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TRANSMISSION OF HUMAN AND ANIMAL ONCHOCERCIASIS AND MOLECULAR DIAGNOSIS OF *ONCHOCERCA* SPECIES IN SOME GHANAIAN COMMUNITIES

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENTOF THE REQUIREMENT FOR THE AWARD OF MASTER OF MPhil ZOOLOGY DEGREE

DEPEARTMENT OF ANIMAL BIOLOGY AND CONSERVATION SCIENCE (DABCS), UNIVERSITY OF GHANA, LEGON

JULY, 2015

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DECLARATION

I do hereby heartily declare that all experimental works described in this thesis were carried out by myself with exception to references made to other people's work published or not but who have all been duly acknowledged. This thesis has never been submitted anywhere else for the award of similar or different degree neither in whole nor in part.

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DEDICATION

With utmost humility and appreciation, I dedicate this work to my father Mr. Mohammed Yakubu and my mother, Amina Mohammed for their support. May Allah bless them.

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ACKNOWLEDGEMENT

With sincere gratitude I want to thank my principal supervisor and sponsor, Dr. Mike Yaw Osei-Atweneboana of Council for Scientific and Industrial Research (CSIR) for giving me the moral and financial support. I also want to thank him for his tolerance and Mentor –mentee relationship he has demonstrated to me for the past four years I have known him. Secondly I would like to thank Dr. Fred Aboagye-Antwi of Department of Animal Biology and Conservation Science (DABCS) University of Ghana for all that he has thought in both in academia and beyond.Special thanks to my lecturers Dr. Langbong Bimi and Dr.Kwansa Bentum for their patience and support. I also want to thank the people of Ohiampe, Mantukwa, Asubende and all the other communities we worked with especially the fly collectors for their immense support. Special thanks to my course mates Mr Eric Kyei-Baffour and Mr John kanamentie. A special thanks to Mr. Kojo Frimpong of Noguchi Memorial Institute and Mr. Francis Veriegh for their support both in the laboratory and on the field. I want to also thank Mr. Edward Jenner Tettevi, Mr Hamidu Bukhari, Mr. Samuel Armoo, Ms Pamela Selormey, Mr Ernest Tawiah Gyan, Mr Bright Idun, Mrs Rhoda Lims Sakyi and all my colleagues at Water Research Institute-(CSIR). I remain indebted to you all. Above all I want to say all thanks and praises are due to Allah for the bountiful mercies and good tidings.

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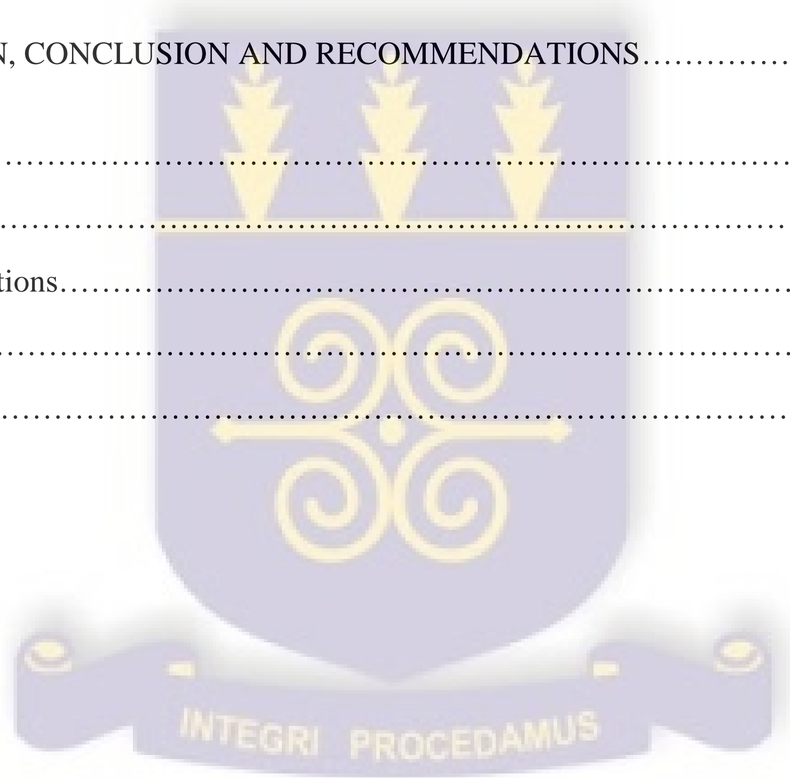

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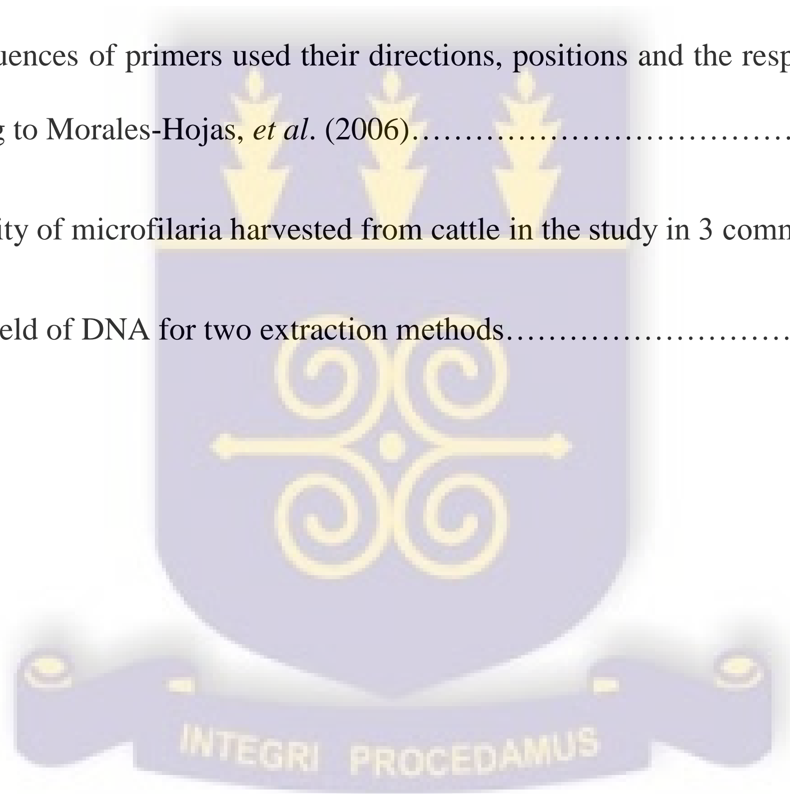

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LIST OF ABBREVIATONS

APOC African Programme for Onchocerciasis

CDTI Community Directed Treatment with Ivernmectin

DEC Diethylcarbamazine citrate

DALY Daily Adjustment Life Years

DNA Deoxyribonucleic Acid

dNTPs Deoxynucleotide Triphosphates

L_1 First Stage Larvae of *Onchocerca volvulus*

L_2 Second Stage Larvae of *Onchocerca volvulus*

L_3 Third Stage Larvae of *Onchocerca volvulus*

MBR Monthly biting Rate

$MgCl_2$ Magnesium Chloride

MTP Monthly Transmission Potential

NADH Nicotinamide Adenine Dinucleotide

OCP Onchocerciasis Control Programme

OEPA Onchocerciasis Elimination Programme for the Americas

OSD Onchocercal Skin Disease

PCR Polymerase Chain Reaction

RAPD Random Amplified Polymorphic DNA

RNA Ribonucleic Acid

SIZ Special Intervention Zone

TDR Tropical Disease Research

UNDP United Nations Development Programme

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UV Ultra Violet

WHO World Health Organization

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ABSTRACT

Control of onchocerciasis over the past 3 decades has brought great relief to millions of people living in endemic communities, by substantially reducing blindness and other dermatological lesions. However, there are still areas where infections in human populations are high and vector transmission is ongoing, requiring a further understanding into the transmission dynamics of the disease in such communities. Furthermore the status of animal onchocerciasis is still unknown in Ghana even though there is a high cattle population in our human onchocerciasis endemic areas who serve as reservoirs for animal onchocerciasis (*Onchocerca ochengi*) as well. Adult female black flies were caught for microscopic assessment of infection in 12 onchocerciasis endemic communities in the dry season months of March, 2014 and February, 2015. Animal onchocerciasis was assessed in nineteen cows from three endemic communities for the presence of *O. ochengi.* Molecular biology techniques were employed to assess the status of infection in cattle while onchocercal larvae was introduced to nulliparous black flies and processed for molecular identification of *onchocerca* spp. A total of 9,343 adult female black flies were collected from all the 12 communities. The Monthly biting rates in all 12 communities were above the WHO threshold biting rate (TBR) of 1000 bites per person per annum in 2014, while in 2015, seven communities had MBR above the WHO threshold. Out of a total of 2,046 parous flies dissected, only 5 infective flies (0.05 %) were found, these flies harboured 11 third stage infective larvae which were all from Agborlekema I. Also, *Onchocerca* microfilarial infections in cattle were found only in Agborlekema I. The PCR-based assay carried out based on *12S* mitochondrial RNA gene using DNA from microfilaria obtained from cattle have

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validated the presence of *Onchocerca ochengi* while DNA obtained from larvae obtained from black flies have validated the presence of onchocercal species in the vector in Ghana. Since black fly vectors transmit various *Onchocerca* spp., including *O.ochengi and O. volvulus*, it is possible that the infected cattle at Agborlekema I might have contributed to most of the transmission recorded in this study. It is therefore necessary that transmission studies should consider molecular diagnoses of infective *Onchocerca* larvae. This will help evaluate the goal of elimination efforts in Ghana. Further studies are required to discriminate O. *volvulus from O. ochengi.*

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 INTRODUCTION

Onchocerciasis commonly known as river blindness is a parasitic disease endemic to 30 countries in sub-Saharan Africa, 6 countries in Latin America, and Yemen. Currently, the disease is known to infect about 37 million people globally while more than 120 million people are at risk of the infection (Basanez *et., al.* 2006; Brattig, 2009; Gustavsen *et al*., 2011). About 99% of the infection occurs in rural Africa (Brattig, 2009; Gustavsen *et al*., 2011; Mackenzie *et al*., 2012; WHO 2009). The disease poses a serious public health problem and creates an obstacle to socio-economic development, particularly in Africa (WHO, 2002).

Onchocerciasis is caused by the filarial nematode *Onchocerca volvulus* and transmitted by the black fly vector belonging to the genus *Simulium.* The black flies breed in fast flowing rivers and streams and thereby predisposing communities living around riverine areas to infection (Katabarwa *et al*., 2008; CDC 2010; WHO, 2011; Mackenzie *et al*., 2012).

Onchocerca volvulus inhabits the subcutaneous and deeper tissues of the human host where the female adult worms release millions of microfilariae into the skin and eyes of the human host (WHO, 1995).These microfilariae are responsible for the pathology of the disease, the clinical features of the onchocerciasis include visual impairment, permanent blindness and pathological changes in the skin and other organs. So far *O. volvulus* is the only species of the genus *Onchocerca* infecting man, while blackflies serve as the intermediate host. The feeding habits of the adult female black flies guarantee their role in

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the transmission of onchocerciasis. The infection process begins with contact between *Simulium* vectors and *O. volvulus* through the ingestion of blood meal from an infected human host. . Within the vector, the parasite develops into the infective third stage larvae (L3) after few morphological transformations. Subsequent contact between an infective black fly and a susceptible human host during a second blood meal culminates in the introduction of the infective larvae (L3) into the skin, where they penetrate into the bite wound, moult twice and develop into the adult worm (Hunter, 1996; Bradley *et al.,* 1993)

The zoophilic mode of feeding among onchocerciasis vectors is an essential factor of epidemiological significance, since all filarial larvae found in man-biting simuliids are not *O. volvulus* but morphologically indistinguishable. Thus, the estimation of transmission indices based on morphological identification of infective filarial larvae is unreliable and might inflate transmission parameters. The animal parasites, *O. gibsoni and O. ochengi,* are closely related to the human-parasitic *O. volvulus* with close taxonomic relationship based on phylogenetic distance (Nelson and Pester, 1962; Bain, 1981; Keddie *et al.,* 1998; Morales-Hojas and Cheke**,** 2006). This reduces the vectorial capacity of *Simulium* spp for *O. volvulus* but make them important in the transmission of animal filariae. In Cameroon two filarial parasites, *O. ochengi* and *O. ramachandrini* have been identified to infect cattle and wart hogs respectively (Wahl *et al.* 1998a), and in Northern Cameroon the microfilariae prevalence of *O. ochengi* in cattle have been observed as high as 66–71 % (Trees *et al.,* 1992; Renz, 1994). However, in Ghana there has not been documented evidence verifying the presence of *O. ochengi* in cattle population. Lack of information on the transmission of animal onchocerciasis in Ghana may frustrate the control of human onchocerciasis, especially since there is a paradigm shift from morbidity control to elimination of onchocerciasis in Ghana.

