EPIDEMIOLOGY, CHEMOTHERAPY OF ONCHOCERCIASIS AND DEVELOPMENT OF *ONCHOCERCA*-SPECIFIC POLYMERASE CHAIN REACTION BASED ON A P-GLYCOPROTEIN GENE.

By

EZEUNALA, MERCY NWAKAEGO

REG. NO. PG/Ph.D/10/57143

DEPARTMENT OF ZOOLOGY AND ENVIRONMENTAL BIOLOGY

FACULTY OF BIOLOGICAL SCIENCES

UNIVERSITY OF NIGERIA, NSUKKA

NOVEMBER, 2014

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A THESIS SUBMITTED TO THE DEPARTMENT OF ZOOLOGY, FACULTY OF BIOLOGICAL SCIENCES, IN FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILISOPHY IN PARASITOLOGY.

SUPERVISORS:

PROFESSOR OKAFOR, F. C.

PROFESSOR EYO, J. E

UNIVERSITY OF NIGERIA, NSUKKA

NOVEMBER, 2014

TITLE PAGE

EPIDEMIOLOGY, CHEMOTHERAPY OF ONCHOCERCIASIS AND DEVELOPMENT OF AN *ONCHOCERCA*-SPECIFIC POLYMERASE CHAIN REACTION BASED ON A P-GLYCOPROTEIN GENE.

CERTIFICATION

EZEUNALA, MERCY NWAKAEGO, a postgraduate student in the Department of Zoology and Environmental Biology, has satisfactorily completed the requirements for research work for the degree of Doctor of Philosophy (PhD) in Zoology and Environmental Biology (Parasitology). The work embodied in this thesis is original and has not been submitted in part or in full for any other diploma or degree in this or any other University.

_____

Prof. Okafor, F.C. Date ----------

Supervisor

_____

Prof. Eyo, J.E. Date ----------

Supervisor

_____

Prof. B. O. Mgbenka Date ----------

Head of Department

_____

External Examiner Date----------

Prof. (Mrs) F. O. Nduka

DEDICATION

This thesis is dedicated to the óI AM THAT I AMô, the Lord Almighty and also to my family who have been my support all through these years. May God make us all Rapturable.

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ABSTRACT

Onchocerciasis is a disease caused by a filarial nematode *Onchocerca volvulus,* which is transmitted to man through the bite of infected black flies of the *Simulium damnosum complex*. In Achi community (South Eastern Nigeria) - ivermectin treatments started since 1990 on a yearly basis under the auspices of a WHO/UNDP/WORLD BANK ONCHO. PROJECT. During this study in Achi and Amansea, the participants medical history, onchocercomata and skin snips were obtained between January, 2009 to December, 2012 from the studied groups. The studied population of 240 subjects was randomly selected thus: Achi (57), Amansea (52) and UNTH (131) (health seeking patients at Oncho. Project, University of Nigeria Teaching Hospital). The studied groups were out patients of the WHO/UNDP/World Bank Oncho Project in the various villages. The patients were grouped into age, sex, occupation, residence, dosage levels, skin microfilariae and degrees of onchodermatitis. The uterine contents of the female worms after ivermectin treatment of patients were qualitatively and quantitatively assessed using embryogram techniques. Sodium Dodecyle Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE) and Immuno blotting techniques were used to separate the protein antigens of the female worms. Chemiluminescence was used to visualize the blots. Genomic DNAs were isolated from various female worms of different dosage levels for the investigations into the presence of mdr genes. Multidrug resistant (mdr) degenerate primers were used with the genomic DNA in polymerase chain reaction (PCR) experiments. The PCR product was isolated, cloned and sequenced and an *Onchocerca*- specific mdr primer designed from homologous and conserved regions of available mdr genes. The *O. volvulus* mdr primer was used to check for amplification on other *Onchocerca* samples using PCR. A laser densitometer was used to read the positive products. A statistical evaluation of the densitometer values was done using t-test analysis. In Achi, 53(93.0%) of the patients were infected thus confirming Achi ï as an

onchocerciasis endemic community, in Amansea 30(58.0%) had onchocerciasis thus confirming it as a meso-endemic region, while 34(25.0%) of the patients from UNTH had onchocerciasis. All prevalence for onchocerciasis were significantly different ($p<0.05$). Out of 57 patients sampled at Achi, 53((93.0%) had nodules which when digested, contained viable adult worms. Similarly, in Amansea, 30(58.0%) patients had nodules that contained little or no worms but rather unidentifiable contents or already calcified worms. The prevalence of onchocercal noodles was significantly different ($p<0.05$) in all the studied groups, suggesting that the presence of nodules might be a good sign for the rapid assessment of onchocerciasis. The embryograms of the uteri of the *O. volvulus* females from ivermectin treated patients revealed that the contents were significantly ($p<0.05$) more deformed than those from non-treated patients. Using C 219 polyclonal antibody, the presence of P-glycoprotein was established in *O. volvulus*. Furthermore, using mdr ï degenerate primers (P3 ï P4) and DNA sequencing, mdr genes has been found in *O. volvulus* for the first time. There was a 53% homology with the available mdr genes. Additionally, an *Onchocerca*-specific mdr primer has been designed from this study. The primers P11 ï P12 showed specificity towards *Onchocerca* species and not to other nematodes. The laser densitometer values when analyzed with the t-test revealed that there was no significant difference ($p > F = 0.8247$) between the two major groups, treated and untreated.

LIST OF ABBREVIATIONS

Ab:	Antibody
Ag:	Antigen
AP:	Alkaline Phosphate
APS:	Amoniumpersulphate
C (%):	Percentage of Bisacrylamide in total Acrylamide solution
cpm:	Counts per minute
dd H_2O:	double distilled water
DEC:	Diethylcarbamazine citrate
DMSO:	Dimethylsulphuroxide
DNase:	Desoxyribonuclease
dNTP:	Mixture of Desoxynucleoxide-Triphosphate dATP, dCTP, dGTP & dTTP
DTT:	Dithiothritol
ECL:	Enhanced Chemoluminescence
EDTA:	Ethylenediaminetetracetic acid
ELISA:	Enzyme Linked Immuno-Sorbent Assay
FCS:	Fetal Calf Serum
G3PDH:	Glycerinaldehyde-3-Phosphate-Dehydrogenase
HBSS:	Hankôs Balanced Salt Solution
HEPES:	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethansulfonacid
IgG:	Immunoglobuline class G
kDa:	Kilodalton
IL:	Interleukine
IVM:	Ivermectin
L3:	Third stage infective larva
L4:	Fourth stage larva

mAb:	Monoclonal Antibody
MDR:	Multi-Drug Resistance
MHC:	Major Histocompatibility Complex
MF:	Microfilaria
Mr:	relative molecular mass
Mrns:	messenger RNS
MW:	Molecular weight
NC:	Nitrocellulose
NaDOC:	Sodiumdesoxycholate
NMWL:	Nominal Molecular Weight Limit
OCP:	Onchocerciasis Control Programme
OD:	Optical Density
Ov:	*Onchocerca volvulus*
OvAg:	*Onchocerca volvulus*-Antigen extract
OvP:	*Onchocerca volvulus* Protein
PAA:	Polyacrylamide
PAGE:	Polyacrylamidegelelectrophoresis
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
p.i.:	Post infection
PMSF:	Phenylmethylsulfonylflouride
RFLP:	Restriction Fragment Lenght Polymorphism
RNase:	Ribonuclease
RPMI 1640:	Roswell Park Memorial Institute, Medium
RT:	Reverse Transcription
SDS:	Sodium Dodecyl Sulphate
T (%):	Total percentage of Acrylamidemonomer

TBS:	Tris Buffered Saline
TDR:	Tropical Disease Research
TEMED:	N,N,Nô,Nô-Tetramethylethylendiamine
TLCK:	Na-p-Tosyl-L-Lysine Chloromethylketone
TNF:	Tumour Necrosis Factor
Tricin:	N-Tris(hydroxymethyl)methylglycine
Tris:	Tris(hydroxymethyl)-aminomethane
UNDP:	United Nations Development Programme
WHO:	World Health Organization

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Onchocerciasis is the disease resulting from infection with the filarial nematode *Onchocerca volvulus*, which is transmitted to man through the bite of the infected black flies belonging to the genus, S*imulium* (Family: Simuliidae) (WHO, 1991, Okuliez, 2008). The global distribution of onchocerciasis includes endemic areas in tropical Africa, where 99% of the total infected persons are found, Central and South America (WHO, 1987). It is estimated that 85.5 million people are at risk and that 17.7 million people are infected with *O. Volvulus* (WHO, 1995). Onchocerciasis is one of the leading causes of loss of sight in the world, responsible for about 270,000 blind and 500 000 partly sighted people (WHO, 1995).

The current global burden of the disease showed that an estimated 123 million persons were at risk of contacting the disease and 17 ï 18 million were infected (WHO, 1999; Hoerauf *et al*., 2003; Boatin and Richrads, 2006). According to IJPD (2009), more than 30 million Nigerians in 32 states and the Federal Capital Territory are estimated to be at risk for onchocerciasis. About 360,000 people are projected to be blind (Abiose *et al*., 1993). The disease is present in all the states including the Federal Capital territory with the exception of Lagos, Rivers, and Akwaï Ibom states where the infections are sporadic.

The vectors breed along fast-flowing rivers where infection intensity and morbidity are highest. Onchocerciasis is also known colloquially as river blindness (WHO, 1985). The manifestations of onchocerciasis are predominantly dermal, lymphatic and ocular in character (Mackenzie *et al.,* 1995), but several other features of uncertain association, etiology or

pathogenesis have also been described, including low body weight, general debility, diffuse musculoskeletal pain and, in Africa, epilepsy and hyposexual dwarfism (WHO, 1987a; Nwoke, 1992; Kipp *et al*., 1994; Shu and Okonkwo, 1998). The consequences of onchocerciasis have repercussions beyond the individual and directly affect the family, community and country. In the usually remote, rural areas of the Savannah zones of Africa, the effects of river blindness have led to the decline and dessertation of villages, where it is without doubt the most important disease afflicting the communities. Blindness rates of 5-10% reduce the viability of communities; when the majority of men over 40 years of age are blind, villages rapidly cease to be economically viable. Populations move to healthier local environments, away from the rivers where the vectors breed but where the soil is usually not so fertile (WHO, 1987, 1995a; Anderson and Fuglesang,1978; Abiose *et al*., 1993; Murdoch *et al*., 2002). Furthermore, since blindness leads to a reduction of some 10 years in life expectancy, onchocerciasis is a disease that not only disables but is indirectly responsible for considerable premature mortality. In persons with prolonged intense infections, the skin lesions and itching are responsible for much chronic misery and disfigurement, and can lead to a degree of social isolation, with detrimental psychological effects (Nwoke, 1990). In Achi, an onchocerciasis-endemic community, in South Eastern Nigeria, the disease effects not only reduces the village economics but also has led to the disruption of most families because the able bodied men flee at the fear of getting blind (Okonkwo *et al*., 1991). In another community also in South Eastern Nigeria, Amazigo (1994) reported that the skin lesions of onchocerciasis causes not only great discomfort but reduces the chances of a girl getting married. Thus, the numerous problems posed by onchocerciasis have led to numerous efforts to control this parasitic disease and treat the millions of people already infected.

Previous efforts at halting the disease include the aerial spraying of biodegradable larvicides in West Africa- a co-operative effort of World Health Organisation -related groups, and the use of drug treatments, Diethylcarbamazine citrate (DEC) and Suramin, which can have severe adverse effects. These drugs were deemed unsuitable for mass use because they produce only short-term or unsatisfactory suppression of microfilariae and patients require close medical supervision during administration. DEC has been the standard theraphy for over three decades (WHO, 1987). It must be given orally daily for seven to ten days and is frequently accompanied by severe reactions, including deterioration of onchocercal eye lesions, and may itself even cause blindness and death. DEC is microfilaricidal only. Suramin kills adult worms and is therefore macrofilaricidal (WHO, 1995). It is/was the only macrofilaricidal drug and has to be given intravenously once a week for several weeks. Its administration may be accompanied by severe rash , diarrhoea, neurotoxicity, nephrotoxicity and sometimes death (Stein *et al*., 1989; La Rocca *et al*., 1990; Voogt *et al*., 1993). It was apparent that the development of a new drug that may be administered orally as a single dose for mass chemotherphy was to be a major research goal. This search for a new drug for onchocerciasis led to the development of ivermectin (IVM) by Merck Sharp and Dohme (MSD). Other drugs which have shown limited promises include Amocarzine (CGP 6140) which is an antifilarial anthelmintic isolated from amoscanate active against adult worms of *O. volvulus*. Amocarzine is toxic to mitochondria and causes inhibition of respiration. The basis for its selective toxicity appears to be preferential drug uptake by the filarial worm (Kohler *et al*., 1992). Moxidectin ï a milbemycin drug used in veterinary medicine has shown macrofilaricidal activity in animal screens and has been shown to be safe in preliminary (phase 1) human trials (WHO, 2000, Cotreau *et al*., 2003). It is being evaluated for Phase II clinical trial, in which 192 persons infected with

onchocerciasis are enrolled. Following the period, moxidectin will hopefully be available to endemic countries by the year 2012 (Eeezzuduemhoi and Wilson, 2008)

Ivermectin, a broad range antiparasitic agent, has been developed for veterinary use and widely used in veterinary medicine. First treatment of onchocerciasis with ivermectin was performed in 1982. IVM has displaced DEC as a microfilaricidal drug (Goa *et al*., 1991). In a single oral dose of 150 ug/kg bodyweight, ivermectin is superior to DEC in elimininating high parasite loads and it has a more prolonged suppressive effect on skin and ocular microfilariae (Goa *et al.,* 1991). Ivermectin causes less severe reactions and importantly, no ocular deficiency, and is therefore useful for mass distribution.

Ivermectin (derived from *Streptomyces avermitilis*) is the current drug of choice for the treatment of onchocerciasis. Ivermectin, a medicine capable of killing the parasite embryos (the microfilariae) circulating in the organism of patients and temporarily interrupting the nematodeôs reproduction, is the only acceptable treatment used for onchocerciasis mass chemotherapy.

Already IVM is being distributed world-wide and several million individuals in several continents (WHO Exp. Committee, 1995a) have been dosed with encouraging results (Chabala *et al.,* 1980; Soboslay *et al.,* 1987, 1991; Taylor and Green 1989). Microfilarial loads are reduced to 20% of pretreatment levels for up to one year after single dose ivermectin therapy (White *et al.,* 1987). In Ghana, skin microfilariae reduced to 96% after 2 months of a large-scale community treatment with ivermectin (Remme *et al.,* 1989). In Liberia, skin microfilaria load reduced by 86% after a large-scale community treatment with ivermectin (Pacque *et al.,* 1990a). Likewise ocular levels of microfilariae in the anterior chamber decreased to 20% of pretreatment values 4 months after ivermectin therapy, but rose 39% at 12 months (Dadzie *et al.,* 1990).

In Achi community, ivermectin treatment started since 1990 on a yearly basis. After the first mass treatment, up to 82% reduction of skin microfilariae load was recorded, followed by the second treatment with 76% reduction observed (Okonkwo *et al*., 1991). However, subsequent treatment did not show much further reduction but rather a small percentage of the treated patients maintained their skin microfilarial load. Thus, it was not clear if this was due to malreabsorption of the drug by the host or reinfection of the parasite or resistance to the drug by the parasite. It is already evident that development of resistance by tissues or organisms to drugs is an evolutionary adaptation that puts at risk every tumouricidal, pesticidal and parasiticidal agent and resistance of parasites and infectious disease organisms to drugs and antibiotics is as old as chemotherapy itself.

Despite all efforts, there is still great frustration in cancer chemotherapy due to development of drug resistance. Malaria is now present in 102 countries, is responsible for 100 million clinical cases and 1 to 2 million deaths each year (Oaks, 1991) because, the lethal form of human malaria caused by *Plasmodium falciparum* has developed resistance to chloroquine in many areas worldwide. The P-glycoprotein (P- signifies permeability), which is a membrane-bound molecule has been implicated in multidrug resistant cancer cells and also in *P. falciparum* resistance to chloroquine. Thus, it was the keeping in mind the possibilty that parasite resistance to ivermectin may develop and pose a big threat both to current control activities and future plans that we thought it necessary to carry out this research work.

1.2 Justification of the Study

In Achi community, ivermectin treatment started since 1990/1991 on a yearly basis. After the first mass treatment, up to 82% reduction of skin microfilariae load was recorded in some villages followed by the second treatment with 76% reduction observed (Okonkwo *et al*., 1991). However, subsequent treatment did not show much further reduction but rather a small percentage of the treated patients maintained their skin microfilarial load. Thus, it was not clear if this was due to malreabsorption of the drug by the host or reinfection of the parasite or resistance to the drug by the parasite. It is already evident that development of resistance by tissues or organisms to drugs is an evolutionary adaptation that puts at risk every tumouricidal, pesticidal and parasiticidal agent and resistance of parasites and infectious disease organisms to drugs and antibiotics is as old as chemotherapy itself.

Despite all efforts, there is still great frustration in cancer chemotherapy due to development of drug resistance. Malaria is now present in 102 countries, is responsible for 100 million clinical cases and 1 to 2 million deaths each year (Oaks *et al.,* 1991) because, the lethal form of human malaria caused by *Plasmodium falciparum* has developed resistance to chloroquine in many areas worldwide. The P-glycoprotein (P- signifies permeability), which is a membrane-bound molecule has been implicated in multidrug resistant cancer cells and also in *P. falciparum* resistance to chloroquine. Thus, it was the keeping in mind the possibilty that parasite resistance to ivermectin may develop and pose a big threat both to current control activities and future plans that we thought it necessary to carry out this research work

Due to the alarming rate in drug resistance, millions of people are concerned about the cause of the increase, the effects it has on humans and how these effects or problems can be controlled. In the treatment of malaria with chlororquine, drug resistance has been a major challenge which has kept malaria disease in the lime light up till today. Several causes has been

attributed of which the most common is the misuse of drugs , malreabsorption of drugs, as well as development of enzymes that inactivate drugs as well as a host of others. Yet, each cause must be proved scientifically.

Since 1990 in Achi (an onchocerciasis-endemic community), South ï Eastern Nigeria, annual Ivermectin treatment has been on-going and some treated patients still harbor a lot of microfilaria (Personal observation, WORLD BANK/UNDP/WHO ONCHO PROJECT, Achi, Enugu state, 1990 -1993). Also, the doubling of cases of infection in certain communities of Ghana between 2000 and 2005, in spite of annual treatments, created fear of the emergence of ivermectin-resistant strains (Flechet, 2008). This phenomenom is not new as it has been experienced in the treatment of malaria with chloroquine, thus the development of resistance to ivermectin is of high relevance especially in health development policies. There is therefore the need to start early and search for the resistant indices as observed in the treatment of other diseases like malaria and cancer.

Using highly molecular approaches to search for resistance indices, I intend to collect nodules and microfilaria from various ivermectin-treated levels of patients in Achi, South ï Eastern Nigeria. Using the collagenase technique, isolate the adult worms. The female worms will be prepared as protein samples and used for SDS-PAGE (Sodium Dodecyle Sulphate Gel Electrophoresis) and DNA analysis. Furthermore, using immune- blotting and PCR (Polymerase Chain Reaction) techniques, the P-glycoprotein related proteins and mdr genes will be sought for and investigations into the possibility of resistance determined using relevant statistical packages.

1.3 Objectives of the Study

The objectives of the investigations described in this research work are threefold. Due to the wide margin of the disease manifestations observed with the patients and the few cases of the persistent microfilaridermiae, we tried to obtain a better understanding of what role epidemiological circumstances (occupational roles, age, sex and proximity to river) plays on the parasitic load of some onchocerciasis patients in Achi and Amansea, South Eastern Nigeria.

Secondly, an investigation was made to evaluate the effect of ivermectin on the uteri status of *O. volvulus* worms collected from treated and untreated onchocerciasis patients in Achi and Amansea.

Thirdly and chiefly, we searched for the presence of the P-glycoprotein (resistant factor) in the *O. volvulus* worms (adults and microfilariae) from these patients. If the P-glycoprotein is present, to determine its level or expression in *O. volvulus* worms collected from responsive and non-responsive onchocerciasis patients treated with ivermectin and investigate if there is any relationship between P-glycoprotein levels in worms and epidemiological circumstances and finally to check if there are already resistance signals. The mdr genes which code for the P-glycoprotein will also be investigated into.

In this write up, the field work was always presented before the laboratory work but a general conclusion which summarized all the discussions was made. The need for a follow up study based on the several questions arising from this research work is also discussed.

Thus, the objectives of this research work include to:

1) Identify the onchocerciasis- affected communities of Achi and Amansea towns and determine their endemicity levels.

2) Investigate the roles of epidemiological circumstances (occupational roles, age, sex, and proximity to river) on the parasitic load of some patients in Achi and Amansea.

3) Evaluate the effect of ivermectin treatment on the uteri status of *O.volvulus.*

4) Evaluate the effect of ivermectin treatment on the uteri status of *O. volvulus* worms collected from treated and untreated onchocerciasis patients in Achi.

5) Search for the presence of the P-glycoprotein (resistant factor) in the *O. volvulus* worms from these patients. If the P-glycoprotein is present, to determine its level or expression in *O. volvulus* worms collected from responsive and non-responsive onchocercerciasis patients treated with ivermectin.

6) Determine the level of homology between the mdr genes found in *O. volvulus* with other mdr genes.

1.4 Literature Review

1.4.1 The biology of the parasite *Onchocerca volvulus*

Onchocerca volvulus is a thin nematode worm found as a parasite in human beings. It is transmitted by black flies of the genus *Simulium*. The adult worms (females 30-80 cm, males 3-5 cm) live in fibrous nodules, some of which are subcutaneous and palpable while others lie deep in the connective and muscular tissues. They have a life span of some 9-14 years. The females produce abundant microfilariae (250-300 µm in length), which migrate from the nodules to invade the skin, eyes and some other organs. They cause most of the disease manifestations of onchocerciasis and have a life span of about 6-24 months. The microfilariae ingested from the skin by blood-feeding *Simulium* vectors develop over 6-12 days, without multiplication, to form infective larvae (L3) which can be inoculated into a new host when the fly feeds subsequently. In the human host they moult twice, again without multiplying, to reach the adult stage; the first

microfilariae produced by adult females may appear in the skin some 10-15 months after infection (Manson Bahr and Bell, 1991). Microfilaria can survive for 30 months in the skin. There is evidence of transplacental transmission of microfilariae so that the foetus is infected in utero (Brinkman *et al*, 1976).

The infective larvae of *O. volvulus* moult to the L4 stage within 3-7 days of arriving in the human host and the moult from L4 to the juvenile adult stage probably occurs 4-6 weeks later. The route followed by immature worms is unknown, they appear to be attracted to existing nodules and may settle on their surface to form satelite or composite nodules. The proportion of infective larvae inoculated that develop into adult worms is unknown. Young, old and calcified dead worms are often associated in the same nodule. Onchocercomata or nodules are found in distinct sites of predilection in the body. On average, 80% of the nodules contain one or two male and two or three female worms. Accumulation of more than 50 worms can occur, but this is the exception. In contrast to the sessile female worms, male *O. volvulus* regularly leave the nodules. In excised onchocercomata, a striking predominance of female worms is often observed as a result of this migration in the host of a proportion of the male worms. It is assumed that the migratory instincts and possibly the reproductive activity of male worms decrease with age in areas where transmission has been interrupted over a long period since a sex ratio of about 1:1 is found in the nodules of persons harbouring an aging worm population. Inactive old male worms are often clearly separated from non-gravid female worms, although they remain in the same nodule (Schulz - Key *et al.*, 1987; Nelson, 1991; Duke, 1990).

With regards to mating, shedding of oocytes into the uteri may be a prerequisite for the stimulation of males to mate since sperm is scanty in female worms with empty uteri. So far, nothing is known about the pheromones or other stimuli that attract males to the females in the nodules. On the other hand, in many females, oocytes are released from the narrow ovaries into the wide lumen of the uteri independently of the presence of male worms. Large numbers of

degenerating and shrinking oocytes may accumulate and subsequently be reabsorbed when the gravid females do not mate. Nematode sperm are short-lived, insemination normally continues during the early phase of embryogenesis. Only 10-15% of worms are found typically entangled in the mating position and, gravid females are regularly found deserted after insemination (Schulz-Key, 1988). Spermatozoa transferred to the female worms show amoeboid movements which enable them to force their way through a stream of embryos or oocytes moving in the opposite direction until they reach the posterior parts of the uteri. Schulz-Key (1988) and Duke *et al.* (1991) variously estimated the development of oocyte into a mature microfilaria within the female to take 3-12 weeks.

The reproduction of *O. volvulus* occurs in asynchronous cycles lasting 2-4 months each (Schulz-Key, 1988). Such cyclic reproduction has been observed for *Onchocerca.ochengi*, *Onchocerca.gibsoni*, and *Onchocerca* species in red deer and roan antelope, and may be typical for species with skin-dwelling microfilariae. However, Duke, (1990) observed that female worms shed oocytes continually while awaiting insemination.

Female *O. volvulus* shows a heterogeneous distribution of uterine/developmental stages. These stages are classified into six groups: Oocytes and five developmental stages of the embryos. Primary oocytes are elongate cells, clusters of which are attached in situ to the rachis of the posterior parts of the ovaries, in all mature female worms but, on average, fewer than two-thirds of the females actually contain embryonic stages and microfilariae. Secondary oocytes are more rounded cells lying separately in the oviduct. Embryonic stages: Two-cell and four-cell stages are co-ordinated to the group of small morulae. Small morulae has an average size of 15:8 µm. Big morulae in the subsequent group are more rounded and measure about 20:13 µm. Normally developed morulae consist of coherent cells filling out the whole space within the egg-shell. Then, is the stage of the advanced embryo which shows a lateral incubation indicating the beginning organization of the embryo. The larvae gradually become longer and slimmer.

Embryos exceeding a circle in the egg-shell are co-ordinated to the subsequent stage of the coiled microfilaria, also called ñbrezel-stageôô. The final stage was represented by the stretched microfilaria which had cast off the egg-shell. However, in all groups, pathological alterations and deformities could be observed occurring naturally or due to drug effects. In the oocytes for example, while normal oocytes showed well defined nuclear membranes, in the deformed oocytes due to shrinkage of the cytoplasm, the shape of the nucleus becomes indistinct and the space within the egg shell will only be partially filled out and sometimes the egg-shell itself will be deformed. The size of the cells in abnormal two- and four-cell stages are often in-equal, the cell membranes are partially dissolved and the cells are sometimes separated from each other, the cytoplasm granular, turbid or caseous. Pathologically altered embryos of the further developed morulae often consist of cells of inequal size some of which do have stunted growth and others might show abnormal increase. Also, single cells are often separated from the embryos which become irregular in outline and disorganised with necrotic areas. The space within the egg shell are not completely filled out, occasionally egg-shells of normal size could be found containing only single cells or remnants of them. In the deformed advanced embryos, a similar segregation of cells can be observed and sometimes longitudinal clefts of the embryos indicates abnormal development and gradual dissolution of cell membranes. Abnormal coiled microfilariae are deformed when they show excrescences, are underdeveloped or have enlarged nuclei. In the pathologically altered microfilariae, the nuclear column seems to be interrupted at several sections by the dissolution of the cytoplasma and sometimes motile microfilariae show abnormal big nuclei and vacuoles (Schulz-Key *et al.*, 1990).

1.4.2 Reproductive potential of *O.volvulus*

Embryogram techniques can be used to assess the reproductive capacity of a female worm because it quantifies the number of intrauterine stages actually present in a female worm, but it cannot indicate how many microfilariae are actually produced or released per day. Schulz-Key (1990) observed worms maintained in-vitro and suggests that 700-1500 microfilariae per female are released into the host on average per day, i.e. only a small proportion of the microfilariae developed in utero actually leave the female worms. In contrast to other filarial species, microfilariae of *O. volvulus* are not expelled by the female worm but leave it actively one by one. It takes at least 5-10 seconds for a microfilaria to leave the female worm when it has arrived at the vulva. Microfilaria that stay in the uteri gradually degenerate and are then reabsorbed. Thus an embryogram can provide precise information on the dynamics of reproduction by assessing the number of intrauterine stages present, the prevalence of abnormal forms, whether a cycle has just started or whether it is expiring, Whether a female worm has recently been inseminated and how many sperms are actually present the effects of drugs.

The reproductive life span of *O. volvulus* has been estimated by longitudinal skin -snip surveys undertaken in villages under vector control and by analysis of trends in community microfilarial load (the geometric mean number of microfilariae per skin snip among persons aged 20 years and over; including those with zero count) using the mathematical model ONCHOSIM. ONCHOSIM uses the technique of ñstochastic microsimulationôô, which involves the explicit simulation of the individual life histories of both human hosts and adult parasites. Models based on microsimulation are flexible in design, which makes it easy to specify and simulate alternative assumptions. Furthermore, they can provide detailed output in the same format as field observations; this is useful in the validation of the model, while it makes the model output more understandable to decision-makers.

In ONCHOSIM, the most important variables are (i) human factors, namely population dynamics (birth, death, immigration), and heterogeneity in exposure to the vector; (ii) vector factors; such as vector density; biting rates and seasonal variation; (iii) the life history of the parasite in the host (life span, pre-patent period, age-specific microfilarial output, mating) ; (iv) larval uptake by flies as a function of human microfilarial load; (v) the development of blindness and associated excess mortality; (vi) the timing and coverage of epidemiological surveys and ivermectin treatment ;(vii) the timing and effectiveness of larviciding and (viii) the microfilaricidal and possible macrofilaricidal effect of ivermectin (Habbema et.al., 1992).

The mean duration of reproductive life has been estimated at 9-11 years, and 95% of adults do not reproduce for longer than 13-14 years (Plaisier et.al. 1991). Furthermore, Schulz - Key (1990) and Duke (1993) from their various observations showed that considerable numbers of female worms are hidden in deep-lying, impalpable nodules, and imply that a high proportion of infective larvae fail to develop into adult worms, and that in many infected persons, tens or hundreds of thousands of microfilariae must die and be disposed in the body each day.

1.4.3 Vector biology

Onchocerca volvulus is transmitted to man through the bite of infected black-flies belonging to the genus *Simulium* (WHO, 1987; Nelson, 1991) which breed along fast flowing water bodies. The female blackfly ingest *O. volvulus* microfilariae (embryonic form) from humans and acts as an intermediary for the development of infective larvae. Transmission occurs when infected flies take a blood meal. Reactions to the bites of Simulium sp themselves can be severe and allergic reactions occur.Very little is known about the development of O. volvulus in man. This is in part due to onchocerciasis being an end of the road disease, with the most infected communities living in remote under doctored areas where there are no facilities for detailed autopsy studies. But of most importance is the lack of a small animal model. Much more information on the development of Onchocerca has been obtained from studies of related

species in Simulium and cattle, especially by Bianco and his colleagues (Bianco *et al*., 1980; Bianco *et al*., 1990). However, Following an approximately 2-week period these larvae can be transmitted back to the host during subsequent bites. The main vectors, *Simulium damnosum*, *S.neavei, S.ochraceum, S.metallicum*, and *S.exiguum*, are complexes of sibling species which do not otherwise form a taxonomically close group of species.

In Africa and the Southern Arabian peninsula, onchocerciasis is associated mainly with members of the *S.damnosum* complex, and to a lesser extent with the *S.neavei* group. These two groups have been known to be broadly distributed (WHO, 1987; Crosskey, 1987 and 1990), but *S.albivirgulatum*, the vector in the ñCuvette centraleôô focus of Zaire, is the only vector species outside these two taxonomic groups. In the Americas, *Simulium ochraceum*, a species complex of at least three cytospecies, is considered to be the primary vector in all five foci in Guatemala and Mexico, while *S.metallicum* s.I. and *S.callidum* play secondary roles. In South America, the known vectors are *S.exiguum* s.I., *S.guianense*, *S. incrustatum*, *S.metallicum* s.I., *S.oyapockense* s.I., and *S.quadrivittatum*, *S.limbatum* is strongly suspected of acting as a vector. The vectors breed in fast flowing and well oxygenated rivers and streams with nutrients in the savannah and rain forest area. They have the ability to travel hundreds of kilometres in flight and wind currents. Their life span is about 4 weeks (Eezzuduemhoi and Wilson, 2008).

The aquatic stages of black flies inhabit a wide variety of streams in different bio-climatic zones that reflect the ecological requirements of each species. These range from tiny rivulets no more than a few inches wide for *S. ochraceum* to very large rivers for some members of the *S. damnosum* complex. In general, breeding occurs in swift running, well oxygenated, unpolluted water. The larvae attach to various submerged supports (vegetation, rocks, debris, etc.) by means of a posterior sucker armed with hooks. The duration of the aquatic stages depends on water temperature and requires 10-12 days for the West African species and up to 30 days for *S. ochraceum* at the higher elevation in Guatemala. Larval simulidae have plumose fans

on their mouthparts that filter out particulate food from the flowing water. Ingestion is indiscriminate and larvae will take in insecticide particles as well as other matter. Female black flies bite from dawn to dusk usually outside of houses. Except for *S. ochraceum*, which feeds mostly above the waist, vector black flies prefer to feed on the lower parts of the body. Biting is not constant throughout the day and unimodal or bimodal biting curves have been described for several species.

The abundance of flies may show marked seasonal variations related to the productivity of the breeding sites. Fly production usually fluctuates according to the water level and flow rate. For example, the high water levels during wet season in Guatemala make the streams unsuitable for attachment of larvae and peak populations occur at the end of the rainy season when streams subside. In Africa, adult fly populations may vary inversely or directly with water level and flow rate depending on the species and the stream type.

Adult females can disperse considerable distances from the breeding sites by flight on a prevailing wind, or by both methods. In Onchocerciasis Control Programme of the Upper Volta river basin, West Africa, flies have travelled distances of 150 km or more to re-invade areas where breeding was apparently controlled. The Central American vectors have maximum flight ranges of 10-15 km. The longevity of adult females is not known precisely but probably does not exceed a month for both *S. damnosum* and *S. ochraceum*. A higher proportion of older females are found within 1-10 km of breeding sites: this makes transmission more intense at the stream banks where people congregate to fish, bathe, wash cloths, and obtain drinking water.

