DIFFERENTIAL EXPRESSION LEVELS OF GLUTATHIONE-S-TRANSFERASE (*OvGST*1) GENE IN THE HOST (HUMAN) AND VECTOR *Simulium damnosum*

BY

Daniel Danladi GAIYA

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AHMADU BELLO UNIVERSITY,
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DECLARATION

I declare that the work in this Dissertation entitled **'DIFFERENTIAL EXPRESSION LEVELS OF GLUTATHIONE-S-TRANSFERASE (*OvGST*1) GENE IN THE HOST (HUMAN) AND VECTOR *Simulium damnosum*'** is the product of my own research effort; undertaken under the supervision of Dr. Idowu. A. Aimola and Dr. Simon A. Yila. No part of this project was previously presented for another degree or diploma in any institution. All sources cited in the text have been duly acknowledged.

Daniel Danladi GAIYA _____ _____

Name of Student Signature Date

CERTIFICATION

This is to certify that the research project report titled **'DIFFERENTIAL EXPRESSION LEVELS OF GLUTATHIONE-S-TRANSFERASE (*OvGST*1) GENE IN THE HOST (HUMAN) AND VECTOR *Simulium damnosum*'** has been examined and approved for the award of the degree of "MASTER OF SCIENCE (MSc.) IN BIOTECHNOLOGY"

Dr. Idowu. A. Aimola_____ _____

Chairman Supervisory Committee **Signature** **Date**

Dr. Simon A. Yila _____ _____

Member Supervisory Committee **Signature** **Date**

Prof. Mohammed Nasir Shuaibu_____ _____

Head of Department **Signature** **Date**

Prof. S. Z. Abubakar_____ _____

Dean, School of Postgraduate Studies **Signature** **Date**

DEDICATION

This work is dedicated to Almighty God and to my lovely late parent Mr. Danladi Barnabas Gaiya and Mrs. Amina Danladi Gaiya.

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Abstract

Onchocerciasis, a neglected tropical disease (NTD), also known as river blindness, the world's second leading cause of preventable blindness is caused by helminths worm *Onchocerca volvulus*. This parasite lives and multiplies extracellularly in the blood and tissue fluids of their human hosts and is transmitted by the bite of infected blackfly(*Simulium damnosum*)of the family *Simuliidae*. *O. volvulus* GST1 (*OvGST1*) modulates immune responses through the synthesis of parasite-derived prostanoids and xenobiotic detoxification, thus preventing effector responses. The *OvGST1*differential expression profile in specific stages of *O. volvulus* quantified using qPCR relative gene expression technique. The results reveal that *OvGST1* is well expressed in the L3 insect developmental stage and microfilaria stage in the human host but poorly expressed in the adult worms of *O. volvulus* in the human host. Differential expression from skin microfilaria revealed 2.55-fold up-regulation relative to the L3 insect stage of the parasite which in turn revealed a higher *OvGST1*differential expression compared to adult worms. Reduced glutathione was higher in the human tissues compared to the *Simulium* proboscis and did not positively correlate with *OvGST1* expression in the life-cycle stages Studied. The distribution of microfilaria from the harvested proboscis showed a significantly high infection load (14.43%) for the L3 infective stage studied, on the other hand, the skin snips examined showed a significantly low infection load (5.55%) for microfilariae compared with the nodule which showed a significant decrease in infection load (3.09%). The results on the quantitative polymerase chain reaction differential expression levels of *OvGST1*gene revealed that *OvGST1*is expressed in the adult worms, skin snip microfilariae and L3 infective stage, of which the skin snip microfilariae *OvGST1*was highly up-regulated.

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LIST OF ABBREVIATIONS

ADM	Administration
APOC	African Programme for Onchocerciasis Control
BDTH	Barau Dikko Teaching Hospital
BLAST	Basic Local Alignment Search Tool
CDC	Certer for Disease Control
cDNA	Complementary Deoxyribonucleic Acid
CDNB	1-Chloro-2, 4-Dinitrobenzene
cds	Coding Deoxyribonucleic Acid Sequence
CDTI	Community Directed Treatment with Ivermectin
DALYS	Disability Adjusted Life Years
ddH_2O	Double Distilled Water
DEC	Diethylcarbamazine
DEPC	Diethylpyrocarbonate
DTNB	Dithio 5, 5-Nitrobenzoic acid
EDTA	Ethylene Diaminetetraacetic Acid
GST	Glutathione-S-Transferase
GSTs	Glutathione-S-Transferases
IDT	Integrated Deoxyribonucleic Technology
KSMH	Kaduna State Ministry of Health

MAPEG	Membrane-Associated Proteins in Eicosanoid and Glutathione
MDA	Mass Drug Administration
MOH	Ministry of Health
mRNA	Messenger Ribonucleic Acids
NAD(P)H	nicotinamide adenine dinucleotide phosphate hydrogren
NCBI	National Center for Biotechnology Information
NGM	Nematode Growth Media
nm	Nanometer
NTD	Neglected Tropical Disease
OCP	Onchocerciasis Control Programme
OEPA	Elimination Program for the Americas
OvGST1	Onchocerca *volvulus* Glutathione-S-Transferase
PCR	Polymerase Chain Reaction
PGDS	Prostaglandins
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROCS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
UV	Ultra-Violet
VOL	Volume
WHO	World Health Organization

WHOTDR World Health Organization Tropical Disease Research

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The filarial parasite *Onchocerca volvulus* is an insect-borne disease and the causative agent of human Onchocerciasis also known as river blindness; a disease characterized by chronic skin and eye lesions (Wanji *et al.*, 2015; Perbandt*et al.*, 2008). This disease is one of the world's major leading infectious causes of preventable blindness. *Onchocerca volvulus* is one member of a genus of tissue dwelling larvae, most of which are parasites of various endemic potential in a wide band of Sub-Saharan Africa, extending from Senegal in the West (Nigeria) through Uganda in the East, isolated foci in North, South, Central America and Asia (Unnasch*et al.*, 2000).

It has been established that 18 - 37 million people are infected, with up to 1-2 million people visually impaired and 270,000 people rendered completely blind (Wanji*et al.*, 2015; WHO, 1995). However, public health efforts by public and private partnerships involving organizations such as the Carter Center and Sightsavers have resulted in significant progress toward elimination of Onchocerciasis, especially in Latin America and West Africa particularly Nigeria(Diemert, 2011 andRichards*et al.*, 2001). As of November 2011, the cycle of transmission of onchocerciasiswas reported to be officially broken in Colombia, Ecuador, Mexico, and Guatemala(WHO, 2015). These countries,especially,Mexico and Guatemala have been declared free of Onchocerciasistransmissionand are undergoing a three year period of post-treatment surveillance by the World Health Organization(WHO, 2015).

However, it remains that more than 90 million people are estimated to be at risk in Africa, with Nigeria and Zaire being the most affected countries (WHO, 2015).

In these highly endemic areas, infection rates can be as high as 80-100% by 20 years of age, with clinical manifestations usually peaking at 40-50 years of age (Duerr*et al.*, 2011). These infections often result in major socioeconomic stigma, as working-aged adults are often the ones affected, leaving the young to care for the adults as well as to provide for the family. Hyperendemic regions are frequently depopulated due to a 3-4 time increase in mortality rate compared to non-infected populations,an average decrease in life expectancy by 7-12 years (Diemert, 2011 and Little*et al.*, 2004).

Human Onchocerciasis is a neglected tropical disease caused by parasitic nematode *Onchocerca volvulus* is transmitted through the bites of female *Simulium damnosum*(blackflies) commonly found in the West African region. Blackflies breed in fast-flowing rivers and streams, increasing the risk of infection to people living nearby (Service, 2008 and Kurtak, 1978). In Sub-Saharan Africa about 99% cases have been reported, where it causes blindness and skin disease, which are together responsible for the loss of over one million Disability Adjusted Life Years (DALYS) every year (Al-Kubatia *et al.*, 2018). Affected persons spend an additional 15% of their annual income on health, children are more likely to drop out of school and farmers have about 30% less land under cultivation (Fischer and Büttner, 2002), thus directly contributing to poverty. The most important pathologies are blindness and skin disease, but skin disease is responsible for 60% of lost DALYs. There are also some reports linking onchocerciasis with epilepsy and dwarfism, and perhaps it increases susceptibility to malaria and reduces the efficacy of vaccinations but not clearly well understood (Basáñez *et al.*, 2006).

The microfilariae are ingested by a Blackfly during a blood meal and move to the midgut as they penetrate the epithelium and migrate, via the hemocoel, to the indirect flight muscles and subsequently to the proboscis as infective (L_3) larvae(WHO, 2015). The microfilariae are released from the proboscis and transmitted directly into the host's bloodstream as the infected fly takes another blood meal from another definitive host (human)(Al-Kubatia *et al.*, 2018; WHO, 2015). Morphological changestake place from $L_3$to L_4 within 2-5 days and the larvae then migrate widely through the body fluid such as the lymph under the skin and between muscles, ligaments,and tendons. The sexually mature female worms release microfilariae which migrate out from the nodules into the skin and other tissues, most significantly into the eye (WHO, 2015).

Onchocerca volvulus glutathione-S-transferase (*Ov*GST1)is sigmaclass of GST glycoproteinwith signal peptide that is cleaved off during the process of maturation (Liebau *et al.*, 1994b). *Ov*GST1is responsible for host immune modulation via synthesis of prostanoids from phospholipids. It is a GSH-dependent enzyme which catalyses reaction similar to the human prostaglandin D_2 synthase (Liebau *et al.*, 1994a Liebau*et al.*, 1994b).

1.2 Statement of Research Problem

Long-termexposure to the infection is often associated with irritating and disabling symptoms which affects the social and economic activities of the inhabitants concerned (Okonkow *et al.*, 2010). It is a major cause of 60% of blindness in different parts of Africa (CDC, 2013).About two decades ago, the African Programme for OnchocerciasisControl began covering another 19 countries, mainly relyingupon the use of ivermectin. Ivermectin treatment is particularly effective because itonly needs to be taken once or twice a year, needs norefrigeration, and has a wide margin of safety; however emerging ivermectin

(mectizan) drug resistance has been observed in Ghana (Osei-Atweneboana*et al.*, 2011; Bundy *et al.*, 2015).In spite of the important role by *OvGST1* of *Onchocerca volvulus* in immune response, it expression is not well understood in the natural environment of the host and vector(Boursou *et al.*,2018; Kuesel, 2016; Vouking *et al.*, 2015).

1.3 Justification

The hallmark of Human Onchocerciasis diseases is the cycle of the parasites betweenthe mammalian host and the insect vector (Coffeng *et al.*, 2013). Glutathione-s-transferase has been intensively studied in helminthes but not in the parasites host and vector environment for differential expression levels. At the moment, there is no *in vivo* validation that *OvGST1*protect the parasite against the host response; this seems highly possible due to the known properties of the enzyme (Krause *et al.*, 2001).

It has been demonstrated that *Onchocerca volvulus*and the host mutually influence various physiological processes by means of *OvGST1* expression (Kampkotter *etal.*, 2003). In order to better understand the functions of *OvGST1* in *Onchocerca volvulus*,*OvGST1* differential expression level was primed for this study. The biochemical pathway and differential expression level of *OvGST1* is poorly understood (Bennuru *et al.*, 2016; Sommer*et al.*, 2003).By virtue of the differential expression levels of *OvGST1*, the gene is a good immune-protective prophylactic target for the development of alternative chemotherapy against the parasite.

1.4 Aim and Objectives

1.4.1 Aim

Tostudy the glutathione-S-transferase (*OvGST1*) differential expression levelsin the life cycle stages critical for the establishment of infection of *Onchocerca volvulus*.

1.4.2 Specific Objectives

i. To determine the microfilariae load in the different environment critical for establishment of infection.

ii. To molecularly identify*OvGST1* gene from the skin snips, human nodule and L3 infective stage of in the proboscis of *Simuliumdamnosum*.

iii. To measure the level of *OvGST1* gene expression level from the skin snips, human nodule and L3 infective stage in the proboscis of *Simulium damnosum*.

iv. To determine the levels of reducedglutathione in the host and vector.

