EVALUATING ONCHOCERCIASIS USING PARASITOLOGICAL, ENTOMOLOGICAL AND SLIDE AGGLUTINATION TECHNIQUE IN KACHIA, KADUNA STATE, NIGERIA

BY

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DECLARATION

I declare that the work in this Thesis entitled "Evaluating Onchocerciasis Using Parasitological, Entomological and Slide Agglutination Technique in Kachia, Kaduna State, Nigeria" has been carried out by me in the Department of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria under the supervision of Prof. (Mrs.) H. I. Inabo, and Prof. S. E. Yakubu, of Microbiology Department and Prof. P. A. Audu, of the Department of Biological Sciences. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

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CERTIFICATION

This Thesis entitled EVALUATING ONCHOCERCIASIS USING PARASITOLOGICAL, ENTOMOLOGICAL AND SLIDE AGGLUTINATION TECHNIQUE IN KACHIA, KADUNA STATE, NIGERIA by Hudu Okankhamame OSUE meets the regulations governing the award of the degree of Doctor of Philosophy in Microbiology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

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Abbreviations

Ab	Antibody
Ag	Antigen
BLAST	Basica local alignment search tool
^{o}C	Degree Celsius
CDD	Community directed distributor
CDTI/M	Community Directed Treatment with Ivermectin/Moxydectin
DALE	Daily adjusted life expectancy
DALYs	Daily adjusted life years
DDBJ	DNA Data Base of Japan
DEC	Diethylcarbamazine citrate
DIA-BA	Dot Immuno-binding Assay-Biotin-Avidin
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
DSIA	Dipstick immuno-binding assay
ELISA	Enzyme-linked Immunosorbent Assay
EMBL	European Molecular Biology Laboratory, Germany.
ESR	Erythrocyte sedimentation rate
fg	Femtogram
γ	Gama
GABA	Gama-amino butyric acid
GU-AG	Guanine Uracil-Adenosine Guanine
IgG or M	Immunoglobulin, Gamma or Miu

in vivo	In the life host
ITS	Internally transcribed spacer
kDa	Kilo Dalton
K*i*	Inhibition constant
LLCMK	Monkey kidney cell 2
LMW	Low molecular weight
µl	Microlitre
MDA	Mass drug administration
M_r	Molecular weight relativity
NAAT	Nucleic acid amplification test
NCBI	National Centre for Biotechnology Information, USA.
ng	Nanogram
nm	Nanometer
NaH_2CO_3	Sodium hydrogen bicarbonate
NPV	Negative predictive values
OEPA	Onchocerciasis Eradication Programme of America
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PELF	Programme for the Elimination of Lymphatic Filariasis
pg	Picogram
PLERM	Probable *L. loa* encephalopathy temporally related to Mectizan-treatment
pMol	Per Mole
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PELF	Programme for the Elimination of Lymphatic Filariasis

PPV	Positive predictive values
RNA	Ribonucleic acid
r.p.m.	Revolution per minute
PCV	Packed cell volume
QLIPS	Quick luciferase immunoprecipitation system
RBD	Relative blood density
SMART	Simple, measurable, accurate, repeatable and timely
SDS	Sodium dodecyl sulphate
ssu	Small subunit
TN	True negative
TP	True positive
WBC	White blood cell

Definitions

Accuracy:	The probability that the results of a test will accurately predict presence or absence of the disease (TP + TN)/(TP + TN + FP + FN).
False negative (FN):	Patients with the disease who have a negative result
False positive (FP):	Patients without the disease who have a positive result.
Negative predictive	The probability that a patient with a negative test result will not have the disease TN/(TN + FN).
Positive predictive value (PPV):	The probability that a patient with a positive test result will have the disease TP/(TP + FP).
Sensitivity	This is true positive rate. The probability that a patient with the disease will have a positive test result TP/(TP + FN).
Specificity	This is true negative rate. The probability that a patient without the disease will have a negative test result TN/(TN + FP).
Likelihood ratio of negative result:	The decrease in the odds of having the disease after a negative result (1-specificity)/Sensitivity.
Likelihood ratio of positive result:	The decrease in the odds of having the disease after a negative result. Sensitivity/(1-specificity).

False negative rate (FNR)	The probability that a patient with the disease will have a negative test result. FP/(TN + FP).
False positive rate (FPR)	The probability that a patient without the disease will have a positive test result. FN/(TP + FN).

Adopted from Elavunkal and Sinert (2007).

Glossary

Amplification	Making copies of DNA sequences using PCR for molecular analysis
Anthropophilic	Affinity or predilection for human
Antigen	Any substance that stimulate antibody production
Antibody	Immunoglobulin protein produced in response to antigenic stimulation
Assymptomatic	Infection without no evidence of clinical signs and symptoms.
Cytoadherence	Mechanism that support attachment of cells to tissue or pathogens.
Diagnostic	Test to determine presence or characteristic indicative of a disease.
Debility	Weakness or incapacity of the body
Demographic	Population related
Elimination	Getting rid of infection to near absence.
Epidemiology	Study of disease occurrence in time, space, transmission and control
Eradication	Absence of the case of infection after 3 years monitoring
Flocculation	Test based on antigen and antibody reaction to produce visible reaction to form aggregate particles that can be observed.
Geographic	Area of coverage or number of communities involved
Glycoprotein	A conjugated protein having carbohydrate component.
Heterologous	Cells or tissues derived from different related or unrelated organism.
Immunoglobulin	Glycoprotein produced by B- lymphocytes in response to antigen stimulation.
Insidious	Subtle and gradual infection with no threat to life.
Ivermectin	A drug produced from bacteria, *Streptococcus ivermetilis*
Microfilaricide	Drug that kills microfilaria or forth stage larvae (L4).
Macrofilaricide	Drug that kill the filarial adult worm.
Metabolomics	Use of metabolic products produced by an organism for identification.

Morbidity	Causing clinical changes or lesions.
Mortality	Number of death in population in specific period.
Nematode	Family of round worms.
Onchocercomata	Nodule produced as a result of onchocerciasis infection where female O. volvulus adult worms are encapsulated.
Onchocerciasis	A disease caused by nematode worm belonging to the Genus and species, *Onchocerca volvulus.*
Ovoviviparous	Production of life motile larvae without embryonic stage.
Pathology	Various lesions and tissue changes as a consequence of disease.
Pruritis	Itching with papules.
Psycho-social	Affecting the mind, mental behaviour and the ways of life of a person.
Reliability	Diagnostic accuracy measured in time of sensitivity and specificity.
Resistance	Loss of the normal response to treatment and is heritable.
Sentinel	Selected study area based on previous knowledge about the situation for which the study will be done.
Stigmatization	Discrimination against someone based on clinical condition.
Surveillance	Investigation of disease presence in time and place.
Sympatric	Belonging to the same family of organisms
Vector	Organism that transmit agent of infection or diseases
Zoophilic	Affinity or predilection for animals

ABSTRACT

It has become necessary to assess the ongoing community directed treatment with ivermectin (CDTI) of onchocerciasis after 18 years of commencement of intervention. This thesis compared baseline and post-treatment data generated in 1994 and 2011 from sample populations (n=531) and (n=593) respectively in six sentinel onchocerciasis meso-endemic communities. Secondly, development and evaluation of a novel *Onchocerca volvulus* slide flocculation test (Ov-SFT) was undertaken. Parasitological analyses of skin snips and engorged blackflies caught by human bait were dissected and examined for microfilaria. Eye and skin clinical examination were performed. In this study, the levels of onchocercal specific serum antibodies reacting with sodium dodecyl sulphate (SDS) extracted low molecular weight (LMW) antigens prepared from adult female worms of *O. volvulus* after collagenase digestion of nodules were assessed at post-control. The Ov-SFT post-treatment data were compared with that of enzyme linked immunosorbent (ELISA) baseline data for the study population. Eight tissue homogenates from rat organs (kidney, liver, heart, spleen, muscle, brain, lungs and testes) were screened for flocculation potential using confirmed onchocerciasis positive and negative sera. The skin microfilaria status of patients were validated using first internal transcribed spacer (ITS1) of ribosomal DNA (rDNA) that lies within 18S and 5.8S gene region in polymerase chain reaction (PCR) amplification of DNA templates extracted from skin biopsies (n=66). The DNA extracted from fragments of *O. volvulus* adult worms from two different nodules and template from three non-infected persons served as positive and negative controls, respectively. A cohort (n=445) of volunteered participants (75%) were examined at both visits. The annual village treatment, 11(64.7%) and population treatment compliance was 92.6% with a range between 88.7 to 100%. Overall mean number of treatment was 2.9±1.6 with a range, 2.0±1.2-3.3±0.6. Post-treatment skin microfilarial prevalence and palpable nodule occurrence were significantly

reduced from 144 (27.0%) and 77 (15%) to 0 (0%) and 4 (0.7%), respectively compared with baseline data ($P<0.05$ t-test of unpaired data). There were significant decrease in cases of papular onchodermatitis from 51 (9.1%) to 2 (0.04%) and onchocercal inducible ocular lesions from 31 (5.8%) to 12 (2.1%), $P<0.05$. Cases of glaucoma 8 (1.4%) and blindness 6 (1.05%) remained unchanged. Those with visual acuity of ≥6/24 in one or both eyes had increased from 26 (4.9%) to 198 (33.45%) with a range 16 (17.4%) – 47 (61.8%) in the villages. The significant increases in cases of cataract 169 (28.5%); pterygium 157 (26.5%) and acute senilis 165 (27.9%) were strongly positively correlated with increase in age (R^2= 0.898 - 0.949). The liver homogenate proved to be the highest, followed by the kidney and heart. 33 (50.76%) of the sera (n= 65) tested positive for onchocerciasis. Profile of antibody titre ranged from 1:2, 1 (1.54%) to 1:32, 13(20%). Three out of the five malaria positive cases were Ov-SFT sero-reactive compared to 9 (63.5%) of the 14 malaria negative. Nanodrop spectrophotometer analyses of DNA concentration of samples and control at 260 nanometer (nm) wave length were between 1.5 to 24.5 nanogram (ng). The 260/280nm ratios were between 1.45 and 1.68 compared to 105 and 10.5ng, and 1.65 and 1.44 nm for control, respectively. Out of the 66 DNA samples analysed using ITS1 primer sequences, 4 (6.1%); (two males and two females) were amplified. Two positive samples had single band of 344 base pairs (bp), while two gave double bands amplification products (amplicons) of 344 and 500bp molecular weight in an agarose gel electropherogram. The 4 amplicons gave DNA nucleotide sequences of between 629 and 639 sequence length. One of the two positive controls gave different amplicons of molecular weight that falls within expected *O. volvulus* 344 bp and other sympatric filarial that lies between 312, 305 and 301bp for *Mansonnela ozzardi*, *M. perstan* and *Wuchereria bancroft*, respectively. Whether the DNA amplified were from dead or living mf remained unknown. The ITS1 PCR amplification of DNA gave greater positive predictive values than skin snip microscopy for microfilaria. Despite varied

compliance to ivermectin treatment, active disease transmission and progression of onchocerciasis induced skin and ocular clinical manifestations have been halted. Ivermectin treatment had no impact on existing cases of glaucoma and blindness, which remained unchanged but halted development of new cases. The Ov-SFT is simple, sensitive, fast and required less skill; it should be suitable for screening exposure to infection. Further validation to establish its specificity using related parasites is required. The need for an algorithm for assessing onchocerciasis treatment control involving antigen-specific antibody screening, molecular diagnosis and parasitological confirmation cannot be overemphasized.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Preamble

Onchocerciasis (synonym: river blindness or 'craw-craw' or Robles disease) is a disease complex caused by the nematode worm belonging to the Genus *Onchocerca*. Of the 34 species known, only *Onchocerca volvulus* (Nematoda: Filarioidea) is anthropophilic, others are zoophilic. The occurrence of other filarial in sympatry with Onchocerciasis has been documented in the Americas and in Africa including Nigeria (Medeiros *et al*., 2009; (Morales-Hojas, 2009; Ta Tang *et al*., 2010). Onchocerciasis is the fourth cause of blindness and second cause of infectious blindness after trachoma the world over (Winthrop *et al*., 2011). Onchocerciasis occurs mainly in Africa with more than 99% of the 26 million infected people living in 31 countries in sub-Saharan Africa (WHO, 2014a). Meri *et al*. (2002) found that the adult worms produce microfilariae, which are responsible for the disease pathogenesis by activating the complement system. The activation stops before the formation of terminal complement complexes. The *O. volvulus* mf may evade human complement by binding factor H (fH) that promote inactivation of C3b into iC3b.

Early control strategy hitherto depends on larviciding, and erstwhile use of diethylcarbamazine citrate (DEC) and Suramin chemotherapy had proved unsuitable for mass drug administration (MDA). In addition, the toxicity of DEC causes “Mazzotti reaction” while Suramin induces serious eye complications (WHO, 1995). Large-scale nodulectomy was also attempted but without success. These methods failed because of several limitations including insecticidal resistance by the vector, hazard to the environment, and cost given the vast landmass to be covered. The onchocerciasis control

Programme (OCP) was established in 1975 and came to an end in 2000 with 11 participating countries. The programme succeeded bringing the disease under control except in Sierra Leon due to internal strife (WHO, 1995). After patenting of Ivermectin for human use in 1987, and the generous donation by the manufacturer, Merck Sharp and Dohme (USA) to provide the drug free of charge to endemic communities as long as required became the very foundation for public and private partnership in disease control. Noma *et al*. (2014) provided the rapid epidemiological mapping of the 20 countries participating in APOC where ivermectin treatment distribution did not cover and areas with *Loa loa* co-endemicity.

Onchocerciasis Elimination Program for the Americas (OEPA) was created in 1991 (WHO, 1995). The distribution of Mectizan started in Nigeria in 1992 under the National Primary Health Care (PHC). The study villages were enlisted into the National Mectizan Distribution Program in 1994. Baseline data were collected before the commencement of treatment (Osue, 1996). African Programme for Onchocerciasis Control (APOC) was established in 1995 to ensure the community directed treatment with ivermectin using community Directed Distributors (CDDs) was put in place in all meso- and hyper endemic communities. There are other non-govermental organizations involved in ivermectin distribution (Bush *et al*., 2011). In Nigeria, the Carter Center (Richards *et al*.,2011), Sight Savers, Theophilus Danjuma Foundation and recently, Emaka Offo have supported effort to control the disease.

Onchocerciasis is among the group of 10 neglected tropical diseases (NTDs) within the programme of the Federal Ministry of Health. The strategic goal is to progressively reduce morbidity, disability and mortality due to NTDs using integrated and cost-effective

approaches with a view to eliminating NTDs in Nigeria (FMoH, 2012). It has a timeframe that aligned with the public and private stakholders London Declaration by public and private stakeholders resolution to eradicate 10 neglected tropical diseases by 2020 (Uniting to Combat NTDs, 2012). Progress in attaining this goal was appraised in 2013 (Uniting to Combat NTDs, 2014); Turner *et al*., 2014a). The disease is transmitted through the bite of the insect vector, a ferocious blood sucking blackfly, *Simulium damnosum s.l.* About 2-3 microfilariae ingested undergo developmental changes from first stage (L_1) to third stage infective larvae (L_3). The classical skin snip test for emerged microfilariae (mf) referred to as the "gold standard" is invasive and less sensitive. Its sensitivity depends on location or site, number of skin snips taken and level of skin mf density (Taylor *et al*., 1989). It is expected to become further less sensitive due to reduction in microfilarial load at the period following post-treatment when surveillance for recrudescence will become inevitable (Guzman *et al*., 2002). The advantage of antibody over antigen and nucleic acid detection is the ability to detect past exposure to infection and current (active) infections (Enwenzor *et al*., 2000). Antibody test can provide the basis to define those to be included for diagnostic and or confirmation tests. The Yaoundé Ministerial Declaration emphasized the need for a sustainable and integrated approach for surveillance and control within strengthened health systems (Amazigo and Boatin, 2006). Economic consideration for lack of market has constrained investment in the development of diagnostics for NTDs.

1.2 Statement of the Research Problem

Following the administration of ivermectin to treat onchocerciasis in six sentinel communities over the past 18 years, the status of onchocerciasis in the said area has not been evaluated to establish the effectiveness of the control programme. Stage of intervention has moved from control to elimination phase (APOC, 2011a). A worrisome

situation is the reports from studies that have shown differences in both geographic and therapeutic coverage and diverse treatment compliance rates (Yirga *et al*., 2010; WHO, 2011a). Added to these is the fear that drug resistance could emerge. All these factors will no doubt hamper the attainment of the set goal to break the disease transmission and eventually eliminate the disease. Deciding when and where to stop treatment will depend on sustained active surveillance and monitoring of control strategy.

A reliable diagnostic test to complement and overcome continued reliance solely on skin microfilaria detection regarded as the 'gold standard' has made the need for standard or quantitative PCR highly desirable. The two tests required specialised skill, costly equipment and rely on invasive skin snipping not suitable for population surveillance. Ethical consideration due to risk of transmitting blood borne diseases such as human immunodeficency virus (HIV) and hepatitis B virus will depend on screening test that will limit the number of participants to undergo diagnostic test involving skin snipping. A diagnostic algorithm with screening, diagnosis and confirmatory tests will ensure effective and efficient surveillance and monitoring onchocerciasis control programme. The screening test should be simple, measurable, accurate, repeatable and timely (SMART) as described by Mabey *et al.* (2004). Antibody detection assays meet the above criteria for a screening test that can readily be applied in the field. A novel onchocerciasis slide agglutination test (Oncho-SAT) is evaluated with samples disease endemic community.

1.3 Justification for the Study

Information about the number of village dosing and individual IVM treatment compliance rates from 1994-2011 to be generated could explain outcome of epidemiological trend. Impact of the control strategy can be adequately appraised with vital empirical clinical (eye

and skin), entomological, geospatial, parasitological and serological data. Comparative analysis of treatment and bio-clinical data will determine the effectiveness of on-going ivermectin treatment intervention. Integration of attribute with geo-referencing data will serve as precursor for the digitizing map of the study area to be produced using geographic information system (GIS) software available online. Effort to address the issue of invasiveness of the "gold standard" will be approached through the evaluation of a novel Ov-SAT to assess the circulating parasite-specific serum antibodies. Impact studies have shown improvement in ophthalmological, dermatological and entomological indicators of treated populations (FMoF, 2012). Invasiveness skin snipping has made the need to have a screening tool that will limit the number to undergo parasitological and molecular diagnostic tests. The sceening test have meet the criteria of been simple, measurable, accurate, repeatable and timely (SMART) (Mabey *et al*., 2004). The need for impact assessment of the on-going CDTI at 18 years post-treatment using the three available test for screening, diagnosis and confirmation will provide a more accurate disease prevalence status.

1.4 Theoretical Framework

Comparison will be made between baseline and current epidemiological bio-clinical data to understand disease transmission dynamics in the study communities. A slide flocculation/agglutination test (SFT) will be developed using homoginised heterelogous tissues (HT) from albino rat organs as antibody binding support. The principle of human immunoglobulin (Ig) binding to specific sites on HT is class and IgG isotype restricted: (i) Among the 5 classes of antibodies only IgG bind to HT, and (ii) out of the 4 IgG isotypes, only IgG2 do not bind to HT (Bennich, 1985). Rat and human immunoglobulin aggregates bound via the crystallisable fragment receptor (FcR) (van der Laan-Klamer *et al.*, (1987).

Unspecific binding and cross reactivity in the diagnosis of parasitic infection are caused by: (i) IgM class, (ii) IgG2 isotype antibodies, and (iii) glycoprotein containing phosphoryl cholin (Lal and Ottesen, 1988). These shortcomings are eliminated using: (i) parasite low molecular weight (LMW) antigens and (ii) antibody detection assay based on IgG4 isotype (Weiss *et al*. 1982; Weil *et al*., 1990; Chandrashekar *et al*., 1996). The half-life of IgG is estimated at 23 days and 80% serum concentration is relatively higher than others. IgG is present in blood plasma and tissue fluids (Prescott, 2002; USCSM, 2007). Apart from measuring the frequency of those antibody reactive, it will also stratify intensity of response by end titre. The nuclear ITS rDNA sequence has been extensively used to define genetic markers for different species of nematodes (Powers *et al*., 1997). On this basis, the primers described by Ta Tang *et al*. (2010) were selected for use in PCR amplification of DNA to validate the skin microfilarial status. Primer sequences for beta actin gene (Zeng and Donnelson, 1992) were used to assess the specificity of the ITS1.

1.5 Aim and Objectives

The aim of this study was to evaluate the current status of onchocerciasis after long-term mass ivermectin treatment of sentinel villages and the implication on public health of the study communities in particular and the onchocerciasis endemic countries where annual CDTI is operational in general.

The objectives of this study were:

i. To evaluate the clinical, parasitological, ivermectin treatment compliance, blackfly population dynamics and infection level in the sentinel villages.

ii. To develop and evaluate *Onchocerca volvulus*-slide flocculation test (Ov-SFT) using Sodium Dodecyl Sulphate (SDS) extracted adult worm antigens.

iii. To validate microfilarial status by amplifying DNA with 18S first internal transcribed spacers (ITS 1) and beta actin (Actin-2B) genes of *O. volvulus*.

iv. To identify *O. volvulus* and sympatric filarial infections in the study area as PCR amplicons of ITS gene region varies among filarial species.

v. To compare and contrast the predictive values and likelihood ratios of Ov-SAT, microfilaria detection and PCR amplification with ITS1 gene.

1.6 Research Questions

i. How does the ivermecin treatment compliance rate affect microfilaria prevalence, serum parasite-specific antibodies and morbidity (eye and skin lesions)?

ii. Can Ov-SAT diagnostic value prove to be suitable, reliable, cheap, simple, easy and serve as a rapid screening tool for population study?

iii. Can ITS1 18S gene primers of *O. volvulus* PCR amplification of DNA more sensitive than microfilaria detection and capable detecting sympatric co-infections?

iv. Is there any case of sub-optimal response to treatment in terms of presence of skin microfilariae despite compliance to treatment from the sample population?

v. Are the likelihood ratios of antibody detection with Ov-SAT, microfilaria detection from skin snip and ITS1 PCR amplification of DNA template somewhat similar.

CHAPTER TWO

2.0 LITERATURE REVIEW

Prior to commencement of control, Onchocerciasis was the fourth leading cause of preventable blindness and second cause of infection blindness. An estimated 125 million people in 37 disease-endemic countries are at risk of infection and 96% of them are in 30 sub-Saharan Africa (Figure 2.1), one in Arabian Peninsula (Yemen) and six Latin America countries (Hodgkin *et al*., 2007) as shown in Figure 2.2. About 99% of the 18 million infected people live in Africa. Figure 2.3 shows the map of onchocerciasis and ivermectin distribution in Nigeria. At least one million are either blind or severely visually disabled and 40,000 blind cases were added each year (WHO, 1995). Nigeria accounts for one third of the global estimates, with about 7 million infections, and 32 states including the Federal Capital Territory are endemic excluding Akwa-Ibong, Bayelsa, Delta and Rivers states (FMoH, 2012). Some cases of onchocerciasis have been reported among migrant and resident of Lagos by Hunponu-Wosu and Somorin (1977). The worst endemic foci may be found in Taraba River valley in Northern Nigeria (Akogun *et al*., 1992). A national programme for mass treatment of onchocerciasis with Mectizan® or ivermectin started in Nigeria in 1992 under the Primary Health Care (PHC) programme. It was later renamed community directed treatment of onchocerciasis with ivermectin (CDTI). Eligible residents of the endemic communities in sub-Saharan Africa receive treatment once a year (annual) while bye-annual treatment was adopted in Onchocerciasis Endemic American Countries (OEAC). With the adoption of this strategy, epidemiologic indices of the disease were expected to change. A study in parts of Cameroon by Duerr *et al* (2004) established there was strong relationship between low infection rate in children which increased with age in adults is evidence that a factor may be responsible. It may be attributed to reduced level of



Countries of the former Onchocerciasis Control Programme (OCP) in West Africa (1974-2002).

Countries of the ongoing African Programme for Onchocerciasis Control (APOC), founded in 1995.

Figure 2.1: Onchocerciasis in Africa – endemic regions and partnership alliances. Annual ivermectin treatment is adopted in the APOC operational areas. Elimination of onchocerciasis have been reported in some foci in Mali, Nigeria, Senegal and Sudan.

Source: Tropical Disease Research (2007) (TDRnews) Pre-view No. 79.

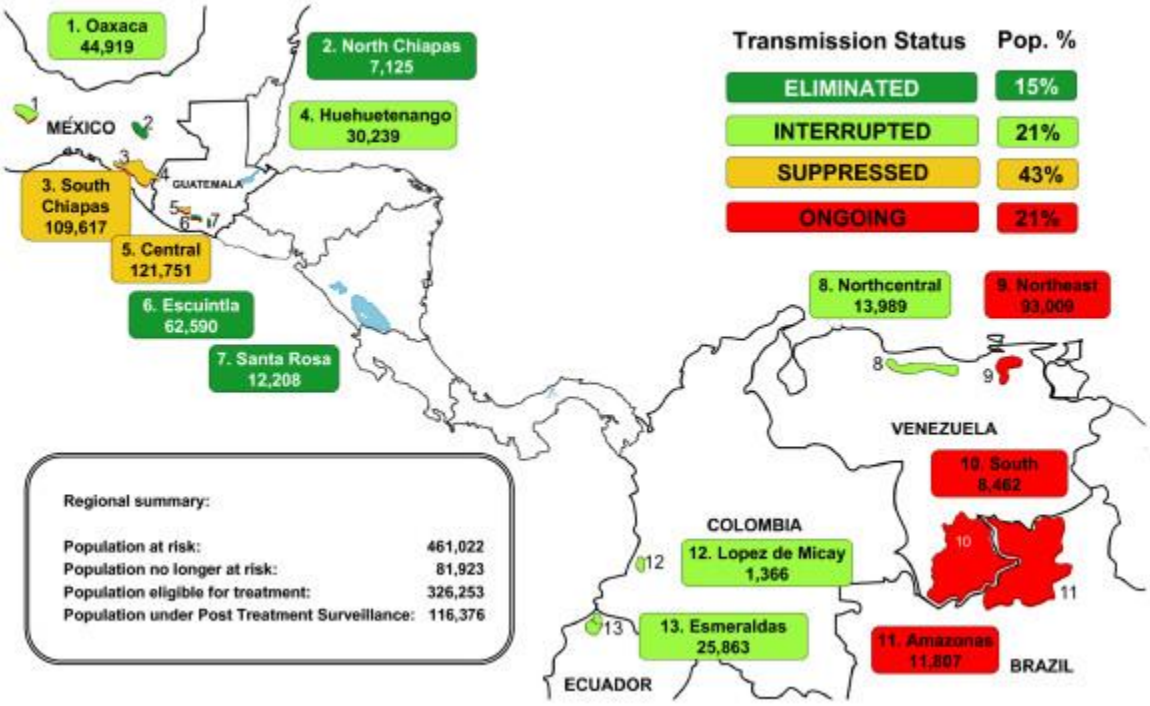


Figure 2.2: Geographic distribution and transmission status of the 13 onchocerciasis foci in the Americas as of December, 2010**.** Biannual treatment is adopted in the Onchocerciasis Endemic Countries of Americas (OECA). Some foci have been declared to be free of onchocerciasis.

Source: Gustavsen *et al*. (2011). *Parasites and Vectors*, **4**: 205. Published online 2011 Oct 25. doi: 10.1186/1756-3305-4-205

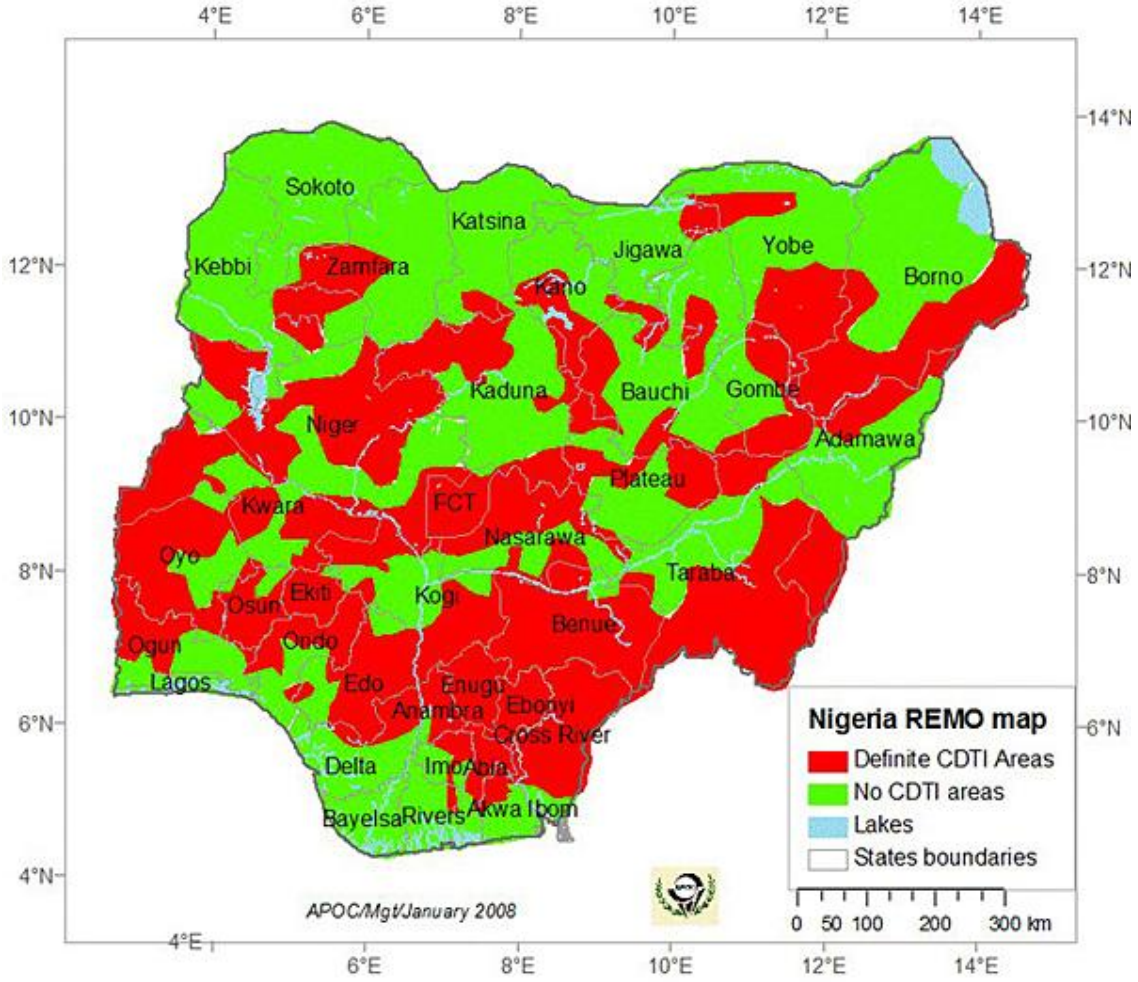


Fig. 2.3: Rapid epidemiological mapping of onchocerciasis in Nigeria 2003. Areas (in red) are where community-directed treatment with ivermectin is needed. Almost all the states are endemic to varying degree excluding Bayelsa, Delta and Lagos states. This notwithstanding the probable mobile cases of the disease may be found in these three states.

Source: WHO/APOC. www.who.int/apoc/countries/nga/en

host-blackfly contact and higher risk of exposure to infection. According to the projection of FMoH (2012) all the 32 endemic States and the FCT have reached the minimum standard of therapeutic coverage (65%). With the huge success already recorded in bringing the disease under control, it is now at the elimination stage. This fit was achieved faster in the OECA where biannual CDTI is practised, while it took longer time period to attain a similar outcome in APOC operational countries where annual CDTI is in place (Turner *et al*., 2013; Turner *et al*., 2014b). One very strong limitation identified so far is the low or non-compliance to treatment that can thwart the effort to achieve elimination and eventual eradication if certain places where this problem exist persist (Katabarwa *et al*, 2011; Katabarwa *et al*., 2013). Interventions to improve compliance in the area should focus on health education using epidemiological data in order to increase risk perception and dispelling misconceptions (Yirga *et al*., 2010). The map of onchocerciasis endemicity levels has proven very valuable for onchocerciasis control in the APOC countries (Zoure *et al*., 2014) particularly for planning treatment, evaluating impact and predicting treatment end dates in relation to local endemicity levels.

2.1 Epidemiology of Onchocerciasis

Although onchocerciasis is insidious and has zero mortality, it causes debility and morbidity associated with various burden of skin clinical manifestations and eye lesions that result in poor vision and blindness (WHO, 1995, Murdoch *et al*., 2002;). The *Onchocercal* skin diseases (OSD) are severe pruritis, nodule or onchocercomata, papular and chronic onchodermatitis, dyspigmentation (hypopigmentation and hyperpigmentation), 'leopard skin', localized onchodermatitis or 'sowda', lichenified skin, thick or 'lizard skin', skin atrophy, lympho-adenopathy, hernia, hanging groin, and elephantiasis of scrotum, labia and leg (Murdoch *et al*., 1993, Murdoch *et al*., 2002). The eye or ocular lesions

comprised of anterior segment lesions, microfilariae in anterior chamber, fluffy opacity, punctuate keratitis, chronic iritis, and secondary glaucoma, early and chronic uveitis. The posterior segment lesions are choroid-retinitis, choroid-retinal atrophy, acute optic neuritis and optic nerve atrophy (WHO, 2014a). Some psycho-social implication of *Onchocercal* skin diseases (OSD) are social isolation, shame and low self-esteem, restlessness/sleeplessness, and marital problems (Okaka *et al*., 2003; Okoye and Onwuliri, 2007; Wogu and Okaka, 2008). The socio-economic effects of the disease are stigmatization, family or community dislocation and relocation, abandonment of fertile arable lands along riverbanks. Onchocerciasis and lymphatic filarial have become a problem of immigrants (Higazi *et al*., 2011; Jimenez *et al*., 2011). Molecular biology diagnostic methods have been shown to be more sensitive than the parasitological diagnostic techniques.