There is a marked difference between the situations in Africa and the Americas with regards to the distribution of *Simulium* spp. Vectors and potential vectors, and that of the disease. In Africa, wherever anthropophilic members of the two vector complexes occur, the human population suffers from some degree of onchocerciasis. In contrast, in the Americas, potential vectors occur widely outside the areas in which onchocerciasis is endemic. In Central

America, the primary and secondary vectors are much more widespread than endemic onchocerciasis. In South America, human-biting blackflies occur over vast areas in the absence of the disease; however, the missing element here is not only the parasite but also a significant human population. It is probable that current changes in settlement pattern together with long-distance migrations of large numbers of people, such as gold-miners, will eventually lead to the establishment of new foci.

1.4.4 Vector ecology

In Africa, the spread of onchocerciasis has resulted from human activity and consequent environmental changes such as deforestation, resulting in the conversion of forest habitats into savanah and the creation of artificial breeding sites. In West Africa, such changes have resulted in a shift of the distribution area of the savannah species into forest zones.

Simulium damnosum was originally considered to be a fairly uniform species, differing biologically in different bioclimatic zones. However, since the mid-1960s it has become apparent that it is a complex of morphologically similar (sibling) species which can be distinguished by the banding patterns of the larval chromosomes. At present, over 40 different cytological forms have been described, half of which have been named without adequate morphological and cytological study.

In West Africa, west of Nigeria, the *S. damnosum* complex has received detailed study, all of these species are either known or suspected (*S. dieguernse*) vectors. In addition to the formally named species, numerous additional cytospecies have been described in West Africa and given vernacular names (Vajime and Gregory, 1990). The situation in Central and East Africa is much more complex. In contrast to West Africa, the cytospecies of the *S. damnosum* complex have not been fully studied and described. Many have very restricted distributions, they are often zoophilic, and many are not known to be vectors of *O. volvulus*. However, it is clear

that species of the *S. damnosum* complex are responsible for most of the transmission of onchocerciasis that occurs in Ethiopia, Malawi and the United Republic of Tanzania.

With regards to vector capacity and transmission in West Africa, under natural conditions, there are significant variations in transmission both between and within species, the differences observed being the result mainly of factors such as longevity, trophic preferences and the relative abundance of the various hosts. In the forest zone of southern Guinea and Sierra Leone, the mean number of infective larvae morphologically indistinguishable from *O. volvulus* and associated with species of the *S. sanctipauli* subcomplex is six. Cytological identification confirms that these areas are populated mainly by *S. leonese*. In most of the basins of the western zone of the OCP, the equivalent values are four L3 larva for *S. yahense* and five for the other forest species. In the Savannah region, *O. volvulus* is associated with *S. sirbanum* and *S. damnosum* s.s., and the average number of L3 larvae per infective fly is just over two.

Extensive experimental studies of vector competence show that all species of the *S.damnosum* complex in West Africa (with the exception of *S.dierguerense*,which has not been studied) are capable of transmitting *O.volvulus*. However, the compatibility of vectors and parasites may depend on their respective origins. Thus the vector competence of the main species of *S.damnosum* s.I. may differ for the main strains of *O. volvulus* (Savannah and forest). The highest parasite yields under normal conditions of transmission occur with species of the *S.sanctipauli* subcomplex and with *S.yahense,* which is consistently a more efficient vector than *S.squamosum*. The lowest parasite yields are found among Savannah vectors (*S.sirbanum* and *S.damnosum* s.s.). Considerable differences exist when vectors and parasites strains are of different geographical origins (cross-transmission). Under experimental conditions, forest parasite strains develop poorly or not at all in Savannah vectors (*S.sirbanum* and *S.damnosum* s.s.).The parasite yields obtained from different species of the *S.sanctipauli* subcomplex are high, in general, irrespective of the geographical origin of the parasite strains concerned. In order

to quantify the relationship between the potential infectivity of *S.damnosum* s.s. and the intensity of *O. volvulus* infection of the human host, the OCP conducted an experiment in the Savannah focus of Asubende, Ghana, in which blackflies were engorged on 40 volunteers with a wide range of microfilarial loads. A clear relationship was found between vector infectivity and skin microfilarial load: persons with a low intensity of infection contributed only little to transmission but the potential infectivity of the vectors increased rapidly with the skin microfilarial load of the host. However, even with the most heavily infected patients, fewer than 50% of the flies became infected. These results, while valid for *S.damnosum s.s*., cannot be extrapolated to other vector species because of differences in the relationship between vector infectivity and skin microfilarial loads. Such differences are of great importance for onchocerciasis control since they should determine the relative effectiveness of large-scale ivermectin treatment programmes in preventing *O. volvulus* transmission (De Leon and Duke, 1966; Dalmat, 1955; Dadzie *et al*., 1989, 1990; Davies and Crosskey, 1991; Dozie and Nwoke, 2002).

Figure 1: The life cycle of *Onchocerca volvulus.*

Ana, (2011)

1.4.5 Onchocerciasis: Disease manifestations

The itchy disease, known as *craw-craw* in the then Gold Coast, was in 1875 associated with onchocercal microfilariae found in the skin of patients by Oô Neil, and in 1893, skin nodules from patients were found by Leuckart to contain adult worms. Sir Albert Cook reported the occurrence of onchocerciasis in Uganda in 1899. Robbles became interested in its manifestation as a serious eye disease in Guatemala in 1915 while Pacheco Luna established its association with blindness in 1918.

1.4.5.1 Basic symptoms of onchocerciasis

The major presenting symptoms of onchocerciasis are dermal, ocular, lymphatic and systemic in nature, arising from the infiltration of microfilariae throughout body tissues. Several other features of uncertain association, etiology or pathogenesis have also been described, including low bodyweight, general debility, diffuse musculoskeletal pain and in Africa, epilepsy and hyposexual dwarfism. A number of individuals, the so-called ôendemic normalsõ, do not show clinical symptoms nor is there any parasitological evidence of onchocerciasis despite ongoing exposure to the parasite (Ward *et al*., 1988) and (Nutman *et al.,*1991). Host-parasite interaction at an immunological level is responsible for the clinical outcome of the infection (Williams *et al.,* 1986 and Mackenzie *et al*., 1987). Marked geographical variations in the clinical picture exist, which may be related to the different pathogenicity of *O. volvulus* parasite strains (WHO, 1987; Zimmerman *et al*., 1992), vector biting habits (WHO, 1987) and host ethnic (Molea *et al.,*1984), genetic (Brattig *et al.,*1986) and immunological factors. Human onchocerciasis differs as regards clinical manifestations in the savannah and the rain forest of West Africa (Anderson *et al.,* 1974). The skin lesions are more pronounced in the forest zones, whereas the blindness rate and the ocular manifestations are severe in the savannah. Microfilariae of the putative savannah strain are more pathogenic than those from the forest strain, if injected into the eyes of rabbits (Duke and Anderson, 1972). The pathogenesis of

onchocerciasis arises from two factors namely the effect due to adult worms and those due to the microfilariae. The adult effects are minimal in early stages of light infection as the adult worms lie freely in the subcutaneous tissues (Duke, 1990). The main significant pathology arises from the reaction to dead microfilariae in the skin and the eyes. In the skin the condition is called onchodermatitis, the basic lesion is the cellular reaction to the dead microfilariae (Manson-Bahr and Bell, 1991).

The extent and distribution of skin and lymphatic lesions permit classification of the disease into generalized and local forms. Generalized onchocerciasis is the usual presentation, characterized by fairly symmetrical lesions which may be more marked in the lower, or less commonly, the upper part of the body. The local form is asymmetric and may be confined to one limb and the adjacent area or to a circumscribed part of the body. Acute manifestations of localised onchodermatitis occur in new residents and in people from outside the endemic areas; the chronic form of localized onchodermatitis is synonymous with hyperactive onchodermatitis or sowda and is characterised by frequent acute exacerbation. The traditional teaching is that microfilariae can be found at all levels in the dermis but tend to be most numerous at the dermal-epidermal junction. Intact microfilariae excite little inflammatory reaction. Vuong *et al.,* (1988) recently studied the skin lesions in people living in the West African forest and savannah and found that the microfilariae of *O. volvulus* were found predominantly in the lymphatic channels of the dermis surrounded by minor areas of inflammatory reaction. Reactions thought to represent successive stages of an inflammatory process were observed around extralymphatic microfilariae (WHO, 1987, 1995b; Chijioke, 2009). The pathogenesis of onchocerciasis arises from two factors namely the effect due to adult worms and those due to the microfilariae. The adult effects are minimal in early stages of light infection as the adult worms lie freely in the subcutaneous tissues (Duke, 1990). The main significant pathology arises from the reaction to dead microfilariae in the skin and the eyes.

1.4.5.2 Skin manifestations

Although some individuals with onchocerciasis may have clinically normal skin, others have intense pruritus which is the most common early manifestation of onchocerciasis, developing after the prepatent period. The itching may lead to excoriation and secondary infection and may involve any part of the body. The first visible changes in the skin other than evidence of scratching, is an alteration in the pigmentation with areas of hyper- and hypo-pigmentation (WHO, 1987, and Duke, 1990). After years of chronic infection, atrophy of the skin develops. In severe cases a condition called *lizard skin*, characterized by a thin epidermis with a shiny fragile appearance, may develop. The normal dermal structure is replaced by a scar tissue and elasticity is lost. Another characteristic aspect of onchocercal skin disease is *leopard skin*, a spotty depigmentation occurring on the anterior part of the lower extremities.(Manson-Bahr and Bell, 1991; WHO, 1995)

In Africa, onchodermatitis is typically ógeneralizedô- diffuse, and characteristically maximal on the lower trunk, pelvic girdle and thighs. Skin lesions in Guatemala and Mexico are in general milder than in Africa. An unusual feature of chronic hyperreactive localized onchodermatitis, often called *sowda*, is found in low prevalence also in Africa and America. Sowda in patients is characterized by an intense itching, usually asymetrical, well-circumscribed onchodermatitis with pustules and crust, oedema, pachydermia, darkening of the skin and considerable enlargement of the local lymph nodes, and no or few microfilariae in the skin (Gasparini 1962).

In most endemic areas of Africa and in Ecuador (Guderian *et al*., 1984), the majority of nodules are found on the pelvic girdle. In Central America, nodules are more common on the head, which may be due to the vectors biting more frequently on the upper parts of the body (WHO, 1987). Nodules measure 0.5 - 2 cm in diameter, but some may be larger with diameters exceeding 6cm. New nodules tend to develop around older nodules. Usually nodules are

painless and cause little trouble. *Onchocerca* nodules may be confused with lymph nodes, lipomas, foreign body granulomas, sebaceous and dermoid cysts, ganglia and histoplasmosis or cysticercosis nodules. Recently a managable clinical classification and grading system of the cutaneous changes has been developed, enabling objective comparism between different geographical areas (Murdoch *et al.*, 1993). Under this classification, the main categories of onchocerca skin disease are defined as follows; acute papular onchodermatitis, chronic papular onchodermatitis, lichenified onchodermatitis, atrophy and depigmentation. But two important points regarding this classification are that, first, it is based on clinical findings consistent with cutaneous onchocerciasis but not necessarily specific or diagnostic of the disease. Secondly, the categories are not mutually exclusive and one pattern may coexist with, or evolve into another. This scheme also includes a method of grading for use in recording the clinical severity of lesions, the clinical activity in terms of pruritus and excoriation, and the extent of distribution over the body. Its practical usefulness is currently being evaluated.

Subdermal nodules called ñonchocercomataò, which are most easily seen over bony prominences are another commonly reported manifestation of onchocerciasis (Okuliez *et al*., 2004). In Africa, onchocercomata are often found over the bony prominences of the torso, and hips, where as in South America, where it is sometimes called ñRobles diseaseò, (WHO, 2001), the predominant strain typically produce nodules in the head and shoulders (Wolf *et al*., 2003). Cases of onchocercomata presenting as a breast mass or as deep nodules in the pelvis have been described (Okuliez *et al*., 2004; Zarich *et al*., 2004). An angiogenic protein produced by the adult female is thought to contribute to the formation of the nodules. The presence of onchocercomata does not correlate with microfilariae load (Okuliez *et al*., 2004).

1.4.5.3 Ocular manifestations

Ocular involvement is common and blindness (resulting from complications such as sclerosing keratitis, iridocyclitis, chorioretinitis and optic atrophy) is the most severe sequela,

occurring particularly in patients carrying a high microfilarial load (living or dead) for prolonged periods. These can be seen with a slit lamp and can involve any part of the eye from the conjunctiva and cornea to the uvea and the posterior segment, including the optic nerve and retina. The earliest sign of occular involvement in onchocerciasis is the invasion of the eye by microfilariae. Conjuctival reactions with hyperaemia, chemosis, and epiphora are often present in severely infected patients. A typical feature of punctate keratitis are snowflake opacities of about 0.5 mm in diameter representing dead microfilariae surrounded by an inflammatory infiltrate. These infiltrates usually resolve in a few weeks without sequelae (O'Day and Mackenzie, 1985). In later stages, sclerosing keratitis may develop. A fibrovascular pannus and an inflamatory infiltrate develop initially in the interpalpebral fissure and inferiorly. After several years, the entire cornea may become opaque, vascularized and pigmented leading to blindness. Anterior uveitis can also be found, which in severe cases often leads to inferior, anterior and posterior synchiae. A characteristic pear-shaped deformity of the pupil, caused by inferior-posterior synechiae, is also called oncho-pupil (Anderson *et al*., 1974b; Dadzie *et al.,* 1990; Murdoch *et al*., 2002). Other complications of severe uveitis include secondary cataract and secondary glaucoma. A higher prevalence of skin snip positive individuals among younger glaucoma patients (10-39 years) compared with older glaucoma patients has been reported (Berghout, 1973). Since these patients lack signs of active uveitis or synechia, a primary role for onchocerciasis in glaucoma has been suggested. However, the relevance of onchocerciasis as a primary cause of glaucoma remains to be established. Onchocercal ocular pathology is usually bilateral but not necessaryl symmetrical and results mainly from local death of microfilariae and the host inflammatory response to *Wolbachia* antigens (Higazi *et al*., 2005)

It was commonly believed at one time that active optic neuritis was rare in patients suffering from onchocerciasis, but this lesion has been observed in a number of subjects in a community-based ivermectin study carried out in Nigeria (Abiose *et al*., 1993). Clinical

experience has shown that the active optic neuritis associated with onchocerciasis lasts from several weeks to one year or more. It appears as a congested suffused disc with or without swelling, while postneuritic optic atrophy is often associated with scarring and pigment disturbance at the disc margin. Associated dense vascular sheathing may extend along retinal vessels for a considerable distance beyond the optic nerve head. Primary optic atrophy may also occur, and may be partial or complete. The reported prevalence of optic atrophy has varied between 1% and 4% in the hyperendemic rain forest and savannah communities of Cameroon to between 6% and 9% in the Guinea Savannah of Northern Nigeria.

Blindness is the most serious consequence of onchocerciasis, and can result from lesions that affect different parts of the eye. In the savannah areas of West-Africa, blindness is prevalent in 2-15%, and was previously attributed mainly to sclerosing keratitis (WHO, 1987), but a recent study has shown that optic nerve disease plays a significant role in some localities (Abiose, 1993). In the rain forest, blindness is less prevalent (up to 2.4%) and is said to be due mainly to posterior segment disease (WHO, 1987, Meyers *et al.,* 1977). Recently, using DNA techniques, an association between ocular pathology and parasite strains has been observed (Zimmerman *et al.,* 1992). Relatively few serious eye lesions occur in South-America (WHO, 1987; Molea *et al.,* 1984). Blindness has an enormous social and economical impact. It strikes mainly at economically active adults in the prime of life, and eventually it may even result in villages and fertile lands being abandoned (WHO, 1987).

1.4.5.4 Other manifestations

In lymphatic onchocerciasis, with heavy microfilarial infection, some of the parasites pass to the lymph nodes draining the area, leading to lymphadenitis with accompanying fibrosis. Enlarged lymph nodes develop especially in the inguino-femoral region, which in combination with wrinkled skin give rise to "hanging groin" and predispose to herniae. Sometimes elephantiasis of the scrotum may occur (WHO, 1987; Duke, 1990).

Onchocerciasis can be considered as a systemic disease. Small numbers of microfilariae can be found not only in skin, eyes and lymph nodes, but also in many deeper organs, including the liver, kidney, spleen, pancreas, lung, peripheral nerves and arteries. Microfilariae have also been found in tears, blood, urine, cerebrospinal fluid, sputum, vaginal secretions, and peritoneal fluid (Meyers et al., 1977). In hyperendemic areas, patients with severe onchocerciasis may lose weight (Burnham, 1991; Kirkwood *et al.,* 1983). The significance of systemic manifestations should not be underestimated. Systemic effects of onchocerciasis may lessen productivity of an endemic region by a process distinct from effects of visual impairment (Burnham, 1991) and may even lead to excess mortality (Kirkwood *et al.,* 1983).

1.4.6 Geographical distribution and epidemiology

1.4.6.1 Geographical distribution

Onchocerciasis is still endemic in 34 countries, 26 endemic areas in tropical Africa, where 99% of the total infected persons are found, 6 in the Region of the Americas, and 2 in the Eastern Mediterranean Region. However, in Africa, in the original core area of the OCP (Cote dõIvoire, Burkina Faso, Mali, Ghana, Togo, and Benin), the disease has declined dramatically in both prevalence and public health importance. The main public health problem remains in the countries of sub - Saharan Africa outside the OCP area, where the disease is both widely prevalent and severe in terms of blindness and skin lesions, and where there is additionally the risk that Savannah blackflies will become established in degraded forest habitats. New foci have been found in the Americas, and the disease may spread still further as infected workers continue to exploit areas of virgin forest.

Current estimates suggest that about 17.7 million are infected, out of which 270 000 are blind; in addition, a further 500 000 are severely visually disabled (WHO, 1995). *Onchocerca volvulus* adult worms can live for over a decade in skin nodules of affected humans, releasing

millions of microfilariae that cause debilitating itching and blindness (Richards *et al*., 2001). An estimated 37 million people are infected and there are 46,000 new cases of blindness annually (http://www.apoc.bf/, 2005).

In Nigeria, currently it is estimated that a few more than three million people are infected of whom about 100 000 are blind as a result of onchocerciasis. Nigeria has the largest population of any sub-Saharan African country, also has the largest number of infected persons, but it is a measure of the lack of accuracy of country-wide endemicity figures that the estimate of nearly 7 million infected persons made by the WHO Expert Committee on Onchocerciasis in its third report (WHO, 1987) was reduced to about 3 million in the national sample survey conducted in Nigeria in 1988-1989. The disease varies in endemicity in Nigeria but it is present in all states and the Federal Capital Territory, with the exception of Lagos, Rivers and Akwa-Ibom states, where infections are sporadic. However, Abanobi and Anosike (2000) observed that there is lack of detailed and accurate statistics on the prevalence of onchocerciasis in Nigeria and can be mostly attributed to the problems encountered in the epidemiological surveys. Some of these problems include language barriers between the investigators and the population at risk, lack of

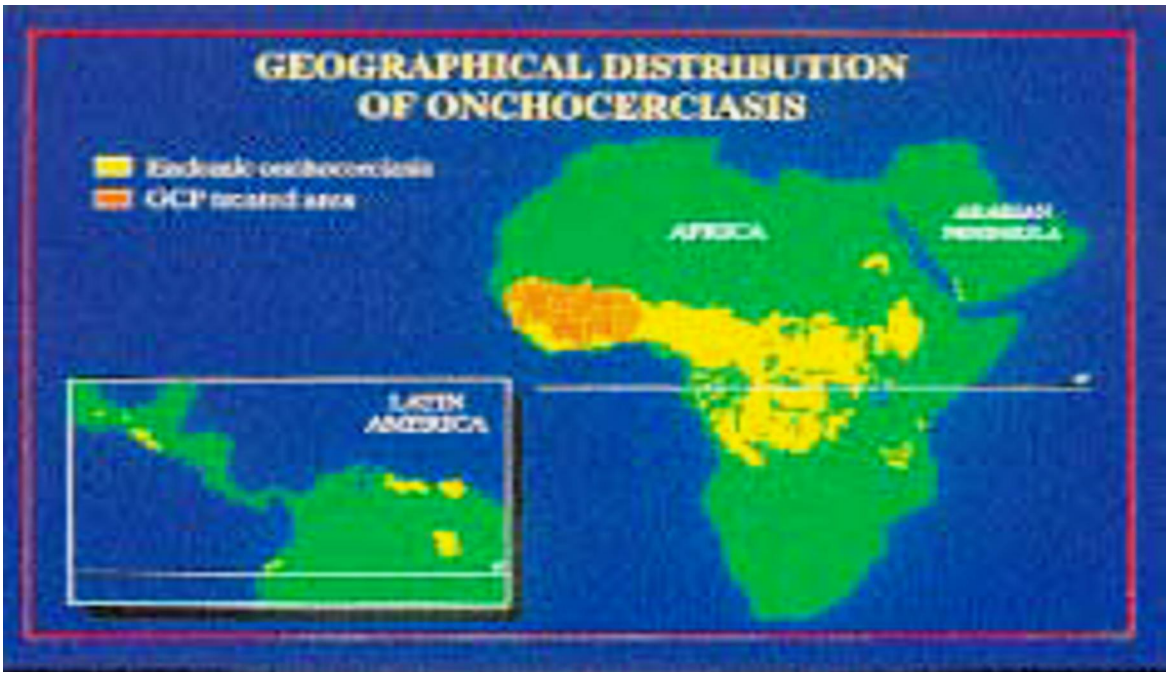


Figure 2. Geographical Distribution of Onchocerciasis

(Bjorn, Onchocerciasis; Elimination on the Horizon. MD, Mectizan Donation Programme, Georgia, USA. GLOBAL REVIEW JOURNAL: ONCHOCERCIASIS, LEPROSY and HIV/AIDS, pp 114 ï 116).

trained man-power, and absence of more sensitive diagnostic techniques to detect sub-clinical infections (Enyenihi, 1982).

In central Africa, the serious blinding form of the disease extends from the eastern Nigerian states of Taraba, Adamawa and Borno across northern Cameroon into the six south-western prefectures of Chad, the three north-western prefectures of the Central African Republic, and south-eastern Sudan. In Uganda, deforestation following subsistence agricultural development and timber exploitation has reduced the cover available to the *S.neavi* vector, thereby reducing the transmission of *O.volvulus*, but if *S.damnosum* colonizes these foci, transmission will return. In Ethiopia , there is substantial disfiguring skin disease, especially in coffee and tea plantations but there appears to be no blindness due to onchocerciasis. In Malawi and Sudan, endemic zones have been identified while in Angola, Liberia, and Zaire, the current situation is uncertain (WHO, 1995).

With regards to the Americas, Studies have revealed the existence of Onchocerciasis in Yemen, and although there are reports of onchocerciasis in Saudi Arabia, transmission in that country has not been confirmed. It is probable that cases in Saudi Arabia have been imported. Furthermore, no vector of onchocerciasis have been identified hence it is unlikely that there is any significant focus of the disease in the country.

About 261 660 people are at risk in Mexico and over 25 000 cases have been reported. In Guatemala, active foci are concentrated on the western slopes of the volcanic range and in the regions of Chimaltenango, Solola and Suchitepequez, 30% of the communities are hyperendemic. In Venezuela, onchocerciasis was first recognised in 1948, In Columbia in 1965, in Brazil, 1967, and in Ecuador in 1982. Since 1985, there has been no convincing evidence of any expansion of the existing foci. In Amazonas state in Venezuela, studies indicate that the geographical distribution of competent vectors is considerably larger than that of the disease, so that, if infected individuals migrate, new foci could be created (WHO, 1995). In Brazil,

onchocercal foci are located in the northern part of Amazonas state and in the western part of Roraima State, which borders Venezuela. In Colombia, the main known focus is the Lopez de Mucay area on the Pacific coast, where 16 300 individuals are at risk of infection in 155 communities. In Ecuador, the onchocerciasis focus is located in the north-western coastal province of Esmeraldas. The major focus involves blacks and Chachi Amerindians living in the Santiago River basin. There were 192 known infected communities in 1993, and 20 089 individuals at risk of infection (WHO, 1995).

1.4.6.2 Epidemiology of onchocerciasis

The epidemiology, of onchocerciasis is that of a vector-borne disease of which human beings are the only vertebrate host. Infection with *O.volvulus*, like other filarial infections, is also characterized by coincidence between the degree of human infection and the intensity of exposure to infected vectors.

However, the epidemiology of onchcocerciasis is not uniform throughout its distribution because different disease patterns are associated with different variants or strains of the parasite, with differences in the vector competence and feeding characteristics of local blackfly populations, with the abundance of the vector and with differences in the human host responses to the parasite. These factors, together with those related to environmental, geographical, social and demographic influences, increase the complexity of the epidemiology of the disease in the different areas of its distribution.

Factors influencing the epidemiology of onchocerciasis can be divided into those relating to the host, the parasite and the vector, but behavioural and community factors also need to be considered.

With respect to host factors, there are no known sex differences in acquisition of infection, and age merely determines cumulative exposure to infection. In individuals with

sowda lesions, there are apparent variations in the immune response to infection. Parasite factors such as genotype may explain the pattern of disease in certain foci, for example, two different types of *O. volvulus* (forest and Savannah) exist in Africa; this is of importance in setting priorities for control measures.

Vector factors are important inasmuch as they affect the transmission of the parasite. Transmission rates may vary both seasonally and by geographical location. Vector abundance depends on hydrological conditions, which determine the number and productivity of blackfly larval habitats. Vector density, is also determined by dispersal habits. Ecological factors such as prevailing winds and humidity, also contribute to passive dispersal and migration, some species of African savannah flies travel up to 400km from their breeding sites. There are also major differences between vector species in their feeding habits, for example, in the degree of preference for human as opposed to animal hosts. Furthermore, the intensity of microfilarial infection in the skin may play a critical role in determining the infection of the vector, since each species has an infection threshold.

Individuals who frequently visit the breeding sites or whose work requires them to spend long periods on the river bank (e.g fishermen, farmers) tend to have very severe manifestations of onchocerciasis. Prevalence of onchocerciasis is lowest in the first decade of life, after which it rises steeply to reach a peak usually, in the third decade of life (WHO, 1976; Onwuliri *et al*., 1987). Male / female differences in prevalence, intensity of infection and clinical manifestation of disease have all been observed (Brabin, 1990). Behavioural and community factors are most important in the planning, implementation, and evaluation of control measures. In the savannah areas, the intensity of exposure to transmission is determined by the distance between a community and a fly breeding site and by the presence or absence of other human settlements in the intervening area; these considerations have led to the characterization of villages as first-,

second-, and third line. Furthermore. Males are generally more affected than females, though gender-related differences may not appear till a certain age.

Another important determinant of intensity of infection is the density of the human population in relation to the vector population emerging from local breeding sites, as is the presence of cattle near rivers, since it reduces the contact of the human population with zoophilic vectors of *O.volvulus*. Additionally, the regular inoculation of the human population with bovine /animal Onchocerca L3 larvae may provide an immunological stimulus to the host and thus help prevent infection with *O.volvulus*.

1.4.7 Economic and public health importance

Wherever onchocerciasis exists at a high intensity and endemicity, it is a serious threat to the health of the populations concerned and an impediment to socio-economic development. The two factors responsible for the major public health impact of the disease are the serious eye lesions, which occur when the intensity of infection is high and where the strain of the parasite is pathogenic for the eye, and the prominent skin lesions. The socio-economic consequences of onchocerciasis are most marked in the hyperendemic belt that extends across sub-Saharan Africa, excluding the West African countries in the original OCP area, where the burden of onchocercal blindness has been greatly reduced as a result of control.

Within Africa, blindness rates in hyperendemic communities not under control may rise to 15%, and up to 40% of adults may show severe ocular impairment. When there are high rates of visual impairment, communities become unstable, their agricultural capacity declines, and eventually the villages are abandoned. Populations move to healthier local environments, away from the rivers where the vectors breed but where the soil is usually not so fertile. Furthermore, since blindness leads to a reduction of some 10 years in life expectancy, onchocerciasis is a disease that not only disables but is indirectly responsible for considerable premature mortality.

In Guinea for example, the impact of onchocerciasis-related blindness on the household is severe, most often resulting in the familyôs inability to support itself. The disruption of family life is directly related to stress within the household, contributing to its destitution. The extra burdens placed on other members of the family, once the main breadwinner is blind and can no longer continue with normal activities, have adverse effects on their physical, psychological and emotional health. The older children often choose to migrate, fearful of becoming blind themselves.

In Nigeria, Nwoke, (1990) reported that in persons with prolonged intense infections, the skin lesions and itching are responsible for much chronic misery and disfigurement, and can lead to a degree of social isolation, with detrimental psychological effects. Also reported in Nigeria is the social stigmatization and rejection of those with skin disease because of the belief that it is caused by dirtiness (Amazigo and Obikeze, 1991). As a result of onchocerciasis, quality of life is reduced and the problems could be compounded by conditions like multiple infections, malnutrition and starvation (Ukoli, 1984).

In the Americas, the socioeconomic consequences of onchocerciasis have not been fully studied and thus requires further study particularly with regard to the impact of blindness and skin disease on productivity.

In the southern Venezuelan foci, cultural interpretation of the origin of some of the disease signs and symptoms, as well as the mechanisms of onchocerciasis transmission, are sometimes a source of conflict between communities. These cultural factors also determine the social acceptance of the changes in physical appearance associated with the disease, although individuals with severe skin manifestations (e.g. hanging groin) are socially isolated (Petralanda *et al.,*, 1993). In the Amazon Basin, a major consequence of the exploitation of forested areas through mining activities will be infection of migrant workers with *O.volvulus*, that over the next decade, the movement of these workers into previously non-endemic areas of South America,

where potential vectors exist may have severe long-term social and economic consequences for several countries in the region. The socio-economic impact of this disease could be summarized as loss of labour (Prost and Prescott, 1984; Prost, 1986) mortality and decreased life expectancy of infected persons (Prost and Vangelade, 1981; Duke, 1990) migration and depopulation of endemic areas which are usually fertile lands (WHO 1976), social stigmatization of persons affected with dermal lesion (Nwoke 1990, Amazigo and Obikeze 1991) as well as loss of productivity due to morbidity (WHO 1997; Benton 1998). Until recently, the blindness and skin pathology caused by heavy infections constituted a major public health problem in many parts of tropical Africa, Yemen and Latin America. This consideration led to the establishment of the Onchocerciasis Control Program (OCP) (Molyneux, 1995) in West Africa, the Onchocerciasis Elimination Program in the America (Blanks *et al*., 1998) and the African Program for Onchocerciasis Control (APOC) (Remme 1995 and WHO 1999). All the three programmes have to rely on the regular (OEPA semi-annually, OCP, both annually and semiannually, and APOC annually) distribution of ivermectin to lower the microfilarial load in affected communities and thereby reduce transmission and mitigate the clinical manifestations of the infections (Goa *et al*., 1991).

Figure 3: Onchocercal blindness: soon to be consigned to the history books?

(Adapted from TDR THIRTEENTH PROGRAMME REPORT, 2001)

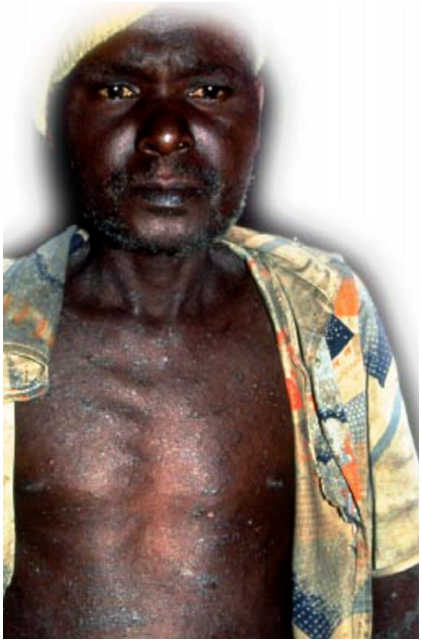


Figure 4: Disfiguring skin lesion due to onchocerciasis.

(Adapted from TDR THIRTEENTH PROGRAMME REPORT, 2001)

1.4.8 Control of onchocerciasis

1.4.8.1 History of early control methods

Previous efforts at halting onchocerciasis include the aerial spraying of biodegradable larvicides in West Africa-(a co-operative effort of World Health Organization-related groups) and the use of drug treatments-(only two anti-onchocerciasis compounds, Diethylcarbamazine citrate (DEC) and Suramin).

Vector control is based on the insecticide treatment of rivers in which the larvae of vector species develop. It rarely takes more than 1-2 weeks for the pre-adult stages to develop from eggs to pupa, and this means that insecticide must usually be applied weekly. In Africa, because of the large areas and number of breeding sites to be treated, insecticides are sprayed mainly from the air. Ground treatment may, however, be undertaken in accessible and isolated areas. Following the eradication of *S. neavei* from the Kodera Valley in Kenya by the application of DDT, French entomologists undertook large-scale trials in West Africa on larviciding of *S.damnosum*. The conclusion of these field studies and pilot projects was that ground control was quite successful in reducing the biting blackfly population as long as control activities were maintained, but the impact on transmission was limited by the regular influx of flies from surrounding untreated areas. Much larger areas would need to be treated to obtain a lasting effect.

With this objective in mind, experiments were carried out by the ORSTOM (Institut Francais de Recherche Scientifique pour le Development en Cooperation) entomologists assigned to OCCGE (Organisation de Cordination et de Cooperation pour la Lutte contre les Grandes Endemies), and a control programme was started in 1962 in an area covering parts of Cote dÕIvore, Burkina Faso (then Upper Volta) and Mali, with financial support from the European Development Fund (EDF) and the French Aid and Co-operation Fund. With the

emerging indications that control was possible, health authorities in the countries most affected invited the scientific community to study the operational aspects of a large-scale control programme. This led to a technical meeting in Tunis in July 1968, of experts in Public health, parasitology, epidemiology, entomology, ophthalmology, economics, sociology and medical geography under the auspices of a joint USAID/OCCGE/WHO.The meeting emphasised that a mass campaign against onchocerciasis should aim at the eradication of this endemic disease. After a series of other joint meetings involving Government of Ghana, OCCGE, EDF, USAID, FAO, the World Bank, UNDP, and WHO, an initial programme known as the PAG Mission (Preparatory Assistance Mission) was initiated with regards to the involved countries-Governments of Dahomey (now Benin), Ghana, Cote dôIvoire, Mali, Niger, Togo and Upper Volta (now Burkina Faso).

