidate genes and seven of the control genes showed genetic polymorphisms using the RFLP and SSCP techniques employed. In all of the control genes and three of the candidate genes which did show polymorphism, there was no significant difference between the drug exposed and naïve groups. This result suggests that the differences found in P-glycoprotein and β-tubulin cannot be accounted for on the basis of inherent population differences between the worms from naïve and ivermectin treated patients.

Significant differences between the two treatment populations in P-glycoprotein and β-tubulin polymorph frequencies were seen by both SSCP and RFLP and the differences were large. β-tubulin and members of the P-glycoprotein family have been identified as genes under selection in ivermectin resistant *H. contortus* (Blackhall et al. 1998; Xu et al. 1998; Blackhall 1999; Le Jambre et al. 1999; Kerboeuf et al. 2003).

P-glycoproteins (PGP) are a group of ATP-binding cassette transporter proteins that are part of a superfamily of integral plasma membrane proteins. P-glycoproteins

are found in many normal and cancerous human cells as well in many groups of organisms, including invertebrates (*Drosophila melanogaster*) (Wu et al. 1991), protozoa (*Plasmodium falciparum*) (Foote et al. 1989; Broeks et al. 1995; Sharom et al. 1995), and the nematodes *H. contortus* (Xu et al. 1998) and *O. volvulus* (Huang and Prichard 1999). PGP functions by non-specifically effluxing a diverse range of molecules from the interior of the cell and the cell membrane to the exterior milieu. Substrates for P-glycoprotein can be quite diverse but do share some similarities, such as being amphiphilic, lipophilic, having a planar shape and usually possessing a ring structure (Ford and Hait 1990). Ivermectin has many of the characteristics described and has been shown to be an excellent substrate for P-glycoprotein (Didier and Loor 1996; Pouliot et al. 1997).

The over expression of P-glycoprotein has been implicated in multi-drug resistance in human cancer cells (Schinkel et al. 1994) and in IVM resistance in the nematode *H. contortus* (Xu et al. 1998). In the mouse model, the *mdr1a* gene codes for a P-glycoprotein, which when knocked out results in a dramatic increase in ivermectin sensitivity (Schinkel et al. 1994). In the nematode, *H. contortus*, allelic selection on PGP has been found in several studies to be associated with IVM resistance (Blackhall et al. 1998; Xu et al. 1998; Le Jambre et al. 1999; Sangster et al. 1999). Recently, differences in genetic polymorphism in two other ABC transporter genes from *O. volvulus* isolated from IVM treated and untreated patients in Ghana were found (Ardelli and Prichard 2004). However, these workers did not analyze P-glycoproteins and did not find differences as pronounced as in the present study. While this study does not prove an involvement of P-glycoprotein with a resistance phenotype, it is possible that

the selection for particular alleles may confer an advantage on the worm to allow it to resist the effects of ivermectin, (e.g., increased rate of drug efflux) or that this gene is linked with another gene involved in sensitivity to ivermectin.

A functional relationship between the mode of action or transport of IVM, and β-tubulin or microtubules is less clear. From previous experiments performed in our laboratory, we believe that ivermectin does not interfere with tubulin polymerization or depolymerization (Oxberry & Prichard, unpublished). One possible explanation for selection on β-tubulin could be genetic linkage of β-tubulin to another gene that is associated with IVM sensitivity, transport or mechanism of resistance. However, microtubules play a large number of roles in cellular physiology, including cellular transport, nerve cell structure and function, the anchoring of ion channel receptors in nerve synapses and other functions which could conceivably influence the action of a neuromuscular ion channel opening drug such as IVM. In this context, recent observations of a marked shortening and derangement of the microtubule bundles in the amphidial neurons in IVM resistant *H. contortus* are interesting (Freeman et al. 2003).

The results that show that PGP alleles from the adult worms obtained from patients under IVM treatment were not in Hardy-Weinberg equilibrium and that the β-tubulin alleles in the same adult worms were close to being significantly out of equilibrium, are interesting because they suggest that the adult worm population, from the patients under treatment, is undergoing an evolutionary or genetic change (Hartl 2000). If a population is in Hardy-Weinberg equilibrium, genotype frequencies will be constant from generation to generation (Griffith J. F. A. et al. 1996). This was the situation observed and expected in the adult worms from the IVM naïve patients.

Recent drug pressure could explain the lack of Hardy-Weinberg equilibrium seen in the IVM exposed samples. The samples were obtained from patients that were taking IVM annually for 6 to 8 years which is likely to have imposed a selection pressure. Another factor that can result in a population appearing not to be in Hardy-Weinberg equilibrium is if an allele is not being amplified and detected. However, in this case one would expect such an undetected allele to be also present in the population from the IVM naïve patients so that they would also not be in Hardy-Weinberg equilibrium for the same gene. This was not the case and this indicates that the two worm populations were genetically different. Finally, a lack of Hardy-Weinberg equilibrium could be due to an inadequate sample size. However, usually a sample size in excess of 30 individuals is adequate to obtain Hardy-Weinberg equilibrium, so that this seems unlikely to account for this genetic result, given the population sizes analyzed.

The rapid onset of IVM resistance in nematode parasites of ruminant animals has been well documented (Shoop 1993). However, anthelmintic resistance has not yet emerged as a defined problem in any human nematode parasites. In the case of human gastrointestinal nematodes, this is perhaps not surprising because there have been few if any control programs in which high levels of coverage of the whole population of communities has been sustained over many years. Furthermore, the level refugia in a parasite population is now recognized as one of the most important elements in the rate of selection for anthelmintic resistance (Coles 2002; van Wyk et al. 2002) and in the case of soil transmitted human nematode parasites, the extent of refugia is likely to be huge in contrast to the situation sometimes found with veterinary nematodes, when resistance has rapidly developed as a result of whole herd treatments and movement of

livestock to worm-free pastures following treatment. The refugia and treatment in human filarial nematodes is, however, far more likely to select for anthelmintic resistance than in soil transmitted human nematode parasites because control programs, for example for onchocerciasis, aim at a relatively high level of community coverage, with treatment repeated year after year. Furthermore, *O. volvulus* occurs almost exclusively in the human population, with only a small fraction of the *O. volvulus* population occurring in the black fly vector as larval stages. These conditions will result in the proportion of the *O. volvulus* population being in refugia being very small and will be conducive to a high selection pressure for the development of IVM resistance. Another factor that will influence the rate of selection for IVM resistance in *O. volvulus* is the fact that IVM causes a very prolonged suppression of reproduction in the adult worms. This is likely to select for a different type of resistance than in veterinary or human gastrointestinal nematodes in which selection depends on death or survival of all of the parasitic stages of the worm. One can hypothesize that in *O. volvulus* a resistance phenotype could be adult worms which can more rapidly return to reproductive fitness following IVM treatment than more susceptible worms and so produce many more progeny (microfilaria), despite treatment, compared with more susceptible adult worms. If the progeny of the more reproductively fit adult worms under IVM treatment are transmitted by the vector there will be selection for IVM resistance. Other factors, such as the density dependent survival and development of *O. volvulus* larval stages in some *Simulium* vectors (Basanez et al. 1996; Demanou et al. 2003) may amplify the selection for resistance.

The potential longevity of *O. volvulus* in the human host may act to delay the

selection for resistance. However, repeated IVM treatment may shorten the life span of the adult worms (Gardon et al. 2002). If this is so, another manifestation of resistance development in *O. volvulus* could be that the more resistant adult worms may survive longer and reproduce more during prolonged periods of IVM control than more susceptible adults. It is also important to keep in mind that the time between vector uptake of a microfilaria and development of a mature adult worm, derived from that microfilaria, following transmission, is about one year. Over a period of a few years of annual IVM treatment, several generations of selection could take place. Nevertheless, despite these biological considerations which argue that if resistance genes are at all present in *O. volvulus*, selection for a resistance phenotype is likely to occur, unequivocal failures in the IVM control programs have not been established.

It has been speculated that the lack of conclusive evidence for a resistance phenotype in *Onchocerca* may be due to low genetic diversity in this parasite which may have resulted from a bottle neck event during evolution as the parasite migrated from a ruminant host to humans (Unnasch and Williams 2000). If the genetic diversity is low, alleles which confer resistance may be absent in the species. Our limited analysis of the *Onchocerca volvulus* genome by RFLP and SSCP indicated that there is genetic polymorphism in many of the *Onchocerca* genes investigated. In fact, in P-glycoprotein, in the limited sequence analyzed (356bp) five alleles were detected.

We have shown genetic evidence that IVM is selecting on *O. volvulus*, and while this does not prove that IVM resistance is present, it suggests that the process of selection for resistance is underway and may lead to the eventual reduced effectiveness of IVM treatment. Genetic selection identified in P-glycoprotein and β-tubulin may

provide useful markers for monitor for genetic selection in *O. volvulus* population under prolong ivermectin treatment. Such markers need not be directly involved in mechanisms of resistance to be useful.

This study has surveyed six candidate and 10 control genes and two of the candidate genes were significantly different between the untreated and ivermectin treated group. Other genes, not so far analyzed, may prove to be more closely associated with IVM selection than the two found here. Further studies are required to see if the genetic selection observed here is associated with higher reproductive fitness or *O. volvulus* survival as microfilaria or greater adult longevity, in the presence of an ongoing IVM control program.

Acknowledgments

We gratefully acknowledge Dr. Kwablah Awadzi for the collection of the *O. volvulus* nodules. This investigation received financial support from the African Programme for Onchocerciasis Control, the Onchocerciasis Control Program in West Africa and the UNDP / World Bank / WHO Special Programme for Research and Training in Tropical Diseases.

Tables and Figures

Table 1. Summary of patient information and Villages in Ghana, West Africa, that were sampled for *O. volvulus*.

Patient Parameter	Untreated Patients	IVM Treated Patients
Age Range (y)	20-56	13-76
Mean age (y)	42.7	44.5
(%) Male	38.2	76.2
(%) Female	61.8	23.8
Number of patients	34	42
Total number of nodules	163	208
Villages	Klave, Kpdze-Sreme, Aflakpe, Kpedze Anoe, Dodome Teleafenu, Kpedze-Todze, Dodome-Awlime, Hoe, Honuta-Gborgame, Kpoeta-Ashanti	Asubin, Okinase, Zodanu, Adumadum, Kunda, Asukawkaw, Katanga
Total number of male worms	79	61

Table 2. Summary of analysis of differences in RFLP and SSCP polymorphism. "NS" indicates no significant difference between *O. volvulus* from IVM exposed patients and from non-exposed patients. "Sig" indicates significant differences found between the worms from the IVM exposed patients and those from non-exposed patients ($\alpha^2 = 0.05$). N/A indicates no data was generated for that analysis.

O. volvulus genes (GenBank Accession Number)	Sense Primer (5' to 3') Anti-Sense Primer (5' to 3')	[d]Genomic position analysed (bp)	[e]cDNA Position analysed (bp)	Number of polymorphs detected (RFLP)	Significant differences (α^2=0.05)	Number of polymorphs detected (SSCP)	Significant differences (α^2=0.05)
Candidate Genes							
P-glycoprotein **(AF083642)**	**CAT CAT TGG TCA AGT CAC AGC** **CCA CTC ATA ATA CTA CCT CGT**	**-**	**1886-2051[c]**	**3 (Dde I)**	Sig	**5**	Sig
Glutamate gated chloride channel **(U59745)**	**GTT TAA TTA CCC AAG TTT GAG** **AGT CAT CGT TAG CAA TGT AGT**	**-**	**492-595[c],** **742-1035**	**4 (Alu)**	**NS**	**3**	**NS**
P-glycoprotein like protein **(AF128532)**	**GAT GGT GTG CCA ATT AGTG** **GTG GCC TAT TAA CAT GTGG**	**1341-1697**	**-**	**3 (Dde)**	**NS**	**3**	**NS**
β-Tubulin (isotype 1) **(AF019886)**	**GCA ACA ATT GGG CTA AGG GAC** **CGA TCC GGA TAT TCC TCA CGA**	**1557-1857,** **1780-2748**	**-**	**2 (Alu)**	Sig	**2**	Sig
Glu/GABA [a]	**TAT TGA AAA AAG GGA AAG AAG** **TCT AAA ATT GAT AAA TGT TCT**	**-**	**352-881[c]**	**N/A**	**N/A**	**2**	**NS**

*Ov-CHE-3*homolog (EST ID [a]: AI130343)	**GAA CTT GAC CGT TCA GTA TAT TCA TCT GAT TGA ATG CTT TAT**	**-**	**-**	**N/A**	**N/A**	**1**	**NS**
Control Genes							
Actin **(M84915)**	**GCG AAC AGA GAA AAG ATG ACG GAA TGA GTA ACC ACG CTC AG**	**520-986**	**-**	**1**	**NS**	**1**	**NS**
α-Tubulin homolog (EST ID [b]:TC2945)	**ATG AGA GAA GTA ATT TCG ATT GTC ACC TCC TGG GAC TAC CGT**	**-**	**-**	**1**	**NS**	**2**	**NS**
Acetylcholine receptor **(L20465)**	**TTA ATT ATT AGC TTG TCG TGT ATA ATA CTT TGT AAT ATT TAA**	**-**	**164-391[c], 809-1048[c]**	**N/A**	**N/A**	**1**	**NS**
Calreticulin **(M20565)**	**TGC TTC TCA GCA ATT AAT GCT CTT TAT CTT TCA CAG CAT CGC**	**-**	**55-471[c]**	**N/A**	**N/A**	**3**	**NS**
Cyclophilin **(U47812)**	**CGG TGG ACA GCA ATC TGG ACG TGA ACC CAC TGC TTC GAT CTT**	**-**	**51-478**	**N/A**	**N/A**	**3**	**NS**
Heat shock protein 60 **(AF121264)**	**CAA TCA TGG GGA AGT CCA AAG CTC AAA ACC TTC CTT TGC AAT**	**-**	**214-590[c]**	**N/A**	**N/A**	**4**	**NS**
Intermediate filament protein **(L31528)**	**CAA TAT GGA TGA AAT TTG GGT TTT CAT ACA TCG TAC GAA CAT**	**-**	**356-741**	**N/A**	**N/A**	**1**	**NS**

Kinesin homolog (EST ID [b]:TC3519)	**TGA GAT TCA GTG GCA GTT GAT TAA ACA CCA ATA TCT GGC CGT**	-	-	**N/A**	**N/A**	**2**	**NS**
Major body wall myosin **(M74066)**	**GTG CCA CAA GTT ACA CAC TTA TCG TCA TAC TCT ACG TCT AAC**	**3229-3816**	-	**N/A**	**N/A**	**2**	**NS**
Retinoid binding protein **(L27686)**	**GGC ATT GAT TGG TGT CAT TAT ATT TAG CAA TGA TGT CTC GAG**	-	**63-457**	**4 (RSA I)**	**NS**	**2**	**NS**

[a]Glu-GABA sequence was obtained from T. Egwang (*pers. comm.*)

[b] EST sequences obtained from TIGR *O. volvulus* Database.

[c] Intron(s) present in the sequence analyzed

[d] Genomic position counted from the first base of the genomic sequence shown in the NCBI database.

[e] cDNA position counted from the first base in the initiation codon of the cDNA sequence.

Table 3. Polymorph frequencies estimated by RFLP and SSCP, for both P-glycoprotein and β-tubulin. Note RFLP and SSCP polymorphs with the same letter designation are not related.

RFLP Raw Data	**Polymorph**	**Non Exposed**	**Ivermectin Exposed**
P-glycoprotein (*Dde I* Digest)	A-Dde	20	17
	B-Dde	19	4
	A/B-Dde	37	2
Total Number		76	23
β-tubulin (isotype 1)(*Alu I* Digest)	A-Alu	69	2
	B-Alu	9	24
Total Number		78	26
SSCP Raw Data	**Polymorph**	**Non Exposed**	**Ivermectin Exposed**
*P-glycoprotein	A	23	17
	B	18	5
	A/B	4	9
	C	1	3
	E	9	6
	B/E	4	0
	F	0	2
Total Number		59	42
β-tubulin (isotype 1)	A	61	18
	B	2	17
	A/B	14	21
Total Number		77	56

P-glycoprotein and β-tubulin SSCP polymorphs were sequenced and the each polymorph corresponds to the allelic composition (e.g. A corresponds to homozygote (allele A/allele A); A/B corresponds to a heterozygote (allele A/allele B), etc).

*Polymorph frequencies for B and B/E, A/B and F were combined in order to calculate the Hardy-Weinberg equilibrium.

Figure 1. Comparison of polymorph frequencies of P-glycoprotein genomic DNA from *O. volvulus* obtained from IVM naïve patients and worms obtained from patients that had been treated with at least six rounds of IVM, in Ghana. Polymorphism determined by RFLP (1A) and SSCP (1C). The RFLP electrophoresis was performed on a 8% 39:1 polyacrylamide gel at 115 V at for 2.5 hours at 23 °C, and the SSCP electrophoresis was performed on a 12% 49:1 polyacrylamide gel at 100 V for 24 hours at 23 °C. The images of the RFLP (1B) and SSCP (1D) polyacrylamide gels containing the representative polymorphs are also shown. The sequence alignment of the five alleles detected by SSCP is presented (1E). Note: RFLP and SSCP polymorphs with the same letter designation are not related.

Figure 2. Comparison of polymorph frequencies of β-tubulin genomic DNA from *O. volvulus* obtained from IVM naïve patients and worms obtained from patients that had been treated with at least six rounds of IVM, in Ghana. Polymorphism determined by RFLP (2A) and SSCP (2C). The RFLP electrophoresis was performed on a 8% 39:1 polyacrylamide gel at 115 V for 2.5 hours at 23 °C, and SSCP electrophoresis was performed on a 14% 49:1 polyacrylamide gel at 100 V for 24 hours at 23 °C. The images of the RFLP (2B) and SSCP (2D) polyacrylamide gels containing the representative polymorphs are also shown. The sequence alignment of the two alleles detected by SSCP is presented (2E). Note: RFLP and SSCP polymorphs with the same letter designation are not related.