2.2 Geospatial Data of Vector-Host Interaction

Blackflies form a uniform family Simulidae in the order Diptera. Entomologists have so recognized and scientifically named about 1554 *Simulium* species. The blackflies comprise only 1.3% of the 119,000 species of Diptera known to Science (Crosskey, 1990). Disease apart, the blackflies are in many regions feared of all biting insects because of the relentless and intolerable nature of their attack –not only on man, but livestock, poultry, and wildlife (Crosskey, 1990). The name *Simulium* meaning 'little snub-nosed being' was created for the blackflies in 1802 by Pierre Latreille, the great father of French entomology. The fly was first associated with the transmission of onchocerciasis by Robles 1917 (cited by Crosskey, 1990). In West and Central Africa including Nigeria, *Simulium damnosum*, which breeds in fast flowing rivers with rapids is the vector of onchocerciasis.

Blackflies are broadly classified into two groups depending on the vegetation of the ecology of their breeding sites (forest and savannah types). A large number of these species are anthrophilic, while few are zoophilic. The 34 known *Onchocerca spp.* are mostly parasites of domestic and wild animals. The blackflies have the ability for long flight, which is associated with intake of carbohydrate through feeding on nectar obtained from flower. The common cytological method of differentiating between these two types is the colour of the wing turf. It is graded as pale or dark in colour with variation in between these extremes. Whether this has any influence on the types of *O. volvulus* causing different clinical manifestations remains unknown. Forest species of the parasite is associated with severe skin manifestations while the savannah type has been known to cause more blindness (Abiose *et al*., 1993). Studies applying molecular biology analytical tools have shown clear biomarkers that distinguished parasites of veterinary importance and between the two human infective strains (Meredith *et al.*, 1991; Herder *et al*., 1994; Adewale *et al*., 2005; Nuchprayoon, *et al*., 2005; Fukuda *et al*., 2008; Fukuda *et al*., 2010a; Fukuda *et al*., 2010b) using genomic finger prints in random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) based on PCR amplification of random DNA fragments. This may not be readily feasible because of difficulty in breeding blackfly in the laboratory.

People mostly at high risk of infection are those having one or more activities close to the breeding sites. They include miners, tourists, farmers, fishermen, hunters, and bathers. It is well established that the fly can travel long distance up to 140 kilometers to establish new breeding sites. Only an infected black fly can transmit the disease. Majority of black flies close to the breeding site are parous (newly emerged young fly) and nulliparous (old fly). When a fly takes its first blood meal it becomes engorged.

2.3 Disease Transmission:

When a blackfly feeds on an infected person microfilariae are imbibed with blood meal and they undergo obligate developmental transformation from first to third larval (L1-L3) stages. In subsequent feeding on a human host, the infective L3 stage (one or more) is then transmitted to the susceptible host. Thereafter, it develops into L4 and then adult worm in about a year within a subcutaneous nodule (onchocercomata) in most cases. The female worms measure between 33 and 50 cm by 270 to 300 μm in diameter, and there may be between 2 and 3 coiled worms in a nodule in subcutaneous connective tissues, while the male worm measure 19 to 42 μm by 130 to 210 μm (Eichner and Renz, 1990). It is capable of moving from one nodule to another to inseminate the females (Albiez *et al.*, 1988). A female adult worm *Onchocera volvulus* undergoes ovoviviparous reproduction. Unlike many other filarial species, the motile larvae called microfilariae are not released continuously, but in regular periodic cycles (Schulz-Key and Karam, 1986). About 200,000-400,000 microfilariae are produced at each cycle with an estimated daily release of 1000-3000 microfilariae per female worm *in vivo* (Engelbrecht and Schulz-Key, 1984). As they migrate out from nodule, large numbers of motile microfilariae invade the skin and eye.

Despite the fact that microfilariae are found in many tissues, the different skin and eye lesions are responsible for its serious morbidity and debilitating consequences. Yet, there is no indication of any direct parasite attrition or mechanical damage involved in the disease process. With time the larval forms (microfilariae) and adult worm (macrofilaria) age and die (Duerr *et al*., 2004). Both dead and living worms elicit host inflammatory responses with bystander effects believed to underlie dermal and ocular changes (Ottesen,

1995; Murdoch *et al*., 1996). Presence of dying, dead and the excretory products of living microfilariae contribute to the lesions of affected tissues.

2.4 Immunopathology of Onchocerciasis

The spectrum of *O. volvulus* infection varies from asymptomatic microfilaridermia often associated with immunological hypo-responsiveness to severe skin and eye diseases including onchodermatitis and blindness. Establishment of infection and disease development are dependent on the specific antigen recognition and immune response of the host. The complex immune response is triggered by numerous different filarial antigens at the same time. To elucidate the mechanism of the immune response, it is essential to explore the specific reactions to individual, native parasite antigens. From all indications, it is obvious that the varied clinical manifestations of onchocerciasis are not due to direct parasite attrition. The co-factors of immune responses implicated in causing dermal and oculars lesions have been reviewed by Eezzuduemhoi and Wilson (2006); Sinha and Schwartz (2006) and Udall (2007). From experiment with rabbits, it has been shown that eosinophils and lymphocytes attach to microfilaria in the stromal cornea. The basic underlying factor in the pathology is due to excessive degranulation of eosinophil thereby releasing protein substances that may be toxic to host tissues.

The role of immune responses in pathogenesis of onchocerciasis has been attributed to involvement of antibody dependent cytoadherence (ADC). This phenomenon very much supported the role eosinophils and lymphocytes play in microfilariae clearance and tissue damage (Cooper *et al*., 1999a). They showed that after treatment with anthelmintic, the eosinophil count falls initially as a consequence of rapid migration from peripheral blood to the skin.

2.5 Clinical Manifestations of Onchocerciasis

The disease is broadly classified into dermatological and ocular manifestations. Briefly, the various skin clinical changes commonly referred to as onchocercal skin diseases (OSD) have been fully described and classified by Murdoch *et al.* (2002). They include acute papular onchodermatitis which involves numerous small pruritic papules that may progress to vesicles or pustules and chronic papular onchodermatitis indicated by larger, flat-topped papules distributed symmetrically over the buttocks, waist, and shoulders. Others are skin atrophy, lichenified skin, leopard skin resulting from patchy dyspigmentation of skin mostly around the shin, hyper-pigmentation or 'Sowda', and palpable nodule or onchocercomata containing worms. Hanging groin or inguinal lymphoadenopathy, elephantiasis (involving the scrotum, labia and legs) are also observed. Important dermatological symptoms associated with the disease include severe itching and musculoskeletal pain (MSP). In addition to contributing to loss in man-hour, the disease has been reported to contribute to increasing the disability adjusted life years (DALYs).

The ocular lesions are categorized as anterior and posterior clinical manifestations, which to a greater extent determines if the blindness they cause is reversible or irreversible. Among the anterior lesions are microfilaria in anterior chamber (MFAC), punctate keratitis which may or may not be inflammatory, corneal opacity, cataract or opacity of the lens, iritis (Kayembe *et al*., 2003). The posterior lesion involves the sclerosing keratitis, uveitis, optic nerve disease or atrophy. Others are glaucoma or cupping of the optic nerve due to high ocular pressure more than 150 mm Hg (WHO 2014b). Similarly, the global estimated visual impairment of over 1 million people and 360,000 blind cases is projected to reduce the disability adjusted life expectancy (DALE) by 10 years (Hodgkin *et al*., 2007). This is apart from the social stigmatization of affected person with disfigurement, family

dislocation and school dropout due to blindness. Okoye and Onwuliri (2007) have reported that low self-esteem, withdrawal syndrome and restlessness and other psycho-social problems were associated with *Onchocercal* skin diseases.

2.6 Serodiagnosis of Onchocerciasis

Among the molecular diagnostic methods, antibody test hold more promise as screening tools. Serodiagnosis is limited by inherent problem of cross-reactivity with other parasites. This shortcoming was overcome by measuring IgE and IgG4 antibodies that do not recognize the ubiquitous immunodominant phosphorylcholine (PC) molecule found in humans and other organisms (Lal and Ottesen, 1988). Cross-reactivity was also overcome by using low molecular weight (LMW) antigens. Most cross-reactants were attributed to high molecular weight (HMW) antigens containing carbohydrate moieties or glycoprotein containing PC, which are not recognized by IgG4 antibody (Weiss and Karam, 1989; Guzman *et al.,* 2002; Osue *et al.,* 2008). Antigen, nucleic acid and antibody detections are adaptable to laboratory investigation and field surveillance. Application of cocktail recombinant antigens in antibody detection ELISA (Bradley *et al*., 1993; Bradley *et al.*, 1998; Nde *et al*., 2002) has increased assay performance. A commercially available serodiagnostic kit called rapid format card test has been developed and evaluated (Weil *et al.,* 2000). Immune serum produced against different recombinant antigens have been used for oncho-dipstick immunobinding assay (DSIA) for antigen detection. The *O. volvulus* recombinant antigen designated Ov1.6 showed high sensitivity (100%) and specificity in urine sample (96.5%), tears (83.4%), dermal fluid (54.76%) and the least (48.5%) in serum (Ayong *et al*., 2005, Wembe *et al.,* 2005, Andrews *et. al*., 2008). The tests that have undergone field trial include patch skin test, which depends on the appearance of erythema within 48 hours after a mixture of 10% diethylcarbamazine citrate (DEC) and cream, is

applied under occlusive dressing. It gave 56-80% sensitivity in skin mf positive cases (Laurent *et al*., 2000; Toe´ *et al*., 2000). In Mazzotti test, patients were treated with a small quantity of DEC at 0.6mg, thereafter observing for hypersensitivity reaction (WHO, 2005). Both tests are less sensitive and are associated with hypersensitivity reactions.

To simplify sample collection, transportation and storage, blood spotted on filter paper (Amuta and Olusi, 2000) has been found to be excellent medium for circulating antibodies. Surveillance for recrudescence is inevitable and is the cornerstone of any disease control and elimination programmes (Guzman *et al*., 2002). Moreover, changes in parasite specific antibodies after short-term ivermectin treatment have been investigated (Osue *et al.*, 2009). Available information on the effect of long-term ivermectin treatment on parasite-specific antibodies has remained equivocal. Lipner *et al.* (2006) reported no change in antibodies 16 years post Ivermectin treatment. On the contrary, a remarkable change from 24% to 4% was observed after a decade of bi-annual ivermectin treatment (Gomez-Priego *et al*., 2005). The existing gap in information on the long term impact of ivermectin treatment on the level of antibodies needed to be clarified.

2.7 Current Control of Onchocerciasis

The best preventive measure for onchocerciasis is eliminating the vectors (*Simulium* spp.) and the *Onchocerca volvulus* microfilariae from the host (Ndyomugyenyi *et al*., 2004). The feat achieved by the Onchocerciasis Control Programme (OCP) in 11 countries of West Africa prompted other donor agencies and stakeholders to initiate the control of the disease in other 19 endemic countries of sub-Saharan Africa and the elimination of the disease in the Americas. Following the discovery of its microfilaricidal activity, and its being well

tolerated, ivermectin or Mectizan® was adjudged suitable for mass treatment (Aziz *et al.*, 1982; Boatin *et al*., 1998a; Remme *et al*., 1995; Remme *et al*., 2002). After undergoing the stages of clinical trials in human, it was patented for use. This evolved a unique public private partnership that paved way for the birth of the African Programme for Onchocerciasis Control (APOC) and Onchocerciasis Elimination Programme of the Americas (OEPA) that institute annual and biannual treatment strategy (Blanks *et al*., 1998; Colatrella, 2003; Gustavsen *et al*., 2011). The suitability of ivermectin for mass treatment depends on its mode of action by disrupting the invertebrate neuro-transmission involving γ-amino butyric acid, i.e. GABA channels (Campbell, 1985). In contrast to the two previous drugs in use, ivermectin (Stromectol, Mectizan®, Appendix II) a semi-synthetic macrocyclic lactone produced by *Streptococcus ivermetilis* was found to be free from serious side effects. Cases of severe adverse drug reaction have been documented (Twum-Danso and Meredith, 2003) and did not differ significantly with history of consumption of alcoholic beverages (Takougang *et al*., 2008). This drug was initially patented and widely used as a broad-spectrum veterinary nematicide. Merck and Co. USA generously donated ivermectin free of charge as long as needed by disease endemic communities.

2.7.1 Community directed treatment with ivermectin

The current control by mass drug administration (MDA) with ivermectin or Mectizan® started in 1988 (Remme *et al*., 2002; Amazigo and Boatin, 2005). MDA later evolved into community directed treatment of onchocerciasis with ivermectin (CDTI). The control strategy has proven effective, disease burden is falling and elimination is planned (Emuka *et al*., 2004; WHO, 2005). There are issues of underdose, therapeutic and geographic under-coverage observed by Remme *et al*. (2007). The search for a macrofilaricide remains

a top priority because sustaining CDTI will continue to remain a major challenge. Effectiveness of this strategy depends on high geographic and demographic therapeutic coverage of endemic communities as observed by Akinboye *et al*. (2010) in small farming settlement in Oyo State, Nigeria. There are reports of prevalence of onchocerciasis and palpable nodules in rain forest south eastern parts of Nigeria by Abanobi 2010; Iroha *et al*. (2010) and Okoro *et al*. 2014). Ivermectin treatment distribution within the study areas and treatment compliance were lacking. Study by Okeibunor *et al*. (2011) the drug had improved social, psychological, and economic well-being of the people.

2.7.2 Possibility of development of drug resistance to ivermectin

So far, no proven resistance to ivermectin treatment has been reported except sub-optimal response to treatment in terms of rapid repopulation of skin with mf as observed in individuals in Ghana (Awadzi *et al*., 2004; Osei-Atweneboana *et al*., 2007; Churcher *et al*., 2009; Osei-Atweneboana *et al*., 2011). There is growing apprehension of *O. volvulus* developing resistance to ivermectin (Awadzi *et al*., 2004a; Awadzi *et al*., 2004b; Osei-Atweneboana *et al*., 2011) attributed to genetic basis associated with selection on ATP-binding cassette (ABC) transporters (e.g. P-glycoproteins), and □- tubulin (Ardelli and Prichard, 2004; Ardelli and Prichard, 2007; Bourguinat *et al*., 2008; Taylor *et al*., 2009; Nana-Djeunga, *et al*., 2012). The ivermectin resistance is found in some parasites of veterinary importance like *Haemonchus contortus* and *Cooperia oonchophera* by Blackhall *et al*. (2003) and Njue *et al*. (2004) to identify cases of sub-optimal response. Similarly, resistance to the related drug, moxidectin has been documented and ivermectin-treated patients have shown decreased diversity at many genetic loci for P-glycoprotein

(Ardelli *et al*., 2005; Eng and Prichard, 2005), suggesting changes in allelic patterns that may lead to resistance.

2.7.3 Macrofilaricidal action of ivermectin

It has been concluded that ivermectin *per se* has direct macrofilaricidal action against adult female worms (Duke, 2005). Many reports indicate the effect of ivermectin on the population of adult worms of *O. volvulus* were found to be in deteriorating condition as observed in Mexico, Guatemala and Ecuador (Nana-Djeunga *et al.*, 2014). This indicated that semi-annual ivermectin treatment of 6 years has had a profound effect on survival and reproduction of this species (Duke *et al*., 1991; Rodríguez-Pérez *et al*., 2008a; Rodríguez-Pérez *et al*., 2008b). Results show that both strategies achieved elimination after 15 to 17 years of treatment (Mackenzie *et al*., 2012). Up to 5% of untreated female *Onchocerca volvulus* filariae develop potentially fatal pleomorphic neoplasms, whose incidence is increased following ivermectin treatment.

2.7.4 Expansion of community directed treatment with ivermectin

In 1998 Merck expanded the donation of Mectizan to include the Programme for the Elimination of Lymphatic Filariasis (PELF) in 28 countries in African and Yemen where both diseases are co-endemic (Alleman *et al*., 2005; Thylefors and Alleman, 2006; Thylefors *et al*., 2008). Nigeria is co-endemic with many neglected tropical diseases (Figure 2.4). The study area is within the zone endemic for leprosy, lymphatic filariasis, malaria, onchocerciasis and schistosomiasis (FMoH, 2012). Mectizan is indicated for the treatment of onchocerciasis caused by *Onchocerca volvulus* and for the treatment of the microfilardermia caused by infection with *Wuchereria bancrofti*, the causative agent of LF

in Africa. Cases of adverse reaction to ivermectin treatment have been documented in areas co-endemic with *O. volvulus* and *Loa loa* as observed by Chippaux *et al*. (1996); Gardon *et al*. 1997; Boussinesq *et al*. (1998); Otubanjo *et al.* (2008) including parts of South-west Nigeria. More intense surveillance and monitoring in the first 2 days after mass distribution in ivermectin-naı¨ve populations would assist in early recognition, referral and management of these cases (Twum-Danso and Meredith, 2003). This severe adverse events include probable *L. loa* encephalopathy temporally related to Mectizan-treatment (PLERM) has not been reported in Nigeria. As observed by Kipp *et al*. (2005) that IVM can be safely used for mass treatment in areas where the prevalences of onchocerciasis and HIV-1 infection are both high.

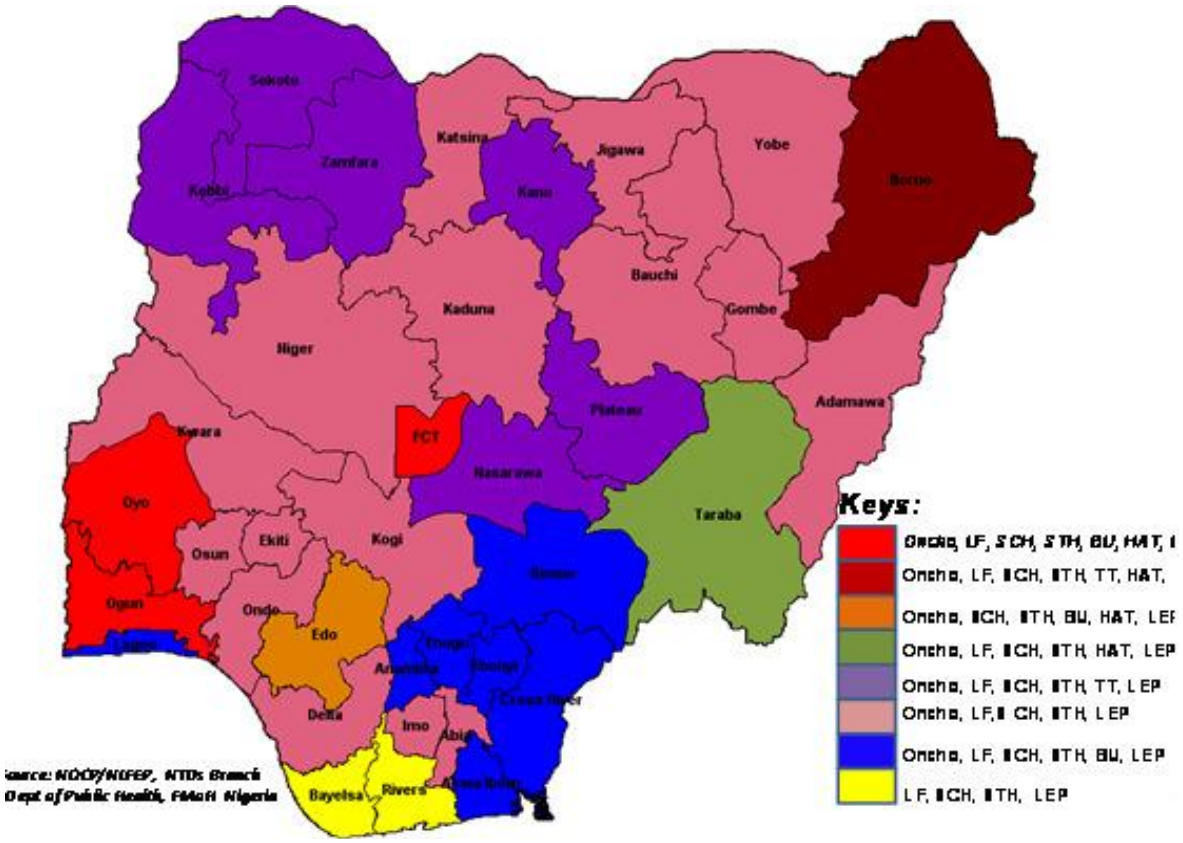


Figure 2.4: Neglected Tropical Diseases Co-endemicity Map of Nigeria. Nigeria is one of the countries that are co- endemic of neglected tropical diseases (NTDs). Only Bayelsa and Rivers States are non-endemic for onchocerciasis. BU= Buruli ulcer, HAT= human African trypansomiasis, LEP= leprosy, LF=lymphatic filariasis, Oncho= onchocerciasis, SCH- schistosomiasis, and STH= Soil transmitted helminthes.

Source: FMoH (2012).

2.7.5 Impact of CDTI on onchocerciasis epidemiological status

Extensive evaluation of the CDTI control strategy carried out in selected foci in Kaduna state, Nigeria and parts of Latin American countries showed that prevalence and skin microfilariae loads were reduced to zero or thereabout (Guderian *et al*., 1997; Lindblade *et al*., 2007; Gonzalez *et. al*., 2009; WHO, 2011; Cruz-Ortiz *et al*., 2012). Although the public health risk of the disease may no longer be the same, availability of macrofilaricides will ensure elimination through a shorter time sustained distribution (Alley *et al*., 2001). Reduction in transmission after 5-8 years of annual ivermectin treatment has been reported (Collins *et al*., 1992; Borsboom *et al*., 1997; Opara and Fagbemi, 2008; APOC, 2011a). Eradication of the disease has been recorded in 3 hyperendemic foci in Mali and Senegal after annual or six monthly ivermectin treatment (Traore *et al*., 2012) and in Abu Hamed focus in Sudan (Binnawi, 2013; Higazi *et al*., 2013). Other area where elimination have been attained are some parts of OEPA operational areas in places like Santa Rosa focus of Guatemala (Lindblade *et al*., 2007; Cruz-Ortis *et al*., 2012), from Northern and Southern Chiapas and Oaxaca focus in Mexico (Rodriguez-Perez *et al*., 2008a; Rodriguez-Perrez *et al*., 2008b; Rodriguez-Perrez *et al*., 2013a) using twice a year ivermectin treatment strategy which commenced in 1994. In these foci, no more evidence of on-going transmission was found from their studies. Ndyomugyenyi *et al*. (2004); Mackenzie *et al*. (2012) had observed that with twice yearly treatment alone or annual treatment with ivermectin coupled with vector control, infection rate continued to fall implying that interruption of transmission could be rapidly attained.

Onchocerciasis is responsible for an estimated one million disability adjusted life years (DALYs) and disability adjusted life expectancy (DALE) reduced by 10-13 years (WHO, 1995). Recently, African Programme for Onchocerciasis Control (APOC) estimated that

between 1995 and 2010, mass treatment with ivermectin averted 8.2 million DALYs due to onchocerciasis in APOC areas, at a nominal cost of about US$257 million. It was expected that APOC will avert another 9.2 million DALYs between 2011 and 2015, at a nominal cost of US$221 million (Coffeng *et al*., 2013). It reduces immunity and resistance to other diseases and interferes with immunization (Cooper *et al*., 1999b; Abraham et al., 2002). Onchocerciasis is responsible for man hour loss, impedes optimal land use for sustainable agricultural and rural development and limits national self-sufficiency in food production.

2.7.6 Effect of ivermectin treatment on adult worm

Lately, there are strong supporting observations from field surveys, the impact of ivermectin treatment on adult worms leading to death as highly significant increase in the number of moribund/dead female worms per nodule were observed (Plaisier *et al*., 1995; Cupp *et al*., 2004; Cupp and Cupp, 2005). Ivermectin had an embryo static effect on *O. volvulus*, but the effect was reduced in the frequently treated cohort compared with the control population (Nana-Djeunga *et al*., 2014). Therefore, complaint of nodule remission or dissolutions or disappearance has been reported from studies carried out at Imo and Kaduna States of Nigeria by Ukaga *et al*., (2000); Emuka *et al.* (2004); Osue *et al.* (2013). Preponderance of higher number of such cases was more prevalent in OEPA areas where bi-annual treatment as against annual treatment adopted in Sub-Saharan Africa. In one study, a long-term repopulation of the skin after single dose treatment was recorded by Awadzi *et al* (2004). Three main possible challenges for onchocerciasis control have been identified: (1) how can adequate treatment coverage with ivermectin be established and sustained in those African settings where MDA is indicated?; (2) what are the means to determine where and when treatment can be stopped?; and (3) how does one ensure effective surveillance in areas where active control has come to an end (Hodgkin *et al*.,

2007). In some quarters, there is growing advocacy for targeted treatment and the ease of identifying target population is very critical as the cost of CDTI per dose delivered may be less than targeted therapy unless screening method is inexpensive (Poolman and Galvani, 2006). The need for macrofilaricide cannot be underscored.

2.7.7 Criteria for cessation of ivermectin treatment

The plan for certification of the elimination of onchocerciasis developed by OEPA is made up of four phases (WHO, 2001b). Phase I includes ivermectin treatment for 2–4 years, which results in suppression of transmission. In phase II, suppression is maintained through treatment of the mean reproductive lifespan of the adult female (approximately 13–14 years). Anti-*O. volvulus* antibodies were not found in samples from non-endemic controls from Mexico, but 3 of 71 samples from residents in the onchocerciasis area of Oaxaca, Mexico, and who have been under ivermectin treatment during the last 10 years were only positive to IgG. No IgG4 isotype was detected and low (4.2%) anti-*O. volvulus* IgG antibody prevalence was found (Gómez-Priego *et al*., 2005). After this, (in phase III), it is expected that the adult parasite population would die by senescence and maintaining the suppression of transmission will no longer be dependent on ivermectin distribution. Thus, in phase III, ivermectin distribution will cease and intensive surveillance will be conducted to document that transmission will not re-develop. Finally, in phase IV, the elimination of the *O. volvulus* infection will be certified.

2.8 Research and Development for Macrofilaricides

Despite the fact that there is control tool in place for onchocerciasis, yet the door for research and development into the disease has not closed. One important aspect is in the

area of drug screening for macrofilaricide. Advances made so far are the clinical trial of vibramycin (doxycyclin) at 100 mg/day for 6-8 weeks eliminate endosymbiotic *Wolbachia* bacteria (Hoerauf *et al*., 2001). Also, trial with rifampicin treatment administered for 2 to 4 weeks have shown some promise of clearing the *Wolbachia* bacteria endosymbiont of *O. volvulus* (Specht *et al*., 2008). It has been established that moxidectin sterilize adult worms (Tagboto and Townson, 1996). Both drugs reduce microfilarial loads and decrease adult worm viability. From mathematical model it has been predicted that availability of a 100% effective macrofilaricide and 100% coverage, elimination could be achieved instantaneous (Alley *et al*., 2001; Hodgkin *et al*., 2007; Turner *et al*., 2014a). Whatever control is in place, there could be emergence of recrudescence and pocket of hypo-endemic foci that may not be enlisted for treatment. Most filarial parasites of humans and domestic animals contain a bacterial endosymbiont (*Wolbachia pipientis*) which is involved in the process of development and reproduction (Taylor *et al*., 2005). Antibiotic treatments that clear *Wolbachia* cause stunted growth, infertility, and eventual death of adult filarial worms (Hoerauf *et al*., 2001; Hoerauf *et al*., 2003a; Hoerauf *et al*., 2009) can readily serve as drug target of biochemical pathways or processes (Slatko *et al*., 2010). Also RNA interference and gene deletion in *C. elegans* further showed that N-myristoyltransferase (NMT) is essential for nematode viability. The effects observed are likely due to disruption of the function of several downstream target proteins (Sheng *et al*., 2010; Wright *et al*., 2010; Galvin *et al*., 2014). The authors have suggested that targeting NMT could be a valid approach for the development of chemotherapeutic agents against nematode diseases including filariasis. Recently, Bhabak *et al*. 2011; Madeira *et al*. 2011; Bulman *et al*. (2015) reported that triethylphosphine gold or deacetylated auranofin, a gold-containing drug used for rheumatoid arthritis, was effective *in vitro* in killing both *Brugia* spp. and *O. ochengi* adult worms and in inhibiting the molting of L3s of *O. volvulus* with IC_{50} values in

the low micromolar to nanomolar range. According to Bulman et al. (2015), auranofin may be beneficial if used in areas where *Onchocerca* and *Brugia* are co-endemic with *L. loa*, to prevent severe adverse reactions to the drug-induced death of *L. loa* microfilariae.

2.8.1 Macrofilaricidal activities of some antibiotics

Treatment with antibiotics to clear the bacterial endosymbionts of the filarial parasites has given rise to superior therapeutic alternative to current anthelminthic drugs (Hoerauf *et al*., 2001; Taylor *et al*., 2005; Johnson *et al*., 2007; Hoerauf, 2008). The rationale for this novel treatment targeting *Wolbachia*- a bacterial, because it has been found to be essential for worm development, fertility and survival and inducer of inflammatory disease pathogenesis. The field trials carried out by Hoerauf *et al*. (2003b) showed that doxycyclin at 100 mg/day when administered for 4-8 weeks demonstrated efficacy in reducing skin microfilarial loads, sterilizes adult worms, and decreases adult worm viability and most importantly death of adult worms. However, the efficiency of using doxycyclin in mass treatment campaigns has been questioned (Sinha *et al.*, 2006). The length of treatment is logistically incompatible with the community-directed strategy used for filariasis control, contraindication for children >9 years and pregnancy (Taylor *et al*., 2009). The slow-kill outcome of doxycyclin treatment has several advantages including elimination of the inflammatory inducing bacteria (Hoerauf *et al*., 2001; Keiser *et al*., 2002; Saint Ande *et al*., 2002) and the avoidance of the potential adverse reactions to nematode products with rapid-kill as observed in co-infection with *Loa loa* (Turner *et al*., 2010; Wanji *et al*., 2009). Taylor *et al*. (2009) opined that the outcome of trials to establish a definition of the minimal effective regimen will provide an important advance in the treatment options for individual cases outside the control areas.

2.8.2 Moxidectin clinical trials and commercialization

Another breakthrough in the search for filaricidal drug was the discovery, just like ivermectin, that moxidectin, a drug used in veterinary medicine was found to have promising macrofilaricidal activity in animal models (Langworthy *et al*., 2000). Moxidectin is a fermentation product from *Streptomyces cyanoeogriseus* var. *noncyanogenus*. It is chemically related to the nematocides, is a milbamycin compound and like Ivermectin it has been used extensively in veterinary medicine. Moxidectin had proven to be more potent than ivermectin (Tagboto and Townson, 2001). It is feared that any resistance to ivermectin may be shared by moxidectin because they are closely related (Shoop, 2003; Townson *et al*., 2006). Like ivermectin, it has microfilaicidal effect and the macrofilaricide action may be cumulative reduction in embryogenesis (Turner *et al*., 2014a). The need for research and development for a macrofilaricide has been emphasized since moxidectin like ivermectin is a microfilaricide (Mackenzie *et al*., 2011; Mackenzie *et al*., 2014). Already, results from the pre-clinical studies carried out showed that the compound fulfils the criteria for a potential macrofilaricide (TDR 2007). This drug has been reported to kill adult worms after a single treatment. It has been found to produce 'slow' death of adult worms in girds and dogs, sterilization of worms in cattle.

The longer plasma half-life of 20 days compared to 2 days for ivermectin will allow for either less frequent treatment or higher efficacy with similar frequency of treatment. Moreover, it has been found to be very effective in infection with animal helminthes that are resistant to ivermectin. The phase III clinical evaluation of Moxidectin as a macrofilaricide was conducted in three African countries. The work ranges from the development of a formulation for human use and initial studies in healthy volunteers, to clinical studies and community studies in Africa (WHO, 2009). Result from the study

proved promising as in Phase II clinical trial, moxidectin reduced skin microfilarial loads to statistically significantly lower levels and for substantially longer than ivermectin (Awadzi *et al*., 2014). Recently, Moxidectin was approved for commercial production by the World Health Organization (FINACIAL, 2015). The Global Health Investment Fund (GHIF) investment will be used to support the manufacture of moxidectin and the compilation of the regulatory dossier required for the registration process for use in humans. Should the drug be approved, Medicines Development for Global Health will work towards ensuring a secure supply of moxidectin for onchocerciasis and GHIF will continue to research other potential human uses of moxidectin for infectious diseases.

Turner *et al*. (2013) projected that when the aCDTM strategy was compared to both aCDTI and bCDTI assumed aCDTM was donated, it will culminate in cost savings estimated at 60% more per year than aCDTI.