CHAPTER TWO

LITERATURE REVIEW

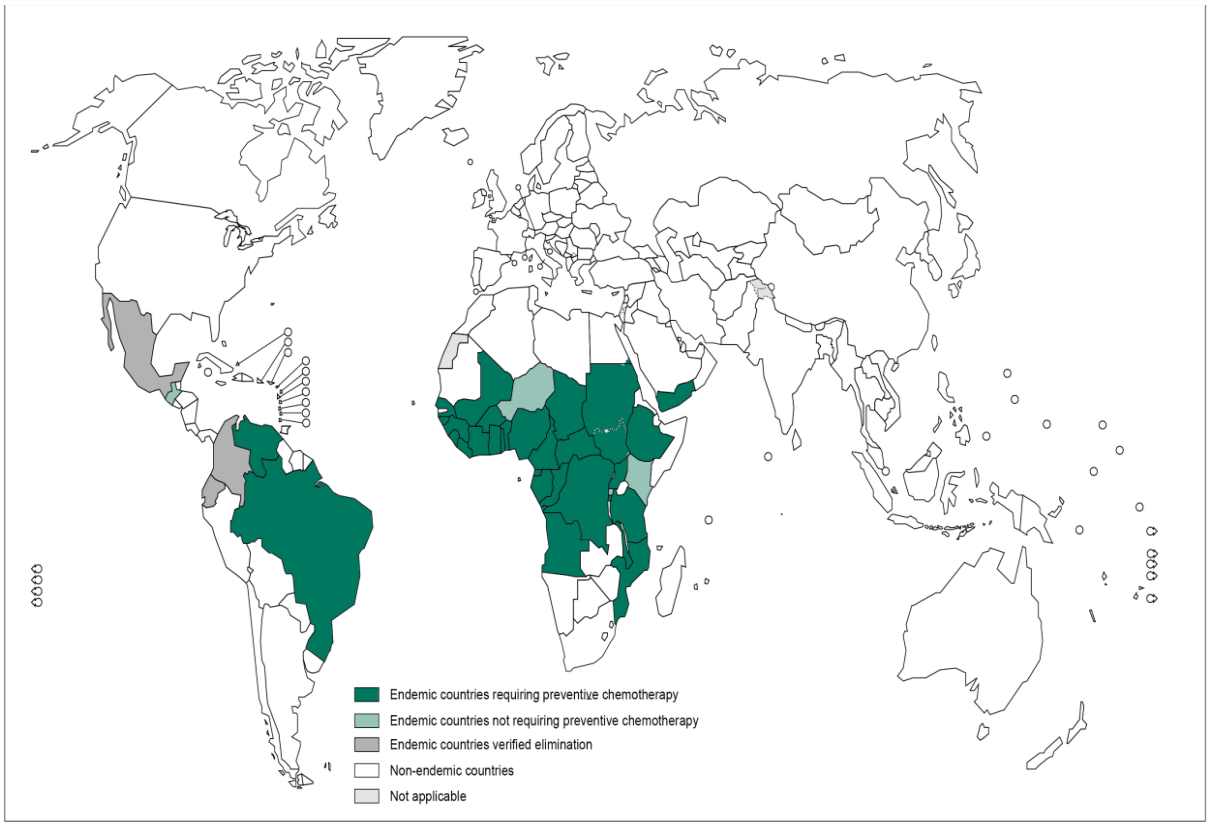
2.1 Human Onchocerciasis

Onchocerciasis often called river blindness because of its most extreme manifestation and because the blackflies (*Simulium damnosum*) that transmit the disease breed in fast flowing waters is a chronic disease caused by infection with filarial worm *Onchocerca volvulus*. It is characterized by the presence of subcutaneous nodules harbouring adult parasites and presentation of dermatitis that can be extremely severe, visual impairment and in severe cases blindness (Wanji*et al.*, 2015 and Perbandt*et al.*, 2008). The etiological agent of the disease is a unicellular helminths parasite of the genus *Onchocerca volvulus* that lives and multiplies extracellularly in the blood and tissue fluids of their human hosts.It is transmitted by the bite of infected blackfly*Simulium damnosum* of the family Simuliidae(Okonkow*et al.*, 2010). The parasite's developmental cycle comprises the long-lived adult stages, skin-dwelling microfilariae (Mf) responsible for most of the pathology associated with Onchocerciasis and the stage infective larvae from the vector blackfly (Armstrong *et al.*, 2016).The Onchocerciasis Community programme started in 1975 and covered seven West African countries: Benin, Burkina Faso, Cote d'Ivoire, Ghana, Mali, Niger, and Togo(Babayan, *et al.*, 2010). However, later evidence indicated that endemic areas outside the initial area posed a threat to the achievement of the OCP and, hence, the programme was extended to include four additional countries, bringing the total number of countries covered by OCP to eleven (Fobi*et al.*, 2015).

Onchocerciasis is basically a rural disease affecting communities residing along fast-flowing rivers (Basanez, 2006). The World Health Organization estimated that more than 18 million people are infected, 500,000 are visually impaired and about 300,000 are blind; however, it is felt that these figures underestimate the true magnitude of the problem (Perbandt*et al.*, 2008; Richards, *et al.*, 2000). Although not generally considered fatal, onchocerciasis causes chronic suffering and severe disability, significantly impeding socio-economic development in affected communities. Nigeria has the largest number of affected people which account for about a third of the global disease burden as reported by Idowu*et al.*, 2013 and Opara*et al.*, 2008.Although ocular damage is the most serious complication, the skin is the principal site of infection and of subsequent cutaneous lesions (Hall and Pearlman, 1999; Murdoch *et al.*, 1993). It is the organ involved in the transmission of the parasite to and from vectors, and the means currently used for parasitological diagnosis by detection of Mf in skin snips. However, microfilariae load (mean number of Mf per milligram of the skin or per snip) constitutes only an indirect and crude approximation to the true parasite burden per host. In general, it is considered that Mf load is roughly proportional to adult worm burden (Duke, 1993).

In tropical Africa the parasite is prevalent over broadly continuous areas of savanna and forest in the west and over more patchy zones in the east, the West African savanna parasite populations being more pathogenic to the eye than their forest equivalents (Zimmerman *et al.*, 1992). However, blinding strains are also found in forest-savanna mosaic and pure forest areas of Central Africa (Bump *et al.*, 2014). There are smaller onchocerciasis foci in the south-western coast of the Arabian Peninsula and in tropical Central and South America (Bulman *et al.*, 2015). The estimated total number of persons living in endemic areas and thus exposed to the risk of acquiring the parasite is 86 million, of which 18 million are

infected, approximately 300 000 suffer from onchocerciasis induced blindness. Africa and Yemen account for 99% of those infected and 99.6% of the blind, whereas the remainder is found in Latin America (Duke, 1990). In the worst afflicted regions, the impact of the disease can be so extreme that fertile valleys are depopulated with serious socioeconomic consequences (Nwoke, 1990; Evans, 1995). For this reason, river blindness has been the subject of extensive research and high expenditure control efforts mainly sponsored through the Onchocerciasis Control Programme (OCP), initiated in 1974 and extended in 1986 to cover 11 countries of the Sudano Guinean savanna belt of West Africa (WHO, 1996). This Onchocerciasis Control Programme (OCP), until recently was based on vector control strategy but now supplemented in some areas with the annual use of ivermectin, a microfilaricidal drug (Molyneux, 1995). As a result of non-inclusion of some endemic countries in the Onchocerciasis Control Programme (OCP), a new African Programme for Onchocerciasis Control (APOC) was launched in order to co-opt the remaining endemic countries. This initiative relies on community-directed ivermectin treatment with vector control in selected foci (Awadzi *et al.*, 2013). The *simuliid* vectors in West Africa belong to the *S. damnosum sensulato*species complex (Crosskey, 1990). In human communities, mean microfilariae intensities may be higher in males than females (but not always).Increase with age in both sexes, reach a plateau in the 15-30 year age group and decline in the elderly. Alternatively, the infection may increase steadily with age and decrease only in the oldest (50+year) age groups (Kirkwood*et al.*, 1983). Broadly speaking, the former pattern is associated with transmission regimes that tend to be seasonal and less intense, whereas the latter seems to occur in places where transmission is heavy and virtually perennial with high intensity of infection (Maizels*et al.*, 2003).



(www.WHO.NTDs.book.org/lecture/images/Africa, 2015).

Figure 2.1: Distribution and Status of Preventive Chemotherapy For Onchocerciasis (River Blindness), Worldwide, 2015. Endemic countries requiring preventive chemotherapy (dark green), Endemic countries not requiring preventive chemotherapy (light green), Endemic countries verified elimination (dark grey), Not endemic countries (white) and Not applicable (light grey) for human onchocerciasis.

2.2 Classification of *Onchocerca*

The nematode parasites *Onchocerca* are grouped in the order 'Spirurida' because of their cylindroid pharynx with an anterior muscular portion and a posterior glandular portion. The males have well-developed alae (a flat, wing-like anatomic process or part) and spirally coiled tails (Lazdins-Helds *et al.*, 2003). This classification in the order Spirurida makes the parasite have a mouth with tooth-like extensions on each side instead of having lips (Otranto*et al.*, 2012). The esophagus of this parasite is undersized and split into two parts. One of these parts is in the front section and is very muscular while the other part is expanded and in the rear of the esophagus. This classification also determines the host for this parasite, requiring an indirect and definitive host (Butler, 2009). Based on the mode of transmission by the insect vector, *Onchocerca* species are potentially zoonotic to some extent.Some species are exclusively dominant among humans as their definitive host while others are found in veterinarians considering geographical locations (Takaoka*et al.*, 2012).In Africa, Central and South America; the human Onchocerciasis is caused by *Onchocerca volvulus,* this blackfly mediated parasitic infection is also called river blindness. *Onchocerca gutturosa, O. ochengi, O. armillata, O. dikei*are species that infect cattle mostly in Cameroon in West Africa (Wahl*et al.*, 1994).*Onchocerca lupi*is a nematode parasite infecting dogs and cats with an unknown arthropod vector, usually found in southern and central Europe and in the United States.It is also being recognized as a parasite that could be responsible for human eye infections (Bennuru *et al.*, 2016; Takaoka*et al.*, 2012; Lazdins-Helds *et al.*, 2003).

In Japan, an infestation of *O. volvulus* or *O. lupi*is not seen, but zoonotic onchocerciasis due to *O. dewittei japonica*, a parasite of wild boar, has frequentlybeen experienced as a reported case ofzoonotic onchocerciasis in Japan (Uni*et al.*, 2015). There are sixother *Onchocerca* species known to be potentially infective tohuman:*O. cervicalis*(infecting horses), *O.*

lienalis(cattle); *O. eberbardi*(sika deer: *Cervus nippon*), *O. skrjabini*(sika deer and serows: *Capricornis crispus*)and *O. suzukii*(serows) (Uni*et al*., 2010).

Domain Eukarya

Kingdom Animalia

Phylum Nematoda

Class Secernentea

Order Spirurida

SuperfamilyFilarioidea

Family Onchocercidae/Filariidae

Genus *Onchocerca*

Species *Onchocerca volvulus*

Figure 2.2: Classification of *Onchcerca volvulus* (Saint-André*et al*., 2002).

2.3*Onchocerca volvulus*

The *Onchocerca volvulus* is found mainly in West Africa and Central and South America and constitute a major public health problem, especially in West Africa (Akpan*et al*., 2012).*Onchocerca volvulus* migrates through the dermis causing itching and skin texture changes and occasionally arrives in the eye where they cause blindness. Detection of these microfilariae is from skin snips or nodule biopsies (Diemert, 2011). When high numbers of microfilariae are present, they can occasionally be found in the blood and urine (Diemert, 2011).

The whitish adult worm lies coiled within capsules in the fibrous tissue. The female can measure up to 50cm while the males are shorter measuring up to 5 cm. The microfilariae of *O. volvulus* are unsheathed and are usually found in the dermis (Fox, 2009). The life cycle of

O. volvulus begins when a parasitized femaleblackfly of the genus *Simulium* takes a blood meal. The microfilariae form of the parasitefound in the dermis of the host isingested by the blackfly. Here the microfilariae then penetratethe gut and migrate to thoracicflight muscles of the blackfly,entering its first larval phase (L1).After maturing into L2, thesecond larval phase, it migratesto the proboscis where it can befound in the saliva. Salivacontaining stage three (L3) *O. volvulus* larvae passes into the blood of the host (Abraham, 2016). From here the larvae migrate to the subcutaneous tissue wherethey form nodules and then mature into adult worms over a period of sixto twelve months. After maturation, the smaller adult males migratefrom nodules to subcutaneous tissue where they mate with the larger adult females, which then produce between 1,000 and 3,000 microfilariae per (Beaver*et al.*, 1989). The normal adult worm lifespan is up to fifteen years. The eggs mature internally to form stage one microfilariae, which are released from the female's body one at a time and remain in the subcutaneous tissue day (Dent and Kazura, 2011). These stage one microfilariae are taken up by blackflies upon a blood meal, in which they mature over thecourse of one to three weeks to stage three larvae, thereby completing the life cycle.The normal microfilariae lifespan is 1–2 years; However, their presence in the bloodstream causes little or noimmune response until death or degradation of the microfilariae or adult worms (Saint-Andr*éet al.*, 2002).

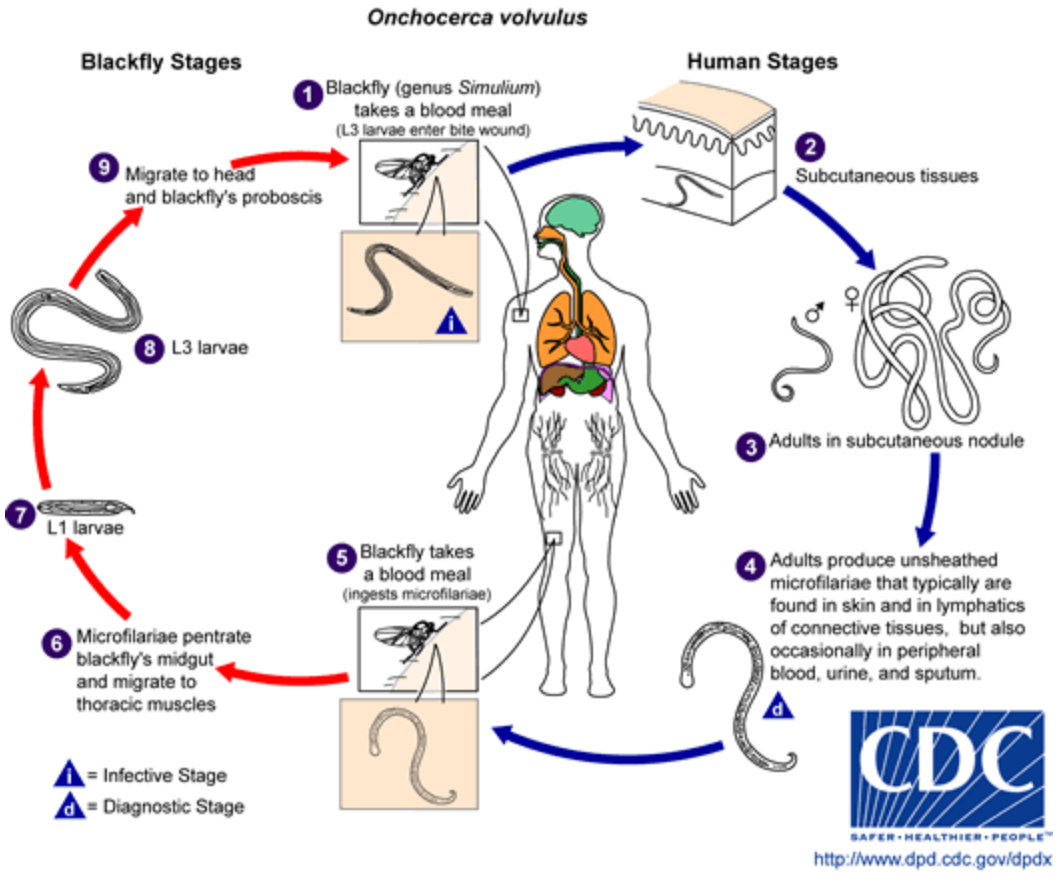


Figure 2.3:Life Cycle of *Onchocerca volvulus* (http://www.dpd.cdc.gov/dpdx),(2005).

2.4 Blackfly(*Simulium damnosum*)

A blackfly(sometimes called a buffalo gnat, turkey gnat, or white socks) is any member of the family*Simuliidae*andrange in size from 3mm to 7mm. Over 1,800 species of blackflies are known (of which 11 are extinct) (Sorrungbe, 2014). Most species belong to the immense genus *Simulium*. Subdivision of the genus *Simulium* in the early days of*Simuliid* taxonomy was based on the following; the structureof the second hind tarsal segment, wing venation in theimago, the structure of the cocoon and the number ofrespiratory filaments in the pupa (Dadzie *et al.*, 2003). They are usually small, black or gray, with short legs, and antennae, most blackflies gain nourishment by feeding on the blood of mammals, including humans, although the males feed mainly on nectar. They spread several diseases, including river

blindness in Africa (*Simulium damnosum* and *S. neavei*) and the Americas (*Simuliumcallidum*and *Simuliummetallicum*in Central America,*Simuliumochraceum*in Central and SouthAmerica) and also *Simulium slossonae*found in Florida in United States (Crosskey, 1990).