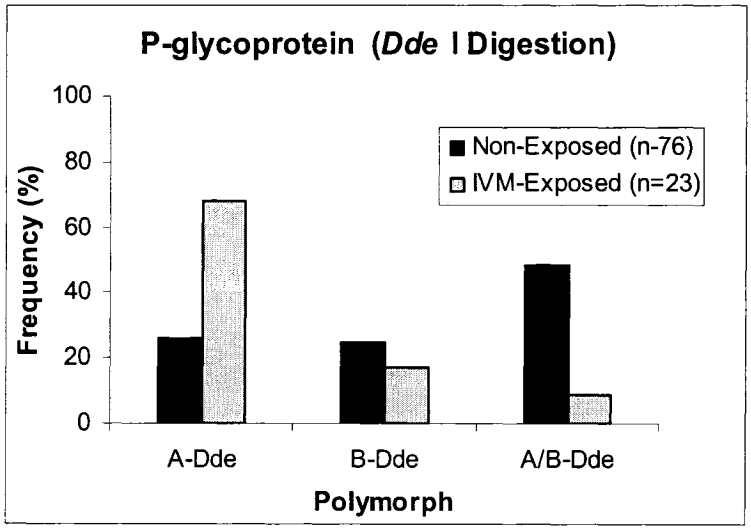

Figure 1A

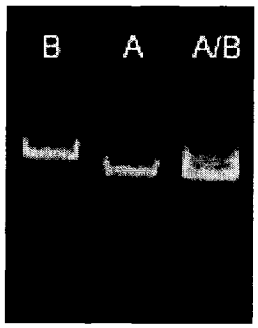

Figure 1B

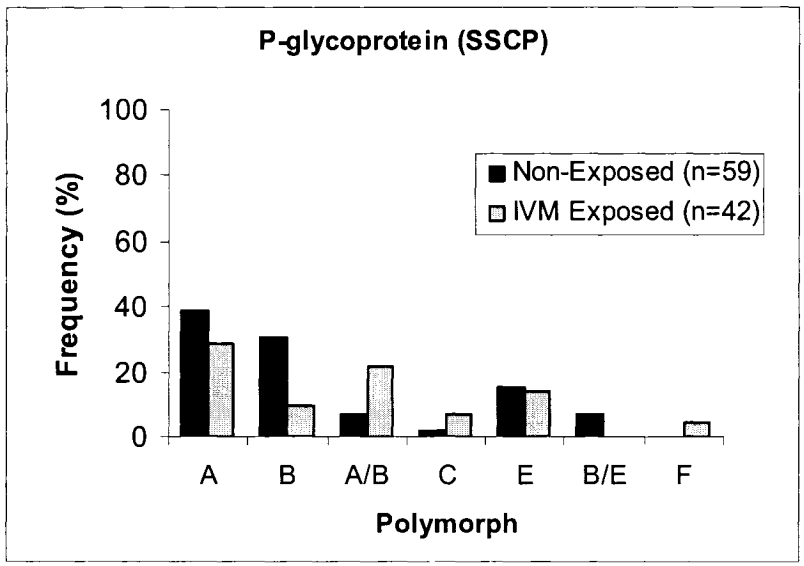

Figure 1C

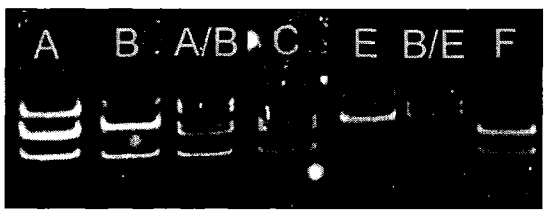

Figure 1D

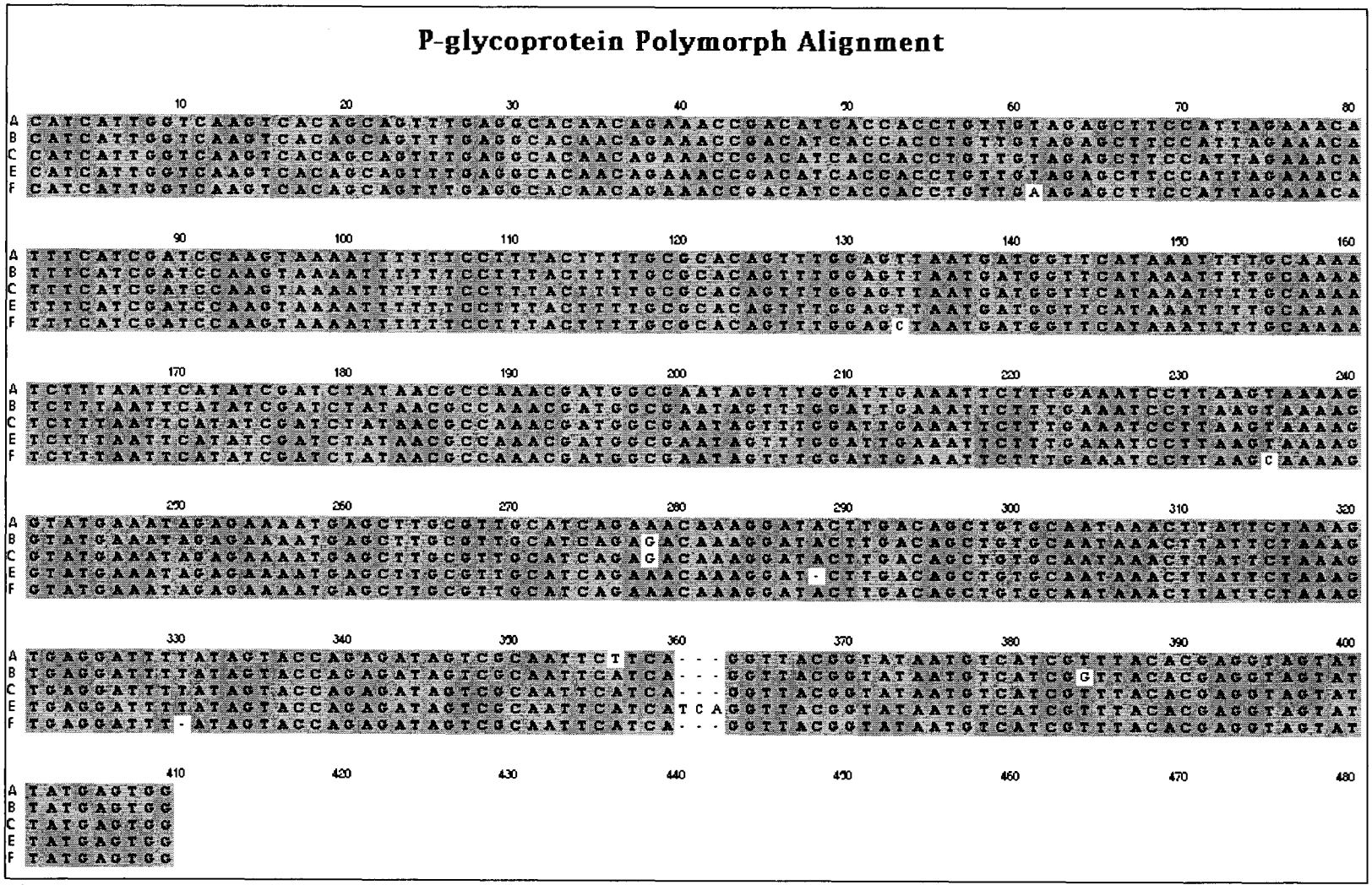
P-glycoprotein Polymorph Alignment
10 20 30 40 50 60 70 80
A CATCATTGGTCAAGTCACAGCAGTTTGAGGCACAACAGAAACCGACATCACCACCTGTTGTAGAGCTTCCATTAGAAACA
B CATCATTGGTCAAGTCACAGCAGTTTGAGGCACAACAGAAACCGACATCACCACCTGTTGTAGAGCTTCCATTAGAAACA
C CATCATTGGTCAAGTCACAGCAGTTTGAGGCACAACAGAAACCGACATCACCACCTGTTGTAGAGCTTCCATTAGAAACA
E CATCATTGGTCAAGTCACAGCAGTTTGAGGCACAACAGAAACCGACATCACCACCTGTTGTAGAGCTTCCATTAGAAACA
F CATCATTGGTCAAGTCACAGCAGTTTGAGGCACAACAGAAACCGACATCACCACCTGTTGTAGAGCTTCCATTAGAAACA
90 100 110 120 130 140 150 160
A TTTCATCGATCCAAGTAAAATTTTTTCCTTTACTTTTGCGCACAGTTTGGAGTTAATGATGGTTCATAAATTTTGCAAAA
B TTTCATCGATCCAAGTAAAATTTTTTCCTTTACTTTTGCGCACAGTTTGGAGTTAATGATGGTTCATAAATTTTGCAAAA
C TTTCATCGATCCAAGTAAAATTTTTTCCTTTACTTTTGCGCACAGTTTGGAGTTAATGATGGTTCATAAATTTTGCAAAA
E TTTCATCGATCCAAGTAAAATTTTTTCCTTTACTTTTGCGCACAGTTTGGAGTTAATGATGGTTCATAAATTTTGCAAAA
F TTTCATCGATCCAAGTAAAATTTTTTCCTTTACTTTTGCGCACAGTTTGGAGCTAATGATGGTTCATAAATTTTGCAAAA
170 180 190 200 210 220 230 240
A TCTTTAATTCATATCGATCTATAACGCCAAACGATGGCGAATAGTTTGGATTGAAATTCTTTGAAATCCTTAAGTAAAAG
B TCTTTAATTCATATCGATCTATAACGCCAAACGATGGCGAATAGTTTGGATTGAAATTCTTTGAAATCCTTAAGTAAAAG
C TCTTTAATTCATATCGATCTATAACGCCAAACGATGGCGAATAGTTTGGATTGAAATTCTTTGAAATCCTTAAGTAAAAG
E TCTTTAATTCATATCGATCTATAACGCCAAACGATGGCGAATAGTTTGGATTGAAATTCTTTGAAATCCTTAAGTAAAAG
F TCTTTAATTCATATCGATCTATAACGCCAAACGATGGCGAATAGTTTGGATTGAAATTCTTTGAAATCCTTAAGCAAAAG
250 260 270 280 290 300 310 320
A GTATGAAATAGAGAAAATGAGCTTGCGTTGCATCAGAAACAAAGGATACTTGACAGCTGTGCAATAAACTTATTCTAAAG
B GTATGAAATAGAGAAAATGAGCTTGCGTTGCATCAGAGACAAAGGATACTTGACAGCTGTGCAATAAACTTATTCTAAAG
C GTATGAAATAGAGAAAATGAGCTTGCGTTGCATCAGAGACAAAGGATACTTGACAGCTGTGCAATAAACTTATTCTAAAG
E GTATGAAATAGAGAAAATGAGCTTGCGTTGCATCAGAAACAAAGGAT-CTTGACAGCTGTGCAATAAACTTATTCTAAAG
F GTATGAAATAGAGAAAATGAGCTTGCGTTGCATCAGAAACAAAGGATACTTGACAGCTGTGCAATAAACTTATTCTAAAG
330 340 350 360 370 380 390 400
A TGAGGATTTTATAGTACCAGAGATAGTCGCAATTCTTCA---GGTTACGGTATAATGTCATCGTTTACACGAGGTAGTAT
B TGAGGATTTTATAGTACCAGAGATAGTCGCAATTCATCA---GGTTACGGTATAATGTCATCGGTTACACGAGGTAGTAT
C TGAGGATTTTATAGTACCAGAGATAGTCGCAATTCATCA---GGTTACGGTATAATGTCATCGTTTACACGAGGTAGTAT
E TGACGATTTTATAGTACCAGAGATAGTCGCAATTCATCATCAGGTTACGGTATAATGTCATCGTTTACACGAGGTAGTAT
F TGAGGATTT-ATAGTACCAGAGATAGTCGCAATTCATCA---GGTTACGGTATAATGTCATCGTTTACACGAGGTAGTAT
410 420 430 440 450 460 470 480
A TATGAGTGG
B TATGAGTGG
C TATGAGTGG
E TATGAGTGG
F TATGAGTGG

Figure 1E

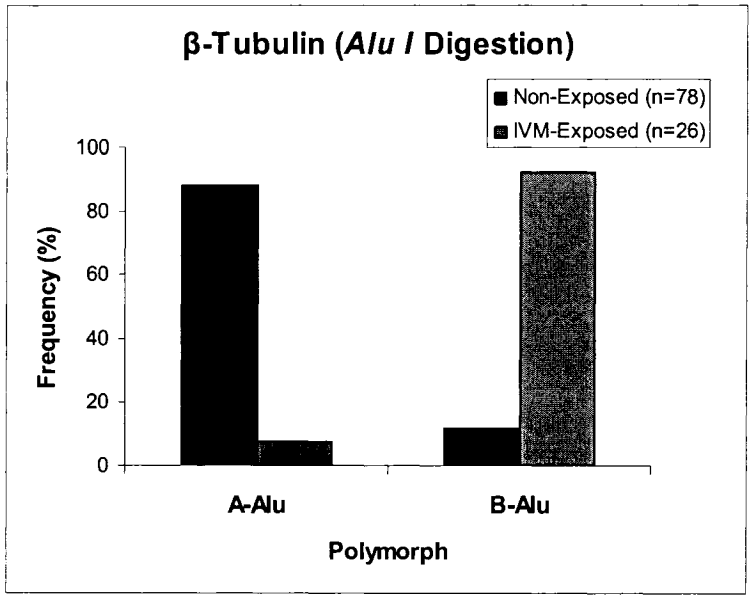

Figure 2A

Figure 2B

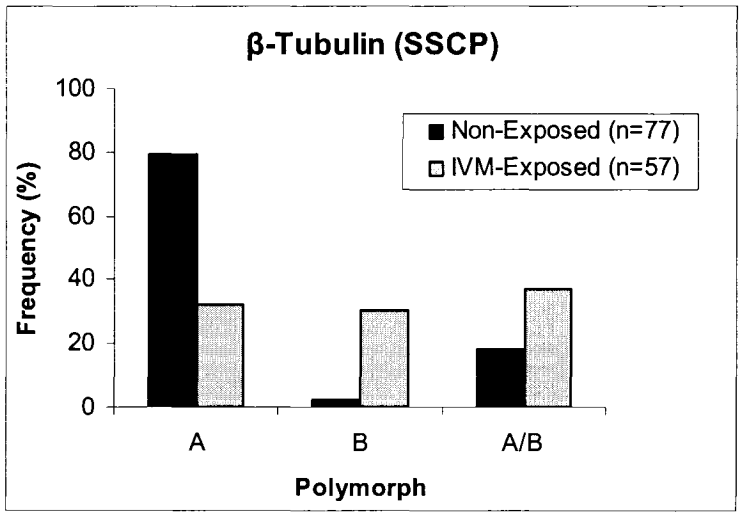

Figure 2C

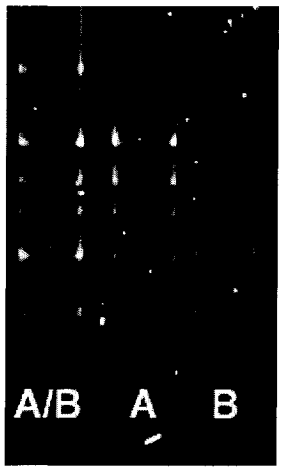

Figure 2D

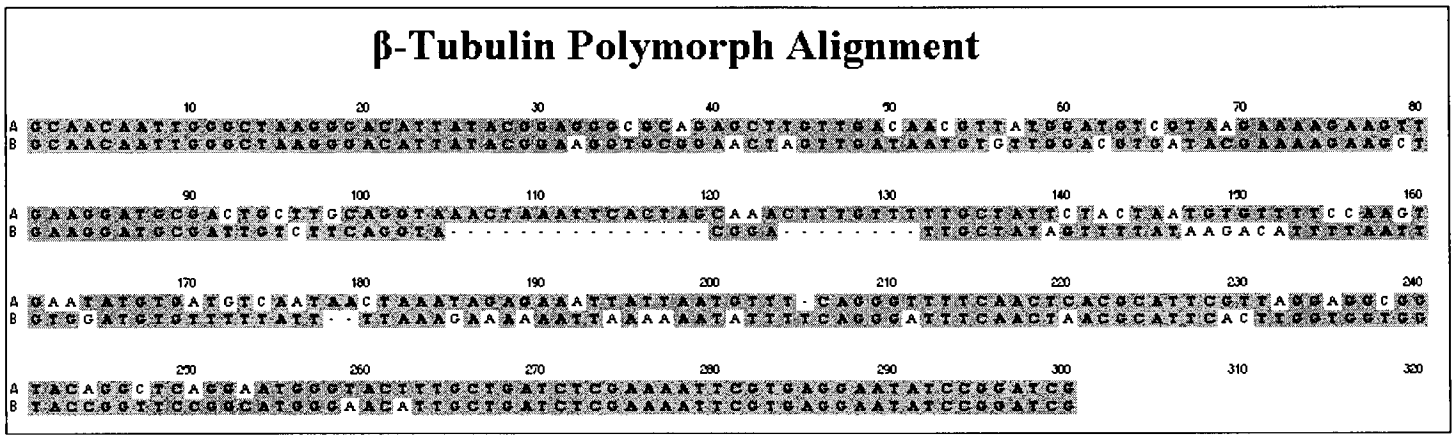

Figure 2E

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Connecting Statement II

The detection of significant changes in allele frequencies associated with treatment would suggest a possible link between a gene under selection and drug action and/or a resistance mechanism. In Chapter II, a gene not initially expected to be associated with ivermectin selection, the β-tubulin isotype 1 gene, appeared to be selected in *O. volvulus* after multiple rounds of ivermectin treatment. Evidence for possible ivermectin selection on β-tubulin was explored further in Chapter III, by comparing the frequency of β-tubulin alleles before any use of ivermectin in several countries in Africa, including Ghana, and the frequency in treated populations of *O. volvulus* from Ghana. Furthermore, an association between β-tubulin allele frequency in *O. volvulus* microfilariae and response to ivermectin treatment in terms of 90 day post ivermectin treatment skin microfilarial count was established. Exploiting sequence differences between the β-tubulin alleles, a simple amplicon fragment length polymorphism assay has been developed to allow rapid genotyping of *O. volvulus* at the β-tubulin locus.

It is of considerable interest that similar evidence of ivermectin selection on β-tubulin has been obtained in ivermectin selected strains of *H. contortus*. Such differences identified in the β-tubulin gene may suggest an involvement of β-tubulin with ivermectin resistance. The genetic differences in the ivermectin selected β-tubulin gene could be used as the basis of an assay to monitor the development of resistance in *O. volvulus*. These results are presented in Chapter III.

Chapter III: Manuscript II

Ivermectin selection on β-tubulin: Evidence in *Onchocerca volvulus* and *Haemonchus contortus*

Eng J.K.L., Blackhall W.J., Osei-Atweneboana M., Bourguinat C., Galazzo D., Beech R.N., Unnasch, T.R., Awadzi K., Lubega W.G. and Prichard, R.K.

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Abstract

Ivermectin resistance is common in trichostrongylid nematodes of livestock, such as *Haemonchus contortus*. This anthelmintic is the only drug approved for mass administration to control onchocerciasis caused by the nematode parasite, *Onchocerca volvulus*. In parts of West Africa up to 18 rounds of ivermectin treatment have been administered to communities and there are reports of poor parasitological responses to treatment. Understanding ivermectin resistance and ivermectin selection is an important step to reduce selection pressure for resistance, and to develop molecular markers which can be used to monitor the development of resistance and its spread. Here we report evidence that ivermectin selection changes the frequency of β-tubulin alleles in both the sheep parasite, *H. contortus*, and the human parasite, *O. volvulus*. In *O. volvulus* we have been able to look at the frequency of β-tubulin alleles in *O. volvulus* obtained before any ivermectin was used in humans in Africa, and following its widespread use. In *H. contortus*, we have been able to look at the frequency of β-tubulin alleles in a strain which has not seen any anthelmintic selection and in an ivermectin selected strain derived from the unselected strain. We have found ivermectin selects on β-tubulin in both of these nematode species. In the case of *O. volvulus*, we had previously reported that ivermectin selects for specific single nucleotide polymorphisms in the *O. volvulus* β-tubulin gene. This polymorphism results in three amino acid changes in the H3 helix of β-tubulin, as well as deletions in an associated intron. We report a simple PCR assay to detect the amplicon length polymorphism, resulting from these intronic deletions, which can be used to monitor the frequency of the β-tubulin allele selected for by ivermectin in *O. volvulus*.

Key words: ivermectin, drug resistance, β-tubulin, nematode, *Haemonchus contortus*, *Onchocerca volvulus*

Introduction

Resistance to ivermectin and some of the other macrocyclic lactone anthelmintics is widespread and increasing in nematode parasites of sheep, goats and cattle, yet we do not adequately understand the mechanisms and genetics of resistance in nematodes of these hosts (Wolstenholme et al. 2004). The most serious problems occur in anthelmintic resistant *Haemonchus contortus* and this nematode has been most widely studied to try to elucidate the mechanisms of ivermectin resistance in nematodes. Ivermectin is the only available drug for mass treatment of onchocerciasis and has been used over many years to suppress clinical manifestations and reduce transmission of the causative agent, *Onchocerca volvulus*, in West Africa. It is now being used in mass treatment programs for onchocerciasis and lymphatic filariasis throughout sub-Saharan Africa as well as for onchocerciasis control in the Americas. Recently, there have been a few reports of poor *O. volvulus* parasitological and clinical responses to ivermectin (Ali et al. 2002; Awadzi et al. 2004; Awadzi et al. 2004) as well as genetic evidence of ivermectin selection on *O. volvulus* (Ardelli and Prichard 2004; Eng and Prichard 2005; Ardelli et al. 2006; Ardelli et al. 2006).

Because ivermectin is not curative for *O. volvulus* infection, but temporarily reduces parasite microfilarial counts by eliminating most of the microfilariae in the skin and reducing the fecundity of the surviving adult parasites, it is difficult to unequivocally show ivermectin resistance in *O. volvulus*. Resistance in the adult worms is likely to be manifested as a much more rapid rebound in skin microfilariae, after treatment, and in the microfilariae by a poor initial response to ivermectin. Unequivocal demonstration of ivermectin resistance in *O. volvulus* would require repeated sequential

determination of skin snip microfilarial counts before and after treatment and the examination of embryogrammes. The studies of Awadzi et al. (1999; 2004) have best approached this type of examination, so far, and their data strongly suggest that ivermectin resistance is developing in female *O. volvulus,* but not in the microfilariae.

The difficulty of unequivocally demonstrating resistance in *O. volvulus* is compounded because there are no adequate *in vitro* biological tests for resistance using this species, because of the long term reproductive effects of the drug and the difficulty of culturing *O. volvulus in vitro*. Furthermore, *O. volvulus* is an obligate parasite of humans and there are no animal hosts available for maintaining the reproductive stages of the parasite. As a result of these great difficulties to measure ivermectin resistance in *O. volvulus*, either *in vivo* or *in vitro*, and the fact that drug resistance is brought about by genetic selection, attention has been given to looking for genetic changes in populations of *O. volvulus* that have been exposed to repeated rounds of ivermectin treatment and comparing these with ivermectin naïve parasite populations (Ardelli and Prichard 2004; Eng and Prichard 2005; Ardelli et al. 2006; Ardelli et al. 2006).