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Large-scale control of onchocerciasis commenced in 1994 by the Onchocerciasis Control Programme (OCP) in savanna regions of eleven West African countries including Ghana The then control strategy was mainly based on vector control using aerial spraying of vector breeding sites with environmentally friendly insecticide until 1987 when ivermectin was approved for treating the disease. Since then treatment strategy has been either ivermectin mass treatment alone or a combination of ivermectin and vector control. Currently community directed treatment with ivermectin is the only available control for onchocerciasis with the recommended annual dose of 150 µg/kg body weight. Ivermectin eliminates skin microfilariae and maintains low levels of microfilaridermias for up to 9 months as well suppressing embryogenesis in the adult female worms. (Awadzi *et al.* 1989; 1985; Duke *et al*. 1992 and Alley *et al*. 1999). The control of onchocerciasis over the past 3 decades has brought great relief to millions of people living in infected communities, by substantially reducing blindness, visual impairments and other dermatological lesions as well as prevalence and intensity of infection (Osei-Atweneboana *et al*., 2007). However, there are still areas where infections are high and transmission is ongoing (Basanez *et al*. 2006).

The World Health Organization's (WHO) road map to accelerate progress for overcoming the impact of NTDs have set goals for the elimination of human onchocerciasis by 2020 in selected African countries (WHO, 2012). To achieve this, there has recently been a shift in onchocerciasis control strategy, changing from prevention of morbidity towards elimination of infection, by switching from annual to bi-annual (twice yearly) ivermectin treatment.

To make the endgame of elimination achievable, there is the need for a sensitive and effective diagnostic tool for detection of *O. volvulus* parasite at low levels of infections in

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both human and vector population. (Awadzi *et al.,* 2004a, b; Osei-Atweneboana *et al*., 2011). However, the detection of *O. volvulus* in the vectors presents a huge challenge because the same vector transmits both human and animal onchocerciasis.

Currently, molecular assays are being used for identification of *Onchocerca* spp present in the vector using the 0 -150 repeat sequence developed over two decades ago (Meredith *et al.,* 1991; Zimmerman e*t al*.1994; Merriweather *et al*., 1996; Unnasch & Meredith, 1996 ; Katholi e*t al.,* 1995;Yameogo *et al*., 1999).These methods involve polymerase chain reaction (PCR) amplification using some selected genes and subsequently species identification using various methods including the application of enzyme-linked immunosorbent assays (PCR-ELISA) (Gopal *et al*., 2012; Guevara *et al.* 2003; Rodriguez-Perez *et al*., 2013). Most of the genes selected for molecular diagnoses of the *Onchocerca* spp are from the mitochondrial DNA sequences. Mitochondrial sequences are often useful tools for phylogenetic studies, as they are generally maternally inherited and therefore not subject to the diversity generating mechanisms associated with sexual re-assortment (Avise *et al.,* 1987). Furthermore, the mitochondrial genome accumulates mutations at a faster rate than nuclearly encoded sequences, making mitochondrial sequences a useful source of phylogenetic data for differentiating closely related taxa (Brown *et al.,* 1979).

Thus, there are different molecular assays using multiple techniques for the identification of *Onchocerca* spp. However, there are insufficient studies that examine a simple PCR based assays to differentiate *Onchocerca* spp as a viable option of diagnosis (Rishniw *et al.*, 2006) for a less resourced research environment.

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1.2 Justification

Almost all vectors of human onchocerciasis are zoophilic, and as such can be infected with animal *Onchocerca* species. For instance, a considerable proportion of infective filarial larvae found in *S. damnosum* s.l. vectors were not of the human parasite *O. volvulus* (Wahl and Schibel, 1998). The presence of these non- *O. volvulus* larvae among vector populations may affect the accuracy of measurements of human onchocerciasis transmission especially during period of control campaigns with the far reaching epidemiological implications of overrunning programmes timelines. Morphologically, filarial parasites of both human and animal origin are indistinguishable from each other. There is therefore the need to develop diagnostic tools particularly using molecular technique for easy species discrimination in order to distinguish between the *Onchocerca* spp. The information generated from this study will be of importance in planning and managing the control of onchocerciasis.

This study seeks to investigate the transmission of human and animal onchocerciasis in some endemic communities, assess the transmission status and the transmission indices using morphological identification of *Onchocerca* spp. Also molecular identification techniques would be carried out to detect *Onchocerca* spp. from the black flies and finally discriminate between *O. volvulus* and *O. ochengi*.

1.3 General Objective

The main objective of the study is to assess transmission of human and animal onchocerciasis, estimate transmission parameters and use molecular diagnostic assay to identify *Onchocerca* spp (third stage larvae) in the blackfly.

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1.3.1 Specific objectives

1. To assess the transmission of human and animal onchocerciasis in the study communities.

2. To assess the relative abundance of black flies and the various transmission indices necessary for the effective monitoring of human onchocerciasis.

3. To compare different DNA extraction techniques for high DNA yield per individual *O. volvulus larvae* and black flies.

4. To optimize various molecular techniques and PCR protocol to identify the presence of *Onchocerca* spp. in black flies.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Human and Animal Onchocerciasis

2.1.1 Etiology and Distribution of Onchocerciasis

Onchocerciasis, caused by infection with *Onchocerca* parasites, is one of the most significant neglected tropical diseases in the world, and is the second leading cause of infectious irreversible blindness. The disease is endemic to 30 countries in sub-Saharan Africa where more than 95% of those infected are found, 6 countries in Latin America, and Yemen. Currently, the disease is known to infect about 37 million people while more than 120 million people are at risk of acquiring the infection (Basanez *et al.,* 2006; Brattig, 2009; Gustavsen *et al*., 2011). The *Simulium* black fly vector which transmits the infection from person to person breeds in fast-moving rivers thereby predisposing communities living around river banks to a higher risk of infection (Katabarwa *et al*., 2008; WHO, 2011). This has been a drastic reduction in socio-economic productivity due to the abandoning of fertile arable land due to the fear of infection (OEPA, 2008). The disease is considered chronic and characterized by itchy skin progressing to serious pathological skin changes and blindness (Bradley *et al*. 2005). Onchocerciasis has also been found to have an effect on the quality of life of infected individuals and has also been reported to shorten the average life expectancy of individuals with high microfilarial load in endemic communities (Udall, 2007).

The genus *Onchocerca* consists of 28 parasitic species (Chaubaud and Bain, 1994), out of which only one *O. volvulus*, the causative agent of human onchocerciasis infect humans while the rest infect ungulate mammals (Morales-Hojas *et al.,* 2006). Infection by these ungulate species

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presents lesions that can affect animal health and degrade the value of meat and carcasses (Muller, 1979).

The human onchocercal disease is transmitted through the bites of haematophagous black flies belonging to the family Simulidae and suborder Nematocera. Microfilariae are ingested by adult female black fly during a bloodmeal and migrate to the flight muscles and develop into infective 3rd stage larvae in about one week. The larvae are inoculated into another human host during a subsequent bloodmeal (Bradley *et al*. 2005). In West Africa, *S. damnosum* species complex, which is the largest of all known species complexes of any vector (Post *et., al* 2007), consists of about 55 named cytoforms, and these are responsible for over 55 percent of the global onchocerciasis transmission (Crosskey and Howard, 2004). In the Western hemisphere the situation is complicated by the presence of a large number of man-biting species. However in the Americas, *O. volvulus* is transmitted by several *Simulium* species complex. Vectors of onchocerciasis in southern Mexico and Guatemala comprises members of the *S. ochraceum* species complex (Ortega and Oliver, 1985), members of the *S. mettalicum* species complex maintain transmission in northern Venezuela (Grillet e*t al* 1994), while *S. exiguum* species complex are the main vectors in Colombia and Ecuador. Transmission within the Amazonian focus between southern Venezuela and northwestern Brazil is maintained by *S. guianense* species complex (Basanez *et al*. 1988). With an additional *S. haematopotum* and *S. oyapockense* in Guatemala and/or Venezuela.

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1997 2002

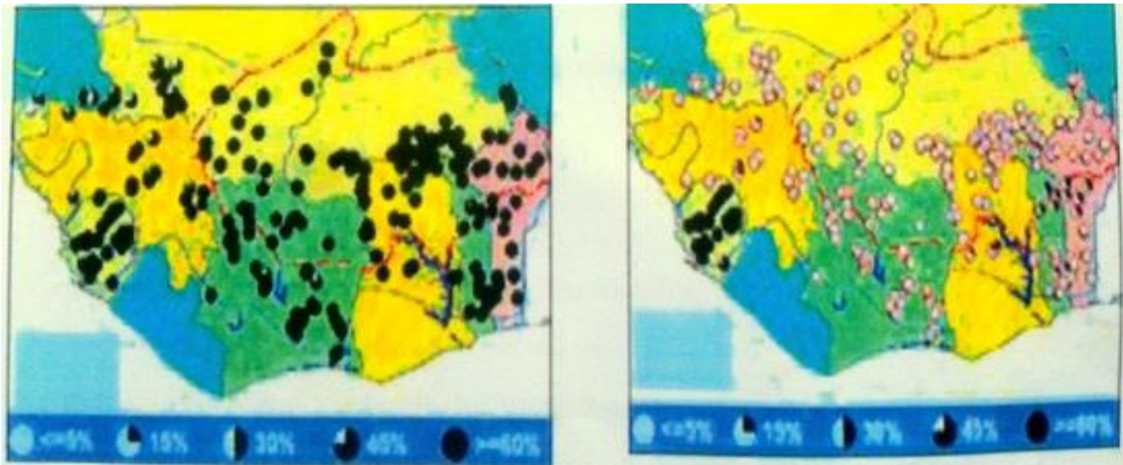

Figure 2.1: The distribution of onchocerciasis in 1997 and 2002 in some parts of the world. Source; Oncho Coordination unit, World Bank

Figure 2.2: Global distribution of Onchocerciasis (WHO, 1995)

The distributional range of onchocerciasis lies between 15° N and 15° S of the equator ranging from Senegal to Malawi. In West Africa, the distribution of the disease is in relation to the topography and river size (Thomsom *et al.,* 2004). The range of each vector species in relation to the West African vegetation type is also influenced by seasonal climatic variations, changes in wind movement as well as river levels (Boakye *et al.,* 1998). The most widely distributed species of the *Simulium damnosum* complex are *S. damnosum* s.s. and *S. sirbanum* which are associated with savannah onchocerciasis whereas some species have limited distribution. In South and

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Central America, the disease is focally distributed with a prevalence of approximately 100,000 (WHO, 1987).

2.1.2 Manifestations and pathogenesis of onchocerciasis

The adult male and female *Onchocerca volvulus* resides in subcutaneous nodules where they produce thousands of microfilariae which invade the skin and eyes (Basanez and Boussinesq, 1999; Bradley *et al*. 2005). The microfilariae survive for a year and must be ingested by a black fly vector to continue development or they die within the skin and eye tissues, provoking an immune response believed to be responsible for inflammatory lesions leading to overt manifestations (Basanez *et al*., 2006). There is growing evidence to show that an obligatory endosymbiotic rickettsial bacteria, *Wolbachia* which lives in *Onchocerca* and other filarial nematodes is largely associated with inflammatory responses that may be involved in the pathogenesis of onchocerciasis (Saint Andre *et al*., 2002).

The main clinical manifestations of the human onchocerciasis are characterized by troublesome itching and skin changes such as early-stage reactive lesions and late-stage depigmentation often referred to as leopard skin and atrophy (Turner *et al*., 2014). The most severe complications of onchocerciasis however is an irreversible ocular lesion of both the anterior and posterior segment of the eye, resulting first in impaired vision and eventually total blindness (Basanez *et al*. 2006).

Quite recently, reports have shown that glaucoma patients living in some endemic Ghanaian communities had a higher prevalence of onchocerciasis even after adjustment for age, region and gender (Egbert *et al*., 2005). Elsewhere in Africa, studies have shown that onchocerciasis is associated with musculoskeletal pains with both men and women weighing less than uninfected cohorts. Similarly, evidence from Uganda and Burundi suggested a possible association between onchocerciasis and epilepsy (Hogarth *et al.,* 1998).

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Animal Onchocerciasis

In the savanna areas of tropical Africa, cattle are frequently infected with the filaria *Onchocerca ochengi*. This parasite is closely related to *Onchocerca volvulus*, the causative agent of human onchocerciasis (river blindness), and is capable of developing in the same vector, *Simulium damnosum* s.l . It is morphologically indistinguishable from the human parasite *O. volvulus* (Bain, 1981); induces like *O. volvulus* and the formation of nodules around the adult worms in Togo, Mali and Cameroon (Denke & Bain, 1978; Omar &Denke et al. 1979). Wahl& Schibel, (1998) reported the identification of identified Type G as *Onchocerca ochengi* from cattle. *Onchocerca. ochengi* has attracted significant attention because its larval stages maybe mistaken for those of *O. volvulus* during entomological assessments of human onchocerciasis (WHO,1993)

In Ghana the presence of animal onchocerciasis has never been confirmed. Most studies conducted in Ghana has extensively dwelled on human onchocerciasis. All infective filariae larvae found in the *Simulium damnosum* vector are considered as *Onchocerca volvulus*. This should not be the case because the cattle population in these endemic communities is high which could serve as reservoir for animal filariae especially *Onchocerca ochengi*.
In North Cameroon,Wahl's (1998a) assessments over the years have shown that a considerable proportion of the infective filarial larvae found in the black fly vectors of human onchocerciasis did not belong to the human parasite, *Onchocerca volvulus*. Since these larvae did not correspond to any of the known filarial parasites of man, they were thought to belong to filariae of animals.