The blackfly is unique in a number of ways with about 1,554 species, described and named (morphological and chromosomal), both larval and pupal stages possess useful taxonomic features for identifying species such as the larval respiratory histoblast and the shapes and sizes of pupal respiratory gills, a high rate of sibling speciation within the genus (Sorungbe, 2014;Crosskey 1990).

*Simulium damnosum sensulato*is a complex group of over 40 sibling species of blackfly distributed throughout Sub-Saharan Africa and the Arabian Peninsula (Akpan, *et al.*, 2012; Cupp, 2011). They are responsible for more than 90 percent of Onchocerciasis cases worldwide and 95 percent of cases in Africa (WHO, 2015). About 54 species of *Simulium* have been reported to bite humans' thus transmitting Onchocerciasis. Members of the *Simulium damnosum*can breed in a variety of aquatic habitats located in the Guinea or Sudan savannah, forest or transitional geo-botanic zones (McCreadie *et al.*, 2005). For example, *S. damnosum sensu strict* and*Simulium sirbanum*are found in large rivers of Uganda, Southern Sudan and across the West African savannahs. In these habitats, their population dynamics are regulated, primarily by seasonal changes in rainfall. Long distance migration of inseminated gravid females also occurs during the rainy season as a feature of the biology of the savannah forms (Akpan*et al.*, 2012; Cupp, 2011). *Simulium yahense*is another cytospecies which are found in small streams and forested areas. Adults of this species remain developmentally active throughout the year. Large swarms of blackflies can often

attack people, causing a considerable nuisance (Butler and Hogsette, 1998). In sensitized individuals, reactions to blackfly saliva injected at the site of feeding may cause a syndrome known as "blackfly fever", which consists of a headache, fever, nausea and, often, inflammation of lymph nodes (Doyle *et al.*, 2016). The presence of fast-flowing streams and rivers is a natural factor, which favours the breeding of *Simulium* species (Canteya*et al.*, 2018; Nwoke, 1990).The distribution of *Simulium* species was studied at 2 breeding sites in Ugbem and UkwepeyiereCommunities after 10 rounds of Ivermectin distribution. At each breeding site, 2 flycatchers worked alternately for 4 hours, both giving a total of 8 hours from 8 a.m. to 3 p.m. each day (Ubachukwu*et al.*, 2001). A total of 3895 *Simulium*flies werecaught and identified using morphologic characteristics as *Simulium sirbanum*(70.5%) and *S. yahense* (29.5). A total of 12.1% of both species were parous while 87.9 % were nulliparous flies. Generally, more flies were caught in the morning (7 a.m. to 11 a.m.) than afternoon (12 noon to 3 p.m.) hours, in both dry and rainy seasons. No larva of *Onchocerca volvulus*was detected after dissection of both parous and nulliparous flies (Akpan*et al.*, 2012).

Immature stages develop in oxygenated water sources; therefore adults are usually associated with slow moving streams, creeks, or rivers where the immature stages develop (Atting, 2000). Flowing water does not necessarily imply white water rapids, but water must be moving (Fasulo*et al.*, 2005). Water in lakes and ponds that are not flowing is unsuitable for blackfly development. *S. damnosum* prefers fairly small, slow-moving streams with an average velocity of 1.5 ft per second. Water is often tea-colored, with ample vegetation, light shade, and a pH of 4.4 to 4.5. *S. damnosum* makes good use of temporary streams that flow seasonally (Pinkovsky and Butler, 1978).

Adult blackflies are small insects that measure1 to 5 mm in length and possess a shiny thorax (middle ofthe fly) that ranges in colour from black to various shades ofgray or yellow (Basanez *et al.*, 2009). Females deposit eggs, 200 to 800 per female,on vegetation just below the water surface. Larvae emergefrom eggs and attach themselves to aquatic or emergentvegetation as well as rocks (Klager*et al.*,2002). They will be particularly abundant near culverts under roads, attached to plantstrailing in the water. Most blackfly larvae are filter feeders,with the larvae feeding on nutrients in the water as it flowsby. Larvae pass through six stages before reaching the pupalstage (Demanou *et al.*, 2003). Pupae are encased in a silken cocoon attached tovegetation or other objects in the stream. Adultsemerge from the pupal case through a slit and float to thesurface on a bubble of air. Some species mate as soon asadults emerge (Klager *et al.*,2002).



Figure 2.4: Blackfly, *Simulium damnosum s.l*, (Butler and Hogsette, 2016).

2.5 Glutathione-S-Transferases

Glutathione-S-transferases (EC 2.5.1.18) are members of acomplex group of protein family of isoenzymes expressed inmost living organisms such as microorganisms, aves, reptiles, amphibians, pisces, insects, plant and mammals (Sherrat and Hayes, 2001). Most glutathione-S-transferase posses the ability to bind structurally to varieties of non-substrate ligands, presumed to function in an array of carrier protein non-catalytic reactions involving modulation of signal transduction pathways and intracellular transport of wide spectrum of non-polar ligands (Laborde, 2010). Before now, glutathione-S-transferaseis known to catalyze the conjugation of reduced glutathione with diverse compounds of electrophilic rich centre resulting in the formation of a thioester bond between the sulphur atom of glutathione (GSH) and substrate (Hayes, and Strange, 2000). These enzymes are known exclusively for their housekeepinga role in cells which is evident in cellular detoxification processes (Laborde, 2010; Johansson and Mannervik, 2001). Recent advances in the molecular biology of the GSTs have revealed a broad spectrum role for these enzymes involved in the biosynthesis and metabolism of prostaglandins, steroids and leukotrienes in the handling of toxic products of lipid oxidation and S-glutathionylated proteins generated by oxidative stress and in the acquisition of resistance to chemotherapeutic agents (Laborde, 2010; Listowsky, 2005; Anuradha*et al.*, 2000; Beuckmann, *et al.*, 2000).

Recently studies underscore several GST isoenzymes possessing the capacity to modulate cell signaling pathways that control cell proliferation and cell death (apoptosis) (Lo and Ali-Osman, 2007; Hayes and Pulford, 1995). Because of their cytoprotective role and their involvement in the development of resistance to anticancer agents making GSTs become attractive drug targets (Romero*et al.*, 2006; Ryoo*et al.*, 2004).

2.5.1 Historical Background of Glutathione-S-Transferases

Glutathione-S-transferases were first studied considering their function in drug metabolism aside their involvement in prostaglandin or leukotrienes metabolism (Hayes, and Strange, 2000). According to Booth *et al.* (1961), it was reported that extracts from rat liver catalyze the conjugation of GSH with either 1, 2-chloro-4-nitrobenzene (DCNB) or bromosulphopthalien. Another investigation shows that the one subunit of the Mu class of the soluble GSTs catalyze the conjugation of GSH to DCNB or bromosulphopthalien, the cytosolic proteins present binds to bilirubin, steroids and carcinogens resulted in the elucidation of the class alpha GST (Harris *et al.*, 1991). The Alpha, Pi, Sigma, Kappa class GST and the members of the Mu class family were identified to bind more specific to the general substrate 1-chloro-2, 4-dinitrobenzene (CDNB). Other class of GSTs such as the class Theta transferases first purified using 1-menapthylsulphate and 1,2-epoxy-3-(p-nitrophenoxy) propane as the substrates while the class Zeta and Omega GST were characterized using Bioinformatics approach (Board*et al.*, 2000).

2.5.2 Classes of Glutathione-S-Transferases

Glutathione-S-transferasesconstitute a super-family of dimeric proteins that catalyze the conjugation of the tripeptide GSH to electrophiles leading to the formation of the corresponding GSH conjugates. These proteins are found in almost all species and are divided into classes, based onsequence similarity (Stenberg*et al.*, 1991; Kong*et al.*, 1992; Ruzza, and Calderan, 2013). These proteins are grouped into three major families which are: cytosolic, mitochondrial, and microsomal also referred to as membrane-associated proteins in eicosanoid and glutathione (MAPEG), however, the cytosolic GSTs constitute the largest family (Hayes*et al.*, 2005).

The currently recognized classes of cytosolic GSTs in mammals include the Alpha, Mu, Omega, Pi, Sigma, Theta and Zeta classes (Mannervik*et al.*, 2005; Van Bladeren, 2000). GSTs from the last two classes have also been identified in plants and other organisms such as the parasites. Other distinct groups have been identified only in insects (Delta and Epsilon), plants (Lambda, Phi,and Tau) or prokaryotes (Beta). A Kappa class mitochondrial GSTs, an independent group of proteins, called "membrane-associated proteins involved in eicosanoid and glutathione metabolism" (MAPEG), are integral membrane components in microsomal and mitochondrial cell fractions with GSH conjugating activities similar, but structurally unrelated, to the soluble GSTs (Ranson*et al.*, 2001; Dixon*et al.*, 2002). In mammals, GSTs are present in virtually all tissues (Ruzza, and Calderan, 2013).

The three-dimensional structures of several classes of soluble GSTs have been resolved by X-rayCrystallography (Armstrong, 1997; Widersten, *et al.*, 1996). Each GST subunit is composed of an *N*-terminal and a *C*-terminal domain. This last domain, that adoptsthe α-helix structure, contains a portion of the GSH-binding site (G-site) and a great part of the binding site for hydrophobic electrophiles (H-site) (Armstrong, 1997). The *N*-terminal domain adopts a thioredoxin-like fold (βαβαββα) and comprises most of the G-site. This domain is quite conserved among the different GST classes, whereas the *C*-terminal domain is more divergent. The variations in hydrophobic amino acid residues of the H-site are strongly related to substrate selectivity (Armstrong, 1997)

The G-site of most soluble GSTs contains a Tyr or Ser residue (e.g., Tyr7 in the Pi class, and Ser11 in the Theta-class) located within hydrogen bonding distance from the sulfhydryl group of the enzyme-bound GSH (Armstrong, 1997; Graminski*et al.*, 1989). When the tyrosine is substituted by a phenylalanine, the catalytic activity is dramatically reduced

(Stenberg*et al.*, 1991), so it has been suggested that tyrosine, acting like a base, could receive the proton from the GSH thiol group, thereby activating it (Awoyemi*et al.*, 2014). Successive studies point to the fact that the GSH glutamyl α-carboxylate group is key for GSH activation (Widersten*et al.*, 1996; Stenberg*et al.*, 1991) whereas others indicate the importance of the active-center water molecules (Parraga*et al.*, 1998). Recently, Dourado *et al.* (2008) proposed a water-assisted proton-transfer mechanism that integrates the suggested roles of the GSH glutamyl α-carboxylate group and the active-center water molecules in GSH activation. After an initial conformational rearrangement of GSH, a water molecule, acting as a bridge was able to transfer the proton from the GSH thiol group to the GSH glutamyl α-carboxylate group. Some GSTs (e.g., GSTO1-1) have instead of Tyr or Ser, a Cys in their G-site that makes a mixed disulfide with GSH (Board*et al.*, 2000). These enzymes participate in redox reactions but with generally poor conjugative activity towards their typical substrates (Townsend*et al.*, 2009; Dixon*et al.*, 2002).

2.6Glutathione-S-Transferase in Helminth

Helminth organisms such as *Fasciola hepatica, Schistosoma mansoni,*and*Ascaris lumbricoides* are prominent parasitesaffecting human and animal health. These organisms are unusualin that they have generally low levels of phase I and otherdetoxification enzyme activities, but express GSTs, especially inresponse to drug treatment (Brophy and Barrett, 1990). GSTs are therefore temptingtargets both for chemotherapy and for vaccine development. Ithas already been pointed out that a helminth GST has been allocated to the Sigma class (Meyer and Thomas, 1995).

Schistosomal GSTs have been studied intensively because they are believed to be responsible for resistance to the major drug called praziquantel administeredalongside with ivermectin

during community-directed treatment. The structure of a non-fusion schistosomal GST, both complexed and non-complexed with praziquantel, was then studied (McTigue*et* al., 1995; Sheehan *et al.*, 2001). It was demonstrated structurally that a single praziquantel molecule binds to a non-substrate ligand binding site. This is located in the inter-subunit cleft, and the manner of binding is such that the ligand can occupy only one of two possible overlapping and symmetry-related binding sites in the cleft(Barycki and Colman 1997). Molecular modeling of the other three enzymes based on this structure showed critical differences in the xenobiotic substrate binding site, which may explain substrate inhibitor differences between these isoenzymes as well as differences in the non-substrate binding site. Surprisingly, the most immunogenic epitopes in Fh-47 (Creaney *et al.*, 1995) were found not to be in the most solvent-exposed region of the protein. From the perspective of classification, it is interesting that, whereas helminth organisms express GSTs that are clearly related to the Mu, Pi and Sigma classes of other organisms, these enzymes contain sufficient regions of structural difference compared with host enzymes to hold out the prospect of the development of parasite-specific vaccines (Sheehan *et al*., 2001).

2.7*Onchocerca volvulus* Glutathione-S-Transferase

The way helminths downregulate host immunityat the molecular level is a subject of intense research (Maizels *et al*., 2004). Immunologists have focused on majorsurface and excreted or secreted antigens, as these arelikely to represent the principal active products thathave the capacity to proactively shape the immunologicalenvironment (Maizels*et al*., 2003). Previous studies haveidentified three different *O. volvulus* GSTs (*Ov*GST-1, *Ov*GST-2,and*Ov*GST-3)

with varying substrate specificities, indicating different physiological functions within the parasite. The *Ov*GST-1 is the major microsomal GST in *O. volvulus*, the general distribution of the highly abundant enzyme in the tissues and life stages of the parasite indicates its essential function (Beuckmann*et al.*, 2000; Liebau*et al.*, 1999). The *Ov*GST1 has moderate sequence similarity to mammalian hematopoietic prostaglandin D synthases (PGDS) and belongs to the σ class. The extracellular glycosylated *Ov*GST-1 is located directly at the parasite-host interface and has GSH-dependent prostaglandin D synthase activity. It, therefore, has the potential to participate in the modulation of immune cell functions by contributing to the production of parasite-derived prostanoids. On the basis of gene structure, amino acid sequence, and immunological and kinetic properties, it was possible to place the *Ov*GST-1 in close vicinity to the sigma class (Sommer *et al.*, 2003; Sommer*et al.*, 2001).