The objectives of the PAG Mission centered on aerial larviciding over an area then believed to be sufficiently large to avoid re-invasion of infective blackflies. The larviciding was to be conducted continuously for 20 years, the period considered necessary for the adult worm reservoir in the human population to die out in the absence of renewed infection. The control area defined by the PAG Mission was confined to the Savannah zones where the blinding form of onchocerciasis was prevalent, and excluded the forest zones characterized by the milder form of the disease. This area delineated for control corresponded largely also to that identified at that 1968 Tunis meeting (WHO, 1995). Following a planning meeting in Accra in October 1972 which discussed the reports of the PAG mission, the representatives of the governments of the seven participating countries signed on November 1, 1973, óAgreement governing the operations of the Onchocerciasis Control Programme in the Volta River Basin areaô with the World Health Organisation as the executing agency of the programme. This thus led the existence of the OCP (Onchocerciasis Control Programme).

So far, the benefits of the OCP are enormous. Currently vector control (OCP operations) has achieved the virtual interruption of transmission in 90% of the original OCP area and the incidence of infection in children has been reduced by 99% . 30 million people have been protected from onchocercal disease - 10 million in the original Programme area where the threat has been virtually eliminated with the disappearance of the parasite reservoir in humans after 14 years of vector control, and 20 million in the extension areas where transmission has been halted by aerial larviciding and where the reservoir will disappear by the turn of the century. It is estimated that 9 million children have been born within the OCP area since operations began and none of them has ever run the risk of contracting onchocercal blindness, and will never do so within the OCP area. Thus, by the year 2000 or before, the numbers of children protected have grown to 15 million. When the programme started, there were those seriously infected with onchocerciasis and therefore in grave danger of developing ocular manifestations, but among them, 1.25 million are no longer infected; this figure is expected to increase to 2 million before or by the year 2000. With regards to blindness, more than 100 000 cases of blindness have so far been averted by OCP control operations, a figure that will rise to 150 000 before the end of the century. Therefore, application of larvicide to the breeding sites of the blackfly is currently the only means of interrupting transmission sufficiently to allow the human reservoir of *O. volvulus* eventually to die out, and will remain so until elimination of the adult worm can be achieved by community-wide application of drugs.

International programs supported by the World Health Organization and many other groups have worked to control the impact of onchocerciasis using vector control with insecticides beginning in 1974 and mass drug administration (MDA) with ivermectin (IVM, brand name Mectizan) beginning in 1987 (Peters *et al*., 2004). IVM is a highly effective microfilaricide and inhibits female worm microfilarial production for several months. Annual IVM MDA reduces morbidity (Ejere *et al*., 2001; Tielsch and Beeche, 2004) and lowers

transmission (Boussinesq *et al*., 1997; Collins *et al*., 1992). From 1974 to 2002, the Onchocerciasis Control Programme (OCP) in West Africa greatly decreased *O. volvulus* transmission in the 11 OCP countries and prevented 600,000 cases of blindness (Molyneux *et al*., 1995, Boatin and Richard, 2006 and Basanez *et al*., 2006). IVM without vector control has been the principal tool for the Onchocerciasis Elimination Program of the Americas (1992ï present) (Boatin and Richards, 2006) and the African Programme for Onchocerciasis Control (1995ï present). In the Americas, where *O. volvulus* is less common, the Onchocerciasis Elimination Program has substantially reduced transmission to eliminate the disease.

Diethylcarbamazine developed in 1947, is a microfilaricide (it kills microfilariae) and must therefore be given repeatedly as long as the patient harbours fertile female worms. It must be given orally daily for seven to ten days. It provokes severe and sometimes dangerous systemic (Mazzoti) reactions and aggravate existing ocular lesions, or precipitate new ones, as a result of the sudden, massive death of the microfilariae. DEC may itself even cause blindness and death (Boatin and Richard, 2006).

Suramin, available as from 1920 for the treatment of sleeping sickness, was shown in the late 1940s also to be effective as a macrofilaricide (it kills the adult onchocercal worm). It is the only macrofilaricidal drug and has to be given intravenously once a week for several weeks up to two months. Its administration may be accompanied by severe rash, anaphylactic shock, kidney, liver and gastrointestinal complications and sometimes death (Manson-Bahr and Bell, 1991).

These drugs were deemed unsuitable for mass use because they produce only short-term or unsatisfactory suppression of microfilariae and patients require close medical supervision during administration. It was therefore apparent that the development of a new drug that is safe and effective, preferably one that may be administered orally as a single dose for mass

chemotherapy must continue to be a major research goal. This search for a new drug for onchocerciasis therapy has led to the development of ivermectin(IVM).

Towards the end of last decade, some other drugs for the treatment of onchocerciasis evolved. Amocarzine (CGP 6140) is an antifilarial anthelmintic isolated from amoscanate. It is active against adult worms of *O.volvulus*. Amorcazine is toxic to mitochondria and causes inhibition of respiration. The basis for its selective toxicity appears to be preferential drug uptake by the filarial worm (Kohler *et al*., 1992).

Moxidectin is a milbemycin drug used in veterinary medicine for the prevention of heartworms and intestinal worms in cats, horses, cattle and sheep. Moxidectin is a fermentation product from Streptomyces cyaneogriseus spp. Noncyanogenic. It is chemically related to avermectins. However, moxidectin has a longer lasting microfilaricidal effect than ivermectin by virtue of its much longer half-life, 20 days as compared to 2 days (Tagboto and Townsend, 1996; WHO, 2000). Moxidectin also shows promising macrofilaricidal activity in animal screens and has been shown to be safe in preliminary (Phase 1) human trials (WHO, 2000; Cotreau *et al*., 2003). Moxidectin is being evaluated in phase II clinical trial, in which 192 persons infected with onchocerciasis are enrolled. Following the period, moxidectin will hopefully be available to endemic countries by the end of this year 2012 (Eezzuduemhoi and Wilson, 2008).

1.4.8.2 Antibiotic therapy

The break-through of using antibiotic to target the bacterial symbiont of the parasite has identified a novel treatment and target that offers a superior therapeutic altermative to current anthelminthic drugs (Johnston and Taylpr, 2007; Hoerauf et al., 2009). The rationale for this novel treatment is the antibiotic targeting of *Wolbachia* a bacterial endosymbiont of filarial parasites. Treatment with doxycycline for six (6) weeks in a controlled trial eliminated

Wolbachia from adult worms resulting in suppression of embryogenesis, and most importantly death of adult worms (Hoerauf *et al*., 2001). In contrast ivermectin only works against late- stage developing microfilariae still in the uterus, and it has little or no effect on early-stage embryos. These suggest that infected patients who permanently leave in areas of enedemicity should be offered, in addition to ivermectin, a 4 ï 6 week course of doxycycline (100-200 mg per day) to achieve long term amicrofilaridermia (Udall, 2007). However, caution should accompany the concurrent use of ivermectin and doxycycline, because these agents have not been formally studied for interactions (Udall 2007). The outcome of doxycycline therapy has several advantages including the elimination of the inflammatory inducing bacteria (Leiser *et al*., 2002), and avoidance of potential adverse reactions to nematode products associated with a rapid- kill as observed in loasis co-infection (Hoerauf *et al*., 2009; Taylor and Hoerauf, 1999; Hoerauf *et al*., 2001, 2003). However, a major obstacle to the use of antibiotic therapy in control of onchocerciasis is the length of the treatment regimen, which is considered to be logistically imcompatible with the community directed treatment strategies used in filariasis control and contraindications for children > 9 yrs and pregnancy (Taylor *et al*., 2009). In consideration of all these, Ivermectin thus remains a more acceptable therapy for onchocerciasis.

1.4.8.3 Treatment with ivermectin

Although five classes of chemotherapeutic agents are potentially available for the treatment of onchocerciasis, only one drug, ivermectin, is actually being used regularly for this purpose. Diethylcarbamazine (DEC) and suramin are considered unsuitable for large-scale therapy, while the two groups of potentially macrofilaricidal compounds, thioureas and benzimidazoles, are still at the stage of experimental drugs. Amocarzin (CGP 6140), the piperazonyl derivative of amoscanate, has been shown to have good macrofilaricidal actvity in animal infections, and has been developed to the stage of Phase II-III clinical trials (Poltera et al., 1991). Most of the trials have been carried out in Ecuador and Guatemala. Benzimidazoles

have always shown good macrofilaricidal activity in screening models for filariasis. However, to date, this activity has not been observed in onchocerciasis patients. Benzimidazoles designed to act as anthelminthic agents against nematodes are poorly absorbed, they bind to the tubulin making up the microtubules of the mitotic spindles of the submembrane network, and so interfere with cell division and substrate transport. Multiple doses of mebendazole and albendazole given orally and flubendazole given by intramuscular injection show little or no macrofilaricidal activity in human onchocerciasis. Their primary action is a toxic effect on the embryonic stages of the parasite. Flubendazole when studied, induced severe inflammation and sterile abscesses at the injection site. A flubendazole prodrug (UMF, 078) has been developed that is macrofilaricidal by both oral and intramuscular routes in animals. When the prodrug is given intramuscularly as a suspension in oil, the inflammatory response seen with flubendazole itself is absent. Two or three doses of UMF 078 are required for full macrofilaricidal activity in animal models; this compound is currently entering the preclinical toxicology phase of development.

IVM is a chemical modification of one of a series of naturally occurring substances designated as avermectins. Avermectins are a family of macrocyclic lactones discovered and developed as anthelmintic agents at the Merck Research Laboratories in the middle to late 1970s (Burg *et al.,* 1979; Egerton *et al.*, 1979). They are naturally fermentation products synthesized by the soil bacterium, *Streptomyces avermitilis*. The avermectin-producing organism was initially isolated from a soil sample collected in Japan as part of a collaborative agreement between the Merck Research Laboratories and the Kitasato Institute in Tokyo (Burg et al., 1979). The anthelmintic activity of the test sample was detected by incorporating the fermentation broth into the diet of mice that had been experimentally infected with the intestinal nematode *Heligmosomoides polygyrus* (*Nematospiroides dubius*) and then monitoring for fecal egg output and presence of worms in the gut as an indication of efficacy. This screen, like many

in vivo screens, allowed for simultaneous assessment of the compoundôs efficacy, oral activity and bioavailability, and absence of toxic side effects. Subsequent investigations revealed that this class of compounds also possessed potent insecticidal activity (Ostlind et.al., 1979) but lacked antibacterial or antifungal properties.

Avermectin nomenclature is based on the biosynthetic variations that occur at the C-5 and the C-22,23 positions and the C-25 side chain. The C-5 hydroxy-substituted avermectin analogs (B series) are generally more potent anthelmintic agents than the C-methoxy derivatives (A series). Ivermectin (22,23-dihydroavermectin B1a)*, a semisynthetic avermectin analogs (Chabala *et al.,* 1980) was introduced commercially in 1981 and rapidly became the drug of choice for treating a broad spectrum of conditions caused by nematode and arthropod parasites. Ivermectin is a highly lipophilic 16-membered macrocyclic lactone, selectively binds to postsynaptic glutamate-gated chloride ion channels in muscles and nerve cells of the invertebrate. Increased permeability of cell membrane to chloride ions leads to hyperpolarization of the nerve or muscle cell, paralysis, and death of the parasite (Eezzuduemhoi and Wilson, 2008).

Ivermectin (IVM) has activity against a wide range of nematode and arthropod ecto- and endo- parasites in domestic animals (e.g Poultry, dogs, goats, sheep, swine, cattle, horses): Example of these parasites include *Strongylus* Spp., *Haemonchus* Spp., *Ancylostoma* Spp. and *Dirofilariae* Spp. It has also been found to be active against human gastro-intestinal nematodes (*Strongyloides* Spp., *Trichuris trichuria*, *Enterobius vermicularis*) and filarial parasites especially *O. volvulus*. Ivermectin has also shown activity against various other nematodes including Wuchereria bancrofti (Diallo *et al.,* 1987; Ottesen *et al*., 1990; Rout *et al.,* 1989), *Loa loa*, *Ascaris lumbricoides* and *Strongyloides stercoralis* (Datry *et al.,* 1991; Freedman *et al.,* 1989; Naquira *et al.,* 1989; Anosike, 2000)

Since its release in 1987, for the treatment of onchocerciasis, ivermectin has gained wide acceptance as a potent anthelmintic for the control of onchocerciasis. Ivermectin is safe, effective and relatively well tolerated drug for the treatment of onchocerciasis. Ivermectin is given as a single oral dose of 150 µg/kg of body weight once or twice a year. It should not be given to children under the age of 5 years or weighing less than 15 kg, during pregnancy, to mothers nursing infants during the first week of life, and in severe illness, as specified in the manufacturerôs exclusion criteria. Different regimens may be required to achieve different objectives, such as the improvement of specific severe ocular or skin manifestations, the control of transmission, and more pronounced effects (macrofilaricidal or sterilizing) on the adult worms. Ivermectin has no pharmacological activity in humans or intrinsic toxicity in single doses of up to 600 µg/kg of body weight or in multiple doses of 100-150 µg/kg of body weight given every 2 weeks, monthly or every 3 months up to a total dose of 1.8 mg/kg of body weight. The reactions seen from patients who took ivermectin were qualitatively similar to those observed following the use of diethylcarbamazine (i.e. the mazzotti reaction) but were much less frequent and less severe. The common side-effects includes itching and rash, musculoskeletal pain, relatively painless swelling (oedema) of the limbs and face, fever, and gland pain and swelling. There have been no life-threatening side effcets or mortality attributable to ivermectin. Severe reaactions are uncommon and those requiring the use of corticosteroids are rare. Most reactions can be managed with simple analgesics and antihistamines. If those receiving ivermectin are advised to rest in bed when feeling weak or dizzy, the incidence of severe symptomatic postural hypotension can be reduced from approximately 1 per 1000 to nearly zero. Reactions commence on the first day after treatment, most of the severe reactions occurring by the second day. There is a direct correlation between infection intensity and the severity of musculoskeletal pain, fever and lymphadenitis, but there is no such relationship for cutaneous reactions. Reactions are prominent in localised onchodermatitis and may be more frequent in

non-resident visitors to endemic areas despite low skin microfilarial counts. Thus previous constraints on the use of diethylcarbamazine do not apply to ivermectin. Even on repeated dosings of individuals or populations , reactions are greatly diminished. However, reactions such as acute laryngeal oedema, attacks of asthma in known asthmatics, bullae, and the late development of abscesses have also been reported, but it might possibly represent pre-existing or coincidental illnesses that appeared at the same time as ivermectin administration. There have been no reports so far on the outcome of pregnancy or on the course of epilepsy.

Although the treatment of patients with concomitant nematode infections has not been fully studied, but investigations to date in onchocerciasis patients who also have loasis have not shown any increased morbidity or adverse experiences and there have been reports of Ascaris spp. and Taenia Spp. excretion from children with multiple worm infections (Okonkwo *et al*., 1991) field observation (Freedman *et al*., 1989; Anosike *et al*., 2007).

Ivermectin is microfilaricidal and thus has a potent, rapid action against skin microfilariae. Although it is still uncertain about the mechanism of action on *O.volvulus*, recent studies of the ivermectin receptor in the free-living nematode *Caenorhabditis elegans* show that in this nematode, ivermectin binds at extremely low concentrations to a membrane chloride channel normally controlled by glutamate.

Massive reductions in microfilarial counts occur during the first few days, and maximum reduction may however, not be achieved for 2 or more weeks. Skin microfilariae migrate into the dermis about 24 hours after ivermectin is given, and elicit little inflammatory reaction (Duke *et al.,* 1991), unlike in the administration of diethylcarbamazine, when microfilariae migrate towards the epidermis as early as 1-6 hours after treatment and become the foci of microabscesses. These differences might account for the much less severe cutaneous manifestations seen after treatment with ivermectin as compared with diethylcarbamazine. Most microfilariae are probably killed in the lymph nodes after treatment with ivermectin, as their

density there increases by a factor of about 1000 (Darge *et al.,* 1991). Suggestively, ivermectin causes them to migrate from the subepidermal layer into the deeper layers of the dermis, then into fatty and connective tissue and finally into the regional lymph nodes. On the other hand, Vuong *et al*. (1988) found that if the microfilariae are predominantly resident in the lymphatics, they could either move actively into the lymph nodes when ñmobilizedôô by ivermectin or be swept passively along the nodes. Almost all microfilariae in the nodes are dead or dying, surrounded by eosiniphils in the early stages, and later by histiocytes and giant cells. With ragards to the adult worms, ivermectin appears neither to be macrofilaricidal for *O. volvulus* nor to affect embryogenesis or spermatogenesis. Its most marked effect in the adult worms is a block in the release of stretched microfilariae from the uterus followed by microfilarial degeneration. These effects last for more than six months and are mainly responsible for the prolonged suppression of skin and ocular microfilariae counts. Other effects include a reduction in the percentage of nodules in which intact microfilariae are present in the nodular tissue. Multiple doses of the standard regimen have been given at varying intervals up to a total of 12 doses and 1.8mg/kg of body weight. Repeated yearly doses appear to have some macrofilaricidal activity, but the greatest effects against adult worms followed 11 doses given at 3-monthly intervals. This resulted in an excess mortality of the female worms (32.6% greater than in the control group who received no drug treatment), a reduction in the number of live male worms and in the proportion of inseminated females, and a cessation of microfilarial production (Duke, 1992). Ukaga *et al*. (2000) and Anosike *et al*. (2007) reported that though Ivermectin is not technically a macrofilaricde but they observed in the course of routine evaluation and monitoring of on-going Community Directed Distribution of Ivermectin (CDTI) in endemic communities in the rain forest of south-eastern Nigeria, that some villagers reported the disappearance or dissolution of onchocercal nodules after repeated treatment with ivermectin.

With regards to ocular changes, during the first 3-4 days after a single dose of ivermectin, the number of microfilariae in the anterior chamber of the eye either remains unchanged or increases temporarily. Corneal microfilarial are not increased in the first few days and a reduction in the number of ocular microfilariae does not occur for at least 2 weeks, and microfilariae may not be eliminated for 3 or more months.

After repeated doses of ivermectin (150µg/kg of body weight given annually or semi-annually): there is a significant reduction in ocular microfilarial loads and a 90% decrease in prevalence after 2-4 years. There is at least a 50% reduction in the prevalence of early iridocyclitis and a reduction of up to one-third in the prevalence of sclerosing keratitis after 2-4 years. There is less marked impact on posterior segment lesions than on anterior segment lesions . There is a significant reduction of about one-third in the overall incidence of optic atrophy, which is suggestive of a substantial reduction in *O.volvulus*-specific lesions. There is no impact on the prevalence of chorioretinitis. No significant benefit in terms of visual acuity is observed but there is a significant reduction in the occurrence of marked loss in paracentral visual fields, especially in those with pre-existing optic nerve disease. There is no evidence of a reduction in the prevalence of blindness and it may take a long time for the effect of ivermectin on the incidence of blindness to become apparent; These were some of the conclusions reached following four community-based studies in Cameroun, Ghana, Nigeria and Sierra Leone on the effect of repeated doses of ivermectin (WHO, 1993, unpublished document). However, so far for the control of onchocerciasis Ivermectin has proved to be both highly effective and well tolerated in clinical and community trials. Ivermectin treatment combined with vector control significantly reduced onchocercal dermatitis, microfilariae carrier rates and nodule prevalence

(Brieger *et al*., 1998; Seidenfaden *et al*., 1998; Emukah *et al*., 2004; Ndymugyenyi *et al*., 2004). This efficacy has made ivermectin to be acclaimed by all and it is being distributed world-wide.

Three main bodies play a significant role in the stimulation and support of ivermectin distribution;

(1)The Mectizan Donation Program which was established in mid-1993 by the Carter Center to expand the activities of the Mectizan Expert Committee in support of non-governmental agencies involved in ivermectin distribution, create regional coalitions of individuals and organizations distributing ivermectin, to generate resources to support distribution, and to review methods and strategies designed to sustain distribution.

NGO Coordination Group for Ivermectin Distribution which is an open-ended group whose membership currently consists of Africare, Christoffel-Blindenmission, Helen Keller International Inc., the International Eye Foundation, Organisation pour la Prevention de la Cecite, the River Blindness Foundation and Sight Savers(formerly the Royal Commonwealth Society for the Blind),the Mectizan Donation Program and WHO. The Group which was established in December 1992 aims to promote world-wide interest in, and support for, the use of ivermectin in the treatment of onchocerciasis in endemic countries, and to assist interested countries or groups of countries in planning, implementing and evaluating ivermectin distribution programmes.

(2) Onchocerciasis Elimination Program in the Americas (OEPA) which is a multinational, multi agency and multi donor initiative, created in September 1991 by the Pan American Health Organization and aims at eliminating the severe pathological manifestations of the disease and to reduce morbidity in the Americas through the mass distribution of ivermectin.

International programs supported by the World Health Organization and many other groups have worked to control the impact of onchocerciasis using vector control with insecticides beginning in 1974 and Mass Drug Administration (MDA) with ivermectin (IVM, brand name Mectizan) beginning in 1987 (Peters *et al*., 2004). Annual IVM MDA reduces morbidity (Ejere *et al*., 2001; Tielsch and Beeche, 2004) and lowers transmission (Boussinesq *et al*.,

1997; Collins *et al*., 1992). From 1974 to 2002, the Onchocerciasis Control Programme (OCP) in West Africa greatly decreased *O. volvulus* transmission in the 11 OCP countries and prevented 600,000 cases of blindness (Molyneux, 1995; Boatin and Richard, 2006; Basanez *et al*., 2006). IVM without vector control has been the principal tool for the Onchocerciasis Elimination Program of the Americas (1992ï present) (Boatin and Richards, 2006) and the African Programme for Onchocerciasis Control (1995ï present). In the Americas, where *O. volvulus* is less common, the Onchocerciasis Elimination Program has substantially reduced transmission to eliminate the disease.

For more than a decade, ivermectin has been distributed world-wide and the number of treatments provided annually continues to grow at a dramatic rate. In 1990, 1.4 million people received ivermectin, in 1991, 2.8 million, in 1992, 5.3 million and in 1993, 9.2 million people (impressive but represents a relatively small proportion of the 100 million people at risk from the disease) (WHO, 1995). Various methods of distribution are being employed, each with its own advantages and disadvantages;

(3) Community-based distribution- where the village leader appoints someone to be trained as a community-based distributor to carry out the yearly treatment within the village. Active treatment campaigns - where organized teams travel to the endemic communities to treat people, a typical example of what obtains with the Oncho. project in Achi, south-eastern Nigeria.

Through passive treatment campaigns - where drugs are left at fixed health posts within the community, and patients are encouraged to visit such posts.

Although ivermectin does not, have such a decisive impact on transmission, as had originally been hoped, encouraging results have been obtained from some large scale community trials (Chabala *et al.,* 1980; Taylor and Greene, 1989; Soboslay *et al.,* 1987, 1991) with regards to skin microfilarial reductions. The efficacy of antifilarial drugs is measured by the

reduction of microfilariae densities in the skin and by the observations of dead or degenerate parasites in the nodules and uteri contents of the female worms (Schulz - Key, 1987). This has been demonstrated in large scale community trials involving tens of thousands of onchocerciasis patients in Ghana comprising a study population of 24 575 people, a total of 14 991 (61.5%) received ivermectin and there was a 92% reductions in skin microfilarial load after 2 months (Remme *et al.*, 1989). In Liberia, in 1987, a total of 7699/7956 were treated and 86% reductions in skin microfilariae was recorded after 6 months (Paque *et al.,* 1990a), and in 1988, out of a total of 13977 people, 8062/8438 (eligible at 96%), 78% reductions was obtained after 12 months(Paque *et al.*, 1990a).

In Nigeria, Ivermectin distribution in Nigeria started as early as in 1989 in different parts of the country under different auspices. The establishment of Onchocerciasis Operation Research (OOR) by TDR in 1990 was a major reorganization that brought success in the disease control in Nigeria (Nwoke and Dozie, 2001).

In 1992, TDR, OOR and Federal Ministry of Health funded a 5-group multicentre, Nigeria Scientists on Rapid Assessment method (RAM) to identify communities/zones eligible for large-scale treatment with ivermectin. Later, Rapid Epidemiological Mapping of Onchocerciasis (REMO) was adapted as a standard protocol for producing acceptable baseline results for the control of disease in Africa and Nigeria using only nodules as a basis for estimating endemicity. Thus an area with the community prevalence rates of nodules > 20% is a priority area where ivermectin mass treatment is indicated (Gemade *et al.*, 1998). The achievements of the Nigerian CDTI programme with the bold back up provided for the National Onchocerciasis Control Programme (NOCP) by the River Blindness Foundation, LION club, The Global 2000 River Blindness Programme, and other NGOs; so far include:

- Treatment of over 17 million people since 1999 (in 1988 only 6270 people treated).

- Training of over 20,848 health staff at all levels.
- Adequate and free flow of ivermectin
- High level of awareness of the programme
- 75% to 80% of all CTDI communities were actively involved in community directed drug distributorsô selection, drug distribution, choice in distribution method, month and CDD training.
- 63% offered suitable incentives to their CDDS.
- Appreciation of the benefits of ivermectin
- Transmission has dramatically declined.
- Incidence of new infection is nearly zero in most foci.
- Today 33 states under treatment (in 1988 only 2 states were covered).
- Oncho control activities gradually intergrated into primary health care.

Treatment coverage rate increased per year by 8 to 10% (Nwoke and Dozie, 2001). Despite these successes recorded there are still other challenges and problems that must be addressed, such as more research needed and development efforts as well as the provision of substancial research grants and other necessary incentives for promoting investigation into various facets of the onchocerciaisis problems. Other crucial considerations such as the looming problem of drug and pesticides resistance as have been variously suggested. This work targets to plunge into the issue of resistance.

1.4.9 Possibility of resistance

In Achi, an onchocerciasis-endemic zone in south-eastern Nigeria, a project supported by the UNDP/WORLD BANK/WHO with the aim of investigating the epidemiology and

controlling onchocerciasis, started ivermectin distribution in October to December 1990 on a yearly basis. Achi, an area described as savannah mosaic revealed that microfilaridermia was found in 76% of the population and nodules in 62%. Skin and ocular lesions of onchocerciasis including sowda were common and *S. damnosum* complex were found breeding along the entire length of the Oji River and its tributaries. Biting occurred all year round but peaked in the months of March, April, and May (Okonkwo *et al.,* 1991, 1992 unpublished).

Despite the wide spread use of ivermectin, there is considerable uncertainty surrounding the mechanism of action. In Achi, ivermectin administration has helped to reduce the microfilarial density of the population but some individuals who have been dosed still show high microfilarial levels. It is also evident that after frequent administration, most organisms (viruses and parasites in particular) seem to have the capacity to become resistant to drug originally used for their control. The reason for this is not known, since ivermectin has been used in Nigeria for quite a while, this might be innate. A further question is whether this is resistance will it be consistent and spread in the course of repeated mass treatments? as in the case of *P.falciparum* malaria and chloroquine.

The trials in Achi showed that in some of the onchocerciasis patients, the drug is not effective. While reductions in the skin microfilarial loads were observed after the first dose, by the second and third doses, further reductions were obtained but there were still some few patients without any reduction in their skin microfilarial loads observed)**Okoli, M.N. personal observations.

One reason for this persistent microfilariae may be reinvasion by infected *Simulium* flies or malreabsorption of the drug by the patients, but there is also the possibility that it may be the early signs of resistance development. Since 1995, the African Programme for Onchocerciasis Control (APOC) has been covering 19 of the continentôs 28 countries hit by the disease. Access to this treatment is possible for 70 million people and has significantly diminished the

onchocerciasis-induced morbidity. However, the doubling of cases of infection in certain communities of Ghana between 2000 and 2005, in spite of annual treatments, created fear of the emergence of ivermectin-resistant strains. Such apprehension appears particularly justified in that a high degree of therapeutic cover is achieved during mass distribution campaigns and hence only a tiny part of the parasite population targeted remains unexposed to drug treatment pressure (Fletchet, 2008)

Parasites developing resistance to drugs directed against them have been one of the few ways that parasites are thriving to defeat man. Drugs which once could be counted on for protection against many parasites and infectious diseases are becoming less and less useful as resistance to them spreads. Drug resistance have been a major cause of setback in the treatment and control of many diseases.

The resistance of parasites and infectious disease organisms to drugs and antibiotics is as old as chemotherapy itself. In malaria chemotherapy, chloroquine was initially acclaimed as the answer to malaria control but currently chloroquine can no longer be used for non-immune patients in most endemic regions because of development of resistant strains of *P.falciparum* (Panisco and Keystone, 1990). Each year 400 million cases of malaria are reported and about two million children die from *Plasmodium falciparum* infection (WHO, 1994). The World Health Organization estimates that 300-500 million new cases of malaria are reported annually causing the death of about 2.5 million people (Van der Westhyzen and Parkinson, 2005). The increasing prevalence and distribution of malaria has been attributed to a number of factors, one of them being the emergence of spread of drug resistant parasites. The chemotherapy of cancer has proved insurmountable because of the development of drug resistance more especially multi-drug resistance. It is already evident that development of resistance by tissues or organisms to drugs is an evolutionary adaptation that puts at risk every tumouricidal, pesticidal and parasiticidal agent. Drug resistance is defined as a change in the gene frequency of a

population that is produced by drug selection whereby more drug is required to exact same effect than was required prior to selection. It is to be expected that when drug pressure is applied repeatedly and intensively, successive populations would move directionally towards less and less susceptibility. If the lack of susceptibility by a population is not the result of drug selection, then it is not resistance but only tolerance. Tolerance is defined as the innate lack of susceptibility that did not result from drug selection. Observations suggest a mechanism for drug resistance, whereby, there appears to be some barrier that kept the drug from reaching the interior of the cell, where it would have its lethal effect. Two possible theories were put forward to account for the evidence. One theory proposed that a permeability barrier prevented drug entry into the cells. The other suggested that an afflux pump, a mechanism that actively pumped drug out of the cell once it had got inside was at work in the resistant cells (Kartner and Ling, 1987). Whatever the actual mechanism, two points seemed clear, one was that the process of keeping drugs out of the cell would need to be rather nonspecific, that is able to cope with drugs of diverse molecular structure. The other was that because the cells surface membrane (the plasma membrane) is the first line of defence against the entry of drugs, the difference between drug sensitive and drug resistant cells would probably be found there. Multi-drug resistance was first noticed in cancer chemotherapy whereby tumour cells were simultaneously resistant - that is , cross resistant to completely unrelated drugs. Greater drug efflux is one of several features resistant malaria parasites share with drug-resistant cancer cells, in which the efflux is mediated by transport protein, the P-glycoprotein (Juliano and Ling, 1976; Di Pietro *et al*., 1999; Hung *et al.*,1998; Rosenberg *et al*., 1997)

1.4.9.1 Molecular nature of resistance

Glycoproteins are compact molecules made up of protein and carbohydrates that are usually associated with the plasma membrane. In P-glycoprotein, P- signifies permeability. P-glycoproteins are evolutionarily well conserved membrane bound proteins which belong to the

family of ATP binding cassette (ABC) transporters (Higgins and Gottesman, 1992) and widely represented in the animal kingdom. The P-glycoprotein located in the plasma membrane, consists of two similar halves, each with six transmembrane domains and an intracellular ATP binding sites (Endicott and Ling, 1989; Gottesman and Pastan, 1988). The P-glycoproteins are coded for by the mdr genes. Reported molecular weights of the P-glycoproteins ranges from 140-170 kDa and 180-210 kDa (Juliano and Ling, 1976; Endicott and Ling, 1989). However, recently, Thevenod *et al.,* 1994, 1996) reported some MDR1 related proteins with molecular weights of about 65kDa and 80kDa. The P-glycoprotein is normally found expressed in the kidneys, adrenal glands, liver, and parts of the gastrointestinal tract of the normal adult (Kartner and Ling, 1989; Schinkel *et al.*, 1994). The most consistent feature of cells with high level of multi-drug resistance (MDR) is the overproduction of the P-glycoproteins, thus increasing the outward transport of the drugs. The roles of the P-glycoproteins remain a mystery or more or less under controversy but they do transport peptides and in yeast, a lipopeptide (McGRath and Varsharsky, 1989). It is also thought that they provide a protective mechanism against exogenous toxins present in the diet and environment (Ames *et al.,* 1990), by actively extruding structurally and functionally unrelated agents, they can rescue the cell, and the organism from toxic drug effects. This mechanism of drug resistance has been found in the resistance of tumour cells to Vinca alkaloids, epipodophyllo-toxins, and anthracyclines (DeVita, 1989). A number of MDR cell lines have been isolated that show a good correlation between the levels of DNA amplification, increased mRNA expression and MDR. Working with MDR cell lines derived from human ovarian SKOV3 cells. At low levels of drug resistance (up to 64-fold relative resistance to vinblastine, and 16-fold relative resistance to vincristine in the cell line SKVLBO.03 selected with vinblastine), increase in P-glycoprotein mRNA and protein occurs without concomitant DNA amplification. P-glycoprotein gene amplification is only observed in

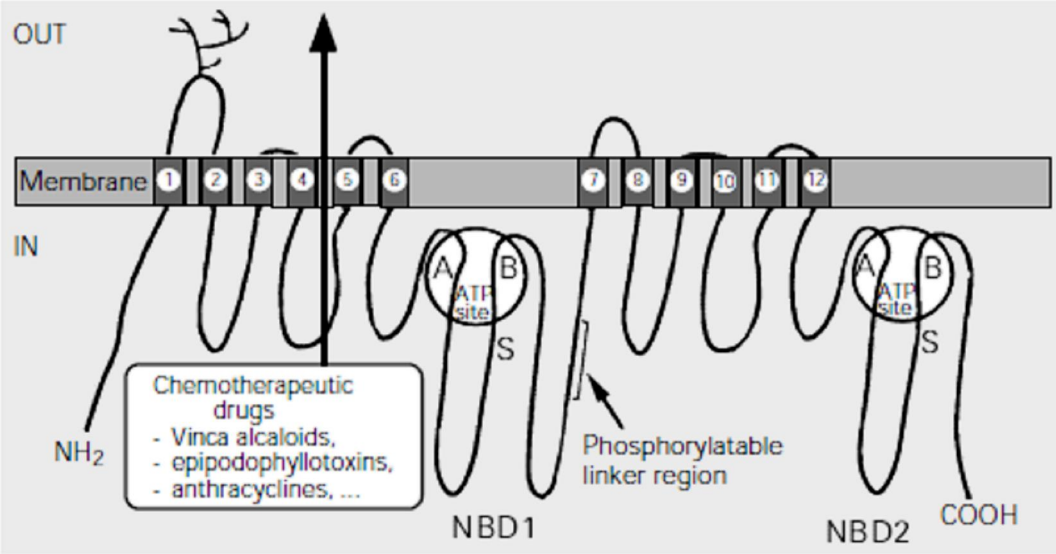


Figure 5: Schematic Structural Organization of P- glycoprotein

Di Pietro *et al*. (1999)

subsequent steps. Thus it appears that MDR cell lines can be selected that show no P-glycoprotein DNA or RNA amplification, but only with low levels of drug resistance. Subsequent selection for high levels of resistance results in DNA amplification, and over expression of P-glycoprotein mRNA. In general the levels of DNA, mRNA, and P-glycoprotein expression correlates reasonably well, but a number of exceptions have been reported. In addition to most commonly observed phenomenon of increased P-glycoprotein mRNA and protein levels as a result of DNA amplification, increase in mRNA and protein expression can also occur without P-glycoprotein gene amplification. This suggests that P-glycoprotein may be transcriptionally and/or translationally regulated and thus not an evolutionary process (Akiyama *et al.,* 1985, Shen *et al.*, 1986; Van de Bliek *et al.,* 1988 and Greenberger *et al.,* 1988). Amplified DNA can often be observed by light microscopy, either as an extended chromosomal region (ECR) or as extra-chromosomal elements, called double minutes (DMs) and the same gene can be amplified either chromosomally or extrachromosomally in a single population of cells, but the two forms do not usually coexist within the same cell (Cherif *et al.*, 1989) although a few examples to the contrary have been noted. A few different molecular mechanisms have been suggested by different workers (Carroll *et al.,* 1988; Passananti *et al.*, 1987). In one of the mechanisms, they proposed that the actual mechanism of DNA amplification on the molecular level occurs in a single cell whereby bidirectional replication at an origin generates a bubble that can undergo further rounds of unscheduled DNA replication, resulting in a nested set of partially replicated duplexes. It is then possible for linear duplex DNA to become detached from the structure if two replication forks can approach one another very loosely. Recombination within the same duplex could generate extrachromosomal circles, while multiple recombination among different duplexes could resolve the structure into an intrachromosomal linear array. Thus, an onion skin structure might give rise either to an ECR or to episome. The episome might then reintergrate giving HSRôs or remain extrachromosomal, perhaps evolving to form DMs (Carroll

et al., 1988). The structural key features of a typical P-glycoprotein which are consistent with biochemical and immunohistochemical data depicts that it consists of about 1280 amino acids arranged in two similar halves (Luurtsema *et al*., 2002).