2.9 *Wolbachia* Bacterial Endosymbiont of *O. volvulus*

Wolbachia is an endosymbiotic bacterium of the major human and animal filarial worms such as *Brugia malayi*, *B. pahangi*, *Wuchereria bancrofti*, *Onchocerca volvulus*, *O. ochengi, O gibsoni*, *O. gutturosa, Dirofilaria immitis*, *D. repens*, *Litomosoides sigmodontis* (Bandi *et al*., 1998; Taylor *et al*., 1999; Taylor *et al*., 2005). *Acanthocheilonema viteae, Chandlerella quiscali*, *L. loa, O. flexuosa* and *Setaria digitata* are *Wolbachia*-free (McNulty *et al*., 2012a). Differences have been reported by Fenn *et al* (2006) on *Wolbachia*, a bacterium that belongs to the Anaplasmataceae in the Rickettsiales, comprise of a diverse group of intracellular symbionts. In other Rickettsiales, the symbiosis is usually parasitic or pathogenic, and many of these bacteria cause significant human and veterinary disease problems (Tamarozzi *et al*., 2011). Wolbachia increase the number and degranulation of mast cells at the site of infection, resulting in greater vascular

permeability (Specht *et al*., 2011). Rickettsiales have also been identified as symbionts of arthropods, and are implicated in causing reproductive manipulations in their hosts similar to those of *Wolbachia*.

Parasitic filarial nematodes of the Onchocercidae, including several major human pathogens, harbour intracellular *Wolbachia* (Sironi *et al*., 1995). No other nematodes are known to harbour *Wolbachia* (Bordenstein *et al*., 2003), though other nematode–bacterial symbioses are common. In the onchocercids, the *Wolbachia* can be divided into two major clades, C and D (Bandi *et al*., 1998), which, unlike the arthropod *Wolbachia* clades, show phylogenetic congruence with their hosts (Casiraghi *et al*., 2001; Casiraghi *et al*., 2004). Killing the bacteria with tetracycline affects nematode growth, moulting, fecundity, and lifespan (Bandi *et al*., 1999; Hoerauf *et al*., 1999; Smith and Rajan, 2000). In arthropods, in most cases, tetracycline treatment yields cured, healthy hosts, and related parasitic nematodes that do not harbour *Wolbachia* are unaffected by treatment (Hoerauf *et al*., 1999; Smith and Rajan, 2000; Casiraghi *et al*., 2001). On the other hand, Sinkins and Gould (2006) and McMeniman *et al.* (2009) concluded that *Wolbachia* is also a potential biological control agent or vector for spreading desirable genetic modifications in insects.

Several filarial species are major human pathogens, and antibiotics with activity against *Wolbachia* offer a promising new therapeutic approach, since the adult worms are relatively refractory to conventional anthelmintics but depend on *Wolbachia* for reproduction and viability. Makepeace *et al.* (2006) found that in a natural filarial parasite of cattle, *Onchocerca ochengi*, intermittent chemotherapy is adulticidal whereas the equivalent dose administered as a continuous treatment is not. The authors recorded the accelerated depletion of bacteria after antibiotic withdrawal relative to the rate of

elimination in the continuous presence of the drug. Comparisons between the mitochondrial genome sequences of *Wolbachia*-dependent and independent filarial worms may reveal differences indicative of altered mitochondrial function (McNulty *et al*., 2012b). The authors found that 9 mitochondrial genomes were similar in size and AT content and encoded the same 12 protein-coding genes, 22 tRNAs and 2 rRNAs. Synteny was perfectly preserved in all species except *Chandlerella quiscali*, which had a different order for 5 tRNA genes. Protein-coding genes were expressed at the RNA level in all examined species. In phylogenetic trees based on mitochondrial protein-coding sequences, species did not cluster according to *Wolbachia* dependence.

2.10 Strategy for Onchocerciasis Drug Discovery Initiative

Ivermecin (patented in 1989 for human use) is not a macrofilaricide and regular administration is required to kill young worms has prompted the need to develop new drug belonging to a different compound. Nwaka and Hudson (2006) proposed that the target profile of the new drug should include the following characteristics: (i) inexpensive and (ii) its safety should be equal or better that ivermecin or combination for lymphatic filariasis. (iii) Short treatment courses ideally single oral dose, (iv) safety profile compatible with use without diagnosis and (v) safe in children and pregnant women (and stable under tropical conditions (shelf life >2 years).

A dire situation in which there is lack of incentive for drug development against onchocerciasis and other filarial and helminthic diseases is due largely to the low investment return that has made research development in this area an unattractive portfolio to big pharmaceutical companies. Hence, the pharmaceutical industry began its withdrawal from the discovery and development of new drugs for tropical diseases in the mid-1970s

(Shoop, 2003). To mitigate this problem a paradigm shift has been championed with the creation of the Helminthes Drug Initiative (HDI) in 2006 and African Novel Drugs and Diagnostics Innovation (ANDI) which came on board in 2009 to stem the tide. A protocol for drug development and drug discovery chain have been designed as described in the reviews by Nwaka and Hudson (2006) and Nwaka *et al.* (2009). The hit criteria for onchocerciasis are observation of *O. lienalis* microfilarial 100% inhibition of motility at 1.25×10^{-5} M and *O gutturosa* adults' 100% inhibition of motility or formazan formation at 1.25×10^{-5}M with no obvious sign of toxicity to monkey kidney feeder cell layer.

The criteria for lead activity are *O. lienalis* microfilaria: active *in vivo* (mice) when given intraperitoneally or subcutaneously in 10% dimethyl sulphoxide (DMSO) formulation at 5x100 mg per Kg as measured by statistically significant reduction in worms (>80% is highly active). It should not be overtly toxic in animals at efficacious dose. Note that values are illustrative (Nwaka *et al.,* 2009). The three strategies for drug discoveries are label extension, which involves the extensions of existing treatments for human and animal ailments to tropical diseases (Witty, 1999). Moxidectin, an analogue of ivermectin, is example of such approach (Cotreau *et al*., 2003). Second, a piggy-back discovery entails exploring molecular targets present in parasites that allow identification of chemical starting points (Gelb *et al.,* 2003). Third, is the *de novo* discovery, which focuses on identification of new chemical entities (Nwaka and Hudson, 2006) both synthetic compounds and natural products as novel anti-parasitic drugs. According to Townson *et al.* (2006) assay for drug screening depends on adult male *O. gutturosa* cultured on a monkey kidney cell (LLCMK 2) feeder layer in 24-well plates with antibiotics and antibiotic combinations (6 to 10 worms per group). The macrofilaricide CGP 6140 (Amocarzine) can be used as a positive control. Worm viability can be assessed by two methods, (i) motility

levels and (ii) MTT/formazan colorimetry. The yellow compound MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] is reduced by the mitochondrial enzyme succinate dehydrogenase of living tissues to produce the blue precipitate MTT formazan (Comley *et al*., 1989a; Comley *et al*., 1989b). Worm motility was scored on a scale of 0 (immotile) to 10 (maximum) every 5 days up to 40 days. On day 40, worm viability was evaluated by MTT/formazan colorimetry, and results were expressed as a mean percentage reduction compared with untreated control values. Marcellino *et al*. (2012) reported new computer based monitoring of hit compound activities to adult worm. Halliday *et al*. (2014) developed a 'pan-filarial' small animal research model with adequate capacity and throughput, to screen existing and future pre-clinical candidate macrofilaricides.

2.11 Diagnostic Challenges in Onchocerciasis Control

With ivermectin control now in place, the need for an alternative to the microscopic examination of skin snips for emerged microfilariae referred to as the "gold standard" cannot be over emphasized. According to Mabey *et al.* (2004) and WHO (2005), the ideal test should be SMART, adaptable for field use and affordable by poor disease endemic countries. Classical diagnosis based on skin snipping is not only evasive, its sensitivity depends on the location or site, number of skin snips taken and level of skin mf density (Taylor *et al*., 1989). Another important disadvantage associated with the use of corneo-scleral biopsy punch is the ethical problem with its attendant serious implications. It is capable of spreading hepatitis A virus and human immunodeficiency virus (HIV), the cause of acquired immunodeficiency syndrome (AIDS). It is expected to become less sensitive due to reduction in microfilaria load at the period following post-treatment with ivermectin when surveillance for recrudescence will become inevitable.

2.11.1 Immunodiagnostic tests

Concerted efforts aimed at developing a definite serodiagnostic test was approached through dot blot immunobinding assay (DIA-BA) based on the biotin-avidin binding system, for the detection of *O. volvulus* specific antigens in body fluids. Compared with the antigen detection assay in urine, dermal fluid and tear specimen over sera that gave 100% in skin Mf positive subjects (Wembe *et al*., 2005). The biotinylated probes were then used to detect *O. volvulus* specific antigens initially blotted onto a nitrocellulose membrane. The smallest amount of blotted antigens detectable by the new test was 0.5ng, 1ng, 1ng and 2ng respectively in urine, dermal fluid, tears and serum samples. Similarly, an oncho-dipstick has been developed and evaluated, and was found capable of detecting up to 25ng antigen in urine and tears by Ayong *et al.* (2005). the sensitivity of the oncho-dipstick assay was 100% in urine and 92% in tears; its specificity was 100% in both. Concordance between urine and tear test results from the same individuals was 87%. Serodiagnosis has continued to attract more attention as a viable screening option (Lipner *et al*., 2006) that complement the less sensitive classical onchocerciasis diagnostic method.

The time preceding when parasite materials are liberated as against when detectable immune responses were invoked decreases the sensitivity of the serological tests. Detection of earlier or pre-patent infection particularly in children was facilitated with antibody serology as reported by Gbakina *et al*., 1992; Ogunrinade *et al* (1992); Ogunrinade *et al*. (1993) and Enwenzor *et al.* (2000). However, a major setback to antigen detection is the need to subject the samples (serum or urine) to one form of pre-treatment or the other (More and Copeman, 1991; Mbacham *et al*., 1992). While serum is treated to remove interference by specific host antibodies, urine is dialyzed to obtain concentrated residue. Hence, this will make the field application of antigen detection somehow a

difficult process to accomplish. Recently, four recombinant *Onchocerca volvulus* antigens (Ov-FAR-1, Ov-API-1, Ov-MSA-1 and Ov-CPI-1) were tested by luciferase immuno-precipitation system (LIPS) allowed for unequivocal differentiation between Ov-infected and uninfected control sera with 100% sensitivity and 100% specificity (Burbelo *et al*., 2009). The mixture of the 4 Ov antigens simultaneously in the standard format or a quick 15-minute format (QLIPS) showed 100% sensitivity and 100% specificity in distinguishing the Ov-infected sera from the uninfected control sera.

2.11.2 Nucleic acid based tests

The nucleic acid amplification test (NAAT) of onchocercal complementary DNA or RNA primer sequences (0-150 base pairs) using PCR technique is dependent on skin snipping or scrapings, requires specialized skill, equipment and is costly. The test has proved to be more sensitive and specific (100%) than skin snips for mf (Zimmerman *et al*., 1994; Rodriguez-Perez *et al*., 2004). An alternative to skin mf detection diagnostic test is the DNA molecular based test, which is more sensitive and specific than the skin microfilariae detection (Boatin *et al*., 1998b; Vincent *et al*., 2000). Ideally, the detection of any stage of the parasite or parasite products will unequivocally indicate current infection status. Other diagnostic approach are based on molecular biology techniques, which depends on detection of DNA or RNA fragment that are capable of distinguishing the parasite from other organisms. Due to minuteness of the nucleic acid (NA) molecule, the parasite-specific DNA and RNA oligo-nucleotide probes are amplified using polymerase chain reaction (PCR) to enhance the level of detection (Zimmerman *et al*., 1994; Rodriguez-Perez *et al*. (2004). Various oligonucleotides that are specific for detection of *O. volvulus* have been produced.

Nuclear genes may provide an alternative or companion to mitochondrial barcode sequences (Floyd *et al*., 2002). The most commonly used barcode regions of nuclear DNA e.g. small subunit (ssu) and large subunit (lsu) rDNA—belong to multigene families, and although these are thought to exhibit concerted evolution there are many cases where intragenomic variation has been detected, especially in the internal transcribed spacer regions e.g. ITS 1 and ITS 2 (Harris and Crandall 2000; Chu *et al*., 2001). The forward primer has been used in a single PCR with a different reverse primer to produce 513 base pairs by Nuchprayoon *et al*. (2005) while the reverse primer has been used in a second reaction of nested PCR to produce 344 bp amplification product by Ta Tang *et al.* (2010). The ribosomal genes are a family of tandem repeated units that despite concerted evolution do not necessarily show complete homogenisation.

One serious setback associated with NA-based molecular diagnostic methods was overcome by replacing the radioactive isotope detection of PCR products with enzyme-based reagents which has improved its field use was envisaged to make it more adaptable for field application. DNA/RNA test using ELISA detection techniques required specialized skill, expensive equipment and costly reagents. Both methods do not fulfill the criteria of SMART. Detection of biotinylated PCR products by DNA probes was performed by ELISA to quantify the PCR product or by DNA detection test strips as a rapid field technique. Although the ELISA is theoretically more sensitive than the test strips for the detection of PCR products, examination of field samples revealed that the test strip method had a higher operational sensitivity and was more convenient to perform (Pischke *et al*., 2002). The DNA Detection Test Strips were reported to be a rapid and low-technology tool for identification of PCR products in laboratories of countries endemic for onchocerciasis.

The complexity of these tests according to Alhassan *et al.* (2014) is the technical barrier which has been surmounted with the development of the loop-mediated isothermal amplification (LAMP). This technique amplifies DNA with high specificity, sensitivity and rapidity under isothermal conditions (Notomi *et al*., 2000). The LAMP reaction includes two sets of primers that hybridize to six sites on the target DNA, and a third set of primers (loop primers) to accelerate the reaction (Nagamine *et al*., 2001). The mixture of stem-loops containing alternately inverted repeats of the target sequence and cauliflower-like structures that are generated result in exponential amplification of the target sequence (>10 µg, >50 x PCR yield). Using three primer sets recognizing eight sites in the target DNA engenders the specificity to discriminate between genomic DNA at both genus and species specific levels (Notomi *et al*., 2000; Nagamine *et al*., 2001; Nagamine *et al*., 2002, Alhasan *et al.*, 2014). LAMP employs Bst DNA polymerase, which provides both strand displacement and target amplification at a single temperature in a simple heat block or water bath at 60–65ºC.

2.11.3 Application of recombinant DNA technology

With the explosion of information on biological research, there are a lot of information stored in databases on DNA, RNA, gene and gene structure, functions and products like enzymes and proteins that are of vaccine, therapeutic and diagnostic importance available on the internet network, world wide web (www). Following sequence analysis by Lobos *et al.* (1991) an immunodominant *O. volvulus* antigen from the cDNA cloned from Lambda gt11 expression library was deduced. The open reading frame (ORF) that encode 152 amino acids, and the deduce sequence predicted M_r16, 850 (was consistent with the apparent M_r 18,000 of the immune-precipitated *in vitro* translation products). The primary translation product contains putative signal peptide of 16 amino acid sequence. The mRNA

coding for this antigen has 950 nucleotides. The antigen coded by this clone was present in hypodermis, cuticle, and the uterus of the filarial worms. The antigen was found to be recognized only by sera from onchocerciasis patients. Many authors have reported construction of recombinant antigens cloned from nucleotide sequences that were amplified with primers containing DNA restriction endonuclease sequences at their 5' and 3" ends to facilitate subsequent ligation and cloning reactions using polymerase chain reaction (Nde *et. al*., 2002).

2.11.4 Enzymes as diagnostic biomarkers

In similar vein, like antigens, immune responses to *Onchocerca* excretory or secretory enzymes have been measured to determine their reliability as diagnostic reagents. Among the enzymes so far tested includes alkaline phosphatase (E.C. 3.1.3.1) with a molecular weight of 90 kDa when in crude extract and dimerises to about 180 kDa upon purification. The enzyme was found to be secreted by both *O. ochengi* and *O. volvulus* worms. Sodium dodecyl sulphate at 2% (w/v) did not inhibit the enzyme activity, but apparently stabilized it during freezing. Inorganic phosphate inhibited the enzyme competitively with an apparent inhibition constant (K_i) of 3.33 ± 0.04 mM, whereas l-phenylalanine inhibited it in a mixed way with a K_i of 3.18 ± 0.03 mM. The apparently unique enzyme which is likely to serve in the nutrition of the parasite could be further characterised as a macrofilaricide target or diagnostic marker in onchocerciasis (Cho-Ngwa *et al*., 2007). Another enzyme of interest is the *O. volvulus* Glutathione S-transferase 1a and 1b (*Ov*GST1a and -1b). These enzymes are unique GSTs in that they are glycoproteins possessing signal peptides that are cleaved off in the process of producing the mature protein. The mature protein starts with a 25-amino-acid extension not present in other GSTs. The ultrastructural localization of the

secretory *Ov*GST1a and -1b in parts of the cuticle and in the outer lamellae of the hypodermis is consistent with the fact that they are secreted proteins (Liebau *et al*., 1994).

The potential significance of *Ov*GST1a and 1b is serving as immuno-prophylactic targets and the immunological relevance of the N-glycans. This higher IgG reaction may be due to similar GST antigen epitopes, which might resemble the N-terminal portion of *Ov*GST1a. There is indication the extracellular glutathione-*S*-transferase gene, *Ov-gst-1*, of *Onchocerca volvulus* has acquired a signal-peptide sequence (Sommer *et al*., 2001). This may be due to a hidden or former infection with *O. volvulus*. The serum pools from patients infected with the trematode *S. mansoni* did not react with the N-terminal extension peptides. Alhassan *et al* (2014) reported on the identification of a new DNA biomarker, encoding *O. volvulus* glutathione S-transferase 1a (OvGST1a), and the development of a simple, single-step, LAMP assay that easily distinguishes between *O. volvulus* and *O. ochengi* DNA represents a significant technical advance.

2.11.5 Metabolomic diagnostic biomarkers for onchocerciasis

Metabolomics, or the measurement of all the metabolites present in an organism, and metabolite profiling, in which a smaller subset of metabolites are measured, have become established as useful tools in the ‘‘real-time’’ measurement of organismal metabolism (Vinayavekhin *et al.*, 2010). A metabolomic approach was applied to the discovery of biomarkers and to create a diagnostic test for identifying and classifying onchocerciasis infection using multivariate statistics and machine learning algorithms. The potential of metabolomic analysis has been demonstrated for uncovering biomarkers for specific determination of not only onchocerciasis infection but holds promise for the diagnosis of other parasitic diseases (Ritchie, 2006, Baek *et al*., 2009).

One important biomarker is the *O. volvulus*-neurotransmitter tyramine (Globisch *et al*., 2013). Ten out of the 14 candidate biomarkers showed excellent performance in the African specific sample set with up to 99–100% sensitivity and specificity when examined with the single machine learning algorithms. Consistent among these 10 small molecular features is that they are all fatty acids and related fatty acid derivatives. Further investigations into the biological roles of these fatty acids and fatty acid sterols in onchocerciasis disease progression and potential interaction with the down-regulated proteins is of distinct interest, not only in the development of a diagnostic but also to more clearly understand the biology of this disease (Denere *et al*., 2010). Almost certainly, other biomarkers could be discovered and validated simply by altering the chromatographic and/or ionization conditions. It is possible that additional markers can eventually be added to the repertoire of biomarkers used for onchocerciasis detection, further increasing assay specificity. Although this appeared to be sensitive and specific, but like the molecular test, it is highly capital intensive and requires specialized skills.

2.11.6 Importance of antibody based serodiagnosis of onchocerciasis

One of the major reasons for the immunology of any disease is to identify those molecules (antigens and antibodies) useful for serodiagnosis (Parkhouse and Harrison, 1989). Cross reactivity with other parasites was a major limitation that was overcome by using low molecular weight antigens (Weiss and Karam, 1989). Secondly, it was further enhanced by measuring IgE and IgG4 antibody (Weiss *et al*., 1982; Lal and Ottesen, 1988; Weil *et al*., 1990). Most of the cross reactants are high molecular weight carbohydrate moieties or glycoprotein containing ubiquitous immunodominant phosphorylcholine (PC). Moreover, IgG4 antibody does not react with PC molecule thereby conferring higher specificity to *O. volvulus* (Nde *et al.*, 2005). Others have used various fractions of *O. volvulus* native antigens (Guederin *et al*., 2004; Osue *et al*., 2008). A four hybrid recombinant antigens

resulted in increased reliability of assays compared to the individual antigens used (Andrews *et al*., 2008). Interesting, the hybrid antigens belong to the relative low molecular weights band.

2.12 Bioinformatics and Genomic Databases for Onchocerciasis

There are huge stores of information in the internet or World Wide Web (www) hosting databases on genomics (DNA, RNA, nucleotide and gene proteomics (peptides/protein structures and functions), metabolomics, transcriptonomics for identification and characterization of expressed sequence tags (EST). There are four main databases: National Centre for Biotechnology Information (NCBI), USA; European Molecular Biology Laboratory (EMBL), Germany; DDBJ of Japan, Swiss-Prot protein sequence database, France. Importance of www is the ease and opportunities it has afforded researchers free access to enormous data and information on designing and comparing of genes, nucleotide or primer, peptide/ protein, and EST. These can be done with either a commercially licensed or an on-line free software DNA workbench to carry out sequence alignment and comparison using the Basic Local Alignment Search Tool (Altschul *et al*., 1997) analysis. With BLAST, you can also perform nucleotide search, construct phylogenetic tree, detect mutagenesis, identify and characterize polypeptide/protein amino acid sequence composition based on messenger RNA, transfer RNA and complimentary DNA.

There are four cDNA libraries for this organism, *Onchocerca volvulus* taxon 6282: the female adult worm, L2, L3 and molting stage (L4) have been hosted. It has been established that *O. volvulus* species contains three distinct genomes. This include the nuclear, mitochondrial and *Onchocerca volvulus*-associated *Wolbachia* genomes (Unnasch

and Williams, 2000). The genomes of parasitic nematode species are between 60 and 250 megabases (Mb) in size (Hammond and Bianco, 1992) and *O. volvulus* has the least. The size of the nuclear genome is roughly 1.5x10(8) bp arranged on four chromosome pairs. Analysis of ETS of different life-cycle stages resulted in the identification of transcripts estimated to be 4000 *O. volvulus* genes (Unnasche and William, 2000). Several of these transcripts are highly abundant, including those encoding collagen and cuticular proteins. Analysis of several gene sequences suggests that its nuclear genes are relatively compact and are interrupted relatively frequently by small introns. The intron-exon boundaries of these genes generally follow the GU-AG rule characteristic of the splice donor and acceptors of other vertebrate organisms. The nuclear genome also contains at least one repeated sequence family of a 150 bp repeat which is arranged in tandem arrays and appears subject to concerted evolution.

The mitochondrial genome of *O. volvulus* is remarkably compact, only 13747 bp in size (Unnasch and Williams, 2000). Furthermore, consistent with the small size of the genome compared to other nematode worm, the author reported that four gene pairs to overlap, eight contain no intergenic regions and the remaining gene pairs are separated by small intergenic domains ranging from 1 to 46 bp. Keddie *et al* (1998) had shown that a total of 20 of the 22 transfer RNAs encoded in the *O. volvulus* mitochondrial genome have the potential to fold into secondary structures lacking the TpsiC arm, as has been reported in other nematodes. Over 14 600 randomly selected cDNA clones have been sequenced from these libraries for expressed sequence tag (EST) analysis (Williams et al 2002). These cDNA clones are said to represent 4208 protein encoding genes. The products of these genes can now be evaluated as vaccine candidates or drug targets in order to develop a product that would eliminate the disease.

2.12.1. Role of expressed sequence tag in onchocerciasis

Over the past decade, the most tractable way of applying genomics to this group of organisms has been by expressed sequence tag (EST) projects (Parkinson *et al*., 2003). Large-scale EST sequencing of the human filarial parasites has registered ESTs that encode proteins with predicted signal sequences. This depends on a complementary DNA (cDNA) library specifically enriched for full-length inserts (Fernandez *et al*., 2002), allowing analysis of amino-terminal signal peptides to be carried out. ESTs are single DNA sequencing reads made from cDNA clone libraries constructed from a known tissue source. Sequencing a large number of these clones from such a library allows one to sample the set of expressed genes, or transcripts, in that particular tissue and experimental state, thus providing a snapshot of the tissue's active genes under those defined conditions (Lizotte-Waniewsky *et al*., 2000). cDNA clones are the biological material from which EST sequences and finished cDNA sequences are produced. A set (or library) of cDNA clones are made from a biological tissue (or pooled tissues) by using routine molecular biological techniques. Cellular mRNA is extracted from the tissue, it is reverse transcribed to produce DNA complementary to the initial mRNA, and incorporated into a plasmid, with appropriate vectors and linkers. The plasmids are then grown up in a suitable host such as *E. coli.*

2.12.2. Possible areas of application of expressed sequence tag

Likely areas where analyses of ESTs may be found to have very important practical application in *O. volvulus,* other filarial and non-filarial parasites are in the screening for potential vaccine candidates. In addition, it can equally be applied for identification of drug targets and proteins or enzymes involved in clinical immunopathology. So far, a new class of helix-rich retinol-binding protein (RBP), Ov20 protein of *O. volvulus* has been described

(Kennedy *et al*., 1997). This protein is known to have strong binding to retinol but differs from previous RBPs in structure and characteristics of its binding site. The nematode spermatozoa contain an abundant protein called major sperm protein (MSP), which is about 15% of the cellular proteins. It is a developmentally regulated gene product produced by the testes and appeared to be highly conserved during evolution. *Onchocerca volvulus* has a limited number of germ-line genes coding for the major sperm protein. The limited number of genes found was similar to what have been reported for the MSP genes in *Ascaris* and several other filarial nematode species. They were vastly different from the more than 50 MSP genes found in the free-living nematode *Caenorhabditis* (Ward *et al.,* 1988; Scott *et al*., 1989). It is presumed that MSP function as a cytoskeletal element involved in motility. Two genes, OVGS-1 (765 bp) and OVGS-2 (1765 bp) were cloned and characterized by restriction endonuclease mapping and sequence analysis. Both genomic clones contain MSP protein coding regions of 99 and 282 bp separated by an intervening sequence of 153 bp. It was apparent the genes showed similarities of 95% nucleotide sequence in the protein coding regions and 79% intron sequences. These genes are in good agreement with consensus sequences in other eukaryotic cells.

Other genes of interest that have been studied is the actin, glyceraldehyde 3 dehydrogenase (GDH), proteinases, proteinase inhibitors, antioxidant or detoxification enzymes, and neurotransmitter receptors, as well as structural and housekeeping genes (Lizotte-Waniewski *et al*., 2000). Other *O. volvulus* genes showed homology only to predicted genes from the free-living nematode *Caenorhabditis elegans* or were entirely novel. They identified potential genes that can be pursued for development of vaccines and/or drug targets against onchocerciasis based on their immunogenicity, up-regulation in larval stages, and/or predicted biological features.

CHAPTER THREE

3.0 MATERIALS AND METHODS

This study entailed field epidemiological surveillance to obtain biophysical, entomological, parasitological and clinical enumeration data and collection of skin snip and blood samples. The study area was geo-referenced with the attribute data generated. The second aspect is the laboratory based analysis of sera (serology), development and validation of slide flocculation test, parasitaemia based on skin snip examination for microfilaria and molecular biologic test using Polymerase Chain Reaction (PCR) amplification of *Onchocerca volvulus* DNA from samples and positive controls. Data obtained from this study were compared with baseline data obtained in 1994 before the commencement of mass ivermectin treatment in these communities.

3.1 The Study Area

Six (6) villages were selected as sentinel study sites and these include: Gantan, Sabon Gantan, Kurmin Gwaza, Bomjok, Ungwan Shaho (K/Gwaza II), and Gidan Tama in Kachia Local Government Area of Kaduna State, Nigeria as shown in Figures 5-7. The villages located within 9° 37"– 9° 45" N and 7° 44"–7°48" E were selected because of the availability of baseline data, which were collected in 1994 (Osue, 1996). The type of vegetation is reminiscent of wooded-shrub grassland with 80–90% farmland (FDF, 1978) and lies within the subhumid climatic zone. Bomjock is farther more in the hinterland and is a small non-autonomous farming settlement under K. G. with which they share ancestral affinity. Sabon Gantan has history of resettlement in a once abandoned site by inhabitants of Gantan and they share ancestral lineage. Bomjock, S. Gantan, and Gantan are situated close to one of the tributaries of River Gurara called River Gantan. Both Gantan and K.G.

are 3Km apart, both are accessible by a federal highway leading to Nasarawa State, while Ungwar Shaho and Gidan Tama are relatively close to the tributary of River Gantan but are far away from the highway.

3.2 The Study Population

Overall projected population of 1600 for the 6 villages was based on 2006 National Population Census (NPC) figures after adjusting with 3% growth rate per year. A target of 50% (n=800) of the study population was set as maximum threshold to be screened. This included those screened at pre-treatment (n=541) to be followed-up and those aged 15-30 years old that were not screened initially. The sample population (n_0) for the study area was derived from the formula:

$$\mathbf{n_0} = \frac{\mathbf{Z^2pq}}{\mathbf{C^2}}$$ to calculate the sample size for proportion (Cochran, 1963).

Where **Z** is desired confidence level at 95% or 1.96, **p** is the estimated proportion of the attribute or prevalence of 37.9% (Osue, 1996), **q** equal **1-p** and **C** is desired level of precision or margin of error at 5% (0.05). The calculated sample size (n_0 =362) served as the minimum threshold.

3.3 Bio-clinical Data.

Each participant was clerked to obtain vital information on biodata (sex, age and height), occupation, residency status and individual ivermectin treatment compliance rate. The reasons for non-compliance and number of ivermectin tablets per dose taken annually, any adverse effect observed after treatment were obtained from individuals. Records of the village dosage since commencement of treatment were extracted from Community Based Distributors' (CBD's) registers.

3.4 Socio-Economic Activities of the Study Area

The people were mostly peasant farmers who grow ginger and soya bean as cash crop, maize, guinea corn, groundnut, and yam as staple food crop. Only adults (aged 15 years and above) were examined during baseline studies. The villagers were briefed in their dialect on the purpose and method of the study through a member of the team who speaks the dialect assisted by Primary Health Care (PHC) staff of the Local Government Health Department assigned to the team. Only persons who freely consented to participate were enlisted for screening. The research protocol was approved and funded by the Nigerian Institute for Trypanosomiasis Research (NITR) which has the national mandate to carry out research into African trypanosomiasis and onchocerciasis. Ethical clearance was obtained from the Kaduna State Ministry of Health for the impact assessment.

3.5 Geospatial and Entomology Data.

A handheld Geographic Position System (GPS), eTrex, Legend, Garmin, was used to capture the coordinates of the study sites and black fly locations. Breeding sites were prospected for along the rivers, streams and rivulets at the villages (Figures 3.1-3.3) during impact assessment study. Three adult males were used as human bait to catch flies in the morning for three days at the Gurara Village, a well-known black fly breeding sites (Crosskey, 1981). The black flies caught were separated into nulliparous and parous (engorged) groups; they were preserved in 80% ethanol. Only the parous flies were dissected under a Wilde dissecting microscope at x4 magnification to determine infection rate. In addition, the flies were broadly characterized into savannah and forest types depending on the colour of the wing turf.

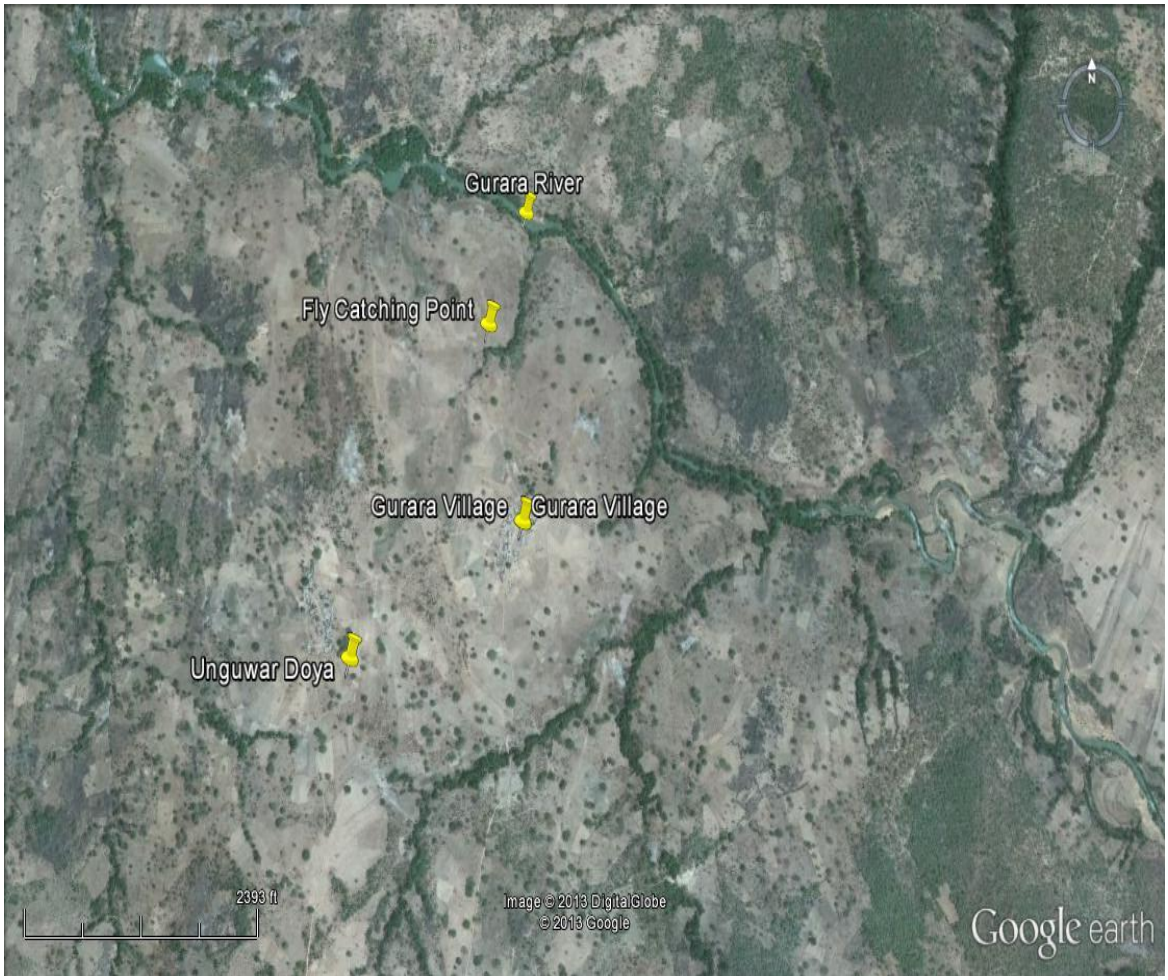


Fig. 3.1: Satellite map of blackfly breeding area along River Gurara. The coordinates of the study villages, fly catching and river points were obtained using Geographic Position System (GPS), eTrex, Legend, Garmin.