Using restriction fragment length polymorphism and single strand conformational polymorphism analyses, we have previously reported significant differences in the genetic polymorphism of β-tubulin between populations of *O. volvulus* from ivermectin treated and ivermectin naïve people (Eng and Prichard 2005). Selection, for allele B, resulted in changes in the coding region which correspond to single amino acid changes, Met117Leu, Val120Ile and Val124Ala (Fig. 1), on the same face of three adjacent coils of the H3 helix of the β-tubulin, in addition to deletion of 24 bp in the adjacent intron, compared with allele A (Eng and Prichard 2005). In the work reported

here, we have made use of the 24 bp of intronic deletion to design a simple amplicon length polymorphism assay to genotype *O. volvulus* β-tubulin and have used this assay to analyze the β-tubulin polymorphism in a number of adult *O. volvulus* worms and microfilariae from ivermectin untreated or repeatedly treated subjects. Some of the *O. volvulus* samples from untreated people were collected from several countries before the general introduction of ivermectin use in Africa, and others after its general use for several years. Upon examination of *O. volvulus* microfilarial counts, 90 days following ivermectin treatment in a community in Ghana, we have found a positive association between the frequency of β-tubulin allele B and high post treatment microfilarial counts. Furthermore, we have found evidence that ivermectin selection on *H. contortus* also exerts selection pressure on β-tubulin. Taken together, these data suggest that β-tubulin may be a useful marker for ivermectin selection in nematodes and a simple assay has been developed so that β-tubulin allele frequencies can be monitored in *O. volvulus*.

Materials and Methods

Onchocerca volvulus nodules

O. volvulus nodules from ivermectin naïve, naturally infected hosts in West Africa (see Table 1) were collected in 1989/1990 and processed, as described previously (Zimmerman et al. 1992) or from Uganda (1996) and Ghana (1998) from ivermectin naïve or treated people (Table 1) as previously described (Eng and Prichard 2005). The samples from Ghana obtained in 1998 all came from the Volta region. The ivermectin exposed nodules obtained from Ghana were from people who had been treated six or more times. The Ugandan samples came from the western part of Uganda, in 1996, prior to the administration of ivermectin in the region.

Adult *O. volvulus* Isolation

Excess host tissue was removed from the nodules and each nodule was placed in a 50 ml disposable conical tube with 10 ml of Medium 199 supplemented with Earle's salt, L-glutamine, and sodium bicarbonate to adjust the pH to 7.0 (Gibco BRL). The digestion medium was also supplemented with 0.2mg/ml of gentamicin sulfate (Sigma) and collagenase (Type 1) purified from *Clostridium histolyticum* (Sigma) at a final concentration of 1.25 mg/ml. The nodules were incubated at 37 °C with constant gentle shaking for a minimum of four hours before removing the individual worms from the digested tissues (Schulz-Key et al. 1977). The individual worms were separated by sex and placed in a 1.5 ml Eppendorf tube with appropriate labeling, quickly frozen on dry ice and stored at -80 °C. Only male *O. volvulus* worms were analyzed. The female worms were not analyzed because of the possibility of DNA "contamination" from

microfilariae and sperm. DNA was extracted as previously described (Eng and Prichard 2005).

Microfilariae Isolation

Skin snips were obtained, using a 2 mm Holth-type corneo-scleral punch, from people in the village of Jagbenbeng, in the Northern region of Ghana, in 2002. They were naturally infected with *O. volvulus*. The skin snips were placed in a 24 well plate containing physiological saline (pH 7.0) and incubated to allow the microfilariae to migrate out of the skin samples over a 24 h period. The individual microfilariae were collected by a pipette and stored in 100% isopropanol until processing. Genomic DNA was extracted from the individual microfilariae. Briefly, DNA was extracted using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) then precipitated with isopropanol:ammonium acetate (2:1). The DNA pellet was air dried, resuspended in 20 µl of TE buffer (10mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) and stored at –20 ° C.

PCR Amplicon Length Test for *O. volvulus* β-Tubulin (isotype 1) alleles

A set of PCR primers was designed that flanked the region of interest in the *O. volvulus* β-tubulin, (H3). This region was amplified by using primers OvTub-5 (5'GCA ACA ATT GGG CTA AGG 3') and OvTub-6 (5' CGA TCC GGA TAT TCC TCA 3'). Amplification was performed in a MJ Research thermal cycler with the following cycling parameters: 95 °C for two minutes; followed by 35 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 45 seconds. The PCR samples were

analyzed on a 2.5 % agarose gel containing ethidium bromide (0.5 μg/ml) by electrophoresis at 100V for 45 minutes.

Haemonchus contortus

The *H. contortus* analyzed were the PF17 or PF23 unselected parental strain and the IVF17 or IVF23, ivermectin selected strain, which was derived from the PF strain with ivermectin selection in infected sheep, at increasing dose rates to achieve an approximate ED_{95}, of successive generations (17 generations in the case of IVF17 and 23 in the case of IVF23) as previously described (Blackhall et al. 1998; Ranjan 2002). DNA was extracted from individual adult male worms as previously described (Blackhall et al. 1998).

Single Strand Conformation Polymorphism (SSCP)

SSCP was conducted on the individual *H. contortus* DNA samples. A 192 bp amplicon was generated from genomic DNA using forward primer RBE31 (5'AGAACACCGATGAAACGT 3') and reverse primer MRβ5 (5'ACCAGACATTGTGACAGA 3'). The pair of primers targets genomic position 2692 bp - 2883 bp of the *H. contortus* β-tubulin gene GRU-1 (GenBank accession no. **X67489**) which corresponds to the protein sequence between amino acids 175-292. Amplification was performed in a MJ Research thermal cycler with the following cycling parameters: 95 °C for four minutes; followed by 40 cycles of 95 °C for 15 seconds, 47 °C for 15 seconds and 70 °C for 30 seconds, with a final extension step at 70 °C for 5 minutes. PCR products were visualized on a 1 % agarose gel containing 0.5

μg/ml ethidium bromide. Individual PCR products were excised from the gels, placed in the tops of 10 μl filter pipette tips which were then microcentrifuged in 1.5 ml Eppendorf tubes at 14,000 rpm for 30 seconds. Two μl of the eluate were then used as template for the following labeling-PCR reactions: 0.5 μl 10X *Taq* buffer, 0.5 μl 50 μM dNTPs, 0.5 μl 25 mM $MgCl_2$, 0.25 μl 2 μM MRβ5 primer (to label the antisense strand), 0.1 μl 1000 Ci/mmol dATP $\alpha^{35}S$, 0.1 unit *Taq* polymerase, and water to a final volume of 5 μl. The reactions were overlaid with a drop of mineral oil and thermal-cycled as above. Six μl of stop solution (10 mM NaOH, 95 % formamide, 0.05 % bromophenol blue, and 0.05 % xylene cyanole) were added at the end of the reactions. The reactions were heated at 80 °C for 2 minutes, placed on ice, and 2.5 μl were loaded onto a 10 %, 39:1 acrylamide:N,N'-methylenebisacrylamide, 1X TBE acrylamide gel and electrophoresed at 50 W for 6 hours in a 6 °C cold room. Gels were dried and exposed to X-ray film overnight. Polymorphs were identified by their differing rates of migration through the gel.

Pyrosequencing (Biotage™ AB)

Sense primer RBE 33 (5' ATGCTACCCTTTCCGTG 3') and antisense primer RBE34-biotin (5' Biotin-TGTGAGTTTCAAAGTGCG 3' (HPLC purified)) were used to amplify the *H. contortus* β-tubulin gene for pyrosequencing. The pair of primers targets genomic position 2662 bp -2765 bp of the *H. contortus* β-tubulin gene GRU-1 (GenBank accession no. **X67489**). All Pyrosequencing reactions were performed on a PSQ™ 96MA instrument from Biotage™ AB. Briefly, sample preparation for the pyrosequencing was as follows: 30 μl of the biotinylated PCR product were immobilized on streptavidin-coated Sepharose™ beads (Amersham Bioscience) with

binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1 % Tween 20, adjusted to pH 7.6) and water to final volume of 80 µl. The immobilized PCR was captured with a Vacuum Prep Tool (Biotage™ AB) and made single stranded by submerging the sample into 70 % ethanol for five seconds, then in 0.5 M NaOH denaturing solution for five seconds, and finally in the washing solution (10 mM Tris-acetate pH 7.6), for 5 seconds. The immobilized beads were then released from the Vacuum Prep Tool into the PSQ™ 96 Plate Low pre-filled with 0.5 µM RBE35 sequencing primer (5' AGAACACCGATGAAACA 3') in 40 µl annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate, pH 7.6). The samples were then heated to 80 °C for 3 minutes and cooled at room temperature for 10 minutes prior to running the pyrosequencing reaction. All reagents for the pyrosequencing reaction were obtained from Biotage™ AB, and used according to their protocols.

Results

The amplicon length assay for β-tubulin alleles (Fig. 2) in *O. volvulus* allowed a clear and rapid analysis of β-tubulin allele frequencies. Using this assay, the frequencies of β-tubulin alleles in *O. volvulus* were obtained from naturally infected people in West Africa and Uganda prior to the introduction of ivermectin, from ivermectin naïve people in Ghana, following approximately eight years of widespread use of ivermectin, and from people who had been treated repeatedly with ivermectin, mostly six – eight times. The results are shown in Fig. 3. In the parasite samples from ivermectin naïve people from various locations in West Africa and Uganda (Table 1), prior to the introduction of ivermectin, only the homozygous allele A (A/A) was

detected. In the 1998 *O. volvulus* samples, after approximately eight years of widespread use of ivermectin against *O. volvulus* in Ghana, parasite samples from ivermectin naïve people revealed that allele B was present, mostly as heterozygotes (A/B), but homozygotes (A/A) still predominated. However, following repeated rounds of ivermectin treatment the frequency of allele B, either as the heterozygous (A/B) or homozygous (B/B) state, was significantly higher (Chi-square, $\alpha < 0.05$) than in any of the ivermectin naïve samples.

Skin microfilariae from people in the Northern Ghanaian community of Jagbenbeng who were not responding well to ivermectin, in terms of 90 day post-treatment skin microfilarial count, had a significantly higher frequency of allele B, as heterozygotes (A/B) than did microfilariae from people who had low counts, as expected, 90 days after treatment (Fig. 4).

A separate SSCP analysis on β-tubulin polymorphism in ivermectin naïve *H. contortus* (PF17) and in *H. contortus* selected from the parental ivermectin naïve strain of *H. contortus* by 17 generations of in vivo selection with an approximate ED_{95} dose rate of ivermectin (IVF17) also revealed significant differences in the frequency of β-tubulin polymorphisms (Chi-square, $\alpha < 0.05$) between the selected and unselected parental strain (Fig. 5). A Pyrosequencing assay was designed to detect changes in the codon for amino acid 200 in β-tubulin, which is known as a SNP site for benzimidazole resistance in *H. contortus* (Kwa et al. 1993). SNP analysis for the phe200tyr polymorphism, revealed that the PF17 strain was 97.5 % homozygous for TTC_{200} (phenylalanine) while 2.5 % of the worms were heterozygotes (TTC_{200}/TAC_{200} (tyrosine)), whereas the IVF17 *H. contortus*, which were derived from the PF strain

following ivermectin selection for 17 generations, were 91.9 % homozygous TTC_{200}, and 8.1 % were heterozygous (TTC_{200}/TAC_{200}) (Fig. 6a). In the PF23 worms, the genotype frequency remained similar to that in the PF17 worms; i.e., 96.4 % were homozygous TTC_{200} and 3.6 % were heterozygous TTC_{200}/TAC_{200}. In contrast, the IVF23 worms, which had been selected from the IVF17 worms, for another six generations, with ivermectin at a dose rate to achieve approximately LD_{95}, were significantly different from the PF23 parasites and only 63.9 % were homozygous TTC_{200}, 33.3 % were heterozygotes TTC_{200}/TAC_{200}, and 2.8 % were homozygous TAC_{200} (Fig. 6b). The PF17, IVF17, PF23 and IVF23 had no exposure to benzimidazole anthelmintics. In field isolates of *H. contortus* that are ivermectin resistant, we have also found high frequencies of the TTC_{200}/TAC_{200} heterozygotes in isotype I β-tubulin (Galazzo, Prichard and Beech, pers. comm.). However, in the case of field isolates, the complete anthelmintic treatment history cannot usually be ascertained and, in addition to repeated ivermectin treatment, treatments with benzimidazole anthelmintics could have occurred, affecting the frequency of alternative codon 200 sequences.

Discussion

Eng and Prichard (2005) found evidence, using RFLP and SSCP analyses, of significant selection on β-tubulin isotype I gene in *O. volvulus* collected in Ghana following several years of widespread ivermectin use and reported sequence differences between two alleles of β-tubulin. Ivermectin selected for an allele (allele B), that was uncommon in *O. volvulus* populations that had not been under ivermectin pressure. This allele is distinguished from the wild-type allele A by having three amino acid

changes in the coding region which correspond to changes on the same surface of three adjacent coils in the H3 helix of β-tubulin and 24 bp of deletion in the intron adjacent to the coding region for the H3 helix. The current study describes an amplicon length assay which rapidly allows individual *O. volvulus* macrofilaria or microfilaria to be genotyped in terms of β-tubulin alleles and extends the information on β-tubulin allele frequencies, by showing that allele B was extremely rare (it was not found in the samples analyzed) in adult *O. volvulus* populations collected prior to the introduction of ivermectin use in a large part of West Africa or Uganda. However, Bourguinat et al. (2006) found β-tubulin allele B in *O. volvulus*, obtained in Cameroon before the introduction of ivermectin for onchocerciasis control in that country, although allele B was relatively rare in these ivermectin naïve parasites. Interestingly, these workers found an association between β-tubulin allele homozygosity or (A/B) heterozygosity and fertility in female *O. volvulus*.

In samples taken in 1998 in Ghana, allele B was quite common in *O. volvulus* macrofilariae obtained from people who had been repeatedly treated with ivermectin and, although uncommon, could also be found in *O. volvulus* from people who were ivermectin naïve (Eng and Prichard 2005). It should, however, be noted that by 1998, ivermectin use was widespread in endemic areas of Ghana. It is of further interest that a higher frequency of allele B (as heterozygotes) was found in a small sample of microfilariae obtained from people in Jagbenbeng, Ghana, in 2002, who responded poorly (greater than 10 microfilariae per skin snip, 90 days after ivermectin treatment (4)), compared with microfilariae from people who responded as expected in terms of 90 day post treatment microfilarial counts following six annual ivermectin treatments.

The lack of B/B homozygotes (Fig. 4) in this sample of microfilariae is surprising as B/B homozygotes were observed in macrofilariae sampled in Ghana in 1998 (Fig. 3) and indicates that the microfilariae were not in Hardy-Weinberg equilibrium. Hardy-Weinberg disequilibrium at β-tubulin was also observed in the *O. volvulus* sampled in Cameroon (Bourguinat et al. 2006) and may indicate non-random mating as has been observed in the closely related filarial nematode, *Wuchereria bancrofti* (Schwab et al. 2006)

The data showing an association of β-tubulin heterozygotes with high microfiladermias following ivermectin treatment adds to evidence (Ali et al. 2002; Awadzi et al. 2004; Awadzi et al. 2004) that suboptimal responses, in terms of post treatment microfilarial counts, may be occurring in some populations of *O. volvulus*. These parasitological data and evidence that ivermectin is causing genetic selection on *O. volvulus* (Ardelli and Prichard 2004; Eng and Prichard 2005; Ardelli et al. 2006; Ardelli et al. 2006), (Bourguinat, Pion, Kamgno, Gardon, Duke, Boussinesq, Prichard, pers. comm.) are suggestive of a developing ivermectin resistance in *O. volvulus* in Africa, possibly involving a subset of the adult parasite population being able to recover fertility more rapidly after ivermectin treatment than was seen when ivermectin was first introduced for onchocerciasis control (Alley 1994; Plaisier et al. 1995; Klager et al. 1996). A longitudinal study of the duration of suppression of *O. volvulus* fertility in areas where sub-optimal responses to ivermectin treatment are suspected is urgently required to establish unequivocally whether ivermectin resistance, in terms of reduced suppression of fertility, is occurring. This should focus on individuals who express the phenotype of suboptimal response such as recurring, significant adverse events

(Mazzotti reactions) despite multiple previous treatments and the persistence or reappearance of readily palpable nodules (Awadzi et al. 2004). Examination of embryogrammes provides direct evidence of adult worm reproductive activity. These clinical and parasitological examinations should be coupled with an analysis of genetic changes in the macrofilariae and microfilariae and compared with parasites from ivermectin naïve hosts. The amplicon length assay described in this report, will allow rapid analysis of individual microfilariae in terms of β-tubulin genotype.

In addition to showing an association between ivermectin treatment and selection on β-tubulin in *O. volvulus*, we also report for the first time, evidence for ivermectin selection on the β-tubulin isotype 1 gene in *H. contortus*. This was seen in the SSCP analysis on the genomic position 2692 bp-2883 bp of the *H. contortus* β-tubulin gene GRU-1, which corresponds to the sequence between amino acid 175 and 292 of the translated protein sequence, and also in terms of a SNP analysis on codon 200 of the β-tubulin gene in an ivermectin selected strain, compared with its parental unselected strain of *H. contortus*. Moxidectin, another macrocyclic lactone anthelmintic, has also been found to select on β-tubulin in *H. contortus* (Blackhall, W.J., 1999, Ph.D. Thesis, McGill University; Galazzo, D., 2005, M.Sc. Thesis, McGill University). The changes seen at codon 200 with ivermectin selection in *H. contortus* do not mean that this amino acid change is directly involved in ivermectin resistance in this parasite, but may be linked to other changes in β-tubulin. What specific changes in β-tubulin, if any, are directly involved in ivermectin resistance in *H. contortus* requires further elucidation, including functional assays. However, it is interesting that Freeman et al. (2003) found a marked derangement in the amphid neurons of *H. contortus* from an ivermectin

resistant strain, compared with an ivermectin susceptible strain, including a shortening of the dendritic processes in the ivermectin resistant worms. A careful examination of the cross sections in this study suggests that the microtubules in the amphid neurons may be altered in the ivermectin resistant nematodes. This may provide a further link for the involvement of tubulin in ivermectin resistance. Whether β-tubulin is genetically linked to another locus involved in a mechanism of resistance to ivermectin or whether β-tubulin may itself be involved in a mechanism of ivermectin resistance still needs to be determined. While microtubules are involved in signal transfer from nerve cells in the neuromuscular system of animals (Maas et al. 2006) it is not established how alterations in β-tubulin might possibly affect signal transfer from ivermectin receptors in nerve cells to muscle in nematodes (Dent et al. 2000) to affect responses to ivermectin, and this will require further investigation.

Benzimidazole resistance is recessive in trichostrongylid nematodes and the homozygous TAC_{200} codon has been shown to produce a benzimidazole resistance phenotype (Elard and Humbert 1999; Prichard 2001). We did not find that ivermectin selected for homozygous TAC_{200} (tyrosine) in the β-tubulin, but appeared to be selecting for β-tubulin TTC_{200}/TAC_{200} heterozygotes which would not show a benzimidazole resistance phenotype. However, some of the progeny of TTC_{200}/TAC_{200} heterozygote mating would show benzimidazole resistance. The genetic changes, including the selection for TAC_{200}, that we have observed in β-tubulin, in *H. contortus* selected by ivermectin, may be linked with other amino acid changes in β-tubulin or alleles of other genes which tend to associate with β-tubulin during meiosis. Considerable work needs to be done to establish which amino acid changes in β-

tubulin, if any, may be functionally important for ivermectin resistance. Benzimidazole resistance, associated with homozygous TAC_{200}, may not itself result in ivermectin resistance if TAC_{200} is not functionally involved in ivermectin resistance, but rather can be linked to another genetic change involved in ivermectin resistance. Benzimidazole resistant nematodes have not been observed to be ivermectin resistant *a priori*. However, ivermectin selection for TAC_{200} may predispose nematodes to benzimidazole resistance and this requires further investigation because it could have long term implications for the sustained use of ivermectin/benzimidazole combination treatments against livestock parasites and for the outcome of albendazole/ivermectin combination treatment against lymphatic filaria in Africa.