Each half is joined by a highly divergent cytoplasmic region. Each half has a short hydrophilic amino-terminal segment, six hydrophobic membrane spanning domains that form three transmembrane loops and a hydrophilic carboxy-terminal region containing consensus sequences for a nucleotide-binding site, presumably responsible for adenosine triphosphate (ATP) binding and hydrolysis. There are several potential glycosylation sites on the first external loop near the amino terminal of the molecule. That each half of the molecule does not act independently to transport the drugs is suggested by the finding that inactivation of either one of the two nucleotide binding sites results in loss of functional activity (Azzaria *et al.,* 1989). The P-glycoprotein molecule presumably forms a channel-like structure through which substrates, including the drugs involved in MDR, are transported actively from the cytoplasm and or the plasma membrane to the outside of the cell. All members of the human and rodent mdr gene families have high sequence homology, indicating a common structural and functional pattern. Mammalian P-glycoproteins are encoded by small gene families containing two members in humans, MDR1 and MDR3 or MDR2, three members in mouse, mdr1a or mdr3, mdr1b or mdr1, and mdr2, and three members in Hamster, Pgp1, Pgp2 and Pgp3 (Chen *et al.,* 1986; Van de Bliek *et al.,* 1986, 1988; Gros.*et al*., 1986b; 1988; Hsu *et al*., 1989; Devault and Gros, 1990; Schinkel *et al.,* 1991). The P-glycoproteins has also been found in many other organisms e.g. invertebrates and parasitic protozoa (Wilson *et al.,* 1989). However, not all P-glycoproteins are involved in drug resistance, human MDR1 and mouse mdr1a and mdr1b can confer resistance, but MDR3 and mdr2 cannot (Gros *et al.,* 1986, 1988; Schinkel *et al.,* 1991; Devault and Gros, 1990). Hamster, pgp1 and pgp2 can confer resistance while pgp3 cannot. The mouse mdr2 gene (and its human MDR3 homolog) is involved in secretion of phosphatidylcholine into bile and is

probably a phospholipid translocator. In *Drosophila melanogaster* three P-glycoprotein genes have been identified and at least one of them (Mdr49) has been suggested to be implicated in drug resistance. The parasite Leishmania has a P-glycoprotein gene family of at least six members, some of which are involved in drug resistance, e.g ltpgpA is involved in low level oxyanion resistance, which includes resistance to antimony, the drug of choice in treatment of leishmaniasis (Callahan and Beverly, 1991), and ldmdr is involved in resistance to several compounds included in the mammalian MDR spectrum. In some resistant malaria parasites, *P.falciparum*, amplification of the P-glycoprotein gene (pfmdr1) was observed and it was concluded that chloroquine resistance was caused by a P-glycoprotein-mediated mechanism (Foote *et al.,* 1989; Wilson *et al.,* 1989) but Wellems *et al.,* 1990, and Wilson, (1993) disproved that when their results from the HB3xDd2 *P.falciparum* cross (Wellems *et al*., 1990) indicate that rapid efflux, chloroquine resistance phenotype is independent of the pfmdr1 and pfmdr2 genes, stating that the history of spread of chloroquine resistance suggests that acquisition of resistance involved very rare molecular events in *P.falciparum*, although the copy number of the pfmdr1 gene does correlate with the level of resistance in mefloquine- and halofantrine-resistant Plasmodium (Wilson, 1993). The malaria parasite's P-glycoprotein homologue, Pgh1, is known to influence the sensitivity of malaria parasites to a diverse range of antimalarial drugs, but the mechanism by which it does so has remained obscure (Saliba *et al*., 2008).

However, it is still unknown whether this P-glycoprotein can be associated with drug resistance in nematodes and in ivermectin and onchocerciasis. Recently, a family of Pgp homologues have been described in nematodes. In the free-living *Caenorrhabditis elegans* and in the sheep parasite *Haemonchus contortus*, there is evidence of Pgp genes. In *C.elegans* four P-glycoprotein homologs have been identified (pgp-1, pgp-2, pgp-3 and pgp-4) and three have been analysed in detail. They share similarities with mammalian P-glycoprotein in their predicted protein structure (Lincke , 1992, personal communication; Broeks, A. ,unpublished

results). The presence of an ivermectin resistant organism was confirmed in South Africa in November 1985, 33 months after the drug was introduced there. Since then, ivermectin resistance has been reported both in the field and in laboratory across the globe. In South Africa, Van Wyk and Malan, (1988) reported that four isolates of *Haemonchus contortus* from sheep to be resistant to ivermectin. In Brazil, resistance to ivermectin was detected in an *H.contortus* isolate from sheep. In New Zealand ivermectin resistance has been reported in *Ostertagia* spp. from goats (Badger and Mckenna, 1990).

Recent studies on P-glycoprotein and ivermectin using mice, further confirms that P-glycoprotein have a wide variety of functions useful to the well being of the cells. The mouse mdr2 P-glycoprotein is indispensable for the secretion of phospholipids into bile (Smit *et al., 1994*). Based on the tissue distribution of the human MDR1 P-glycoprotein (Cordon-cardo et al., 1989) it has been proposed that MDR P-glycoprotein plays a role in the protection of organisms against toxic xenobiotics, by active excretion of these compounds into bile, urine or intestinal lumen and by preventing accumulation in critical organs such as the brain. This postulation was proved when Schinkel and his co-workers (1994) generated an mdr1a P-glycoprotein-knock-out mice and administered it with ivermectin. The mice died of ivermectin toxicity at a concentration very low to affect the heterozygous mouse whose P-glycoproetin was intact (Schinkel *et al.,* 1994). Furthermore, mice without mdr1a P-glycoprotein and orally injected with ivermectin (0.2mg/kg) retained the drug in especially brain tissue more than the heterozygous mice with their mdr1a intact. Thus, suggesting that the P-glycoprotein coded for by the mdr1a might confer tolerance to ivermectin in other organisms with a blood-brain barrier. Meanwhile, *O. volvulus* have no blood-brain barrier.

The possibillity that Pgp is involved in anthelmintic resistance is real. At least two common anthelmintics. Ivermectin and closantel, are potential substrates and resistance to both occurs independently in strains of trichostrongylid nematodes from grazing sheep. In *H.*

contortus, no evidence of mdr gene amplification has been found in benzimidazole-resistant isolates. Levamisole-resistant isolates nor ivermectin-resistant isolates, rather Benzimidazole-resistance was found to entail a minimum of two genetic steps at separate ß-tubulin loci - first, through a series of steps at which different isotype 1 alleles are lost, followed by the loss of different isotype 2 alleles and other possible mechanisms (Roos *et al.*, 1995). Drugs that cause gene amplification in tumour cells do not cause amplification of P-glycoprotein genes in *C.elegans* even at much higher concentrations (Lincke, personal communication). On the other hand, of the four P-glycoproteins genes (pgp-1, pgp-2, pgp-3, pgp-4) identified in *C.elegans*, three have been analysed in detail. Pgp-1 and pgp-3 are expressed through out the life cycle, and exclusively expressed in the intestinal cells and pgp-3 was found to be involved in colchicine and chloroquine resistance in *C.elegans* (Broeks *et al.,* 1995).

Onchocerca volvulus is a parasitic nematode. Ivermectin is the current drug of choice for the treatment of onchocerciasis and ivermectin is a potential substrate. The possibility of resistance manifestations in the treatment of onchocerciasis patients with ivermectin is very real and it would be worth genuine practical consideration to ensure early surveillance so as to prevent another ócheмotherapy shockôor resistance outbreak like in the treatment of malaria.

However, the life cycle of *O. volvulus* (review in 1.1, and Schulz-Key et.al., 1987) is not as simple as that of *C.elegans* or *H.contortus* where you can maintain easily in-vitro or in-vivo in animal models. Up to date, only controlled efficacy tests requiring necrospy or FECR (Fecal egg count reduction) tests have been used to detect ivermectin resistance and because of practical considerations and expense, manufacturers recommended use-level has been employed as the threshold of resistance. Reliance upon necropsy following treatment cannot be routine procedure for resistance monitoring and it is unknown how sensitive FECR tests are at detecting ivermectin resistance at low frequencies. Five in-vitro tests to detect ivermectin resistance have been reported but non is used routinely (Gill *et al.*, 1991). All were tested against isolates that

are resistant to the use-level of ivermectin and whether they are able to detect resistance when gene frequencies are low is unknown, moreover these methods cannot be easily applied in humans.

The use of the PCR pool screening technique will make it possible to detect now the level of transmission in a given area with a limited investment of human and material resources. This technique may thus be a valuable tool for the surveillance activities to be conducted in the OCP area following the cessation of active vector control in 2002. In addition, the ability to accurately measure infection rates in the vector population may prove useful in monitoring the effect of ivermectin-based onchocerciasis control programs (such **as** the Onchocerciasis Elimination Program in the Americas and the African Program for Onchocerciasis Control) on transmission. Furthermore, the pool screen PCR is an efficient means to screen large numbers of flies for the presence of *O. volvulus* larvae. It may thus prove useful in establishing that a given area is freed of *O.* volvulus transmission following successful control.

Drug resistance has been defined as a loss of the normal response to treatment and is heritable (Prichard *et al*., 1980). Ivermectin affects both the microfilariae, removing them from the skin, and the adult worms, inhibiting their reproduction for many weeks. Because of these various and prolonged effects, and because ivermectin activity involves host immunity, it is not reliable to assess efficacy *in vitro*. Phenotypic assessment of resistance needs to consider both skin microfilarial loads (repopulation of the skin by microfilariae), and worm fertility (by embryogram). A meta-analysis was conducted of both these outcomes after single-dose ivermectin following a systematic review of early clinical and field trials and fitted a mathematical model to the data . Results were compared with those obtained in a study of 10 repeatedly treated communities in Ghana (with >10 annual treatments) (Osei -Atweneboana *et al.,* 2007). This study indicated continued high microfilaricidal activity of ivermectin but suggested that inhibition of reproduction by adult worms was impaired in some repeatedly

treated communities in contrast to an ivermectin-naïve community. Subsequent repopulation of skin by microfilariae was faster than expected even after considering the inter-study variability of the (also ivermectin-naïve) meta-analysis. A model for repopulation rates was fitted to microfilarial temporal profiles after treatment for each person examined in one of the communities (treated for 10 years) to quantify the level of inter-individual variability in parasitological response. Ivermectin resistance is common in veterinary parasites and has a genetic basis associated with selection on ATP-binding cassette (ABC) transporters (e.g. P-glycoproteins) and ß-tubulin. *Onchocerca volvulus* samples from communities in Ghana and Cameroon that have received many treatments have been found to have significant changes in similar genes (ß-tubulin, P-glycoproteins and other ABC transporters) compared with worms isolated from treatment-naïve subjects or the same subjects prior to treatment (Bourginat *et al*., 2006). These genetic changes should be useful markers for ivermectin resistance.

Isoenzyme analysis has also been used to characterize ivermectin-resistant and susceptible strains. Using starch-gel electrophoresis it was found that one ivermectin-resistant isolate of *H.contortus* did not show activity for propionyl esterase when the susceptible isolate did (Echevarria *et al*., 1992), in the same test, another isolate multiply resistant to ivermectin and benzimidazoles did have propionyl esterasse activity. Whether the loss of propionyl esterase activity in the ivermectin-resistant isolate had anything to do with its resistance or whether it was only coincidental is not clear. Thus a major obstacle facing investigators searching for an effective monitoring against ivermectin resistance in *O. volvulus* is the lack of an elaborate, complete and controlled maintenance system for *O. volvulus*. But, since some of the factors responsible for most mdr resistance manifestations (like the P-glycoprotein coded for by the mdr genes or the loss of isotype 1 and 2 alleles respectively) have been discovered in some other organisms, it will be of primary importance to investigate also in *O. volvulus* for such factors. To date, resistance of *O. volvulus* to ivermectin has not been clearly described but constant

surveillance must be maintained to ensure that any such development is detected after the cessation of control activities. The *Onchocerca* genome has to be studied (Jolodar and Brattig, 2009; Jolodar, 2010).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Description of the Project Area

The project areas, Achi and Amansea towns are located in South-Eastern Nigeria, a rainforest region described as savannah-mosaic area. Achi is a town under Oji River Local Government Area in Enugu state. Achi lies between Latitude 6^{o} 05 ñ to 6^{o} 16ò North and Longitude 7^{o} 16ò East to 7^{o} 25ò East (Latitude six degrees five seconds to six degrees sixteen seconds to the North and Longitude seven degrees sixteen seconds to seven degrees twenty five seconds to the East). It is about 45 km from Enugu town, the state capital of Enugu State, Nigeria). Achi is made up of 12 communities or villages popularly known as ósii na uno, isii na aguô with a population of about 40, 000. Although there are indications that each community was unique in the severity and infection pattern of the disease, as well as in their knowledge of and attitude to the disease, a preliminary survey found 76% microfilaridermiae in the population and nodules in 62% Skin and ocular lesions of onchocerciasis, including ósowdaô were common. Thus Achi is described as an onchocerciasis endemic area (Okonkwo *et al.,* 1991).

Amansea lies between Latitude 6^{o} 13 ñN to 6^{o} 19 ñ North and Longitude 7^{o} 06ò to 7^{o} 11 ñ East. It is about 70 km from Enugu town and about 20 ï 25 km from Achi. Amansea is an onchocerciasis meso-endemic area although there has not been much detailed report on onchocerciasis prevalence in Amansea and there was also no history of ivermectin treatment until now.

Ivermectin administration has started on a yearly basis since October 1990 in the Achi community. This was a project sponsored by UNDP/WHO/TDR on the epidemiology and chemotherapy of onchocerciasis in Achi. The principal investigator is Prof. P.O.Okonkwo. This project is a multi-factoral team project involving clinicians, ophthalmologists, parasitologists,

medical entomologists, pharmacologists, nurses and community health workers. Centres accessible to all were designed at the different villages while another centrally located centre was made for our project team and the various assessments and ivermectin dosing took place at these centres. Permission was obtained from the town chief after the objectives of the project was clarified, thus all the patients gave their informed consent.

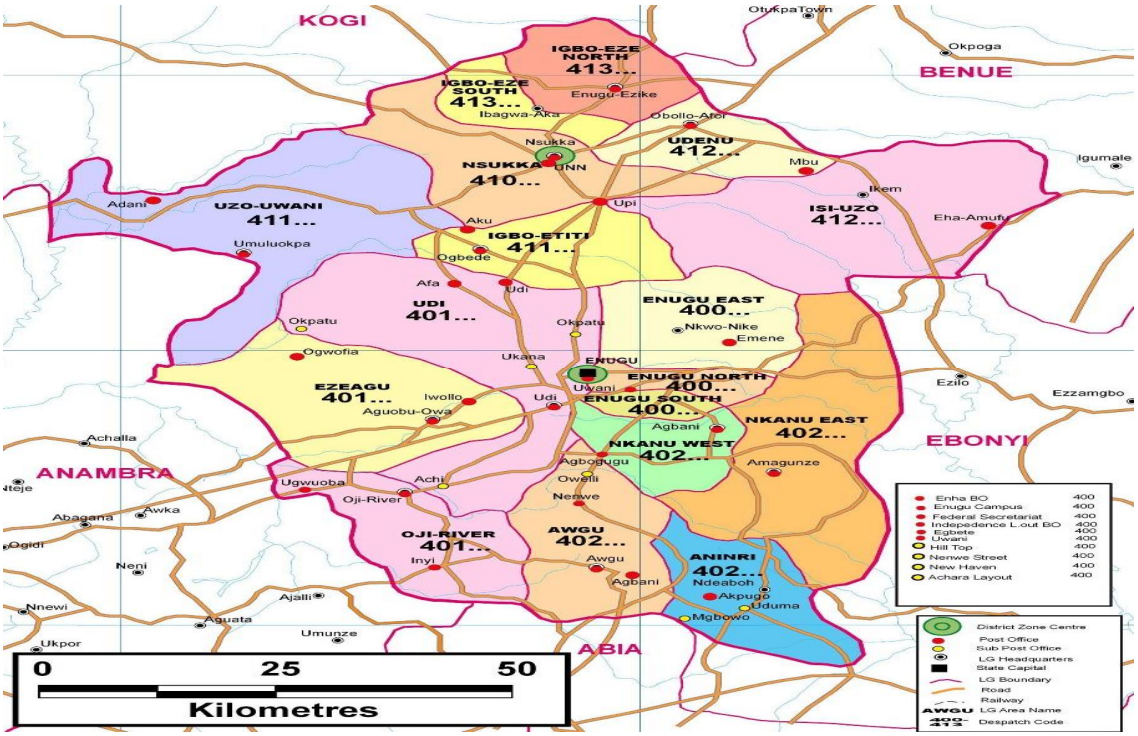


Figure 6 : Map of Enugu State showing the different local governments and neighbouring states. Note Oji River and Achi.

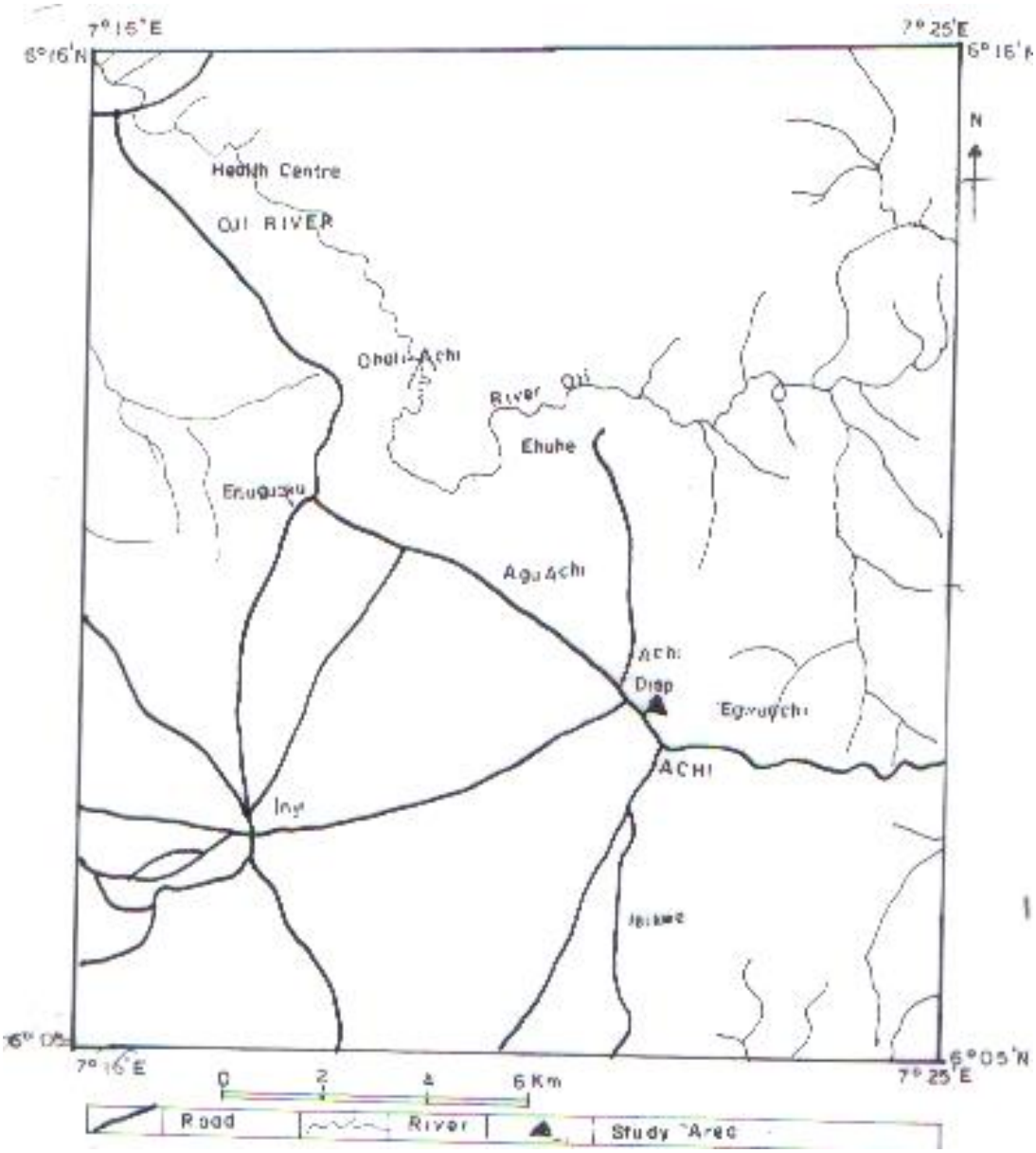


Figure 7: Map of Achi and its environs

Source: Topographic maps of Nigeria. Sheets 361 Published by Federal Survey 1967.

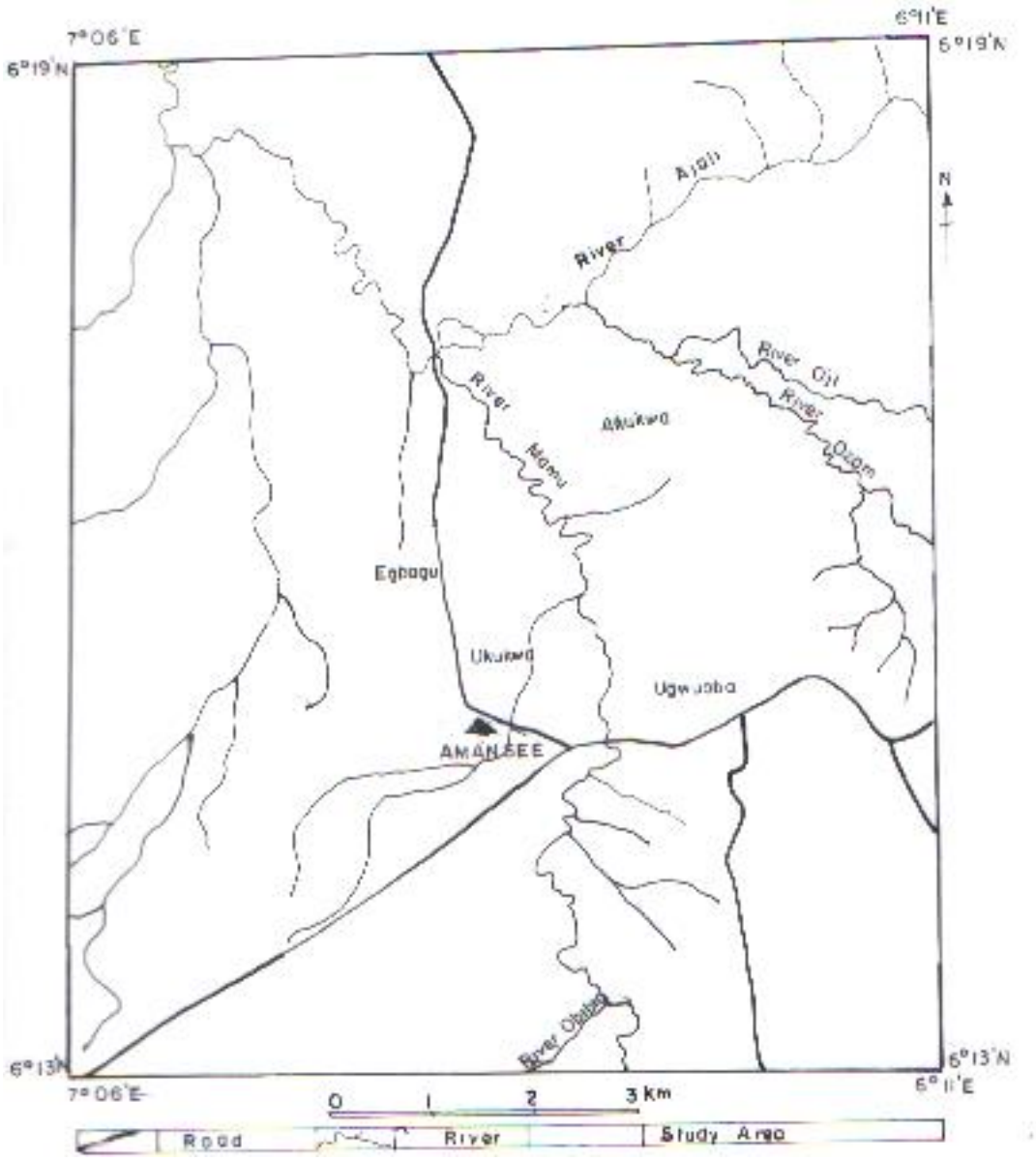


Figure 8. Map of Amansea and environs

Source: Topographic maps of Nigeria. Sheets 361 Published by Federal Survey 1967.

2.2 Collection of Study Materials

The patients were classified according to age, sex, school attendance, marital status, occupation, duration at place of work, and duration in the study area and parasitic load. They were medically examined with emphasis on height, weight, presence and sites of nodules, number of nodules, presence of acute skin manifestations such as urticarial rash, chronic manifestations of onchocerciasis such as depigmentation and lizard skin. Skin snips were obtained, on the day of examinations, from the right iliac crest and right calf of each subject between 11.00 and 13.00 hours, using a 2 mm corneoscleral punch. The skin snips were placed in normal saline in wells of microtitre plates, covered and incubated at ambient temperatures. They were examined 24 hours later for microfilariae, using an inverted Zeiss microscope. Nodules were surgically removed under local anaesthesia by a specialist surgeon from some of the patients. The nodules were collected for various investigations and preserved according to the required use.

Preservation of Nodules: Nodules used for the study of the intra-uterine stages were freshly preserved in RPMI 1640 medium and used immediately for the collagenase technique on return to the laboratory or preserved in 99% ethanol for later use. Nodules used for the DNA work were preserved in 99% ethanol and were analysed in the Netherlands. Nodules used for protein analysis were freshly subjected to the collagenase technique on return to the laboratory and the female worms isolated were frozen in liquid nitrogen. All these different preservation methods were in order to obtain very viable and fresh material for the various investigations.The nodules were randomly selected and used either for embryogram studies or for the DNA investigation or for protein analysis.

In Achi, some of the patients had received ivermectin treatments either once or twice or thrice while some others had not been treated at all. While in Amansea, the patients have had no previous ivermectin treatments. Some of the assessments were also made based on treatment

levels. Regular visits were undertaken by the team to Achi and Amansea where the clinical assessments were made. All the patients who reported to the project centre were clinically examined and skin snips were taken from the crest and calf. Nodules were also excised from the patients irrespective of their treatment levels. The nodules were randomly selected for the different studies. It should be noted that nodules used for one study could not be used for the other because a different kind of material was needed from the worms for each study, e.g, the genomic DNA of a worm sample cannot be isolated again after the protein antigen has already been isolated from that particular worm. Microfilariae were collected from some skin snips although the quantity was not be significant.

Furthermore, some health-seeking patients who do come to the Project unit in the teaching hospital were included in the study with informed consent. These patients who had onchocerciasis and on hearing about our project came to the hospital UNTH (to our Oncho.project unit) to seek treatment with ivermectin. Clinical examinations were made and skin snips taken. Nodules were not collected from some of them. All the observations and informations were analysed to ascertain the level of infections among the different groups of patients, to determine what role, factors like sex and occupational roles play on the epidemiology of the disease and to see the trend of ivermectin treatment on the control of the disease.

2.3 Embryograms

Nodules collected for the morphological and embryogram studies were freshly collected and preserved in RPMI 1640 under 4°C and later subjected to the collagenase technique. Individual adult *O. volvulus* can be examined and studied using the collagenase technique. The collagenase technique developed at the Liberia Research Unit of the Tropical Institute Hamburg by Schulz-Key *et al*. (1977, 1980) during the seventies, has been an essential approach to several new fields of research in onchocerciasis. This fundamental technique has successfully been

applied to isolate living and intact adult *O. volvulus* from excised nodules for various biological, morphological, biochemical, immunological and in-vitro studies.

For this technique, palpable nodules were excised and only the loose tissue of the surface carefully removed. The nodules were incubated into tissue culture medium RPMI 1640 about 5mls per nodule, supplemented with 0.3% - 0.5% collagenase (Preferably collagenase from *Clostridium histolyticum*, Boehringer) and with antibiotics for example gentamicin in a final concentration of 0.2 mg/ml. The ideal temperature and duration of incubation ranged from 30°C - 35°C and 2 - 4 days but incubation was interrupted as soon as the worms became visible. Continuous or repeated gentle shaking of the tubes was advantageous but not necessary. For ethanol-fixed nodules, they were put in Phoshpate buffered saline (PBS), pH 7.4 for 24 hours before incubating in the collagenase medium. This initial immersion in PBS was very necessary so as to remove the alcohol.

The worms were carefully isolated using blunt forceps and then cleaned-off of nodular tissues with fresh medium. The worms were morphologically examined under the microscope and then depending on the objectives of the studies, embryograms were performed. For the embryograms, only female worms were used, the males were preserved in 100% ethanol. Morphological observations were recorded, which include weights of nodules, colour of worms, calcification, males and female surface differences. Age of worms were assessed based on the colour of their cuticle.

For the embryograms, clean and intact female worms were individually cut into pieces of about 1-2 mm in an embryogram mortar using clean tweezers. I ml of normal saline was added and the pestle of the mortar turned gently to squeeze out the uteri contents but not hard enough to squash them. Two drops of the solution were added onto a Fuchs-Rosenthal counting chamber and observed under the microscope (using x10). The normal and pathologically altered forms of the different developmental stages (intra-uterine stages) were counted and recorded and

at the end the mean and percentages of these stages calculated. The developmental stages in the females include:

Oocytes: Primary oocytes are elongated cells, clusters of which are usually attached in situ to the rachis. They are often aggregated in bundles after grinding in the mortar. Secondary oocytes are more rounded cells lying separately in the oviduct.

Embryonic stages: Two and four cell stages form the group of small morulae. Small morulae have an average size of 15: 8 µm. Big morulae are more rounded and measure 20:13 µm. Normally developed morulae consisted of coherent cells filing out the whole space within the egg shell. The stage of the advanced embryo is a lateral incubation indicating the beginning of organization of the embryo. The larva gradually gets longer and slimmer. Embryos exceeding a circle in the egg shell are co-ordinated to the subsequent stage of the coiled microfilariae also called ôbrezelô stage. The final stage is represented by the stretched microfilariae which had cast off the egg-shell.

Pathologically altered stages: In all groups, pathological alterations and deformities were registered. Normal oocytes showed well defined nuclear membranes. In pathological forms, there was shrinkage of the cytoplasm with the nucleus becoming indistinct. The space within the egg-shell was only partially filled out. The egg-shell was sometimes deformed. The size of the cells in abnormal two and four cell stages were often unequal. The cell membranes were partially dissolved and the cells sometimes separated from each other, the cytoplasm is granular, turbid or gaseous. Pathologically altered embryos of the further developed morulae often consistes of cells of unequal size some of which might be stunted growth, others showed abnormal increase. Single cells were often separated from the embryos which becomes irregular in outline and disorganised with necrotic areas. The space within the egg-shell was not completely filled out, occasionally egg-shells of normal size could be found containing only single cells or remnants of them. In the advanced embryos, a similar segregation of cells was

observed. Sometimes, longitudinal clefts of the embryo indicate the abnormal development and the gradual dissolution of the cell membranes. Abnormal coiled microfilariae that are deformed showed excrescences, under-development or had enlarged nuclei. The nuclei column of pathologically altered microfilariae were interrupted at several sections by the dissolution of the cytoplasm. In some cases still, motile microfilariae showed abnormal big nuclei and vacuole (Schulz - Key *et al.,* 1977, 1980).

All the stages observed were distinguished and recorded. Some of the remaining mixtures for the embryograms were preserved for future use in practical classes.

2.4 Molecular Biology Work; Search for the PGP Gene.

2.4.1 DNA extraction

Nodules collected from Achi and preserved in 99% ethanol were submerged in PBS (PH 7.4) and subjected to collagenase digestion as described in 2.4. The female *O. volvulus* worms were properly cleaned of human tissue and used. *O. volvulus* females collected from Sierra Leone and provided by Dr. S.Meredith were also used. The worms cryopreserved, were properly cleaned in PBS and used for genomic DNA extraction. Some worms were pooled together for the DNA extraction while others were prepared separately but their identities with regards to treatment levels of the patients were noted. For the genomic DNA extraction, the worms were freeze-thawed three times and homogenized with a mortar and pestle. The homogenate was transferred to a 50ml tube in NET buffer or lysis buffer (150 mM Nacl, 10 Mm EDTA, 50 mM Tris-Hcl, Ph 7.4), 1 ml worm homogenate in 10 ml buffer. Ribonuclease A (Sigma) was added to a final concentration of 100 mg/ml and incubated for 30 min at 60°C. Proteinase K was added to a final concentration of100 µg/ml and the solution was further incubated for 3 hours at 60°C. The DNA was extracted with phenol/chloroform and dialyzed extensively against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The yield of DNA varied from 3.4 µg from a small

female worm to 1083 µg from a worm pool. The amount of host DNA contamination was monitored using a human b. globuline primers PC03 - PC04 in PCR reactions. Genomic DNA were also isolated from uterine microfilariae. The DNA from (from the *O. volvulus* worm pool from ivermectin-treated patients) Sierra Leone was labelled OV 92 while the DNA isolated from untreated patients in Achi was labelled OV 0. These two genomic DNA samples were used for the first series of PCRs in order to check for and isolate the mdr genes.