Source: Google earth map 2013 images download.

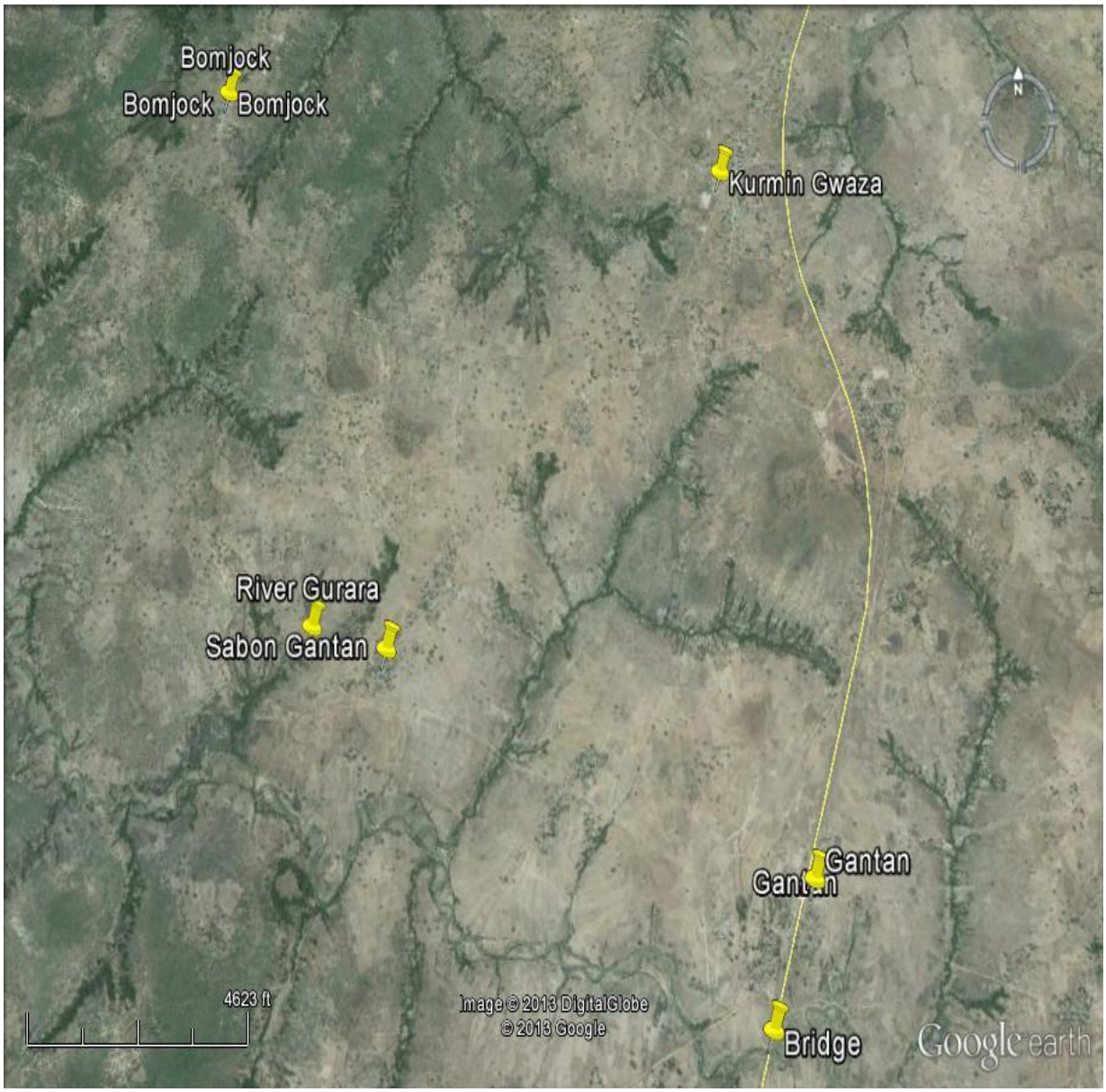


Fig. 3.2: Satellite map of a tributary of River Gurara and four study villages. The coordinates of the study villages and rivers points were obtained using Geographic Position System (GPS), eTrex, Legend, Garmin.

Source: Google earth map 2013 images download.

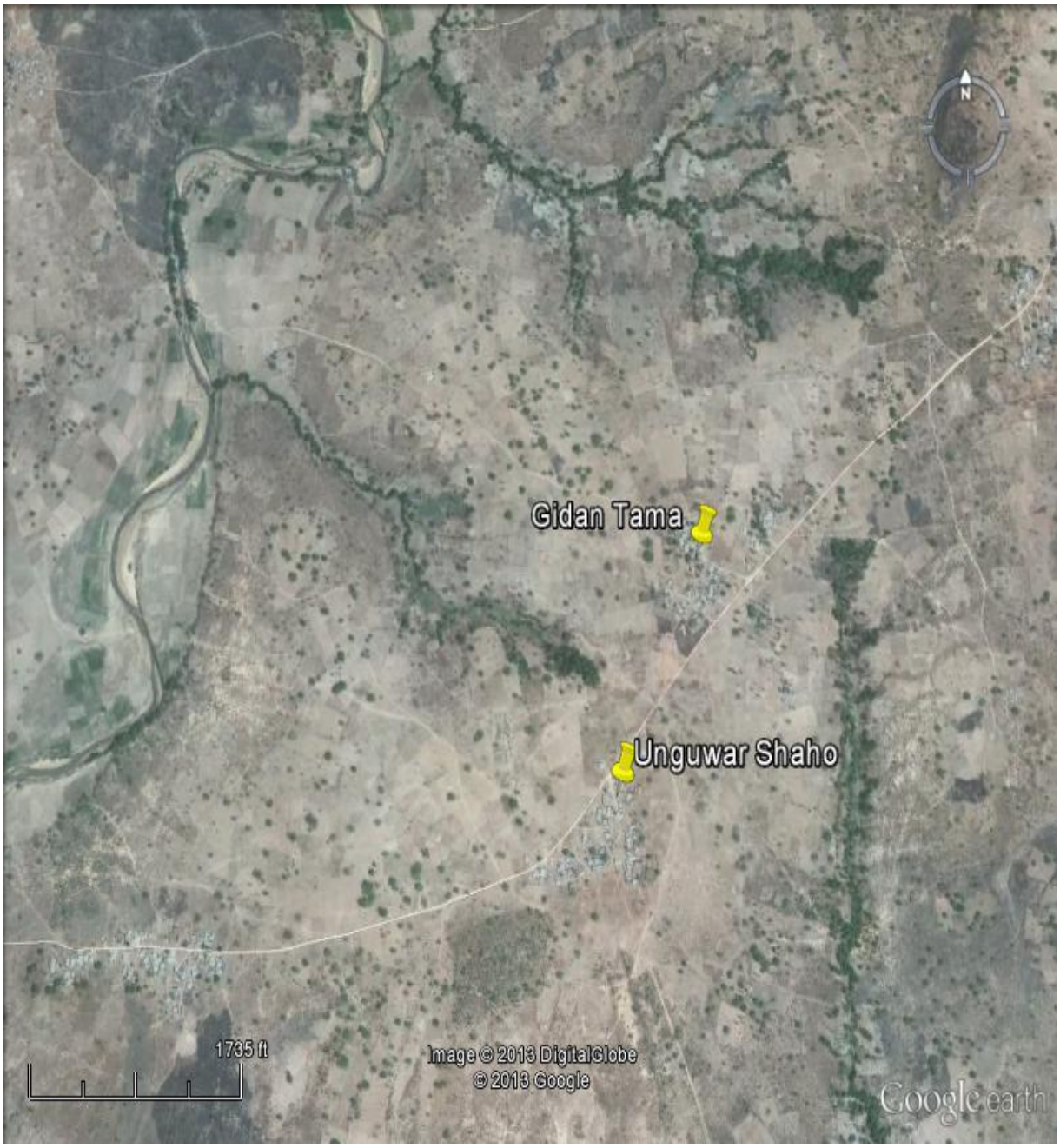


Fig. 3.3: Satellite map of a tributary of River Gurara and two study villages. The coordinates of the two study villages which are about 500 metres apart were obtained using Geographic Position System (GPS), eTrex, Legend, Garmin.

Source: Google earth 2013 images download.

3.6 Experimental Design

This study entailed field epidemiological surveillance to obtain biophysical, entomological, parasitological and clinical enumeration data and collection of skin snip and blood samples. The study area was geo-referenced with the attribute data generated. The second aspect is the laboratory based analysis of sera (serology), development and validation of slide flocculation test, parasitaemia based on enumeration of emerged microfilaria from skin snips under an inverted microscope at x 40 magnification. Molecular biologic test was performed using Polymerase Chain Reaction (PCR) amplification of *Onchocerca volvulus* DNA in samples and control DNA templates.

Only those who volunteered to participate after the protocol had been clearly explained to them in their native dialect were enlisted for the study. Endemic control serum samples were selected from among residents that had showed no evidence of onchocerciasis infection either now or before commencement of treatment. None-endemic samples were obtained from NITR staff known to be onchocerciasis free. Experienced and qualified personnel performed the clinical examination, blood sample collection using sterile disposable syringes and needles and skin snipping with Holth corneoscleral biopsy punch sterilized by flaming and dipping into 70% alcohol and glycerol in between patients. Statistical relationship between the measurable data was analyzed with respect to the number of ivermectin treatments received by the individuals. The relationship of individual and community treatment compliance and coverage rates *vis-a-vis* the parasitological, clinical and levels of parasite antigen specific antibodies were assessed.

This study also measured the reliability (sensitivity and specificity) and determined the true positive and false positive, true negative and false negative results were used in computing the assay positive and negative predictive values (PPV and NPV) of serum antibody reaction with SDS extracted antigens. In addition to the primary data that were generated from this study, the baseline data obtained before mass distribution of Mectizan® commenced in 1994 served as secondary materials that provided basis for comparison. Enumeration of emerged Mf from skin snips taken from both iliac crests using Holth corneoscleral biopsy punch served as "gold standard" for the serodiagnostic tests. A subset of sample population from participants that were skin snipped were randomly selected for serology/PCR. This subset was analyzed on the bases of current and previous history of skin microfilaira and palpable nodule and clinical status. Each skin snip preserved in 80% ethanol was subjected to polymerase chain reaction (PCR) test using the 18S *O. volvulus* ITS-1 r RNA and beta actin (Actin-2B) primer sequences.

Data generated from field epidemiological surveys were double fed into Microsoft Excel. Descriptive statistical analysis were done using Microsoft Excel software statistical package to compute mean and standard deviation of replicate assays, percentage sensitivity and specificity, binomial confidence interval of antibody level amongst the study population and predictive values of assays using the Bayesian method. Variable were subjected to F-test for paired and unpaired data. Tabular forms and graphical illustrations were made using Chart in Microsoft Word software.

3.7 Field Epidemiological Surveys

A pre-survey was undertaken to meet with LGA officials, District and Village Heads. The people were mobilized and the purpose of the study explained to them in their native dialect through the LGA Health Official assigned to the team. A hand-held Geographical Positioning System (GPS, Garmin) was used to locate the bearings of the studied communities. Participants' biophysical and clinical data were recorded in a form. The data were double fed into computer and cleaned for areas of conflicts.

3.8 Case Definition of Onchocerciasis

A suspected case of onchocerciasis is defined as the presence of nodule(s) in subcutaneous tissues and/or 'leopard skin' and the presence of one or more microfilariae in a skin snip (WHO, 2002). Participants were examined for onchocercal related skin diseases essentially as described by Murdoch *et al.* (1993). Visual acuity (VA) tests on both eyes were performed using illiterate E-chart. Anterior and posterior eye lesions were evaluated using touch loop and handheld ophthalmoscope (WHO, 2005).

3.9 Collection of Samples

3.9.1 Parasitological Sampling

Two skin snips were taken from left and right iliac crests using sterile biopsy punch with 2mm bite. Skin snips were incubated in normal saline overnight at ambient temperature (28-30º C). Snips were removed and few drops of formalin added per each well of microtitre plate. Emerged microfilariae were counted under x40 objective of inverted microscope using hand held tally counter (WHO, 1974). Individuals were randomly selected and skin snips obtained were placed inside tubes containing 80% ethanol as preservative and stored at ambient temperature before use in polymerase chain reaction.

3.9.2 Collection of Blood Samples and Serum Extraction

The second phase of the study was serology to determine the levels of onchocercal antigen-specific serum antibodies among the residents. Onchocerciasis slide flocculation tests (Oncho-SFT) was developed using rat tissue homogenate as flocculating particles. The anatomic site was disinfected with 70% alcohol and about 5 ml blood was collected from individuals using sterile disposable syringes and needles. Blood was allowed to clot under ambient temperature. Serum was decanted into screw capped tubes and stored frozen at -20° C before use.

3.9.3 Collection of Experimental and Control Sera Samples

Sera samples were obtained from residents of five (5) sentinel villages located within Gurura River basin in northern savannah onchocerciasis meso-endemic foci in Kachia Local Government Area of Kaduna State, Nigeria. The study population comprised of individuals whose parasitological, clinical and serological baseline data were obtained in 1994 prior to commencement of ivermectin treatment of the study sites. Sera from mf positive onchocerciasis patients (n=5) were obtained from NITR Onchocerciasis Serum Bank and from non-infected persons (n=10) resident since birth at Kaduna Metropolis, a non-endemic area. Sera samples (n=19) from proven cases of malaria which was co-endemic with onchocerciasis in the study area were used for cross-reactivity test.

3.10 Sodium Dodecyl Sulphate Extracted Antigens

Adult female worms of *O. volvulus* were dissected from nodules preserved for several years at -20ºC in deep freezer (Gomez-Priego *et al*., 2005) were placed in 1ml of 150mM phosphate-buffered saline containing protease inhibitors, 1mM phenylene sulphonyl

chloride and 50mg/ml N-tosyl –phenylalanine chloromethyl ketone. Adult worms were grinded under liquid nitrogen in PBS containing 1% SDS in a sterile porcelain mortar and pestle, centrifuged at 1300 g for 5 minutes and supernatant fluid was designated SDS-extract as described by Engelbrecht *et al*. (1992).

3.11 Slide Flocculation Test

Laboratory bred rats (3) obtained from NITR small animal colony were anaesthetized with ether and dissected. Tissues were teased separately from the heart, kidney, muscle, spleen, liver, testes, lung and brain, washed 3-5 times with phosphate buffered saline (PBS), and homogenized in Potter glass tissue grinder. After centrifuging for 5 minutes at 10,000 r.p.m, supernatant fluid was decanted and few drops of 0.01% sodium azide added as preservative. Ten (10µl) each of tissue homogenate and antigen (SDS-extract) were thoroughly mixed and shared into two wells, and 10µl antibodies (serum) was added at neat to the wells and mixed. Thereafter, serial double diluted in phosphate buffered saline (PBS). The protocol for slide flocculation test (Norman and Kagan, 1963) was adopted (Figure 3.4). Serum samples (n=10) were heat inactivated at 56ºC for 30 minutes. Flocculation reactions of serum at neat were subjectively graded as no reaction or negative (0), very low (1), low (2), medium (3), high (4) and very high (5) positive reactivity. Series of serial dilutions were performed to determine prozone effects and working concentrations of reactants. The highest dilution with flocculation reaction was taken as the assay titre. The Ov-SFT reactivity of serum samples were analysed by age, sex (male n=28 and female n=37), skin microfilaria status and treatment doses taken. The Ov-SFT reaction of a sub-set of malaria positive (n=5) with negative (n=14) sera were compared. Pre-treatment baseline IgG ELISA antibody were compared with the Ov-SFT results. The differences were subjected to t-test analysis at 0.05% confidence level.

Homogenate of HT (10µl) placed on Wellcome Microscope Slide



Add 10µl of antigen (Ov-SDS extract and mix thoroughly

(shared into two wells of 10µl each)



Add 10µl serum (antibodies) mixed and double diluted



Rocked gently for 1-3 minutes



View reactions in wells against bright light

All steps were carried out at room temperature (25-30º C)

Fig. 3.4: *Onchocerca volvulus* slide agglutination test protocol

HT= Heterologous tissue, Ov-SDS= *Onchocerca volvulus* sodium dodecyl sulphate

3.12 Collection of Skin Biopsies for PCR

Sterilized Holth corneoscleral biopsy punch with 2mm bite was used to obtain skin biopsies from iliac crest region according to WHO (2001a) guideline. The study population for molecular assessment with PCR amplification of template DNA were from randomly selected persons (n=66); male (n=32) and female (n= 34) who volunteered to participate in the study. The snips were preserved in 80% ethanol at ambient room temperature before DNA extraction as recommended by Toe´ *et al.* (1998). Genomic DNA was extracted from 3 different nodules with adult filarial worms of *O. volvulus* provided by NITR Onchocerciasis Bank to serve as positive controls. Three samples from individuals residing in a non-endemic area, two tubes containing distilled water and mastermix without any template served as internal assay and negative controls, respectively.

3.12.1 DNA Extraction from Skin Biopsies

DNA was extracted from skin biopsies (n=66) and from three adult worm fragments using DNeasy® Blood and Tissue Kit (QIAGEN®, Germany) by adhering to the manufacturer's instructions. Briefly, samples were digested with 15µl of Proteinase K in 1.5ml microcentrifuge tube, mixed by vortexing, incubating at 56ºC until completely lysed. Vortex was done during incubation and for 15s before 200µl Buffer AL was added. The mixture was incubated for 10 minutes at 56ºC and 200µl ethanol (96-100%) was added and mixed thoroughly by vortexing in between. The mixture was transferred into the DNeasy spin column in a 2ml collection tube and centrifuged at ≥6000xg (8,000 rpm) for 1 min. Flow-through and collection tube were discarded. Spin column was placed in a new 2ml collection tube and 500µl Buffer AW1 added and centrifuged for 1 min at 6,000 x g. Flow-through and collection tube were discarded. Spin column was placed in a new 2ml

collection tube and 500µl Buffer AW2 added and centrifuged for 3 min at 20,000 x g (14,000 rpm). Flow-through and collection tube were discarded. Spin column was placed in a new 2ml microcentrifuge tube and DNA eluted by adding 200 or 100µl Buffer AE to centre of spin membrane and incubated for 1min at room temperature (15-25ºC). It was centrifuged for 1 min at ≥6,000 x g and DNA extract in elution buffer was stored at -20ºC in deep freezer until used.

3.12.2 Determination of DNA Concentration

Each sample and positive control DNA extracts were subjected to spectrophotometric analysis at 260 and 280 nm wavelength using NanoDrop 2000C (Thermo Scientific, England). To assess the purity of the DNA extract, the concentration in nanogram per microlitre (ng/µl) were measured and the 260/280 nm ratios of samples were obtained. Sample analysis was done as recommended by the manufacturer. Briefly, the software icon on desktop was double clicked and the application (Add to report) on menu bar was selected and the instrument was allowed to initialize. A blank was established using the distill water in which the DNA sample was suspended. About 2 µl was drawn with pipette onto the bottom pedestal and the upper arm was lowered and clicked on the blank button. The Blank was wiped, the sample ID was entered in appropriate field and 2 µl was pipette again and Measure on the menu bar was selected. Both pedestals were wiped after each sample with dry, lint-free laboratory wipe. Blanking cycle was completed with 4 repeats as above and obtained a spectrum readings not more than 0.04 A (10 mm absorbance equivalent). After blanking, both pedestal surfaces were wiped and samples were anaysed. A new blank was taken every 30 minutes before the completion of sample analysis.

3.13 Onchocerciasis Primer Data Mining

GenBank database of National Centre for Biotechnology Information (NCBI), Atlanta, USA were searched for primer sequences specific for *Onchocerca volvulus.* The possibility of mis-match occurrence depends on the length of the primer, 18 was set as minimum, while 23 bp was taken as maximum to avoid dimer-dimer formation.

3.13.1 Primer design

The primers used were ribosomal DNA (rDNA) first internal transcribed spacer (ITS-1) that lies between 18S (small subunit ribosomal RNA) and 5.8S gene region sequenced by the Integrated DNA Technologies (IDT), USA. Both the forward and reverse primers as described by Nuchprayoon *et al* (2005) and Ta Tang *et al* (2010) with accession numbers AF228575 and AF228575 with positions in reference sequence (gene) at 173-192 (18S) and 512-493 or 609-590 (ITS) were selected. The sequences had been aligned using ClustalW software (Thompson *et al*., 1994) and the primer selection was based on general primer design criteria, including, where possible, Tm = 60ºC, at least 40% guanine-cytosine content and less than 60% homology with non-filarial species for the family-specific primers (Ta Tang *et al*., 2010). The 20 base pairs primers were reconstituted as recommended by adding molecular biology grade de-ionized distilled water. The melting temperatures for the forward and reverse primers were 56º and 48.6ºC, respectively.

3.14 PCR Analyses of Sample and Control

Skin snips obtained from residents in study areas (n=65) and persons living in non-endemic areas as negative control (n=3) were tested essentially as described by Ta Tang *et al*. (2010) with slight modification. The PCR protocol established at the Nigerian Institute for Trypanosomiasis Research Molecular Biotechnology Laboratory as described in the

Standard Operating Procedure (SOP) was strictly followed. The PCRs were performed in a 50-µl reaction containing PCR buffer.

Briefly, the PCR Master mix was made up by 10µl PCR buffer added to 32.1µl of de-ionized water in an eppendorf tube, followed by 5.0µl of $MgCl_2$. Thereafter, 1µl of dNTP (1mM dATP, dCTP, dGTP and dTTP, 0.5 mM dUTP, 0.5 U uracil-N-glycosylase and 0.5U *Taq* DNA polymerase was added. Amplification of DNA extract using PCR was performed as described by Morales-Hojas *et al*. (2006) and Ta Tang *et al*. (2010). The forward and reverse primers (18S-F; 5-GGTGAACCTGCGGAAGGATC-3 and ITS1-R; 5-TGCTTATTAAGTCTACTTAA -3 at 5 pmol each and 10–20 ng of parasite DNA template were used at a concentration of 20 pmol per 50 µl each. The primers used satisfied the critera given for selecting sequence primers by the kit manufacturer, QIAGEN®, Germany (Appendix III). One microlitre of the sample and 1µl of the internal control *O. volvulus* adult worm DNA were added to the Mastermix. Eppendorf tubes containing the reagents were vortexed at low speed (6,000rpm) then pulse centrifuged and placed in a thermal cycler (Techne, Germany). Volume and concentration of reagents used for PCR are shown in Appendix IV. The PCR was performed at the Centre for Biotechnology Research and Training (CBRT), Ahmadu Bello University, Zaria. Amplification was performed in 30 cycles at 94° C denaturation, 55°C annealing and 72 °C extension of 30 s each.

The duration of the thermal cycling process was about 2 h. Hot start polymerase (TaQ Polymerase, Qiagen, Hilden, Germany) was used for PCR. An activation step of 5 min at 94°C preceded the cycling programme. The complete set of the PCR is represented as follows: <94°C5:00[94°C0:30; 60°C 0:45; 75°2:00]35; 72°C7:00>. In PCR assays,

samples were analysed with well that contained an adult worm *O. volvulus* DNA fragment, Mastermix with water instead of sample DNA and three skin microfilaria negative served as positive, internal assay and negative controls, respectively. To amplify filarial-specific DNA, the 18S rRNA ITS1 and 5.8S genes forward and reverse primers (Morales-Hojas *et al*., 2007; Ta Tang *et al*., 2010; Albers *et al*., 2012) were used.

Onchocerca volulus actin-2B specific primers; F- GTGCTACGTTGCTTTGGACT and R-GTAATCACTTGGCCATCAGG were used to amplify samples (n=15) included the 4 PCR ITS1 positive. PCR were performed along with the positive, negative and internal (blank) controls

3.14.1 Gel electrophoresis

The 2% w/v gel (2g of agarose powder was added to 100ml 1X TBE buffer) was dissolved by boiling in a microwave oven. It was allowed to cool to 60ºC and 10µl Syber Green was added and swirled gently using magnetic stirrer. The liquid was poured into electrophoresis tank with comb in place and ensured no air bubble was formed to obtain 4-5mm thick gel. It was allowed to solidify for 20-25 minutes and the comb was removed. The tray was placed in the electrophoresis tank and TBE buffer (216 g Tris base, 110 g boric acid, 16.6 g EDTA added to 2 litres H_2O) was poured into the tank with surface gel completely covered. A 15 µl of sample was mixed with 2 µl of loading dye were carefully loaded into wells created by the combs using a 20 µl pipette tip. Finally, 5 µl of the PCR products were checked by agarose gel electrophoresis with appropriate molecular weight markers (1000 bp ladder) were inserted to determine the expected size relative to the marker. The marker, positive and negative controls and samples were loaded. Electrodes were connected with negative terminal at the loading end. Electrophoresis was run at 60-100V till migration of

dye reached three-quarter of gel. Current was turned off and the electrodes disconnected. Gel was observed under a UV Trans-illuminator and the molecular weight band documented in a Syngene Digigenius Gel Documentation System with camera linked to a computer.

3.15 PCR Product Extraction and Sequencing

Bands of DNA in gel were cut, melted in Stratagene Gradient PCR and amplified products were extracted using QIAGEN® PCR Purification Kit essentially as described by the manufacturer. Amplification of PCR products were carried out using the same forward and reverse primers. DNA sequencing includes several methods and technologies that are used for determining the order of the nucleotide bases adenine, guanine, cytosine and thymine in a molecule of DNA. The BigDye termination sequencing with chain terminator ddNTPs was used in a single reaction, rather than four reactions as in the labeled-primer method. Each of the four dideoxynucleotide chain terminator was labeled with a fluorescent dyes, which emit light at different Wavelength.

3.15.1 BigDye terminator cycle sequencing with quick start kit

Sequencing reaction was prepared in a 2.0ml tube and all reagents kept on ice, were added in the following order: dH_2O 0 - 9.5µl, DNA template 0.5 – 10.0µl, primers 2.0µl and dye terminator cycle sequencing (DTCS) quick start Mastermix 8.0ul (Appendix V). The sequencing reaction was set in the PCR machine with thermal cycling program: 96ºC for 20 sec, 50ºC for 20 sec x 30 cycles and at 60ºC for 4 minutes. The protocols for purifying PCR fragments, preparing extension products, spin column purification and sequencing of single strand DNA were strictly according to the manufacturer's instruction detailed in

Appendices VI-IX. Some core equipment used for DNA extraction and PCR assay at the NITR and CBRT are shown in Appendix X.

3.15.2 Ethanol precipitation

Sterile PCR tubes of 0.5ml volume were labelled for each sample. Fresh stop solution/glycogen mixture per sequencing reaction was made up of 2µl 3M sodium acetate, 2µl 100mM of Na_2-EDTA and 1 µl, 20 mg/ml of glycogen (provided in the kit). To each labelled tube, was added 5 µl of the stop solution/glycogen mixture. The sequencing reaction was transferred to an appropriately labeled tube and mixed thoroughly by vortex. Thereafter, 60 µl cold 95% (v/v) ethanol from -20ºC freezer was mixed thoroughly and immediately centrifuged at 14,000 rpm at 4º C for 15min. The supernatant was carefully removed with a micropipette (the pellet should be visible) and the pellet was rinsed with 200µl sodium acetate and 70% absolute ethanol (200% assured molecular grade) from -20º C freezer and centrifuged at 14,000rpm at 4º C for a minimum of 2 minutes. The supernatant was carefully removed with a micropipette. The pellet was vacuum dried for 10 minutes or until dry and re-suspended in 40µl of sample loading solution (provided in the kit).

3.15.3 Sample Preparation for Loading into the Instrument

The re-suspended DNA pellet was transferred to the appropriate wells of the sample plate (PN 609801). Each re-suspended sample was overlaid with one drop of mineral oil from the kit and the sample plate was loaded into the instrument. Sequencing of the PCR products were carried out with SDS-PAGE Beckman Coulter CEQ 2000XL DNA Analysis System (Appendix XI) at the DNA Laboratory, Kaduna.

3.16 Analysis of Primer Sequence BLAST Hits

The National Centre for Bioinformatics (NCBI) search engine was used to perform BLASTn (Altschul et al., 1997) for the primers sequences of *O. volvulus*. Also, analyses of data were generated to see the percentages and sequence length or number of nucleotide similarities scored was subjected to statistical analysis. Frequency of homology or similarity with sequences from sister (animal infective) *Onchocerca* species, other filarial and non-organisms were presented.

3.17 Statistical Analysis

Individual biophysical and clinical data, and optical density (OD) values from ELISA reader were entered directly into a computer using Microsoft Excel spreadsheet. Statistical tests for trend were performed using Cochrain-Armittage test (Armitage and Berry, 1994). The sensitivity and specificity were tested on positive and negative control samples, respectively. The influence of prevalence rate on positive and negative predictive values (PPV and NPV) were calculated using Bayesian analysis as described by Killeen (2007). The mean, variance S^2, standard deviation and t-test for groups and sub-groups were calculated using STAT-CALC (EPI-INFO 3.4.1 version, Atlanta GA, USA) computer software programme. Descriptive statistics and tabular data presentations were made.

3.18 Ethical Clearance

This study was backed by ethical and Institutional clearance from Kaduna State Ministry of Health and Nigerian Institute for Trypanosomiasis Research (NITR), respectively. The provisions of the International Ethical guidelines for epidemiological research involving humans prepared by the WMA (2008) Declaration of Helsinki and the Committee for International Organizations on Medical Surveillance (CIOMS, 2002) were followed.

CHAPTER FOUR

4.0 RESULTS

4.1 Study Population

The overall study populations of 900 and 1230 were based on figures provided by the community directed distributors (CDDs) in the sentinel villages. Baseline sample population, n = 532 (male: 297 and female: 235), and impact assessment sample population, n =593 (male: 208 and female: 385), volunteered to participate in the two studies. The mean ages of the sample populations were 42±19, with a range between 5 and 90 yrs old. The demographic composition of the study population by gender and age-class distribution are shown in Figure 4.1.

4.2 Geographic Information System and Entomology Data.

River Gurara has a network of tributaries and rivulets that extend to the study area. A total of 1222 black flies were caught with 1022 were at Gurara Village in Kachia Local Government Area of Kaduna State, Nigeria. Twenty two (22) flies were caught at Unguwar Shaho within the vicinity of the Primary School (9∘424'6.13N "and 7∘55'40.20"E) used as screening centre. The coordinates of the study villages and blackfly catching locations are shown in Table 4.1. None of the engorged flies was infected. All the 222 parous blackflies had grey wing turf.

4.3 Parasitology

At eighteen years post-treatment with ivermectin, the skin and blood microfilaria prevalence was 0% and the number of nodules had significantly decreased to 4 (0.7%). Two nodules each were seen in Ungwan Tama and Gidan Tama vilages.