In the dry season, there was an increase in the flies' biting rate on cattle resulting in a 36-fold increased daily transmission potential of *O. ochengi* on cattle as compared to the rainy season. This is probably due to the flies taking many more bloodmeals on cattle in the dry season, because in this season the cattle are brought daily to the river.(Wahl 1998a,1998b).This informed

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our decision to carry out our study in the dry season in Ghana as well to increase our chances of detection of animal onchocerciasis as this has never been confirmed in Ghana.

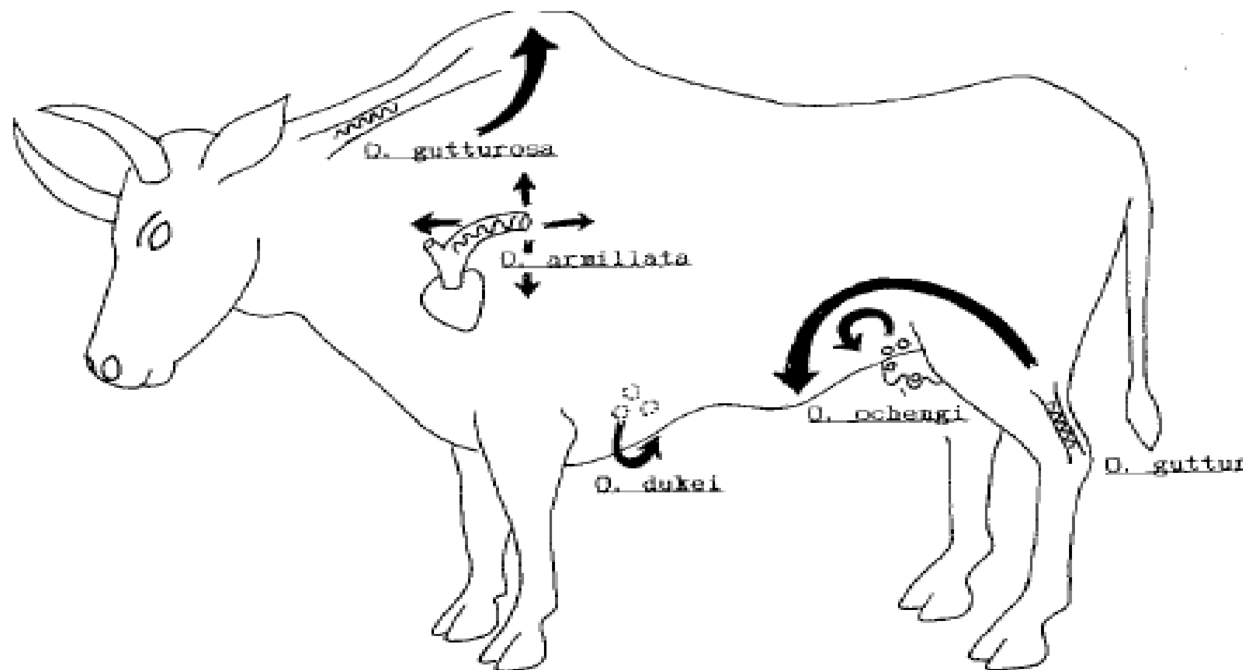

Figure 2.1.2: Predilection site of Onchocerca adult worms and microfilaria in Zebu cattle in Northern Cameroon. Source: Wahl *et al*., (1994)

2.1.3 Onchocercal Ocular Disease

Corneal inflammation (keratitis) is a major cause of visual impairment in *O. volvulus* infection. Two distinct forms of corneal diseases are recognized: reversible punctate keratitis and the irreversible more severe sclerosing keratitis, which has a permanent effect (WHO, 1987). Even though the exact role of *Wolbachia* in the pathogenesis of onchocerciasis is not exactly known, some information suggests that neutrophil activation by *Wolbachia* organisms contribute to the pathogenesis of ocular onchocerciasis (Gillette-Ferguson *et al*., 2004). These obligatory bacteria live inside *O. volvulus* in a symbiotic relationship and are essential for female worm's fertility and reproduction (Taylor and Hoerauf, 1999). Therefore the depletion of these bacteria has been targeted as a novel therapy human onchocerciasis (Saint-Andre *et al.,* 2002; Taylor, 2003).

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The exact mechanism of invasion of the eye by microfilariae from the skin is not yet been understood but, Duke and Anderson (1972) demonstrated that when microfilariae migrate from the conjunctiva or the skin into the cornea, a punctate keratitis develops around dead microfilariae. Continual exposure to heavy worm load results in the development of sclerosing keratitis and iridocyclitis, causing visual impairment and eventual permanent blindness. Opacification of the cornea progresses centrally along with deep vascularization that obscures the entire cornea, causing a complete loss of vision (Abiosse, 1998; Pearlman and Lass, 1994). Although onchocerciasis is associated with eye and skin lesions, in West Africa savanna areas, eye lesions are more prevalent leading to blindness whereas skin lesions are more predominant in the forest bioclimatic zones.

Inflammatory responses may differ predominantly from person to person depending on length of exposure to antigens and the down-regulating activities by the host immune system. The incidence of blindness of onchocerciasis increase as prevalence and intensity of infection increases in a given community, especially in the savanna areas. The relative impact of both ocular and dermal symptoms to the burden of onchocercal disease and their socio- economic consequences are recognizably high (Kale, 1998).

2.1.4 Dermal Manifestation

The skin is the principal site of infection and a broad spectrum of onchocercal-induced skin manifestations. Skin lesions appear to be the most pervasive consequences of onchocerciasis because some surveys show that between 40-50 % of adult populations from selected endemic

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African countries had severe itching with some patients compelled to sleep on their elbows and knees (Gleich *et al*. 1993). Onchocercal skin disease (OSD) is therefore strongly correlated with psychosocial problems, ostracism as well as stigmatization of infected individuals (Vlassoff *et al.* 2000). The pathology of onchocercal skin diseases (OSD) is associated with generalized, lichenified onchodermatitis and depigmented skin condition known as “leopard skin”. With prolonged exposure to high intensity of infection, degenerative skin changes usually set in, destroying elastic fibres and leaving the skin thinned with a wrinkled appearance. The atrophied skin begins to sag, the most extreme state being “hanging groin” (Greene *et al.* 1983).In the more heavily infected persons living in endemic areas, scratching and excoriations to the point of bleeding is common. Localized rash and erythema–may be superimposed on the ongoing dermatologic manifestations at essentially any stage of the disease (WHO, 1999, Udall, 2007).Easily-appreciable socio-economic impacts therefore arise from onchocercal skin disease, especially on the productivity of farmers, breastfeeding mothers and school attendance by children (Kim *et al*. 1997).

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2.1.5 Epidemiology and Disease Burden of Onchocerciasis

Worldwide, it had been estimated that onchocerciasis is the second leading infectious cause of blindness (Sommer, 1998). Out of the 36 endemic countries, 30 are in sub-Sahara Africa where about 99% of all those infected live (Etyalale, 2002). Africa has about 96% of the total at-risk population, and at least one million are either blind or severely visually disabled. Recently, Basannez *et al.* (2006) made a surprising finding which counteracted what was formerly seen as the most reliable estimate by the World Health Organization in 1995, which showed that 17.7 million people were infected with the disease and of whom about 270,000 were blind and another 500,000 were severely visually impaired (WHO, 1995). They revealed that 37 million people are having onchocerciasis, with another 90 million at risk in Africa, with about 400,000 people at risk of contracting the diseases in the South America Arabian Peninsula.

Countries with the highest historical prevalence of onchocerciasis include 11 sub-Sahara Africa nations such as Nigeria, Ghana, and Cameroon. Onchocercal blindness may affect one-third of the adult population of the most highly affected communities, however skin disease is responsible for 60% of lost Disability Adjusted life years (DALYs) (WHO, 2009). However, the prevention of blindness was the main clinical reason for initiating the Onchocerciasis Control Programme (OCP) in West Africa in 1974 (Benton, *et al.* 2002).

Males are more exposed to infective bites from the vectors by the nature of their occupation sites, which are usually located around the vector breeding areas. There is a steady build-up of microfilariae in their skin and elsewhere and so adult males primarily go blind. High microfilarial loads also have negative effects on the life expectancy of infected ones who have not yet lost their sight (Little *et al.* 2004). There are therefore obvious social and economic implications of such a high prevalence of blindness, affecting the working age population. The high rate of mortality among the blind, particularly among males may be significantly high in endemic communities (Pion *et al.* 2002).

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Onchocerciasis is deemed responsible for the annual loss of approximately 1 million DALYs, healthy life years lost due to disability and mortality, more than half of them due to skin diseases (Remme, 2004). It demonstrated to have more than just a negative effect on the quality of life, it also appears to shorten life span. Little *et al*., (2004) found an association between *O. volvulus* microfilarial load and all–cause mortality, stating that 5% of the deaths in the temporal and regional boundaries of their study areas were attributable to *O. volvulus* infection and that blindness per se did not have a significant effect on mortality when adjusted for microfilarial load. Low productivity, low income and higher health related costs are found among infected persons (Prost, 1986; Pion *et al.,* 2002).

2.1.6 Disease Burden and Socio-economic Impact of Onchocerciasis

The true burden of onchocerciasis on the economies of affected communities has largely been underestimated (Noma *et al.,* 2001). The World Health Organization over the past four decades has revealed that more than 60% of people in some savanna populations investigated were infected with *O. volvulus*, 10% of the adult population, half of which are males aged over 40 years were blind, with an additional 30% of the people suffering from visually impairment, with early signs of the disease becoming common among children (WHO, 1991).

These socio-economic consequences of onchocerciasis on the development and public health systems and of endemic communities have been debilitating. This situation leads to depopulation of otherwise fertile arable lands for fear of acquiring the infection (Hervouet and Prost, 1979). The annual loss of over 1 million adjusted life years, greatly reduced income generating capacity of the affected communities (Oladepo *et al.* 1997). Children are more likely to drop out of school due to stigma associated with onchocercal skin disease and farmers have about 30% less land under cultivation (leading to an estimated loss of US $300 million on annual basis (WHO, 2004).

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2.2 Vectors of Onchocerciasis

Black flies (Diptera: Simuliidae) of the *Simulium damnosum* Theobald complex are the only vectors of human onchocerciasis in West Africa (WHO, 1995; Boakye *et al*. 1998). Ever since the pioneering work incriminating the *S. damnosum* as vectors of onchocerciasis, it was recognized that *S. damnosum* was a species complex and Vajime and Dunbar (1975) described nine distinct species in West Africa using variations in the banding pattern of larval polytene chromosomes. Two of the nine species namely *S. damnosum* s.s. and *S. sirbanum* have been designated as savannah cytospecies whereas *S. squamosum, S. sanctipauli, S. yahense, S. soubrense, S. konkourense, S. leonense* and *S. dieguerense* are described as forest cytospecies on the basis of the difference in the larval polytene chromosomes (Boakye and Meredith, 1993). In contrast to cytospecies, cytotypes are chromosomally distinctive populations of unknown specific status. Cytospecies and cytotypes are together known as cytoforms (Post *et al.* 2011). The *S. damnosum* complex is made up of at least 55 named cytoforms, forming the largest sibling species of any known disease vector (Post *et al.* 2007). All nine species described in West Africa are vectors of human onchocerciasis however at varying degrees of competence (Boakye and Meredith, 1993).

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2.2.1 Life Cycle of *Simulium damnosum* species complex.

The *Simulium* fly is a holometabolous insect whose developmental life cycle passes through four different stages namely; egg, larva, pupa, and adult stages. All stages except the adult stage have an association with fluvial ecosystems. A fertilized adult female black fly deposits between 150-800 eggs on partially submerged objects such as stones, woods, vegetation etc., glued by means of sticky substances. The eggs hatch in about 1-3 days Members of the *S. damnosum* s.l. have seven larval instars (Petry *et al.,* 2006). The puparium emerges from the seventh larval stage into an adult in about 2-5 days. The larval stage takes about 7 to 14 days to moult into the pupal stage and the adult emerges after 2 to 5 days in the puparium (WHO, 1991). The properties of the breeding habitat sustaining the juvenile fly during these periods of development depend largely on the species and temperature (Hadis, 2005; Petry *et al.,* 2006). Pharate stages, a situation in which the fly has reached a physiological stage of pupa or adult but for the time being, still remains the seventh larval or pupa. The larvae later pupate under water and the emerging adult floats to the surface and then emerges in a bubble of air, and takes a mating flight (WHO, 1991).

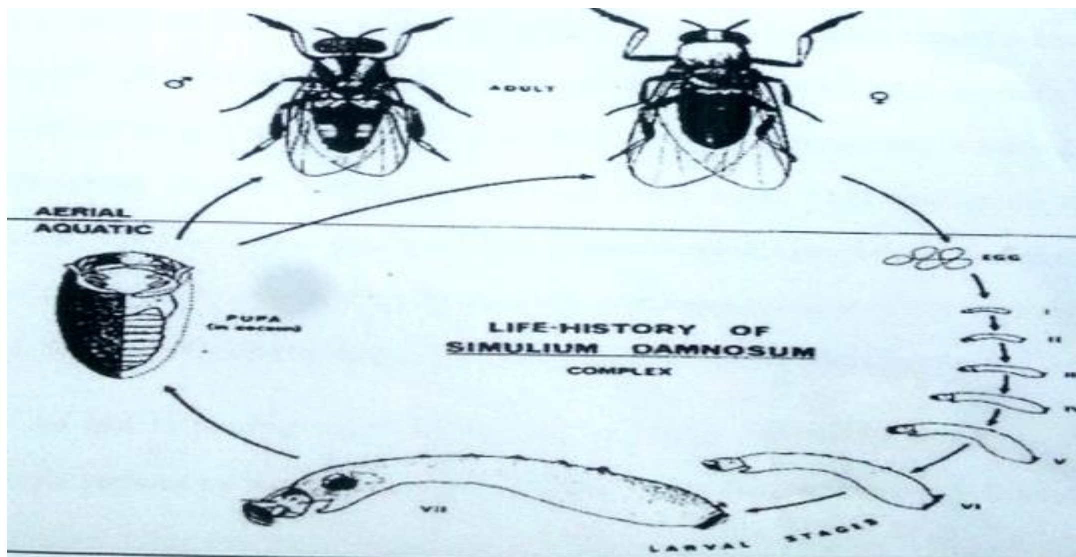

Figure 2.2.1: The life cycle of *S. damnosum* (WHO, 1991b).