Acknowledgements

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Table 1. Location of villages where *O. volvulus* nodules were obtained

Country	Village	Date of collection	Number of Annual Ivermectin Treatments
Sierra Leone	Bonjeima	1989-1990	None
	Kambama	1989-1990	None
	Mawule	1989-1990	None
	Nitty	1989-1990	None
	Palima	1989-1990	None
	Yisaia	1989-1990	None
Cote d'Ivoire	Ahininkro	1989-1990	None
	Assereko	1989-1990	None
	Hemakono-Avo	1989-1990	None
	Louga	1989-1990	None
	Mamorodougou	1989-1990	None
	Oua	1989-1990	None
Ghana	Bielikpong	1989-1990	None
	Aflakpe	1998	None
	Asubende	1998	None
	Dodome-Awlime	1998	None
	Dodome-Teleafenu	1998	None
	Hoe	1998	None
	Honuta-Gborgame	1998	None
	Honuta-sifiafe	1998	None
	Klave	1998	None
	Kpoeta-Ashanti	1998	None
	Kpedze-Anoe	1998	None
	Kpdze-Sreme	1998	None
	Kpedze-Todze	1998	None
	Nakong	1998	None
	Asubin	1998	4 - 7
	Okinase	1998	6 – 7
	Zodanu	1998	3 - 6
	Adumadum	1998	4 – 7
	Kunda	1998	6 – 8
	Asukawkaw	1998	6 – 8
	Katanga	1998	6 – 8
Guinea	Morigbedougou	1989-1990	None
Senegal	Morougokoto	1989-1990	None
Uganda	Western region	1996	None

Fig. 1. Amino acid differences between *O. volvulus* β-tubulin allele A and allele B in the H3 helix. Amino acid differences, between the alleles, are indicated in bold.

Fig. 2. Gel electrophoresis of the amplicon length assay for β-tubulin allele identification in *O. volvulus*. Lane 1 shows electrophoresis of a homozygous allele A, β-tubulin sample. Lane 2 shows a homozygous allele B, β-tubulin sample and lane 3 shows a heterozygous (A/B) sample. Lane 4 is a 100 Bp DNA ladder (Invitrogen).

Fig. 3. A comparison of β-tubulin (isotype 1) genotype frequencies between ivermectin naïve populations of *O. volvulus* from across Africa before the introduction of ivermectin for onchocerciasis control (1989-90, West Africa; 1996, Uganda) and ivermectin naïve and ivermectin exposed populations of *O. volvulus* from Ghana collected in 1998. Note that the 1989-90 genotypes were analyses on individual *O. volvulus* nodules and each nodule could be expected to average 5 adult worms per nodule. 1996 and 1998 analyses were on individual adult parasites.

Fig. 4. β-tubulin genotype frequencies of microfilariae in skin snips collected from people in Jagbengben, Northern region of Ghana, in 2002, according to skin microfilarial count 90 days after ivermectin treatment, as an index of response to treatment. All the people sampled and the community had received six annual treatments of ivermectin. The people sampled were allocated to two ivermectin response groups, normal (good) responders (90 day post treatment skin microfilarial

(mf) loads of <10 mf/mg) (n = 7) and poor responders to treatment (90 day post most recent treatment, after 5 previous annual treatments, mf loads of >10 mf/mg) (n = 8).

Fig. 5 . Polymorph frequencies for *H. contortus* β-tubulin analyzed by SSCP. PF17 = unselected parent strain; IVF17= ivermectin selected strain, derived from the PF strain by ivermectin selection over 17 generations by increasing dose rates of ivermectin to achieve approximately an ED_{95} effect, in vivo. The polymorph frequencies were significantly different from each other by Chi-square analysis ($\alpha < 0.05$).

Fig. 6. SNP analysis of codon 200 of β-tubulin isotype I, in *H. contortus*, by Pyrosequencing. Comparison of the frequency of TTC_{200} (phenylalanine) or TAC_{200} (tyrosine) in the PF17 and IVF17 (Fig. 6a) and PF23 and IVF23 (Fig. 6b) strains. With each group of *H. contortus*, 36 to 40 adult male worms were individually genotyped at codon 200 of β-tubulin.

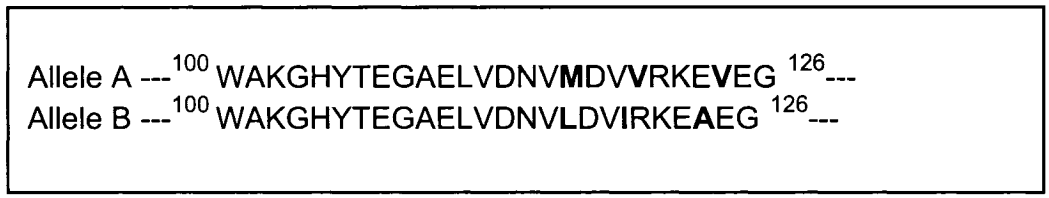
Allele A ---100 WAKGHYTEGAELVDNVMDVVRKEVEG 126---
Allele B ---100 WAKGHYTEGAELVDNVLDVIRKEAEG 126---

Fig. 1

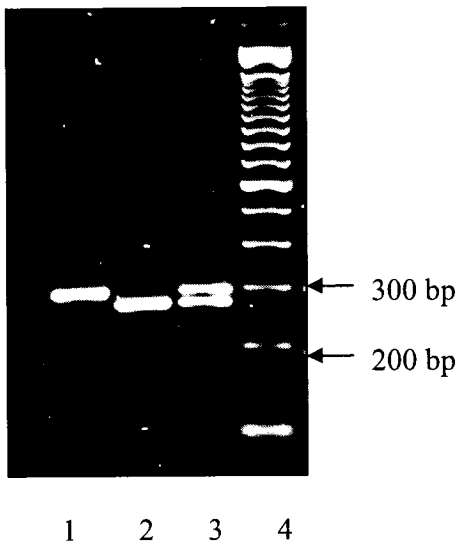

Fig. 2

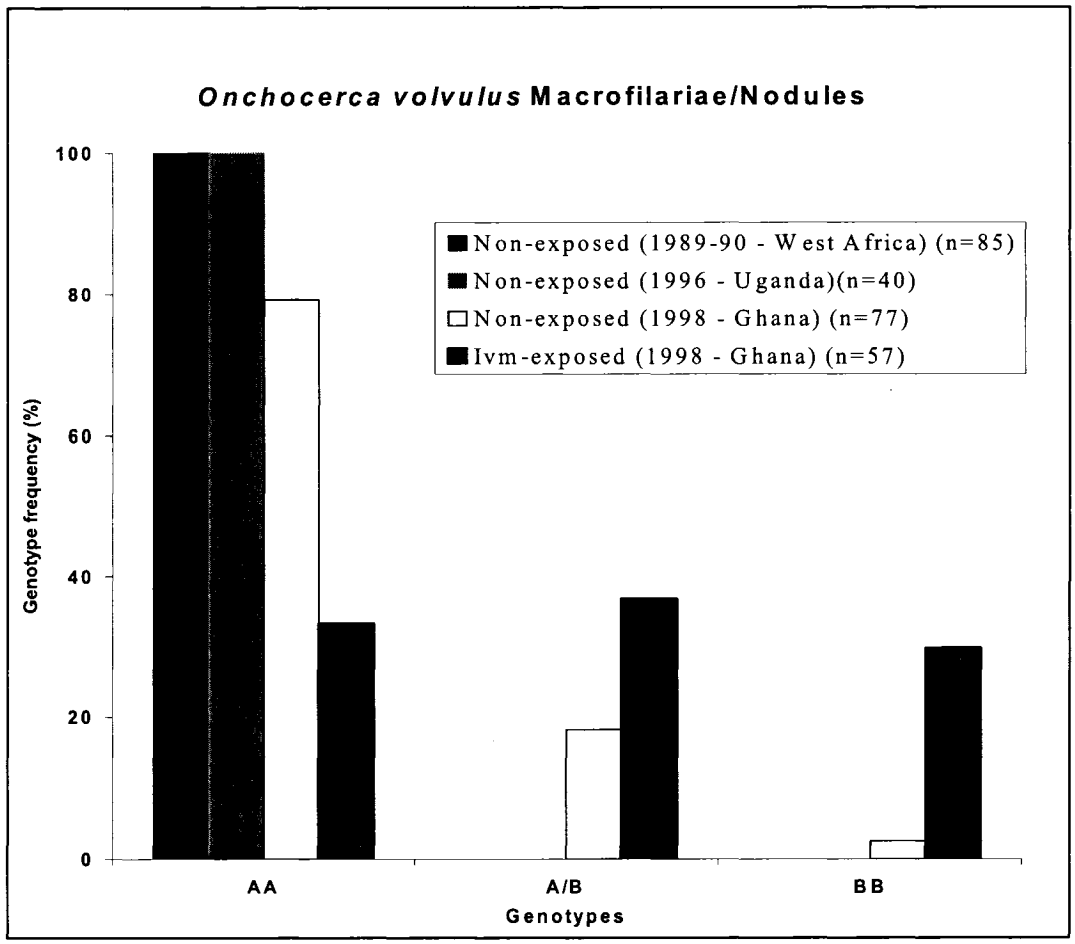
Onchocerca volvulus Macrofilariae/Nodules
100
80
60
40
20
0
Genotype frequency (%)
Non-exposed (1989-90 - West Africa) (n=85)
Non-exposed (1996 - Uganda)(n=40)
Non-exposed (1998 - Ghana) (n=77)
Ivm-exposed (1998 - Ghana) (n=57)
AA
A/B
BB
Genotypes

Fig. 3

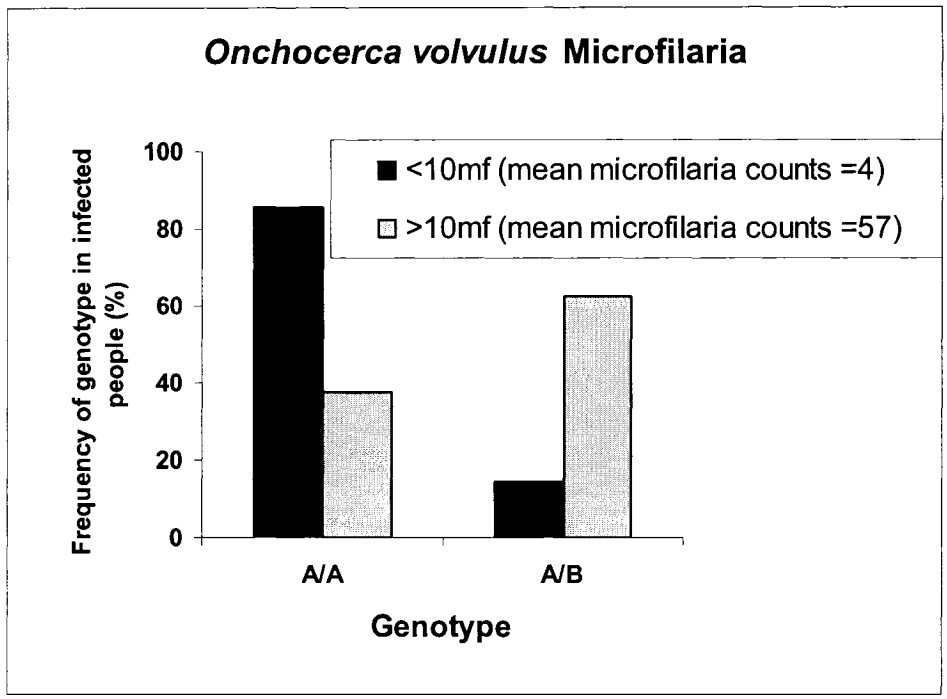
Onchocerca volvulus Microfilaria
Frequency of genotype in infected people (%)
100
80
60
40
20
0
<10mf (mean microfilaria counts =4)
>10mf (mean microfilaria counts =57)
A/A
A/B
Genotype

Fig. 4

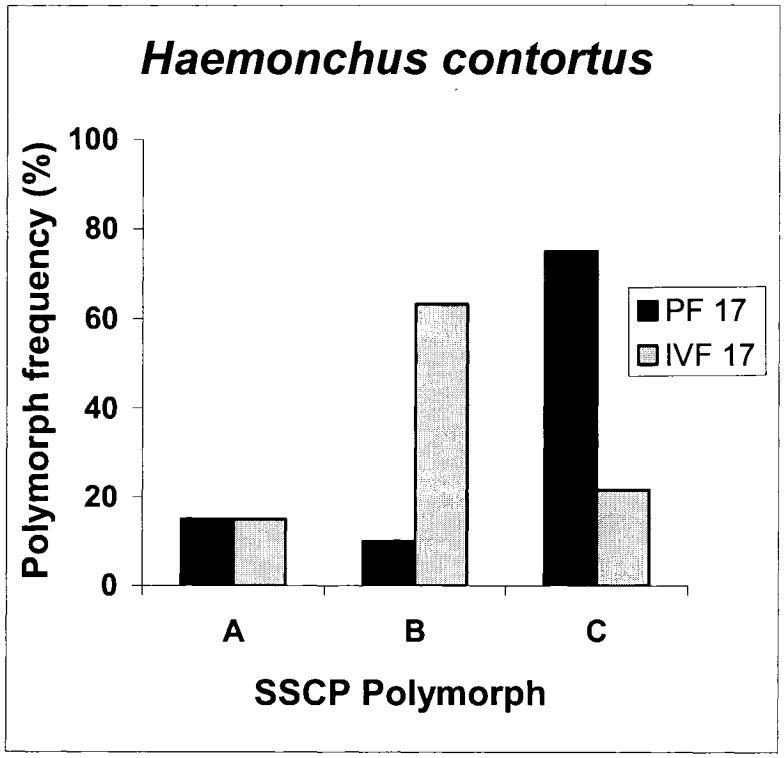
Haemonchus contortus
Polymorph frequency (%)
100
80
60
40
20
0
A
B
C
SSCP Polymorph
PF 17
IVF 17

Fig. 5

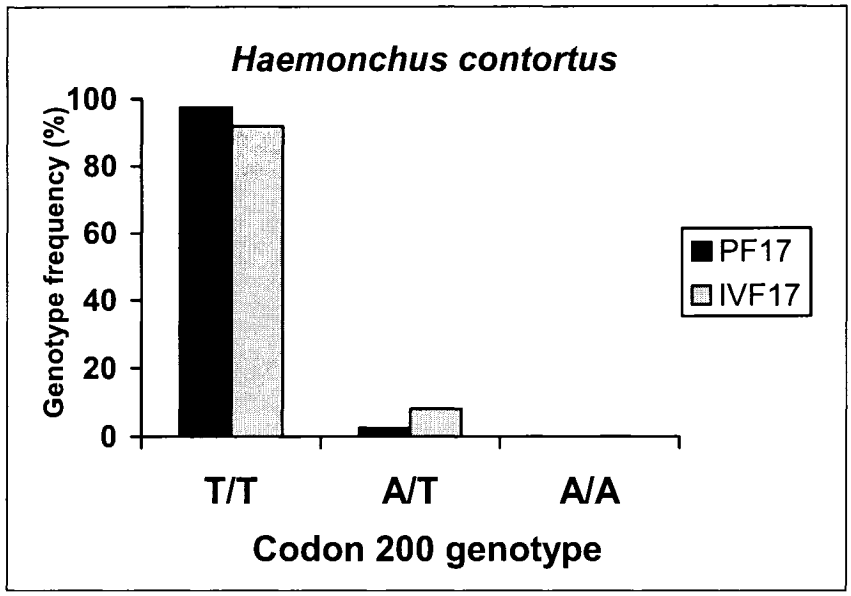
Haemonchus contortus
Genotype frequency (%)
100
80
60
40
20
0
T/T
A/T
A/A
Codon 200 genotype
PF17
IVF17

Fig. 6a

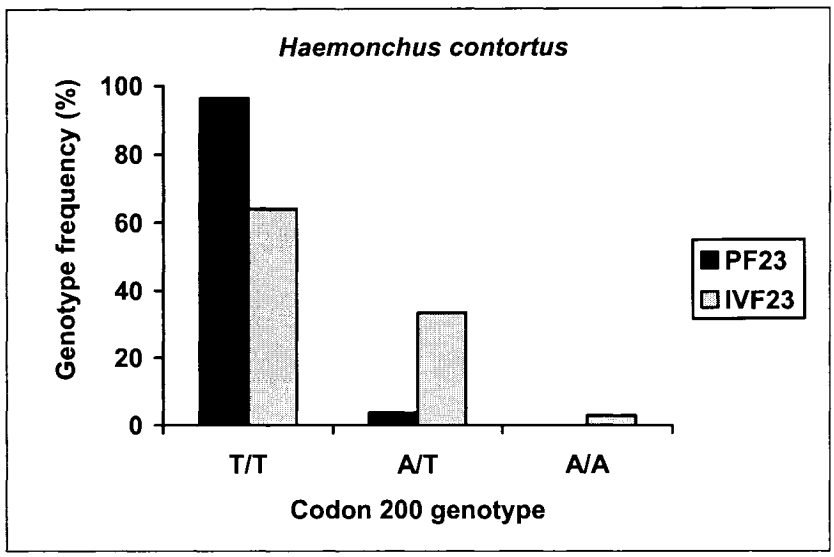
Haemonchus contortus
Genotype frequency (%)
100
80
60
40
20
0
T/T
A/T
A/A
Codon 200 genotype
PF23
IVF23

Fig. 6b

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Connecting Statement III

In Chapters II and III genetic polymorphism in several genes in *O. volvulus* obtained from ivermectin naïve and repeatedly treated people was investigated, with selection on a P-glycoprotein and a β-tubulin gene being shown. A rapid assay for examining genetic polymorphism in β-tubulin was developed. The assay was used on additional parasite samples, including samples obtained before any general use of ivermectin against onchocerciasis, and microfilariae obtained 90 days after the last treatment with ivermectin of people who had been repeatedly treated with ivermectin; some of whom appeared to be showing sub-optimal parasitological responses to treatment. Furthermore, β-tubulin also appears to be interesting as a marker of ivermectin selection in *H. contortus*. In Chapter IV, *O. volvulus* α-tubulin and the two β-tubulin alleles were cloned, expressed in *Escherichia coli* and purified. Polymerization and depolmerization along with ivermectin binding experiments, using equilibrium dialysis, were conducted in order to elicit a better understanding of the role of the β-tubulin gene product in ivermectin resistance.

Chapter IV: Manuscript III

Onchocerca volvulus β-tubulin: functional relationship to ivermectin resistance

Eng J.K.L., Osei-Atweneboana M, and Prichard, R.K.

*Manuscript in preparation for submission

Abstract

Onchocerca volvulus is a parasitic filarial nematode which is responsible for human onchocerciasis, a disease commonly referred to as "River Blindness". It is a major cause of preventable blindness and skin disease. Ivermectin is used annually or semi-annually to remove parasite microfilariae and inhibit reproduction by the adult parasites. However, it is not curative. We have shown that repeated ivermectin treatment results in selection on β-tubulin in *O. volvulus*. Analysis of the ivermectin selected *O. volvulus* β-tubulin allele identified three amino acid substitutions in the helix 3 region. Further analyses were conducted to investigate a possible direct relationship between ivermectin and β-tubulin. Recombinant *O. volvulus* β-tubulin alleles and α-tubulin were expressed and used in polymerization and depolymerization assays. These assays showed differences in the extent of polymerization when the different β-tubulin alleles were polymerized with α-tubulin. Furthermore, it was found that 50 ng/ml ivermectin significantly reduced the rate of depolymerization of the polymeric tubulin, composed of the ivermectin selected β-tubulin allele with α-tubulin, but not of the wild-type β-tubulin allele. Data obtained from the equilibrium dialysis experiments, with BODIPY FL ivermectin indicated that BODIPY FL ivermectin not only bound to the purified β-tubulin but also to the α-tubulin protein. Non-fluorescent ivermectin and taxol were able to compete with the BODIPY FL ivermectin. The data suggests a putative binding site for BODIPY FL ivermectin in the hydrophobic taxol binding domains in β- and α-tubulin. Alterations in the β-tubulin protein, caused by the identified amino acid changes in the ivermectin selected β-tubulin allele, might lead to disorganization of the microtubules in the amphidial neurons. Shortening and

disorganization of the amphidial neurons has been reported in ivermectin resistant *Haemonchus contortus*, another nematode in which tubulin is implicated in ivermectin resistance. Alteration in β-tubulin, and subsequently in microtubules, may also affect the functioning of ligand-gated chloride channels in neurons and transmission of a hyperpolarizing signal to muscle cells.

Introduction

Onchocerciasis is the disease caused by the human filarial nematode *Onchocerca volvulus*. The disease is not lethal but is very debilitating. It is estimated that 18 million people around the world are infected, 500,000 are visually impaired and another 270,000 are blind as a result of this infection. In total, it has been estimated that 123 million people are at risk of infection (Luder et al. 1996; Raff et al. 1997; Dull and Meredith 1998).