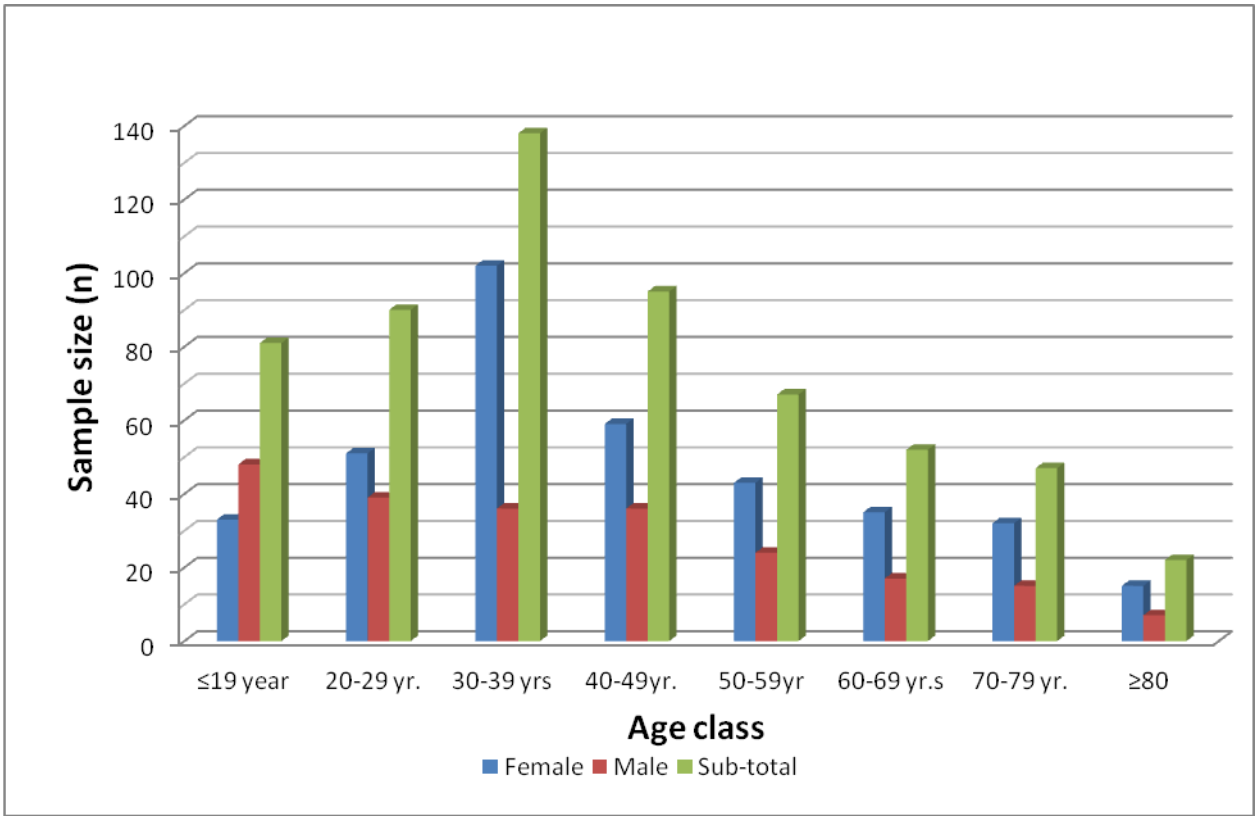


Figure 4.1: Gender and age-class distribution of sample population. The population is normally distributed.

Table 4.1: Coordinates of study locations.

S/No.	Location	Coordinates	
		Longitude	**Latitude**
1.	Kachia LGA Headquarters	9º 51.536"N	7º57.890"E
2.	Gantan	9º 38'37.03"N	7º57'10.58"E
3.	Sabon Gantan	9º 39'16.07"N	7º55'38.01"E
4.	Kurmin Gwaza	9º 40'37.51"N	7º56'57.41"E
5.	Bomjock	9º 40'56.09"N	7º54'59.63"E
6.	Gidan tama	9º 43' 1.84"N	7º55'47.86"E
7.	Unguwar Shaho	9º 42'46.13"N	7º55'40.20"E
8.	GuraraVillage	9º 39'57.02"N	7º39'57.02E
9.	Fly catching spot	9º 40'13.18"N	7º52'58.04"E
10.	Gurara River	9º 40'22.67"N	7º53'24.41"E

4.4 Onchocercal Skin Clinical Signs and Symptom

There was significant reduction (P <0.01) to 2 (0.4%) in skin clinical changes at post-treatment (Table 4.2). Other notable observations of public health importance were guinea worm infection and bilateral lymphoedema possibly by *Wuchereria bancrofti* in two males: 65 and 72-year-old at Sabon Gantan. There were 5 (0.84%) cases of corneal scars, allergic ophthalmitis (0.4%), and trachoma (n = 1; 0.2%). Midge infestation in 2 adult males (0.4%) was seen in Unguwar Shaho Village.

4.5 Annual Ivermectin Treatment Coverage and Compliance Rate

The study population was apparently stable as migration into the village within the period by residence of ≤10 years ranged between 5.3 and 14.1% as shown in Table 4.3. All the villages had 11(64.7%) annual treatment coverage (Table 4.4). The sample population treatment compliance rate varied from 85.6 to 100% with mean dose of 2.9±1.6 (with a range 2.1 ± 1.7–3.4 ±1.6). Those who received at least treatment once were 89.0%. Figure 4.2 shows the distribution of annual number of ivermectin treatment doses taken from inception comprising of those that did not receive treatment 65 (11.0%) and those who received 1 to ≥7 treatment doses. The participants who received 4 treatment doses were 180 (30.4%) the highest followed by those who received 3 treatments 140 (23.6%).

4.6. Analyses of Ocular Clinical Signs by Gender and Village

Among the female (n=373) and male (n=220) sub-groups the frequency of ocular clinical signs varied between female and the male subgroups as shown in Tab. 4.5. The female had significnantly higher (p<0.05) cases of cataract and pterygium than the male, while the male had significnantly higher cases of glaucoma. Only in S. Gantan the female had significantly higher (p<0.05) cases of cataract, pterygium and A. senilis than the male.

Table 4.2: Changes in skin microfilaria and clinical signs.

S/No.	Parameter	Baseline Data, Expected n=531 (%)	Post-control Data, Observed, n=592 (%)	Impact of Treatment, %	Statistics Test
1	Skin mf prevalence	144 (27.0)	0	100	T-test of unpaired data, P<0.01
2.	Nodule positive	77 (14.4)	4 (0.7)	95.1	P<0.05
3.	Scratch marks	24 (4.5)	22 (3.7)	17.8	P>0.05
4.	Skin clinical manifestations	51 (9.1)	2 (0.4)	96.2	P<0.05
6	Optic nerve disease	24 (4.5)	4 (0.7)	77.8	P<0.05
7.	*Inducible eye lesions	31 (5.5)	0	100	P<0.05
8.	Blindness (No perception of light)	6	6	0	Unchanged, no new case detected

* Inducible eye lesions include punctate keratitis, sclerosing keratis and cornea opacity.

Table 4.3: Participants years of residence at post-treatment.

S/No.	Village	≤10 Years n (%)	≥11 Years n (%)	Sample population n (%)
1.	Gantan	10 (10.9)	82 (89.1)	92 (15.5)
2.	Sabon Gantan	16 (11.4)	124 (88.6)	140 (23.6)
3.	Kurmin Gwaza	4 (5.3)	72 (94.7)	76 (12.8)
4.	Bomjock	10 (14.1)	61 (85.9)	71 (12.0)
5.	Gidan Tama	17 (13.5)	109 (85.5)	126 (21.3)
6.	Ungwar Shaho	10 (11.5)	77 (89.5)	87 (14.7)
Total		**67 (11.3)**	**525 (88.7)**	**592 (100)**

Table 4.4: Eighteen years of ivermectin post-treatment coverage of the study area from 1994-2011.

S/No.	Study area	Population treatment coverage (%)	Total village treatment doses	Mean treatment doses
1.	Gantan	91.3	11	3.2±2.8
2.	Sabon Gantan	100	11	3.3±0.6
3.	Kurmin Gwaza	85.6	11	2.1±1.7
4.	Bomjock	88.7	8	2.0±1.2
5.	Gidan Tama	96	11	3.1± 1.7
6.	Ungwar Shaho	94.2	11	3.4±1.6
Total		**92.6**	**11**	**2.9±1.6**

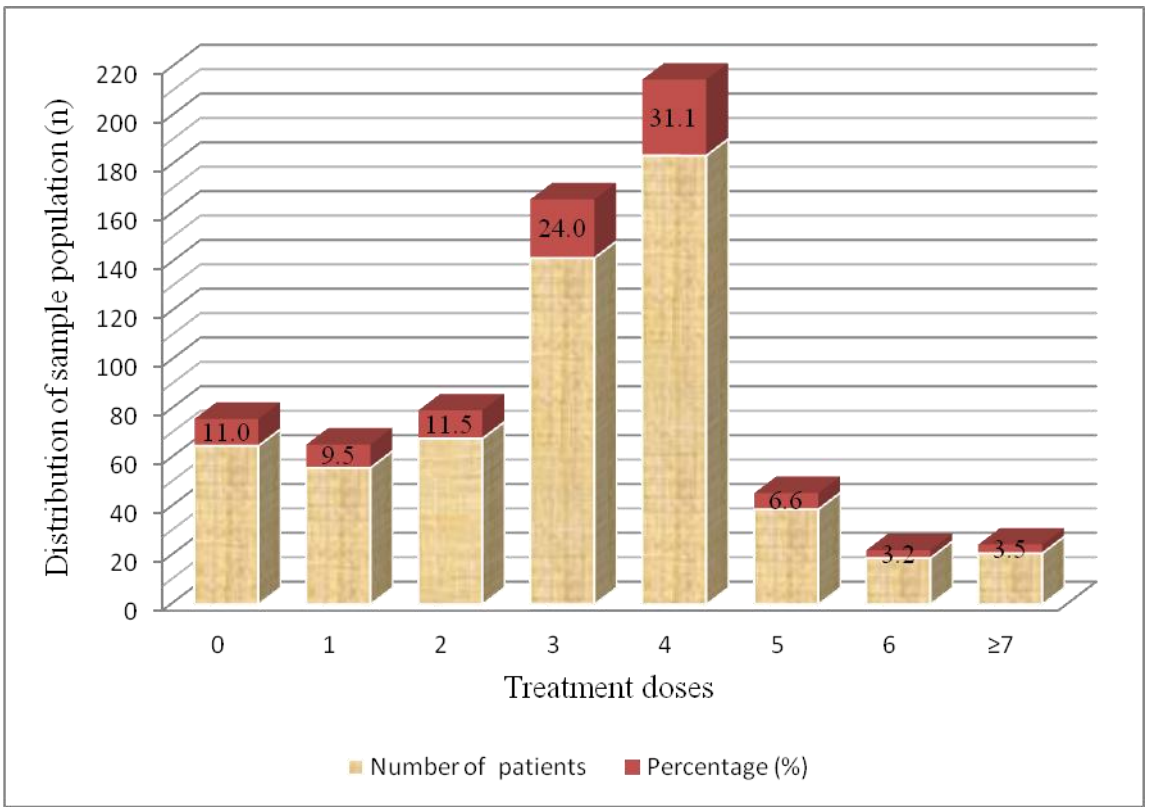


Fig. 4.2: Doses of ivermectin taken by participants from 1994-2011.

Table 4.5: Gender and village prevalence of ocular clinical manifestations.

S/No.	Study Area	Gender (n)	Glaucoma (%)	Cataract (%)	Pterygium (%)	Acute Senilis (%)
1.	Gantan	Female (63)	0	28 (44.4)*	27 (42.9)	17 (27.0)
		Male (29)	0	5 (17.2)*	11 (37.9)	9 (31.0)
2.	Sabon Gantan	Female (69)	0	21 (30.4)φ	20 (37.7)§	30 (43.5)α
		Male (61)	0	9 (14.8)φ	6 (9.8)§	12 (19.7)α
3.	Kurmin Gwaza	Female (59)	1 (1.7)	32 (54.2)	21 (35.6)	32 (54.2)
		Male (17)	2 (11.8)	9 (52.9)	6 (35.3)	10 (58.8)
4.	Bomjock	Female (47)	0	13 (27.7)ø	9 (19.1)	8 (17.0)
		Male (24)	4 (16.7)	4 (16.7) ø	3 (12.5)	3 (12.5)
5.	Ungwar Tama	Female (82)	0	15 (18.3)	16 (19.5)	12 (14.6)
		Male (44)	0	9 (20.5)	8 (18.2)	7 (15.9)
6.	Ungwa Shaho	Female (53)	0	12(22.6)æ	14 (26.4)~	16 (30.2)
		Male (33)	0	11 (33.3)æ	12 (36.4)~	10 (30.3)
	Total	Female (373)	1 (0.3)	121 (32.7)#	107 (34.7)¤	99 (26.5)
		Male (220)	6 (2.7)	47 (21.4)#	46 (20.9)¤	51 (23.2)

The *, φ, □ , ø, and # signs indicated that observed cases in the female subgroups were significant higher ($p<0.05$) than the respective male subgroups. The æ and ~ signs indicated the males had significantly higher cases of cataract at U. Shaho.

4.7 Ocular Onchocerciasis

Post-treatment impact study showed there was a significant reduction (*t*-test, $P < 0.01$) in prevalence of optic nerve disease to 0.7% (n=4) and skin clinical manifestations to 0.4% (n=2). There was no change in cases of glaucoma 1.4% (n=8) and blindness 1.0% (n=6), and no new blind case was recorded. Among the villages, the frequency of visual impairment were varied; poor visual acuity (Figure 4.3), cataract, and acute senilis were highest in Kurmin Gwaza, while in Gantan village the highest rate of pterygium was observed (Table 4.6). Those with two or three ocular manifestations in an individual were as follows: cataract and acute senilis (n=114; 32.0%), the highest combination followed by acute senilis and pterygium (n=89; 25.0%), cataract and pterygium (n=88; 24.0%), and those with three lesions (n=69; 19.0%) are shown in Fig. 4.4. Prevalence of visual impairment and the three other ocular clinical manifestations were strongly and positively associated with increase in age (Figure 4.5) with regression square (R^2) values ranging from 0.898–0.949 as shown in Appendix I. Generally, the number of visual impairment increased remarkably to 168 (32.3%). Figure 4.6 shows that those with visual acuity ≥ 6/24 (n=198; 33.45%) and the 6/60 (n=26; 4.9%) were severely visually impaired among the older age group.

4.8 Screening of Tissue Homogenates from Rat Organs

Tissue homogenate from liver was most reactive, followed by spleen, and then heart in slide flocculation test. Among the tissues (n=8) evaluated, muscle, lung and blank negative control (without tissue) homogenate did not show flocculation. The homogenate from testes was sticky and not suitable for use. Performances of different tissue homogenates from rat organs in Ov-SFT flocculation tests with positive control serum are shown in Table 4.7.

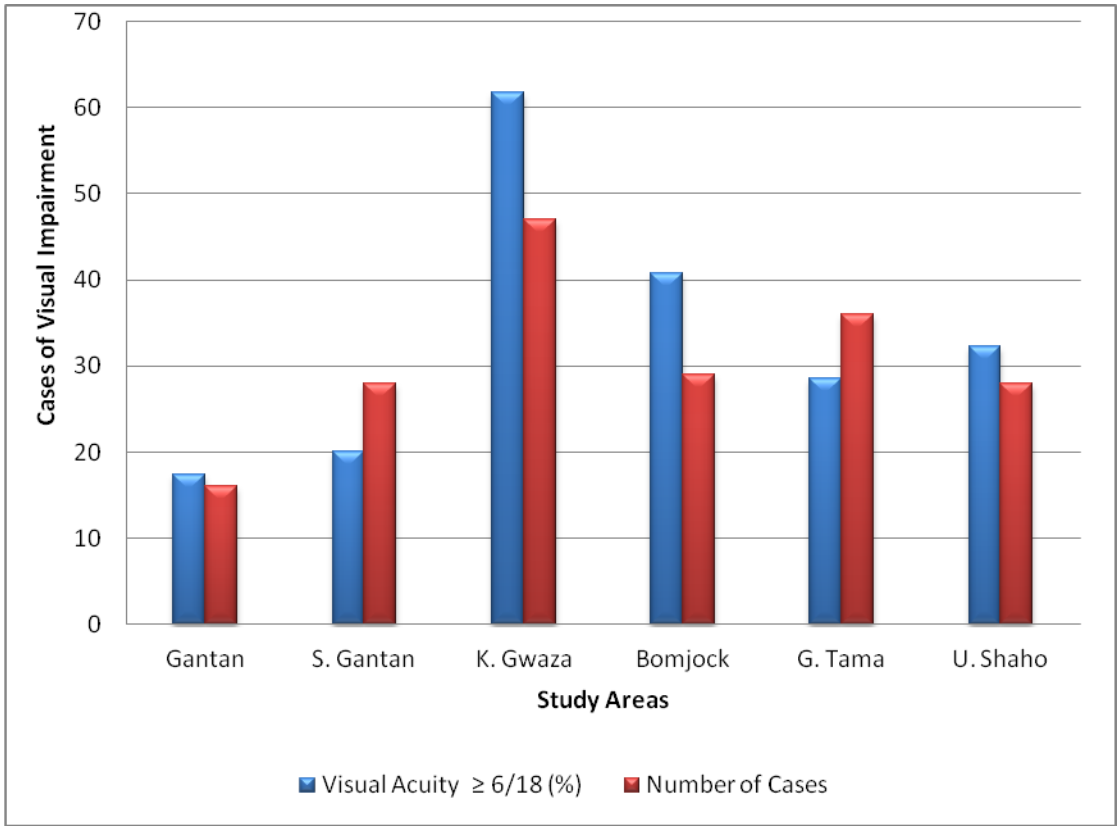


Fig. 4.3: Post-treatment village prevalence of visual impairment.

Table 4.6: Prevalence of onchocercal related ocular clinical manifestations in study population.

S/No.	Study Area	Sample Size (n)	Acute Senilis n (%)	Pterygium n (%)	Glaucoma n (%)	Cataract n (%)
1.	Gantan	92	26 (28.3)	38 (41.3)	0	32 (34.8)
2.	Sabon Gari Gantan	140	42 (30.0)	26 (18.6)	0	29 (20.7)
3.	Kurmin Gwaza	76	45(59.2)	27 (35.5)	3 (3.9)	41 (53.9)
4.	Bomjock	71	12 (16.9)	12 (16.9)	4(5.6)	17 (23.9)
5.	Ungwar Tama	126	20 (18.0)	26 (23.4)	0	25 (22.5)
6.	Unguwar Shaho	87	20 (30.3)	28 (42.4)	1 (1.5)	25 (37.9)
Total		**592**	**165 (27.9)**	**157 (26.5)**	**8 (1.4)**	**169 (28.5)**

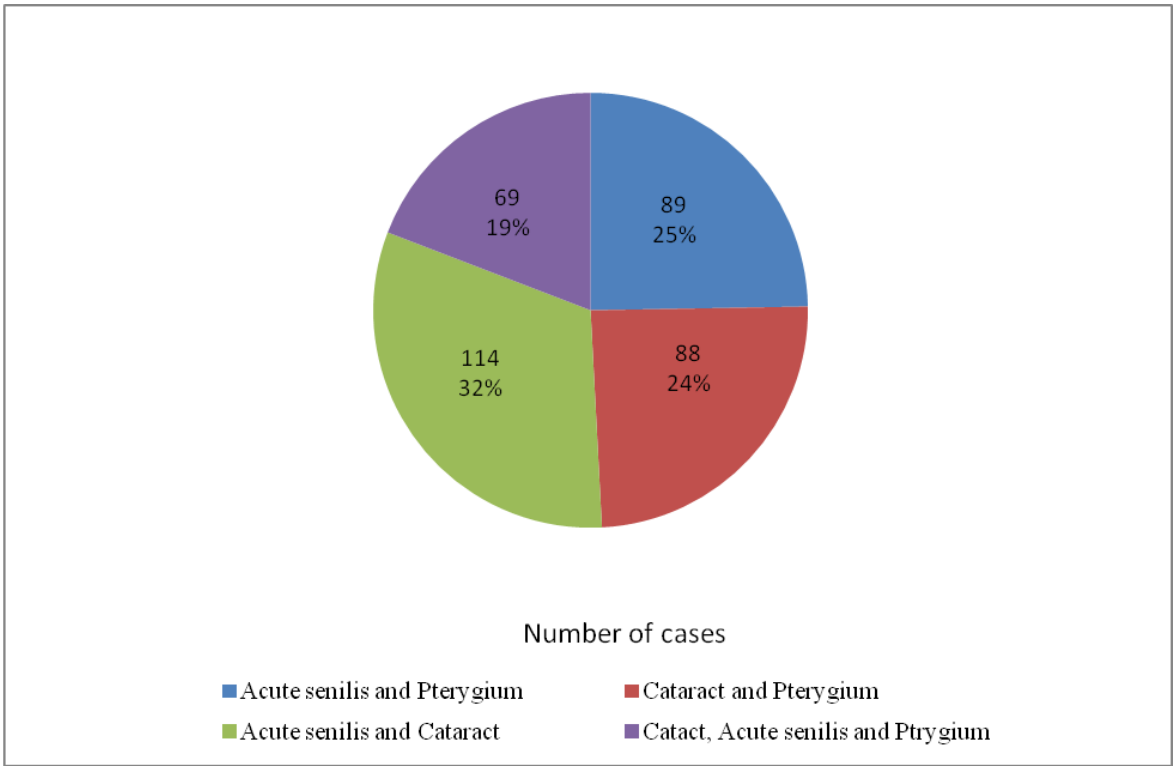


Figure 4.4: Proportions of individuals with multiple ocular clinical manifestations.

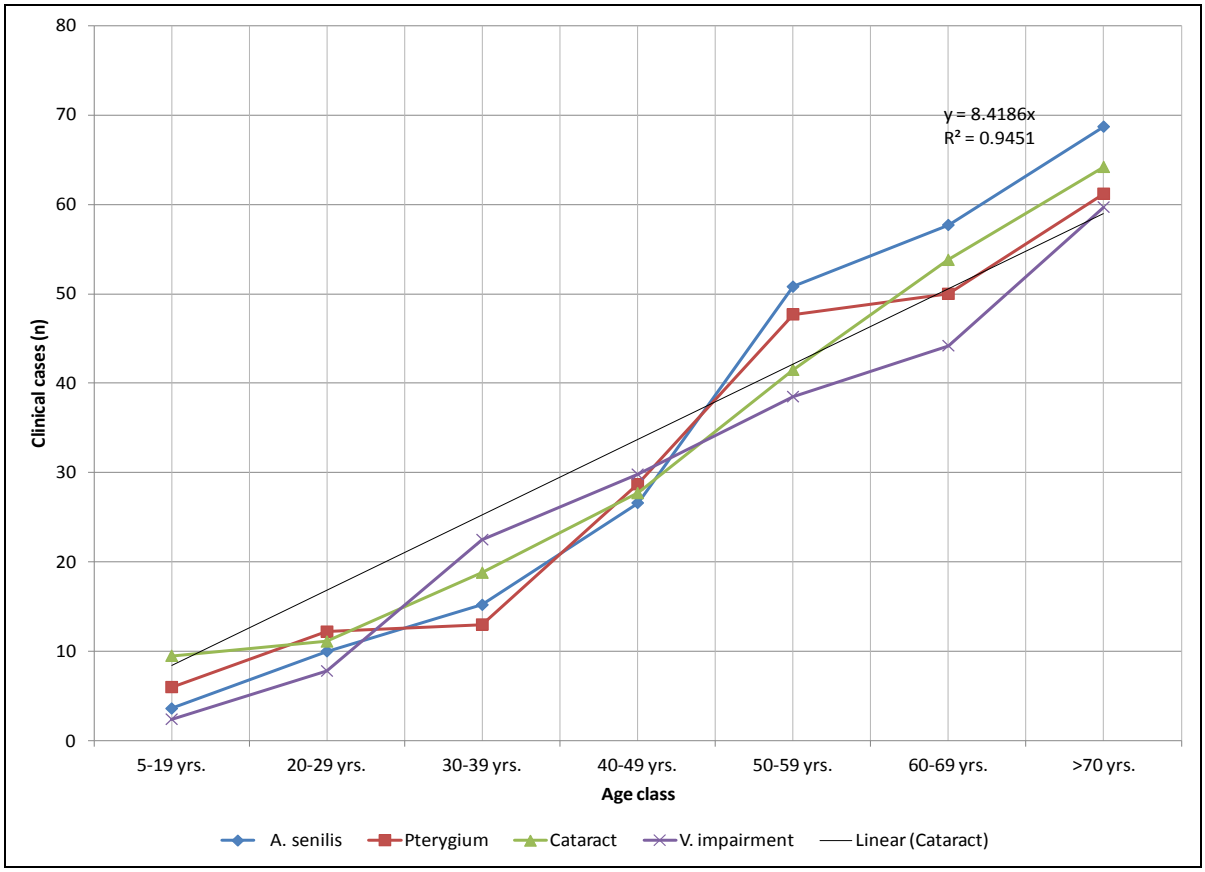


Fig. 4.5: Age-related prevalence of ocular onchocerciasis.

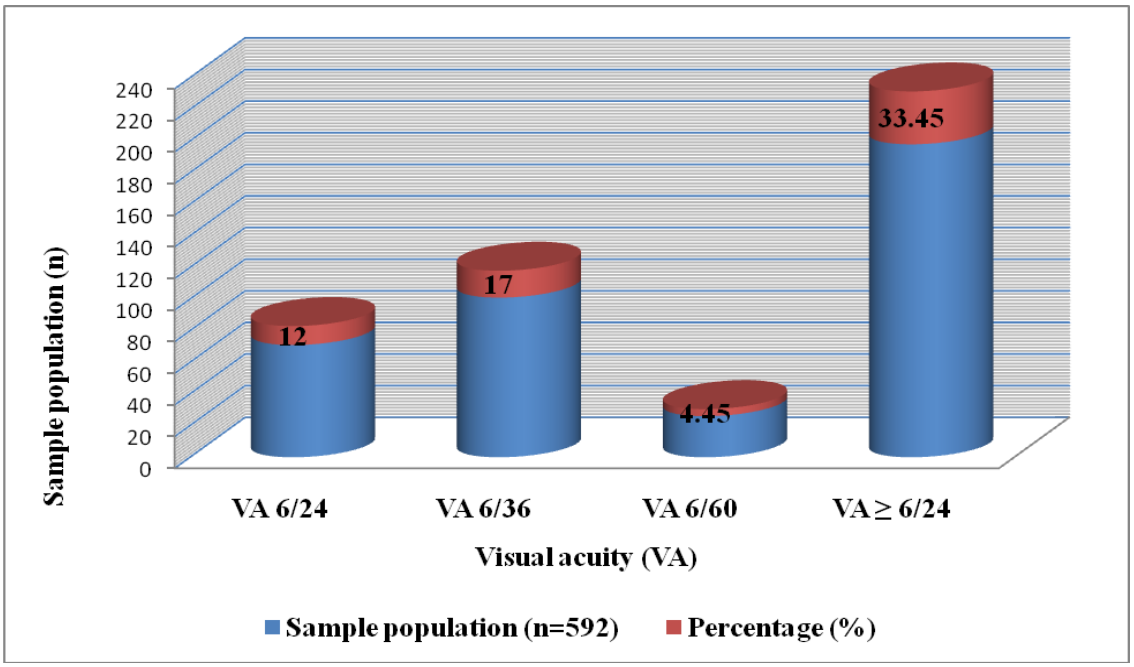


Fig. 4.6: Cases of visual acuity measured with Snelle's illitrate E-chart.

Table 4.7: Screening of tissue homogenates from rat organs

S/No.	Homogenate of HT+Ag (10 µl +10 µl)	1/2 (10µl) HT:Ag +10 µl Ab	1/4 (10µl) PBS	1/8 (10µl) PBS	1/16 (10µl) PBS	1/32 (10µl) PBS
1.	**Kidney**	++	+	-	-	-
2.	**Liver**	++++	+++	++	+	-
3.	**Heart**	+++	++	+	-	-
4.	**Spleen**	+++	++	++	+	-
5.	**Brain**	++	±	-	-	-
6.	**Muscle**	-	-	-	-	-
7.	**Testes**	ND	ND	ND	ND	ND
8.	**Lungs**	-	-	-	-	-
9.	**Blank**	-	-	-	-	-

Ab= antibody, Ag= antigen, HT=Heterologous tissue, ND= Not done and PBS= phosphate buffered saline.

4.9 *Onchocerca volvulus* Slide Agglutination Test

Ov-SFT repeatability tests with 10 replicates of positive and negative control sera were both 100% reactive and non-reactive, respectively (Table 4.8). Three out of five malaria positive cases compared to 9 (63.4%) of the 14 non-infected malaria were Ov-SFT sero-reactive. There was no difference in reactivitiy between the heat inactivated serum complement and the paired unheated serum samples. Overall, the number of sero-positive among sample population (n=65) using Ov-SFT was (n=33; 50.8%). There was significant difference ($p<0.05$) between male and female with antibody titre of ≥1:2 shown in Table 4.9. Profile of antibody titre ranging from 1:2, (n=1; 1.54%) to 1:32, (n=13; 20%) are shown in Figure 4.6. There was bimodal increase in Ov-SFT positive cases in 31-40 and 51-60 age groups (Table 4.10). Number of ivermectin treatment doses did not have significant effect on mean titre (Table 4.11.) Among the paired sera and skin biopsies (n=30) tested with Ov-SFT and PCR amplification gave 12 (40%) sero-reactivity and 0% positive, respectively.

4.9.1 Comparing the Baseline ELISA with Ov-SAT Data

Prevalence of antibody at baseline measured with ELISA compared to Ov-SAT showed significant decrease ($p<0.05$). There were no signifant differences between male and female at baseline and post-treatment. Both tests showed significant difference between those that were micrifilaria positive and negative at baseline as shown in Table 4.12.

Table 4.8: Repeatability of *Onchocerca volvulus* slide flocculation test.

S/No.	Sera	1/2 (%)	1/4 (%)	1/8 (%)	1/16 (%)	1/32 (%)
1.	**Positive control (n=10)**		10 (100)	10 (100)	10 (100)	0 (0)
2.	**Negative control (n=10)**		0	0	0	0
3.	**Malaria positive (n=5)**		3 (60)	3 (60)	1(20)	0
4.	**Malaria (n=14)**		9 (64.3)	9 (64.3)	5 (35.7)	4 (28.6)

Table 4.9: Slide flocculation test antibody analyses by gender and titre among the sample population

S/No.	Titre	Male, n=28 (%)	Female, n=37 (%)	Total, n=37 (%)
1.	Negative	12 (42.9)	20 (50.1)	32 (49.2)
2.	1:2	1 (3.6)	0	1 (1.5)
3.	1:4	3 (10.7)	1 (2.7)	3 (4.6)
4.	1:16	3 (10.7	3 (8.1)	6 (9.2)
5.	1:32	4 (14.3)	5 (13.5)	9 (13.8)
6.	1:64	5 (17.9)	8 (21.6)	13 (20.0)
Total	**Positive**	**16 (57.1)***	**17 (46.0)***	**33 (50.8)**

*There is overall significant difference ($P<0.05$) between male and female with t-test of unpaired data

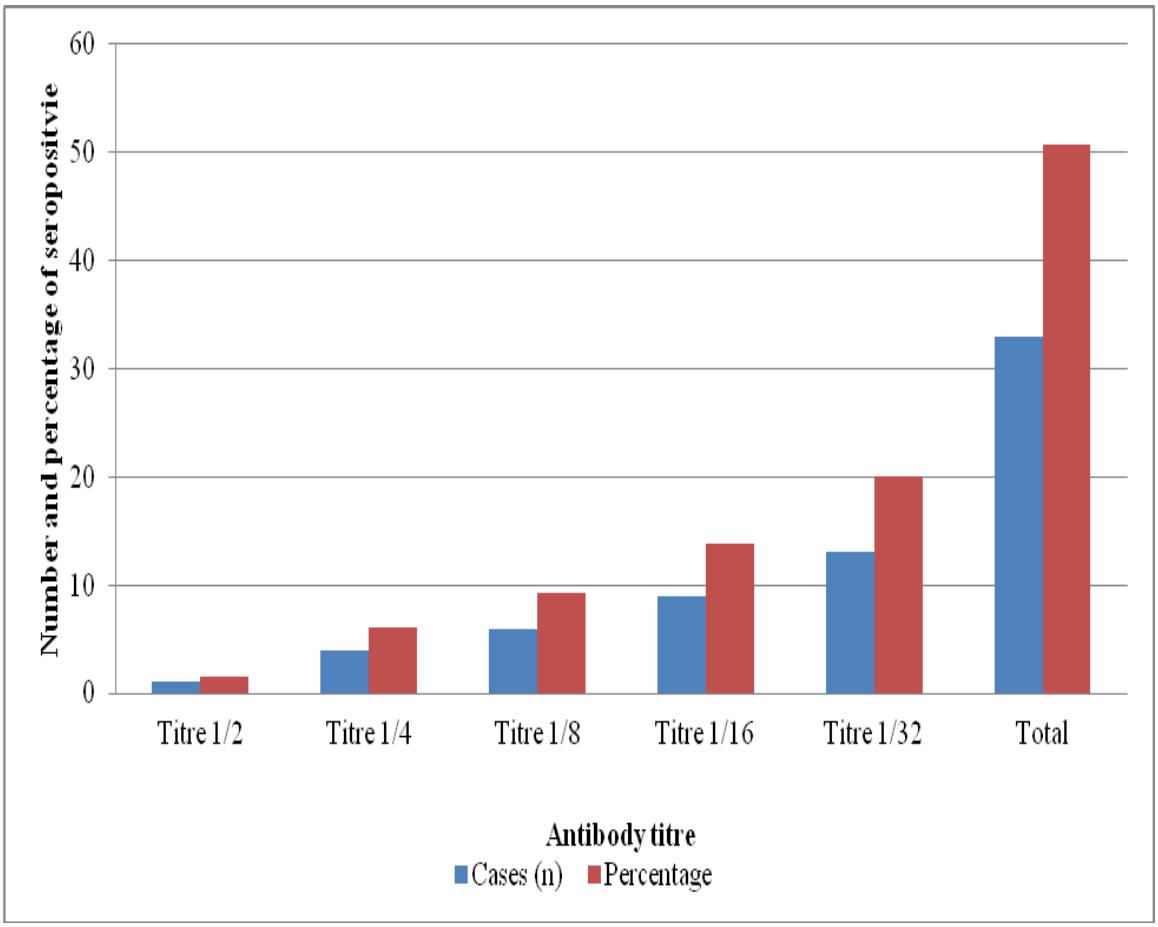


Fig. 4.7: Slide flocculation test analyses by antibody titre levels among the sample population.