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2.3 Vector-Parasite Interaction in Onchocerciasis

Vector-parasite interaction plays an important role in the epidemiology of vector-borne diseases. Vector-parasite interaction is an epidemiological term used to describe vector-parasite combinations that result in the development and transmission of the local parasite population (Duke, 1966). In terms of human onchocerciasis, the term *Onchoerca-Simulium* complexes describes the well-adapted parasite-vector systems in which the savannah form of the parasite develops successfully within the savannah species of the *S. damnosum* sensu lato (Duke, 1966; Basanez *et al*. 2009). Evidence from DNA analysis of a tandemly repeated sequence family of *O. volvulus* (O-150) has confirmed the existence of distinct rain forest and savanna parasite populations in West Africa with some barriers inhibiting genetic exchange between these two populations (Zimmerman *et al.* 1994). A series of cross-experimental studies has shown that the operation of *Onchocerca-Simulium* complexes is not limited to West Africa (Basanez *et al*. 2000). These results therefore suggest the operation of strong ecological adaptations between parasites and their local vectors within endemic areas (Basanez *et al*. 2009). These vector-parasite interactions in human onchocerciasis obviously have implication for the spread of infection outside current endemic areas (Basanez *et al*. 2000).

2.4 Identification of Simuliid flies

The widely practiced method for the identification of members of this family has been on the basis of their morphological variation. However, there are problems associated with the morphological identification of this group especially those belonging to the *Simulium damnosum* complex. The difficulties differ with species, for example the African *S. damnosum* s.l. can be readily recognized by morphological characters at the larval and pupal stage but not at adult stage. Other problems include the differences in behavior, ecological requirements, variations in habitat preference and genetic diversities of gene from one species to another.

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Onchocerciasis vector species are seldom isolated from other members of the Simuliidae that have not yet been implicated as vectors. In most zones where they occur, they usually cohabit with other innocuous members of the family (WHO, 1991). In 1926, Blacklock first incriminated a species of the *Simuliidae* as a vector of the parasite, *Onchocerca volvulus* .He described the development of the various stages of the causative filarial parasite in black flies of the *Simulium damnosum* complex (Blacklock, 1926).

2.4.1 The use of adult morphology in identification

Morphotaxonomy remains a crucial tool for the identification of adult black flies of the *S. damnosum* species complex. Where it works, morphotaxonomical identification is more preferable since it is simple, convenient for field use and its cheaper compared to other techniques (Wilson and Gomulski, 1993; Beebe and Cooper, 2000). This method of identification was reviewed by Wilson and Gomulski, (1993), who developed a new system of identification. They used a discriminant function analytical system (LDF) in discriminating the various species of the complex. The LDF proved efficient in the identification of the *S. damnosum* sub-complex, *S. squamosum* and *S. yahense* with an overall correct classification of over 98% (Wilson, *et al.,* 1993). This identification system was employed for routine use by the Onchocerciasis Control Programme (OCP) (Ibeh *et al*. 2008).

To understand the sources of recrudescence in areas where vector control have been successful, the identification of adult flies is imperative (Baker *et al.* 1987). Considering the rapid rate of locality related changes in population structures of female adult flies, proper identification of adult flies caught during human bait collection is imperative (Post and Boakye, 1992).

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Epidemiological contributions are species-dependent and so not all sibling species are equally important.

The relative simplicity with which the use of morphological identification is related (Wilson *et al.* 1993) makes it an efficient tool especially for use in the field. Generally, the following characters have proven very essential:

1. The colour of the fore coxa, scored as pale or dark (Wilson *et al.,* 1993).
2. The arculus scored as pale, intermediate or dark (Wilson *et al.,* 1993).
3. The stem vein setae (wing tufts) also scored on a scale A-E (A, all pale; B, up to five dark hairs; C, mixed ;D, up to five pale hairs; E, all dark; and O, character missing) (Kurtak *et al.* 1981).
4. The scutellar hairs, ninth abdominal tergite setae, also scored in a similar way but considering all mixtures together (A, all pale; C, mixed; E, all dark; and O, character missing), as described by Wilson *et al*. (1993).

Detailed research have shown that even when different populations of simuliid flies look morphologically similar, some further morphological, behavioral and even physiological differences can still be found (Lewis,1965). Possible adjustments in applied interventions and control measures may be required when dealing with variation in breeding sites preferences and larval ecology.

In West Africa, among the *S. damnosum* complex, savanna and forest species differ in their morphology and physiology and as such are associated with varying strains of the parasite they transmit (Duke *et al*., 1966).Variations in breeding sites requirement in different bioclimatic zones and altitude was validated by the work of Grunewald (1976). New approaches have therefore evolved since morphology alone does not produce scientifically reliable identification of members of this group. These approaches include:

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- Cytotaxonomy.
- Cuticular hydrocarbon analysis.
- Molecular methods- microsatellites analysis, Random Amplified Polymorphic DNA, Restriction Fragment Length Polymorphism, Standard Polymerase Chain Reaction, DNA barcoding among others.

2.5 Life Cycle of *Onchocerca volvulus*

The life cycle stages take place in two hosts, the human definitive host and the black fly as the intermediate host. Infective third stage larvae (L3) are inoculated into the skin of a human host during a blood meal culminating in the introduction of the larvae into the skin. The larvae penetrate into the bite wound after the fly withdraws its biting mouthparts. The larvae moult twice and develop into adult male and female worms which normally reside in nodules in the subcutaneous connective tissues (Bradley *et al*. 2005). Within 1-3 years after infection, onchocercal nodules appear in subcutaneous tissues, usually at sites of bony prominences such as the hips and ribs. Adults can live in the nodules for up to 15 years (Dadzie *et al.* 1990), with some nodules containing numerous male and female worms.

In the subcutaneous nodule, the female worms produce unsheathed microfilariae with a lifespan that may reach between 1-2 years for a period of about ten (10) years which invade the skin and eyes (Basanez *et al.,* 2006). The fly ingests microfilariae from the skin during a bloodmeal. The microfilariae migrate from the fly's midgut through the haemocoel to the thoracic muscles where they continue their development into the first-stage larvae, second stage (pre-infective stage) and subsequently into third -stage infective larvae (Kirkwood *et al*., 1983). The third-stage infective larvae then migrate to the proboscis of the fly and can infect another human when the fly takes a

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blood meal (McCarthy *et al*., 1994). The onchocercal disease condition manifests itself after the buildup parasite intensity following infective bites by the black flies. An infective larva takes about a year to mature into adult male or female worm after entering the skin of the human host. Therefore, the process of parasite acquisition must recur many times over, and many years of exposure are usually required before a heavy load of adult worms and hence the onset of pathogenesis in the human hosts (WHO, 1987). In the human host, there exist differential infection intensities among residents in endemic areas. These differences cannot possibly be accounted for by differences in exposure alone (King and Nutman, 1991).

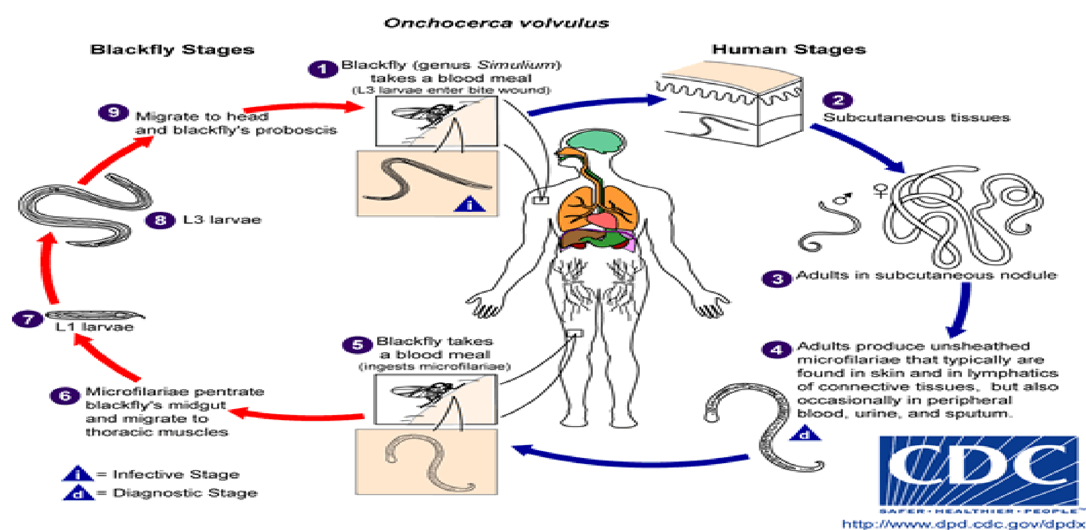

Figure 2.5: Life cycle of *Onchocerca volvulus.*

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2.6 *Onchocerca* Genome

2.6.1 The Genome of *Onchocerca volvulus*

2.6.2.1 Coding Sequences

The nuclear genome of *O. volvulus* has been estimated to be approximately 1.5×10^8 bp distributed among four pairs of chromosomes (Donelson *et al.* 1988). The karyotype consists of three pairs of autosomes, and a pair of dimorphic sex chromosomes (Hirai *et al*. 1987; Post *et al*. 1989). Work on the genome of *O. volvulus* has been focused on the identification of parasite proteins that might have utility as diagnostic markers, components of a potential vaccine, or may represent potential chemotherapeutic targets. The genome of *O. volvulus* is relatively AT rich with an overall AT content of 68% (Unnasch and Williams, 2000). The exon sequences are slightly less AT rich (61%) than are the intron sequences (73%) and this observed difference is as a result of an increase in the proportion of thymidine residues in the coding strand of the intron sequences (40%) when compared with that of the exon sequences (28%) (Keddie *et al.* 1998).

The 0-150 family is apparent characteristic of most species of the genus *Onchocerca*. Within the 0-150 family, variation within the individuals appears to be constrained, probably through the processes of concerted evolution (Arnheim *et al*. 1983). Cross hybridization and degenerate PCR experiments have revealed sequence similarity between *O. volvulus* and other *Onchocerca* species (Meredith, 1989, 1991; Zimmerman *et al.,* 1993). To date, 5 high quality cDNA libraries have been constructed from mRNA isolated from the following stages:

(1) Adult females isolated from skin nodules from human patients

(2) Microfilariae (first-stage larvae shed from adult females)

(3) L2 larvae (these result from the molt of first-stage larvae in the black fly to L2 larvae)

(4) Infectious stage (L3) larvae isolated from black flies

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(5) Larvae undergoing the molt from the L3 stage to the L4 stage (L3M), the first molt in the human host (Source: Filarial Genome Project 1999, *Parasitol Today*).

To date, the most extensively studied library from *O. volvulus* has been that derived from infective-stage larvae. Analysis of the infective stage larval genome (L3) indicates the exclusiveness of several genes that are expressed (Unnasch & Williams, 2000). The table 2.6 below show many of the most abundantly expressed transcripts in L3 infective stage that encode structural proteins or housekeeping enzymes. However, several other proteins of interest are also expressed in appreciable levels. For example, there is a homologue of an *Ancylostoma caninum* secreted antigen which represents 2% of the L3 infective stage larvae dataset (Keddie *et al.* 1998; Unnasch and Williams,2000).

Keddie *et al*., (1998) found that the most abundant transcript seen in the molting L3 dataset is a homologue of cuticulin, one of the two important proteins that make up the cuticle of nematodes (the other is collagen). Several collagen genes also seem to be expressed exclusively in the L3 larval stage or the infective L3M (moulting) stage. A gene encoding a protein of unknown function is the most abundantly expressed gene identified to date in *O. volvulus* (Unnasch & Williams, 2000). One other gene known as alt-2 for abundant larval transcript is expressed only in the L3 and L3M stages. An isoform of alt-2 called alt-1 is also quite abundant. Most of the highly expressed genes in these critical stages of the parasite are of unknown function and therefore are important candidates for future study. These and similar molecules may prove to be excellent vaccine candidates or drug targets for chemotherapeutic intervention (Keddie *et al.* 1998).