The *Ov*GST-2 is the major cytosolic GST and structurally similar to the π-class GSTs and constitutes about 0.1% of the total protein content in adult filarial worms (Perbandt*et al.*, 2005). In comparison to the host's counterpart, it has significant structural differences, particularly in the binding site of the electrophilic substrate. The enzyme possesses limited glutathione peroxidase activity but can readily detoxify toxic secondary products of lipid peroxidation (Perbandt*et al.*,2008).According to Wildenburg*et al.*, (1998) proposed that one of the biological functions of the *Ov*GST-2 is to neutralize, via GSH conjugation, glutathione peroxidase activity, or passive binding, the cytotoxic lipid peroxidation products arising from immune initiated an attack on parasitic membranes. Homology modeling and sequence alignment revealed the *Ov*GST-2 to be topologically related to the mammalian π-class GSTs, and it was suspected that the hydrophobic substrate binding site of the parasitic enzyme is more solvent accessible than the typical π-class enzyme of the host (Brophy *et al.*, 2000).

The *Ov*GST-3 belongs to the ω-class and is dramatically up-regulated in response to oxidative stress, presumably providing a defense against immune initiated peroxidation of parasite membranes (Liebau *et al.*, 2000).*Ov*GST-3 is dramatically up-regulated at the steady-state transcript level in response to oxidative stress (Krause *et al.*, 2001).Interestingly, the inducible *Ov*GST-3 (AF203814) shows significant homology to stress response proteins that contain a GST domainbut only 14% and 21% sequence identity with *Ov*GST-1 (AF265556) and *Ov*GST-2 (X77393), respectively (Liebau *et al.*, 2000).According to the molecular phylogenetic analysis of nematode GSTs, the *Ov*GST-3 is classified as a member of the recently discovered omega GST class (Board *et al.*, 2000).The level of up-regulation of *Ov*GST-3 depends on the nature of oxidative stress applied to the parasite. In response to an intracellular oxidative stress, only a moderate increase in transcript level was observed, whereas an external or extracellular environmental oxidative stress results in a dramatic boost (Campbell *et al.*, 2001).This difference in gene expression observed in response to internal and external oxidative stress leads to the speculation that *Ov*GST-3 not only plays a role in protecting *O. volvulus* against cellular generated ROS but also is important for the defense against the host-derived ROS (Liebau *et al.*, 2000). It is not possible to completely elucidate the function of *Ov*GST-3 or any other gene in the filarial nematode *O. volvulus* since this human parasite cannot be maintained in the laboratory and genetic manipulations cannot be performed at the moment (Hashmi*et al.*, 2001).

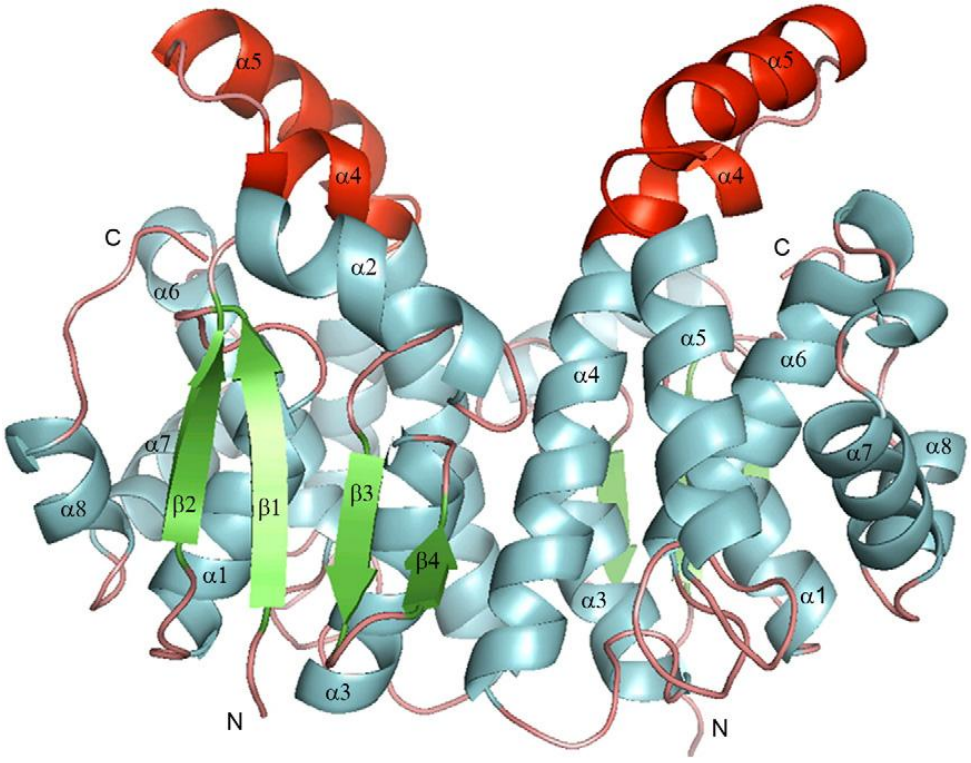


Figure2.5: Ribbon diagram of the homodimeric *Ov*GST1. β-Sheets are green and α-helices blue. The largest structural deviations between the parasitic enzyme and the human host counterpart are shown in red and concern helix α-4 and α-5 (Perbandt*et al.*, 2008).

The entire structure of*Ov*GST1 also possesses a 25-aminoacid stretch prior to the homology to other known GSTs with 50% of the residues polar, 33% are hydrophobic, 4% are negatively charged, and 13% are positively charged (Liebau*et al.*, 1994). The positively charged residues (lysines) are at the carboxy terminus at positions 41, 43, and 45. In the same region, at positions 42, 48, and 44, there are amino acids with alpha-helix-breaking properties (two prolines and one glycine, respectively) (Liebau*et al.*, 1996; Perbandt*et al.*, 2008; Daniel, 1993).

2.8 Glutathione Binding site

Glutathione has the same conformation and binding mode in both PGDS and *Ov*GST1. The G-site is formed by helix α-2, by residues connecting helix α-2 and strand β-3 and by a segment connecting strand β-4 to helix α-3 (Oakley*et al.*, 1997). The molecular recognition of the tripeptide GSH is facilitated by nine hydrogen bonds and three nonpolar interactions (Angeli *et al.*, 2001). The catalytic tyrosine 32 is located at the end of the C-terminus of strand β-1 and is followed by a conserved loop connecting β-1 and α-1. It is generally accepted that GSH predominantly reacts as the anionic thiolate and a network of hydrogen bonds is responsible for the activation of the GSH sulfur (Semnani, and Nutman, 2004). The α-carbonyl group of the γ-glutamyl moiety isaligned by two hydrogen bonds of Ser88. Theα-amino group interacts with Glu121 from theother monomer, and the backbone of the γ-glutamylmoiety is stabilized by van der Waals contacts toArg38, which is also in close proximity to the H-site.The backbone of the Cys moiety of GSH forms twohydrogen bonds with Val75, a residue in cis conformation,and reveals non-polar interactions withHis74. The Gly moiety is bound by Trp63 and Lys67,the backbone being further stabilized by van derWaals contacts to Phe33 (Pinzar *et al.*, 2000).

2.9 Glycosylation of *Ov*GST1

A wide variety of eukaryotic membrane-bound and secreted proteins possess covalently bound carbohydrates. Posttranslational modification of the polypeptide *Ov*GST1, in the form of N-glycosylation, was investigated because of the 4-kDa size difference between the native mature enzyme (without a signal peptide) and recombinant enzyme encoded by the cDNA (without a signal peptide), which were expected to be equivalent (Liebau *et al.*, 1996). The *Ov*GST1 amino acid sequence possesses four potential N-glycosylation sites (Liebau *et al.*, 1994b). Here, the presence of N-linked oligosaccharides on *Ov*GST1 was detected by the

sensitivity of *Ov*GST1 to the enzyme N-glycosidase F (N-glycanase F), which cleaves the N-glycan linkage of glycoproteins between asparagine and the carbohydrate chain (Rademacher, *et al*., 1988).

For the native *Ov*GST1, four N-glycosylation sites at positions Asn50, Asn79, Asn134 and Asn144 have been reported (Sommer*et al*., 2001). The prokaryotic system has been used to express the *Ov*GST1 and the structure reveals no glycosylation sites; however, the structural environment of the corresponding asparagines can describe (Liebau*et al*., 1994a). Site 1 (Asn50) is located in the loop following helix α-1 and site 2 (Asn79) in the sheet β-1. Both sites are 15 Å away from the first visible residue of the N-terminus (Sommer*et al*., 2001). The other two sites are located at the opposite side of the molecule. Site 3 (Asn134) is in the neighbourhood of some important residues of the catalytic pocket, directly at the loop connecting helix α-4 and α-5. Site 4 (Asn144) is located near the kink in helix α-5 (Sommer*et al*., 2001). Site 4 is especially highly glycosylated, bearing large mannose-type carbohydrate structures (Man5 to Man9). However, both sites do not interfere with the catalytic pocket (Perbandt*et* al., 2008). The function of the 25-amino-acid extension cannot be sufficiently elucidated by the presented structure because the 25 N-terminal residues remain disordered (Sommer*et al*., 2001). Nevertheless, there are conspicuous similarities to sequences capable of forming amphipathic helices with a membrane anchor function (Cotton*et al* ., 2017). The periodical occurrence of hydrophobic and hydrophilic residues correlates with the potential to form an amphipathic helix with clusters of hydrophilic residues at one side and clusters of hydrophobic residues at the other side. It was recently shown that the helical conformation depends on anionic lipids and the random coil state is favoured in their absence (McGarry *et al* ., 2005).TheN-terminal sequence may interact with lipids of the hypodermal membrane

and/or the cuticle and therefore has a function as a structural motif that is important for the localization and orientation of the enzyme (Wang, *et al.*, 2000). A potential membrane anchor function would orient the molecule in a way that the catalytic binding site is turned away from the membrane side (Herve *et al.*, 2003). Moreover, also the large glycosylation site 4 (Asn144), bearing Man5GlcNAc2 to Man9GlcNAc2, would be orientated in the same manner (Perbandt*et al.*, 2008). Glycosylation is widely known to be an effective viral strategy of shielding the virus from immune recognition (Reitter *et al.*, 1998).The eukaryotic parasite also makes use of glycosylation as a tool to protect potential protein antigenic epitopes(Angeli*et al.*, 2001). However, the function of the N-terminal tail, the function of glycosylation and the orientation of *Ov*GST1 at the parasite-host interface have to be further investigated (Perbandt*et al.*, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents/Kits

All reagents used were of molecular biology and analytical grade: RNA extraction Miniprep kit (DNALand Scientific, Green BioResearch, Baton Ruoge, Los Angles, USA), AccuPowerRocketplex RT-PCR cDNA synthesis PreMix kit (Bioneer Corporation, Daejeon, Republic of Korea), *OvGST*1 synthesized primers (Bioneer Corporation, Daejeon, Republic of Korea), AccuPower PCR Hotstart PreMix (Bioneer Corporation, Daejeon, Republic of Korea), RNAlater 250ml Sigma Aldrich, *AccuPower*qPCR Dual HotStart preMix kit (Bioneer Corporation, Daejeon, Republic of Korea), agarose gel powder (Thermofisher, Waltham MA, USA).

3.2 METHODS

3.2.1 Ethical Considerations

Prior to recruitment, the nature and objectives of the study were explained to potential participants and those who agreed to take part in the study signed a consent form while an assent was obtained from parents or guardians of children who were enrolled in the study; communal meetings were held with the local authorities and community members to sensitize them on the importance of the research work and also mobilize them for full participation, and inform them about their right to either participate in the examination or not. Participation was voluntary. All volunteers were handled in accordance with the Helsinki declaration on the use of humans in biomedical research. This study was approved by the

Health Research Ethical Committee Kaduna State Ministry of Health, Kaduna (MOH/ADM/744/VOL.1/268). Fly collectors were given a dose of ivermectin as prophylactic treatment against filariasis before the entomological survey.

3.2.2 Parasitological Evaluation

Nodule palpation and skin snipping were carried out to determine the presence of the parasite; this was done according to a method described by Prost, (1986). Before parasitological examination, participants' socio-demographic information and duration of stay in the community were collected using a structured questionnaire.50 subjects were examined based on voluntary consents from three collection pointsand only 11 subjects were confirmed positive for Onchocerciasis.

3.2.3 Nodule Palpation

Participants who gave their consent were examined individually in a private room. Clinical examinations were performed on the partially undressed participants, paying attention to bony prominences of the torso, iliac crest and upper scapular, arms and legs. Participants were examined for the presence of nodules and nodule prevalence according to the method of Wanji *et al*., 2015.

3.2.4 Skin Snipping

After the clinical examination, two skin biopsies from the posterior iliac crest, scapular and gluteal folds (buttocks) were aseptically taken using a 2mm corneoscleral punch (CT 016 Everhard 2218–15 C, Germany) during daytime, the scleral punch was sterilized sequentially in sodium hypochlorite solution, distilled water and then autoclaved by pressure for 15 minute. The skin samples from each participant were placed in two separate sterile 2ml Eppendorf tube containing 1000µl of sterile normal saline according to the method of Wanji

et al., 2015. The corresponding Eppendorf tube numbers were reflected on the participant's form. The tubes were properly capped to prevent any spillover or evaporation and incubated at room temperature for 24 hours. Subjects predominantly farmers and fishermen were considered to be infected if more than one microfilaria were found in the two skin snips. All emerged microfilariae were counted using an inverted microscope at x40 magnification and expressed per skin snip while the intact motile microfilariae were stored in 1000µl RNAlater at -20^{o}C.

3.2.5 Blood Sample Collection

Phlebotomy was conducted using sterile 5ml syringe to siphon 2ml of blood and stored in 5ml free EDTA tube and specimens were examined under low power (x40 objective) for microfilariae according to the method of Wahl *et al.*, (1994).

3.2.6 Entomological Evaluation

Capture of wild *Simulium* flies collection took place in the month of January to March, 2016. The fly collection was conducted by the researcher a trained personnel at three alternating sites between 4.00pm and 6.30pm in the evening daily each week as described by Maikaje *et al.*, 2015. Female *Simulium damnosum s.l.* flies coming to the exposed legs for a blood meal were captured using plane EDTA free tubes before they bite and 388 female blackflies were caught.