Table 4.10: Analysis of slide flocculation test by age

S/No.	Age group (years)	Sample size (n)	Positive cases	(%)
1.	**20-30**	13	5	45.5
2.	**31-40**	12	8	75.0
3.	**41-50**	11	5	45.5
4.	**51-60**	8	7	85.7
5.	**61-70**	9	4	44.4
6.	**≥71**	12	3	25.0
Total		**65**	**33**	**50.8**

Table 4.11: Analysis of antibody titre in relation to ivermectin treatment doses

Number of doses	**N**	**Cases**	**%**	**Mean titre**	**Mean positive titre**
0	2	1	50	2.0±4	4
3	22	10	45.5	1.95±2.26	4.3±0.8*
4	41	22	53.7	1.98±2.08	3.7±1.3*
Total	**63**	**33**	**49.7**	**1.98±2.09**	**4.0±1.18**

* Difference not significant ($p>0.05$) by t-test

Table 4.12: Comparing baseline IgG ELISA with *Onchocerca volvulus* slide flocculation test data.

S/No.	Parameters	n	Post-treatment Ov-SAT	Baseline ELISA	n	Remarks
1.	Prevalence of IgG antibody	n=114	50.14%§	98.5%§	65	§($p<0.05$)
2.	Male (n=42)	n=42	2.04±0.82 Φ	0.58±0.16*	28	*($p>0.05$)
3.	Female (n=53)	n=53	1.92±0.76Φ	0.56±0.14*	37	Φ($p>0.05$)
4	Baseline mf positive	n=63	2.2±0.79□	0.61±0.14#	43	#($p<0.05$)
5.	Baseline mf negative	n=15	1.3±01.07□	0.46±0.14#	21	□ ($p<0.05$)

Enzyme linked immunosorbent assay (ELISA) and *Onchocerca volvulus* slide flocculation test (Ov-SFT). The § sign showed difference between baseline ELISA and post-treatment Ov-SFT data was statistically significant while * and Φ signs indicated no significant difference by t-test ($p>0.05$). The # and □ signs implied that differences between microfilaria (mf) positive and negative were statistically significant by t-test ($p<0.05$).

4.10 Data Mining for *O. volvulus* Primer Sequences

A total of 153 hits for *O. volvulus* primer sequences were obtained from National Centre for Biotechnology Information (NCBI), Atlanta, USA nucleotide (nt) database available in the World Wide Web (www). Of these, 118 primer sequences were identified with accession numbers AF281481-AF281598 belonging to the O-150 bp repetitive sequences (Meredith *et al*., 1989; Unasch *et al*., 1997). There were 22 primers from ITS region so far identified. There were 10 primer sequences derived from the mitochondria (mito) genes. The *O. volvulus Wolbachia* genome has 3 primers, which included cell wall (FtsZ), surface proteins and serine protein genes (Casiraghi *et al*., 2001). The frequency of nucleotide length of the O-150 DNA primer sequences from 18/18 to 23/23 base pairs (n=100) are shown in Fig. 4.7.

4.11 Molecular weight of Extracts

Gel electrophoresis analysis of the SDS extracted antigens revealed that the component were mainly of low molecular weight (15 to 350 ng). NanoDrop test of the protein content gave a reading at 280nm wavelength showing they were mostly proteins. The mean concentration of DNA was 7.43±13.69ng/µl with a range from 0.3 to 25.7ng/µl for samples. The two positive controls were 11.5 and 106.5 ng/µl not included in the scatter diagram (Figure 4.8). The R^2 value (0.003) indicated the concentrations were evenly distributed.

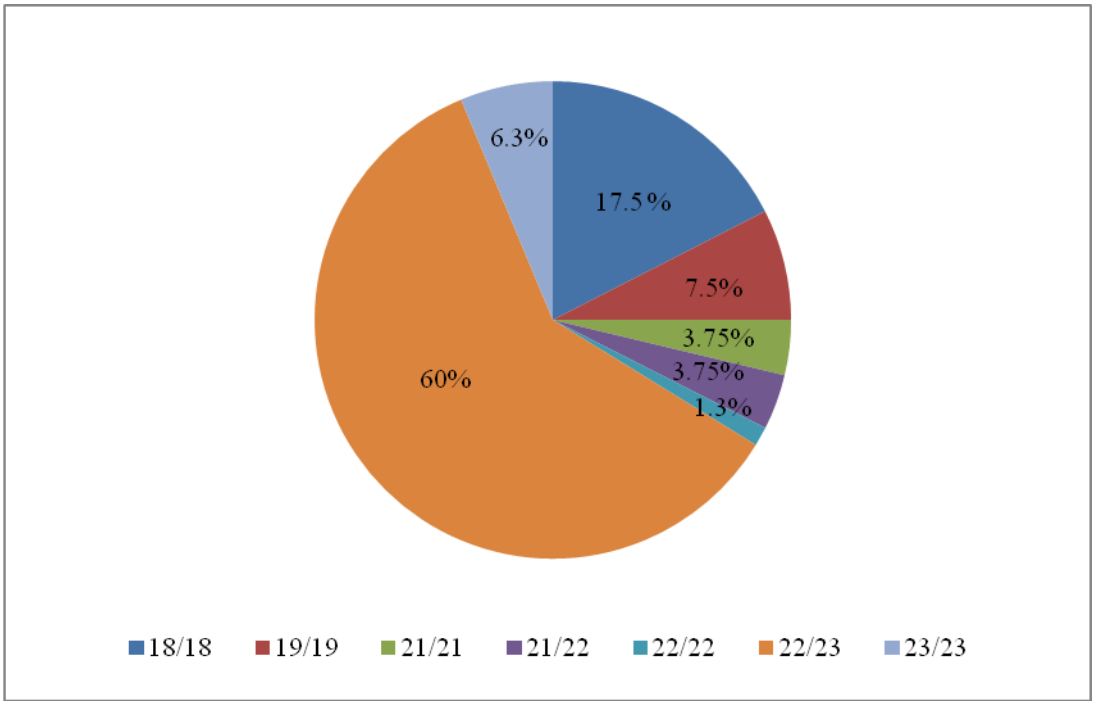


Fig. 4.8: Frequency of *Onchocerca volvulus* specific primer sequences with 18-23 base pairs.

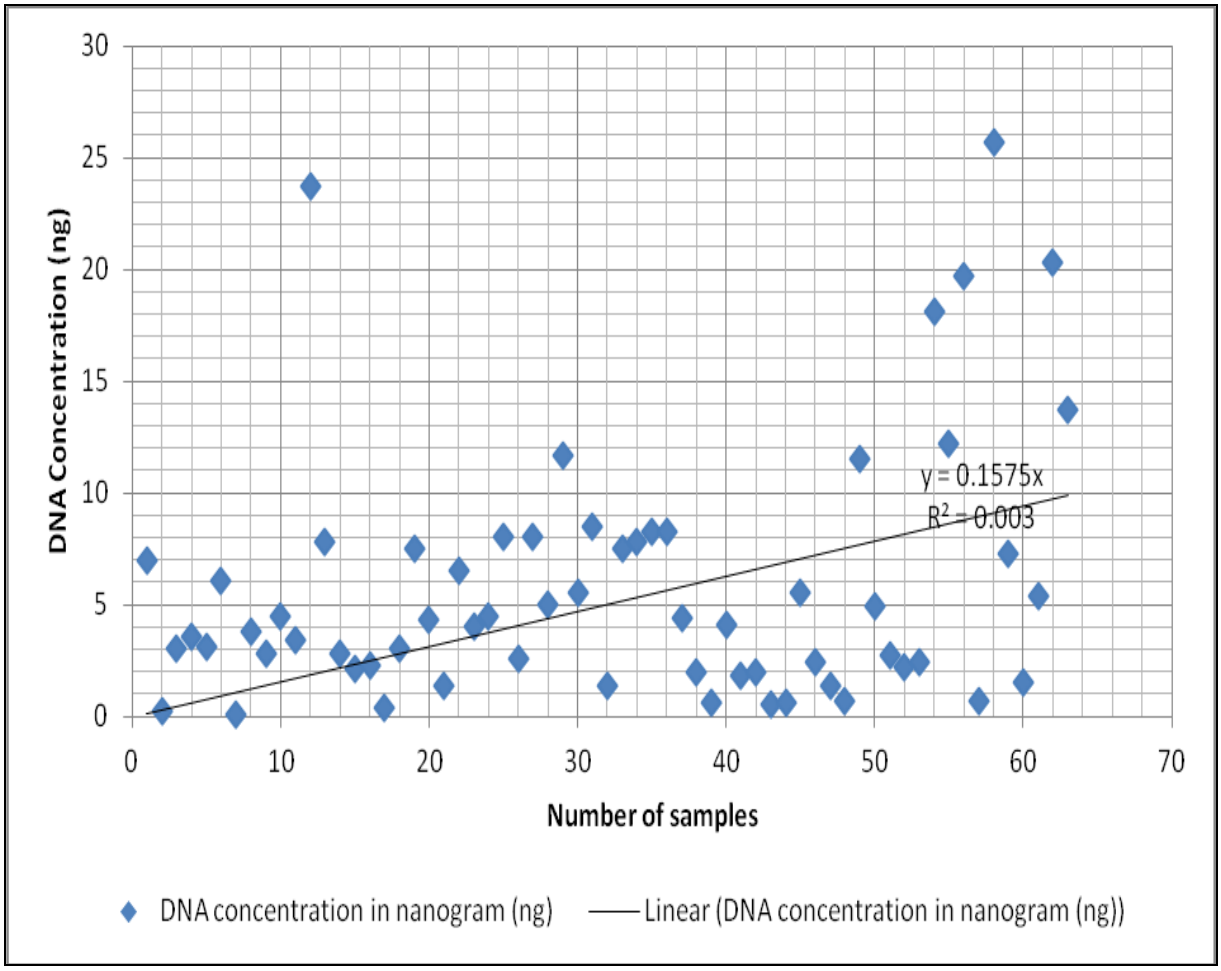


Figure 4.9: Estimated DNA concentration of samples in nanogram (ng) usingNanoDrop at 260 nanometre (nm) wave length.

4.12 PCR Amplification of DNA

Of the 66 samples amplified, there were double bands of amplicons observed in two out of the four PCR amplified templates and in one of the two positive controls DNA from adult worm's fragments during optimization. The negative and internal controls did not show any amplification. Amplification of the sample templates using ITS 1 primer sequences were carried out thrice and sequencing of amplification products were performed twice. Two templates with double band had molecular weight of about 500 and 700 base pairs, and the other two templates had single bands of ~350bp. Only sample 15 of the 30 templates had 350bp amplicon (Plate I) and no amplicon out of the 28 samples (Plates II) that were analysed. Three of the 8 templates and the positive control had PCR amplicons. The three negative controls and blank (master mix without template) had no amplicon (Plate III). The standard molecular weight ladder (M) is 1 kilobase (Kb) or Kb plus in multiple of 100 base pair.

4.12.1 Repeated DNA Amplification

A repeat of the PCR assay for 3 samples excluding No. 15 gave similar pattern of double and single bands of 500bp and 700 bp (Plate IV). Pre-sequencing repeat of the PCR analysis produced double and single bands of 350 and 500bp. The replicates of positive controls 1 and 2 gave 3 single bands of ~350 and 1 band of ~310bp, respectively (Plate V). DNA extraction from gel bands for three sample templates and the positive control (PC2) were within 350 and 500bp molecular weight. The PC2 had double amplicons of very sharp and intense 500bp trailed by a 350bp band (Plate VI). Actin-2B gene of *O. volvulus* specific primer sequences of a subset of samples (n=15) inclding the 4 ITS1 PCR positive, two positive and three negative controls had a 200 bp amplicons (Plate VII).

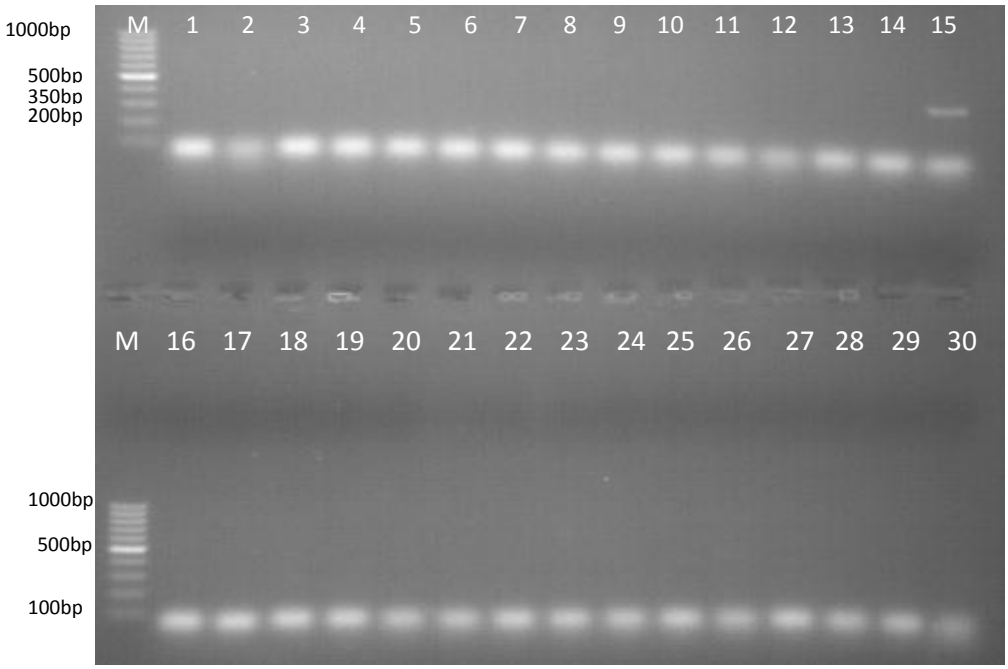


Plate I: Gel electrophoregram of DNA amplification of samples 1-30. Molecular weight marker (M) of 1 kilobase in multiple of 100 base pairs (bp) and a positive well, sample 15 of 344 bp.

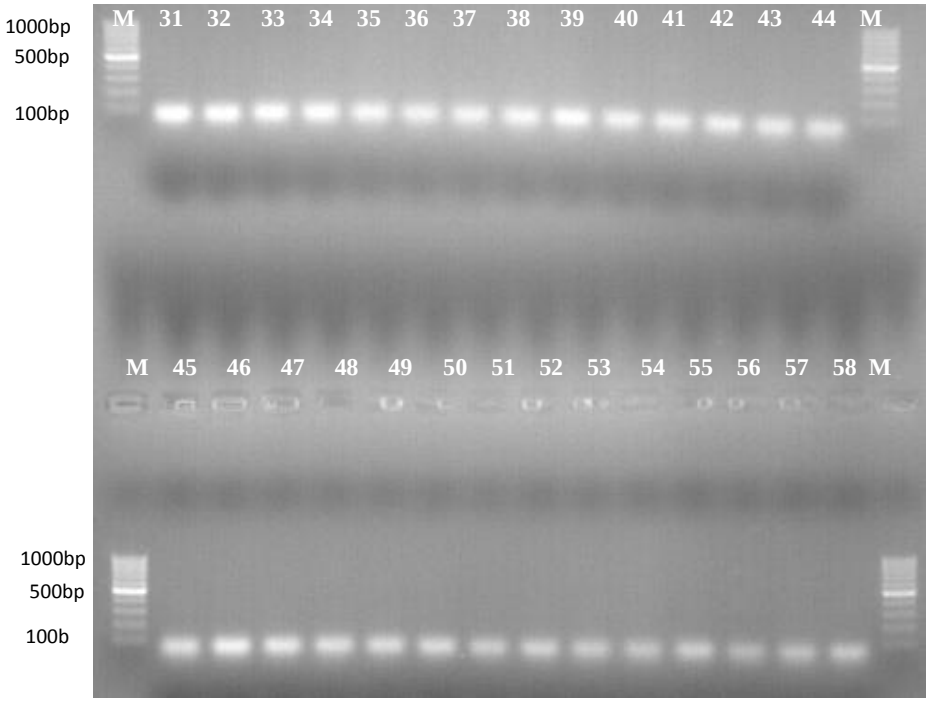


Plate II: Gel electrophoregram of PCR amplification of samples, 31-58. All samples were negative. M is molecular weight ladder of 100 bp (1kilobase).

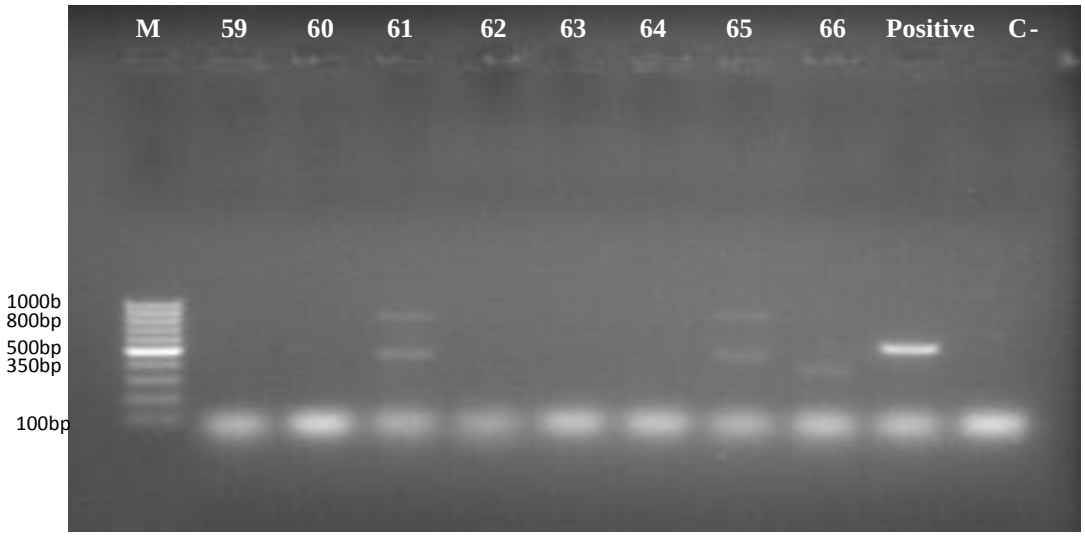


Plate III: Gel electrophoregram of PCR amplified samples (59-66). Samples 61 and 65 had double bands of 500 and 800 bp. Sample 66 had single faint band of about 350 bp. The positive (C+) control had a sharp band of 500 bp and the negative (C-) control showed no amplification. M represent 1 kilobase molecular weight marker of 1 kilobase in multiples of 100 bp.

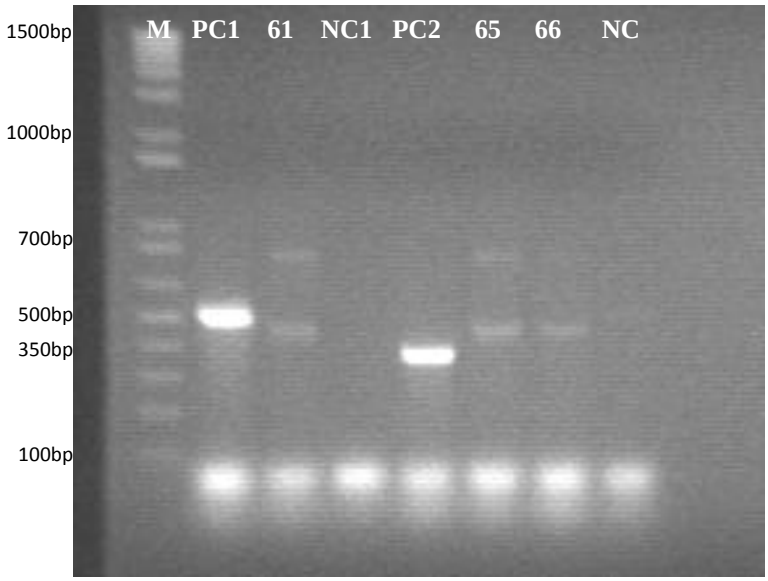


Fig. IV`: Gel electrophoregram of PCR re-amplification of samples and controls: samples (61, 65 and 66), positive controls (PC1 and 2) and negative control (NC). Samples (61 and 65) had double bands of 450 and 700 bp, 66 had single band of 450 bp. The positive controls (PC1 and 2) gave single bands of 500 and 350 bp, respectively. M represents 1Kilobase plus molecular weight ladder (in multiples of 100bp).

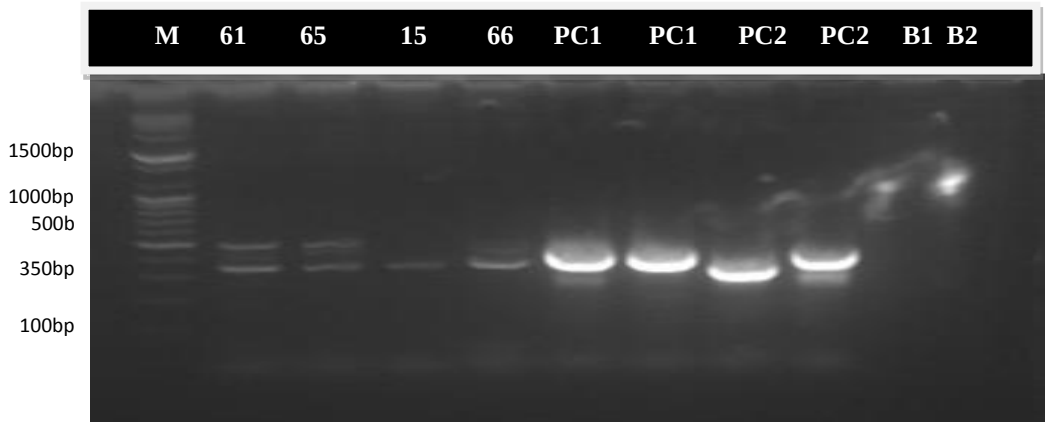


Plate V: Gel electrophoregram of PCR re-amplified positive templates: two samples (Nos. 61 and 65) had double amplicons, two samples (Nos. 15 and 66) and replicate positive controls (PC1 and 2) had single amplicons with molecular weight of 500 and 350bp. One of the PC2 had ~300bp. The two blank wells (B1 and 2) had no amplicon. Molecular weight marker (M) is 1Kilobase plus in multiples of 100bp.

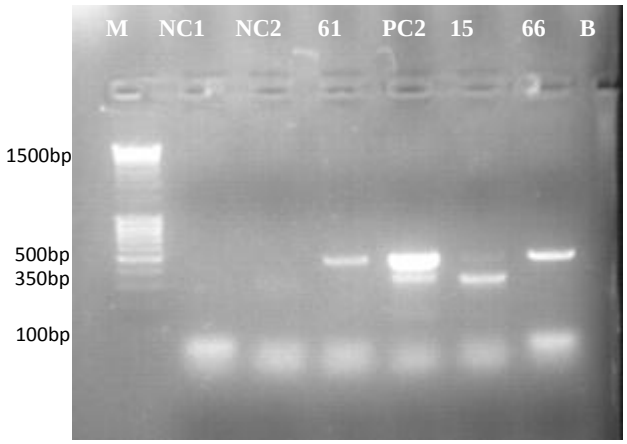


Plate VI: Gel electrophoregram of PCR re-run of DNA extracted from bands. A: samples (61 and 66) were 500bp and 15 was 350 bp. The positive control (P2) had double band of a large and very sharp band (500bp), and a trailing band of ~350bp, respectively. The two negative controls (NC1 and 2) and blank well (B) did not show any amplicon. The molecular (M) weight ladder is 1kilobase plus in multiples of 100bp.

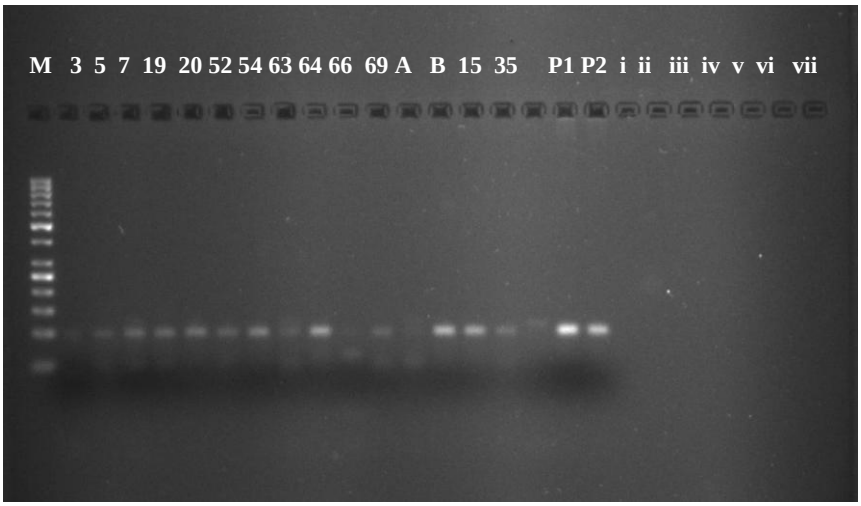


Plate VII: Gel electrophoregram of beta actin gene primers PCR amplicons. The DNA templates (n=18) including 4 of the ITS1 PCR positive samples, 3 positive and 3 negative controls. The blank wells contain master mix without template. The molecular (M) weight ladder is 1kilobase in multiples of 100bp. Out of the 11 bands, there are 5 sharp and 7 faint bands. The sharp bands included the 2 positive controls (P1 and P2) and the 3 ITS1(15, 35 positive samples.

4.12.2 Gender, Age and Village Analysis of PCR Results

Our findings showed that two males and two females were positive by PCR with no significant difference between them. The gender and age distributions of PCR positive cases are shown in Table 4.13. Two of the five study villages accounted for the few cases recorded in this study. The two villages are within proximity of 500 meters apart. The DNA concentrations of 8, 18.1 and 25.7ng for PCR positive samples 33, 34 and 45 and those of the positive controls (n= 2) were 106.5 and 11.5 ng, respectively. One out of the four PCR positive cases had not taken ivermectin, while two had taken 4 and one 5 doses. Extraction of DNA in 100µl (n=31) and 200µl (n=37) volumes of elution buffer had 6.45% and 5.4%. All the paired skin snips from a subset of sera (n=30) were PCR negative compared to 12 (40.0%) that were Ov-SFT serological test positive.

4.13 DNA sequencing

The first DNA sequencing of PCR amplification products with ITS1 primers gave 187, 629, 639 and 1155 nucleotide sequence lengths for the 350, 500, 500 and 700 bps. The second sequencing gave 440, 613, 613 and 673 bases for the 350 and 450, 450 and 500 bp, respectively.

4.13.1 DNA sequence alignment

The sequenced PCR amplification products of 350 single bands had 440 bases and the two bands of 500 bp gave nucleotide sequence length of 629 and 639. The 700 bp band gave 1155 bases did not show significant alignment with any sequence in the GenBank Databases.

Table 4.13: Analysis of PCR amplification of samples by gender and age

S/No.	Age (n)	Gender ITS-1 Positive cases		Total
		Male n=26 (%)	Female n=42 (%)	n=66 (%)
1.	**<30 yr. (n=15)**	1	0	1 (6.7)
2.	**31-40 yr. (n=14)**	1	1	2 (14.28)
3.	**41-50yr. (n=11)**	0	1	1 (9.1)
4.	**51-60yr. (n=7)**	0	0	0
5.	**>61 (n=16)**	0	0	0
Total		**2 (7.69)**	**2 (4.76)**	**4 (5.88)**

When subjected to the GenBank Database of the NCBI BLAST analysis (Altschul *et al*., 1997), the 629 sequences aligned with the *O. volvulus* AF 228574 and AF228575 in a discontinuous similarity alignment with corresponding number of nucleotides homology of 94% identities. The 673 bp aligned with 1.9 E-value and 100% identity to HG738137.1 *O. volvulus* Cameroon_v3 genomic scaffold, OVOC_OM1b, whole genome shotgun sequence. The print out of the BLASTN analysis is shown in Fig. 4.9. The alignment of the nucleotide sequences of three samples and positive control with ITS 1 gene, which served as reference sequence are shown in Fig. 4.10.

4.14 Determination of Likelihood Ratios of PCR Tests

The result of Ov-SAT showed 50.8% positive cases, which indicated possible exposure to infection. These groups may be regarded as false positive (FP) since there was no skin microfilaria positive and the 4 (6.1%) PCR positive are true positive (TP). The overall true negative (TN) for Ov-SAT is 49.2%. There was no PCR positive among the paired sera and skin snips (n=30) tested and the Ov-SAT positive (n=12) cases were taken to be false positive (FP). The criterion standard test and the operating characteristics of Ov-SFT, skin snip microscopy detection of microfilaria, PCR amplification and result of DNA sequence analysis are shown in Tab. 4.14. Only the 350bp PCR positive templates of ITS 1 and Actin-2B showed significant sequence alignment with GenBank Database. The computed positive and negative predictive values for Ov-SFT are 0.96 and 0.06 and for ITS 1 are 0.55 and 0.45, respectively. Both ratios are strongly indicative of more likely the patient(s) are at lower risk of the disease.

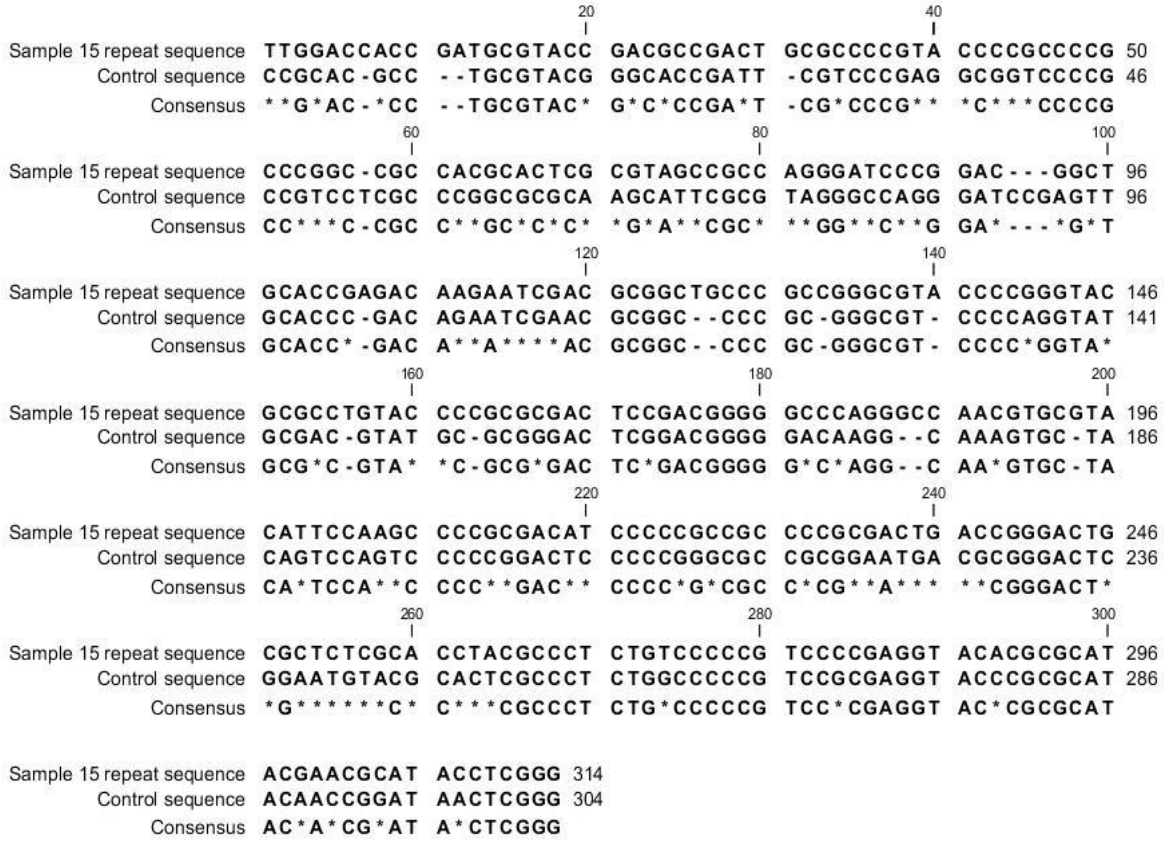


Fig. 4.10: DNA sequence alignment of sample 15 and positive control using ClustalW (Thompson *et al*. 1994). The consensus sequence derived shows (-) and (*) signs representing gaps and points of conflict, respectively. There was strong homology (69.1%) between the two sequences.