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Table 2.6: Highly expressed genes in the infective larvae expressed sequence tag dataset (Unnasch and Williams, 2000)

Clone name	Description	% of L3 Dataset
alt-2	Abundant larval transcript	12.6
col-1	Collagen	3.5
cpi-1	Cysteine protease inhibitor	2.3
asp-1	Ancyclostoma secreted protein	2.0
tpx-2	Thioredoxin peroxidase	1.6
tin-2	Troponin	1.4
alt-3	Abundant larval transcript	1.3
col-3	Collagen	1.2
alt-1	Abundant larval transcript	1.0
ant-2	Abundant novel transcript	0.8
thi-1	Thioredoxin	0.8
fba-1	Fructose bisphosphate aldolase	0.8
col-2	Collagen	0.7
act-1	Actin	0.6
tin-1	Troponin	0.6
cpl-1	Cathepsin	0.6

2.6.2 The Mitochondrial Genome

The mitochondrial gene order is most preferred in examining phylogenetic relationships among relatively distantly related organisms because it is relatively stable across large evolutionary distances (Keddie *et al.* 1998). Therefore the mitochondrial sequences have become useful tools for phylogenetic studies. They are generally maternally inherited and therefore not subject to the diversity generating mechanisms associated with sexual re-assortment (Avise *et al.* 1987). Furthermore, the mitochondrial genome accumulates mutations at a much faster rate as compared to nuclearly encoded sequences, making mitochondrial sequences a useful source of phylogenetic data for differentiating closely related taxa (Brown *et al.* 1979; Vawter and Brown, 1989; Boore *et al.* 1995). Similarly, since the mitochondrial genome is generally not subject to genetic recombination, the gene arrangements therefore tend to be highly conserved across long evolutionary distances (Wolstenholme *et al.* 1992).

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The total mitochondrial genome of *O. volvulus* is just 13 747 bp in size, making it the smallest metazoan mitochondrial genome described to date (Keddie *et al.* 1998). In keeping with the compact nature of the genome, the intergenic regions of the *O. volvulus* mitochondrial genome are quite small. Four gene pairs overlap while eight gene pairs lack an intergenic region altogether. There are a total of 17 intergenic regions ranging from 1 bp to 46 bp in size. The genome contains two ribosomal RNA genes, genes for 12 mitochondrial proteins and genes for 22 transfer RNAs (Keddie *et al.* 1998).

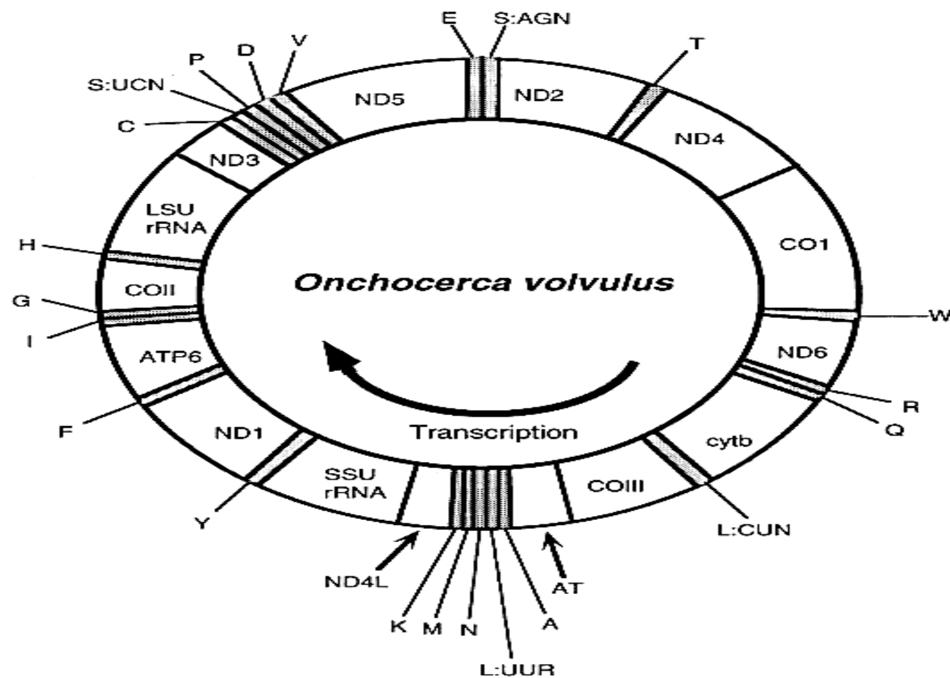

Figure 2.6: A map of *Onchocerca volvulus* genome Keddie *et al.* (1998).

The observed difference between the nuclear and the mitochondrial phylogeny is surprising, and it's speculated to be due to the greater rate of change in the mitochondrial genome of *O. volvulus* than in other nematode species (Keddie *et al*. 1998). However, the hypothesis supporting the rapid rate of mutation in the mitochondrial genome of *O. volvulus* is not supported by an analysis of the level of genetic diversity in the mitochondrial genome of *O. volvulus*. Thus, the reason for the discordance of the mitochondrial and nuclear-derived phylogenies remains to be deduced (Keddie

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et al. 1998). One plausible deduction for the discordance in the nuclear and mitochondrial phylogenies may be found in an analysis of the codon usage of the protein synthesis in the *O. volvulus* mitochondrial genome. Thirteen of the 15 amino acids codons have a T in the third (wobble) position. This preference for a T in the third position is reflected in the overall strong bias for T in the coding strand of the mitochondrial genome (54% T) (keddie *et al.* 1998 Interestingly, it appears that apart from the O-150 sequence, the level of genetic variability within *O. volvulus* genome is quite limited (Unnasch and Williams, 2000). For example, in a study of polymorphisms in the actin genes of *O. volvulus*, only a single nucleotide polymorphism was detected in comparison to gene sequences from parasites collected from Mali and Cameroon (Zeng *et al.* 1992). Similarly, no restriction site polymorphisms in parasites collected from four geographically separated areas were noted in genomic Southern blots employing probes derived from the actin genes (Zeng *et al.* 1992). More recently, a study has been conducted comparing cDNA clones from blinding and non-blinding strain parasites that encode antigens either implicated in the induction of ocular onchocerciasis or which are commonly recognized by the host's immune system (Keddie *et al.* 1998). No sequence polymorphisms resulting in an amino acid change were found in any of the antigens (Unnasch and Williams, 2000).

Furthermore, very few silent polymorphisms were seen, resulting in an overall level of calculated diversity of roughly 0.1% (Unnasch and Williams, 2000).The study was extended to examine the level of genetic diversity in the mitochondrial genome of *O. volvulus*. Using a combination of polymerase chain reaction (PCR) ± restriction fragment length polymorphism (RFLP) and direct sequencing of the hyper-variable AT domain of the mitochondrial genome and the results show that a very limited level of genetic variation was seen in the O. *volvulus* mitochondrial genome among 11 individual parasites examined from six geographically distinct allopatric foci located in East, West Africa and the Americas. The level of diversity seen was roughly 10 fold less than that

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seen in studies of other nematode species. These results, when taken together, suggest that the level of genetic heterogeneity in the coding regions of the nuclear genome and the mitochondrial genome of *O. volvulus* is extremely low (Keddie *et al.,* 1999). It has been proposed that this low level of genetic diversity in *O. volvulus* is because the species suffered a genetic bottleneck in the recent past (Keddie *et al*., 1998) .In this regard, it is interesting to note that, other members of the genus *Onchocerca* are generally parasites of ruminant animals, while *O. volvulus* is an obligate parasite of humans. It is tempting to speculate that *O. volvulus* might have developed as a result of a recent host switch from one of the endemic ruminants in Africa to humans. Sexual reproduction of *Onchocerca* parasites in the vertebrate host, have resulted in a founder effect, limiting the number of reproducing individuals in the population (Keddie *et al.,* 1998).

The lack of genetic diversity seen in *O. volvulus* may be exploited for immunotherapy and chemotherapy against the parasite since little heterogeneity may exist in the parasite antigens that confer protective immunity against *O. volvulus*. Furthermore, if the natural genetic diversity in the targets for various chemotherapeutic agents is also limited, it is less likely that a pre-existing genotype encoding a drug-resistant phenotype will be present in the natural population. This may serve to slow the development of resistance to drugs such as ivermectin, which is currently being used in mass chemotherapy campaigns to combat onchocerciasis (Cupp *et al*. 2011).

2.6.3. Translation Initiation and Termination

Three different initiation codons are used in the mitochondrial genome of *O. volvulus*. The most frequently used initiation codon is ATT (eight genes). TTG is used three times as an initiation codon. The codon GTT is used for translation initiation once in the ND1 gene (Keddie *et al.* 1998). Most genes in the mitochondrial genome of *O. volvulus* end in the complete stop codons

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TAG (6 genes) or TAA (4 genes). The 3% end of the COII gene overlaps the start of tRNA by a single A, completing a TAA stop codon. The gene for ND1 ends with a single T. Many examples of the use of T or TA as termination nucleotides are found in metazoan mitochondrial genomes. These partial termination codons are believed to be completed by polyadenylation after transcript cleavage (Ojala, *et al.,* 1981).

2.6.4 Codon Usage

Codon usage in the *O. volvulus* mitochondrial genome, based on the 12 mitochondrial protein genes, Codon assignments are as previously identified for invertebrate mitochondrial DNAs (Osawa *et al.* 1992). All codons are used except ACC (Thr), ACA (Thr), CGC (Arg), and CGG (Arg) (Ojala, *et al.,* 1981). A strong preference for T is reflected in a strong codon bias in the mitochondrial genome of *O. volvulus.* For 15 of the 20 amino acids, a single member of the codon family encoding a given amino acid is used greater than 70% of the time (Keddie *et al*. 1998).
Some other studies have suggested that, as in many other organisms, the rate of nucleotide change in the mitochondrial genome of nematodes is greater than that seen in nuclearly encoded sequences (Thomas *et al.* 1991). The availability of the complete sequence of the *O. volvulus* mitochondrial genome will make it possible to investigate the level of diversity in the mitochondrial genome among the different strains and populations of this important human parasite.

2.7 Identification of *Onchocerca* Species

Estimation of the transmission intensity by the *Simulium* vector populations has relied on manual dissection of flies for the detection of filarial larvae, though this process does not discriminate among *Onchocerca* species in *refugia* (Rodriguez-Perez *et al*., 1999). A reliable and rapid taxonomic identification of parasitic disease agent is basic for their correct diagnosis; hence

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molecular assessment of species diversity has become increasingly popular (Alhassan *et al.,* 2014). Several species of filarial nematodes are agents of tropical diseases of both human and other veterinary animals. These filariae are transmitted through haematophagous insect vectors within which different developmental stages are produced (Besansky *et al.,* 2003; Power, 2004; Blaxter *et al.,* 2005). The exact identification of these juvenile developmental stages is a necessary condition for assessing transmission indices in endemic areas.

Ever since Blacklock (1926) incriminated *S. damnosum* as vectors of human onchocerciasis, it has mostly been assumed that all filarial larvae found in man-biting simuliids are *O. volvulus* and this make the estimation of transmission indices from unidentified infective filarial larvae unreliable given the close morphological characteristics among *O. volvulus, O. gibsoni and O. ochengi* (Bain, 1981).

2.7.1 Molecular Identification of *Onchocerca* spp

Monitoring infections in the vector population offers some advantages over repeated blood examinations of the human population (Bradley *et al.* 1996; Boatin *et al*. 1993). Infection rates in black fly population can sometimes be misleading especially given the high prevalence of animal *Onchocerca* species in West Africa which is home to a large number of game animals (Wahl *et al*. 1998b). Consequently, the use of molecular tool to discriminate between human and animals parasites is essential in assessing the impact and success of campaign programmes based on MDAs. Currently, molecular assays based on analysis of the O-150 repeat sequence of *Onhocerca volvulus* have been developed for species identification and this has proven useful of species discrimination (Meredith *et al.* 1991; Zimmerman e*t al*. 1994; Merriweather *et al*. 1996; Katholi e*t al.* 1995; Yameogo *et al*. 1999). The availability of new technologies including an enzyme-linked immunosorbent assay (PCR-ELISA) that aid the detection of parasite genomic

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amplification products has provided more impetus in the control of human onchocerciasis (Gopal *et al*. 2012; Guevara *et al.* 2003; Rodriguez-Perez *et al*., 2013).

In Northern Cameroon, prevalence *O. onchengi* of microfilariae in cattle can be as high as 66–71% in some areas (Trees *et al.,* 1992; Renz, 1994). However, since *O. ochengi* closely related to *O. volvulus*, and the routine used of O-150 as diagnostic marker for *O. volvulus* clusters with other *Onchocerca* species species discrimination is problematic (Krueger *et al.* 2007). But molecular phylogenetic analysis of three mitochondrial DNA gene fragments [12S, 16S rRNA genes, and NADH dehydrogenase subunit 5 (ND5)] from five *Onchocerca* species has shown promise for use in species separation (Morales-Hojas *et al.* 2006). Moreover, the development of DNA probes specific for *O. volvulus* and *O. ochengi* have become additional tools for discriminating among *Onchocerca* species (Wahl and Schibel, 1998).

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in 12 onchocerciasis endemic communities located in the Pru and Banda districts in the Brong –Ahafo region. The study was conducted in the communities between March, 2014 and February 2015. The geographical co-ordinates indicating the locations of all 12 communities are shown in table 3.1 The Brong-Ahafo Region has many fast flowing rivers including the Bia, Black Volta, Pru, Tain, Tombe and Tano Rivers. The region thus provides vast water bodies which serve as breeding sites for the *Simulium* vectors of onchocerciasis. Currently, there are nineteen districts in the region and twelve districts are endemic to onchocerciasis, nine of which occur towards the northern part of the region and experience the savanna type of the disease. The primary occupation of these communities is farming and fishing.