Ivermectin (Mectizan™) has been successfully used to treat human onchocerciasis for almost two decades without ivermectin resistance being unequivocally described. However, in 2002 reports from Sudan and Ghana indicated high numbers of residual microfilaria in a number of infected patients after multiple treatments with ivermectin (Ali 2002; Awadzi and Edwards 2004) indicating possible sub-optimal responses to ivermectin. In addition, genetic analysis of 16 genes in *O. volvulus*, by Eng and Prichard (2005), indicated a genetic change in allele frequency in two genes from nematodes exposed to multiple treatments with ivermectin. Of interest, the results from the single strand conformational polymorphism (SSCP) conducted by Eng and Prichard (2005), showed that the *O. volvulus* β-tubulin isotype 1 gene showed a change in allele frequency with positive selection for a particular allele (allele B). In addition, Eng et al. (2006) described genetic selection on the β-tubulin gene isotype 1 in ivermectin exposed *O. volvulus* and an ivermectin resistant strain of *Haemonchus* contortus.

Experimental results with *C. elegans* and *H. contortus* suggest that ivermectin enters the nematodes via an oral route and/or via transcuticular uptake mechanism (Ho

et al. 1990; Geary et al. 1993). Once the drug enters the nematode system, it then binds to its target(s), i.e. the glutamate-gated chloride channels and/or GABA-gated chloride channels causing flaccid paralysis of the nematode (Dent et al. 2000; Feng et al. 2002). In case of *O. volvulus*, the pharyngeal pumping is believed to be much reduced compared with non-filarial nematodes and possibly non-functional (Franz and Buttner 1983; Schulz-Key 1988). Therefore the possible lack of an oral uptake mechanism for ivermectin in *O. volvulus*, suggest that ivermectin may enter the nematode via alternative routes, such as a cuticular uptake mechanism (Ho et al. 1990; Cross et al. 1998) or via chemosensory pores such as the anterior amphids (Kotze and Bagnall 2006).

The nematode amphid sensory neurons are used for chemical and thermal signaling (Dent et al. 2000). The structural integrity of the amphids is very dependent on microtubules (Nikolic 2004). Experimental data from Freeman et al. (2003) showed distorted amphid structures and microtubule bundles in the amphidial neurons from ivermectin resistance strains of *H. contortus.* Literature searches reveal no reports of any direct interaction between the tubulin and ivermectin. Therefore to develop a better understanding of the possible role of tubulin in ivermectin resistance we expressed and purified the two β-tubulin alleles that were reported by Eng and Prichard (2005) and an *O. volvulus* α-tubulin gene (accession number: AY936208) and performed drug binding experiments and tubulin polymerization/depolymerization assays. The results presented in this paper provide evidence that multiple exposures to ivermectin treatment may select for a β-tubulin allele that alters the functioning of the β-tubulin compared with the wild-type β-tubulin allele. β-tubulin may be included as a contributing factor in the

mechanism of ivermectin resistance.

Material and Methods

Cloning of α-tubulin

To clone the full length α-tubulin cDNA of *O. volvulus*, total RNA was extracted from two adult female *O. volvulus* worms using methods described in the Trizol® procedure (Invitrogen). Reverse transcription to generate the α-tubulin cDNA was performed following the protocol described by Invitrogen for M-MLV Reverse Transcriptase. A 1347 bp full length cDNA of α-tubulin was generated using sense primer (5'-ATGCGCGAGGTGATATCAGTA-3') and anti-sense primer (5-TCAATACTCTTCGCCTTCGCC-3') (accession number **AY936208**). The full length α-tubulin was then cloned into the pJC45-MCS expression vector (6xHis-tagged) and the complete construct was transformed into *E. coli* strain ER2566. The full length cDNA was sequenced to confirm its identity.

Cloning of β-tubulin

The 1344 bp full length cDNAs of the two β-tubulin alleles were generated using and anti-sense primer from male *O. volvulus* samples previously genotyped using methods described by Eng et al. (2006). The two β-tubulin alleles were cloned into the 6xHis-tagged PQE-30 expression vector (Invitrogen) and the complete construct was transformed into *E. coli* strain ER2566 and sequenced.

Tubulin Expression and Purification

All three tubulin constructs were grown at 37 °C shaking at 250 rpm in 500 ml Lauria-Bertani medium (LB) containing 100 μg/ml ampicillin. In addition, 25 μg/ml kanamycin was added for the α-tubulin construct only. When the bacterial sample reached an optical density of 0.6, induction of tubulin synthesis was initiated by the addition of 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). Incubation continued for another 5 hours. The samples were then spun at 2000 rpm to pellet the bacteria.

Lysis of bacteria was performed by the addition of 15 ml binding buffer (1x phosphate buffer, pH 7.5), to resuspend the bacterial pellet, followed by three cycles of freeze thaw in liquid nitrogen. The lysis was then sonicated for six minutes (10 second pulses with 20 second resting periods) on ice. A second spin was performed at 14,000 rpm for 20 minutes at 4 °C. The soluble fraction was collected and 2 ml of pre-washed Ni Sepharose™ 6 Fast Flow slurry (GE Healthcare Life Sciences) was added and incubated over night at 4 °C, under rotation.

The samples were loaded onto a disposable column (BioRad Laboratories) and the samples were allowed to move through the column by gravity flow. The nickel resin was washed three times with 15 ml Wash Buffer (1x phosphate buffer, pH 7.5, 18 mM imidazole) and then eluted with 10 ml Elution buffer (1x phosphate buffer, pH 7.5, 60 mM imidazole), with collection of 4 ml fractions. All eluted samples were analyzed on a 10 % tris-glycine sodium dodecyl sulfate polyacrylamide gel by electrophoresis (SDS-PAGE) to ensure purity. Monoclonal antibodies to α- and β-tubulin (GE Healthcare) were used in Western blots to verify the presence of the respective tubulin proteins.

Polymerization Assay

One mg/ml of purified α-tubulin was added to 1 mg/ml of purified β-tubulin and mixed in polymerization buffer (0.1 M MES pH 6.4, 10 % glycerol, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP) to a final volume of 1ml on ice. The samples were transferred to a 1 ml quartz cuvette (Beckman Coulter) and the rate of polymerization, indicated by the increase in turbidity, was monitored on a Beckman Coulter DU® 640 spectrophotometer at 340 nm at 37 °C (Shelanski et al. 1973). Optical density readings were taken every 15 seconds. A working stock solution of ivermectin (Sigma-Aldrich) was prepared to a final concentration of 1.75 μg/ml in 100% DMSO (Sigma-Aldrich) and used at a final concentration of 50 ng/ml (2.85% final concentration of DMSO).

Depolymerization Assay

In the depolmerization assay, the products of the polymerization assay were incubated on ice for five minutes while the cuvette holder in a Beckman Coulter DU® 640 spectrophotometer was cooled to 10 ° C. Once cooled the samples were placed back into the Beckman Coulter DU® 640 spectrophotometer and sample turbidity was monitored at 340 nm. Optical density readings were taken every five seconds.

Equilibrium Dialysis

1 mg/ml of purified tubulin protein, 1 ml total in PBS (pH 7.2), was pipetted into presoaked dialysis tubing (Spectropor, 10 kDa cut off) and sealed with dialysis clips. The dialysis tubing was inserted into a modified 15 ml round bottom tube containing 1 ml of dialysis solution, plus or minus FITC (1 ug/ml), 1 μg/ml BODIPY

FL ivermectin (Invitrogen™), BODIPY FL ivermectin (1 μg/ml) plus non-fluorescent ivermectin (5 μg/ml) and/or taxol (Sigma) (1 μg/ml). The modified round bottom tube was wrapped with Parafilm and the sample was incubated for 48 hours at 4 °C while shaking gently. After 48 hours, both the samples from the interior of the dialysis tubing and the dialysis solution were separately removed by pipette. Both solutions were analyzed in a Varian Cary Eclipse fluorescence spectrophotometer at room temperature. The fluorescence spectrophotometer was set to scan from 500 nm to 530 nm. The peak excitation for the FITC-ivermectin is 520 nm. Three replicas of each experiment were performed. The net fluorescent reading of BODIPY FL ivermectin bound to the protein was determined by subtracting the fluorescent reading of the solution exterior to the dialysis tubing from the fluorescent reading of the solution inside the dialysis tubing.

Results

Three amino acid substitutions were identified in the helix three domain of allele B at amino acid positions 117, 120 and 124. We then calculated the hydrophobicity changes in the helix three domain between the two β-tubulin alleles and determined a slight increase in the hydrophobicity value with the β-tubulin B allele compared with allele A (Table 1). The two β-tubulin alleles were modeled using Swiss-Prot and Table 2 shows the overall structural profile of the two β-tubulin alleles. The data from Swiss-Prot suggests a slight overall structural change in the β-tubulin allele B compared with allele A.

The expression and purification of the recombinant *O. volvulus* α- and β-tubulins are shown on an SDS-PAGE (Figure 1). The molecular size of the purified α-tubulin and β-tubulins were calculated, on the basis of their putative amino acid sequences, to be 49280 Da and 49390, respectively. The size of all three recombinant proteins was confirmed by the SDS-PAGE (Fig 1). Commercially produced monoclonal antibodies for the α- and β-tubulin (GE Healthcare) were then used in a Western blot analysis to confirm the identity of the recombinant proteins (Figure 2a and b).

Once the Western blot confirmed the presence of tubulin, an assay was performed to test the relative rates of polymerization of the recombinant tubulins (Figure 3). The addition of colchicine, which inhibits polymerization, acted as a negative control in the polymerization assay.

The linear portion of the slopes for polymerization and depolymerization were analyzed by linear regression using Prizm software (GraphPad Software Inc. V.4.00). In the polymerization assays, Figures 4 and 5, three replicas of each assay were

performed. A representative of each assay is presented. No statistical differences were measured for the polymerization rates of the two β-tubulin alleles in either the assays containing α- + β- tubulin alone, α- + β-tubulin + DMSO, or α- and β- tubulin + ivermectin (50 ng/ml). However, it was noted that all samples containing α + β tubulin (allele A) reached a significantly higher plateau of polymerization than did the samples containing α + β-tubulin (allele B) ($P < 0.05$).

In the depolymerization assays in the absence of ivermectin, no significant differences were found between the α- + β (allele A)-tubulin and α- + β (allele B)-tubulin (Figure 5A) nor in assays containing DMSO as a solvent control (data not shown). However, in the depolymerization assays containing 50 ng/ml of ivermectin, a significant difference ($P < 0.05$) was detected in the slopes of the two curves (Figure 5B). The average rates of depolymerization (based on the linear portion of the curves) are presented in the Figure 5B insert.

Equilibrium dialysis was performed to determine whether ivermectin and BODIPY FL ivermectin are able to directly interact with the tubulin proteins. Three controls were used, PBS plus 1 μg/ml FITC, PBS, and 0.5 mg/ml of purified guanosine monophosphate reductase (GMPR) (as a protein control). After 48 hours of dialysis against 1 μg/ml BODIPY FL ivermectin for all incubations, except the FITC control, no significant change in the fluorescent level was detected, between the samples retrieved from the interior of the dialysis tube verses the solution outside the dialysis tube, in any of the three controls (Figure 6A). On the other hand, dialysis samples which contained the α-tubulin, β-tubulin (A) or β-tubulin (B) proteins showed a significant increase of fluorescents signal in the samples retrieved from the interior of the dialysis tube (Figure

6A). The increase in the fluorescent signal from the BODIPY FL ivermectin which associated with the tubulin protein was significantly diminished by the addition of a non-fluorescent (NF) ivermectin as a competitor (Figure 6B). With respect to the α-tubulin sample, the fluorescent decrease to roughly 350 a.u. which represents an approximate 50 % decrease in the fluorescent signal. In the β-tubulin samples, the decrease in the fluorescent reading was roughly 300 a.u. (decrease of ~ 60 %) for the allele A and a difference of 119 a.u. (decrease of ~30 %) for allele B.

Taxol was added to determine whether there was any interaction between the ivermectin and taxol binding sites. When 1 μg/ml of taxol was added to the dialysis solution, a decrease in the fluorescent, attributable to the binding of BODIPY FL ivermectin was observed (Figure 6C). BODIPY FL ivermectin fluorescence decreased for α-tubulin by approximately 45.7 %. The decrease in BODIPY FL ivermectin fluorescence associated with β-tubulin allele A was 16.5 % and for allele B it was 61.9 %.

Discussion

Previous reports from Eng and Prichard (2005) and Eng et al. (2006) identified genetic selection on the β-tubulin isotype 1 gene in *O. volvulus* exposed to multiple yearly treatments with ivermectin, and in ivermectin resistant *H. contortus*. An increase in frequency of the allele B was present in *O. volvulus* macrofilariae obtained from patients who had been multiply treated with ivermectin compared with *O. volvulus* obtained from ivermectin naïve patients, in which the allele B was present at a low frequency. Furthermore, microfilariae obtained from multiply ivermectin treated patients who responded poorly to ivermectin in terms of microfilarial suppression also showed a higher frequency of allele B compared to microfilariae from patients responding well to ivermectin. However, there is lack of information which could explain the relationship between repeated ivermectin treatment and genetic selection on the β-tubulin gene. One explanation, suggested by Blackhall et al. (PhD thesis), speculated that the selection on the β-tubulin gene in *H. contortus*, could be the result of genetic linkage of β-tubulin to a gene on the same chromosome which is directly involved with ivermectin resistance and therefore selection on an unknown gene could also select on the β-tubulin gene.

The objective of this study was to determine if ivermectin, at a concentration of 50 ng/ml, which is a pharmacologically relevant concentration *in vivo*, could bind to tubulin and directly affect tubulin polymerization or depolymerization. Recombinant *O. volvulus* α- and two β-tubulin alleles were successfully expressed and purified from an *Escherichia coli* bacterial expression system. Polymerization and depolymerization rates were calculated by measuring regression slopes for each reaction. Initial experiments without ivermectin indicated that there were no significance differences in the rate of

polymerization between α + β-tubulin (allele A) compared with α + β-tubulin (allele B). The polymerization assays also confirm experimental data published by Oxberry et al. (2001), that recombinant nematode tubulins could polymerize in the absence of microtubule-associated proteins.

Although no differences in the polymerization rates were determined with or without the presence of ivermectin, it was interesting to note that the extent of polymerization was different when β-tubulin allele A and allele B were used in the assay. The slightly different putative three dimensional conformation of the two β-tubulin alleles may account for the differences seen in the plateau region in the polymerization data and in the depolymerization assay. The depolymerization assay did not show a statistical difference in depolymerization rates between the α+β(A) and α+β(B) in the absence of ivermectin. However, in the presence of 50 ng/ml ivermectin, there was a marked difference in the rate of depolymerization between the polymers containing allele A and allele B. Ivermectin markedly slowed the rate of depolymerization of the α+β(B) polymers compared with the polymers containing allele A.

Since it is believed that the H3 domain of the β-tubulin interacts with the M-loop of the adjacent β-tubulin protein (Lowe et al. 2001), we hypothesize that the changes in the overall hydrophobicity in the helix three domain may cause a change in the lateral interaction of the tubulin units in microtubules and possibly result in an altered form of microtubules. This hypothesis is consistent with the analysis of the amphid structures in ivermectin resistance strains of *H. contortus* which showed a shortened and abnormal structural arrangement of the dendrites in the amphid structures (Freeman et al. 2003).

The integrity of the amphid structures and many other cellular structures depend on

the protein tubulin. Alterations of the tubulin protein, i.e. coding SNPs, could affect not only the structure but the function of the amphid. Such functional changes could include neurotransmission which could decrease the sensitivity towards ivermectin. We also postulate a possible direct mechanism involving β-tubulin. It is believed that the pharyngeal organs which are present in filarial nematodes, are non-functional in terms of nutrient uptake (Franz and Buttner 1983; Schulz-Key 1988). It is therefore likely that ivermectin enters the nematode via an alternative route to the pharynx. It has been suggested that the anterior amphid region may be involved in the entry of ivermectin (Dent et al. 1999). Grant and Hunt (pers. commun. cited in Starich et al. (1995)), observed that all ivermectin-resistant *C. elegans* larvae were dyf mutants, showing a dye-filling defect, and suggested that the amphidial neurons played a significant role in determining ivermectin sensitivity. Johnson et al., (pers. commun. cited in Starich et al. (1995) proposed the same hypothesis and added that amphid defective *C. elegans* mutants were able to grow in culture medium containing 5 ng/ml of ivermectin. The amino acid changes in the H3 domain in the β-tubulin allele B protein (Table 1 and 2) may causes a structural change in resulting microtubules which could alter the overall structure and functioning of the amphid, and render the worm less permeable to ivermectin.

Interaction between ivermectin and tubulin has not been previously reported. The equilibrium dialysis experiments have shown an increase in the fluorescence in the dialysis samples which contained tubulin proteins, whereas when GMP reductase was dialyzed against the BODIPY FL ivermectin there was no significant increase in fluorescence inside the dialysis tube compared with the external solution. It was of

interest that the α-tubulin had the greatest level of fluorescent compared with the two β-tubulin samples. Equilibrium dialysis data along with the depolymerization data indicate that ivermectin can indeed bind to and interact with tubulin. Though the exact mechanism and location of the binding site has not yet been determined, it is possible that the hydrophobic helix 3 domain, which extends out of the β-tubulin structure to interact with the M-loop of an adjacent tubulin, may be involved. Taxol binding occurs near the M-loop and at the site of lateral interaction (Nogales 2000) and it is of interest that taxol reduced the binding of the BODIPY FL ivermectin to the tubulins.

Though the β-tubulin subunit has been implicated as the major target for taxol (Nogales et al. 1995; Nogales et al. 1998), photoaffinity labeling experiments by Dasgupa et al (1994) and Leob et al (1997), with a taxol analogue showed that the taxol analog also bound to the α-tubulin subunit. In addition, experiments by Banerjee and Kasmala (1998), studying the *in vitro* assembly of different α-tubulin isoforms in the presence of taxol concluded that part of the taxol binding domain was on the α-tubulin subunit. If ivermectin does binds to the hydrophobic taxol binding domain, it may explain why both the α- and β-tubulin dialysis experiments indicated binding of BODIPY FL IVM, and a decrease in fluorescent signal when taxol was present.

It can be hypothesized that the SNPs identified in the β-tubulin allele selected with ivermectin expose in *O. volvulus*, could cause structural changes in the *O. volvulus* amphids, similar to those seen in ivermectin resistant *H. contortus* and this may limit the uptake of ivermectin or affect transmission of the signal from ivermectin sensitive ion channel receptors in neurons to the muscles. This is the first study to show ivermectin binding to and a functional effect on tubulin. However, additional experiments will be

required to fully comprehend the relationship between ivermectin and tubulin and further studies will be requires to determine whether there are morphological changes to the amphids in *O. volvulus* selected by ivermectin.

Acknowledgements

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Figures and Tables

Figure 1. Purification of recombinant *O. volvulus* α- and β-tubulin from *E. coli*. White arrows indicate purified tubulin proteins. Lane 1 represents the column flow through (FT), Lane 2 represents the wash fraction (W), and Lanes 3 and 4 represents the column elution fractions (E1-2).

Figure 2. A) Western blot of the recombinant α-tubulin. A β-tubulin sample was included as a negative control sample. B) Western blot of the recombinant β-tubulin, βT(A)= allele A and βT(B)= allele B. The α-tubulin (αT) was included as a negative control sample.

Figure 3. Tubulin polymerization assay. Colchicine was added at 0.5 μg/ml to inhibit tubulin polymerization.

Figure 4.Tubulin polymerization assay with samples containing the purified tubulins (-|-, -◊-), with 2.85% DMSO (-●-, -Δ-) or 50 ng/ml ivermectin in DMSO at a final concentration of 2.85% (-■-, -▲-).

Figure 5. A comparison of tubulin depolymerization rates between the β-tubulin allele A and allele B samples without ivermectin (A) and in the presence of 50 ng/ml of ivermectin (B) (calculated depolymerization rates calculated from the linear portion of the depolymerization curves).

Figure 6. A comparison of the net fluorescent reading (differences between samples retrieved from the interior and exterior of the dialysis tubing), (A) PBS control dialyzed against 1 μg/ml FITC, PBS control dialyzed against 1 μg/ml BODIPY FL ivermectin, a control protein sample, 0.5 mg/ml GMPR, dialyzed against BODIPY FL ivermectin and the purified tubulin samples (1mg/ml) dialyzed against BODIPY FL ivermectin. (B) Competition study with 5 μg/ml of non-fluorescent ivermectin and 1 μg/ml of BODIPY FL ivermectin, and (C) competion study with 1 μg/ml of taxol and 1 μg/ml of BODIPY FL ivermectin.