2.4.2 PCRs

MDR degenerate primers P3 - P4 (P3 5ô GGACGAAGCTACGTCAGC 3ô, P4 5ô CGGACCATCAGGATGTGGAA 3ô) were used for the PCRs. These degenerate primers are designed from homologous sequences of Mouse mdr 1, mdr 2 (Gros *et al.*, 1986, 1988), Human MDR 1 and MDR 2 (Chen *et al.*, 1986; Van der Bliek *et al.,* 1986) and Hamster pgp 1 and pgp 2. PCR was carried out according to the protocol of the manufacturer (Promega), in an Omnigene automated cycler (BIOZYME). First, one cycle denaturation of 2 mins at 94°C 30 cycles of: 40 secs denaturation at 94°C, 30 secs primer annealing at 46°C and 1 min 40 sec Primer extension at 72°C. The PCR products were cut out carefully from the Agarose gel and gene-cleaned (Quiagen Protocol) for cloning experiments.

2.4.3 Cloning and sequencing of the *O. volvulus* mdr genes.

The *O. volvulus* mdr PCR product was subcloned into the PCR^{Tm} vector (3.9kb) according to the manufacturers directions (TA cloning® kit, INVITROGEN, catalogue no K 2000-01, version 2.1). Different ligation ratios of the vector and the insert were used for the ligation experiments. The ligation mix was kept at 37°C over night and then plated onto LB agar plates containing ampicillin (50 µg/ml) with 25 µl of X-Gal (40 mg/ml stock in dimethylformide). Several white as well blue colonies were obtained after incubation of the plates at 37°C overnight.

Twenty six (26) white colonies/clones were randomly selected, incubated and grown up overnight. Plasmid (Miniprep.) DNAs were prepared from the clones and the following three different methods were used to determine whether they are the right or expected clones.

First, all the 26 miniprep DNA were each cut with 5 units Hind III restriction enzyme. Secondly, all the 26 miniprep DNAs were each cut with 5 units Hind III and 5 units Eco R1 restriction enzyme (RFLP: Restriction Fragment Lenght Polymorphism) so as to see the sizes of the vector and the insert differently. Thirdly, a PCR was carried out using 5µl (1:500 dilution) of the miniprep DNA as templates. PCR conditions described in 2.5.1 was used but on block control.

On confirmation of the right clones and for the purpose of nucleotide sequencing, single stranded DNA were prepared from 4 clones (1, 5, 6 and 10) and sequenced using the T7 sequencing kit, Pharmacia. (Maniatis *et.al.,* 1982; Hattori and Sakaki, 1986). Based on the position of the primers (Figure 1), one nucleotide binding region (NBD) of the whole structure was cloned and sequenced. The nucleotide sequences were read manually several times for confirmation purposes. This is a routine protocol in the Laboratory of Dr. Marleen Roos. Also for confirmation purposes, a sequencer machine was used.

2.4.4. Computer analysis

The PC/GENE DNA Sequence Analysis System (IntelliGenetics, Inc./GENOFIT SA., Mountain View, CA) was used to analyze nucleotide and deduced peptide sequences. The amino acid sequences will be compared with known mdr genes.

In order to screen for possibly more mdr genes, the PCR products of the plasmid DNA from the clones were digested with various restriction enzymes so as to see if there are more mdr genes which might be observed from their different restriction sites. 303 ng plasmid DNA of some clones were each cut with six different restriction enzymes (Mbo 1, Hin f 1, alu 1, Hae III,

Msp 1 and Rsa 1, according to the directions of the manufacturers) and the digestion products were visualized on 1.5 % agarose gel.

2.4.5 Designing of *O. volvulus* mdr primers (P11 - P12)

As sequel to the revealed *O. volvulus* mdr amino acid sequences, *O. volvulus* mdr primers (P11 - P12) will be designed from both conserved and identical subtitutes but with an aim of showing specificity for *Onchocerca*. The P? (forward reading) and the P? (reverse complememt) primers each had one conserved substitute. Upon successful completion, the new primers were then used for PCRs with several other *O. Volvulus* DNA samples as well as other parasites to check for specificity.

2.5 Protein Analysis; Search for the P-Glycoprotein in *O. volvulus*

2.5.1 Preparation of protein antigens (OvAg/OvP)

The cryopreserved *O. volvulus* female worms (3-8 worms as a pool or single worm) were homogenised using a tissue Grinder (Fischer Scientific, Pittsburgh, U.S.A.), in homogenisation buffer on ice. The homogenisation buffer consists of 1% Sodium deoxycholate (NaDOC) in 10 mM Tris-HCl, pH 8.0 with I mM Ethylenediaminetetraacetic acid (EDTA), 100 µg/ml Phenylmethylsulfonylflouride (PMSF) and 50 µg/ml Na-p-Tosyl-L-Lysin Chloromethylketone (TLCK) all properly mixed together. The homogenate was sonicated on ice for 10 mins with the Ultra sound sonifier (30% Cycles and 20 Output control, Branson Sonifier 250) and then centrifuged for 30 mins at 16000 g under 4°C. The supernatant was collected, aliquoted and stored under -20°C. The pellet were also preserved at -20°C.

The protein content of the antigen were determined using Bio-Rad-Protein determination assay, (after Bradford, 1976) in microtitre plates using Bovine IgG standard at 25 to 500 µg/ml. Also BCA Protein Assay -Enhanced Protocol (PIERCE, Rockford, IL, 1991) was used to determine protein concentrations in microtitre plates.

2.5.2 Separation of *O. volvulus* worm antigens (SDS-PAGE)

The *O. volvulus* protein samples were separated according to their different molecular weights on a 1 mm thick polyacrylamide gel held onto a GelBond® PAG film (FMC BioProducts, Rockland) in a Multiphor II Horizontal electrophoresis unit (LKB/Pharmacia, Freiburg). The samples were added on the 4.5% T (total polyacrylamide concentration) loading gel. The protein samples were then separated in the linear form on the gradient gel containing 7.5% to 17.5% SDS separating gel over a distance of about 9 cm. The mixture contents of the loading gels and separating gels is given in Table 1. After the pouring of the loading gel (in a gelkassette comprising of two glass plates with 1 mm spacers in between and held tightly at three sides with clamps) the separating gel was then poured carefully with the help of a gradientmixer (Modell 385, Bio-Rad, Munchen). The gel was overlayed with the overlayer solution comprising of 0.375 M Tris-HCl, PH 8,8 and 0.1 % SDS mixed together. The gel was left for 30 mins at room temperature for the gradient to stabilise and at 4°C overnight for the polymerisation.

Table 1: Mixture contents and Final concentrations (in brackets) of solutions for the Preparation of analytical SDS-Glycine-Gel Electrophoresis.

	4.5% T Loading Gel	**7.5% T Separating Gel**	**17.5% T Trenngel**
60% Glycerine	2.4 ml (36%)	1.5 ml (12.5%)	------(0%)
dd H_2O	-----	2.1 ml	1.2 ml
M Tris-HCl, pH 8.8, 0.4% SDS	1.0 ml (375 mM Tris, 0.1% SDS)	1.8 ml (375 mM Tris, 0.1% SDS)	1.8 ml (375 mM Tris, 0.1% SDS)
30% T, 3% C PAA	0.6 ml (4.5% T)	1.8 ml (7.5% T)	4.2 ml (17.5% T)
100% TEMED	5.0 µl (0.125%)	5.0 µl (0.069%)	5.0 µl (0.069%)
40 % APS	2.5 µl (0.025%)	6.2 µl (0.034%)	4.0 µl (0.022%)
Total volume	4.0 ml	7.2 ml	7.2 ml

Before the electrophoresis itself, the protein samples were lypholised in a vacuum concentrator to obtain about 25 mg/ml of protein solution where possible. The samples were then mixed with SDS-Sample buffer (2X conc.) at a ratio of 1:1. The protein samples were then heated for 30 mins at 37°C and the molecular weight markers were heated for 5 mins at 100°C before being applied onto the sample slots on the gel. A protein concentration of about 0.5 mg/cm was applied with a precaution of not overloading the gel. Four pieces each set of Whatman 3M (cut to size) filter papers were soaked in the electrode buffers and then carefully laid onto the gel at the two electrode ends to complete the circuit between the gel and buffer. The electrophoresis was left to run for about 2 h 45 mins under 10°C at a constant current of 500 Volts. Molecular weight markers (protein standards) were run along sides to the protein samples and the relative molecular weights of the proteins were determined using the markers (Dalton Mark VII-L: Mr 66 - 14.2 kDa and High Molecular Weight Standard mixture: Mr 205 - 29 kDa), as standards.

The SDS-Sample buffer and the Electrode buffer comprises of ;

SDS-Sample Buffer(2x conc.) :	Electrode Buffer (pH 8.5):
12 % Glycerine	25 mM Tris
4 % SDS	192 mM Glycin
65mM Dithiothreitol	10 % SDS
00.02 % Bromonphenol blue	
75 mM Tris-HCl, pH 8.8	

After the electrophoresis, the gels were either silver-stained or Coomassie Brilliant blue-stained to visualize the separated proteins. In other cases, the proteins were transfered onto nitrocellulose membranes or polyvinyldiflouride membranes (PVDF) for further imunoblotting.

2.5.3 Staining of the SDS - gels to visualize the separated proteins

If the gels are not required for western blotting, the gels were stained either with Coomassie Brilliant Blue or with silver stain so as to visualize the separated proteins and determine their molecular weights.

For the Coomassie-G250-Staining, the gel was fixed for 30 mins in 20 % Trichloro acetic acid, stained for 30 min in 0.15 % Coomassie Brilliant Blue G250, 20 mM $CuSO_4$ x 5 H_2O in 10% Acetic Acid and 45 % Methanol. The blue background was destained until clear in 20 mM $CuSO_4$ x 5 H_2O in 10 % acetic acid and 25 % methanol. Finally, the gel was impregnated for 5 mins in 10 % glycerine, 10 % Acetic acid and 25 % methanol and then wrapped in a transparent cellophane foil and left to dry at room temperature.

For the silver staining of the gel, the very sensitive method according to Blum *et al.* (1987) will be performed as follows: The gel will be;

Fixed in 50 % methanol, 12 % acetic acid, 0.0185 % formaldehyde	1-2 hrs
Washed in 50 % ethanol	2 x 20 mins
Washed in 30 % ethanol	20 mins
Treated in 0.8 mM Sodiumthiosulphate-Pentahydrate	1 min
Rinsed in dd H_2O	3 x 20 sec
Stained in 11.8 mM silvernitrate, 0.0278 % formaldehyde	20 min
Rinsed in dd H_2O	2 x 20 sec
Developed in 0.57 M Sodiumcarbonate, 0.0185% Formaldehyde and 0.016 Mm Sodiumthiosulphate-Pentahydrate	max. 10 min
Rinsed in dd H_2O	2 x 2 min

stopped development in 50 % Methanol and 12 % Acetic acid	10 min
Washed in 50 % methanol	20 min
Impragnated finally in 10 % glycerine	10 min

The gel was then wrapped in a transparent cellophane foil and left to dry at room temperature.

2.5.4 Western blotting and immunoblotting

Following the electrophoresis (whereby the gel was not stained), the separated proteins were then transferred electrophoretically through semi-dry blotting (Kyhse-Andersen, 1984) onto Nitrocellulose (NC) or Polyvinyldiflouride (PVDF) membranes (Western Blotting). NC membranes with pore size of 0.45 µm and Hybond-PVDF membranes from Amersham Life Science, Amersham plc, England, were used in this study.

For the western blotting protocol, two Graphite-electrode plates were needed, cathode and anode, three transfer buffers, Anode I (0.3 M Tris-HCl, PH 10.4, 20 % Methanol), Anode II (25 mMTris-HCl, pH 10,4, 20 % Methanol), and Cathode (40 mM6-Aminohexanoic acid, pH 7.6, 20% Methanol).18 pieces of filter papers were cut to size of the gel to be blotted, 6 were soaked into Anode I buffer, 3 soaked into anode II and 9 was soaked into anode three buffer, shaken for about 10 - 15 min and then removed (carefully and rolled over with a roller to remove excess liquid and possible air bubbles) to make the blotting assembly. The NC or PVDF membrane were cut to size of the gel and soaked in Anode II buffer. For PVDF, the membrane was first made wet with Methanol, washed off in plenty of dd H_2O before soaking in Anode II.

The PVDF membrane were laid on top of the gel carefully to avoid air bubbles, 3 Anode II filters were placed on top of the PVDF membrane, 6 anode I filters on top of the anode II filters, the gel is carefully scraped off the GelBond PAG film by rolling a thin wire underneath, and the 9 cathode filters were then laid on the other side of the gel. This assembly were carefully

placed onto the wet electrode plates with the filters lying on their respective electrodes. The circuit was completed and transfer is left to run for 90 mins under a current of 0.8 mA/cm^2 of gel. For control purposes a pre-stained molecular weight marker was used so as to see the success of the Western Blotting (Dalton, Mol Weight Marker, 7B).

Indian Ink staining procedure was carried out to see the transferred proteins on the membranes or Ponceau S staining can be used also. For the Indian ink staining, the membrane was incubated in 0.2 M NaOH for 5 min, washed 4 times 10 min each in 0.05 % Tween 20 in PBS, and stained for 3 hrs. In 0.1% Fount Indian Ink + 1 % Acetic acid in Tween-PBS. The membrane was then washed 2 x 2 min each in dd H_2O. The Indian-ink staining is stable for several months.

For the Ponceau S staining, Ponceaus S was diluted in dd H_2O at a ratio of 1:1 and the membrane was immersed in the dilution for 15 mins and then washed with dd H_2O so as to visualize the transferred proteins. Ponceau S staining is not stable for several months.

For the immunoblotting, the NC membranes were cut into 3-4 mm stripes and put into special immunoblotting plastic plates. The PVDF was not cut into stripes. The membrane was rinsed after the western blotting with TBSTM, incubation medium and was incubated with blocking medium for 6 hrs or overnight at 4°C so as to block none-Specific binding sites. After blocking, the memebrane was washed with TBSTM 1x 15 min, 2x 5 min and then incubated in the primary Antibody overnight (or for about 12 hrs or more) at 4°C. The primary antibody was diluted with TBSTM to the required concentration shortly before incubation. The membrane was washed again 1x 15 min and 2x 5 min with TBSTM and incubated for one or two hours under 4°C in the Secondary antibody-Alkaline phosphatase-conjugated (anti-mouse IgG) or Horseradish peroxidase-conjugated as the case may be. The secondary antibody was also diluted with TBSTM to the required concentration shortly before the incubation.

For the alkaline phosphatase conjugated antibody, detection follows after washing again as usual and incubating in Substrate solution for 30 min or more and then stopped the detection by washing in TBS pH 7.0. The NC membranes were then left to dry at room temperature. The PVDF membranes were wrapped and sealed in transparent plastic foils.

Incubation Medium (TBSTM)	Blocking Medium
TBS, PH 7.0	TBSTM
0.05% Tween 20	3 % Nonfat dry Milk
0.1% Nonfat dry Milk	

Substrate Solution

0.33 mg/ml Nitroblue Tetrazolium Chloride

0.165 mg/ml 5-Bromo-4-Chloro-3-Indoxylphosphate

5 Mm mgCl2

0.1M Tris-HCl, pH 9.5

For the horseradish peroxidase-conjugated secondary antibodies, the ECL detection system from Amersham was used (ECL™ Western Blotting Protocols, Amersham) for detection. The secondary antibodies which was used with this detection system were ECL Amersham NA 931-Mouse Ig, horseradish peroxidase-linked whole antibody (from Sheep) and NA 934 Rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey). After incubation with the secondary antibody, the membrane was washed as usual and the ECL detection reagents 1 and 2 were mixed at a ratio of 1:1 and carefully laid over the membrane for 1 min. The membrane was then exposed to an X-ray film in a film cassette for 1 - 3 mins, depending, and the X-ray film was developed immediately so as to visualize the signals. This

detection procedure was carried out in a dark room. The X-ray films were then stored for reference purposes.

Several antibodies (both Polyclonal and Monoclonal) were used for the search for the P-glycoprotein of the *O. volvulus* female worms. However, reproducible results was obtained with C219 (P-glycoCHEK C219^{TM} MAB, Centocor, Diagnostics Division, PA, USA) monoclonal antibody and Pgp 389 Polyclonal antibody (kindly donated by Dr. Jörg Striessnig, Universität Innsbruck, Austria.) Pgp 389 polyclonal antibody is not commercially available at the moment.

The Amersham ECL western blotting system is a light emitting none-radioactive method for detection of immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. ECL means Enhanced chemiluminescence. Luminescence is the emission of light resulting from dissipation of energy from a substance in an excited state. In chemiluminescence, the excitation is effected by a chemical reaction. The ECL protocol has been optimized for use with different membranes and is a highly sensitive none-radioactive detection system. Detection of less than 1 pq of antigen, stable hard copy results on film and detection of antigen with small amount of antibody or low affinity antibody are few of the several advantages of this protocol. Moreover, signals generated can be quantitated with a densitometer (ECL^{TM} western blotting protocols, Amersham LIFE SCIENCE, 1994).

2.6 Statistical Analysis

The data obtained from the densitometer readings were analysed using the Whole model test and T ï test analysis of variance. This was a software package available at the laboratory of Prof. K. Dietz, Institut fur Medizinische Biometrie, Universitaet Tuebingen, Germany.

2.7 Classification of the Worm Samples used for the Study

The *O. volvulus* female worms, both protein and DNA samples used for the studies were labelled/ given numbers and identified according to the patients (from whom the worms were

isolated), identity and treatment level with ivermectin. Sample numbers which starts with 0 meant that the patient has not been treated with ivermectin while sample numbers which starts with 1 or 2 or 3 meant the patient had been treated once, twice or thrice with ivermectin. Sample numbers which ended with a P means, it is a protein while samples numbers ending with PP means it is a pellet of the protein sample. U at the beginning of some samples meant undosed while D meant dosed patients.

Comparisons were made among the results obtained from the different studies. The comparisons were chiefly carried out among the different treatment groups as well as different age groups, parasitic level, occupation and epidemiological circumstances.

Table 2: Identities of the parasite materials used for DNA work

Sample	Source of Parasite	Method of Isolation of Nodule(dna)	Stage of Parasite	Treatment with IVM.
OV 92	Adult blind male in Lunsar eye clinic, Sierra Leone.	Collected from a contact	Matured female worm	Once treated
OV 005	60 yr. old female from Achi, Nigeria	Nodulectomy by the Oncho. Team(isolated by me)	Old female worms	Not treated
OV 018	20 yr. old female from Achi, Nigeria	,,	Matured female worms	Not treated
OV 023	55 yr. old male from Achi, Nigeria	,,	Matured female worms	Not treated
OV 024	3 yr.old male from Achi, Nigeria	,,	Young female worms	Not treated
OV 1	From 2 adult males and 1 female from Achi, Nigeria	,,	Matured female worms	Once treated
OV 102	56 yr. old female from Achi, Nigeria	,,	Mature female worm	Once treated
OV 105	Adult male from Achi, Nigeria	,,	Young female worms	Once treated
OV 3	From a 38 yr.old female and 60 yr. old male from Achi Nigeria	,,	Adult female worms	Thrice treated
OV MfF	Adult female from Sierra Leone	..	Uterine mf from mature worms	Not treated
OV MfS	Adult female from Sierra Leone	,,	Uterine mf from young worms	Twice treated
OV S	Adult female from Njala district, Sierra Leone	,,	Mature worms	Once treated
H.c.	*H.contortus* plasmid DNA in Utrecht, Netherlands	,,		
Human	Human ovarian DNA from Amsterdam, Netherlands	..		

OV T	From Togo	,,	Old female worms	Not treated
O.gibsoni	From Australia	,,	matured female worms	
O.ochengi	Cameroun	,,	matured worms	
L.carinni	From infected cotton rats in Tropenmedizin Institut, Tübingen	,,	matured worms	
Anopheles DNA	Female anopheles mosquito from Mali	,,	matured flies	
Salmon sperm	BOEHRINGER, MANNHEIM	,,		
L.striatus	Plasmid DNA from infected mice in Tropenmedizin Institut, Tübingen	..	Plasmid DNA	
P.vivax	From a malaria patient in Tropenmedizin Institut, Tübingen	,,		
PßR 322	Plasmid DNA from another lab. in Tropenmed. Institut, Tübingen	..	Plasmid DNA	
A.vitae	From Max-Planct Institute, Tübingen	,,	Adult worms	

Table 3a : Identities of parasite materials used for the protein analysis

Sample	Source of parasite material	Method of isolation of nodule(ovp)	Stage of parasite	Treatment with ivermectin
OVP 1	From some males and females in Achi, a worm pool	Nodulectomy by Oncho.Team (Protein prep by me)	Young and matured female worms	Not treated
OVP 2	From some males and females in Amansea, a worm pool	,,	Young and matured female worms	Not treated
OVP 3	From females in Achi, a worm pool	,,	Young and matured female worms	Once treated
OVP 4	From females in Achi, a worm pool	,,	Young and matured female worms	Not treated
OVP 5	From males in Achi, a worm pool	,,	Young and matured female worms	Once treated
OVP 6	From males in Achi, a worm pool	,,	Young and matured female worms	Twice treated
OVP 7	From males in Achi, a worm pool	,,	Young and matured female worms	Thrice treated
OVP 055	From a 40 yr.female in Achi	,,		Not treated
OVP 059	From a 40 yr. male in Achi	,,		Not treated
OVP 061	From a 40 yr. female in Achi	,,		Not treated
OVP 069	From a 24 yr. female in Achi	,,		Not treated
OVP 070	From a 40 yr. female in Achi	,,	Young female worm	Not treated
OVP 071	From a 30 yr. female in Achi	,,	1 young and 2 matured worms	Not treated
OVP 073	From a 22 yr. female in Achi	,,	1 matured worm	Not treated
OVP	From a 25 yr. female in	,,		Once treated

107	Achi			
OVP 202	From a 10 yr. male in Achi	,,		Twice treated
OVP 203	From a 14 yr. female in Achi	,,		Twice treated
OVP 204	From a 11 yr. male in Achi	,,		Twice treated
OVP 206	From a 65 yr. female in Achi	,,		Twice treated
OVP 210	From a 60 yr. male in Achi	,,		Twice treated
OVP 211	From a 42 yr. female in Achi	,,	2 matured worms	Twice treated
OVP 212	From a 50 yr. female in Achi	,,	2 matured worms	Twice treated
OVP 301	From 11 yr. female in Achi	,,		Thrice treated
OVP 302	From a 48 yr. male in Achi	,,		Thrice treated
OVP 305	From an 11 yr. male in Achi	,,		Thrice treated
OVP 306	From a 75 yr. female in Achi	,,		Thrice treated
OVP 308	From a 40 yr. female in Achi	,,		Thrice treated
OVP 309	From a 50 yr. male in Achi	,,		Thrice treated
OVP 310	From a 50 yr. male in Achi	,,	1 matured worm	Thrice treated
OVP 311	From a 63 yr. male in Achi	,,	2 matured worms	Thrice treated
Cancer cells	Donated by Dr. Gekeler, Phsio/Chem. Institut, Tuebingen	,,	CCRF/CEM cancer cell lines with 5µg/ml adriamycin	
Cancer cells adr.5000	Donated by Dr. Jörg Striessnig, University of Innsbruck, Austria	,,	adr. 5000 membrane homogenate	
TP 1	From male or female in Togo	,,	Matured female worms	Treated

TP 2	From male or female in Togo	„	Young female worms	Treated
TP 3	From male or female in Togo	„	Adult female worms	Treated
TP 4	From male or female inTogo	„	Young female worms	Treated

Table 3b: Morphiological observations on isolated *O. volvulus* worms from some ivermectin treated and non-treated patients in Achi.

No.	Nodule No.	Nodule Weight(g)	No. of Male Worms	No. of Female Worms	Colour of Female Worms (age)	Clacification	Uferi Content	Other Observation
				Untreated Patients				
1	U1	1.4g	0	1	Yellowish (Mature)	_	All embryonic stages	
2	U2	1.4g	0	6	3 Transparent (young) 3 yellowish (mature)	_	Few embryonic stages	
3	U3	1.2g	0	1	Yellowish (Mature)	_	Empty Uterus	Non-intact female
4	U4	0.99g	_	3	1 Calcified 2 Brownish (Old)	+	Few embryonic. Stages	
5	U5	0.48g	_	_	_	_	Degenerated worms Brownish oily Liquid Content Observed	No embryonic stages Seen
6	U6	0.11g	_	1	Brownish (Old)	_	Empty Uterus	
7	U7	0.55g	_	2	1 Yellowish (Mature) 1 Brownish (Old)	_	Very few stages	
8	U8	0.56g	_	1	Brownish (Old)	_	Empty Uterus	
				Treated Patients				
9	D1	0.15g	1	1	Yellowish (Mature)	_	No Oocytes but all otherembryomc stages	

10	D2	0.99g	_	1	Yellowish (Mature)	_	Oocytes and remnant mf	
11	D3	1.0g	_	2	Brownish (Old)	_	Empty Uteri	
12	D4	1.9g	_	_	_	_	Lipome	Yellowish oily/fatty tissue
13	D5	2.2g	_	_	_	_	Lipome	,,
14	D6	2.6g	_	_	_	_	,,	,,
15	D7	1.7g	_	_	_	_	,,	,,
16	D8	1.75g	_	_	_	_	,,	,,
17	D9	0.93g	_	_	_	_	,,	,,

Expected Outcome

It is expected that the results of this work will show the level of endemicity of Onchocerciasis in Achi and Amansea communities.. It will also reveal the effect of occupational roles in determining the prevalence or degree of infection on the members of the community. It will reveal what goes on beyond the skin snip level. There will also be a clear knowledge of the presence of mdr genes and P-glycoprotein in *O. volvulus* for the first time. This will guard towards taking precautionary measures against early development of resistance.

The results obtained from this work will go a long way in discovering the early development of resistance to ivermectin in the treatment of onchocerciasis. Also the procedures applied here can be used in other diseases of importance most especially in malaria research. The procedures and processes can be used as a template to check on the treatment of other diseases of medical importance. The results obtained will assist relevant authorities in making health policy statements.

CHAPTER THREE

RESULTS

3.1 Prevalence of Onchocerciasis in three Different Communities

3.1.1 Clinical manifestations of onchocerciasis

In order to have an overview of the prevalence of onchocerciasis in the three different study groups, an analysis of clinical manifestation in the patients was made. This was a blind study and the patients gave their informed consents.

In Achi community, a total of 57 patients were examined, out of which 40% were males and 60% were females. 26% were children and 74% adults. 58% of the patients tested skin microfilariae positive while 42% tested negative. However, nodules were present in 93% of the patients while 7% had no nodules, meaning that some patients with nodules exhibited no microfilaridermiae. Onchodermatitis as well as secondary stages of onchocerciasis (lizard and leopard skin) were registered in 21% and 47% of the patients respectively. The patients had various occupations but farmers were highest (54%) of the other occupations.

In Amansea community, a total of 52 patients were examined in the study out of which 38% were males and 62% females. Half of the patients (50%) were children and the other half were adults. 17% of the patients had skin microfilariae while 83% had no skin microfilariae. 58% had nodules. Suprisingly the nodules on collagenase digestion contained very few worms instead, they contained unidentified materials as well as very old remnants of calcified worms. The patients had various occupations but this time there were more students/pupils involved in the study while 40% were farmers. 19% of the patients had onchodermatitis while 4% had lizard or leopard skin.

In the UNTH study group, the patients came from different towns to our onchocerciasis office in UNTH. It was thus difficult to classify this community as endemic or non-endemic or meso-endemic. A total of 131 patients were analysed, out of which 39% were males and 61% females. 21% were children under 20 years while 79% were adults over 20 years. Very few of the patients (10%) had skin microfilariae. 87% had no skin microfilariae, 15% had nodules and 85% had no nodules, however, the nodules from this group were not removed due to facility reasons. Again, very few of the patients were farmers (5%), while mostly students (49%) and civil servants (32%) were involved. 50% of the patients had onchodermatitis while 18% had lizard or leopard skin (Figures 9 and 10).

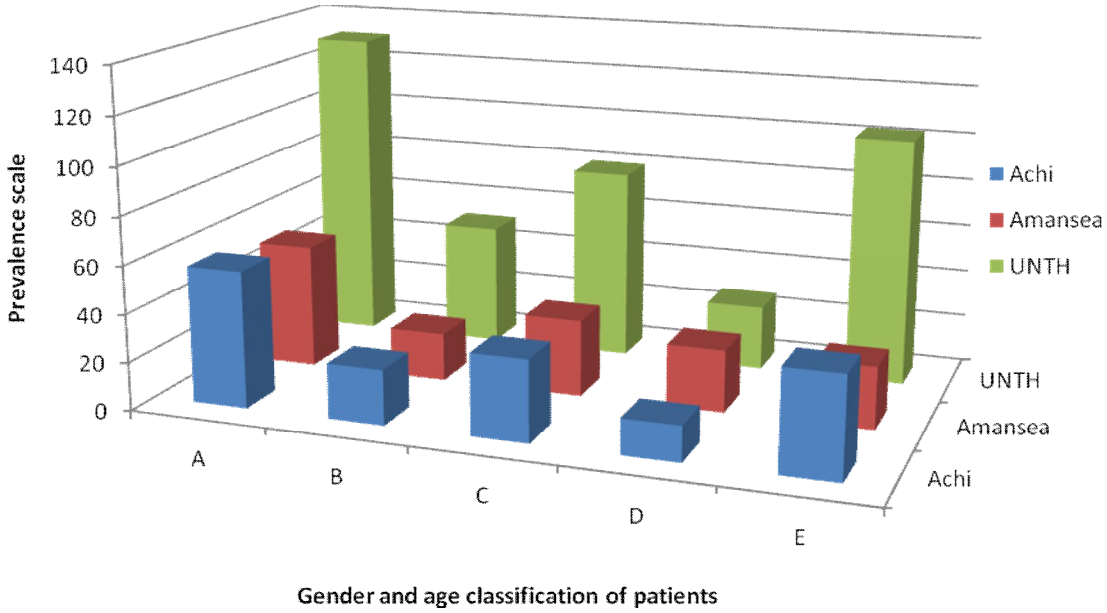


Figure 9: Gender and age classification of patients

A: Total number of patients B: Males C: Females

D: Children E: Adults

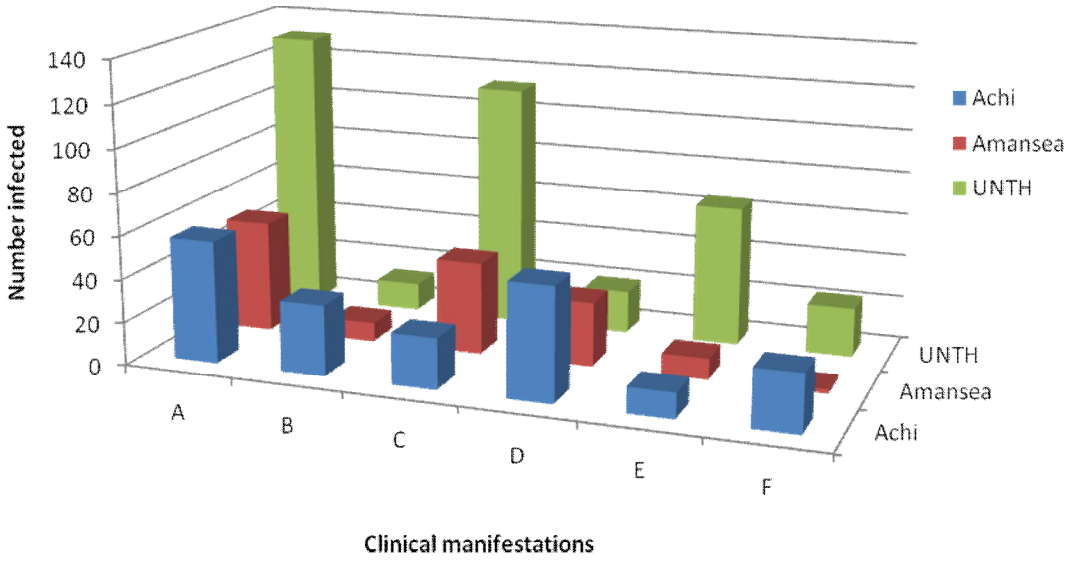


Figure 10: Clinical manifestation of Onchocerciasis in three different communities

A: Total number of patients

B: Patients with skin MF

C: Patients with no skin MF

D: Patients with nodules

E: Patients with Onchodermatitis

F: Patients with lizard/leopard skin

3.1.2 Variation in content of nodules in Achi and Amansea

Nodules collected from the study groups and digested in 0.4% collagenase revealed various consistencies. In Amansea, out of 52 patients, 30 had nodules which were removed and out of the 30 nodules, only 5 contained *O. volvulus* worms in them, while 20 had various consistencies but no worms. 5 were lipomes. In Achi, out of 10 patients, 9 had nodules and 8 of the 9 nodules had *O. volvulus* worms. Only 1 nodule did not contain *O. volvulus* worms. In U.N.T.H., out of 64 patients, 12 had nodules. However, the nodules were not isolated (Figure 11).