3.2.7 Dissection of *Simulium* flies

Live captured flies in plane EDTA free tubes were made immobile using deionized water and carefully picked with forceps and dissected in physiological saline under a dissecting microscope. Flies caught were recorded and dissected on a daily basis to determine their parity and infection. The dissection technique consisted of holding the fly with a dissection

needle in the thorax, piercing the abdomen with a dissecting needle at the posterior end and then pulling out the different internal organs to examine the quantity of fat bodies, the state of the malpighian tubules and the ovaries in order to distinguish parous from nulliparous flies. The proboscis, head, thorax and abdomen of parous flies were further dissected separately and examined for *Onchocerca volvulus* developing larvae (L1, L2 and L3) as described previously byMaikaje *et al.,* 2015. Any infections found were counted and recorded on a dissection sheet. The infected *Simulium* proboscis and heads were separated and pulled together and stored in sterile Eppendorf tube using RNAlater and kept at -20^{o}C.

3.3 Reduced Glutathione Assay

The reduced glutathione content of the tissue as non-protein was estimated according to the method described by Sedlak and Lindsay (1968). A volume of 500µl 10% TCA was added to the tissue homogenate. The samples were centrifuged at 3000 rpm for 30 minutes. 250µl of supernatant was treated with 150µl of Ellman'sreagent (19.8mg of 5, 5-dithiobisnitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate) and 3.0ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412nm as described by Saliu and Bawa-Allah, 2012 andBlum and Fridovich, 1983.

3.4 RNA Extraction

3.4.1 RNA Extraction of *Onchocerca volvulus* from Nodule

RNA was extracted using miTotal RNA Extraction MiniPrep Kit (DNALand Scientific, Green BioResearch, Baton Ruoge, Los Angles, USA) was employed as follows: 400µL of lysing buffer (1000µL of RX buffer and 10µL β-mercaptoethanol fresh preparation) was dispensed into a microtube containing 4mg of adult *O. volvulus* sample. The solution was incubated at 25^{0}C for 5 minutes to ensure complete dissociation of nucleoprotein complexes

after which it was gently vortexed for 1 minute. The lysate was centrifuged at 15996 x g for (Eppendorf 5402 Refrigerated Centrifuge with F-45-18-11 Rotor) 5minutes and 400µL supernatant carefully transferred into a new microfuge tube by pipetting and 300µL of 75% of cold ethanol was added and mixed by pipetting. The solution was transferred into an *O*-ring Mini spin column placed in 2mL collection tube and centrifuged at 15996 x g for 1 minute and the filtrate discarded. A volume of 500µL Wash buffer 1 was dispensed into the Mini spin column and centrifuged at 8161x g for 1 minute. Five hundred microliters of wash buffer 2 was dispensed into the column and centrifuged at 8161 *g* for 1 minute. The column was centrifuged at 8161 x g for 1 minute to dry the column 4^0C. The column was placed in a new microfuge tube and 15 µL of diethylpyrocarbonate (DEPC) -RNase free water (ddH_2O) was dispensed directly to the centre of the spin column membrane and spun at 15996 x g for 60 seconds. The fifteen microliters of eluate was then quantified using a Nanodrop spectrophotometer to determine RNA yield and purity to be used for subsequent RT-PCR (cDNA synthesis) and Real-Time PCR.

3.4.2 RNA Extraction of *Onchocerca volvulus* from Infected Skin Snip

RNA was extracted using miTotal RNA Extraction MiniPrep Kit (DNALand Scientific, Green BioResearch, Baton Ruoge, Los Angles, USA) was employed as follows: 4mg tissue stored in RNA*later* was disrupted and homogenized in 200µL of lysing buffer (1000µL of RX buffer and 10µL β-mercaptoethanol fresh preparation) using sterile homogenizing plastic stick by vortexing for 15 minutes.The lysate was centrifuged at 15996 x g for (Eppendorf 5402 Refrigerated Centrifuge with F-45-18-11 Rotor) 5minutes and the supernatant carefully transferred into a new microfuge tube by pipetting and 400 µL of 100% of cold ethanol was added and mixed by pipetting. The solution was transferred into an *O*-ring Mini

spin column placed in 2mL collection tube and centrifuged at 8161 x g for 1 minute and the filtrate discarded. Five hundred microliters of wash buffer 1 was dispensed into the Mini spin column and centrifuged at 8161 x g for 1 minute. Five hundred microliters of wash buffer 2 was dispensed into the column and centrifuged at 8161 x g for 1 minute. The column was placed in a new microfuge tube and 20 µL of RNase free water was dispensed directly to the centre of the spin column membrane and spun at 15996 x g for 60 seconds. The twenty microliters of eluate was then quantified using a Nanodrop (GeneQuant) spectrophotometer for subsequent RT-PCR (cDNA synthesis) and Real-Time PCR.

3.4.3 RNA Extraction of *Onchocerca volvulus* from Proboscis of *Simulium* flies

RNA was extracted usingmiTotal RNA Extraction MiniPrep Kit (DNALand Scientific, Green BioResearch, Baton Ruoge, Los Angles, USA) was employed as follows: 4mg tissue stored in RNA*later* was disrupted and homogenized in 200µL of lysing buffer (1000µL of RX buffer and 10µL β-mercaptoethanol fresh preparation) using sterile homogenizing plastic stick by vortexing for 15 minutes.The lysate was centrifuged at 15996 x g for (Eppendorf 5402 Refrigerated Centrifuge with F-45-18-11 Rotor) 5minutes and the supernatant carefully transferred into a new microfuge tube by pipetting and 400 µL of 100% of cold ethanol was added and mixed by pipetting. The solution was transferred into an *O*-ring Mini spin column placed in 2mL collection tube and centrifuged at 8161 x g for 1 minute and the filtrate discarded. Five hundred microliters of wash buffer 1 was dispensed into the Mini spin column and centrifuged at 8161 x g for 1 minute. Five hundred microliters of wash buffer 2 was dispensed into the column and centrifuged at 8161 x g for 1 minute. The column was placed in a new microfuge tube and 20 µL of RNase free water was dispensed directly to the centre of the spin column membrane and spun at 15996 x g for 60 seconds.

The twenty microliters of eluate was then quantified using a Nanodrop spectrophotometer for subsequent RT-PCR (cDNA synthesis) and Real-Time PCR.

3.4.4 RNA Quantification

RNA was quantified using the Nanodrop UV- Spectrophotometer (GeneQuant). Briefly, samples were lightly vortexed and centrifuged before checking the concentration with the nanodrop. The quartz cuvette was wiped clean with Kimwipe and 1µL of RNase free water dispensed into the cuvette to initialize the machine. The sample type RNA was selected for RNA quantification. 1µ L of elution buffer (buffer solution in which RNA was eluted in) was placed on the cuvette to blank the machine. The quartz cuvette was again wiped thoroughly with Kimwipe. 1µL of RNA sample was dispensed and the concentration of RNA measured in µg/ mL at the absorbance ration of 260/280 nm and 260/230 nm, which ranged between 1.76–1.82 and 2.0–2.1 respectively.

3.5 cDNA Synthesis

cDNA was synthesized using cDNA Synthesis Kit (Bioneer Corporation, Daejeon, Republic of Korea) as follows: 15 µL of the total RNA eluted was transferred into the nuclease free cDNA synthesis pre-mix microtube containing 1µl of primers, dNTP Mix and reverse transcriptase enzyme. The solution was vortexed for 60 seconds. The mix was heated at 42^0C for 1 hour to synthesize cDNA and RTase inactivation at 94^0C for 5min.

3.5.1 Primer Design

*Ov*GST1 specific primers for detection of *Ov*GST1 mRNA (cds) expression were designed by alignment of *Onchicerca volvulus* sequences from the NCBI database using Genious software and Integrated DNA Technology (IDT). Accession numbers of the *Ov*GST gene was *Ov*GST1 (X75029.1) (Liebau, *et al*., 1994). The specificity of the primers was verified

using a Primer-BLAST search against the human and *Simulium* flies gene sequencesto rule out non-specific matches to vector-derived sequences. Forward primer (***OvGST*1 F-**CCTGAAGGAGAGCACTACCG) and reverse primer (***OvGST*1 R-**AACAAAGCTTGCTAGTTGCGG) synthesized by Bioneer Corporation, Daejeon, Republic of Korea.

3.6 Conventional PCR

Polymerase chain reaction is a biochemistry and molecular biology technique foramplification of target DNA across several orders of magnitudes,generating millions or more copies of target DNA pieces.There are three major steps at different temperatures in a PCR, whichare repeated for 30 or 45 cycles. Double-stranded target DNA is heatdenatured(denaturation step), the two primers complementary to thetarget segment are annealed at low temperature (annealing step), andthe annealed primers are then extended at an intermediate temperature (extension step) with a DNA polymerase.

Conventional PCR was performed using a 25uL reaction volume containing 1µl of each primer (100 pmoles), 5.5µl DEPC-RNase free, 12.5µl Bioneer Hot Start PCR Premix contains hotstart*Taq* DNA Polymerase in an optimized 1x PCR buffer with 1.5mM Mg^{2+} and 250µM of each dNTPs. The master mix retains all features of the *Taq* DNA polymerase (Bioneer Corporation, Daejeon, Republic of Korea). 5µl cDNA was used as template in the reaction. The cycling conditions were as follows: 1 cycle at 94^0C for 5 minutes followed by 45 cycles at 94^0C for 15 seconds, 57^0C for 15 seconds, 72^0C for 30 seconds and a final extension 72^0C for 3min. The PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel prepared with 1µl of ethidium bromide (GibcoBRL Life Technologies, USA) and bands were visualized using Gel Doc 200Imaging System (BIO-RAD, USA).

3.7 Real-time PCR Amplification (qPCR)

Quantitative Polymerase Chain Reaction uses the TaqMan assays, also referred to as 5'-nuclease assays; use the 5' to 3'exonuclease activity of *Taq* DNA polymerase. Each reaction contains agene specific primer and a fluorescence dye labeled TaqMan probe. Theprobe contains a 5' reporter dye (e.g. FAM) and a 3' quencher dye (e.g.TAMRA). Duringamplification, *Taq* DNA polymerase cleaves the probe and displaces itfrom the target, allowing extension to continue. Cleavage of the probeseparates the reporter dye from the quencher dye, resulting in anincrease of fluorescent intensity. The increased fluorescence onlyoccurs if the target sequence is amplified and is complimentary to theprobe, thus preventing detection of non-specific amplification. For anygiven cycle within the exponential phase, the amount of product, andhence fluorescence signal, is directly proportional to the initial copynumber as calculated using Ct (threshold cycle) values.

qPCR was performed with a LightCycler Real-Time PCR System software version 3.5 (Roche Molecular Biochemical LightCycler , Sandhoferstrabe 116D-68298 Mannheim. Germany). The qPCR was performed in a 2mL Eppendorf tube containing 1µl of 1µg cDNA, 1µl of each primer, 12.5µl 2x Green Star qPCR Master mix, (Bioneer), 9.5µl of diethylpyrocarbonate (DEPC-H_2O). The reaction mixes were transferred into each pre-cooled LightCycler Capillary and spun at 700 x *g* for 60sec and loaded into the 96 capillaries sample carousel of the LightCycler Instrument (Roche Molecular Biochemical LightCycler, Sandhoferstrabe 116D-68298 Mannheim. Germany) and covered with LightCycler Instrument lid. The qPCR cycle protocol started with the initial denaturation at 95^0C for 15s, 45 cycles of 95^0C for 15s, 57^0C for 15s and 72^0C for 30s. All qPCR amplifications were reproduced in triplicates.

CHAPTER FOUR

RESULTS

4.1 Infection Load of *O. volvulus* Life Cycle Stages from Human Skin Snip, Human Nodule and Proboscis

The present results highlightthat microfilaria uptake by the vector is not strongly dependent on the skin and nodule infection load in human. In blood-fed flies dissected after being caught, we noted the ingestion of a high number of microfilariae by the blackflies at each catching time however most of the microfilariae were trapped within the peritrophicmembrane. The low infection load of microfilaria in the skin snip and adult worm from the nodule in humans could be attributed to the ongoing mass drug treatment with Ivermectin within the community of study. The 14.43% infection load observed out of the total blackflies caught and 5.55% infection load observed from the human skin snip critical for the establishment of infection showed that the morbidity and ongoing transmission of Onchocerciasis is at large which possibly suggest the re-emergence of the disease. The distribution of microfilaria from the harvested *S. damnosum* proboscis showed a significantly high infection load for the L3 infective stage studied. On the other hand, the human skin snips examined showed a significantly low infection load for microfilaria compared with the human nodule which showed a significant 3.09% decrease in infection load. This distribution directly impact on the fecundity and endemic nature of Onchocerciasis from the sample collection centers.

The morphological features associated with skin snip microfilaria describe it as unsheathed, with space at the anterior end and sharply bent tail and possessed no nuclei which did not extend to the tip of the tail. The plate (See Appendix9) showed adult *O. volvulus* from the

human nodule, human skin snip microfilaria and L3 infective stage from the proboscis. These differences in their morphology also affect the susceptibility of the parasites to live conveniently in the environment where they reside and alternate between the definitive human host and intermediate *Simulium damnosum* thus maintaining the developmental stages critical for the establishment of infection.

Table 4.1: Infection Load Distribution of Life Cycle Stages in Human Skin Snip, Human Nodule and Proboscis.

Tissues	Number of Tissues Examined	Infection Load (%)	Life Cycle Stages
*S. damnosum*Proboscis	97	14 (14.43)	L3 cycle infective stages
Human Skin Snip	97	5 (5.55)	Microfilariae
Human Nodule	97	3 (3.09)	*O. volvulus* worm

4.2 PCR Identification of *OvGST1* from Skin Snip Microfilaria, Nodule,andProboscis L3 Infective Stage.

The electrophoregram result (See Plate I) showed the amplification product of *OvGST1* gene form infected samples collected from hypo-endemic foci sites in Kaduna State, Nigeria. Amplicon products observed in lanes 1, 2 and 3 indicated successful amplification of *OvGST1* gene on adult *O. volvulus* from the human nodule, microfilariae from human skin snip and L3 infective stage larvae from the proboscis of S. damnosum, housekeeping gene β-tubulin (lane 4 and 8) and 100bp plus ladder (M), *O. volvulus* from human nodule, microfilariae from the skin snip and microfilariae larvae L3 from proboscis sample at un-annealed temperature (5, 6 and7) and negative control with (-). The result presented in this study (See Plate I) provide evidence that *OvGST1* gene appears to be present in the adult worm from the nodule, microfilariae from the skin snip and L3 infective stage from proboscis of *Simulium damnosum*. The molecular maker designated as M is DNA a 100bp plus ladder with a fragment size ranging from 100-1000bp which helped us track the position of the amplicon band size alongside the β-tubulin housekeeping gene which is constitutively expressed in the human nodule adult worm, skin snip microfilariae and L3 infective stage present in humans (definitive host) and *Simulium damnosum* (intermediated) while the negative control which contains water and the *OvGST1* primers showed no band. The amplification of *OvGST1* gene by the primers and tracking of the band sizes on the agarose gel revealed the specificity of the primers to amplify the *OvGST1* gene at the target thus ruling out any form of mismatch within and outside the gene. This validated the primer sequence compatibility test conducted on *OvGST1* nucleotide sequences.