Table. 1. Hydrophobicity values for the three amino acid differences between the two β-tubulin alleles. Amino acid hydrophobicity values were obtained from (Eisner, D. *et al* (1982) *Faraday Symp. Chem. Soc.* **17**:109-120.)

	Amino Acid	**Hydrophobicity Value**	**Total Hydrophobicity Value**
A Allele	Methionine [117]	1.1	5.7
	Valine [120]	2.3	
	Valine [124]	2.3	
B Allele	Alanine [117]	1.0	6.3
	Isoleucine [120]	3.1	
	Leucine [124]	2.2	

Table 2. Structural values for the two β-tubulin alleles, generated by Swiss-Prot.

Allele	Number of H-Bonds	Number of Helices	Number of Strands	Number of Turns
A	206	20	15	46
B	205	21	15	47

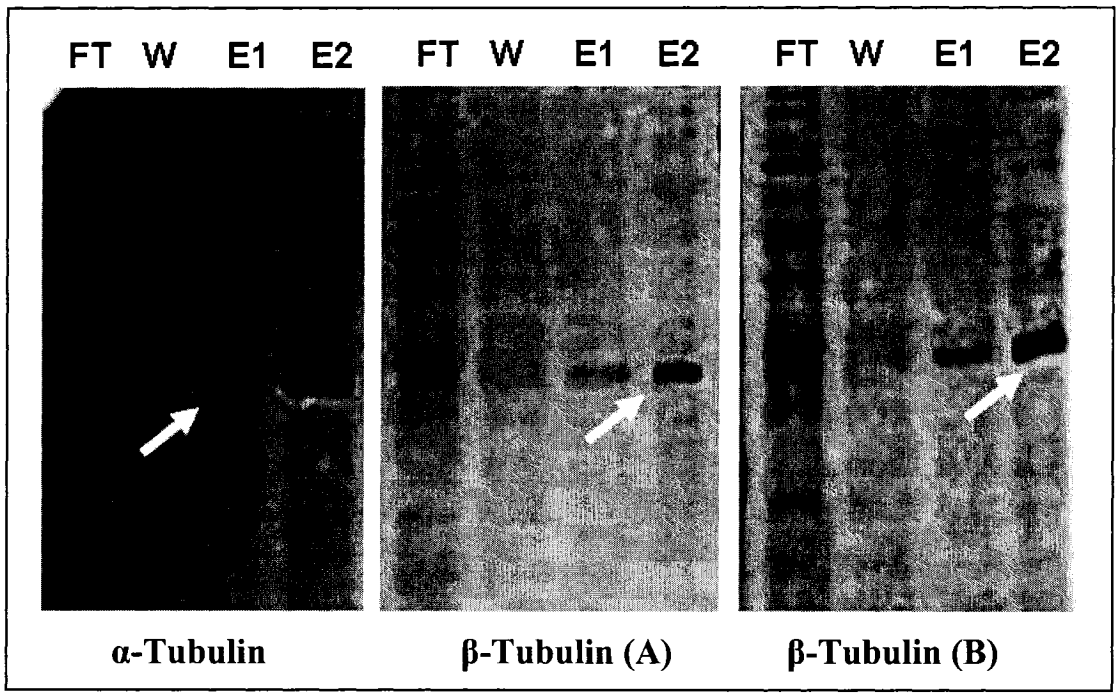
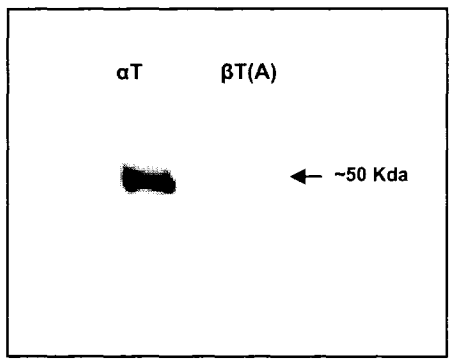
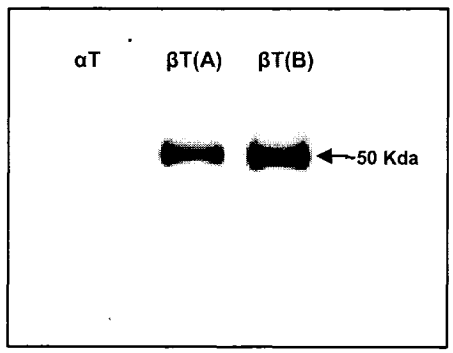


Figure 1



A



B

Figure 2

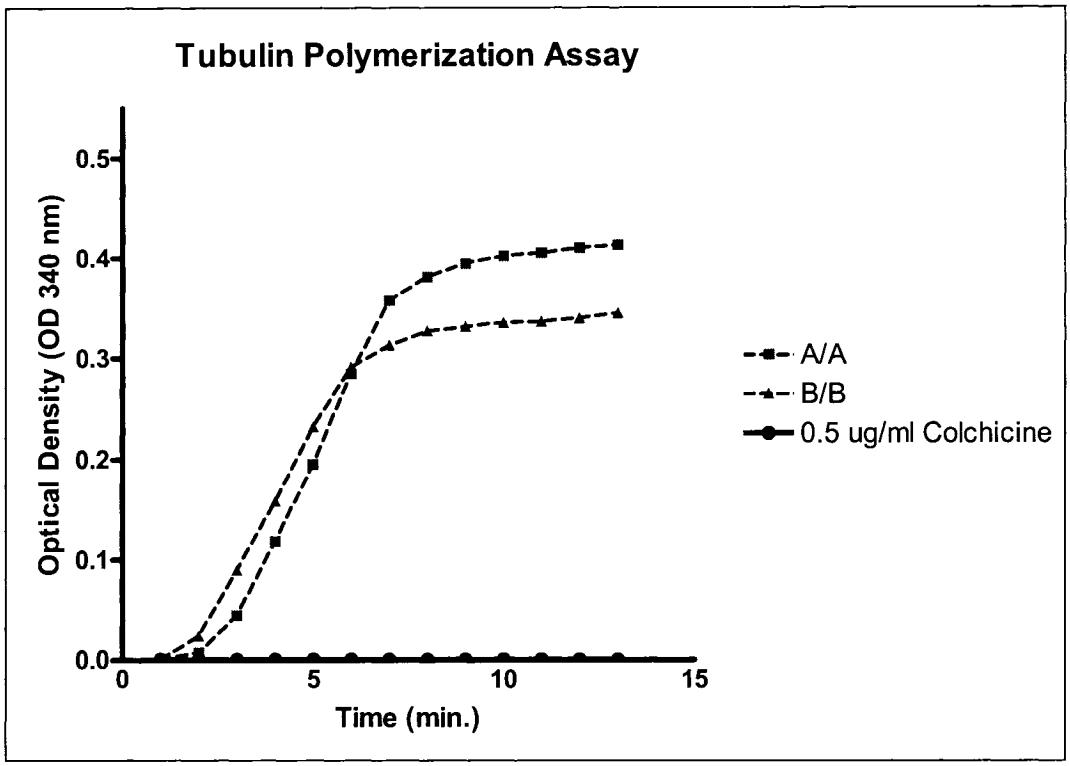

Tubulin Polymerization Assay
Optical Density (OD 340 nm)
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
Time (min.)
A/A
B/B
0.5 ug/ml Colchicine

Figure 3

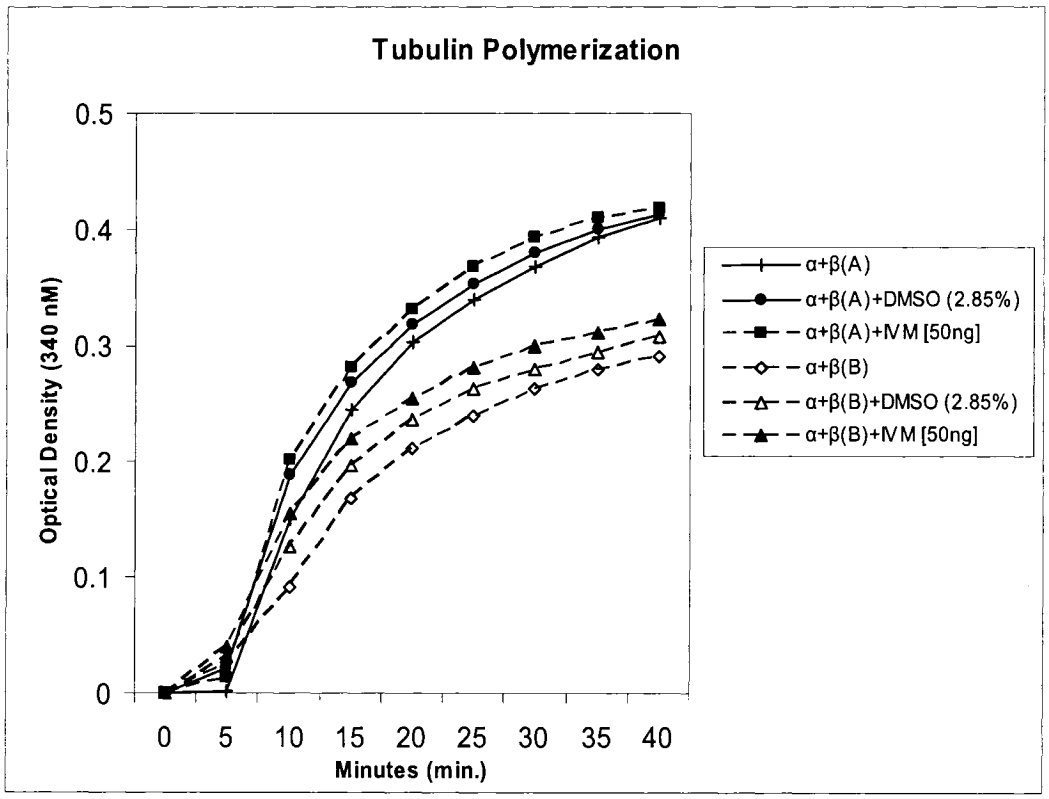

Tubulin Polymerization
Optical Density (340 nM)
0.5
0.4
0.3
0.2
0.1
0
0 5 10 15 20 25 30 35 40
Minutes (min.)
α+β(A)
α+β(A)+DMSO (2.85%)
α+β(A)+IVM [50ng]
α+β(B)
α+β(B)+DMSO (2.85%)
α+β(B)+IVM [50ng]

Figure 4

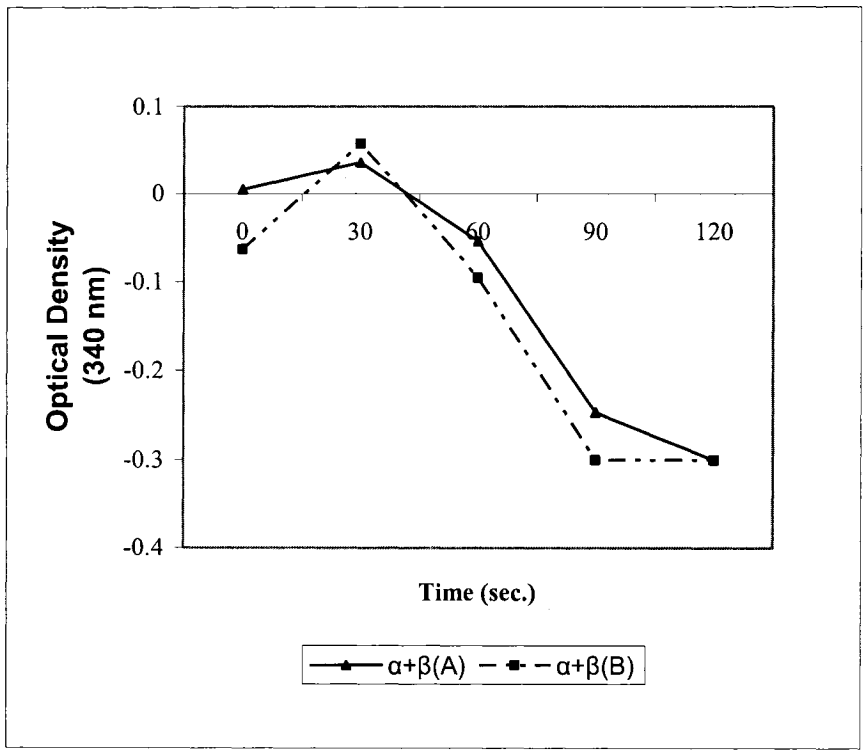
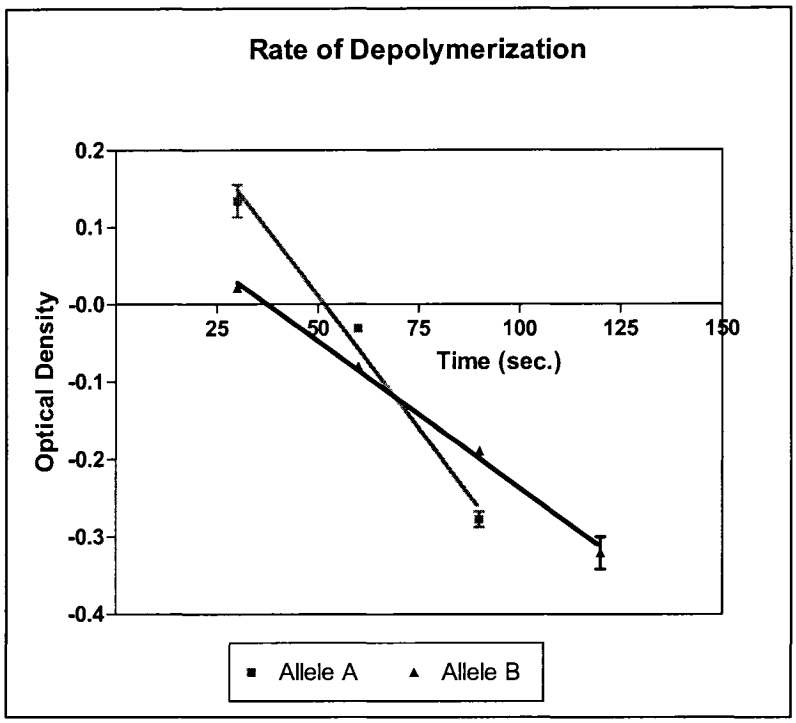

0.1
0
-0.1
-0.2
-0.3
-0.4
0
30
60
90
120
Optical Density (340 nm)
Time (sec.)
α+β(A)
α+β(B)

Figure 5A



	Average slope (x3)
α+β(A) + IVM [50ng]	$-6.85 \times 10^{-3} \pm 4.17 \times 10^{-4}$
α+β(B) + IVM [50ng]	$-3.78 \times 10^{-3} \pm 1.58 \times 10^{-4}$

Figure 5B

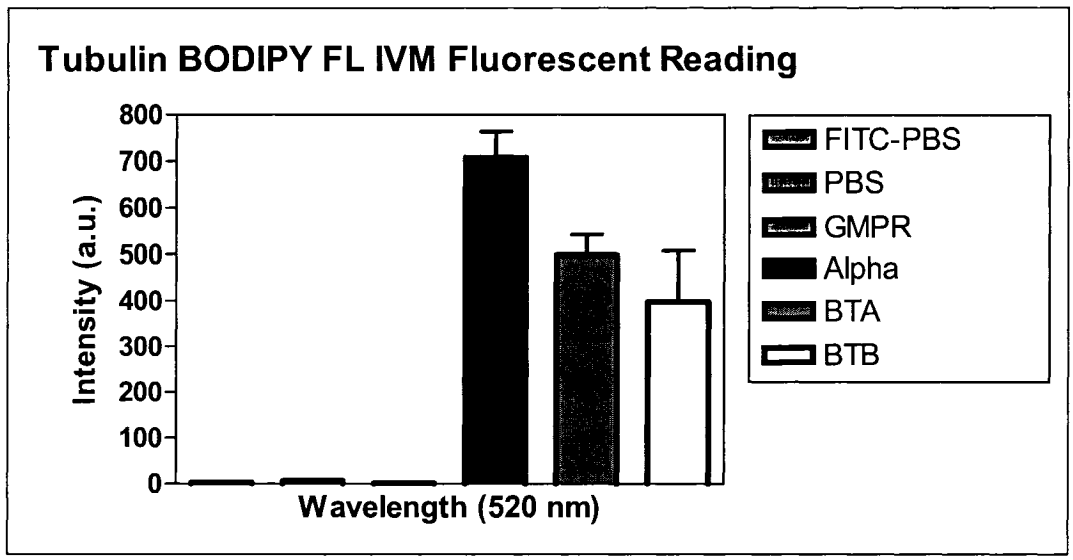

Tubulin BODIPY FL IVM Fluorescent Reading
Intensity (a.u.)
800
700
600
500
400
300
200
100
0
Wavelength (520 nm)
FITC-PBS
PBS
GMPR
Alpha
BTA
BTB

Figure 6A

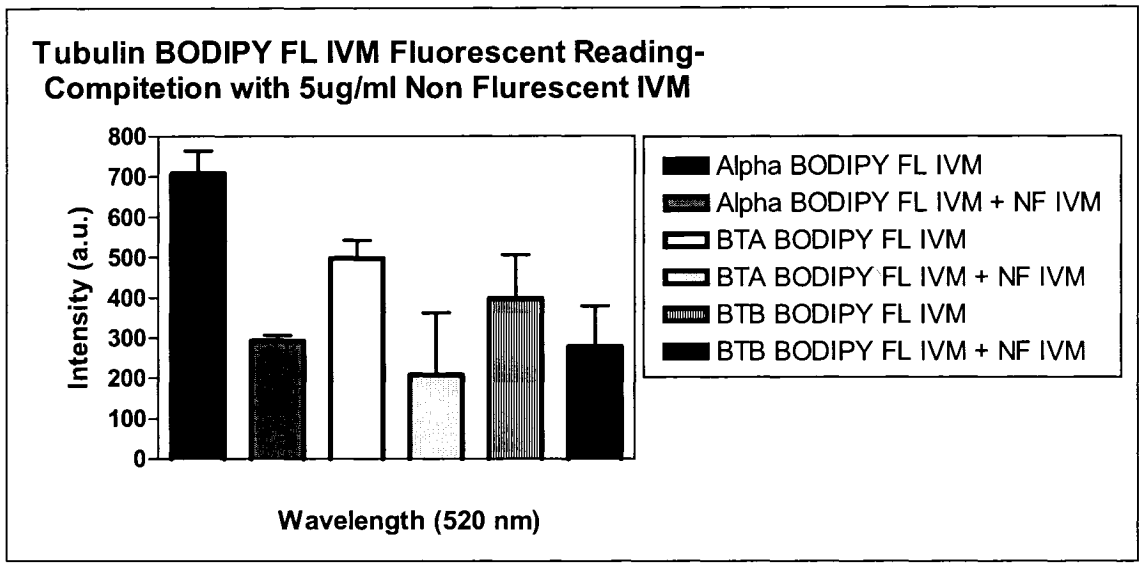

Tubulin BODIPY FL IVM Fluorescent Reading-
Compitetion with 5ug/ml Non Flurescent IVM
Intensity (a.u.)
800
700
600
500
400
300
200
100
0
Wavelength (520 nm)
Alpha BODIPY FL IVM
Alpha BODIPY FL IVM + NF IVM
BTA BODIPY FL IVM
BTA BODIPY FL IVM + NF IVM
BTB BODIPY FL IVM
BTB BODIPY FL IVM + NF IVM

Figure 6B

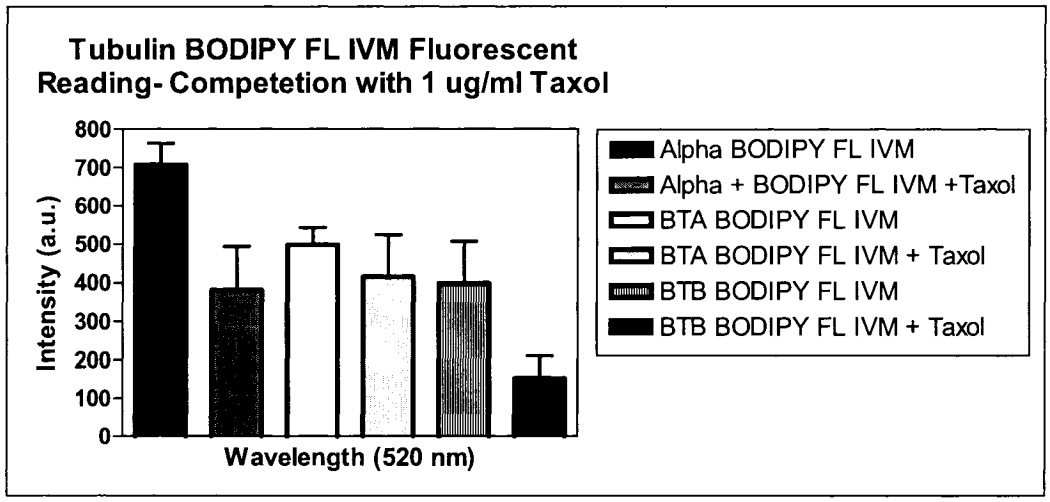

Tubulin BODIPY FL IVM Fluorescent Reading- Competetion with 1 ug/ml Taxol
Intensity (a.u.)
800
700
600
500
400
300
200
100
0
Wavelength (520 nm)
Alpha BODIPY FL IVM
Alpha + BODIPY FL IVM +Taxol
BTA BODIPY FL IVM
BTA BODIPY FL IVM + Taxol
BTB BODIPY FL IVM
BTB BODIPY FL IVM + Taxol

Figure 6C

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Section III

General Discussion

In *Onchocerca volvulus*, the phenotype of ivermectin resistance is currently not clearly defined. We predict that the resistance phenotype may include characteristics such as an increased number of residual microfilariae after ivermectin treatment, a reduction in the period of time female worms fail to produce microfilariae following IVM treatment or the rapid return of skin microfilariae following treatment (Grant 2000; Awadzi et al. 2004). We hypothesize that the mechanisms of resistance to ivermectin in *O. volvulus* would mostly likely be similar to those described in other nematodes, such as *H. contortus* and *C. elegans*, in which putative mechanisms such as an increased rate of drug removal from within the nematode (possibly due to overexpression of P-glycoprotein(s)) (Kerboeuf et al. 2003), alterations in the binding site of the drug target which either abolish or decrease the effectiveness of the drug (i.e. glutamate-gated chloride channels) (Wolstenholme and Rogers 2005) and possibly other mechanisms which reduce the entry of the drug to the target tissues and cells (e.g. via chemosensory pores) (Starich et al. 1995; Freeman et al. 2003).