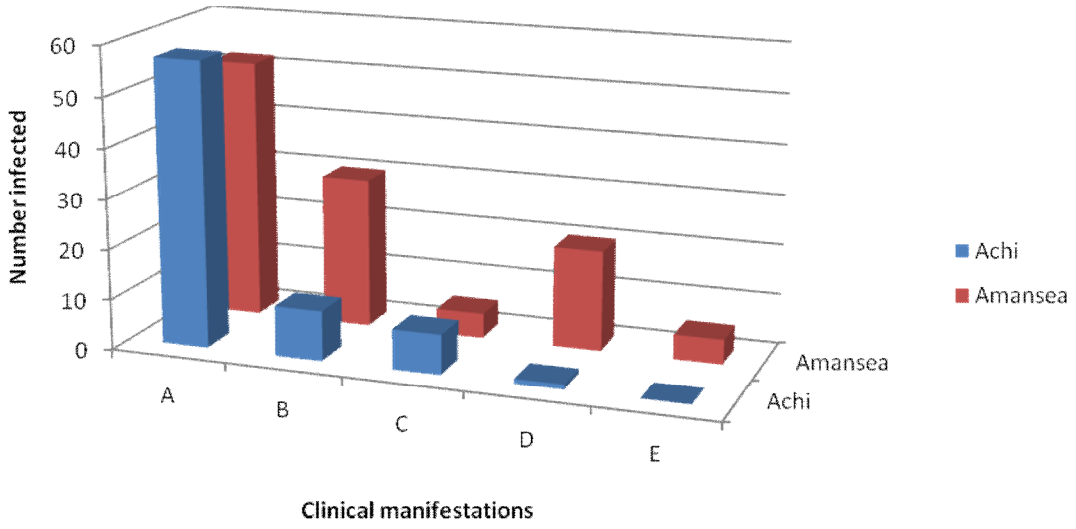


Figure 11: Worm population in nodules (A tale of two communities)

A: Total number of patients

B: Patients with nodules

C: Nodules with worms

D: Nodules without worms

E: Lipomes

3.1.3 Influence of occupation on the prevalence of onchocerciasis

In Achi, out of the 57 patients, 30 were farmers among which 65% had skin microfilariae. Eleven (11) patients were civil servants among which 64% of them had skin microfilariae and fourteen (14) were students/pupils among which 43% had skin microfilariae.

In UNTH, out of the 131 patients that came, 6 (5%) were farmers and none had skin microfilariae. 42 (32%) were civil servants out of which 10% had skin microfilariae and 64 (49%) were students out of which 11% had skin microfilariae. Figure 12

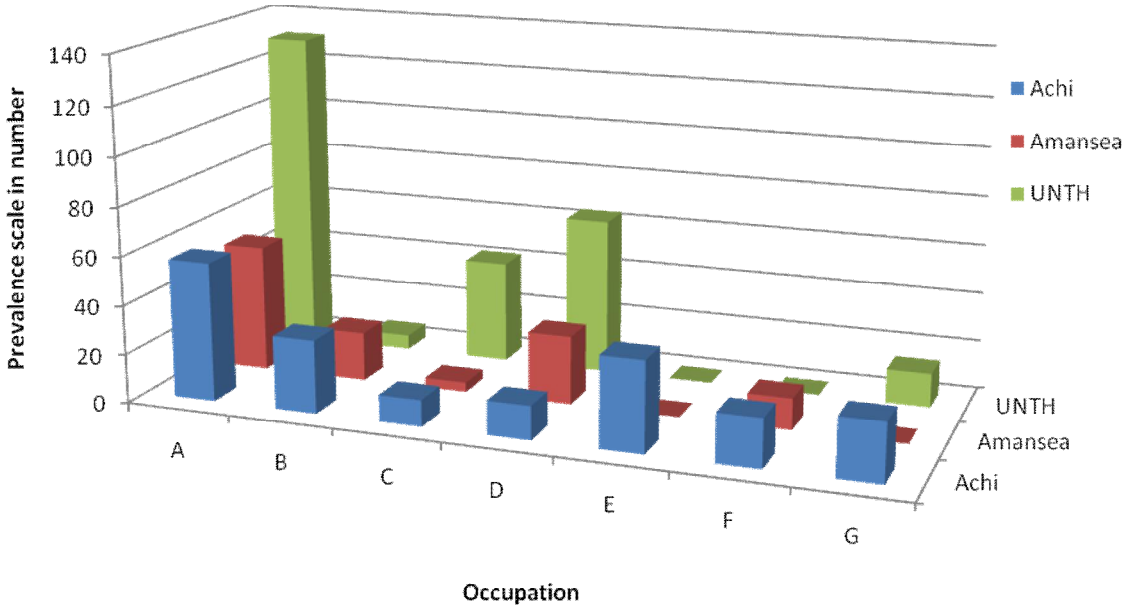


Figure 12: Influence of occupation on the prevalence of onchocerciasis.

A : Total no of Patients *B : Farmers*

C : Civil Servants / Traders D : Students / Pupils

E: Farmers with MF F: Civil servants with MF

G: Students with MF

3.1.4 Microfilaridemia level of ivermectin treated patients (possibility of resistance)

A total of 57 patients in Achi were analysed, 31 patients already took ivermectin once or twice or thrice. Whereas 19 (61%) of the number that took ivermectin still had skin microfilaria, 12 (39%) of them did not have skin microfilaria. With the treated 24 had been treated more than once, 14 out of them still have microfilaria (Figures 13 and 14).

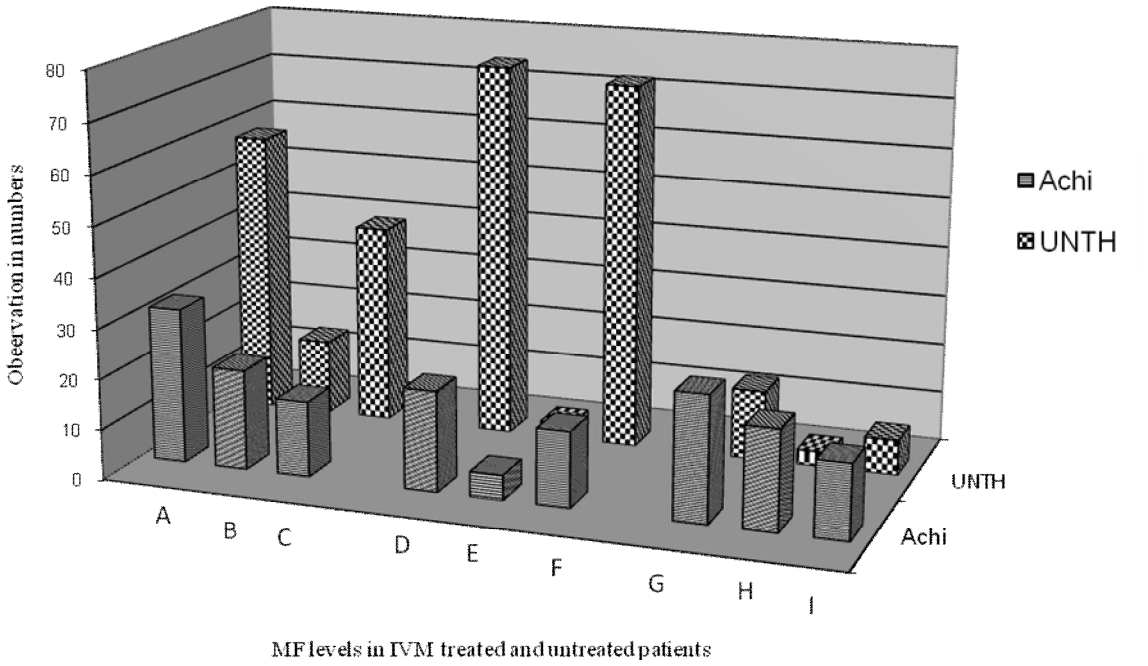


Figure 13: Possibility of Ivermectin resistance

A: IVM TREATED PATIENTS *B : TREATED and TESTED MF. +VE*

C: TREATED and TESTED MF ï VE, D: UNTREATED PATIENTS,

E: UNTREATED and MF +ve, F: UNTREATED and MF ï VE,

G: PATIENTS TREATED 2x OR 3x, H: 2X OR 3X TREATED and MF +VE,

I: 2X OR 3XTreated and MF ï ve

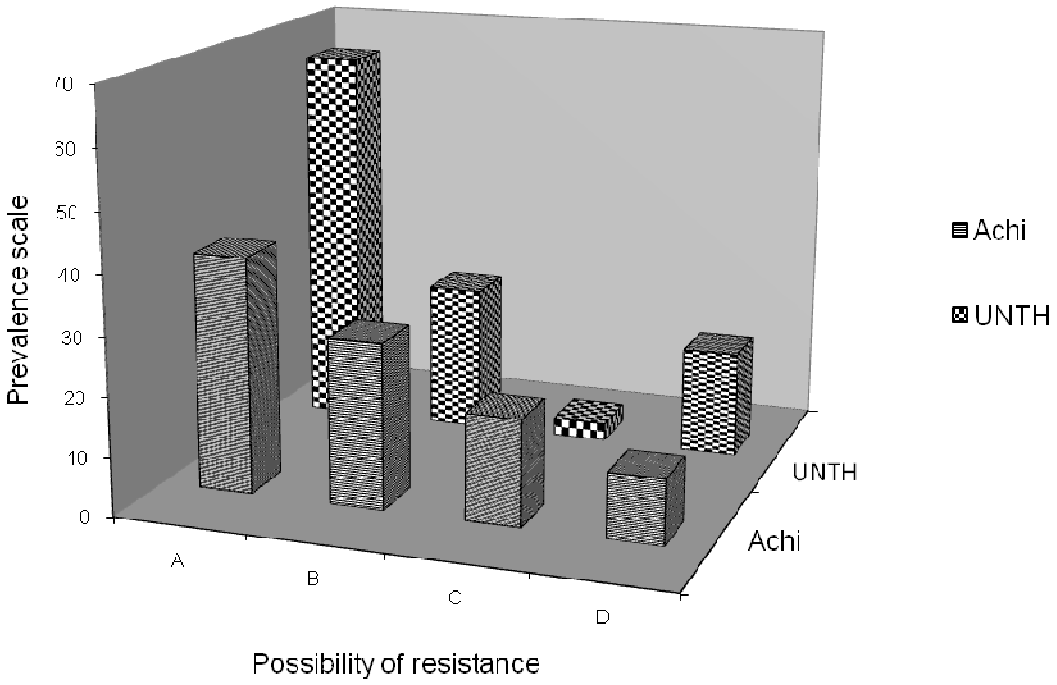


Figure 14: The Possibility of ivermectin resistance in the treatment of onchocerciasis.

A: Patients treated with IVM (1x, 2x, 3x) B: IVM-Treated patients with +ve MF

C: IVM ï Treated patients with - ve MF D: 2x or 3x IVM Treated Patients still +ve MF

3.2 Embryonic Status of *O. volvulus* Female Worms

From the treated patients, more of the normal oocytes and early morulae were observed than their abnormal counterparts, the later stages (late morulae, horse-shoe shaped stage and stretched microfilariae) recorded more pathological stages than normal stages. Equal numbers of coiled microfilariae were observed from both normal and abnormal/pathological stages.

From the worms collected from untreated patients, the embryonic status revealed more pathological forms in the later stages (late morulae, horse-shoe shaped, coiled microfilariae and stretched microfilariae). No normal stretched microfilariae as observed but more normal oocytes as well as early morulae were observed than pathological oocytes and early morulae. Thus, the embryonic stages contained in female worms both ivermectin treated and untreated patients were almost similar. On the average, very few embryonic stages were recorded from both groups (Figures 15 and 16).

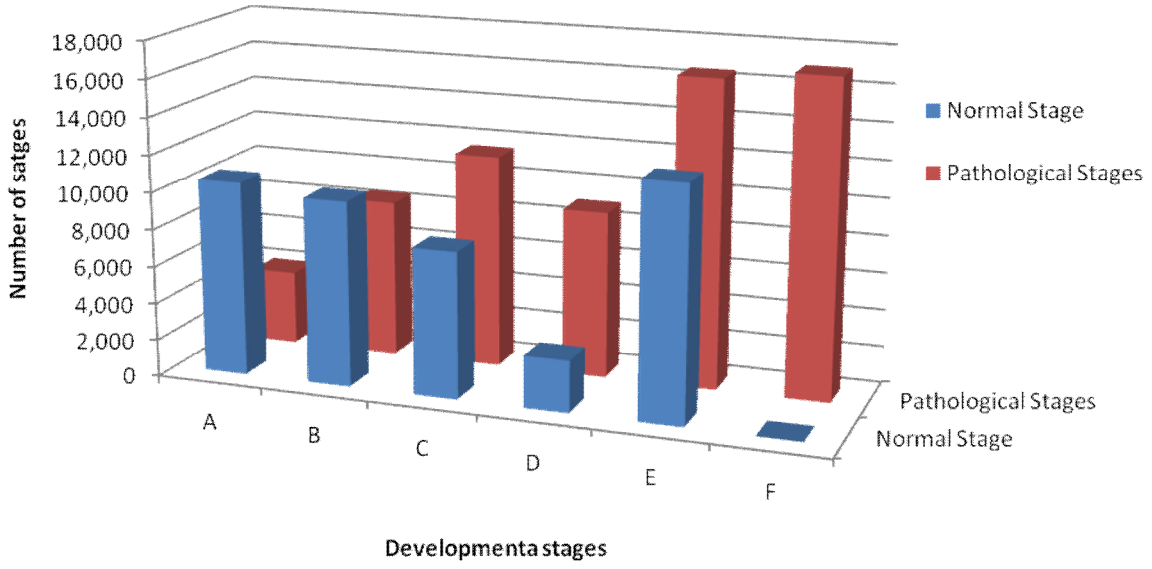


Figure 15: Embryonic Stages in *O. volvulus* from non-ivermectin treated onchocerciasis patients

A: Oocytes **B**: Early morulae **C**: Late Morulae

D: Horse-shoe shaped stage **E**: Coiled MF **F**: Strechted MF

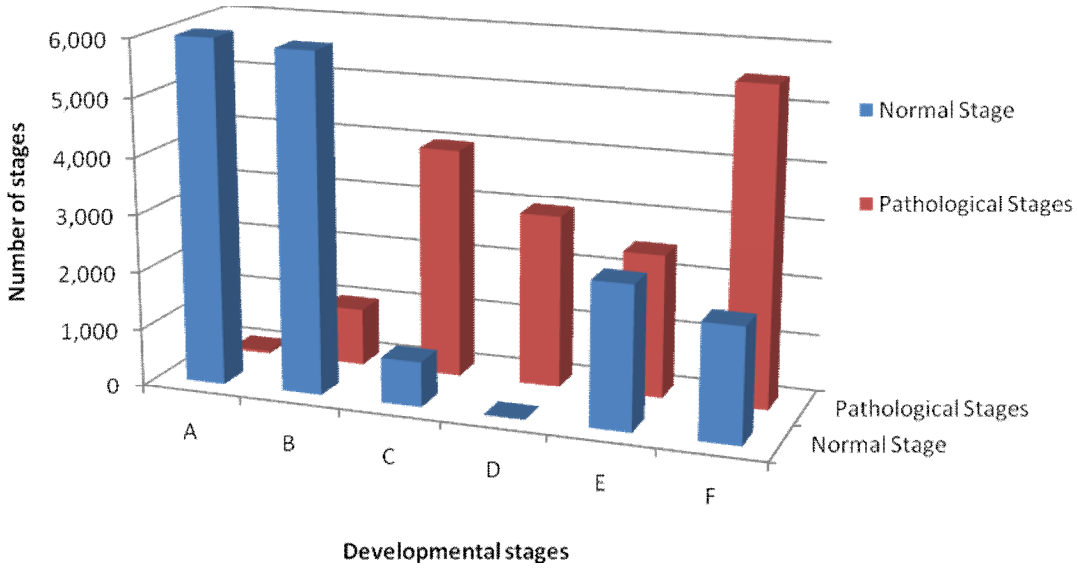


Figure 16: Embryonic Stages in *O. Volvulus* from Ivermectin treated onchocerciasis patients.

A: Oocytes **B**: Early morulae **C**: Late Morulae

D: Horse-shoe shaped stage **E**: Coiled MF **F**: Strechted MF

3.3 Molecular Biology Studies

3.3.1 Isolation of the *O. volvulus* PGP genes.

The P-glycoprotein coded for by the mdr genes is an evolutionarily conserved molecule. Figure 17 shows the structure of the P-glycoprotein as described by Gottessman and Pastan (1988).

The positions of the P3-P4 mdr degenerate primers have been circled for clarity purposes, so as to show the part of the gene that was sequenced. The PCR results using the primers P3-P4 on two *O. volvulus* templates yielded a product of about 1100 bp (Figure 18). The success of the cloning of the PCR product was confirmed with the RFLP and PCRs with the plasmid DNA (Figure 19 a and b, Figure 20 a and b).

The results of the sequencing revealed the deduced peptides and amino acid sequences. Two similar but slightly different amino acid sequences were observed *O. volvulus* pgp1 and *O. volvulus* pgp2. Comparisons with six other known mdr genes showed a 53% homology so far. See figures 21, 22, 23, 24 and 25. From the *O. volvulus* Pgp1 sequence, primers P11-P12 were designed to be used in further PCRs on other *O. volvulus* templates as well as other different templates. The PCR on several *O. volvulus* templates using the P11-P12 *O. volvulus* Pgp primers yielded a product of about 950 bp (not suprising due to the positions of the primers). PCR with P11-P12 on other *Onchocerca* templates (*O.gibsoni*, *O.ochengi*) were positive but on other filarial templates (e.g *A.vitae, L.carinii*) as well as other different sources (e,g, Anopheles, salmon sperm) were reproducibly negative. See Table 7. The heat-shock protein primers (HSP 25-1, HSP 25-2) used also in PCRs on *O. volvulus* templates served as an internal control in order to check for any signs of gene amplification, since it is expected that amplification of genes is a rare phenomenom only found in mdr genes and thus should not be expected with HSP primers. The PCR with the HSP primers yielded a product of about 130 bp.

In order to check for any signs of gene amplification of the *O. volvulus* pgp, the PCR products (from the two different sets of primers P11-P12, HSP 25-1,HSP 25-2) were measured under the laser densitometer/video camera and their various densities were compared. Comparisons were made between the densities of the different primers as well as the treated and untreated groups. The values were analysed statistically in a whole model test and a probability value of > F = 0.8247 was obtained. This was not significant since a Probability value of P>0.05 is non-significant. Our analysis showed that there was not yet any significant difference between treated and untreated patients with regards to ivermectin effect on *O. volvulus* worms. Figures

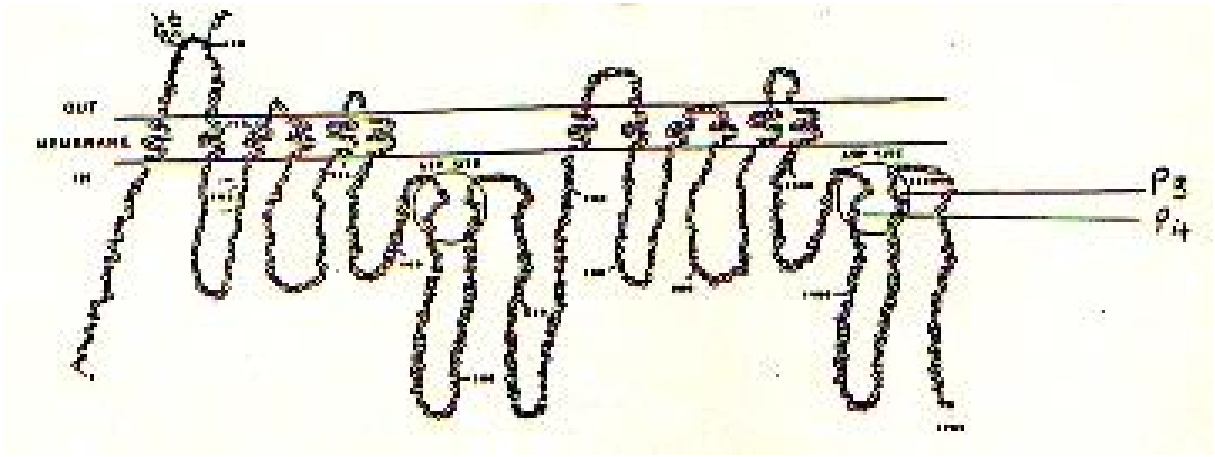


Figure 17: P-GLYCOPROTEIN purported structure as described by Gottesman and Pastan (1988).

The model of the human multidrug transporter clearly shows the location in the plasma membrane with the positions of the ATP sites within the two identical halves. Our P3 - P4 primers are located on the ATP binding sites on Amino acids 1187-1204 and 1062-1082 respectively.

P3 DNA sequence 5ôGGACGAAGCTACGTCAGC 3ô
P4 DNA sequence 5ôCGGACCATCAGGATGTGGAAA 3ô

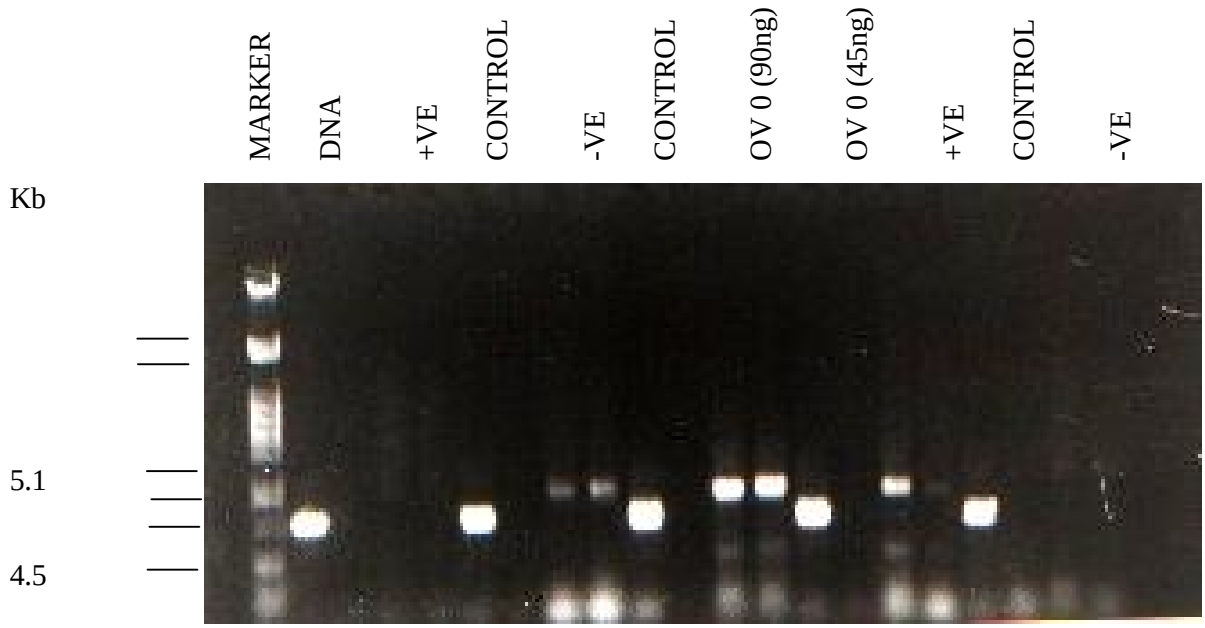


Figure 18: Isolation of the mdr genes from *O. volvulus.*

0.8 % agarose gel showing result of PCR using degenerate primers P3-P4 on two templates Ov 0 and Ov 92 (45 ng, 90 ng DNA conc.). Positive control is *H.contortus* mdr. Negative control is dd H_2O.

Amplification product yields a fragment of about 1.1 kb. The PCR was carried out according to the protocol of the manufacturer (PROMEGA) at standard conditions, (2.0 mM Mg2+), 30 cycles were performed with 2 min denaturation at 94°C, 30 sec. Annealing at 46°C and 5 min final extension at 72°C.

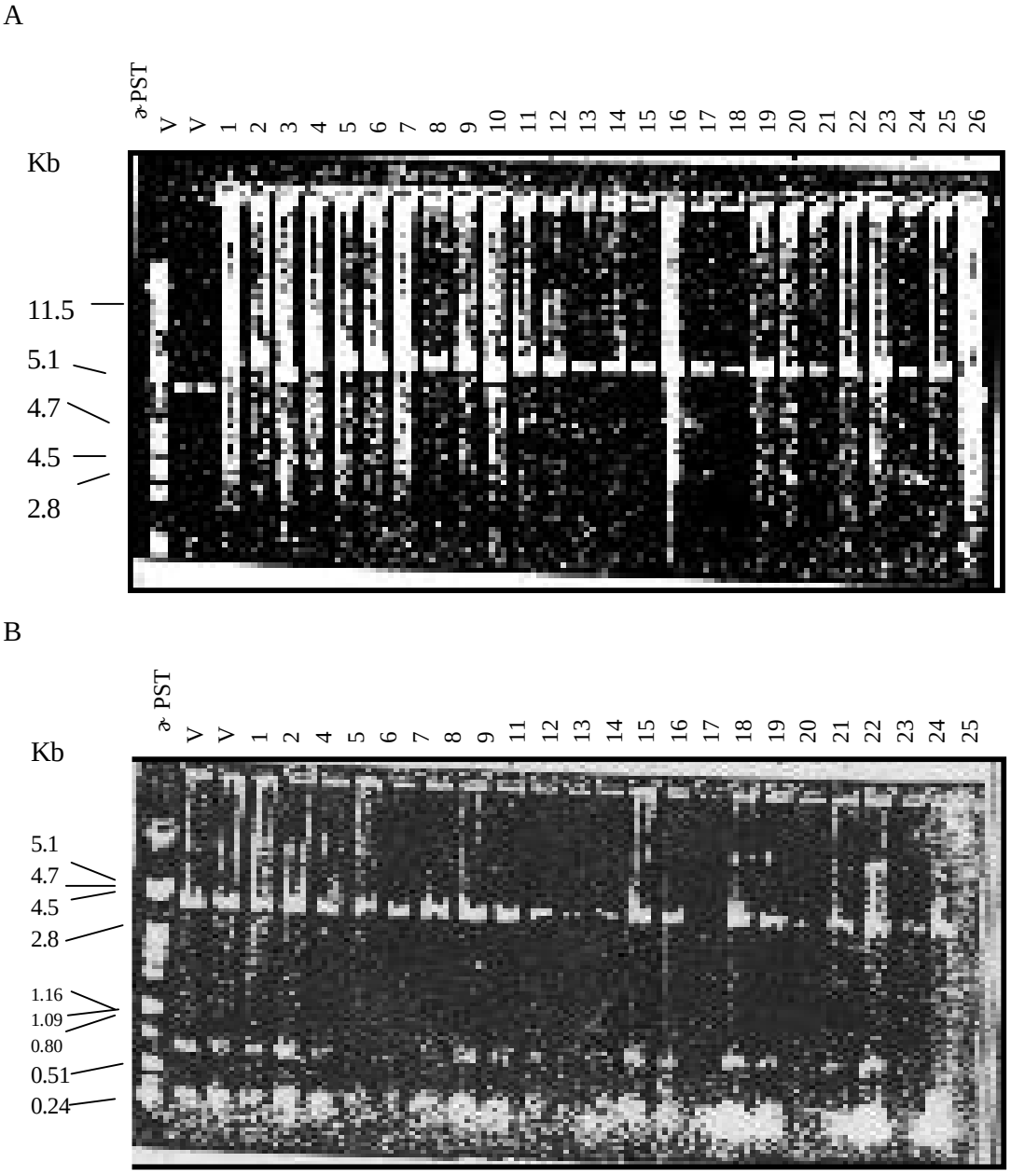


Figure 19: (a and b) Restriction Fragment length analysis of clones (RFLP)

(a) RFLP analysis of clones 1 - 26 obtained by preparation of plasmid DNA from the white colonies before the digestion. About 2-3 µg plasmid DNA from the clones were digested with 5 units Hind III and run on 0.8 % agarose gel. Lane 1 is Marker DNA: Phage lambda cut with Pst 1, Lane 2 and 3 are the vectors (about 3.9 kb) while Lanes 4 - 29 are digested clones of about - 5.0 kb except for clones 3 and 10 (about 4.5kb), as well as clone 26 which is almost same size as that of the vector.

(b) RFLP analysis of clones 1 - 25 (excluding clones 3, 10,26), digested with two restriction enzymes, Hind III and Eco R1, 5 units each and run on 0.8 % agarose gel. Observed fragments 3.9 kb is the vector. Possibly the *O. volvulus* mdr insert might have possesed a restriction site, thus we observe two fragments of about 0.75 kb and 0.30 kb approx.

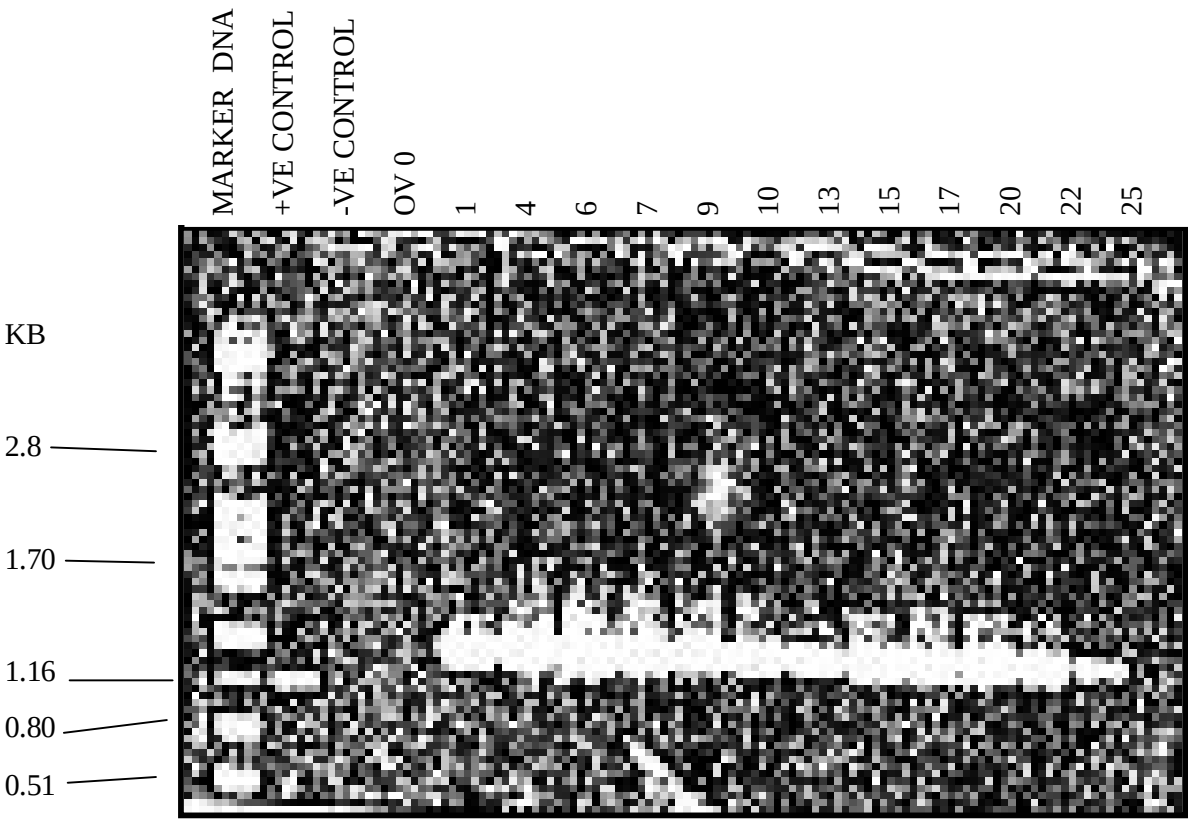


Figure 20 (a): Analysis of a PCR with degenerate primers P3 - P4 on Plasmid DNA of clones 1,4,6,7,9,13,15,17,20,22, 25 (about 0.5 µg each) and Ov 0 genomic DNA (about 90 ng) run on 0.8 % agarose gel. Positive control is *H.c.* mdr. Negative control is dd H_2O.

Observe that all the clones gave a positive reaction and the size of the PCR product (about 1.1 kb) is same as that obtained with the genomic DNA templates.

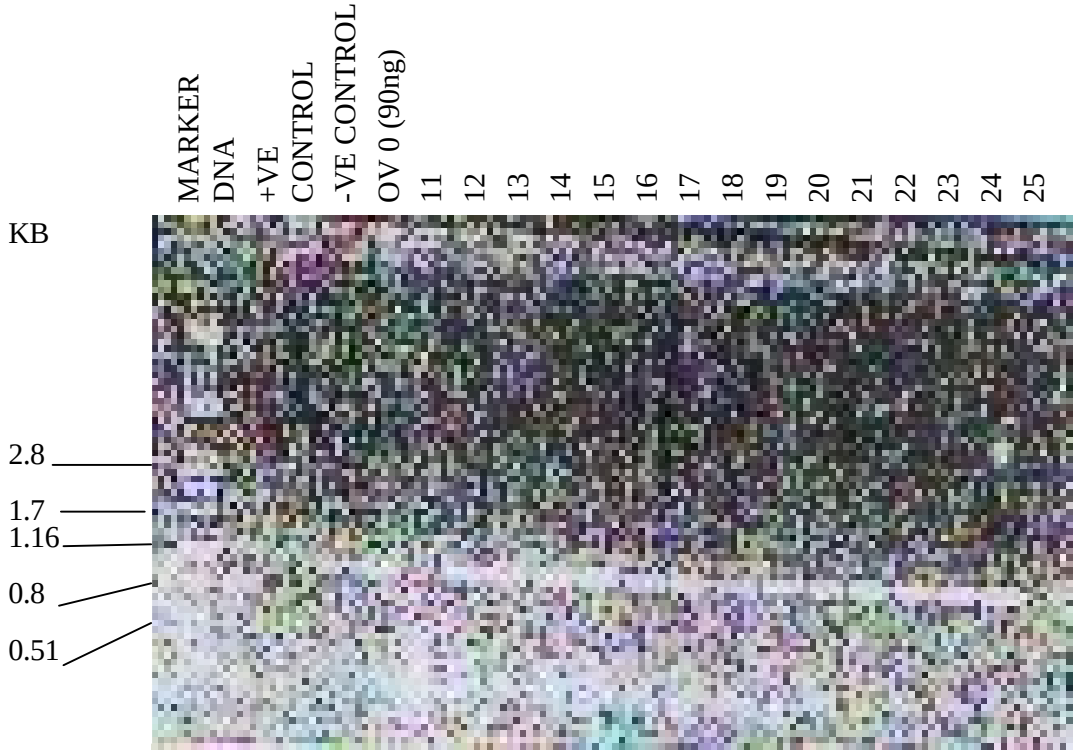


Figure 20 (b): Analysis of PCR with degenerate primers P3 - P4 on plasmid DNA from clones 11,12,13,14,15,16,17,18,19,20,21,22,23,24,25 (about 0.5 µg each) and Ov 0 genomic DNA (about 45 ng) run on 0.8 % agarose gel. Positive control is *H.c.* mdr. Negative control is dd H_2O. Observe product sizes.