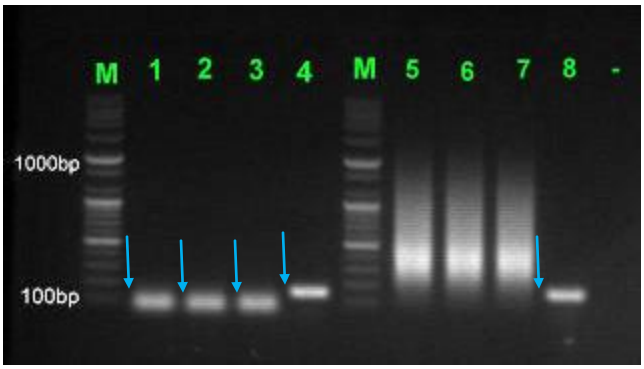


Plate I. RT-PCR amplification of *Ov*GST1 cDNAnodule, skin snip, nodule and *Simulium damnosum*proboscis against 100bp plus DNA ladder (Bioneer 100bp plus ladder).

Lane 1 = Adult *O. volvulus Ov*GST1

Lane 2= *O. volvulus* microfilariae *Ov*GST1

Lane 3 = L3 *O. volvulus* larvae *Ov*GST1

Lane 4and 8 =β-tubulin

(–) = Negative Control with water

Onchocerca volvulus from nodule, microfilariae skin snip and microfilariae larvae L3 from proboscis samples at unannealed temperature(lane 5, 6, 7).

Arrows showing *OvGST1* gene and β-tubulin house keeping gene.

4.3 *OvGST1*Quantification by Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR).

Using *OvGST1* specific primers RT- qPCR, Figure 4.1 showed the relative quantification of the mRNA gene expression pattern. Our results reveal that *OvGST1* is well expressed in the L3 insect developmental stage and microfilaria stage in the human host but poorly expressed in the adult worms of *O. volvulus* in the human host. Expression from skin microfilaria revealed 2.55-fold up-regulation relative to the L3 insect stage of the parasite which in turn revealed a higher 0.38 fold *OvGST1* expression relativeadult stages of *O. volvulus* parasite with less *OvGST1* expression 0.14 fold relative to the β-tubulin gene.We observed that *OvGST1* mRNA was detected in all the developmental stages studied. The results of the study revealed a developmental expression pattern of *OvGST1* in the life cycle stages critical for the establishment of infection of *O. volvulus*. The findings are similar to those reported by Waniewski *et al.*, (2000) who used *in vitro* cDNA analysis and immunoscreening to characterize potential vaccine targets in *O. volvulus*. They reported the up-regulation of *OvGST1* in the L3 and skin microfilarial stages of *O. volvulus*. Based on the findings, *OvGST1* is expressed in the adult worms, skin microfilaria and L3 infective stage in the parasite life cycle, critical for the establishment of infection in the host and vector, of which the *OvGST1* from the skin snip microfilariae was highly up- regulated.

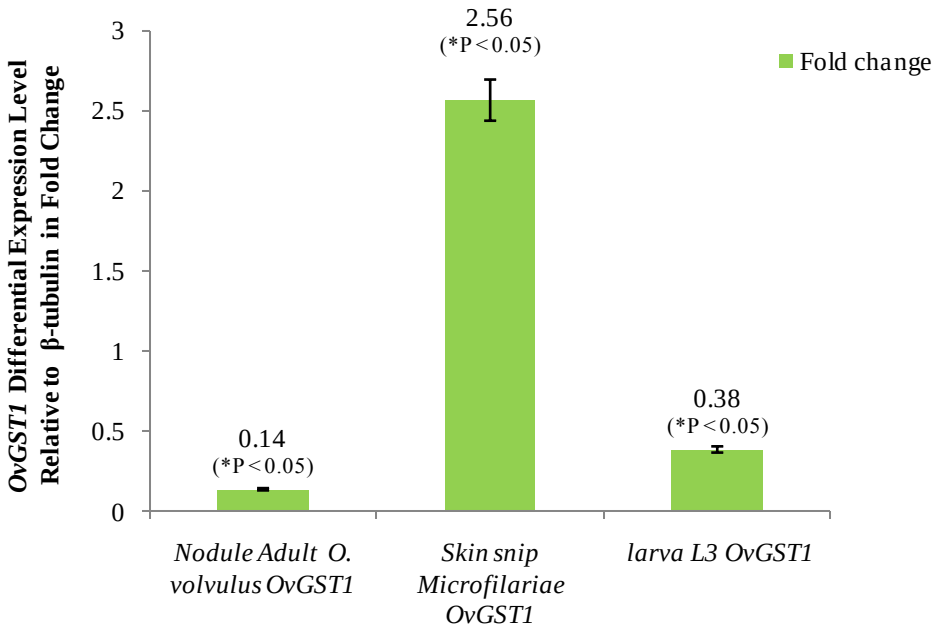


Figure4.1:Differential expression levels of *OvGST1*inadult *O. volvulus* (nodule), microfilariae (skin snip),L3 infective stage (*Simuliumdamnosum*).

4.4 Reduced Glutathione Content from Human Tissue and *Simulium* Proboscis.

The result of Figure 4.2 showed the various level of glutathione concentration present in the different tissues critical for the survival of the parasite *O. volvulus*. The human skin tissue revealed significant higher amounts of reduced glutathione content compared with the reduced glutathione amounts from the human nodule while on the other hand, the *S. damnosum* proboscis showed significant low amounts of reduced glutathione content. The human tissues revealed higher concentration of reduced glutathione (GSH) compared to the insect tissue. Export of reduced glutathione (GSH) rather than glutathione disulfide (GSSG) from parasite to host cell may serve an important physiological role in the parasitized tissues; the human cells may rely on a supply of glutathione (GSH) in order to maintain antioxidant defense.

The observed high amounts of reduced glutathione in the human skin snip tissues relative to the human nodule suggest the cellular strategy deploy by the host tissue so as to protect host cells from cellular damage caused by the parasite *O.volvulus* which can be seen in relation to the significant skin snip microfilariae infection load as revealed in Table 4.1. on the the other hand, the *Simulium damnosum* proboscis which harbours the L3 infective stage larva revealed low amounts of reduced glutathione relative to the human nodule which suggest the protective and symbiotic relationship that exist between the L3 infective stage larva and *Wolbachia* bacteria present in the digestive tract (midgut) of the blackfly which in turn create room for ongoing Onchocerciasis transmission*Simulium damnosum* to human thereby promoting morbidity of Onchocerciasis among people and thus leading to re-emergence of new*O.volvulus* infection and delaying the possibility of the disease been eradicated.

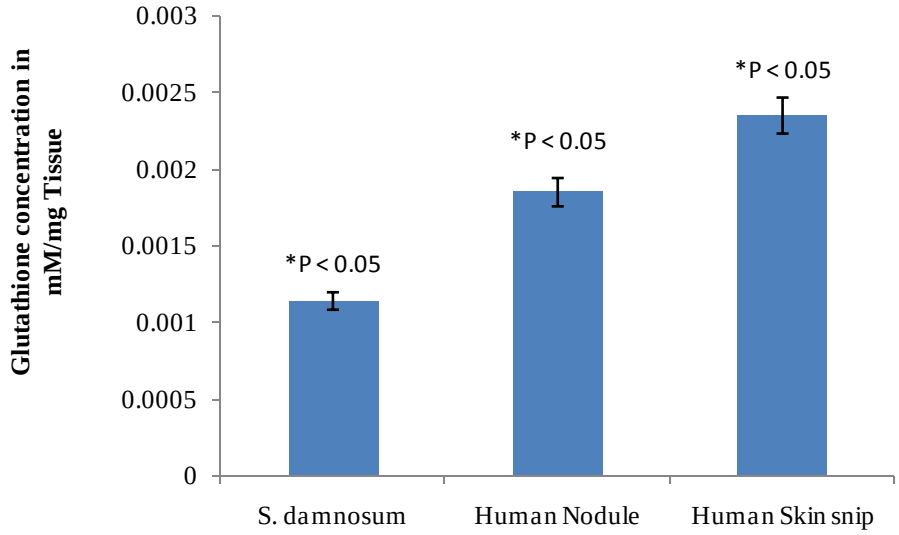


Figure 4.2: Reduced Glutathione concentration levels in the host and vector.

CHAPTER FIVE

DISCUSSION

The principal aim of this research was to determine thedifferential expression levels of *OvGST1*gene in the adult worm from the human nodule, human skin snip microfilariae and L3 infective stage,critical for the establishment of infection both residual in the host and vector *Simulium damnosum*. Therefore, it was necessary to study and evaluate the prevalence of infection load of Onchocerciasis in endemic communities within and the level of microfilaria intensity and the specific sites for vector collection. This prompted a critical study of the skin snip, nodule and L3 infective stage towards understanding the pathogenesis of *Onchocerca volvulus* for a feasibility outcome. The distribution of microfilaria (See Table 4.1) from the harvested proboscis showed significant high infection load for the L3 infective stage studied. On the other hand, the skin snips examined showed significant low infection load for microfilariae compared with the nodule which showed a significant decrease in infection load. This distribution directly impact on the fecundity and endemic nature of Onchocerciasis from the sample collection centers. The amplification result (Plate I) has concisely revealed the *OvGST1* transcript undoubtedly present in the developmental stages critical for the establishment of infection in the life cycle (vector-host) of the parasite. RT-qPCR gene expression pattern result clearly shows that this enzyme is developmentally regulated and is expressed in the lifecycle. To authenticate these results, RNA samples which had a 260/280 and 230/260 ratio of ~2.0 and above were used to ensure purity. Using RT-PCR, we could detect mRNA encoding *OvGST1* in Skin snips, nodule and probosci's samples from both hosts and infected *Simulium damnosum* (see plate I).

This study employed the use of *O. volvulus* isolated from their natural infection environment (host-vector) and thus represents a cross-sectional view of the differential gene expression profile of *OvGST1* mRNA. We observed that *OvGST1* mRNA was detected in all the developmental stages studied. The results of the study revealed a differential gene expression pattern of *OvGST1* in the life cycle stages critical for the establishment of infection of *O. volvulus*. The findings are similar to those reported by Waniewski *et al.,* (2000) who used *in vitro*cDNA analysis and immunoscreening to characterize potential vaccine targets in *O. volvulus*. They reported the up-regulation of *OvGST1* in the L3 and skin snip microfilarial stages of *O. volvulus*. This study also revealed significant2.56 foldup-regulation of *OvGST1* expression *in vivo* in skin snipmicrofilarial stages relative to the L3 stage *OvGST1* expression from the proboscis of the vector which in turn showed significant0.38 foldincrease of *OvGST1* expression compared to the adult stages of *O. volvulus* parasite with less*OvGST1* expression 0.14 fold relative to the β-tubulin gene demonstrating a role of *OvGST1* in the infection cycle of *O. volvulus* (Figure 4.1). *OvGST1* is a sigma class GST which is unique unlike the GST found in the free-living nematode *Caenorhabditis elegans* which has a cytosolic localization and enzyme activity, hence suggesting that the unique features of the *OvGST1* are probably evolutionary designs of parasitism to enable the parasite evades host defenses through GST detoxification activities.

The animal body including human beings and insect are known to use tightly regulated mechanisms to prevent free radical induced tissue cell damage; it is accomplished by a set of endogenous antioxidant enzymes and protein such as GSH, GST, SOD,and CAT (Blokhina*et al.*, 2002). When the balance between ROS production and antioxidant defense is lost oxidative stress set in. Cellular proteins are believed to be the target of free radical-induced

oxidative injury; which through a series of events deregulates the cellular functions leading various pathological conditions (Wammes*et al*., 2012). Increased reduced GSH concentration from the skin snip, nodule compared to the *Simulium* proboscis was in response to the host immune responses in an effort to protect host cells (Figure 4.2). Export of glutathione (GSH) rather than glutathione disulfide (GSSG) from parasite to host cell may serve an important physiological role in the parasitized tissues; the human cells may rely on a supply of glutathione (GSH) from the parasite in order to maintain antioxidant defense. Thus the transfer of glutathione in reduced form from the parasite to the host cell, and then from the host cell to the extracellular environment, may be a mechanism whereby the infected tissues limits exposure to oxidative stress. Interestingly the correlation of high amounts of reduced glutathione relative to increased gene expression of *OvGST1* suggests a major role of host glutathione in host antioxidant defense against *OvGST1* induced tissue damage suggesting L4 skin snip microfilaria *OvGST1* as a promising chemotherapeutic target to interrupt the developmental cycle of *O. volvulus*.

Sequel to the down-regulation of *OvGST1* in the adult *O. volvulus* stage, this could be a potential target for chemotherapeutic agent owing to the fact it is at the viable stage for larvae reproduction. Therefore interrupting the life cycle at infective stage L3 will definitely go a long way in breaking the normal developmental cycle to the*O. volvulus* microfilaria skin snip L4 thus curbing the possibility of vector to host transmission since ivermectin kills the microfilaria in the blood other than adult in the subcutaneous tissue as reported by Osei-Atweneboana*et al.*, (2011). And for the relative up-regulation spotted on the adult *O.volvulus* stage and L3 it can be utilized as a candidate for vaccine development.

CHAPTER 6

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

This work was aimed at studyingdifferential levels of *OvGST1* gene expression in the adult *O. volvulus* from the nodule, microfilaria and L3 infective stage critical for the establishment of infection in the *Simulium damnosum* vector and subsequent transmission to the mammalian host. Therefore, well-controlled laboratory experiments with the evaluation of the situation in the field through molecular and biochemical approaches have been deployed to investigate the expression pattern of *OvGST1* gene in the infection cycle of *Onchcerca volvulus* in the human host and vector *Simulium damnosum*.

- *OvGST1* was found to be expressed in the skin snip microfilariae, L3 infective stage and adult *O. volvulus* critical for the establishment of infection.
- *OvGST1* was found to be highly expressed in the skin snip microfilariae of *O. volvulus*.
- The reduced glutathione content from the human skin snip showed significant increase.