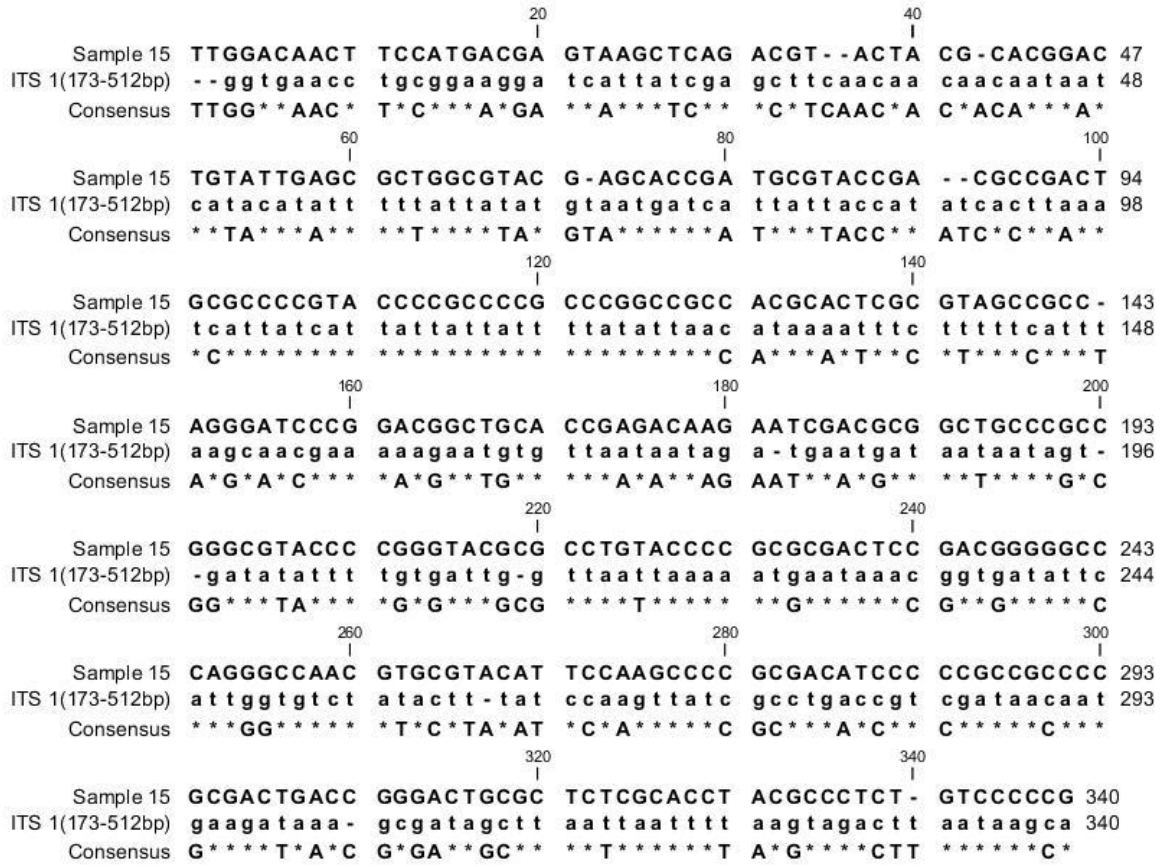


Fig. 4.11: DNA sequence alignment of sample 15 and AF228575 ITS 1 gene sequence using ClustalW. Consensus show - and * representing gaps and points of conflict. There was no strong alignment with ITS 1 gene region sequences of GenBank Accession Numbers g |AF228566-AF228576 located at 173-192 and 512-493 (340bp)

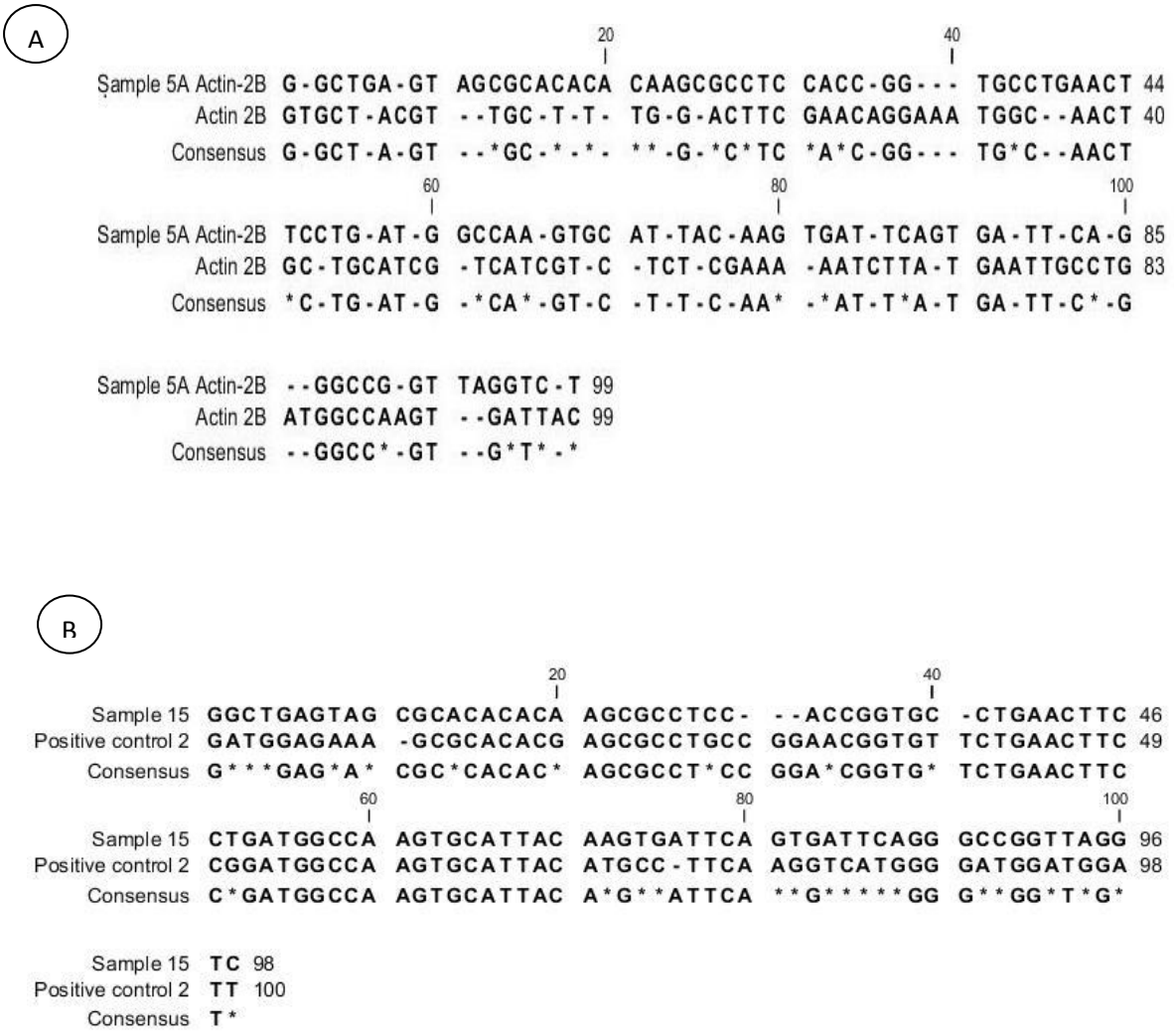


Fig. 4.12: DNA sequence alignment of sample 15 and *O. volvulus* Beta Actin (Actin-2B) gene (A) and postive control 2 (B) using ClustalW. A: Consensus sequence with (-) and (*) signs representing gaps and points of conflict, respectively. There were about 60% alignments with GenBank Accession Number ID: gb|M84916 located at 1033-1052 and 1131-1120 (99bp) or 1675-1694 and 1773-1754; ID: gb|M84916.1|ONGACT1A. B: Consensus sequence showed 68% homology between sample 15 and postive control 2.

Table 4.14: Disease prevalence and predictive values of the three tests

S/No.	Test	Test Positive (%)	Test Negative (%)
1.	Skin Mf detection (n=592)	0 (0)/TP	592 (100)/TN
2.	Sera and snips, Ov-SFT (n=65)	33 (50.8)/FP	42 (49.2)/TN
3.	Skin snips, PCR ITS-1 (n=66)	4 (6.1)/TP	62 (93.9)/TN
4.	Paired sera and snips, Ov-SFT (n=30)	12 (40)/FP	18 (60)/TN
5.	Paired sera and snips, PCR ITS-1 (n=30)	0 (0)/TP	30 (100)/TN
6	Sub-set of sera, PCR Actin- 2B (n=15)	10 (66.6)	5 (33.33)
7.	Ov-SFT and PCR (n=108)	37 (34.3)/FP	72 (65.7)/TN
8.	Pre-treatment mf positive Ov-SFT	30 (75)/FP	10 (25)/TN
9.	Pre-treatment mf negative, Ov-SFT	3 (13.04)/FP	20 (86.94)/TN
10.	HRP-2 positive Ov-SFT (n=5)	3 (60.0)*	2 (40.0)*
11.	HRP-2 negative Ov-SFT (n=14)	9 (64.3)*	(35.7)*

FN= false negative, FP= false positive, HRP-2= Histidine rich protein-2, ITS1= first internal transcribed spacer, Ov-SFT= Onchocerca volvulus slide flocculation test, PCR= polymerase chain reaction, TN= true negative, TP= true positive and * represent absence of cross-reactivity as difference between HRP-2 negative and positive for Ov-SFT was not statistically significant ($p>0.05$).

CHAPTER FIVE

5.0 DISCUSSION

Impact of 18 years of IVM treatment of onchocerciasis was assessed in six sentinel villages. Pre-treatment data and post-treatment parasitological and clinical data were compared. The study villages were geo-referenced with spatial and temporal (attribute) data captured; there was no baseline entomological study. The fact that no breeding site was located during impact assessment undertaken at the peak of raining season may be due to over flooding and the breeding sites may have been submerged was similar to observation by Adeleke *et al*. (2011) of seasonal fluctuations of *Simulium damnosum* complex. The study area is no doubt within previously active onchocerciasis endemic savannah foci of Nigeria (Osue, 1996). The blackflies caught in three days showed that high fly population density will be a sustained source of ensuring onchocerciasis transmission and its attendant severity of biting nuisance (Crosskey, 1990). With availability of environmental friendly blackfly control using a Esperanza trap designs trapping techniques (Rodriguez-Perez *et al*., 2013) could allow enlisting of endemic communities' participation and possibly ownership. According to Rodriguez-Perez *et al*. (2014) the traps represent a viable alternative to using humans as hosts for the collection of vector flies as part of the verification of onchocerciasis elimination.

Absence of no infection in flies has been acclaimed as alternative means of establishing a break in disease transmission. Molecular diagnostic method applied for pool-screening described by Oskam *et al.* (1996); Guevara *et al.* (2003) and Marchon-Silva *et al*. (2007) may have given better result than the parasitological diagnosis. This study has provided the need to intercept man-simuliid vector contact at the study area as suggested by Adeleke *et*

al. (2010). The persistence presence of huge fly population is inimical to full utilization of the highly needed arable fertile riverine alluvial land for agricultural crop farming. Extensive vector studies to determine if blackflies have the capacity to continue transmission of *O. volvulus*, which was observed to be higher in dry season than the rainy season by Adewale *et al.* (2008) cannot be overemphasized. More so, only few parous flies (n= 220) were caught and dissected in this study. There was a strong possibility of peridomestic transmission among the ginger farmers in Bomjock and Sabo Gantan, both situated at the upper course of the river. Vector host contact was strongly suggested by catching of blackflies at the vicinity of the primary school used as screening centre at Unguwar Shaho. River Gurara and its tributaries and rivulets are undoubtedly the major and minor breeding sites for the black flies. Hence, agricultural productive capacity of these farmers may be adversely affected as noted by Ufamadu *et al.* (1994) among rice farming communities elsewhere. It was the growing need for more fertile farmlands that made inhabitants of Sabo Gantan to resettle in once abandoned location and Bomjok settled in virgin lands (Osue, 1996). Lamberton *et al*. (2015) has however, highlighted vector species influences on transmission through biting and parous rates including vector competence, and suggested it should be included in transmission models.

Optic nerve disease was ranked highest in frequency (4.5%) among the ocular lesions detected in sampled population. In view of the above, the enlistment of the communities in the nationwide mass treatment coverage in 1994 was timely. It was envisaged that the huge reservoir of parasites will be cleared and many of those with 10 or more mf per skin snip at pre-ivermectin treatment that were at risk of developing optic nerve disease as suggested by Abiose *et al.* (1993) have been prevented. Again, treatment would have mitigated

reported impaired anti-tetanus IgG response in those with heavy infections compared to those with light infections or no infection (Cooper *et al*., 1999a). Unexpectedly, high prevalence rates of cataract, pterygium, and acute senilis were observed after long-term post-treatment. Cataract is associated with visual impairment as reported by Nmorsi *et al.* (2002) despite ivermectin treatment. This was corroborated by data from this study with overall prevalence of cataract 169 (28.5%), acute senilis 167 (27.9%), and visual impairment 157 (26.5%). The absence of punctate keratitis (PK) and microfilariae in anterior chamber (MFAC) recommended by WHO (2000) as criteria for assessing ivermectin treatment success, have been challenged by Winthrop *et al.* (2006) not to be good indicators. Combined mass treatment with ivermectin and Albendazole started in 2005 based on key informants' deposition.

Co-endemicity of onchocerciasis, lymphatic filariasis, malaria and guinea worm had coexisted in the study area (Osue, 1996). The effect of combining the two drugs and IVM alone for treatment annually or biannually potentiates clearing of microfilaria and the reduction in their release by female adult worms of *O. volvulus* (Awadzi *et al*., 1994). How this may have played a role in the outcome of this study cannot be fully deduced. An isolated case of guinea worm disease (GWD) observed in this study calls for caution in declaring eradication of the disease. The study area was a co-terminal falariasis and guinea worm focus (Osue *et al*., 2013). Richard *et al*. (2005) reported ivermectin annual therapy for onchocerciasis has not interrupted transmission of *Wuchereria bancrofti* in Nigeria.

This study has shown that the disease dynamics in the sentinel villages have changed with a possible break in active transmission and halted the development of new lesions (Table 4.1). This is in agreement with similar findings in two different foci within the same state

by Tekle *et al.* (2012) with median prevalence pre-treatment infection levels of 52 per cent was reduced to 0 per cent after 20 years post-treatment. These outcomes were achieved despite the varied rate of annual community coverage and individual treatment compliance rates. Finding from this study conformed to that reported by Emukah *et al.* (2004) that differences in community coverage did not appear to influence the benefit from treatment of individual residents. It is important to note that one of the study communities, Bomjock in Kachia LGA of Kaduna State, Nigeria despite being hyperendemic with the least number of doses as at 2003 follow up study, overall microfliarial load had reduced to 1.7mf per skin snip with 4.6% skin mf prevalence, compared to baseline data of 17.7mf and 70% respectively. Prevalence of palpable nodules was drastically reduced from 14.5% to 5.4% (Osue *et. al*., 2005) after a decade of ivermectin MDA. It was obvious that low treatment compliance may not have seriously jeopardized the control strategy. It appears that the geographic and population coverage are more critical, as observed in Bomjock village. There are instances where the standard therapeutic dose of 150 mg/kg of bodyweight culminated in achieving microfilaricidal effect of IVM leading to 98% clearance of the skin mf within 2–3 weeks (Basa´n˜ez *et al*., 2008). Vieira *et al*. (2007) reported major impact of IVM treatment on the prevalence and transmission of infection, and elimination of ocular morbidity. Except the reports of Katabarwa *et al*. (2011) and Katabarwa *et al*. (2013) where 15 and 17 years of treatment have not succeeded in halting diease transmission in parts of Cameroon. This might be attributed to levels of IVM treatment coverage of the areas.

This long-term longitudinal studies have provided practical evidence for empirical benefits of annual IVM treatment in breaking disease transmission dynamics and prevented development of new onchocerciasis induced skin and eye clinical lesions (Ozoh *et al*.,

2011). Where control has been achieved as in OCP operational areas, the need for continued surveillance cannot be underscored (Tekle *et al*., 2012; WHO, 2015). The low rate of clinical cases may be in part due to the fact that some of the early patients who had died or were not present for re-evaluation had no significant influence on the outcome of this study. Noteworthy, the zero prevalence has proved that there were no new onchocerciasis infection and clinical manifestations of the disease within the study areas.

The ten-year post-treatment assessment of the study area confirmed reduction in these parameters (Osue *et al*., 2005). It is now understood that mass drug administration using annual IVM treatment in West Africa is showing promise of eliminating the disease (WHO, 2001; WHO, 2005; Diawara *et al.*, 2009) therefore the need to consider using targeted drug distribution proposed by Poolman and Galvani (2006) or biannual CDTI or CDTM (Turner *et al*., 2010; Katabarwa *et al*., 2014) is virtually unattractive. Moreover, the time period to achieve elimination in an area is said to vary depending on the level of endemicity. Communities with low level may take 10 years and those with high level could take 15-25 years (APOC, 2011b). It is obvious that we have to contend with the problem associated with logistic distribution and apathy on the part of the people to the control strategy of MDA. The logistic and cost limitation of adopting bi-annual treatment based on its proven impact in Latin American countries in achieving a break in onchocerciasis transmission within a short term (Rodriguez-Perez *et al*., 2008a; Rodriguez-Perez *et al*., 2008b) may not be feasible in Africa, including Nigeria. Elimination and eradication scenarios would require a shorter treatment phase and a smaller amount of ivermectin than the control scenario, mainly because community-directed treatment with ivermectin could be ended earlier thanks to regular active surveillance (Kim et al., 2015). When alternative drug is available it will require the revaluation of on-going annual CDTI as annual moxidectin and biannual ivermectin treatment would achieve similar reductions in

programme duration relative to annual ivermectin treatment (Turner *et al*., 2015). Annual moxidectin treatment would not incur a considerable increase in programmatic costs and, therefore, would generate sizeable in-country cost savings, assuming the drug is donated. Similar to target for annual CDTI, treatment coverage of 65% will be adequate to achieve a break in transmission of the disease using annual CDTM (Turner *et al*., 2014b). This will require strong political and administrative support and commitment at all tiers of government. According to Abanobi (2010) and Binnawi (2014), continued community awareness creation, active participation and ownership remain as a panacea to ensure attainment of the WHO (2001a) recommended 100% geographic coverage and 85% treatment compliance.

This study had shown that mass treatment of onchocerciasis patients had succeeded in reducing prevalence of parasite-specific IgG antibodies is in agreement with reports of reduction in antibody levels following IVM treatment (Rodriguez-Perez *et al*., 1999; Gonzalez *et al*., 2009; Gomez-Priego 2005). When compared with the baseline antibody levels measured with IgG ELISA antibody (Osue *et al.*, 2008) clearly indicated the high levels of those exposed to infection in the study population (n=114) was 98.5% as against the 50.8% recorded in this study using Ov-SFT. Although the comparative analyses of the sensitivity of the two tests have not been made, the ability to demonstrate presence of specific antibodies is not in doubt. More importantly, this study showed that apparently no difference between the intensity of antibodies of males (n=28) and female (n=37). The observed differences in antibody reaction in male (57.1%) and female (46.0%) was statistically significant ($P>0.05$) by t-test of unpaired data is similar to trend of baseline ELISA data. Intensity of antibody among the males and female groups did not show any significant difference ($p<0.05$). According to Lovato *et al*. (2014) both reduction of

infection rate to zero and absence of anti-Ov-16 antibodies in children and adolescents showed that transmission had been interrupted. These were the basis of cessation of mass drug treatment with ivermectin in the endemic foci of Ecuador. The need to compare Ov-SAT with existing tools like the Rapid-Format Antibody Card Test (Weil *et al*., 2000), the four-Ov-antigen standard or 15-minute format quick luciferase immunoprecipitation system (QLIPS) that has been found to show sensitivity and specificity of 100% (Burbelo *et al.,* 2005; Burbelo *et al.,* 2008; Ramanathan *et al*., 2008; Burbelo *et al*., 2009) cannot be overemphasized. Similarly, to compare it with the recent commercially available lateral flow test (Golden *et al*., 2013) a novel, inexpensive, and simple approach to actuating the detachment of the blood separation membrane from the nitrocellulose test with no impact on the performance characteristics of the test.

The Ov-SFT is simple, sensitive and fast and required no specialized skill. Flocculation test has long been recognized for screening of parasitic diseases like schistosomiasis, filariasis, amoebiasis and others as reported by Allen *et al*. (1972). It has been used for syphilis serologic screening with a non-treponemal test, such as the rapid plasma reagin (RPR) to identify persons with possible untreated infection (Radolf *et al*., 2011). The test is cheap for population screening before diagnosis and to detect exposure to infection. Further research and development on the Ov-SFT general application will require the much needed evaluation in different clinical disease entities. A unique feature of Ov-SFT that stand it out from others is the possible mechanism of binding could be by crystallisable fragment receptor (FcR). Immune complexes (ICx), rat and human immunoglobulin aggregates bound via an FcR demonstrated on spleen, macrophages and on kidney interstitial cells (van der Laan-Klamer *et al*., 1987). This area need further research to improve on the Ov-SFT. One major problem encountered with Ov-SFT is that minimal

reactive and slight roughness is often misinterpreted as positive or negative because of the subjectivity of visually interpreting the results (Castro *et al*., 2012). This may lead to variation among observers and laboratories performing the test. Observed levels of antibodies in this study measured with Ov-SFT is in agreement with Mai *et al.* (2007) who reported the persistence of *O. volvulus*-specific IgG1 and IgG4 subclass reactivity at higher levels in onchocerciasis patients than in *O. volvulus* exposed but microfilariae-free endemic controls. Comparing results of Ov-SFT paired heat treated and untreated sera strongly indicate the reaction is independent of complement factor. This study showed that it is the antibody Fc and not that of complement factor that bind to the FcR on tissue homogenate. Therefore, to perform the Ov-SFT do not require heat pre-treatment of sera.

The least DNA concentration of our samples (0.4ng) was above minimal detectable level as PCR can amplify down to 0.003 and 0.005 ng/µl, which corresponds to less than a microfilaria worm (Leroy *et al*., 2003). Secondly, the reliability of PCR to detect only few cases of infections (n=4) after long-term treatment intervention is in agreement with similar finding reported by Fink *et al* (2011); Gopal *et al*. (2011) and Higazi *et al*. (2011). The sensitivity of PCR to detect the presence of microfilaria in skin snip sample has been found to be very high (Toe´ *et al.*, 1998). The findings from this study has confirmed the probable presence of microfilaria in the sample population (n=66) drawn from a lager sample population (n=592) reported to be skin snip microfilaria negative. It is therefore expedient to caution sole dependence on parasitological test to declare sample population free of microfilaria as observed by Tekle *et al* (2012); Osue *et al* (2013). Where there is low infection during control and pre-infection period, PCR appeared to be more sensitive than reliance on presence of mf in skin snip, which are very scanty in the skin (Zimmerman *et al*., 1994; Lindblade *et al*., 2007; Cruz-Ortis *et al*., 2012). Similarly,

Convit *et al.* (2013) used PCR amplification and antibody reaction with *O. volvulus* antigens to support assessment of onchocerciasis control in female insect head and school children in endemic areas of Northern Venezuela.

The primer sequences used in this study, 18S ITS-1 gene gave both single and double band in DNA templates of two positive samples. The controvertible outcome of ITS1-PCR double amplicons is subject to further research. Implication of the forward primer also found to be located in a fungi family *Cortinariaceae* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (Liimatainen *et al*., 2014) remained unanswered. The fungus has been reported to belong to Agaricales, GenBank accession No. gbKF732628 which is regarded as the largest family of fungi.

The rRNA ITS1 gene region analysis provides a multi-species-specific diagnosis by a single PCR (Salim *et al*., 2011). Furthermore, Njirun *et al.* (2005) and Cox *et al.* (2005) documented specific PCR product length corresponding to each *Trypanosoma species*, which was the basis for differentiation. In an experiment to detect coagulase (coa) gene, the PCR products showed double bands, three bands and single PCR band (Tiwari *et al*., 2008). Secondly, available evidence from the GenBank database, the ITS1 genes are in two or more loci. Therefore, it is very likely that some DNA amplifications could give rise to double band amplicons either due to presence of different loci. This observation of double band in gel electrophoresis, which re-occurred in a repeated PCR appeared to be a unique characteristic that have never been reported for *O. volvulus* or other filarial species. In addition, nonspecific amplification may occur at other sites with similar sequences (Vierstracte, 1999) or reduced amplification may occur at primer-template mismatched sites. The ITS1 forward and reverse primer set showed specificity as no amplicon was

produced in 3 control reactions that contained human DNA and blank without any DNA. A similar observation was reported by Nuchprayoon *et al.* (2005) who had used the same ITS1-F and a different reverse primer was used in this study. Another study by Higazi *et al*., (2001) in Sudan revealed that *O. volvulus* endemic to the northern focus at Abu Hamed were significantly different from all other *O. volvulus* populations examined to date.

There is documented evidence for ITS1 PCR amplification, which resulted in a double-band pattern in all *Leishmania tropica* cases, while a sharp single band was observed for *L. infantum* and *L. major* isolates (Schonian *et al*., 2001, Ghatee *et al*., 2013). The double-band pattern was attributed to a 100 bp fragment difference within ITS-rDNA alleles. Thirdly, the BLASTN analysis from the sequences derived from both the forward and reverse sequences generated did not show any significant alignment with mega blast for *O. volvulus* nucleotides in the GenBank database. But there were somewhat similar alignment with *O. volvulus* genomic DNA from Cameroon isolates. More importantly, not much reports on primers designed available are from Nigeria parasites. Therefore, whether this is due to new sequences that have not been reported is subject to further investigation.

The outcome of both PCR and sequencing that confirmed the amplicons were those of *O. volvulus* proved that ITS1 primers used have greater positive predictive value than skin snip microscopy. A similar finding has been reported by Fink *et al.* (2011) using real time PCR with 2 out of 218 samples positive by microscopy and PCR and 12 samples were positive by PCR only. PCR based assays targeting specific genomic sequences are theoretically 100% specific. In practical terms, PCR assays are subject to false positive results that are caused by contamination with amplicons. This obstacle can be overcome by employing a confirmatory assay that targets an independent portion of the target

organism's genome. However, in individuals with low mf densities, whose skin samples may be devoid of whole or partial mf, the PCR will be negative because free parasite DNA is not stable in the tissue (Fischer *et al*., 1996). Therefore, the PCR on tissue samples is a sensitive, direct method to prove the presence of intact, disintegrating mf or remnants of them. This brings us to the question whether a gene copied during PCR was the right size? According to Vierstracte (1999) some problems that make every PCR not to be successful are probably due to the poor quality of the DNA, that one of the primers doesn't fit, or that there is too much starting template. Second, is the product the right size as the band of 500 base pair, and the expected gene for *O. volvulus* should be 344 bp molecular weight (Morales-Hojas *et al*, 2010). It could be possible one of the primers probably fits on a part of the gene is either closer or farther to the other primer. It is also possible that both primers fit on a totally different gene. Third, where more than one band is formed, it is possible that the primers fit on the desired locations, and also on other locations. In that case, you can have different bands in one lane on a gel. Moreover, the *Taq* polymerase used has no proofreading function (3'-5' exonuclease activity), therefore is prone to generate errors during DNA synthesis (Suchman, 2013). Other thermo stable archeal DNA polymerases such as *Pfu* which has 3'-5' proofreading function can be used to overcome such problem.

A combination of screening tool, standard skin snip microscopy of emerged microfilaria and application of LAMP assay for the amplification of specific target DNA detectable using turbidity or by a hydroxyl naphthol blue colour change described by Goto *et al.* (2009) and Alhassan *et al.* (2014) will provide a better epidemiological situational assessment for the determination of control and certification of eradication status of

ivermectin treated communities. The results indicated that the assay is sensitive and rapid, capable of detecting DNA equivalent to less than one microfilaria within 60 minutes.

Observed greater positive predictive values of the screening test over the two diagnostic tests were attributed to false positive rate. Implication of cross-reactivity of Ov-SFT particularly with other co-endemic nematode parasitoses and malaria may interplay in the outcome of serological test. Fairlie-Clarke *et al.* (2010) reported cross-reactive antibody responses in two murine models of malaria-helminth analysed in singly- or co-infected with *Plasmodium chabaudi chabaudi* and *Nippostrongylus brasiliensis* or *Litomosoides sigmodontis*. Ov-SFT detected patients exposed to infection that have no detectable skin microfilaria at post-treatment. Predictive values are not stable characteristics of a diagnostic test (Elavunkal and Sinert, 2007) and are dependent on disease prevalence. In this study, the zero prevalence was recorded based on the gold standard. The same diagnostic test will have varying predictive values in different populations. Both conventional and real-time PCR-based assays are significantly more sensitive than current methods for diagnosing *Onchocerca volvulus* infection, and it overcomes many difficulties in identifying active onchocerciasis (Nutman *et al*., 1996). Fink *et al.* (2011) averred that since chemotherapy is widely used to treat onchocerciasis, PCR is important for assessing responses to treatment, predicting recrudescence and detecting filarial infections in mobile populations. Cases of positive reaction among the infected and non-infected malaria control sera from participants are not indicative of cross reaction.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

This study has shown the remission of some onchocerciasis inducible ocular (punctate and sclerosing keratitis) and skin (onchocercomata or nodules and leopard skin) lesions. Despite the observed varied compliance to ivermectin treatment, the study area can be certified to have reduced *O. volvulus* infection to threshold level needed to institute the second phase of disease elimination drive. Absence of any incidence of infection and eye or skin clinical manifestation clearly demonstrate the break in disease transmission. An *Onchocerca volvulus* slide flocculation test (Ov-SFT) developed and evaluated in this study is a novel adaptation of classical method using heterologous tissue in place using bentonite, latex or cholestrol. The test is qualitative and semi-quatitative, it will be very cheap to produce, easily deploy for field application and suitable for point of care use. Superior sensitivity of molecular diagnosis using primers of conserved ITS1 18S ribosomal DNA and actin-2B genes for PCR amplification of template invalidated the 0 prevalence of skin microfilaria observed in this study. It appeared that the Actin-2B primers may be more sensitive than the ITS1or could this be due cross-rection with host actin-2B which share similar homology. This study has provided unequivocal evidence of probable sympatric co-infection or a case of heterogenous strain or occurrence of different primer recognition sites. There is no doubt that the non-amplification of the negative and blank well has excluded the possibility of contamination, as it is very unlikely in this circumstance. Specificity of the ITS1 primers was confirmed with the non-amplification of negative control and the amplification of all the replicate positive controls. Outcome of this study has reinforced the need for the establishment of an algorithm for surveillance and monitoring onchocerciasis and sympatric filarial infection. A combination of antibody

detection, single PCR analysis and the gold standard for confirmation deserved further research and development. The different likelihood of Ov-SFT, ITS1 primers PCR analysis and microfilaria detection will provide better epidemiological information showing those exposed and active infections.

6.2. Conclusions

1. An in-depth study of the existing blackfly population infection rate using molecular based diagnostic methods will be needed. A comprehensive and elaborate investigation will be required to obtain large number of parous flies becomes imperative to confirm the result from the pilot entomological study that was carried out.
2. The observed skin microfilarial prevalence of zero has practically demonstrated the effectiveness of the annual CDTI in this area despite varied level of individual compliance to IVM treatment.
3. Similarly, antibody assay with the novel Ov-SFT was able to demonstrate continued presence of antibodies among the participants. The Ov-SFT is amenable for use not only for onchocerciasis; it could be adapted in the screening of other parasitic and infectious diseases.
4. One major constraint is sourcing for *O. volvulus* female adult worm antigens, which may be overcome as there are many recombinant antigens with proven sensitivity and specificity that could be adopted for developing Ov-SFT as a screening tool.
5. A possible valid explanation for the double band and different 350 or 500bp amplicons may be attributed to variable location of primer site. The four PCR amplified samples and the positive controls have the expected 344bp band weight that corresponds to *O. volvulus*.

6.3 Recommendations

1. Sustained routine active surveillance and monitoring of treatment coverage and compliance rates will rely on the use of screening, diagnosis and confirmation tests.
2. Further work to develop the Ov-SFT as a screening tool will be need determination of the specificity and absence of cross-reaction filarial and nematode infections.
3. Research work to determine the mechanism of antibody binding is solely FcR dependent and the most suitable between heterologous tissue and lympoid cells (macrophages, monocytes, PMN's and some lymphocytes) as binding support.
4. Further research to verify that the double band PCR amplicons with ITS1 gene was not a chance occurrence, since it occured in two samples and a positive control.
5. As antibody test measures exposure to infection, it can only serve for screening purpose. Sensitivity and specificity of Ov-SFT could further be enhanced with well defined low molecular weight antigens for onchocerciasis.

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APPENDICES

Appendix I: Regression square values for age class of ocular manifestations

S/No.	Clinical Parameters	Equation on chart	Regression square values	Remarks
1.	Village mean age	y=9.517x	-1.40	Negative association
2.	Visual impairment	y= 7.716x	0.949	Positive association
3.	Acute senilis	y=8.976x	0.898	Positive association
4.	Cataract or lens opacity	y=8.418x	0.945	Positive association
5.	Pterygium	y=8.222x	0.918	Positive association

Appendix II: Illustration of *Onchocerca volvulus* slide flocculation test.