Table 3.1: The geographic coordinates of the communities of the study area.

Community	**Prang Transmission zone coordinates.**	
Asubende	N 08° 01' 08.8"	W 000° 59' 01.5"
Mantukwa	N 08° O1' 06.4"	W 001° 00' 14.0"
Ohiampe	N 08° 00' 26.22"	W 001° 63.49.5"
Beposo	N 08° 00' 26. 7"	W 000° 57' 40.2"
Abua	N 07° 58' 42.1"	W 000° 53' 4'41.5"
Fawoman(Prang)	N 08° 01 11.4"	W 000° 53'41.5"
Community	**Banda Transmission zone coordinates.**	
Nyire	N 08° 06'43.7"	W 002° 17'45.4"
Fawoman (Banda)	N 08° 07'10.7"	W 002° 14'13.3"
Tainso	N 08° 06'03.4"	W 002° 06'54.1"
Kyingakrom	N 08° 05 58' .4"	W 002° 03' 27.6"
New Longoro	N 08° 08'13.5"	W 002° 02' 07.6"
Agborlekema I	N 08° 14' 01.7"	W 002° 12'18.8"

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3.2 Study Design

A 12 month epidemiological, entomological and molecular study was conducted to assess the transmission of human and animal onchocerciasis in the study communities between March 2014 and February 2015. Cattle were examined for the presence of subcutaneous nodules and skin snip taken to assess for *Onchocerca* infection. Adult Black flies were caught at specific catching points along the river basins, using human landing method as described in Walsh (1983) and WHO (1991a). The flies were kept in ice chest and transported to the laboratory for dissection and identification of the infective stage (L3) of *Onchocerca* spp. Molecular studies were then carried out to determine the presence of *Onchocerca* spp in the cattle and black fly populations.

3.3 Ethical Clearance

Before the commencement of the study, ethical approval was obtained from the Institutional Review Board of the Council for Scientific and Industrial Research, Ghana. Informed consent was sought from all vector collectors after the purpose and schedules for the study was explained to all fly collectors. All those who agreed to collect the black flies signed the informed consent forms and a copy given to them.

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3.4 Collection of Adult Female Black Flies

Prior to the beginning of the study, some onchocerciasis endemic communities were visited as part of the community entry procedure, to interact with the chiefs and elders of the villages and also explained the objectives and schedules of the study. A total of 12 communities were selected from two districts in the Brong-Ahafo region. Community volunteers were selected for each participating community and two of those who gave informed consent were trained in vector collection procedures. One vector catching site was selected for each community and flies were collected using the OCP protocol for human landing catches technique with slight modifications (Davies *et al*., 1978; Walsh, 1983; WHO, 1991). Two trained community volunteers worked alternately on hourly basis from 06:00 to 18:00 hours to compensate for differences in their relative attractiveness to biting flies. Each fly was caught and stored in a separate capped plastic tube and the number of flies caught were collated, counted and recorded. The plastic tubes containing flies were wrapped with moist cotton wool to provide low temperature and high humidity in order to preserve them alive until dissection in a field laboratory. Fly sampling was done for four consecutive days per month in March 2014 and February 2015. Data obtained from adult black flies were used in determining transmission parameters such as biting rate and transmission potential.

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3.5 Dissection of Adult Female Black Flies for Onchocerca Parasites

The entomological assessment involved the identification and dissection of flies caught. Female adult flies were anaesthetized with chloroform and placed dorso-ventrally on a microscope slide containing a drop of physiological saline to aid in visibility. Dissection was carried out under a dissection microscope using two fine dissecting pins, beginning from the posterior-ventral end of the abdomen, from where the ovaries were removed for determination of parity. The internal organs were observed and recorded as parous or nulliparous according to Cupp & Collin,1979;Morky, (1980). The nulliparous one were those that had never had a blood meal yet and had not completed a gonotropic cycle, therefore could not harbor the parasite larvae, they have tightly coiled ovary tracheal systems, without follicular relics, nor retained eggs. The parous flies had taken at least a blood meal and had completed at least one gonotropic cycle resulting in the presence of follicular relics below the maturing oocyte and loosely stretched condition of the ovary tracheal systems (Cupp & Collins ; Morky, (1980).They also have a pale intestine and may contain retained eggs.

The whole essence of dissection is to expose the internal organs of the flies which could possibly be harbouring the parasite. All parous flies were then further minutely dissected to search for other developmental stages of *onchocerca* spp. according to the number of larva found in the head, thorax and abdomen. The number of various larval stages of *Onchocerca* species found in different parts of the flies was all noted and their stages of developments and sites were all noted.

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3.6 Assessment of *Onchocerca ochengi* Transmission in Cattle Populations

Cattle populations in three of the study communities; New Longoro Beposo and Agborlekema I were assessed for the presence of animal *Onchocerca* spp. The cattle varied in age from 1 to 6 years. Four cows were sampled from Beposo, 4 from New Longoro and 11 from Agborlekema I. The animals were tied and held to the ground by the herdsmen for the skin snips to be safely taken. *Onchocerca ochengi* microfilaria is known to be concentrated at the belly, penis and scrotal regions (Wahl *et al*., 1994), while the nodules containing the adult worms can be found in the subcutaneous tissues. A total of 19 cows from the study area were examine and palpation carried out to locate *Onchocerca* nodules (see plate 3.1). Skin microfilarial load was assessed for each cattle by taking two superficial skin sliver (mean surface: 10 mm) with a corneoscleral punch (see plate 3.2) from the belly (midline between umbilicus udder and scrotum), where most *O. ochengi* mf are concentrated (Wahl *et al*., 1994). Each skin snip was incubated in medium RPMI 1640 at 32 °C and emerged mf counted. Some snips were also minced and incubated at 32 °C in 0.5% collagenase RPMI 1640 digestion medium for 48 hrs. Every 12 h the mf released from the disintegrating snip were counted and the snip transferred to a new medium. The mf density was estimated as number of mf per mg of snip.

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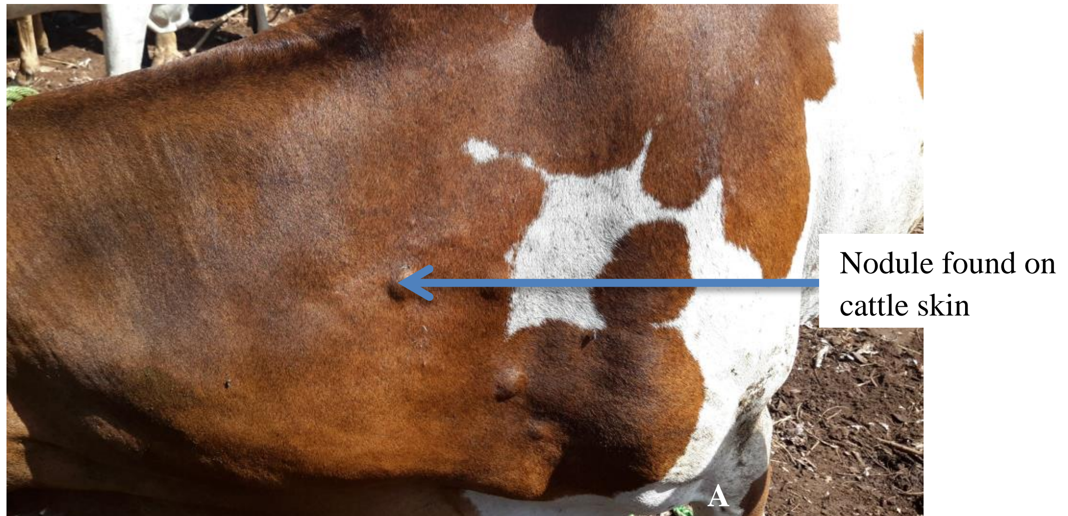

Figure3.6: (a) A cow in Agborlekema having multiple nodules.

Figure 3.6: (b) Taking skin snip from cattle at Agborlekema I.

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3.7 Processing of Black flies and *Onchocerca* Samples for DNA Extraction

Three types of parasite samples were available for this study; a) *Onchocerca* L3 larvae preserved on microscope slide (obtained from infective black flies after dissection), b) black flies preserved in 95% ethanol c) *Onchocerca* microfilariae obtained from cattle.

Only the head capsules of the black flies were used because this part habours the infectious stage of the parasite which contributes to transmission of the disease. It was important to reduce the insect biomass because the whole fly limits the sensitivity of the assays. Very efficient methods for collecting black fly heads have been developed (Yameogo *et al*., 1999; Rodriguez-Perez *et al*., 2013). A total of 80 black flies preserved in 95% ethanol were used for the study, with the heads separated from the thorax.

3.8 DNA Extraction Techniques

DNA was extracted from various *Onchocerca* spp samples using two DNA Extraction Techniques:

1. Qiagen kit Extraction technique (Qiagen Tissue and Blood Kit CA, USA, 2006).
2. Lyses buffer Extraction technique (Viagen Biotech,CA,USA ,2013).

For each technique, three sets of samples were processed for DNA extraction

i Non-infected black fly heads obtained from nulliparous flies

ii. *Onchocerca* L3 larvae obtained from infected black fly

iii. Microfilaria of *Onchocerca* spp obtained from cattle

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3.8.1 Qiagen kit Extraction Technique (Qiagen Tissue and Blood Kit CA, USA, 2006)

Each of the samples from above were homogenized in a 1.5 unit Eppendorf tube. In the case of Onchocerca the head of the nulliparous black fly was added. Also, the *Onchocerca* infective larvae (L3) from dissected fly and *Onchocerca ochengi* microfilaria were each placed into different 1.5 ml microcentrifuge. An amount of 180 μl Buffer ATL (Tris, pH 8.6) was added. Approximately 20 μl of proteinase K was added to each tube and each mixed thoroughly by vortexing. This was incubated at 56°C until the tissue became completely lysed and then vortexed for 15 seconds.

Twenty sets of samples for each group were prepared as above with each set labelled from 1 to 20. Each set was incubated at the 56°C for 5 minutes (min). About 200μl of Buffer AL (4M Gu Cl) was added and then mixed thoroughly by vortexing. This was followed by the addition of 200 μl of ethanol (96-100 %) and then mixed thoroughly by vortexing This was pipetted into the DNeasy Mini spin column, centrifuged and the process repeated twice then 500 μl Buffer AW1 (Quanidine) was added centrifuged for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane and DNA eluted about 200 μl of Buffer AE (1OmM Tris-Cl, 0.5Mm EDTA,pH 9.0) DNA was stored at -20° C freezer until ready for amplification by PCR method.

3.8.2 Lyses Buffer Extraction Technique (Viagen Biotech,CA,USA ,2013).

The four sets of 20 samples each used for the Qiagen extraction method were also used for the Direct lyses buffer method. 19.07 μl of the Lyses buffer was added to 0.03 μl of proteinase k and a single infective larva (homogenized with a black fly head by grinding with a pestle) was added to the total 20 μl solution in an Eppendorf tube. The solution was then placed in a thermocycler at conditions of 65°C for 18 hours and then 85°C for 1 hour.

After DNA extraction using both the Qiagen Extraction Kit and Direct Lyses buffer Technique, the DNA from both techniques were assessed by comparing the concentration of DNA yield as

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shown in table 4.4. Also gel electrophoresis was carried out and gel images compared for both technique as shown in Figures 4.4. The assessment of DNA yield using the Direct Lyses buffer technique was important because it was about 10 times cheaper than the Qiagen Extraction Kit, so getting a better yield would have saved the study huge cost.

3.9 Molecular identification of *Onchocerca* spp from blackfly and cattle using Polymerase Chain Reaction

The molecular identification of *Onchocerca* spp involved the molecular analysis of three mitochondrial DNA gene fragments [12S and 16S rRNA genes, and NADH dehydrogenase subunit 5 (ND5)] using DNA samples extracted from *Onchocerca* spp from black flies and cattle.

The PCR amplifications of DNA gene fragments [12S and 16S rRNA genes, and NADH dehydrogenase subunit 5 (ND5)] were carried out separately using the primers below;

Table 3.9.1: sequences of primers used their directions, positions and the respective genes they amplify according to Morales-Hojas, *et al*. (2006).

Name	Sequence	Direction	Gene	Position
12SOvC	TCGGCTATGCGTTTTAATTTT	Forward	12S	7496-7517
12SOvB	CAACTTACGCCCCTTTAGGC	Reverse	12S	7996-8015
16SOvC	AGCCTTAGCGTGATGGCATA	Forward	16S	10976-10995
16SOvB	ACCCACATTGCATTCCTTTC	Reverse	16S	11442-11461
ND5OvA	TTGGTTGCCTAAGGCTATGG	Forward	ND5	12697-12716
ND5OvC	CCCCTAGTAAACAACAAACCACA	Reverse	ND5	13145-13167

The primer design was based on the *Onchocerca spp.* mitochondrial genome (Keddie *et al.,* 1998).PCR reactions were performed in a total volume of 10µl each containing 1X buffer (Promega), 1.5µl $MgCl_2$ (GeneOn) and 2µl of the DNA template.

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Amplifications consisted of a first denaturation step at 94°C for 1 minute, followed by 32 cycles at 94°C for 30 seconds at 50°C (annealing) for 45 seconds, extension at 72°C for 1minute, with a final extension step of 5 minutes at 72°C.PCR amplification products were run on 1.5%(w/v) agarose gel stained with ethidium bromide and visualized under UV light.