3.3.2 The *O. volvulus* PGP sequences

The next figures 3.3.5 (a - e) shows the oligonucleotides as well as the amino acid sequences of the two clones that were sequenced. Two mdr genes from *O. volvulus* might have been possibly been isolated. a and b are the sequences obtained from Ov 0 clone 1 using the Universal primer and M13 reverse primer respectively. c and d are the sequences obtained from Ovô92 clone 20 using the same set of primers as in a and b. Observe the slight differences in the sequence. (e) shows a comparisom of the amino acid sequences of six other mdr genes with that of the *O.volvulus*. A 53% homology obtained clearly signifies that the P-glycoprotein is really a conserved molecule across the animal kingdom. However, the conserved subunit has enabled us to design the *O. volvulus* mdr specific oligos. Which will be used as the primers for further PCRs (P11 - P12).

The *O.v.* mdr primers were designed in a way that it will be used for further PCRs on other *O. volvulus* templates to check for the presence of the mdr genes and thus should be specific but our experiments showed that our *O.v.* mdr primers are specific for *Onchocerca* species but did not react with the other filariae like *L.carinii*, *A.vitae*, *H.contortus* and human ovarian DNA to mention but a few.

P11 5' CGGGAAATCAACTATAATTG 3'

P12 5' TTCGGATTACGAACTAAAAT 3'

The screening of the PCR products of the different clones with different restriction enzymes revealed that the clones were more or less highly identical. With Mbo 1 enzyme, clone 20 looked different so it was included for sequencing. See Figure 3.4.5, c and d. Restriction digestion with the other 5 enzymes, yielded similar results.

Figure 21: DNA sequence of OV 0 (Forward primer)

Figure 22: DNA sequence of OV 0 (reverse complement)

Figure 23: DNA sequence of OV 92 (Forward primer)

Figure 24: DNA sequence of OV 92 (reverse complement)

Figure 25: Comparison of Different mdr Genes.

Comparison of the amino acid sequences of eight different mdr genes. Note the conserved substitutes and identical amino acids. O.V 92 showed a 53% homology.

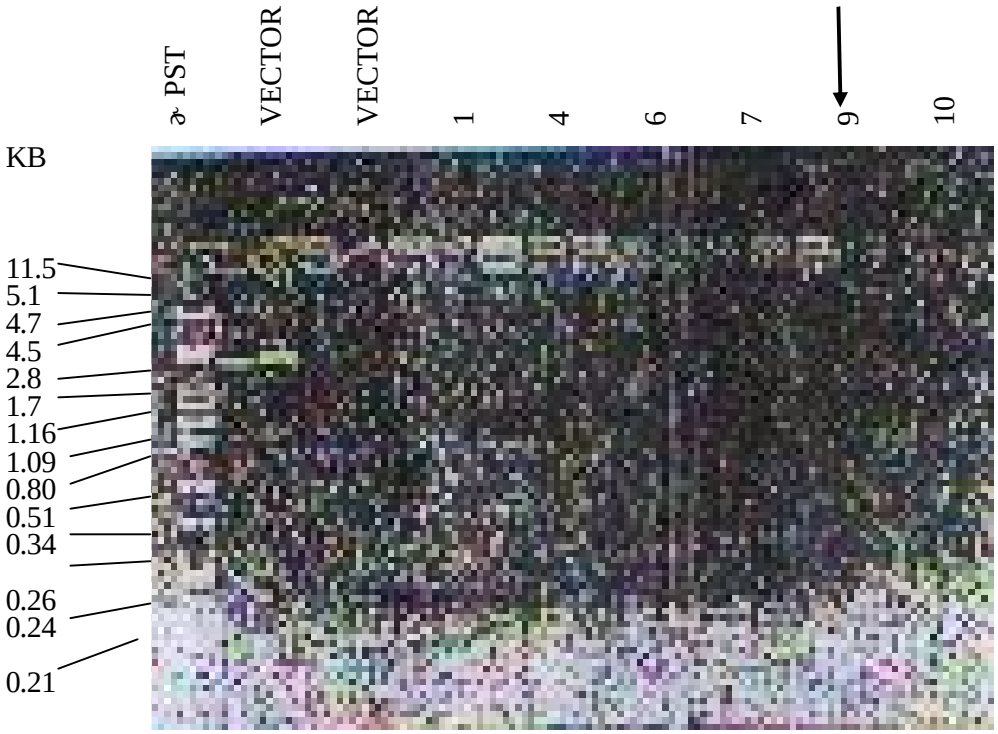


Figure 26: PCR product analysis of digested clones.

Analysis on a 1.5 % agarose gel. Restriction digestion of the PCR products (clones 1,4,6,7,9,10,13,15,17,20,22,and 25) each with 6.5 units Mbo 1 enzyme. Observe 3 different fragments of about 600 bp, 260 bp, and 247 bp on each clone except for clone 20 with fragments of about 600 bp, 264 bp, 216 bp. This shows that clone 20 posseses a different restriction site and might be another mdr gene. This is confirmed by the slight difference in the oligonucleotide sequence as well as the amino acid sequence.

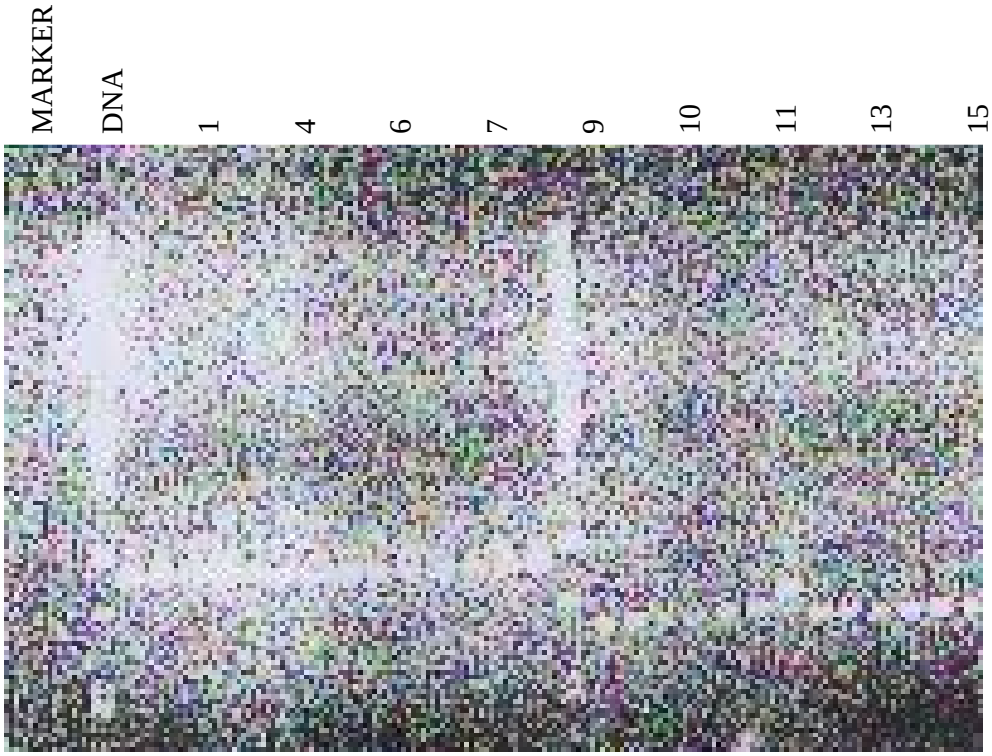


Figure 27: Restriction Fragment Lenghth Polymorphism (RFLP) analysis.

Analysis on a 1.5 % agarose gel. Restriction enzyme digestion of some of the clones PCR-products, with Msp 1 and Rsa 1 enzymes respectively. Labelled numbers depicts clone identities.

Restriction digestion with Msp 1; all yielded similar results and shows 2 fragments of about 0.950-1.0 kb and the other smaller fragment of about 100 bp not visible on the gel.

Restriction digestion with Rsa 1 enzyme yielded a fragment of about 720 bp and the other smaller part of about 250 bp cannot be seen. Note that Clone 11 looks different with a fragment size of about 820 bp seen and the other fragment not seen.

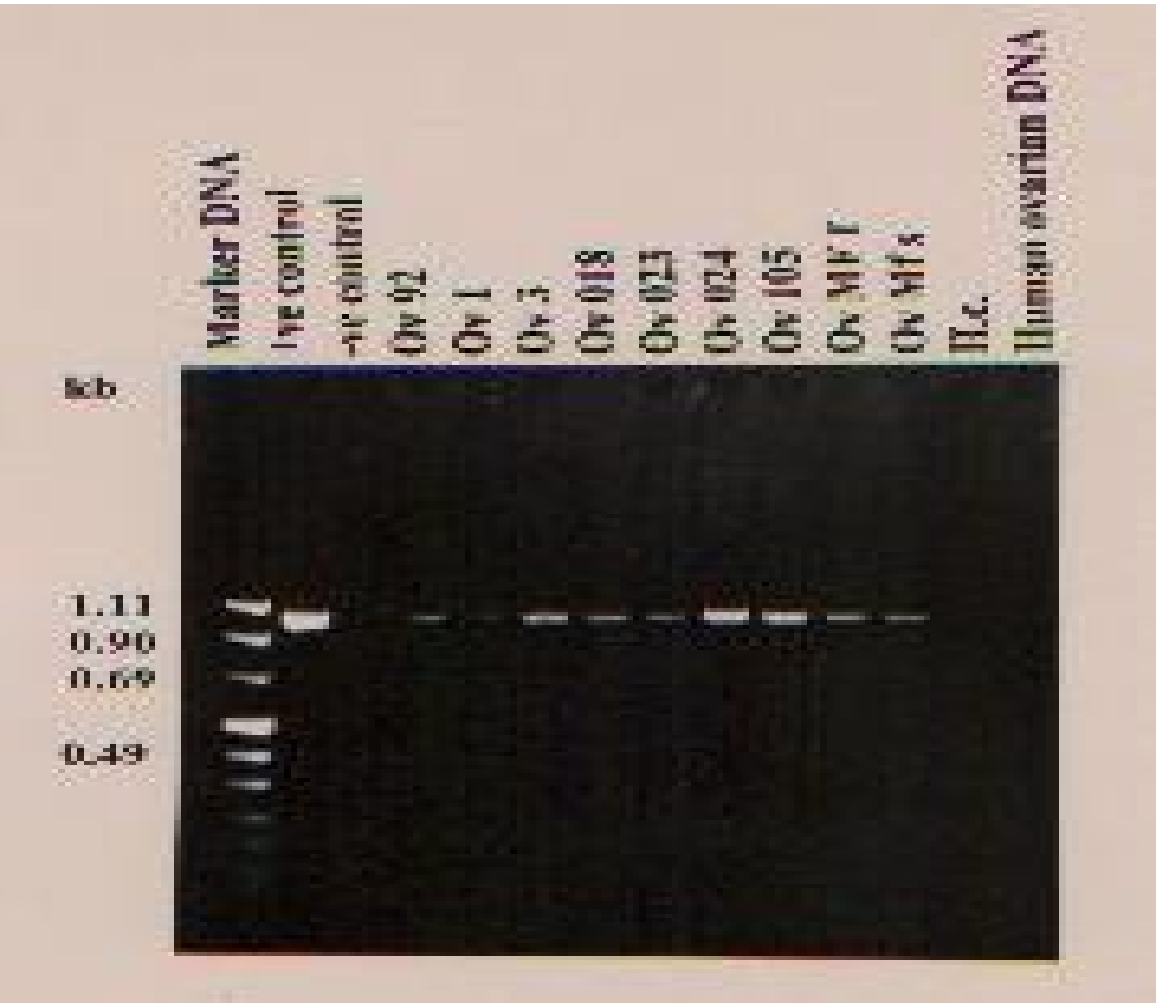


Figure 28: PCR product analysis with P11 – P12 primers.

PCR test with O.v. mdr primers P11 - P12 on several O.v. templates. Analysis on a 1 % agarose gel. Amplification yields a fragment of about 950 - 980 bp. No amplification product obtained with Human ovarian DNA template as well as H.c.

PCR was carried out according to the protocol of the manufacturer (Promega) at standard conditions (1.5 mM Mg2+), 30 cyycles were performed, 40 secs denaturation at 94°C, 30 min annealing at 46°C and 1 min 40 secs primer extension at 72°C.

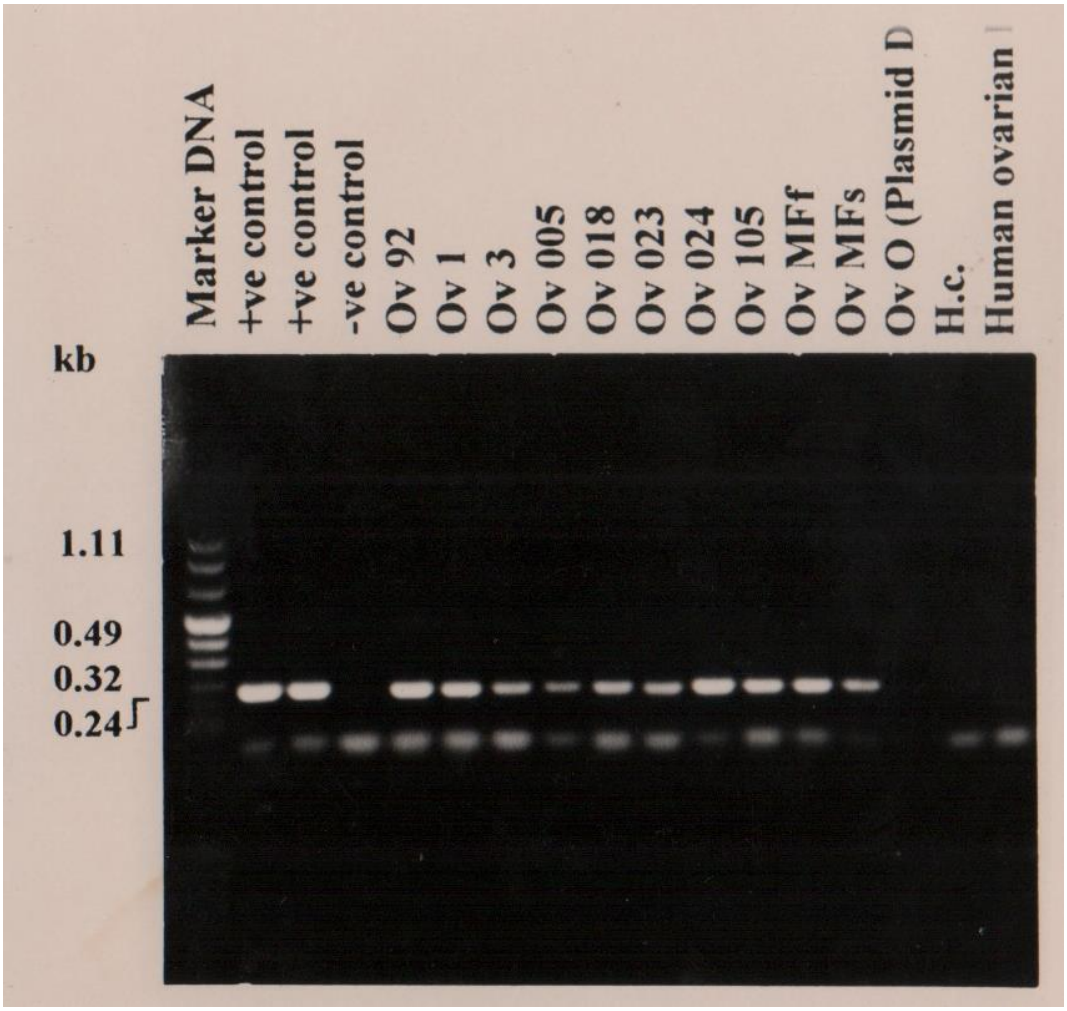


Figure 29: PCR product analysis of heat shock protein (hsp) primers.

PCR with Heat-Shock protein primers (HSP 25 - 1, HSP 25 - 2) on other *O.v* templates. Amplification yields a product of about 130 bp to 220 bp. PCR was carried out according to same protocols described above in Figure 3.3.7 (a).

Table 4: Specificity of *P11 – P12 O. volvulus* MDR primers

Samples	Result of PCR with P11 – P12
Positive Control (*O. volvulus* mdr plasmid DNA)	+ve
Negative Control (ddH_20)	-ve
OV 92	+ve
OV 018	+ve
OV 023	+ve
OV 024	+ve
OV 105	+ve
OV 1	+ve
OV 3	+ve
OV MFf	+ve
OV S	+ve
OV P	+ve
O. gibsoni	+ve
O. ochengi	+ve
Human Ovarian DNA	-ve
H. contortus	-ve
L. carinii	-ve
***Salmon* sperm**	-ve
Anopheles	-ve
P. vivax	-ve

PCR with P11 ï P12 primers on *Onchocerca* as well as other DNA templates, purposely to see how specific the primers are. PCR was carried out according to the protocols already described in fig. 3.4.7 a. Marker DNA is molecular Weight Marker VIII (BOEHRINGER MANNHEIM, Cat. No.1336045). Analysis on a 1% agarose gel. Positive templates showed a product of about 1kb.

(+) = Positive (-) = Negative

Table 5: Values expressing the relative density of the amplification products (Video Camera or Densitometer measurements)

PCR	DNA (µg)	Primers	+ve	+ve	-ve	Ov 92	Ov 005	Ov 018	Ov 023	Ov 024	Ov102	Ov 105	Ov 1	Ov 3	Ov mfF	Ov mfS	Ov S	Human DNA	H.c.
	0,13 - 0,18	P11P12	100,00			73,28	0,70	3,03	69,96	93,31	2,13	16,48	62,99	80,93	11,42	5,15	3,27	1,26	1,30
	0,26 - 0,36	P11P12	100,00		11,52	40,03	0,09	56,77	40,36	73,52	6,49	63,15	39,67	70,47	40,08	36,61	_	0,00	0,00
	0,26	P11P12	100,00			89,76	5,40	44,38	24,34	53,82	1,98	41,35	42,28	58,16	22,21	1,98	_	_	_
	0,26	P11P12	100,00			88,99	1,98	39,65	19,08	44,90	0,90	34,42	37,07	53,26	17,14	0,89	_	0,00	0,00
	0,26 - 0,36	P11P12	100,00		-2,34	72,33	1,22	52,51	59,06	77,07	_	84,16	52,96	22,18	55,97	31,73	86,57	0,00	_
	0,13 - 0,18	HSP25-1,-2	100,00	177,91		163,11	107,79	84,05	142,07	182,70	7,73	97,34	145,59	105,59	123,00	172,25	_	0,09	3,63
	0.26 - 0.36	HSP25-1,-2	100,00	88,95	29,74	63,67	28,00	54,04	33,07	97,18	18,82	61,83	60,49	40,51	57,15	67,47	_	0,00	0,00
	0,26 - 0,36	HSP25-1,-2	100,00	100,67		80,57	52,78	51,22	62,67	77,77	2,61	77,75	75,35	43,50	77,24	66,03	_	_	_
	0,26 - 0,36	HSP25-1,-2	100,00	106,33		80,30	56,78	49,06	50,62	82,69	9,25	71,38	75,13	39,80	71,31	61,54	2,89	_	_
	0,26 - 0,36	HSP25-1,-2	92,84	103,75	- 13,76	82,87	22,91	55,43	44,15	65,71	_	60,82	42,14	31,72	67,13	59,73	_	0,00	_

This table shows the values of the laser densitometer/ video camera readings of the PCR products. Dates of the PCR experiments, concentrations of the genomic DNA used as well as which primer are all shown. It is important to note that genomic DNA is not easily homogenous in ddH_20 or TE buffer so this explains the concentrations range. A correction factor was added to the values in order to eliminate the negative values for the purposes of statistical analysis.

Figure 30: Statiscal analysis of the densitometer values; Whole-Model Test.

The densitometer readings, which expressed as values the mdr PCR product bands, was used for analysis of variance with regards to the different treatment levels of the onchocerciasis patients. The P-values was calculated and a linear model was fitted to the logarithms of some ratio taking into account different patients and different treatments with ivermectin. The T-test analysis showed there was no significant difference between the two major groups (treated and untreated). Combination treatment ; Prob. >F = 0.8247. However, it should be noted that, the model explains about 63% of the total variation.

The 0.8247 probability value suggests that at the moment there is no significant difference among the different treatment groups from our sample collection.

3.4 Protein Analysis and Immunoblotting

3.4.1 SDS page analysis

Seperation of the *O. volvulus* protein antigens on a SDS-PAGE system revealed several proteins with different molecular weights ranging from 205 kDa to 13.5 kDa. While most of the proteins were reproducible and appeared to be present in all the *O. volvulus* samples, few other proteins were quite irreproducible and not contained in all the samples. However, these few others might thus be taken as a result of minor errors in antigen preparation but it is also important to point out the few differences. Figures 31a and b.

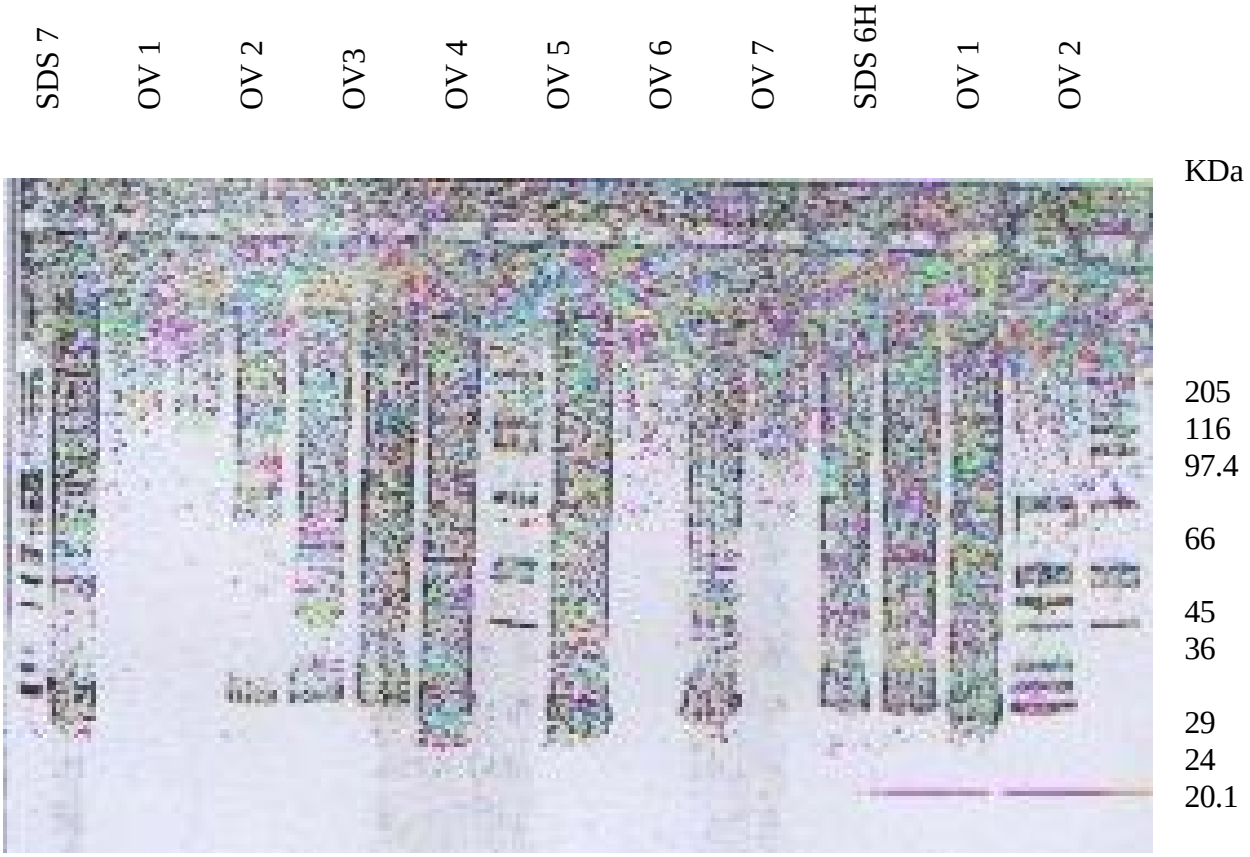


Figure 31 (a): SDS-Polyacrylamide gel electrophoresis and Silver staining of *Onchocerca volvulus* protein antigens

Adult worm homogenates prepared from both worm pools and individual worm samples and separated according to the different proteins - molecular weights through Glycin-SDS-PAGE in a gradient gel (7.5 % - 17.5 %). The gel was silver-stained in order to visualize the separated proteins. Molecular weight markers run parallel to the protein samples were used to determine the molecular weights of the separated proteins. Protein of various molecular weights ranging between 14kDa to 205kDa were observed among the worm samples.

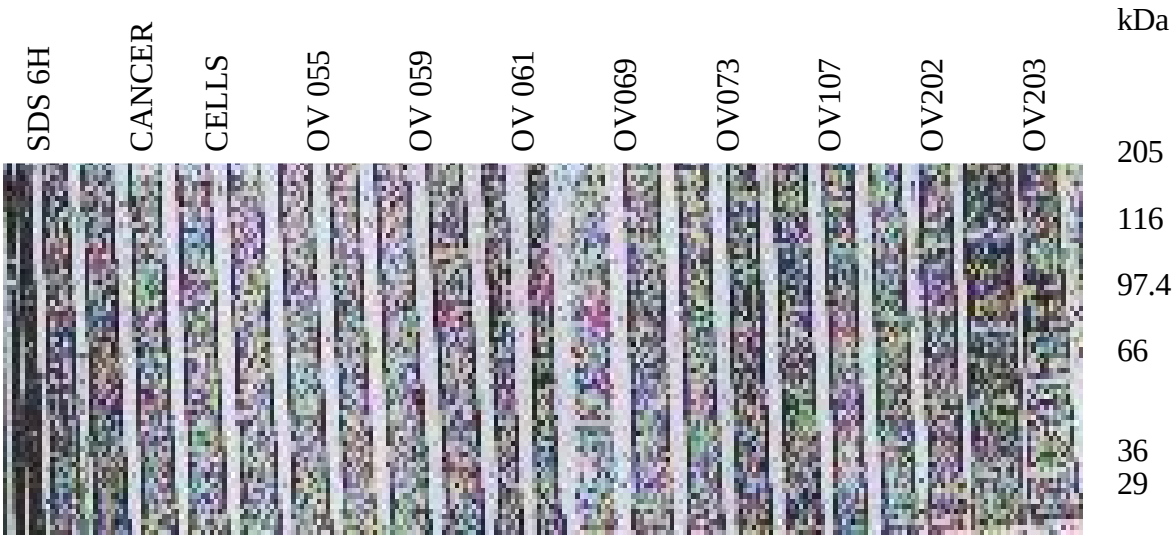


Figure 31 (b): SDS-Polyacrylamide gel electrophoresis and Silver staining of *Onchocerca volvulus* protein antigens

Adult worm homogenates prepared from both worm pools and individual worm samples and separated according to the different proteins - molecular weights through Glycin-SDS-PAGE in a gradient gel (7.5 % - 17.5 %). The gel was silver stained in order to visualize the separated proteins. Molecular weight markers run parallel to the protein samples were used to determine the molecular weights of the separated proteins.

Different proteins of different molecular weights were observed among the different worm samples.

3.4.2 Western blotting and Immunoblotting

Immunoblotting with the mdr polyclonal Ab from DIANOVA (Mannheim - Germany) gave a positive signal with a protein of about 62 - 68 kDa in a pool of *O. volvulus* protein antigen. The immunoblotting with C219 monoclonal Ab (P-glycoCHEK C219^{TM} MAB, CAT.NO 800-661, CENTOCOR DIAGNOSTICS, Centocor, Inc., Malvern, Pa., USA) gave a positive signal with samples OVP 1 and OVP 059. OVP 1 is a worm pool sample from once-treated patients while OVP 059 is from one untreated male adult. The positive signal was quite reproducible, a protein of about 51 - 65 kDa. See Figures, 32, 33, 34.

Immunoblotting with anti Pgp 389 polyclonal Ab gave a much stronger positive signal with the same samples OVP 1 and OVP 059 signalling a protein of about 51 - 65 kDa (Figure 33 and 34).

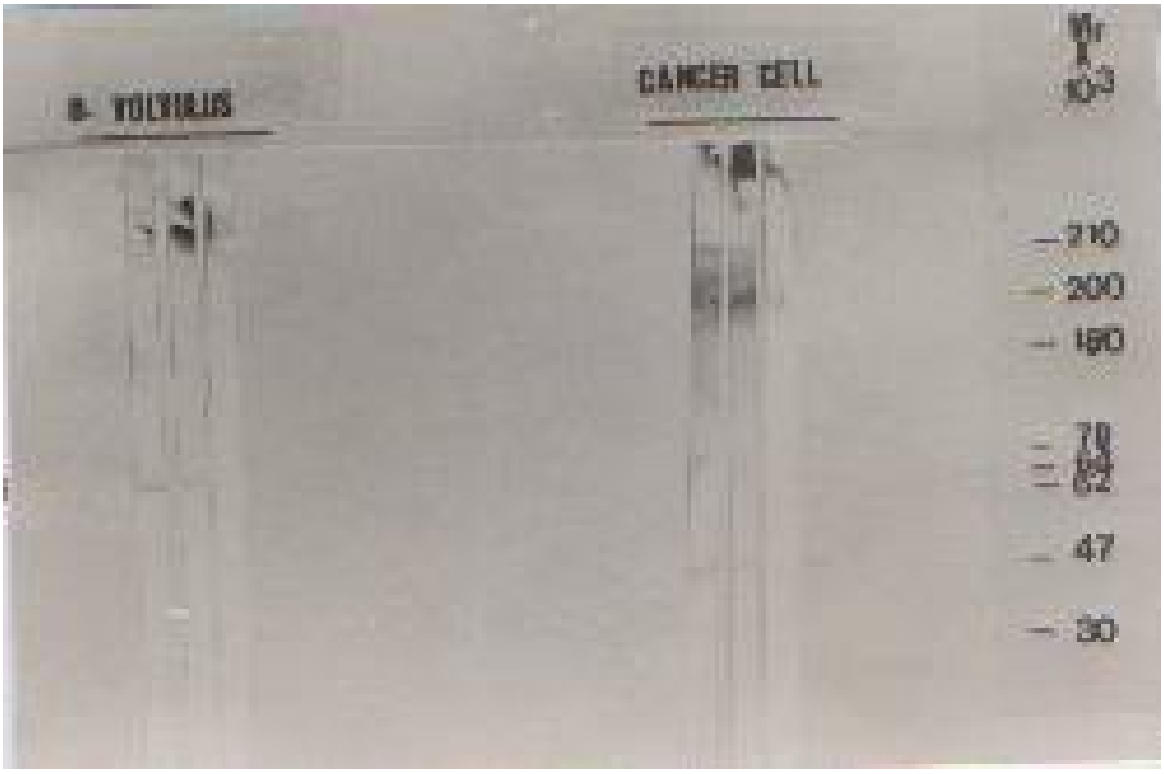
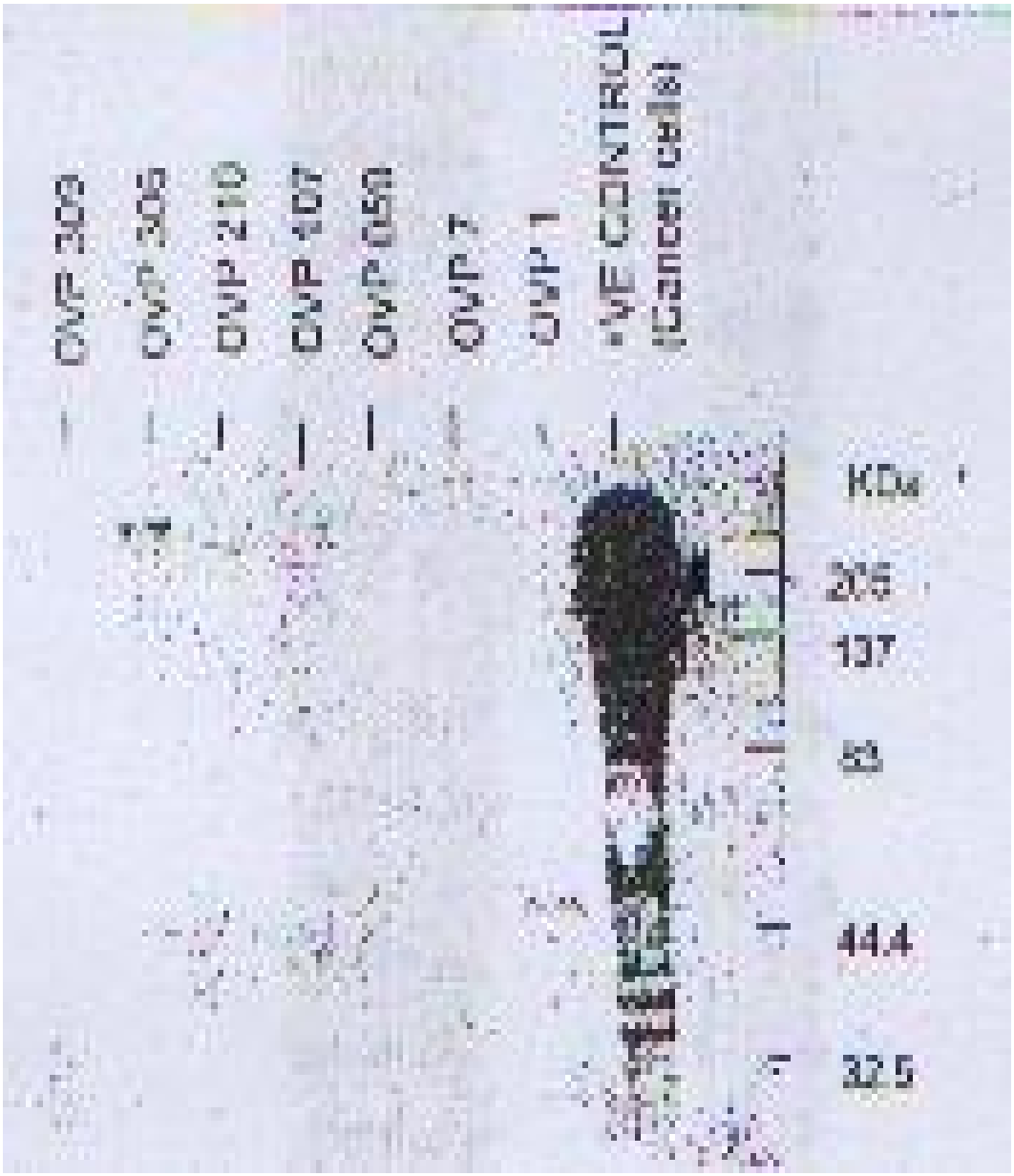


Figure 32: Western Blot analysis of *O. volvulus* antigens

Western Blot analysis of *O. volvulus* antigens using mdr Polyclonal antibody (DIANOVA, Mannheim, Germany). 10 - 12 µg protein samples were separated by SDS-PAGE, transferred to Nitrocellulose membranes and probed with a 1:100 dilution mdr polyclonal antobody and detected with anti mouse Ig G secondary Antibody, BCIP and NBT substrate solutions were used for development of the NC foils. The third NC strip was incubated without the primary antibody but with the secondary antibody so as to confirm that the positive signals were not due to cross reaction from the secondary antibody. A positive signal in the 35 ï 70 kDa range was observed.