The basic phenotype of an organism is based on its genotype. Regrettably, the importance of identifying genetic changes early in the development of resistance is often overlooked in favor of visual traits. Unfortunately, by the time a measurable resistance trait is identified, the genetic changes have already occurred and the drug resistance gene(s) has/have already spread throughout the population.

Remarkably, and in contrast to the veterinary field, ivermectin has been used for almost 20 years to treat human onchocerciasis without ivermectin resistance being

unequivocally documented, although there were indications that it may be present. In the mid 1990's the phenomenon of sub-optimal ivermectin responses in patients infected with *O. volvulus* began to emerge (Awadzi et al. 2004) . Certain patients did not clear the microfilarial load after their annual treatment of ivermectin, leading to the speculation that a resistance phenotype was possibly emerging.

In this thesis, following the initial concern over the sub-optimal responders, an extensive genetic study was performed on various *O. volvulus* genes. A genetic comparison between parasites from ivermectin naïve and multiple drug exposed patients was used to identify possible changes in allelic frequencies due to drug selection. We identified polymorphisms in many *O. volvulus* genes, but in addition identified three genes, P-glycoprotein, β-tubulin and *dyf-8* (unpublished, please see appendix I), which showed changes in allele frequencies in worms exposed to multiple treatments of ivermectin compared with worms from ivermectin naïve patients. The results of this analysis on genetic polymorphism are presented in Chapter II, manuscript I.

SSCP and RFLP analysis of 17 different genes, based on available sequences at the commencement of the study, showed different allele frequencies in P-glycoprotein, β-tubulin and *dyf-8* genes in *O. volvulus* obtained from patients following multiple exposures to ivermectin compared with worms from ivermectin naïve patients. No significant differences were observed between these worm populations in the other 14 genes examined. In P-glycoprotein, SSCP uncovered seven polymorphs in comparison to the three obtained using RFLP. Two alleles were of interest, as there were large decreases in the frequencies of alleles B, A and B/E in the IVM exposed group in comparison to the naïve group and a large increase in the frequencies of alleles A/B, C and F in the

ivermectin exposed worms compared with the IVM naïve group. With β-tubulin, two alleles, A and B, were identified and sequenced. In the naïve population, allele A predominated with a low frequency of allele B. In contrast, the ivermectin exposed population had a significantly higher frequency of allele B.

Ivermectin has been used for almost 20 years in some parts of Africa. The long term use of ivermectin and the identification of a change in genetic frequency in three of the 17 genes examined were initial clues that ivermectin could be changing the genetic composition in the *O. volvulus* population. Though the phenotype for ivermectin resistance has yet to be fully defined, changes to genes previously linked to the ivermectin resistance phenotype in other parasitic nematodes (Xu et al. 1998; Eng et al. 2006) were the first clues that resistance may be emerging.

Chapter III, manuscript II further develops work described in manuscript I with the focus being on the *O. volvulus* β-tubulin gene. The significant increase in the β-tubulin allele B frequency identified in the multiple treated microfilarial samples and from microfilarial samples from skin snips collected from people in Jagbengben, Northern region of Ghana, made it an ideal candidate for a biological diagnostic marker for ivermectin selection. Therefore in manuscript II, we describe a straightforward polymerase chain reaction (PCR) test that was developed to genotype individual *O. volvulus* (micro and macrofilariae) for the β-tubulin alleles. The premise of the DNA test was the identification of a 24 bp deletion in intron three of the β-tubulin gene. The 24 bp deletion only occurred in allele B. The total amplicon was 299 bp for allele A and 275 bp for allele B. The deletion was large enough that differences could be resolved in a 2.5-3 % agarose gel. Since we have previously linked the β-tubulin gene to ivermectin treatment, this test could easily be

used to monitor populations under treatment with ivermectin and control methods could be adjusted if resistance selection was suspected, e.g. add vector control or use antibiotics against the symbiotic *Wolbachia.*

In addition, in manuscript II, we reported for the first time evidence for ivermectin selection on a β-tubulin gene in laboratory strains of *H. contortus.* Interestingly, we identified an increase in the frequency of the SNP associated with resistance to benzimidazole anthelmintics (TAC_{200}) in the ivermectin selected *H. contortus.* The data presented indicated a significant increase in the level of heterozygosity, T(T/A)C , in subsequent generations of an ivermectin resistant strain of *H. contortus* (with selection increasing from 17 to 23 generations). This suggests that prolonged use of ivermectin may be selecting for β-tubulin heterozygotes which may be linked to the benzimidazole resistance codon. This may predispose nematodes to benzimidazole resistance and requires further investigation given that it could have long term implications for the sustained use of ivermectin/ benzimidazole combination treatments against livestock parasites and for the outcome of albendazole/ivermectin combination treatment against lymphatic filariasis in Africa.

In chapter IV, manuscript III explores the functional relevance of the three amino acid changes in the helix three domain of the β-tubulin allele B. Protein translations of the two alleles identified three amino acid substitutions at 117, 120 and 124. The three amino acid changes resulted in a slight increase in the overall hydrophobicity within the helix three domain of allele B compared with allele A. Although the amino acid substitutions are within the same family group (aliphatic amino acids), we did not expect to find any large changes due to the fact that tubulin is a very conserved protein and is involved in a

multitude of functions (Dustin 1984; Zabala et al. 1996; Correia and Lobert 2001). Therefore, more drastic changes to conserved regions of this protein would most likely result in deleterious effects. As a result, the slight changes in the three amino acids of the H3 helix may be sufficient to show a possible phenotypic effect, yet small enough to maintain functional homeostasis.

The helix three domain has been speculated to interact with the M-loop of the adjacent β-tubulin subunit (Nogales et al. 1998; Nogales 2000). We therefore speculate that the amino acid changes identified in the helix three domain may have an effect on the tubulin protein interaction(s) and hence result in a functional difference in the microtubules. Tubulin polymerization and depolymerization assays were performed to determine the functional relevance of the three amino acid substitutions. Although no significant changes were observed in the polymerization rates, the plateaus of the curves were significantly different. It is possible that the differences in the final polymerized protein structures may lead to different three dimensional protofilaments being formed.

Although we lack visual evidence in *O. volvulus*, the possibility that changes in β-tubulin may affect microtubule structures is supported by electron microscopy observations in *H. contortus*, in which disorganized and shortening of the amphid structures, composed mainly of microtubules, were observed in an ivermectin resistant strain of *H. contortus* (Freeman et al. 2003). If the observed amphid phenotype in *H. contortus* is correct, one could postulate that the shortening and disordering of the microtubules, if it also occurs in *O. volvulus*, may result in a decrease in spectrophotometric absorbance when tubulin containing the allele B derived protein is polymerized.

The initial identification of genetic selection on the *H. contortus* β-tubulin genes was

by Blackhall W.J. (1999). Blackhall hypothesized that genetic selection on the β-tubulin gene was not due to a direct mechanism but rather the result of an indirect mechanism of genetic selection via a hitch hiking phenomena to a gene within the same chromosome that was being selected. To determine whether ivermectin can directly interact with tubulin proteins, equilibrium dialysis was performed using fluorescein conjugated to ivermectin (BODIPY FL ivermectin). The results are presented in manuscript III and suggested that the two β-tubulin alleles were able to bind BODIPY FL IVM. Interestingly, α-tubulin also showed binding of the BODIPY FL IVM. None of the dye controls, nor the protein control sample (*Leishmania donovani* GMP reductase) showed any significant binding to fluorescein conjugated ivermectin.

Non-fluorescent ivermectin was able to compete against the fluorescent labeled ivermectin in all three purified recombinant *O. volvulus* tubulin proteins, resulting in a net decrease of the fluorescent signal. At the moment, we do not know the exact binding location(s) nor do we have direct information on the binding kinetics for ivermectin, but due to the hydrophobic nature of ivermectin and the effect of taxol on the binding of ivermectin, we speculate that a possible binding site exists in or around the hydrophobic region of the taxol binding domain. A taxol binding site has been previously identified to be located on the β-tubulin protein near the M-loop and at the site of lateral interaction with the adjacent tubulin molecule (Nogales 2000).

The taxol competition assay showed that 1 μg/ml of taxol was able to significantly decrease the net fluorescent signal of the two β-tubulin alleles compared to the samples with BODIPY FL IVM sample alone. The results suggest that the BODIPY FL IVM may share the same binding site as taxol. Interestingly, the taxol competition assay also

decreased the net fluorescent signals in the α-tubulin sample. The α-tubulin results may be explained by the work of Dasgupa et al (1994) and Leob et al (1997), in which both studies used analogs of taxol for photoaffinity labeling experiments and concluded that the α-tubulin subunit was able to bind taxol. In addition, experiments by Banerjee and Kasmala (1998) studying the *in vitro* assembly of different α-tubulin isoforms in the presence of taxol concluded that part of the taxol binding domain was on the α-tubulin subunit. If ivermectin does indeed bind to the hydrophobic taxol binding domain, it may explain why both the α- and β-tubulin dialysis experiments indicated binding of BODIPY FL IVM and a decrease in net fluorescent signal when taxol was present.

Much emphasis has been placed on the glutamate gated chloride channels and GABA channels in other nematodes species such as *H. contortus*, *C. elegans* and *C. oncophora* in the mechanism of ivermectin action (Cully et al. 1994; Blackhall et al. 1998; Dent et al. 2000; Feng et al. 2002; Blackhall et al. 2003) or involved in a mechanism of ivermectin resistance (Blackhall et al. 1998; Feng et al. 2002; Blackhall et al. 2003; Njue and Prichard 2004). It is thought that the glutamate-gated chloride channel receptors regulate pharyngeal pumping and thus feeding in non-filarial nematodes (Dent et al, 2000). Although the *O. volvulus* ligand-gated chloride channel genes examined were polymorphic, no significant changes in allele frequency were identified. One of the major effects of ivermectin is its ability to temporarily halt microfilarial production in female worms. Although the mechanism is not fully understood, we speculate that ivermectin causes paralysis to the uterine muscle in a similar manner to that seen in the neuro-muscular system of the nematode pharynx. It is therefore possible to speculate that there is an unknown type of inhibitory receptor on the uterine muscle or uterine neurons, possibly another ligand gated

ion channel, to which ivermectin can bind.

In *O. volvulus*, the selection for ligand-gated chloride channels which are less sensitive towards ivermectin may contribute less significantly to the survival of the nematode due to the following factors:

1. Unlike other nematodes, *O. volvulus* is believed to have a non functional pharynx (Strote et al. 1996).

2. Nutrient uptake: In nematodes such as *H. contortus* and *C. elegans*, nutrients are obtained via the oral-pharyngeal route. Ivermectin is believed to inhibit the pharyngeal muscle by binding to the glutamate gated chloride channels resulting in the hyperpolarization of the pharyngeal muscle. In *O. volvulus*, the majority of the nutrient uptake is believed to be via passive and/or active mechanisms through the cuticle. Inhibition of a non-functional pharynx may be of no consequence to adult *O. volvulus*.

3. Locomotion: In nematodes such as *H. contortus* and *C. elegans*, locomotion is used to obtain nutrients and or stabilize the parasites in their environment. If ivermectin is then added to these nematodes, flaccid paralysis will occur due to the hyperpolarization of the muscles from it binding to the ligand-gated chloride channels. In the case of *H. contortus*, the paralysis will prevent the nematode from holding on to the abomasal wall of the sheep and hence the parasite will be flushed out with the gut content. With *C. elegans*, the flaccid paralysis will prevent it from finding food and therefore the animal will starve to death. In comparison, *O. volvulus* adult female worms are encased in a fibrous capsule called a nodule. Ivermectin may also cause flaccid paralysis to *O. volvulus* but no serious consequences will occur since it is protected within the nodule and it is able to obtain nutrients from the environment via cuticular absorption.

Since the pharyngeal organ in the filarial nematodes is believed to be non-functional (Strote et al. 1996), the entry of ivermectin must be via an alternative route. Experimental work produced by Cross et al. (1998) reported binding of [3H]ivermectin to the cuticle surface of *Onchocerca ochengi*, but internalization of the drug from the cuticle was never investigated. A more detail analysis was undertaken by Ho et al. (1990), where they investigated the role of the *Ascaris suum* cuticle in ivermectin uptake. Using lipid extraction cuticle preparations from *A. suum*, they determined that ivermectin was indeed able to translocate through the cuticle. Interestingly, with unextracted cuticle preparations, only 2 % of the available ivermectin was able to cross the cuticle. In addition, the hypodermal layer was physically removed in both the extracted and unextracted cuticle preparations. According to experimental results from Martin et al. (1992), injections of bodipy-ivermectin into *A. sums* resulted in an accumulation of the drug in the hypodermal layer. The hypodermal layer is believed to be a "sinkhole" for hydrophobic molecules where they can be accumulated and then removed from the organism (Martin et al. 1992). Therefore if only 2% of ivermectin is able to translocate through the unextracted cuticle of *A. sums* (without the hypodermal layer), it is unknown what the actual percentage of ivermectin that can pass through the hypodermal layer to cause an effect.

A possible alternative routes of drug entry could be the anterior amphidial neurons (Martin et al. 1992; Dent et al. 1999). Previous experimental work by Starch et al. (1995) has shown that the anterior chemosensory organs called the amphids were capable of taking up hydrophobic dyes such as fluoroisothiocyanate (FITC). Grant and Hunt (pers. commun. cited in Starich et al. 1995) determined that the amphidial neurons played a significant role in determining ivermectin sensitivity. In addition, analysis of the amphid structures in

ivermectin resistant strains of *H. contortus* indicated an abnormal structural arrangement of the amphids (Martin et al. 1992; Freeman et al. 2003). The structural integrity of the amphids and many other cellular structures depend on tubulin protein. Alterations to the tubulin protein, i.e. coding SNPs, could affect not only the structure but the functioning of the amphids.

We therefore suggest that in *O. volvulus* the anterior amphid chemosensory neurons could be a possible alternative route of entry for ivermectin. We were able to clone and carry out SSCP analysis on a fragment of a putative *O. volvulus dyf-8* gene and determined that there was a significant change in the allele frequency from a population of naïve worms compare to worms derived from patients exposed to multiple treatments with ivermectin.

A revised model is therefore proposed in which there is a possible direct mechanism of selection for the β-tubulin gene due to the role of the chemosensory amphids. It is proposed that in unselected *O. volvulus*, the structural integrity of the amphids is intact and therefore ivermectin can enter into the worm to cause effects on the nervous system and hence on the muscles of the worm. In a situation where coding mutations are present in the β-tubulin protein, these mutations may cause a slight structural change which alters the overall function of the amphid, i.e. shortening and/or disorganization of the amphid neurons as seen in *H. contortus*, and thereby a reduction in the uptake of ivermectin. We have generated some primary evidence for a structural change in the β-tubulin alleles from a SWISS PROT model and by calculation of the hydrophobicity change in the H3 domain in the two β-tubulin alleles (Manuscript III). Furthermore, the three amino acid substitutions identified in the H3 domain are located in the exterior surface of the domain

which interfaces with the adjacent β- tubulin at the M-loop (Lowe et al. 2001).

Based on the evidence generated with *O. volvulus* and *H. contortus*, the following diagrammatic outline of five possible multifactorial resistance mechanisms in *O. volvulus* is proposed. The proposed five mechanisms include, P-glycoprotein, β-tubulin, ligand gated chloride channels, nematode amphids and cuticle uptake of IVM. In the ivermectin naïve *O. volvulus* nematode (Figure 1), molecules of ivermectin can enter the nematode via the amphid pores and most likely via a cuticle uptake mechanism(s). Once the drug enters the worm, it is proposed that the ivermectin accumulates in the nervous system in a manner similar to the observed effect in *Ascaris* (Kass et al. 1984), in which the accumulation of ivermectin can potentially compromise the nematode signal transduction systems. Active pumps such as P-glycoprotein can efflux the drug out of sensitive cells. In sensitive worms, the presence of the drug may lead to paralysis of the nematode somatic and reproductive muscular systems such as the uterine muscular system and hence prevent the release of microfilaria. In addition, β-tubulin proteins are involved in two aspects. First, the β-tubulin polymerizes to form microtubules which are involved in a complex that aids in the anchoring of surface ligand receptors. The receptor-anchoring complexes are also believed to be involved in the signal transfer from nerve cells in the neuromuscular system of animals (Maas et al. 2006). Secondly, the microtubules are involved in the structural integrity of the chemosensory pores (Freeman et al. 2003).

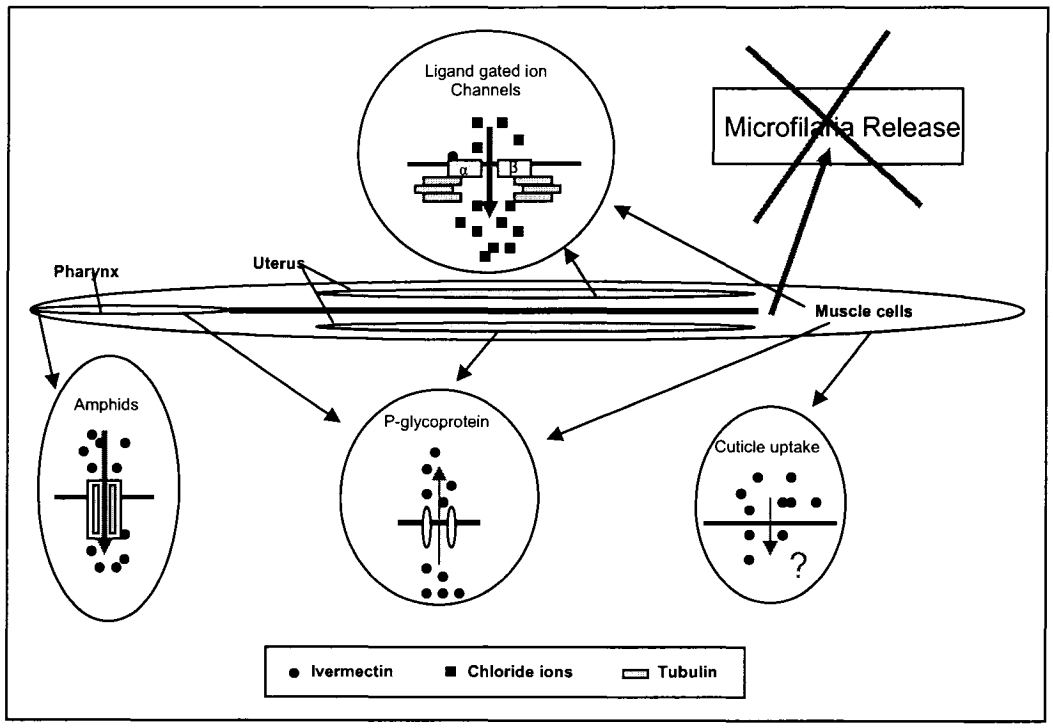

Figure 1. Proposed impact of ivermectin on IVM-sensitive *O. volvulus* nematodes.