3.9.2 Statistical Analysis

3.9.2.1 Entomological indices and their estimation.

Biting rates and transmission potentials represent the most widely used and the most reliable transmission indices. Planning, execution and assessment of the success of disease control efforts rely heavily on proper estimation of these parameters. The estimation of density and level of onchocerciasis transmission also relies to a greater extent on these two indices

MBR (monthly biting rate) =theoretical number of *Simulium* bites received by a person who remains stationary at a catching site during the twelve hours of the daylight for one complete month in a given community.

MTP (monthly transmission potential) =Total number of infective larvae found in the head of the black flies received in one month by an individual who remains stationed at a catching point for twelve hours in a given community (WHO, 1991).

Man biting rate

$$\textbf{MBR}=\frac{\text{number of flies caught x number of days in a month}}{\text{Number of catching days}}$$

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Transmission potential

$$\text{MTP} = \frac{\text{MBR x No. of } 3^{rd} \text{ Stage larvae in the head}}{\text{Number of flies dissected}}$$

The monthly relative abundances of *S. damnosum* were evaluated using the two way analysis of variance (ANOVA).

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CHAPTER FOUR

RESULTS

4.1 Relative Vector Abundance

A total of 9,343 adult female black flies were caught from all 12 study communities in the dry season months of March, 2014 and February, 2015.A comparison between o flies recorded in in the duration of this study showed that March, 2014 had a significantly ($P < 0.05$, 0.021) higher relative fly abundance than February, 2015. A significantly higher proportion of the flies (80.0 %) were collected in the March 2014 as compared to 20.0% in 2015. Out of a total of 1865 flies collected in 2015, 252 flies representing 13.5 % were from the Banda catchment area whilst 1613 (86.5 %) were collected from six communities in the Pru area. In 2014 however, 5380 flies (71.9 %) out of a total of 7478 were collected from the six communities in the Banda area whereas the Pru catchment area had only 28.1 % of the flies collected. Generally the number of flies collected per community varied considerably as shown in Figure 4.1 below. Agborlekema recorded the highest fly numbers of 2324 in March, 2014 while in February, 2015, no black flies were recorded in two communities.

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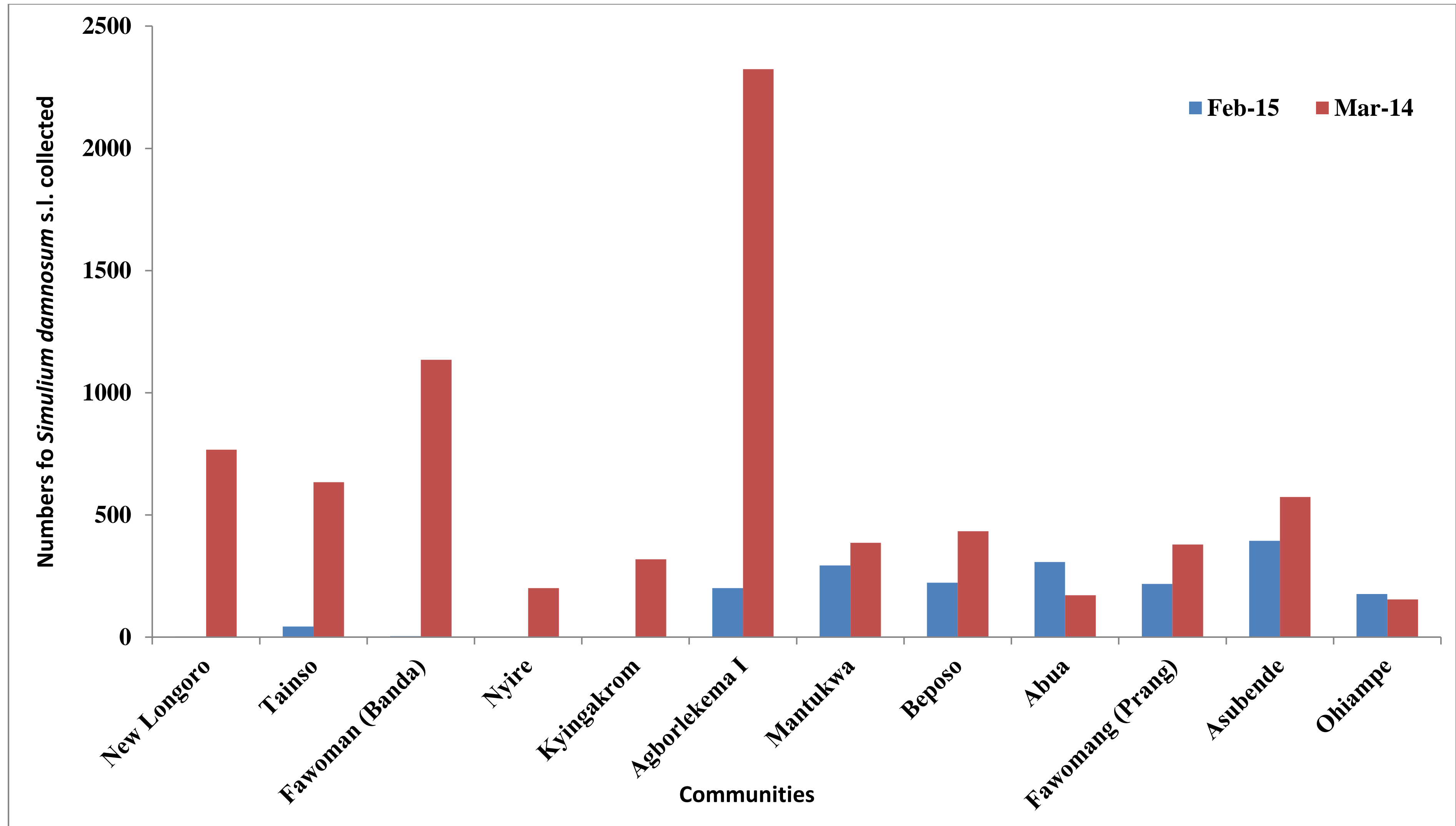

Figure 4.1: Relative abundance of black flies collected from 12 onchocerciasis endemic communities.

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4.2 Entomological Indices and Vector Infection Rates

A total of 2014 flies (21.9 %) dissected were parous with only 5 infective flies (0.05 %) from Agborlekema I. Monthly biting rates (MBRs) were estimated for the twelve study communities using fly collection data as shown in Figure 4.2 below. In general, the MBR varied significantly across all 12 villages studied. Moreover, since the MBR is directly proportional to the number of *Simulium* flies collected in a month, the pattern of variation of the MBRs were similar to the vector population densities. During the two months of vector collection, Agborlekema I in the Banda transmission zone had the peak MBR of 16268.0 bites per person per month whiles Abua in the Pru area had the minimum MBR in 2014. For the 2015 surveys, Asubende in the Pru catchment area had the maximum MBR of 3677.3 bites per person per month with Nyire and Kyingakrom recording no fly biting activities. The Monthly biting rates in all 12 communities were above the WHO threshold biting rate (TBR) of 1000 bites per person per annum in 2014, while in 2015, seven communities had MBR above the WHO threshold. Out of a total of 2,046 parous flies dissected only 5 infective flies (0.05 %) were found in Agborlekame I, and these flies harboured 11 third stage infective larvae The general variation of the MBRs as well as the monthly transmission potential (MTP) across all 12 endemic communities for the two months of sampling are shown in Figure 4.2 and 4.3 respectively.

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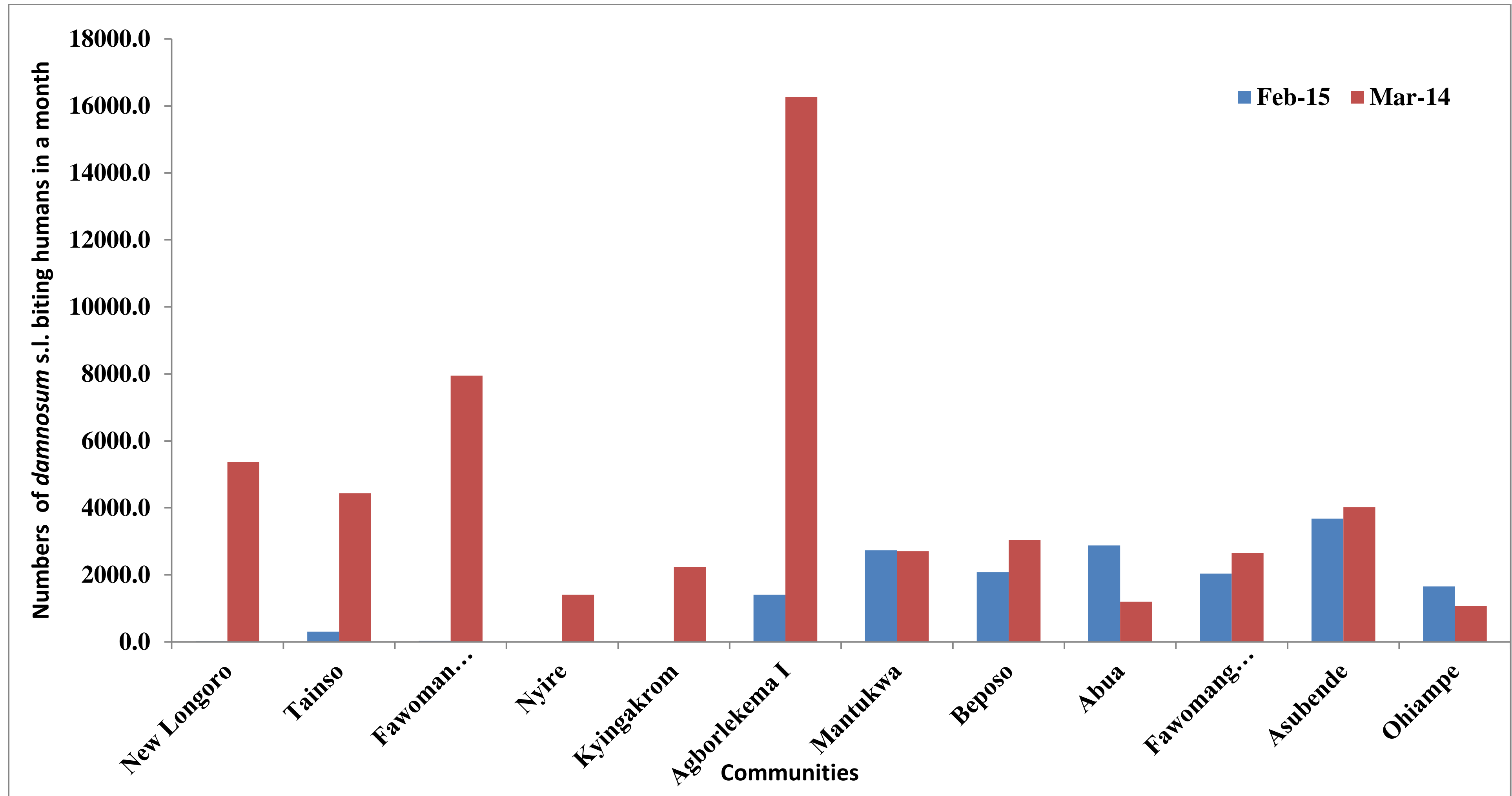

Figure 4.2a: Monthly biting rates of 12 onchocerciasis endemic communities in the Brong-Ahafo region .