Figuree 33: Western blot and immunoblotting analysis of *O. volvulus* protein antigens using monoclonal antibody against P-glycoprotein.

(C219 Mab) monoclonal antibodies, about 5 ug of the O .v. protein antigen were loaded. Proteins were separated by SDS-PAGE, transferred to polyvinyldiflouride membranes, and probed with 4 µg/ml C219 Mab. Immunoreaction was assessed by the enhanced chemiluminescence technique as already described in methods. Positive control is *cancer cells*. Positive signal in the 55 ï 70 kDa range was observed in some samples and not in others.

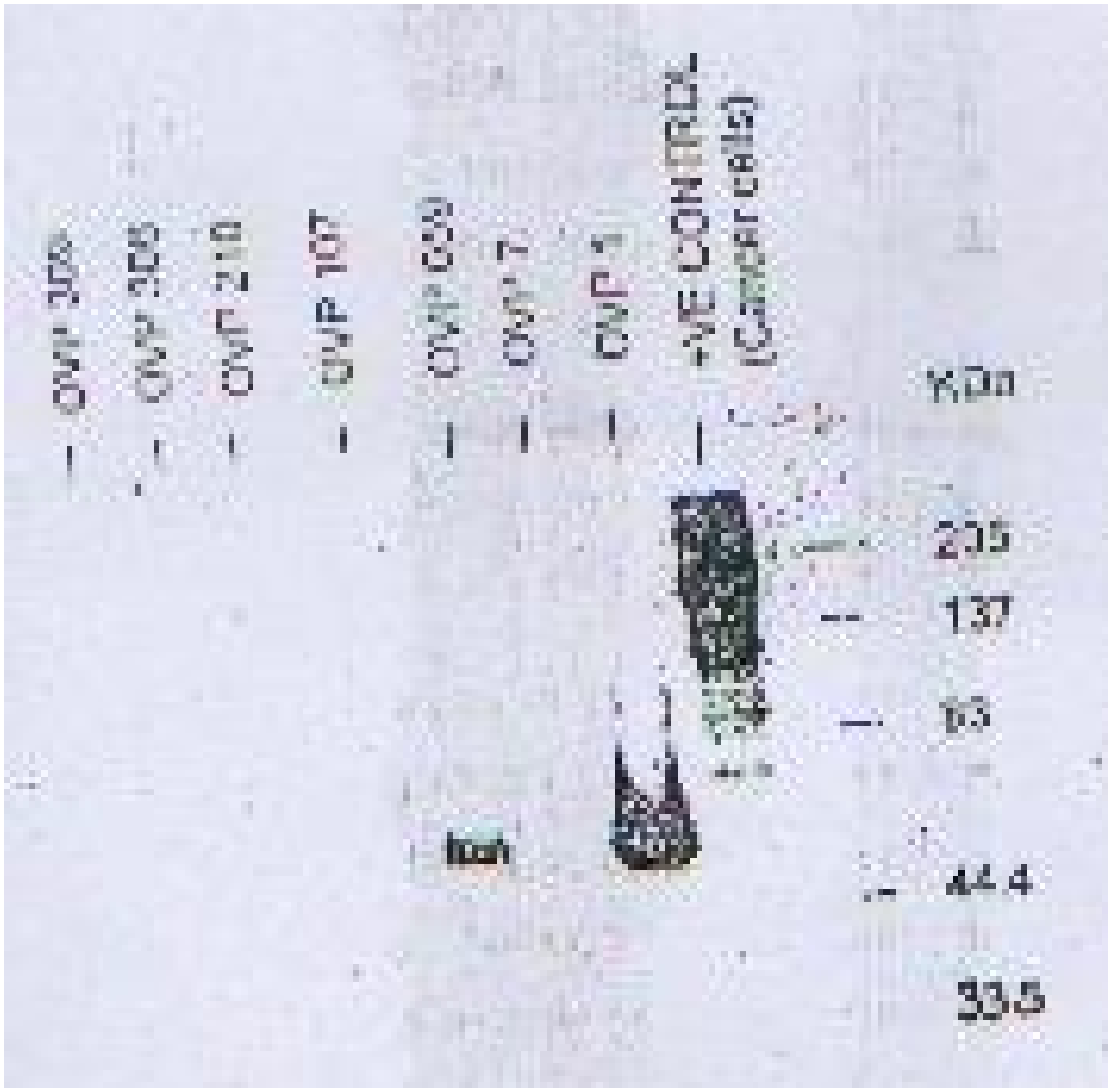


Figure 34: Western blot analysis of *O. volvulus* protein antigens using Anti pgp 389 polyclonal antibody.

About 5 ug of the *O.v.* protein antigen were loaded. Proteins were separated by SDS-PAGE, transferred to polyvinyldiflouride membranes, and probed with 1:5000 dilution anti-pgp 389. Immunoreaction was assessed by the enhanced chemiluminescence technique as already described in methods. Positive control is cancer cells (adriamycine 5000).

The anti-pgp-389 polyclonal antibody is not commercially available but was kindly donated to us by Dr. Jörg Striessnig , University of Innsbruck, Austria. The anti-pgp 389 polyclonal antibody is directed against amino acids 381 - 406 of the MDR1 gene. It is not commercially available. Positive signal in the 55 ï 70 kDa range was observed in some samples and not in others.

CHAPTER FOUR

DISCUSSION

4.1 Field Study

Results of these studies have shown that distribution and occurrence of onchocerciasis is most times focal in affected areas, that you have serious endemicity levels within an area or a community. Our study areas, Achi is about 25-30km from Amansea, the Oji River (along which the *Simulium* vectors breed) runs chiefly through Achi but has its tributaries to Amansea. Entomological studies by Okonkwo *et al.* (1991), showed that breeding occurs throughout the length of the Oji river and its tributary streams and springs. Yet our analysis revealed that Achi is an onchocerciasis hyperendemic region while Amansea is a mesoendemic region. Out of the 57 patients in Achi, 93% had onchocerciasis while out of the 52 patients from Amansea, 58% had onchocerciasis. Although these numbers are not representative of the two populations, it should be taken that characteristics of the participating patients represent what is to be expected from the whole population and moreover, the analysis of the module contents from Achi and Amansea clearly signifies the difference in the endemicity levels. Studies on onchocerciasis in Amansea have been quite scanty but the present study (carried out with an intention to treat) revealed that though situated very close to Achi, onchocerciasis prevalence in Amansea is small. Achi is a town with a population of about 25,000 people (Okonkwo *et al.,* 1991), made up of 21 villages. In one of the villages, Gbaragu, Okonkwo *et al.* (1991) reported a 76% microfilaridermiae in the populace and indicated that within Achi, each community was unique in the severity and infection pattern of the disease as well as in their knowledge of and attitude to the disease. A similar observation had been seen in south central Sierra Leone where Gbakima (1996) reported that the prevalence of *O.*

volvulus infection in the village community ranged from a low 7.4% among the international school children to a high 97% in Gbessebu. Also in Nigeria, Ogumba and Gemade (1992) reported that within four Nigerian states, of a total of 118 925 individuals, onchocerciasis pre-treatment prevalence ranged between 28% and 90%. Furthermore, in Nigeria, Akogun *et al.* (1994) reported that within 14 communities situated along the major road passing through Bali district in Taraba state, a range of between 1.1% and 45.5% prevalence was recorded, and infection was recorded in every community. In a study on the immunoepidemiological profiling of onchocerciasis patients, Arndts et al.(2014) maintained that in hyperendemic areas, 95% of individuals are MF^{+}, whereas in hypoendemic areas the rate is <30%. However, in areas that have been part of MDA programmes, individuals have been identified that present nodules but are amicrofilaridermic (a-MF). These two groups they studied their immune responses and found out that the data imply that this ñman-madeò group has a distinct immune profile but also suggests that immune responses are affected by the amount of IVM intake on both the individual and community level (region).

Thus there is little to wonder about the prevalence range of 93% and 58% in Achi and Amansea respectively, rather there is more to wonder about the nodule contents of the two areas. In Achi, 70% of the nodules isolated contained *O. volvulus* worms and 28% contained no worms, however, in Amansea, only 17% (5 out of 30) of the nodules isolated contained *O. volvulus* worms and another 17% were lipomas but the rest of 67% contained unidentified objects and old calcification-like materials. Apart from lipomas which have also been occasionally encountered in mass nodulectomy campaigns (Schulz - Key and Albiez, 1977; Albiez *et al*., 1984), very few reports if at all have reported the presence of unidentified objects in nodules. Looking for a rapid way of diagnosing onchocerciasis in the field, Remme (1992) suggested that presence of nodules can be used for onchocerciasis diagnosis since it has been found in different studies that

microfilaridermiae level is directly proportional to the presence of palpable nodules. But the analysis of Amansea in the present study is totally in contrast with this suggested method of diagnosis. In Amansea, within the 52 patients, 58% had nodules and 17% had skin microfilariae. This is not proportional. Meanwhile, 17% of the nodules contained worms and this looks in proportion to the 17% which had skin microfilariae, so it seems that the proportion of the patients with skin microfilariae is same as that of nodules with worms, but it should be noted that contents of nodules cannot be known immediately on the field but only after digestion. So do nodules persist after worms have become senescent or moribund? Presence of nodules may therefore under conditions yet to be clarified be a misleading indicator in the rapid diagnosis of onchocerciasis. Proper diagnosis of onchocerciasis is as important since it gives an idea of the epidemiology of the disease so that mass chemotherapy campaigns can be properly implemented.

Among the epidemiological factors which have been considered as having an enormous influence of onchocerciasis prevalence are occupation and sex. Kirkwood *et al.* (1983), reported lower infection rates in females in OCP areas and in Sierra Leone, in both savannah and forest areas, males had a higher rate than females, Okoye and Onwuliri ((1997), also reported higher prevalence of onchocerciasis in males among the inhabitants of Hawel River valley, here in Nigeria, but on the contrast, Okonkwo *et al*. (1991), reported no significant difference in infection rates between males and females in Gbaragu. In this study, females showed more infection rates than males in both Achi and UNTH group but the difference was also not significant. Reasons for this were not known as it might be a coincidence that more infected females had turned out during the study period. Achi town as well as Amansea consist mostly of subsistence farmers, fishermen and traders and their families. In Achi, onchocerciasis prevalence and intensity was more among the farmers. Among the patients in Achi, 54% were farmers and 65% of them had skin microfilariae.

19% of the patients were civil servants and 64% of them had also skin microfilariae. The students constituted 25% of the Achi patients and 43% of them had skin microfilariae. In Opi-Agu community, a tropical semi-urban area of Enugu state, Eyo *et al*. (2013) reported that the males had higher infection than the females and this can be explained on the bases of occupational differences and degree of exposure to blackfly. While the males are normally involved in agricultural work in farms where they spend most of the day, the females are engaged in petty trading in the local market and this reduces the chances of contact with the disease vector among females.

In Amansea, 40% of the patients were farmers, 44% students and 4% civil servants. In UNTH, 5% of the 131 were farmers and none had skin microfilariae. 32% were civil servants among which 10% had skin mf and 49% were students among which 11% of them had skin mf. Since it is expected that farmers and fishermen will have the highest infection prevalence and intensity (due to the fact that their occupation keeps them in close contact with the *Simulium* vectors, thus increasing their chances of infection) as seen in Achi, but it seems also that nearly same percentage of civil servants in Achi were infected too, and in the UNTH patients, none of the farmers were infected (the farmers were not from Achi). It points out or suggests that occupation might not actually be the decisive factor in infection prevalence and intensity but rather the overall endemicity of the area or community. So from this study, sex and occupation to play much role on infection prevalence and intensity but rather what determines is the overall infection level of an area which includes the abundance of infected black files, degree of man-fly-contact as well as transmission potential and intensity. It is therefore most beneficial to combine vector control with mass chemotherapy in the control of onchocerciasis.

Severre ocular manifestations and blindness which is the most devastating effect of onchocerciasis (Amazigo and Leak, 2008), were not prominently recorded among the patients who

participated in this study but in Achi community, other manifestations such as lichenified onchodermatitis, leopard and lizard skin, palpable nodules, and various degrees of onchodermatitis were observed. These findings agree with some other reported clinical signs of onchocerciaisis (Okoye and Onwuliri, 1997; Ukaga, *et al*. 2000; Ubachukwu and Anya, 2003; Okuliez *et al*. 2004; WHO, 2006; Bari and Rahman, 2007; Amazigo and Leak, 2008; Adeleke *et al*., 2010; Eyo *et al*., 2013). It is therefore most likely to be the forest strain.

Currently there are plans to use mass treatment with ivermectin to clear all Africa of the worst ocular and cutaneous effects of onchocerciasis. Several million people have already been dosed and ivermectin have shown encouraging results in the control of onchocerciasis-both transmission and morbidity (Karen *et al*., 1991), and this is shown from various reports on the evaluation of treatment programmes. The efficacy of antifilarial drugs is measured by the reduction of mirofilarial densities in the skin and by the observation of dead or degenerate parasites in the nodules and uteri contents of the female worms. Ivermectin is microfilaricidal, unlike suramin which is macrofilaricidal-showed a statistically significant macrofilaricidal efficacy quantitatively but not qualitatively (Wolf *et al.,* 1980)

Ivermectin does not only cause the prolonged microfilarial suppression by immediate killing of skin microfilariae (Duke *et al*., 1991) but also prevents the release of more microfilariae from the uterus of the adult female (Schulz-Key *et al.,* 1986). Thus, an investigation into the embryonic status of female *O. volvulus* is being used often for the evaluation of ivermectin efficacy. From our investigations into the uteri contents of *O. volvulus* worms collected from ivermectin treated and untreated patients in Achi, there were no significant difference between the two groups with regards to distribution of developmental stages. Both groups registered almost a similar pattern of distribution of developmental stages (normal and pathological forms) so it is difficult to draw any

conclusion as to the effect of ivermectin (on the average, very few developmental stages were recorded per female), even considering other observations like presence of calcified worms as well as presence of more females than males in the nodules. already made Similar observations have been made on the distribution of developmental stages from their various studies and also reported that embryos were obviously not produced continuously but in intervals of unknown length and frequency, about two thirds of females contain developing embryos or released microfilariae and the other females either contained eggs or showed empty uteri (Schulz Key and Albiez, 1977; Schulz-Key *et al*., 1980; Schulz-Key *et al*., 1986). They observed also degenerating worms and calcified worms and from one of their studies registered a male: female sex ratio of 1:1.5. In this study females were significantly more than males, a male: female sex ratio of 0:1.1 was registered. In a recent study carried out by Ben Li *et al*. (2014), in their quest to find out the mechanism of AVM anthelmintics in reduction of microfilaria production and release in filarial worms, through Glutamate ï gated chloride channels (GluCL) as targets for avermectins/milbemycin study. They showed that two GluCL subunits genes are expressed in developing embryos, and reproductive tissues of adult worms. The avr = 14 gene is highly expressed in *Brugia malayi* developing embryos and reproductive tissues and they provide evidence for the involvement of GluCL in gamete production and embryogenesis in filarial worms. This may explain the observed suppression of microfilaria production by female worms following treatment with avermectins/milbemycin anthelmintics.

However, in as much as ivermectin effect might not be totally/clearly/qualitatively observed in the embryonic status of *O. volvulus* females, ivermectin reduces both dermal and ocular microfilariae - in the skin by active killing of microfilariae and in the eye by passive elimination (Dadzie *et al*., 1989). Efficacies of ivermectin (given annually or six-monthly) in reducing dermal

and ocular microfilariae have been reported from several mass chemotherapy trails. A mass chemotherapy campaign to treat onchocerciasis with ivermectin was carried out in Northern Cameroun, in the North Vina valley, where all levels of onchocerciasis endemicity existed. The patients were grouped into two, one group received ivermectin at 6 month interval and the other group received annually. One year after the first treatment, skin microfilariae loads were down by more than 90% in the six-monthly group and more than 60% in the annual treatment group (Prod'hon *et al*., 1991).

In Ghana, in a hyperendemic Savannah onchocerciasis community, an ivermectin treatment trial to determine the effect of mass treatment on the microfilariae reservoir and on transmission of *O. volvulus* was carried out by Remme and his co workers. They reported that mean skin microfilariae load in treated persons fell by more than 96% two months after treatment and during the next two months, there was an increase in microfilarial loads. Entomological results indicated a reduction in transmission of 65%-85% during the first three post treatment months, thus showing that mass ivermectin chemotherapy can reduce skin microfilariae as well as onchocerciasis transmission significantly (Remme *et al*., 1989).

In another hyperendemic focus in Sierra Leone, Whitworth *et al*. (1996) followed up 948 onchocerciasis patients (treated annually or six monthly) for 6 years in a double-blind, randomized, controlled study of ivermectin for onchocerciasis. They reported that microfilarial loads were well suppressed but using a intention-to-treat analysis, they found a microfilarial prevalence of 16% months after up to 4 annual doses of ivermectin and 13% prevalence in the group receiving up to 10 doses of ivermectin at 6 - monthly intervals. This persistence of microfilarial they attributed to the presence of life adult female worms.

In Liberia, Pacque and his co-workers, after a large scale community ivermectin treatment reported an 86% skin microfilarial reduction at 6 months and in 1988 recorded a 78% skin microfilariae reduction at 12 months (Pacque *et al*., 1990a). In Malawi, ivermectin proved to be effective in the cure and improvement of papular, edematous or lichenified skin lesions of onchocerciasis (Burnham, 1995). Pacque *et al*., (1991) also reported improvement in severe onchocercal skin disease after a single dose of ivermectin.

In Achi, ivermectin distribution started since 1990 on a yearly basis. Distribution was chiefly conducted through the centralized system, (which was shown to be most cost effective after being compared to other methods of distribution like door-to-door and clinic-based, Akpala *et al*., 1993) so all eligible patients were dosed. Post-treatment biopsies in Achi revealed that skin microfilarial densities were reduced to a range of 20% - 0% pre-treatment levels among the various villages. Onchocerca eye diseases were also improved after ivermectin treatment (Okonkwo *et al*., 1993, unpublished data). However, I observed that there were still some patients without reductions in their skin microfilarial densities, just like the skin microfilarial persistence already reported by some workers (Remme *et al*., 1989, Plaisier *et al*., 1995, Whitworth *et al*., 1996). Ivermectin has been shown to be well tolerated in mass treatment campaigns (De-sole *et al*., 1989, Remme *et al*., 1990, Pacque *et al.,* 1991, Chijioke and Okonkwo, 1992; Ogunba and Gemade, 1992).

The emergence of drug-resistant *O. volvulus* has been suggested by reports of patients failing to respond to ivermectin treatment (Awadzie *et al*., 2004). A recent report from Ghana has provided the first proof of ivermectin resistance in *O.volvulus*. Mike Osei ï Atweneboana and colleagues showed that the ability of ivermectin to suppress skin microfilariae repopulation was reduced in some communities that had received 6 ï 18 yrs of ivermectin mass dosage administrations ï MDA (Osei *et al*., 2007).

From our study analysis in Achi, out of 31 patients who had received invermection treatment 61% of them had skin microfilariae and 24 of them had received ivermectin twice or thrice and yet 58% of these 24 still retained skin microfilarial. The reason(s) for this is not known. In the UNTH group, 33% of the twice of thrice treated group still had skin microfilariae. One reason though may be re-invasion by the infected *Simulium* flies or presence of more live females of which most of the other workers blamed the mf persistence upon or mal-reabsorption of the drug by the patients, although Okonkwo *et al*. (1993) in an effort to find a way of checking compliance, reported that ivermectin binds specifically to blood- human serum albumin but not in urine or saliva. Elkassaby (1991) also reported ivermectin uptake in plasma and tissue of Sudanese and Mexican onchocerciasis patients. So the drug is being absorbed, and currently, Baraka *et al.,* (1996) showed that infection with *O. volvulus* does not affect the pharmacokinetics of ivermectin and that filarial infected tissue and parasites themselves do take up the drug. So this leaves us with another `fragile' reason and it is the possibility that this persistence of skin microfilariae is an early sign of resistance development and for this reason, the further search for resistance factors and possible manifestations were performed in this study.

4.2 Molecular Biology Studies

The resistance of parasites and infectious disease organisms to drug and antibiotics is as old as chemotherapy itself. Drug resistance has been a major cause of setback in the treatment and control of most diseases of which typical examples are in cancer chemotherapy (Shen *et al*., 1986, Schimke, 1988; Marx, 1986; Kartner and Ling, 1989) and malaria chemotherapy (Payne 1987, Foote *et al.,*, 1989, Wellems *et al.,* 1990, Cornelissen *et al*., 1991). The P-glycoprotein coded for by the mdr genes have been associated in most of the resistance instances, although it is not certain at the moment if, when and how the resistance of *O. volvulus* (adult land microfilariae) to ivermectin

will develop. The key issue in the development of resistance is the percentage contribution that helminths surviving therapy make to the next generation. Factors that select for resistance included: mass treatment; frequent use of the anthelmintics; use of same class of anthelmintics over long periods and underdosing. Coles *et al*. (1995) have drawn attention to the risk of resistance developing even when only one or two doses of anthelminthic are used yearly. According to Shoop (1993), the important question is not whether resistant alleles can arise, but whether the putative residual population of microfilariae carrying them is large enough to contribute to the fixation of the resistance gene(s) in the population. Unlike *H. contortus, O. volvulus* has an indirect life cycle. Drug- selected resistant mf must first be transmitted to the insect vector and survive there, and then return to the human host before having a chance to mate and multiply (Shoop, 1993).

Till date, no safe macrofilaricidal drug for onchocerciasis existed. This changed with the identification of Wolbachia endobacteria (order Rickettsiales) in filariae of medical importance in the late 1990s (Taylor *et al*., 1999; Henkle ï Duhrsen *et al*., 1998), which prompted a series of experiments in animal models demonstrating that antibiotics (especially the tetracyclines) not only impede the growth and embryogenesis of filarial worms (Horauf *et al*., 1999; Bandi *et al.*, 1999) but also can kill the adult parasites (Langworthy *et al*., 2000). Such effects were not observed at clinically relevant doses in filarial species that naturally lack Wolbachia symbionts (Horauf *et al*., 1999; Brougui *et al*., 2001). Clinical trials of doxycycline (DOX) for human onchocerciasis were implemented rapidly, which achieved sterilization of female worms using a regimen of 200 mg/day for 4 weeks or 100 mg/day for 5 weeks (Hoerauf *et al.,* 2009). However, significant macrofilaricidal/ adulticidal activity (killing of 60 to 70% of female worms) required a regimen of 200 mg/day for 6 weeks. This relatively protracted course of treatment, coupled with contraindications in children below 8 years of age and in pregnant or lactating women, have

prevented approval of DOX for MDA to date (Germanus *et al.,* 2014), so ivermectin still remains more reliable than the other options.

The fear of ivermectin resistance is of high relevance. Ivermectin resistance has been reported in some parasitic nematode livestock (Coles, 2006; Coles *et al*., 2006). At present, there are no alternative drugs for ivermectin for use in the onchocerciasis control Programmes (mass chemotherapy) that reduce microfilariae or suppress adult worms, which can live up to 15 yrs in the human host. There is thus the need for the distributors of ivermectin to exercise a tougher and a firmer control over the mass distribution of ivermectin so as to make it difficult to enter unauthorised channels. Ivermectin should not be an over-the counter drug. If it becomes so, the abuse will readily enhance the development of resistance as has been recorded in some cases of malaria treatment.

However, our results show for the first time the presence of mdr genes (OVpgp l and OVpgp 2) in female *O. volvulus* and also a P-glycoprotein related protein of about 65 KDa in some of the female worms. Various functions of this P-glycoprotein abound, for example. *Leishmania* has a P-glycoprotein gene family of at least six members, and some of them have been shown to be involved in drug resistance (1tpgpA in low- level resistance to antimony). In some resistant strains of *Plasmodium falciparum*, amplification of pfmdr1 (a P-glycoprotein gene) is observed, and although the copy number of pfmdrl correlates with levels of resistance in mefloquine- and halofantrine- resistant *Plasmodium*, it does not correlate with chloroquine resistance.

So far these *O. volvulus* mdr gene have about 53% homology with that of *H.contortus* mdr1, *C.elegans* pgpAa, Human MDR 1a MDR 1b, Mouse and Hamster mdr genes. Two mdr genes in *O. volvulus* were identified although there might possibly be more since we did not search extensively.

The P11 and P12 primers, although designed from only one clone, were quite specific for *Onchocerca* species which renders it a useful tool for diagnostic purposes more especially in the field. Most important aspect of this primers P11 and P12 is the fact that it does not react with DNA from human, *H.contortus, L.carinii. Anopheles, L.striatus* and *P. vivax* (Figure 4). These are few sources of natural contacts with *O.volvulus*. At the moment, with the comparison and the calculations of the amplification products densities, there seem to be no significant difference between the ivermectin treated and the untreated patients although it can be argued that only 3 yearly doses of 150 g/kg of ivermectin might be too early to trigger off resistance. But it is known that large scale use and improper dosage has cause selection for resistant parasite population, which render previously effective l doses ineffective (Jackson, 1993). In Table 8 , with the Ov mdr primers, Ov 92 seem to have higher values than the other Ov samples and with the HSP primers Ov 3 has values, but on calculation as a combined treatment group against nontreatment group, there was significant difference. Thus, it is not yet ripe to conclude that there is amplification.

Furthermore from this study, the P-glycoprotein related protein was detected in some and not in others while the OVpgp genes was detected in all the female *O. volvulus* worms analysed. Already, it is known that overexpression of the P-glycoprotein is a result of the mdr gene amplification. So this suggests further that there is no amplification. Yet given the rapid development of ivermectin resistance in helminth of sheep and goats (Egerton *et al*., 1988, Echevarria *et al*., 1992; Shoop, 1993; Coles *et al*., 1994), the possibility of this resistance occurring in *O. volvulus* should be analysed.

However, it is not clear at the moment in what way resistance to ivermectin in *O. volvulus* will manifest as gene amplification which may or may not be observed as P-glycoprotein overexpression or as point mutation of promoter modification which would be more difficult to

assess? Drug resistance has followed chemotherapy as a faithful shadow and the history of chemotherapy is the history of drug resistance.

In *H. contortus* no evidence of mdr gene amplification has been found in benzimidazole-resistance isolates, levamisole-resistant isolates nor ivermectin- resistant isolates, rather Benzimidazole-resistance was found to entail a minimum of two genetic steps at separate β-tubuli loci - first, throughout a series of steps at which different isotope 1 alleles are lost, followed by the loss of different isotope 2 alleles and other possible mechanisms (Roos *et al.,* 1995).

In *C. elegans*, four P-glycoprotein genes have been identified (pgp-1. pgp-2, pgp-3, and pgp-4) and three have been analysed in detail. They share similarities with mammalian P-glycoprotein in their predicted protein structures. pgp-1 and pgp-3 are expressed throughout the life cycle, and exclusively expressed in the intestinal cells. *C.elegans* is a free-living soil nematode and it is assumed that soil nematodes have P-glycoproteins to protect themselves against toxic compounds made by plants and microbes in the rhizosphere. But pgp-3 was found to be involved in colchicine and chloroquine resistance in *C.elegans* (Broeks *et al*., 1995). Currently, Broeks and his coworkers identified four multidrug resistance associated protein (MRP) homologues in *C. elegans* and studied one member mrp-1 in detail (MRP belongs to the ABC transport superfamily. ABC transporters play a role in the protection of organisms against exogenous toxins by cellular detoxification processes). They found out that nematodes were hypersensitive to heavy melts when both the MRP homologue, mrp-1, and a member of the P-glycoprotein (Pgp) gene family, pgp-1, were deleted and thus concluded that nematodes have multiple proteins, homologous of mammalian proteins involved in the cellular resistance to chemotherapeutic drugs, that protect them against heavy metals. since *O. volvulus* is a nematode (but not free living), the P-glycoprotein related found might be playing its natural role of detoxification but the role of keeping out ivermectin poses a

great threat in the treatment of onchocerciasis since the interpretation is resistance. In order to avoid this happening, it might be a good consideration to include reversal agents in the treatment of onchocerciasis with ivermectin or direct more efforts towards a new and effective macrofilaricide

Additional, studies have associated ivermectin resistance with genetic markers (Kohler, 2001; Ardelli *et al*., 2006), particularly the ϐ-tubulin gene in human *O. volvulus* and the livestock nematode parasite haemonchus contortus (Eng and Prichard, 2005; Eng *et al*., 2006). However, previous *O. volvulus* genotyping studies were non-longitudinal using worms collected from different ivermectinï naïve and treated individuals. A new study by Catherine Bourguinat and colleagues reported and concluded not only that ivermectin caused genetic selection in *O. volvulus* worms but that this selection was also associated with a lower reproductive rate of female parasites (Bourguinat *et al*., 2007). This pointed to the daunting possibility of the spread of IVM resistant parasites in endemic regions already treated with ivermectin. Thus in the control of onchocerciasis with ivermectin treatment, there is an urgent need to develop tools for detecting ivermectin resistance that could be easily deployed in large scale ivermectin treatment programmes or already established yearly treatments. This is because, if resistance is detected early, strategies can be deployed to contain it before it becomes a widespread menace as we see today in cancer and malaria chemotherapy.

4.3: Conclusion

The discovery of mdr genes in *O. Volvulus* and the design of P 11ð P12 onchocerca-specific primers are novel innovations and a major contribution to knowledge. These primers can be used as a tool for monitoring resistance. In as much as the need for onchocerciasis persists, it is still of utmost importance to make sure that the drug ivermectin currently in use still remains effective and proper monitoring be maintained so as to detect early selection for resistance to ivermectin in *O.*

volvulus when it occurs. Although one of the major obstacles in onchocerciasis is the lack of available model systems to study host-parasite interactions, and how sensitive are the drug-parasite *in-vitro* tests at detecting resistance at low levels, the findings from this study have revealed the basic questions. *O. volvulus* is a parasitic nematode. Pgp genes have been found in *O. volvulus*. A P-glycoprotein related homologue had also been isolated in some *O. volvulus* worms. Ivermectin is the current drug of choice in the treatment of onchocerciasis. Ivermectin helps in the clearing of some other human gastrointestinal parasites, like *Necator americanus*, *Trichuris trichuria* and *Ascaris* (Behnke *et al*., 1994; Okoli, Mercy personal observations in Achi) and ivermectin has been found to be effective in the treatment of scabies (Elgart, 1996) although not yet approved, so there are numerous benefits from ivermectin and there is need to retain these benefits.

However, ivermectin is a potential substrate (Pouliot *et al*., 1997). Therefore the possibility of resistance manifestation in the treatment of onchocerciasis with ivermectin is very real and more research on this is urgently needed. The detection of the Pgp genes in *O. volvulus* can serve as a first step towards the monitor for ivermectin resistance. More work should be directed at locating the tissue distribution of P-glycoprotein in *O. volvulus* and analysis of all the Pgp genes present so as to shed more light on structure and function and in order to hamper this resistance development, ivermectin can be doubled at intervals.

4.4 Future Studies

There is need to follow up the participants who have received up to five, ten or even fifteen years annual ivermectin treatments. The observation of nodule content in Amansea requires further investigations to ascertain the implicationsfor the findings and more villages should be

involved. There is also a need to isolate and sequence the complete *O. volvulus* mdr/pgp genome and if possible identify existing variants.

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APPENDIX

Appendix 1: Showing the Prevalence of Onchocerciasis in three (3) different communities. (Age classification 0-20 yrs children 21 and above adults)

	No of Patients	Males	Females	Children	Adults	+VE MF	-VE MF	+ve nodules present	-Ve nodules absent	Onchodermatitis	Lizard/ Leopard skin	Total Prevalence
Achi	57	40% 23	60% 34	26% 15	74% 42	58% 33	42% 24	93% 53	7% 4	21% 12	47% 27	93% 53
Amansea	52	38% 20	62% 32	50% 26	50% 26	17% 9	83% 43	58% 30	42% 22	19% 10	4% 2	58% 30
UNTH	131	39% 51	61% 80	21% 27	79% 103	10% 13	87% 113	15% 20	85% 111	50% 65	18% 23	5% 6

Appendix 2: A tale of 2 communities; Worm content of nodules

	Total no of patients	No with nodules (no collected)	Nodules with worms	Nodules without worms	Lipomes	Others
Achi	57	10(9)(15%)	8(88%)	1(11%)	-	-
Amansea	52	30(57%)	5(16%)	20(66%)	5(16%)	-

Appendix 3: Influence of occupation on the prevalence of onchocerciasis in three areas.

	Total no patients	Farmers (%)	Civil Servants (%)	Students Pupils	+ve MF farmers	+MF Civil Servants	+MF students
Achi	57	30 (52)	11 (19%)	14(24%)	37 (65%) 19/30	36(64%) 7/11	24 (43%) 6/14
Amansea	52	20 (40%)	4(7%)	28(53%)			
UNTH	131	6(5%)	42(32%)	64(49%)	-	10% (4/42) 13	14 (11%) 7/64

Appendix 4: Showing quantitative distribution of embryonic stages in Onchocerca vovulus worms isolated from ivermectin treated and untreated patients.

EMBRYONIC STAGE	**NORMAL FORMS**	**PATHOLOGICAL FORMS**	
FROM UNTREATED PATIENTS			
Oocytes	10, 500	4, 000	
Early Morulae	10, 000	8, 500	
Late Morulae	7, 900	11, 500	
Horse-Shoe shaped	2, 800	9, 000	
Coiled MF	12, 500	16, 500	
Stretched MF	0	17, 000	
FROM IVERMECTIN TREATED PATIENTS			
Oocytes	6, 000	50	
Early Morulae	5, 900	1, 000	
Late Morulae	0, 800	4, 000	
Horse-shoe Shaped	0	3,000	
Coiled MF	2,500	2,500	

Streched MF	2, 000	5, 500	