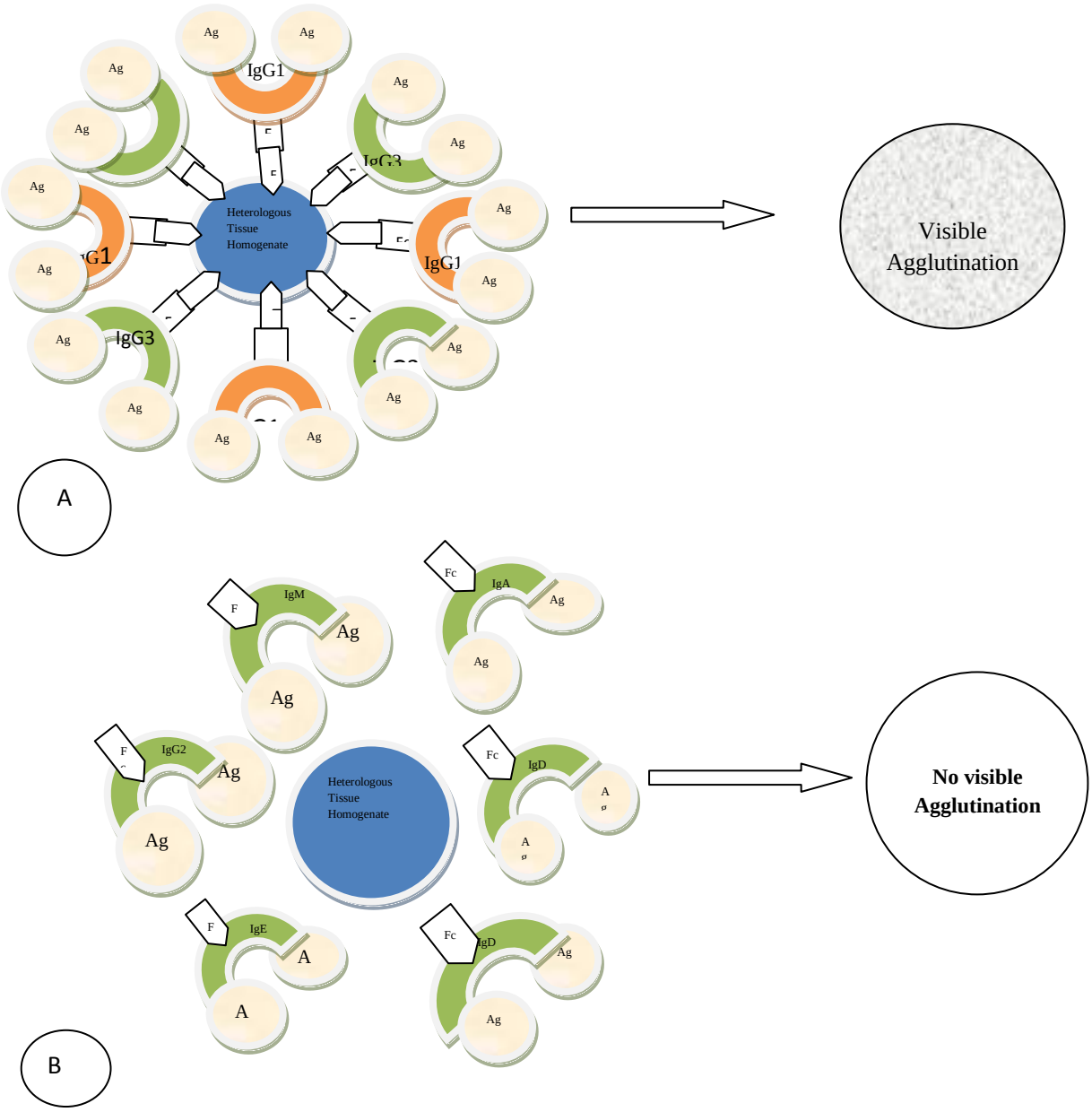


Diagram A (above) illustrates the binding of immunoglubolin gamma (IgG) subclasses 1 and 3 through the fragment crystallizable (Fc) to the Fc receptor (FcR) on the homogenate of heterologous tissue/mast cells. The variable region of the Ig bind to adult worm extracted antigens (Ag). B: The Fc of the other Ig classes (IgA, IgD, IgE and IgM) and IgG subclasses (2 and 4) do not bind to heterologous tissue, so no vissible agglutination is seen.

Appendix III: Optimizing Primer Selection

The following recommendations are provided to help optimize primer selection:

(i) Primers should be at least 18 bases long to ensure good hybridization.

(ii) Avoid runs of an identical nucleotide, especially guanine, where runs of four or more Gs should be avoided.

(iii) Keep the G-C content in the range 30–80%.

(iv) For cycle sequencing, primers with melting temperatures (Tm) above 45 °C produce better results than primers with lower Tm.

(vi) For primers with a G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the Tm>45 °C.

(vii) Use of primers longer than 18 bases also minimizes the chance of having a secondary hybridization site on the target DNA.

(viii) Avoid primers that have secondary structure or that can hybridize to form dimers.

(ix) Several computer programs for primer selection are available. They can be useful in identifying potential secondary structure problems and determining if a secondary hybridization site exists on the target DNA.

Appendix IV: Volume and concentration of reagents used for PCR

S/No.	Materials	Stock Solution 50ul	Optimization (50ulx5)	Final Assay 50x80ul
1.	PCR buffer	0.5ul	2.5µl	200µl
2.	Magnesium chloride (MgCl2)	2.5ul	12.5µl	200µl
3.	dNTP (deoxyribo-nucleoutide triphosphates)	0.5ul	2.5µl	40µl
4.	Primers (Forward and Reverse)	0.5/ul (20pmol)	2.5µl	40µl each
5.	Dream Taq DNA polymerase	2.5ul	12.5µl	200µl
6.	Sample/Control DNA (template)	1ul	5µl	80µl
7.	Sterile deionized water	19.5ul	97.5µl	1.576ml

Appendix V: Reagents used to prepare Dye Terminator Cycle Sequence (DTCS) Quick Start Mastermix.

S/No.	Materials	Quantity
1.	10× PCR buffer	1.0
2.	25mM $MgCl_2$	1.0
3.	5pMol forward primer	0.5
4.	DMSO	1.5
5.	2.5Mm DNTPs	0.8
6.	Taq (homemade)	0.3
7.	Template 10ng/µl	2.0
8.	H_2O	2.9
Total		**10µL**

Appendix VI: Purifying PCR fragments by ultra-filtration

1. Assemble the Centricon-100 column according to the manufacturer's recommendations.
2. Load 2 mL deionized water onto the column.
3. Add the entire sample to the column.
4. Spin the column at 3000 × *g* in a fixed-angle centrifuge for 10 minutes.

   Note: The manufacturer recommends a maximum speed of 1000 × *g*, but 3000 ×*g* has worked well in Applied Biosystems laboratories. If you are following the manufacturer's guidelines, increase the time to compensate.
5. Remove the waste receptacle and attach the collection vial.
6. Invert the column and spin it at 270 × *g* for 2 minutes to collect the sample. This should yield approximately 40–60 μL of sample.
7. Add deionized water to bring the purified PCR fragments to the original volume.

Appendix VII: Preparing Extension Products

Overview This section provides instructions for adding a sodium dodecyl sulphate (SDS)/heat treatment to the spin column and spin plate purification methods. This SDS/heat treatment effectively eliminates unincorporated dye terminators from cycle sequencing reactions.

WARNING: CHEMICAL HAZARD. **Sodium dodecyl sulphate (SDS).** Exposure causes eye, skin, and respiratory tract irritation. Exposure may cause severe allergic respiratory and skin reaction. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Preparing extension products:

1. Prepare 2.2% SDS in deionized water. This SDS solution is stable at room temperature.
2. Add an appropriate amount of the 2.2% SDS solution to each sample to bring the final SDS concentration to 0.2%. For example: Add 2 µL of 2.2% SDS to each 20-µL completed cycle sequencing reaction.
3. Seal the tubes and mix thoroughly.
4. Heat the tubes to 98 °C for 5 min, then allow the tubes to cool to ambient temperature before proceeding to the next step.

   Note: A convenient way to perform this heating/cooling cycle is to place the tubes in a thermal cycler and set it as follows:

   • 98°C for 5 min

   • 25°C for 10 min

5. Spin down the contents of the tubes briefly.
6. Continue with spin column or 96-well plate purification.

Appendix VIII: Spin column purification

1. Prepare the extension products according to "Preparing Extension Products" in the Appendix VI above.
2. Gently tap the column to cause the gel material to settle to the bottom of the column.
3. Remove the upper end cap and add 0.8 mL of deionized water.
4. Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.
5. Allow the gel to hydrate at room temperature for at least 2 hours.

   Note: Hydrated columns can be stored for a few days at 2–6 °C. Longer storage in water is not recommended. Allow columns stored at 2–6 °C to warm to room temperature before use.
6. Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.
7. Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity.

   Note: If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.
8. Insert the column into the wash tube provided.
9. Spin the column in a microcentrifuge at 730 × *g* for 2 minutes to remove the interstitial fluid.
10. Remove the column from the wash tube and insert it into a sample collection tube (for example, a 1.5-mL microcentrifuge tube).

Appendix IX: Sequencing of Single Stranded DNA

To sequence single- and double-stranded DNA on the GeneAmp® PCR System 9700 (in 9600 emulation mode), 9600, or 2400:

1. Place the tubes in a thermal cycler and set to the correct volume.
2. Perform an initial denaturation.

a. Rapid thermal ramp to 96 °C

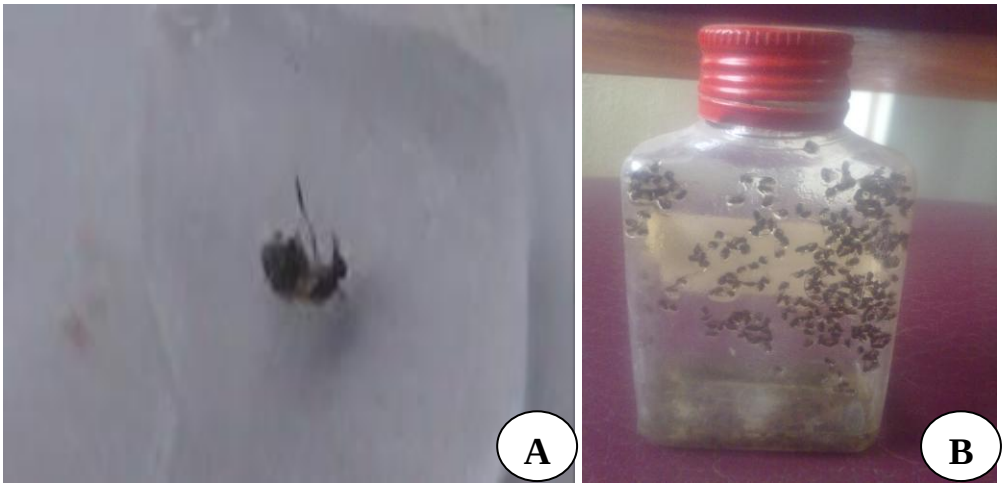
b. 96 °C for 1 min

3. Repeat the following for 25 cycles:
   - Rapid thermal ramp* to 96 °C
   - 96 °C for 10 sec
   - Rapid thermal ramp to 50 °C
   - 50 °C for 5 sec
   - Rapid thermal ramp to 60 °C
   - 60 °C for 4 min

   *Rapid thermal ramp is 1 °C/second.

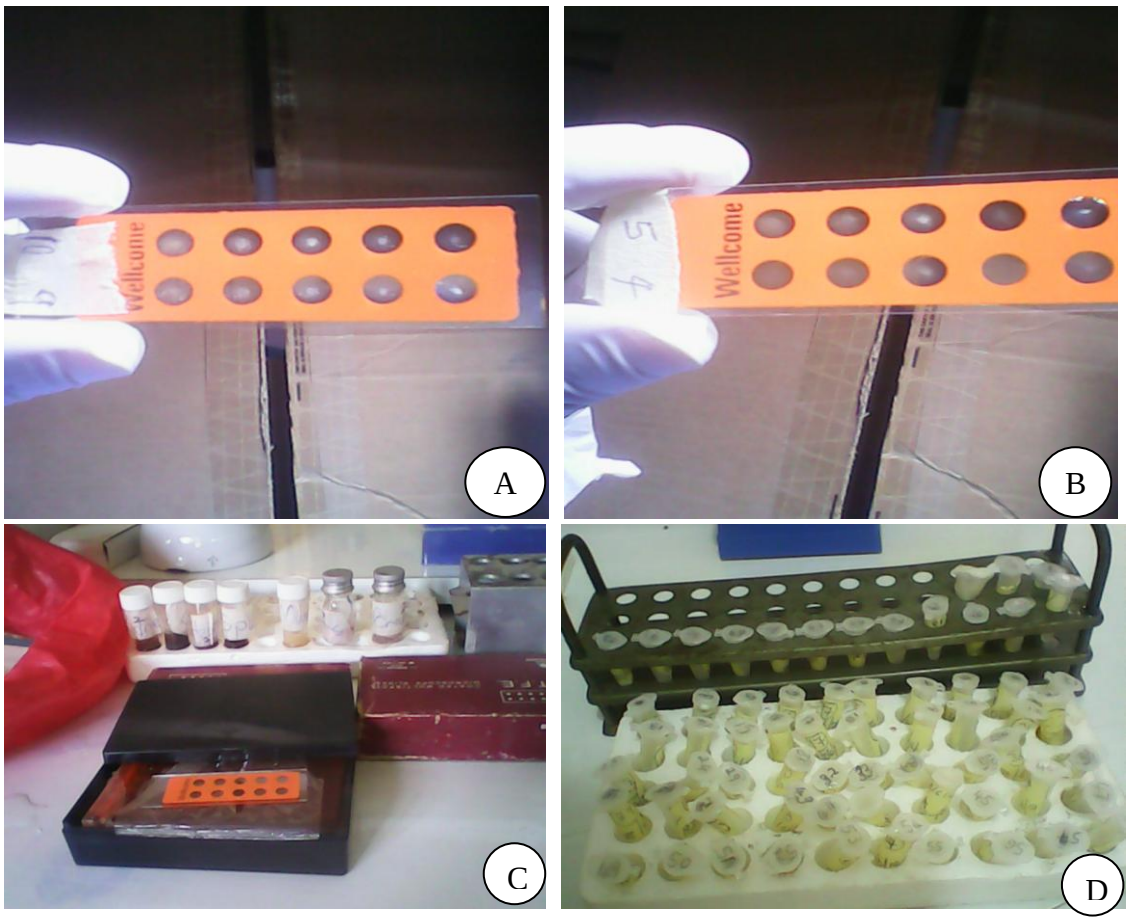
4. Rapid thermal ramp to 4 °C and hold until ready to purify.
5. Spin down the contents of the tubes in a microcentrifuge.
6. Proceed to “Purifying Extension Products.”

Appendix X: Photographs of a Blackflies caught at the Study Area.



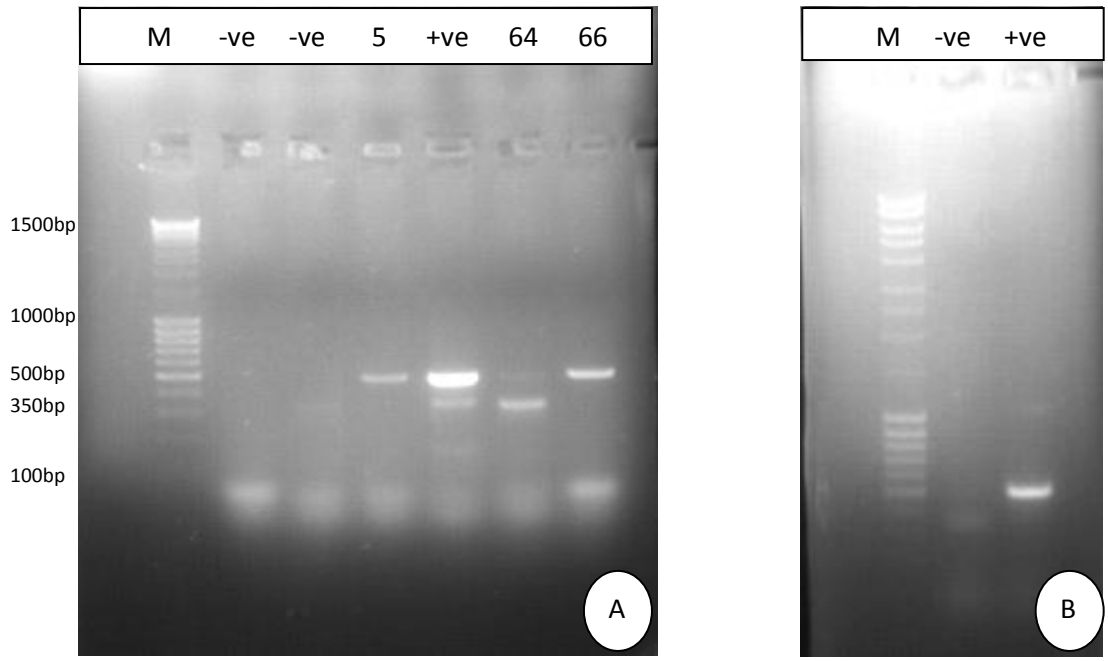
A: Blackfly caught at the vicinity of the Unguwar Shaho Primary School inmersed in phosphate buffer saline. B: A bottle containing 200 parous blackflies preserved in 70% ethanol.

Appendix XI: ***Onchocerca volvulus*** **Slide Flocculation Test**



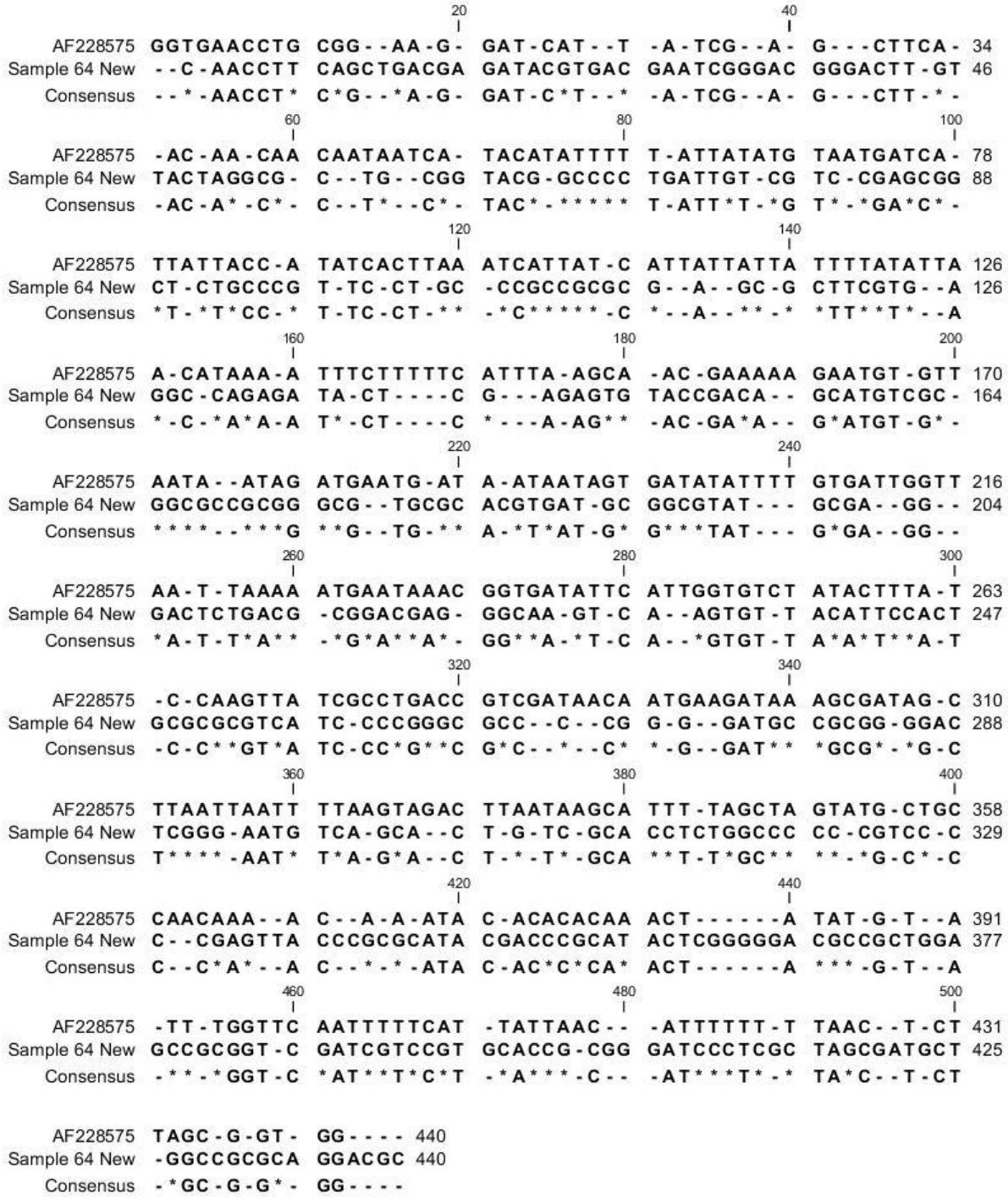
A: positive slide B: negative slide, C: tubes and bijou bottles containing homogenized tissues from organs used for the tests. D: Skin snip samples in eppendorf tubes

Appendix XII: Gel electrophoregram of repeated amplified template DNA



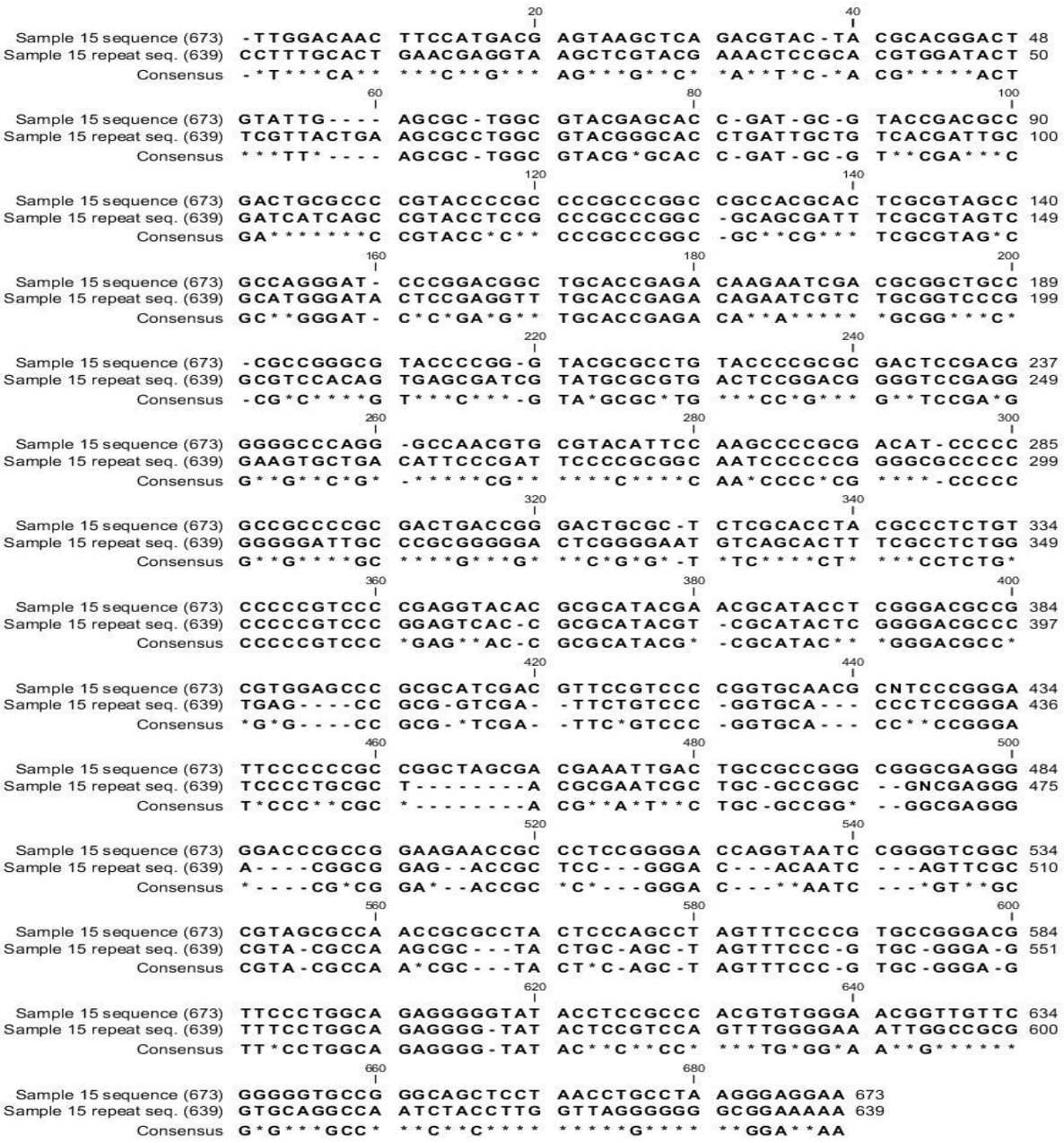
Electrophoregram A: Two single bands of 500bp and one 350 bp. The positive control had double bands of very sharp 500 and faint 350bps. M is the molecular weight ladder (1Kb plus). B: Negative (-ve) and positive (+ve) control with Actin-2B gene sequence.

Appendix XIII: DNA sequence alignment of sample 64 and sample AF228575 ITS 1 gene sequence using ClustalW.



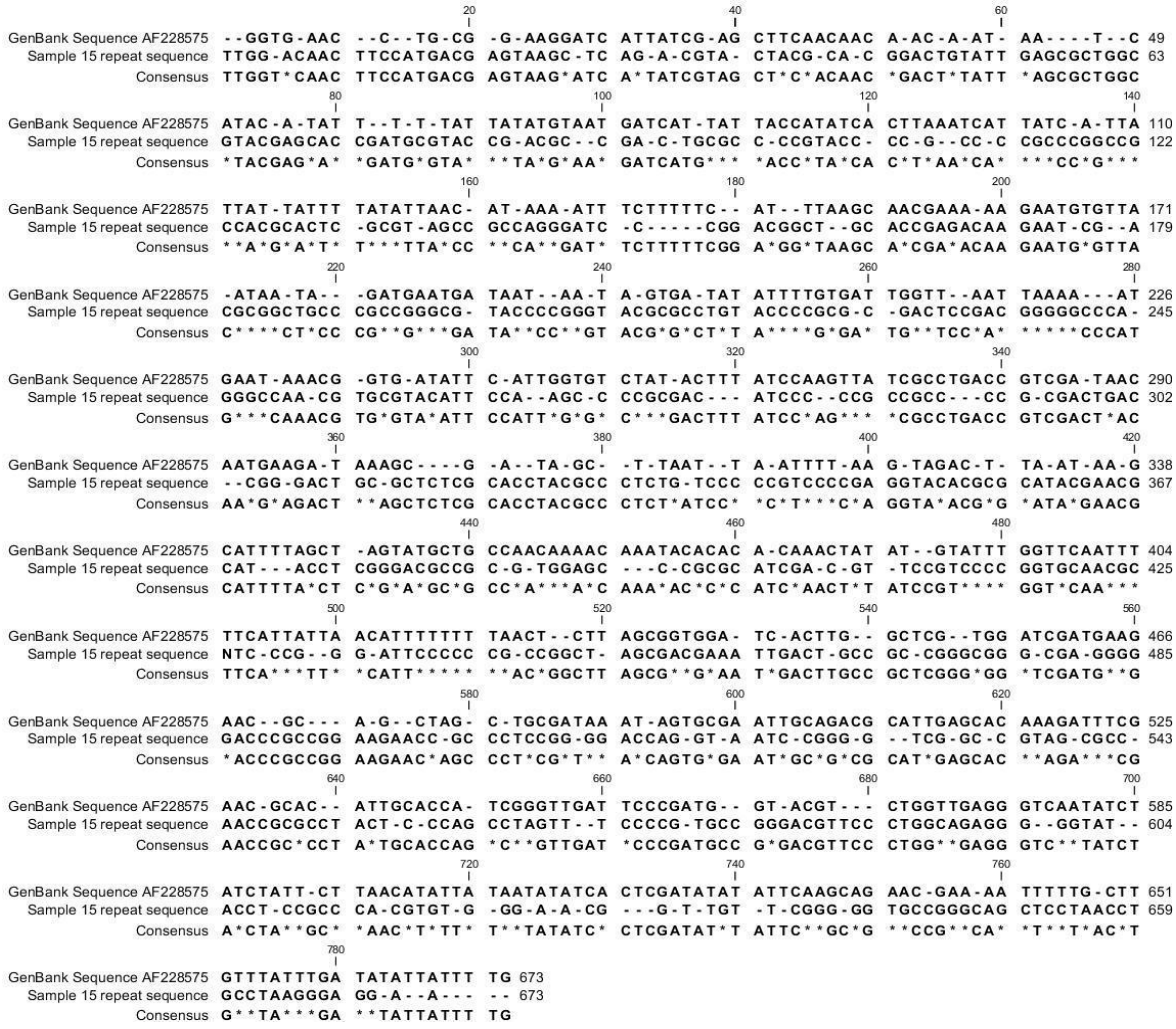
The consensus sequence derived shows (-) and (*) representing gaps and points of conflict, respectively. There was no strong alignment.

Appendix XIV: Basic Local Alignment of Sample 15 initial and repeat sequences.



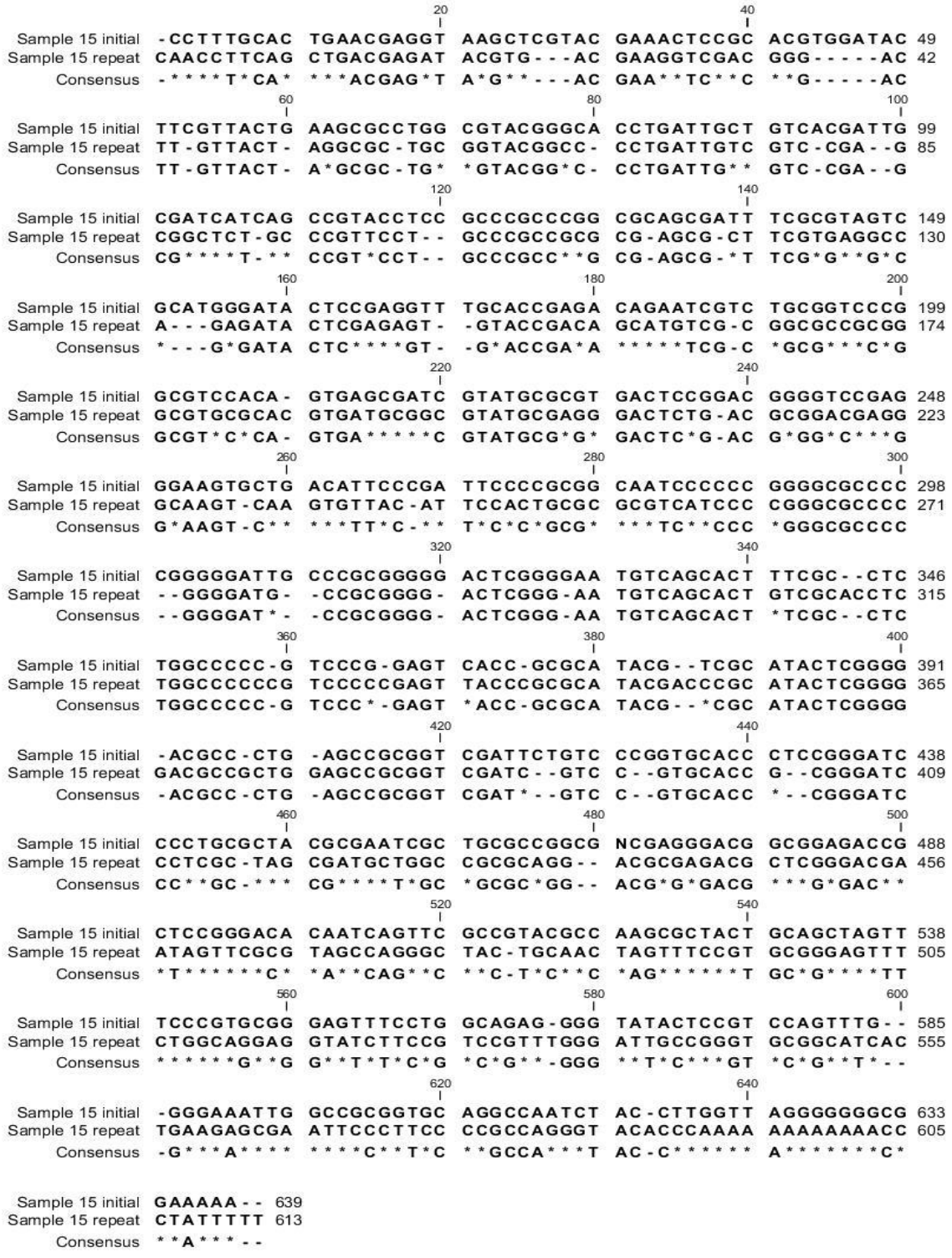
There is very strong homology between the initial and repeated sequences of sample 15.

Appendix XV: Basic local alignment of sample 15 and reference sequences.



The first internal transcribed spacer (ITS1) gene sequence with sample 5 did not show any significant homology.

Appendix XVI: Basic local alignment of replicated sequences of sample 15.



There was very strong homology between the sample 15 initial and repeat sequences.