With respect to a "resistant" *O. volvulus* nematode (Figure 2), we again can presume that ivermectin can possibly enter via a cuticle uptake mechanism(s), but limited uptake occurs via the amphid pores due to the dysfunction of the amphid structures as a result of a change in the dynamics of the microtubules. The limited amount of ivermectin that enters the worm is efflux out of sensitive cells via the P-glycoprotein pumps and paralysis of the worm is limited due to the decreased concentration of ivermectin reaching targets such as the ligand-gated chloride channels. In addition, it is not established how alterations in β-tubulin protein might possibly affect signal transfer from ivermectin receptors in nerve cells to muscle in nematodes to have an effect on responses to ivermectin. We can speculate that the alterations identified in the β-tubulin allele B might also result in an alteration of function of the microtubule of the anchoring complex and therefore possibly change the way signals are transfered from nerve

cells in the neuromuscular system of animals. At this point, there is no evidence that the cuticular uptake mechanism(s) is/are altered in ivermectin "resistant" worms (Geary et al. 1993). The end result is a resistant worm which will be less sensitive to ivermectin and capable of releasing newly formed microfilaria after a dose of ivermectin.

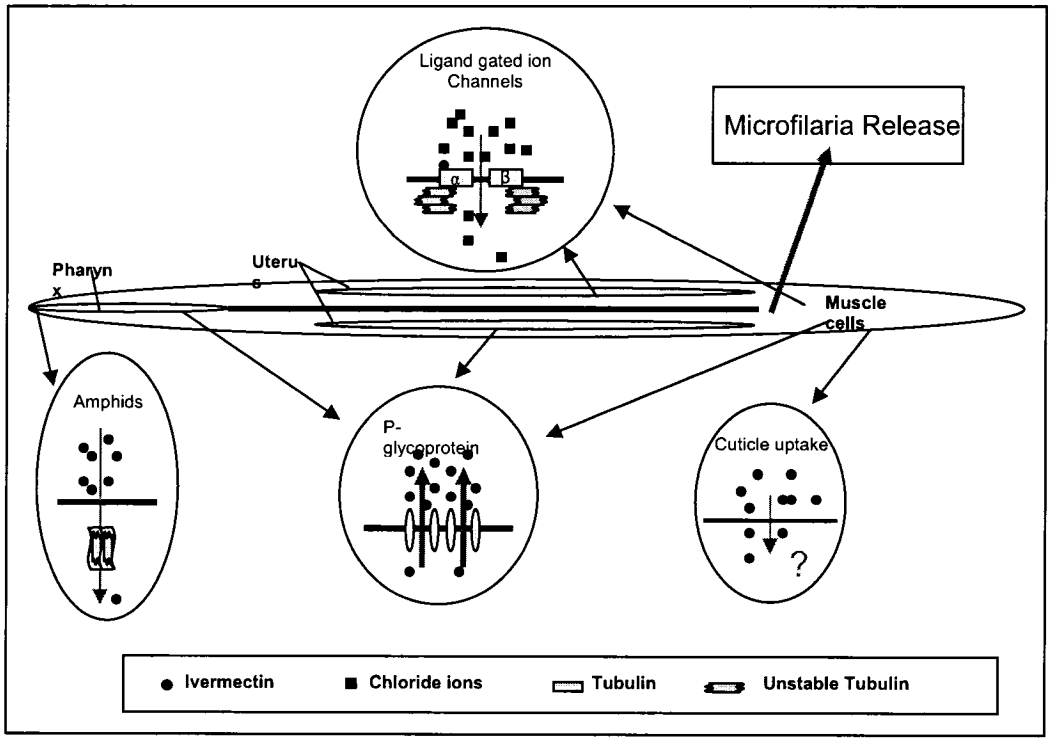

Figure 2. Proposed impact of ivermectin on 'resistant' *O. volvulus*

The results obtained in this thesis have only begun to explore the possible resistance mechanisms towards ivermectin in *O. volvulus*. Although many genes were analyzed for genetic selection, only a limited number of genes have been identified to interact and/or are possibly linked to a resistant phenotype. The data supports the concept of multifactorial resistance mechanisms and that there may not be a single contributing factor that describes the resistance mechanism. Each individual mechanism of resistance may contribute to only a fraction to the resistance phenotype. Some mechanisms may contribute more and some less. It

will be the totality of all the mechanisms which will result in a resistant phenotype. In addition, the mechanisms of resistance to ivermectin will most likely be conserved throughout the nematode family resulting in similar mechanisms of resistance.

After almost 20 years of ivermectin treatment for onchocerciasis, the people in West Africa have relied heavily on a safe and effective treatment. Our analysis of a small handful of *O. volvulus* genes, compared to the predicted greater than 4000 genes in its genome (http://www.tigr.org), have revealed genetic selection which can be an early indicator that the long term exposure to ivermectin is causing a change in the profile of certain genes which may indicate the development of resistance. The emergence of ivermectin resistance could again devastate the rural villages once endemic for *O. volvulus* which would again have to be abandoned, and the associated rich fertile land near the rivers, with the people having to move to less fertile areas to avoid contact with the insect vectors. The end result could be turning the clocks back more than 20 years where the African proverb 'Nearness to rivers can eat the eyes' would once again be told.

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Appendices

Appendix I

Unpublished data

A putative *Onchocerca volvulus dyf*-8 gene

Introduction

Nematodes have a pair of sensory organs, laterally located in the anterior head region, which are open to the outside environment. Soluble chemicals can access the amphid neurons via pores, the amphid pore. Within each pore lie dendritic processes. It is by these processes that the nematode can "taste" the environment. The amphids aid in seeking out and reacting to food, avoiding toxic substances, such as drugs, and in finding suitable mates (Ashton et al. 1999; Wicks et al. 2001).

In the nematode *C. elegans*, 13 dye-filling (*dyf*) defective mutants have been identified (www.wormbase.org). *Dyf* defective nematodes are so-named because of their inability to take up lipophillic dyes, e.g. fluoroisothiocyanate (FITC), into the amphids and phasmid neurons, from the environment via the amphid sensory endings (Starich et al. 1995). Phenotypical consequences of being a dyf mutant is that the amphid organ becomes highly disorganized; there is a profound defect in behavior. The worm requires an intact amphid for chemo-sensation, such as drug avoidance and mating (Starich et al. 1995; Dent et al. 2000).

Grant and Hunt (pers. commun. cited in Starich et al. (1995)), observed that all ivermectin-resistant *C. elegans* larvae were *dyf* mutants and determined that the amphidial neurons played a significant role in determining ivermectin sensitivity. Johnson et al., (pers. commun. cited in Starich et al. (1995)) also proposed the same hypothesis and added that amphidial defective *C. elegans* mutants were able to grow in

culture medium containing 5 ng/ml of ivermectin.

We therefore have cloned a putative *dyf-8* gene fragment of 557 bp from *O. volvulus*, and conducted from which single strand conformational polymorphism (SSCP) analysis between worms obtained from ivermectin naïve and ivermectin exposed patients. Data from SSCP suggested a significant change in the allele frequency, in terms of heterozygotes, in worms derived from multiply exposed hosts compared with worms from IVM naïve people. Since the oral-pharyngeal route in *O. volvulus* is believed to be non-functional (Strote et al. 1996), a loss of function in the amphid neurons may allow *O. volvulus* to limit the amount of ivermectin taken up.

Material and Methods

DNA extraction

Individual worms were washed twice with cold PBS (pH 7.0) and transferred to a clean Eppendorf tube containing 100 µl STE (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0)), 1 mM EDTA (pH 8.0), 0.6 M β-mercaptoethanol and 200 µg/ml Proteinase K. The worms were incubated for two hours at 65 °C. DNA was extracted using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) then precipitated with isopropanol: ammonium acetate (2:1). The DNA pellet was air dried, resuspended in 20 µl of TE buffer (10mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) and stored at –20 °C.

PCR amplification of genomic DNA fragments

The sequence of the putative *Onchocerca volvulus dyf-8* gene was obtained by aligning the *C. elegans dyf-8* homolog (accession number NM_077229) to the *O. volvulus* EST database from TIGR (www.tigr.org). A putative *O. volvulus dyf-8* gene cDNA fragment was obtained (EST ID AI540037). The putative *Onchocerca volvulus dyf-8* genomic fragment was amplified by using primers Dyf-8F ('5-ACG ATT CAA TGA TAT TTC CAG-3') and Dyf-R2 ('5-TAG CAG CTT GAA GAG TTT TTC-3') on total genomic DNA (see appendix II). PCR products were amplified from the purified genomic DNA samples from the individual worms on a PTC200 DNA Engine Thermalcycler (MJ Research). The standard PCR conditions were as follows: 95 °C for 1 min, 50 °C for 30 sec, and 72 °C for 1 min. The 557 bp amplicon was generated and cloned into PCR 2.1 (Invitrogen).

Single Strand Conformation Polymorphism

Two μl of the PCR product were diluted with 10 μl of SSCP buffer (95% formamide, 10 mM sodium hydroxide, 0.25% bromophenol blue, and 0.25% xylene cyanole), denatured for 5 minutes at 95 °C and quickly cooled on ice for two minutes. The denatured samples were analyzed on a 15 % 49:1 polyacrylamide gel and subjected to an electric field for 48 hours at room temperature. The gels were stained with ethidium bromide (0.5 μg/ml), and a scanned image was obtained from a Molecular Imager FX Pro Fluorescent Imager (Bio-Rad).

Result

Individual male and female *O. volvulus* worms were obtained from naïve and ivermectin treated patients in Ghana. Total genomic DNA was extracted from each *O. volvulus* worm and SSCP was performed. In the SSCP analysis, three polymorphs were identified in both the males and females worms. In both the male and female population of nematodes, the AA polymorphs predominated whereas the BB and AB appeared in lower frequencies. Chi square analysis (χ^2=0.05) indicated a significant difference in the polymorph frequency between the naïve and ivermectin exposed nematode populations for both males and females. With respect to the ivermectin exposed population of male worms, the AA polymorph still predominated but decrease from 75.5 % found in the IVM naïve group to 47.5 %. There was also a significant change in the AB polymorph which increased from 13.5 % in the naïve group to 40 % in the ivermectin exposed worms.

The female SSCP polymorph profiles were similar to those in the males. In the naïve and ivermectin exposed female samples, the AA predominated but decrease from 70.5 % in the IVM naïve to 46.2 % in the ivermectin exposed population. Also similar to the male samples, the AB polymorph increased in frequency from 21.5 % in the naïve females worms to 41.5 % in the ivermectin exposed female worms.

Discussion

The mode of nutrients uptake in filarids, such as glucose uptake, have been speculated to be via cuticle uptake (Brattig and Erttmann 1992), whereas ivermectin uptake via the cuticle ins, to on the other hand, is still uncertain. Experimental work done by Cross et al.(1998) have has previously shown interesting binding of [3H]ivermectin to the cuticle surface of *Onchocerca ochengi*, but actually internalization of the drug from the cuticle was never investigated. Possible alternative routes of drug entry could be the anterior amphidial neurons (Dent et al. 1999). The nematode amphid sensory neurons are used for chemical and thermal signaling in *C. elegans* (Dent et al. 2000), *H. contortus* (Ashton et al. 1999) and *O. volvulus* (Strote et al. 1996). Since there is evidence that the amphid organs are used for "tasting" the environment, it is therefore reasonable to think that the amphid mutants, such as *dyf-8* mutants, may result in a drug resistance phenotype, i.e. ivermectin resistance, because they can render the worm less permeable to the drug. A limited amount of information is available pertaining to the *dyf-8* protein. The *dyf-8* gene product is believed to be required to form the proper connection between sheath and socket cells, during the brief period in late embryogenesis when the amphid is being formed, which may mediates the stabilization of the cilia/support cell interactions (Wicks et al. 2001).

Phenotypical consequences of being a dyf mutant is that the amphid organ becomes highly disorganized (Starich et al. 1995). Supporting evidence came from Freeman *et al.* (2003) who compared the amphid structure between ivermectin naïve and resistance strains of *H. contortus*. Their findings showed a significant shortening and diffused morphology of the amphidial dendrites in ivermectin resistant, compared with

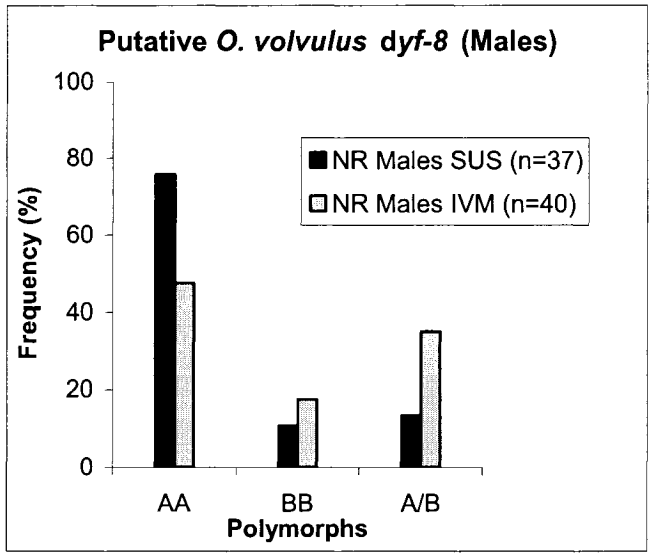
wild-type strains. This is a similar phenotype to that identified in *dyf* mutants in *C. elegans* (De Riso et al. 1994). The authors suggested that the shortening and possible dysfunction of the amphid dendrites may prevent access of the drug into the nematode. Therefore, the presence of the *dyf* phenotype, resulted in a defect in the uptake of the drug, may lead to a resistance phenotype.

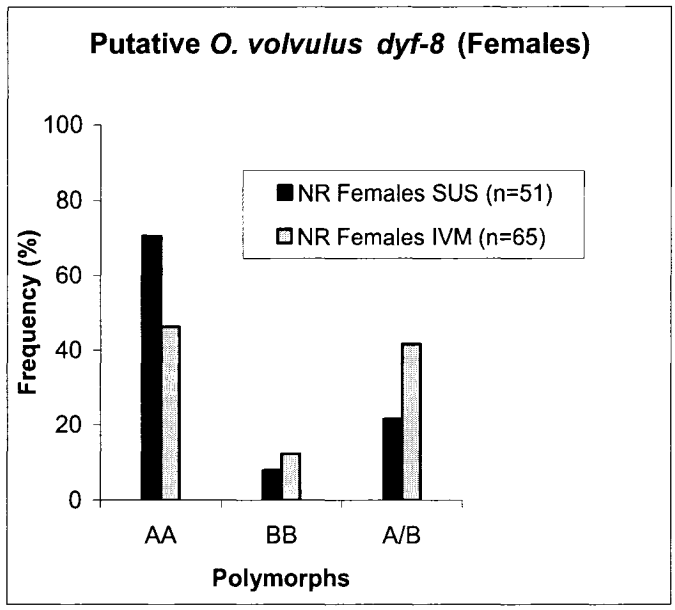
The data presented on the putative *O. volvulus dyf-8* genomic gene fragment shows a significant decrease in the level of A/A heterozygote homozygotes and an increase in the level of A/B heterozygote A/B in ivermectin exposed nematodes (males and female). The full length of the two putative *dyf-8* alleles will be required to fully identify the significance of the two alleles in terms of protein structure and/or function. Currently, the functional relevance of the two putative *O. volvulus* dyf-8 alleles is unknown, but in genetic terms, increases in the level of heterozygosity can potentially indicate an outbreeding population and negative assortative mating, i.e. between the drug resistance and susceptible worm populations. Thought functional relevance is important, the genetic information pertaining to this gene and genetic analysis by Eng and Prichard (2005), i.e. β-tubulin isotype 1, can provide insight of the population dynamics influenced by ivermectin exposure. The genetic selection identified in both the putative *dyf-8* gene and the β-tubulin gene, the basic structural components of the amphid neurons, is an interesting characteristic which may indicate how *O. volvulus* or any other nematode, may adapt to decrease ivermectin intake and sensitivity.

Figures

Figure 1. OvDyf-8 SSCP results from individual male *O. volvulus* nematodes. SUS = worms from IVNM naïve patients, IVM = worms from IVM multiply treated patients.

Figure 2. OvDyf-8 SSCP results from individual female *O. volvulus* nematodes. SUS = worms from IVNM naïve patients, IVM = worms from IVM multiply treated patients.


Putative O. volvulus dyf-8 (Males)
100
80
60
40
20
0
Frequency (%)
NR Males SUS (n=37)
NR Males IVM (n=40)
AA
BB
A/B
Polymorphs
Figure 1.


Putative O. volvulus dyf-8 (Females)
100
80
60
40
20
0
Frequency (%)
NR Females SUS (n=51)
NR Females IVM (n=65)
AA
BB
A/B
Polymorphs
Figure 2.

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Appendix II

Genbank submissions of *Onchocerca volvulus* sequences

A.II.A Onchocerca volvulus alpha-tubulin

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DEFINITION Onchocerca volvulus alpha-tubulin mRNA, complete cds.
ACCESSION AY936208
VERSION AY936208.1 GI:60549976
KEYWORDS
SOURCE Onchocerca volvulus
ORGANISM Onchocerca volvulus
Eukaryota; Metazoa; Nematoda; Chromadorea; Spirurida; Filarioidea; Onchocercidae; Onchocerca.
REFERENCE 1 (bases 1 to 1347)
AUTHORS Eng,J.K.L. and Prichard,R.K.
TITLE Onchocerca volvulus alpha-tubulin mRNA
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1347)
AUTHORS Eng,J.K.L. and Prichard,R.K.
TITLE Direct Submission
JOURNAL Submitted (16-FEB-2005) Institute of Parasitology, McGill
University, 21 111 Lakeshore Road, Ste Anne de Bellevue, QC
H9X 3V9, Canada
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PQVSTAVVEPYNSILTTHTTLEHSDGAFMVDNEAIYDICRRHLDVE
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PLATYAPVISAEKAYHESLSVADITNACFEPANQMVKCDPRHGKY
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KRAFVHWYVGEGMEEGEFSEAREDLAALEKDYEEVGIDSLEGEG
EGEEY"

ORIGIN
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121 caaaaccaag tcgatgactc attttctacg ttcttctcgg aaacgggcac tggtcgccat
181 gttccacgag cgattatggt tgatttggaa ccaacggtag tagatgaaat caggaccggg
241 acatacaagc aacttttca tcctgaacaa atggtcaccg gcaaggaaga tgctgcgaac
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421 ggtggtggga ctggttcagg tttcacatca ttattgatgg aacgattatc tgtcgattat
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1261 cgtgaagact tggctgctct cgaaaaggac tatgaagagg tcggaattga ttcattggaa
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A.II.B Onchocerca volvulus putative dyf gene

LOCUS AY934537 557bp DNA linear INV 13-MAR-2005
DEFINITION Onchocerca volvulus putative dyf gene, partial cds.
ACCESSION AY934537
VERSION AY934537.1 GI:60617324
KEYWORDS .
SOURCE Onchocerca volvulus
ORGANISM Onchocerca volvulus
Eukaryota; Metazoa; Nematoda; Chromadorea; Spirurida; Filarioidea; Onchocercidae; Onchocerca.
REFERENCE 1 (bases 1 to 557)
AUTHORS Eng,J.K.L. and Prichard,R.K.
TITLE Direct Submission
JOURNAL Submitted (15-FEB-2005) Institute of Parasitology, McGill University, 21 111 Lakeshore Road,
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 121 cattgtataa ttaatttatt tgtttattgt gacctaatta attttattaa tgcaatatga
 181 ttaatatttg gaagaagcga aaaatgaagc atgcatttgc ataaatatga taataaagta
 241 aagagccgtt aagtcgaact tgtgaatttt gcattttatt ttaaaatttt gatggagaaa
 301 aatttcattt taaaatttta tttaaaaatt ttgatgaaga aaaacaaaat tccaataaag
 361 aaatttttaa caacttttt gctttatatc ttacaaatta taaaaatttc aaaaaaaaaa
 421 tagtatgatc agtctcggat aaaataataa tttgaattat agtggtcatg aagttgttgc
 481 atcaactggt ggaaaaccaa agcctaaagt tgaaatgata tttcgtagta ttgatggacg
 541 acctttagct gctgcta
```

Appendix III

Environment Safety Documentation

In accordance with McGill University policies, I have included the appropriate certification documents.