6.2 CONCLUSION

Based on the results obtained, it was inferred that *OvGST1* is expressed in the skin snip microfilariae, adult *O. volvulus* and L3 infective stage studied which is critical for the establishment of infection in human host and *Simulium damnosum*, of which the *OvGST1* from the skin snip microfilariae was highly up- regulated.

6.2 RECOMMENDATIONS

Enzyme activity of *Ov*GST1 in the different tissues critical for establishment of infection should be investigated to understand the relationship between the enzyme activity and *OvGST1* gene expression.

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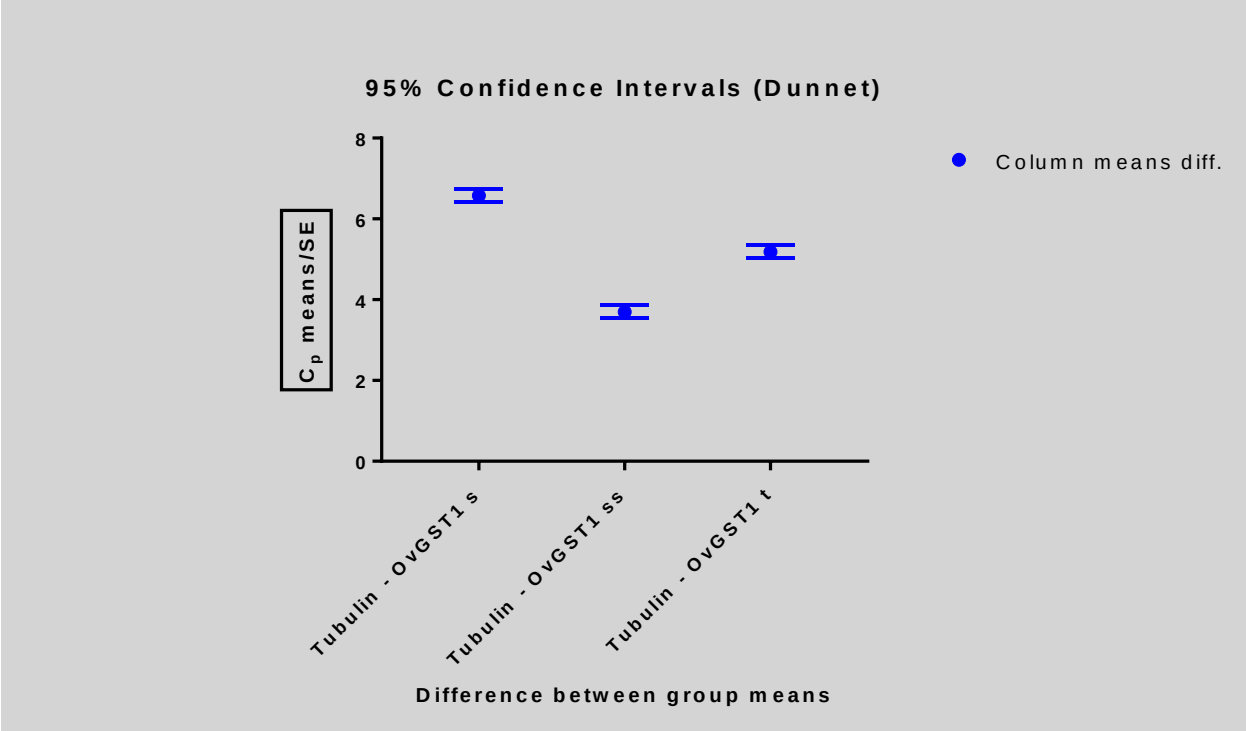
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APPENDICES

Appendix 1: Dunnett's multiple comparisons test at 95% confidence interval depicts significant difference in group Cp values relative to Tubulin with P < 0.05 post hoc test with Dunnet (GraphPad Prism version 7.02).



Appendix 2: Distribution of *O. volvulus* **infected** *Simulium damnosum* **segment body parts**

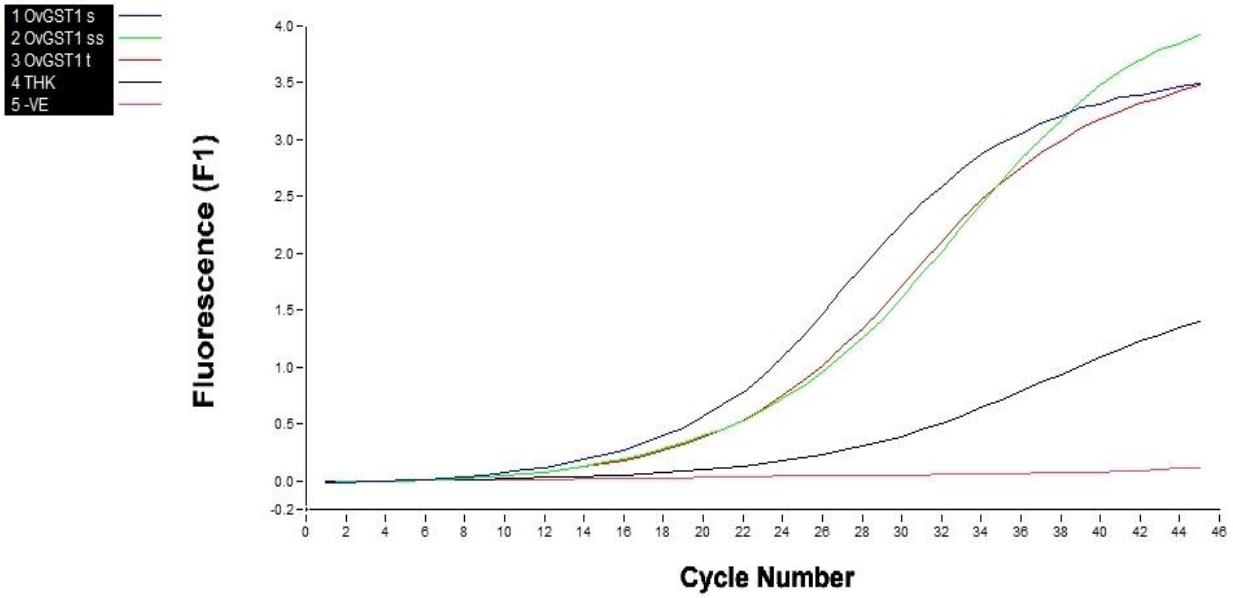
Body segment of *S. damnosum* **isolated**	**Number of** *S. damnosum* **examined**	**Number of** *S. damnosum* **body parts Infected (%)**
Head	388	56 (14.43)
Thorax	388	55 (14.17)

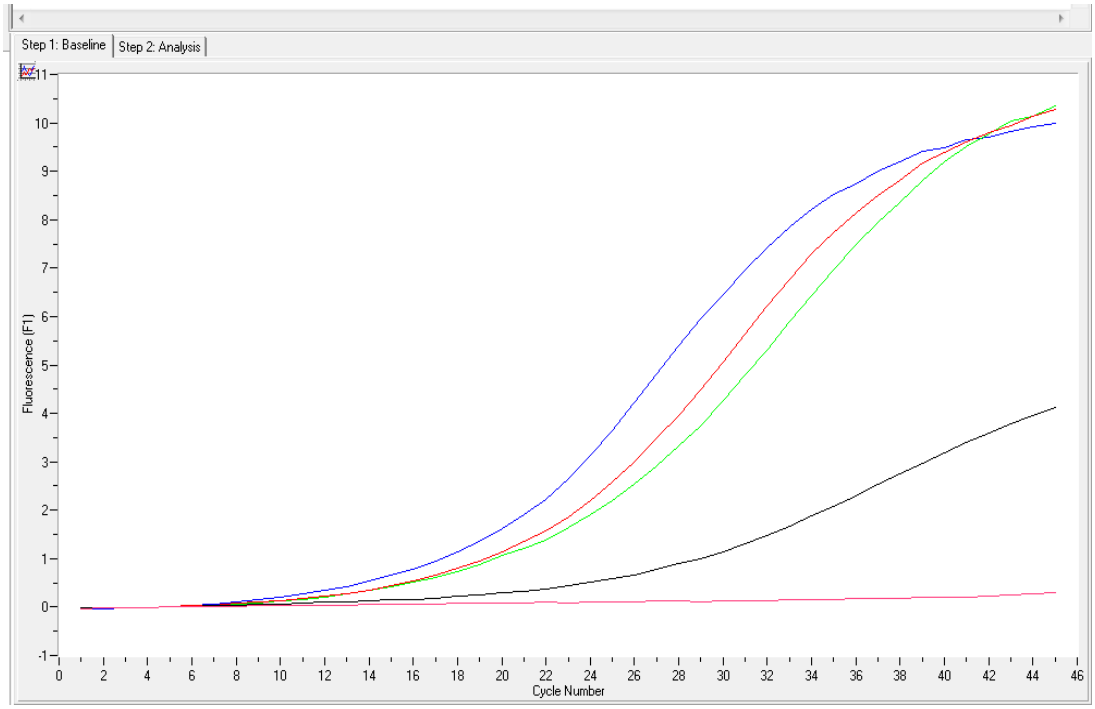
Abdomen	388	89 (22.93)
Total	388*	200(51.53)

*Not additive

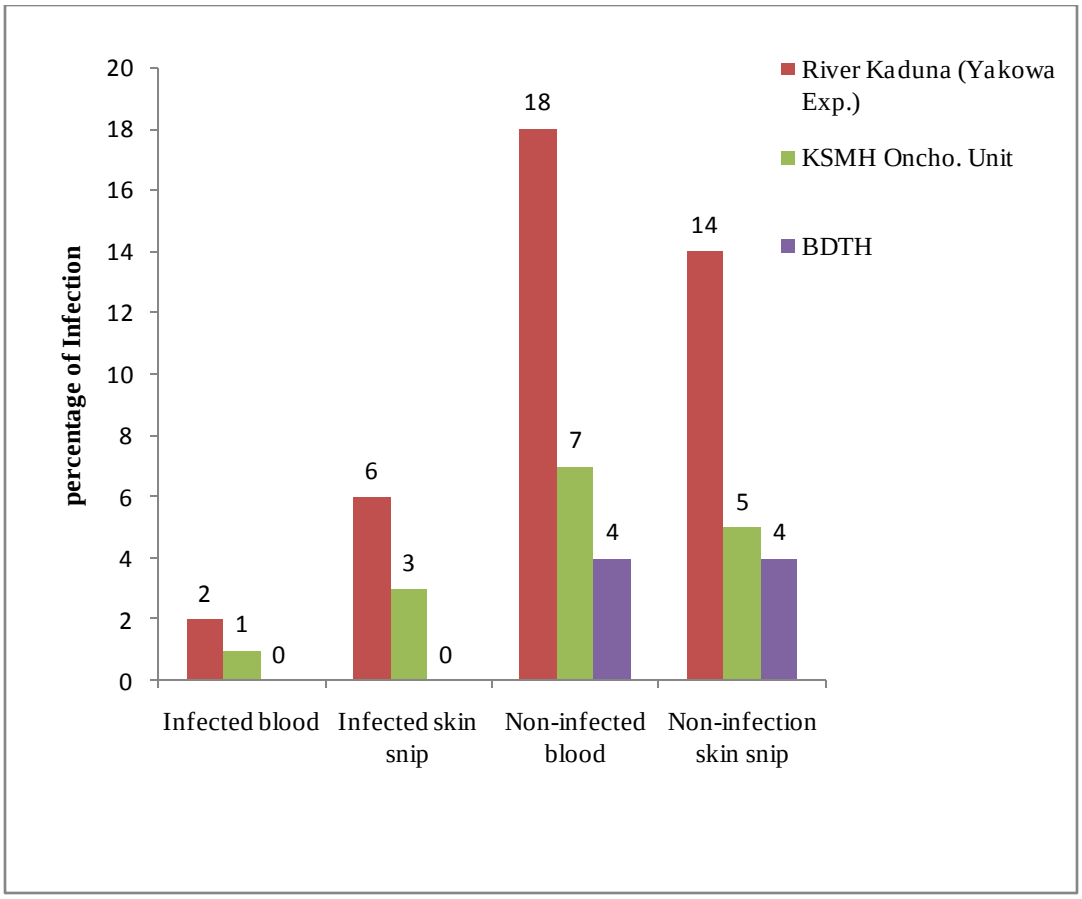
Appendix 3:TheRT-qPCR amplification plots of *Onchocerca volvulus***glutathione-s-transferase cDNA from** *O. volvulus***nodule, microfilariae skin snip and microfilariae larva of proboscis whichwas assayed using Syber Green Star qPCR Master mix and primers (*Ov*GST1) targeting glutathione-s-transferase gene. The assay was performed**

with a LightCycle Real-Time PCR System software version 3.5 (Roche Molecular Biochemical LightCycle).





Appendix 4: Epidemiological Distribution of Infection Load in Ungwan Pada, River Kaduna, Onchocerciasis Unit, Kaduna State Ministry of Health and Barau Dikko Teaching Hospital



Appendix 5: Weekly distribution of *Onchocerca volvulus***infected** *Simulium damnosum* **in the Bank of River Kaduna Yakowa Express Bridge**

Total of two hundred (200) adult female *S. damnosum* was caught during the twelve weeks of study. The weekly fly harvest of *S. damnosum* and the isolated *O. volvulus* from the dissected female flies are represented in Figure 4.2. Most of the blood sucking flies infected were caught between week four and week eight; while a general decrease in the number harvested was observed between week one to three and week ten to twelve ($P < 0.05$).

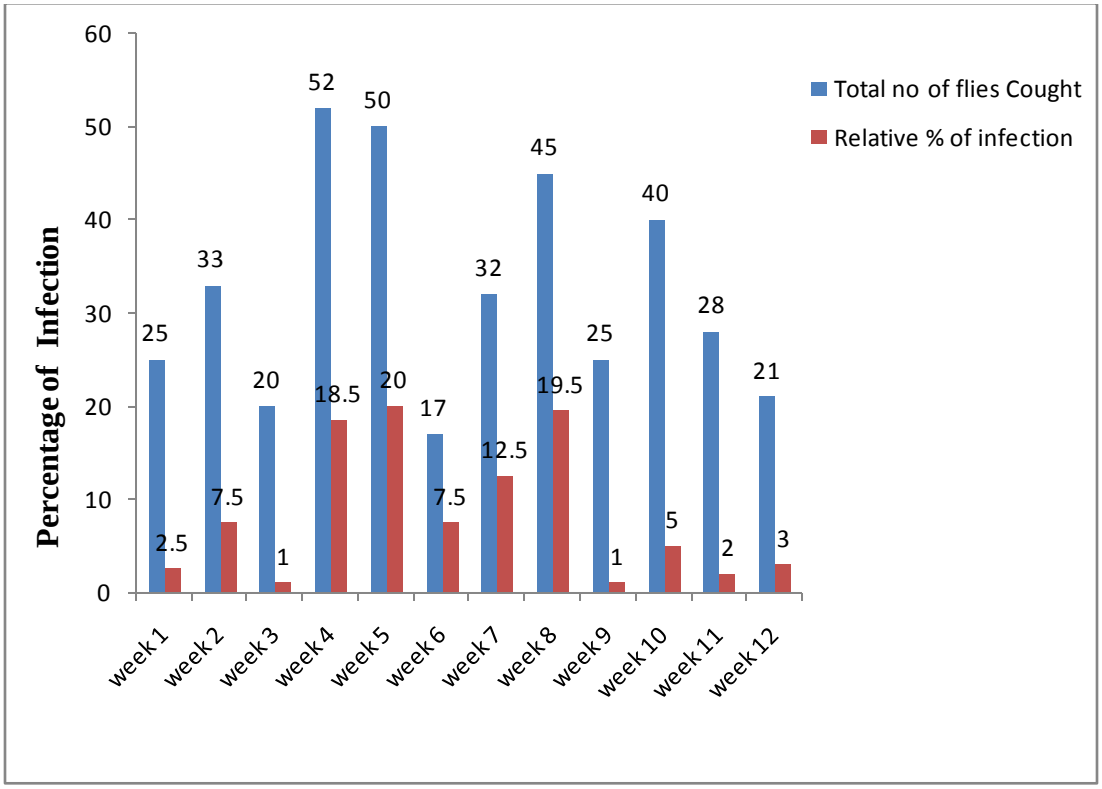


Figure 4.2: Weekly distribution of *Onchocerca volvulus* infected *Simulium damnosum*

Appendix 6: Distribution of isolated *Onchocerca volvulus***larvae in different segments of** *Simulium damnosum***harvested from the bank of River Kaduna under Yakowa Express Bridge and environs.**

The distribution of *O. volvulus* larvae in the three segments (head, thorax, and abdomen) of *S.damnosum*caught and dissected during the survey showed highest weekly and total *O. volvulus* larval infection rates in the abdomen, followed by the head and thorax. There was a gradual increase in the number of *O. volvulus* isolated in the heads and thorax of these flies from week four to week eight. An average of 2 to 4 infective stages of *O. volvulus*larvae was isolated from each morphological segment of each dissected female blackfly.

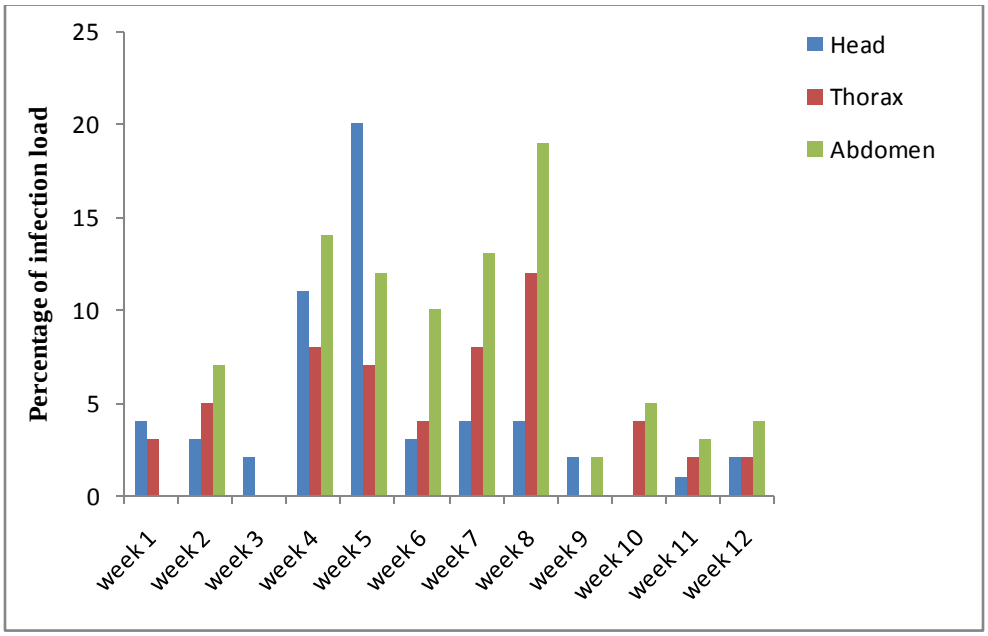


Figure 4.3: Weekly Isolation *Onchocerca volvulus* larvae in different segments of *Simulium damnosum*

Appendix 7: Optimization of Polymerase Chain Reaction for Detection of *OvGST1* Gene

Specific primers were used to amplify ***OvGST1*** gene of cDNA from *O.volvulus* with a typical size product, the observed band size was 114. Furthermore, primers were designed for species-specific amplification for confirmation and higher sensitivity. After optimization lanes 1, 2 and 4 represent amplicons from 1µg amounts of *O.volvulus* cDNA, no amplification was observed in the negative control.

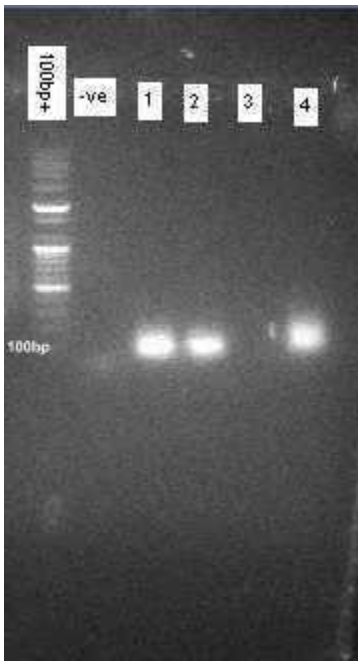


Plate II. The PCR products were analyzed via gel electrophoresis on a 1.5% Agarose gel stained with ethidium bromide (GibcoBRL Life Technologies, USA).

Lane (1) - *O. volvulus* microfilariae

Lane (2) - Adult *O. volvulus*

Lane (4) - L3 *O. volvulus* larvae

(–ve) - Negative Control with water

Appendix 8: Primer Sequence Compatibility Test Outcome against *OvGST1* Nucleotide Sequences

The *OvGST*1 gene is made up of 800 nucleotide sequences in length (size) and contains predominantly the coding sequences (CDS) that regulate *OvGST1* gene expression.

Primer Map results

Results for linear 800 residue sequence "gi|437890|emb|X75029.1| O.volvulus mRNA for glutathione S-transferase"

```
       1 TTTTATTACGATGAATTTTGTTGTTGAAGCTGCATCTAGCAATGCTAATCAGGCGATAAC
       1          10        20        30        40        50
       1 AAAATAATGCTACTTAAAACAACAACTTCGACGTAGATCGTTACGATTAGTCCGCTATTG
      61 TTCGGAAAACTCTATTAAGCCCAAAGGAAAGCTACAACCTCAAATGGAAAAGTACACATT
      61          70        80        90        100       110
      61 AAGCCTTTTGAGATAATTCGGGTTTCCTTTCGATGTTGGAGTTTACCTTTTCATGTGTAA
     121 AACTTATTTTAACGGTCGCGGTCGTGCCGAAGTAATACGATTATTATTTGCTCTGGCAAA
     121          130       140       150       160       170
     121 TTGAATAAAATTGCCAGCGCCAGCACGGCTTCATTATGCTAATAATAAACGAGACCGTTT
     181 TGTAAGTTACGAAGACAACCGTATCACTCGAGATGAATGGAAATATTTGAAACCCAGGAC
     181          190       200       210       220       230
     181 ACATTCAATGCTTCTGTTGGCATAGTGAGCTCTACTTACCTTTATAAACTTTGGGTCCTG
     241 ACCGTTTGGGCATGTTCCAATGTTGAACGTTAGTGGCAATGTATTAGGAGAGAGTCATGC
     241          250       260       270       280       290
     241 TGGCAAACCCGTACAAGGTTACAACTTGCAATCACCGTTACATAATCCTCTCTCAGTACG
     301 TATCGAACTTCTTCTAGGTGGAAGATTCGGTTTGTTGGGAACAAATGATTGGGAAGAAGC
     301          310       320       330       340       350
     301 ATAGCTTGAAGAAGATCCACCTTCTAAGCCAAACAACCCTTGTTTACTAACCCTTCTTCG
     361 AAAAATTATGGCAGTTGTTCTGAATATAGACGAACTTTTCCAGAAATTAATACCATGGAC
     361          370       380       390       400       410
     361 TTTTTAATACCGTCAACAAGACTTATATCTGCTTGAAAAGGTCTTTAATTATGGTACCTG
     421 ACATGAAAAAAACACTACCAAAAAGGCTGAATTATTCCGAAATCTTTCTGAAAGCGATGT
     421          430       440       450       460       470
     421 TGTACTTTTTTTGTGATGGTTTTTCCGACTTAATAAGGCTTTAGAAAGACTTTCGCTACA
                                  >>>OvGST1 F>>> 511 to 530
     481 AATGCCATTCCTTGGACGGTATGAAAAGTTCCTGAAGGAGAGCACTACCGGACATATTGT
     481          490       500       510       520       530
     481 TTACGGTAAGGAACCTGCCATACTTTTCAAGGACTTCCTCTCGTGATGGCCTGTATAACA
     541 CGGAAATAAGGTATCCGTAGCAGATTTGACTGTTTTTAACATGCTCATGACTTTGGATGA
     541          550       560       570       580       590
     541 GCCTTTATTCCATAGGCATCGTCTAAACTGACAAAAATTGTACGAGTACTGAAACCTACT
                            <<<OvGST1 R<<< 623 to 643
     601 TGAAGTTAAGCTTGAGGAATATCCGCAACTAGCAAGCTTTGTTAACAAAATTGGACAAAT
     601          610       620       630       640       650
     601 ACTTCAATTCGAACTCCTTATAGGCGTTGATCGTTCGAAACAATTGTTTTAACCTGTTTA
     661 GCCCGGAATCAAGGAATGGATCAAAAAAAGACCAAAAACGTATTTCTAGATTGTTTGTTG
     661          670       680       690       700       710
     661 CGGGCCTTAGTTCCTTACCTAGTTTTTTTCTGGTTTTTGCATAAAGATCTAACAAACAAC
     721 TGATTCTCTCATTCAAGATTTTTCATTTAAAAAAAAGACATTTTGAATAAATTTTTCATC
     721          730       740       750       760       770
     721 ACTAAGAGAGTAAGTTCTAAAAAGTAAATTTTTTTTCTGTAAAACTTATTTAAAAAGTAG
     781 AAAAAAAAAAAAAAAAAAAA
     781          790
     781 TTTTTTTTTTTTTTTTTTT
```

Primer:	Sequence:
OvGST1 F	5'-CCTGAAGGAGAGCACTACCG-3'
OvGST1 R	5'-AACAAAGCTTGCTAGTTGCGG-3'

Appendix 9. Morphology of Life Cycle Stages of *O. Volvulus* Critical for the Establishment of Infection

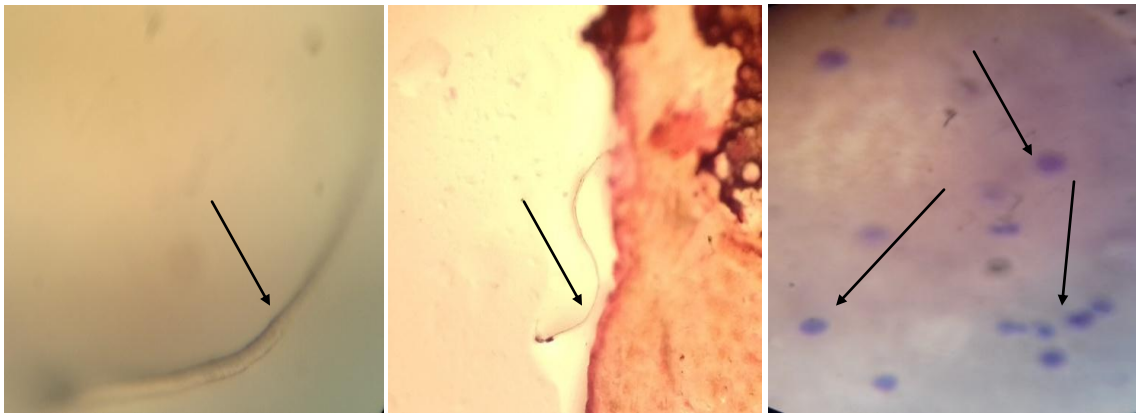


Plate III. (A). Adult *O. volvulus* under oil immersion at x100 (B). *O. volvulus* microfilaria migrating from skin snip at x50 (C). Giemsa Stained L3 microfilariae larvae from proboscis of *Simulium* flies at x50.

Appendix 10: Ethical Clearance Obtained from Kaduna State Ministry of Health.

MINISTRY OF HEALTH, KADUNA STATE

All Communication to be addressed to:
THE HON. COMMISSIONER
Quoting Reference and Date
Telephone: 234-248048
Website: ttp://www/moh.kd.gov.ng.
Email: info@moh.kd.gov.ng.

Independence Way,
P.M/;B 2014
Kaduna.
Kaduna State, Nigeria.

MOH/ADM/744/VOL.I/268

22nd April, 2015

Health Research Ethical Committee Kaduna State Ministry of Health.

To.....

Ministry of Health Research Ethical Clearance.

RE: COMPARATIVE STUDY OF GLUTATHIONE-S-TRANSFERASE (OvGST) IN THE DEVELOPMENTAL STAGES OF *Onchocerca volvulus* IN THE HOST (HUMAN) AND VECTOR (*Simulium flies*)

This is to inform you that the research described in the submitted protocol, the consent forms and other participant information materials have been reviewed and given full approval by the Health Research Ethics Committee.

Name of Investigator	Galya, Daniel Danladi
Address of Investigator:	Biochemistry Department, Ahmadu Bello University, Zaria
Date of Receipt of Application:	22nd March, 2015
Date of Ethical Approval:	1st April, 2015
Research Period	9 months

You are kindly requested to give researchers maximum cooperation during the period of research.

However, you are kindly requested to submit a copy of your findings to the state Ministry of Health please.

F. A. Kida (Mrs.)

Secretary, Research Ethical Committee

MOH/ADM/744/VOL.I/268

22nd April, 2015

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To.....

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F. A. Kura (Mrs.)

Secretary, Research Ethical Committee