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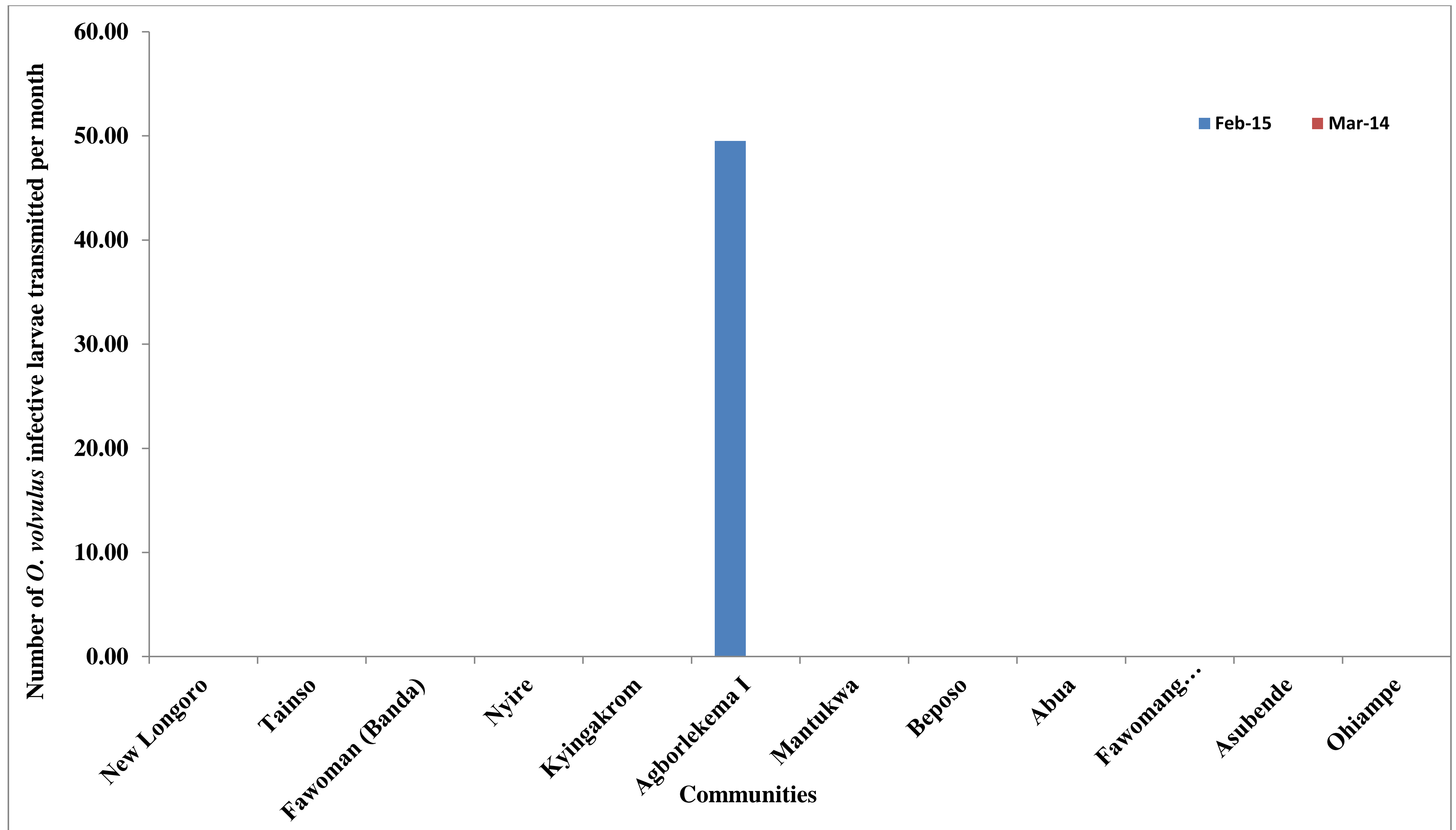

Figure4.2b: Monthly transmission potential of 12 onchocerciasis endemic communities in the Brong-Ahafo region

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4.3 Transmission of Animal Onchocerciasis in Study Communities

Transmission of animal onchocerciasis was assessed in three onchocerciaisis endemic communities; Abgorlekema I, Beposo and New Longoro. Out of the 19 cattle snipped, only one cattle was found positive for microfilaria. Though *Onchocerca* nodules were found on the cattle palpated in both Beposo and New Longoro, no microfilaria was isolated from all 11 cattle samples in both communities. A total of 12 microfilaria were harvested from the skin snip taken from one cattle from Agborlekema I.

Table 4.3: Quantity of microfilaria harvested from cattle in the study in 3 communities.

Community	Cow ID	No.of snips taken	Body part snipped	No of Mf contained from Normal saline	No. of Mf from Collagenase
Abgorlekema I	Ag 01	3	belly,rare flank	0	0
	Ag 02	2	belly,groin	0	0
	Ag 03	2	belly,rare flank	6	6
	Ag 04	3	belly	0	0
	Ag 05	3	nodule on rare flank	0	0
	Ag 06	3	belly	0	0
	Ag 07	3	belly	0	0
	Ag 08	3	belly	0	0
Beposo	Bp 01	2	nodule by rare flank	0	0
	Bp 02	3	belly	0	0
	Bp 03	3	belly,	0	0
	Bp 04	3	nodule on rare flank	0	0
	Bp 05	2	udder,belly	0	0
	Bp 06	3	belly,groin	0	0
New longoro	Nl 01	4	groin,udder	0	0
	Nl 02	4	belly,udder	0	0
	Nl 03	3	groin,nodule on rare flank	0	0
	Nl 04	2	belly,groin	0	0
	Nl 05	3	udder,belly	0	0

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4. 4 Comparison of Efficiency of Qiagen Extraction Kit and Direct Lyses Buffer Technique

The Qiagen Extraction Kit gave a significantly higher DNA yield with a concentration of up to 60 ng/ul compared to Direct Lyses buffer technique which gave a maximum yield of 9 ng/ul. DNA extracted from 15 out of 20 (75%) infective *Onchocerca* larvae gave using Qiagen Extraction kit yielded good quality DNA while the Direct lyses Buffer technique gave a good yielded for (N=20) 3 samples out of the 20 (15%)

Table 4.4: The yield of DNA for two extraction methods

Onchocerca L3 larvae sample	Qiagen Extraction	Lysis Buffer Extraction
L1	21.08	0
L2	24.34	0.63
L3	25.62	0
L4	28.54	0
L5	22.34	0
L7	22.4	0
L8	50.33	0
L9	60	9
L10	0	0
L11	33.82	0
L12	29	0
L13	28.33	0.74
L14	0	0
L15	20	0
L16	26	0
L17	21.02	0
L18	0	0
L19	0	0
L20	25.4	0

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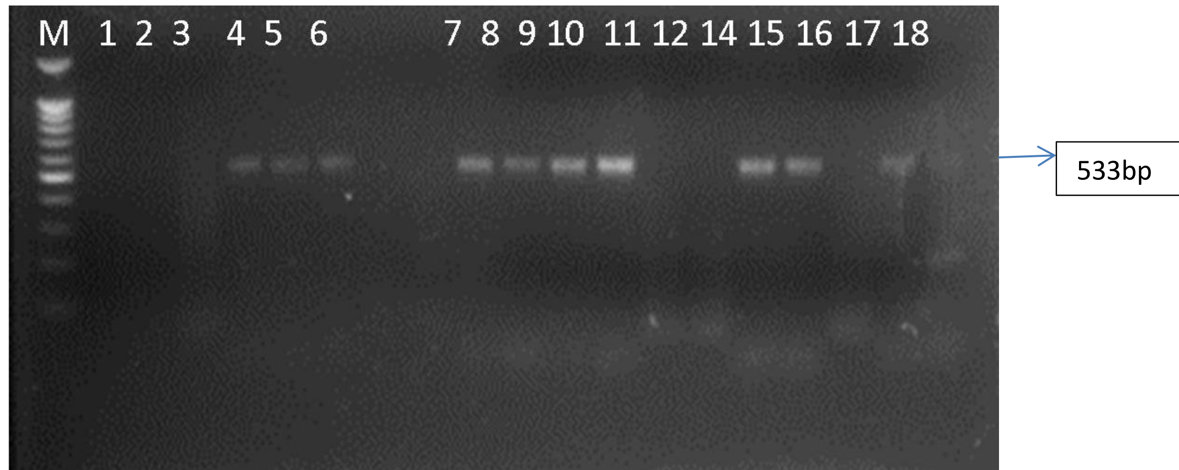

Figure 4.4 Image of bands of PCR products run on agarose gel with Qiagen kit extraction method using the 12S primer (533bp). M=Ladder (100 bp),Sample 4,5,67,8,9,10,11,15, and 18 and were positive DNA amplification of larvae of *Onchocerca volvulus* and sample 16 was *Onchocerca ochengi* and the rest were negative.

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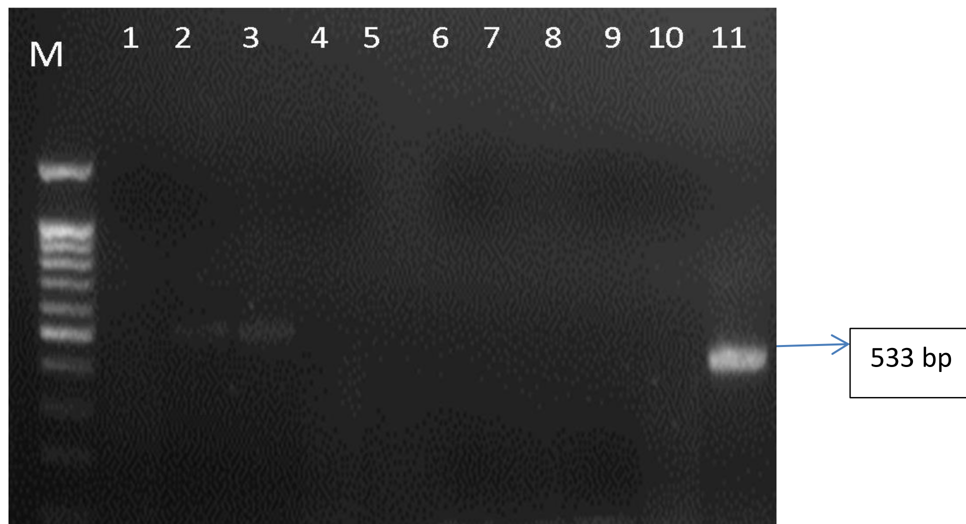

Figure 4.5 Image of bands of PCR products run on agarose gel with Lyses buffer extraction method *O.volvulus* larvae using 12S primer where M=Marker (100 bp).Sample 2,3 and 11 only showed positive DNA amplification for *Onchocerca volvulus.*

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CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Transmission of Human and Animal Onchocerciasis

Onchocerciasis control intervention employed by the Onchocerciasis Control Programme (OCP) of West Africa and the African Programme for Onchocerciasis Control (APOC) has led to drastic reduction in disease morbidity and prevalence (Osei-Atweneboana *et al*. 2007). However areas of persistent transmission still persist in Ghana. As the goals of onchocerciasis control programmes shift from morbidity control to elimination (WHO, 2000), knowledge of ongoing transmission and the *Onchocerca* spp. carried by the black fly vectors is very essential to help achieve this goal. This will enable entomological monitoring of programmes' progress and inform decision making on intervention strategies.

Despite three decades of ivermectin treatment, human onchocerciasis transmission still persist in Ghana (Osei-Atweneboana *et al.,* 2011; Lamberton *et al.,* 2014), therefore assessing infection transmission in humans as well as transmission in cattle populations would give insight into the real picture of onchocerciasis transmission in Ghana toward effective control and eventual elimination of the disease. This is important because the status of animal onchocerciasis in Ghana is unknown (Osei-Atweneboana, unpublished data); moreover the routine assessment of human onchocerciasis transmission is mainly based on morphological identification of *O. volvulus,* which cannot discriminate between *O. volvulus* and *O. ochengi*.

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Out of the twelve communities assessed for *O. volvulus* transmission during the dry season, only Agborlekema I reported infections. The absence of infection during the dry season does not necessarily mean that onchocerciasis transmission has been interrupted in these communities, since there have been reports of ongoing transmision in these and other communities especially during the rainy season (Osei-Atweneboana *et al.,* 2011; Lamberton *et al*., 2014). Also this is not surprising because these communities had taken ivermectin treatment in January, 2014 and January 2015, therefore it is expected that the drug would have cleared the skin of mf, leaving insignificant numbers for vector uptake during blood meal.

Unlike the rest of the communities, Agborlekema I was the only community that had detectable ongoing transmission despite ivermectin treatment. At the same time it was observed that only the cattle from Agborlekema I had infections since black fly vectors transmit various *Onchocerca* spp., it is possible that the infected cattle at Agborlekema I might have contributed to the reported ongoing transmission in this community. In the light of this information, it is important to carry out epidemiological study on animal onchocerciasis in Ghana to help map out areas of mixed infections of both animal and human *Onchocerca* spp. This will help delineate transmission zones so that areas where molecular diagnosis is required to confirm vector transmission are mapped out. This is necessary because in Northern Cameroon the microfilariae prevalence of *O. ochengi* in cattle can be as high as 66–71 % (Trees *et al.,* 1992).Furthermore there have been reports of an overwhelming majority of all filarial infections found in the *Simulium damnosum* s.l. vectors throughout the year, of up to 89% of *O. ochengi* infections (Wahl *et al.,* 1998a). This has huge implications on the epidemiology of human onchocerciasis.

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This therefore calls for urgent need for molecular identification of *Onchocerca* spp found in vectors during transmission studies. However, in limited resource countries such as Ghana, a much simpler and cost effective methods is need for diagnosis of *Onchocerca* species rather than a much complicated assays based on probes and other options which are much difficult to secure in our setting (Guevara *et al.,* 2003; Gopal *et al*., 2012; Rodriguez-Perez *et al*., 2013).

The sampling was done during the dry season where black fly numbers and infections levels were low, during this period fly numbers could be managed and the risk of infection is very low. In general, the population of flies collected in 2014 was significantly higher than in 2015; however the Banda catchment area which comprises the Lower Black Volta, the Tombé River, the Tain River and their tributaries had significantly more vector abundance and monthly biting rates than the Pru catchment area where the communities are located on the Pru River basin and its tributaries. This observation is in sharp contrast to that of Diawara *et al.* (2009) who reported no fly catches in the dry season in Senegal and Mali. During the commencement of the study in 2014; the Bui hydroelectric dam had huge volumes of water and the discharge of water downstream caused apparent rise in water level in the Lover Black Volta creating more rapids to aid in fly breeding and resultant significantly increased in fly populations when compared to 2015. This could explain the high fly numbers in the Banda areas in 2014 compared to 2015.

The intensity of exposure to infection transmission as measured by the monthly biting rate (MBR) is commensurate with vector population density and thus varied considerably across all 12 villages studied. It has been indicated that a threshold biting rates (TBR) of 1000 bites per person per annum was considered as significantly tolerable for a person living in onchocerciasis

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hyperendemic area (WHO, 1995). However, throughout this study, the average MBR per community was in excess of 4000 and 1000 bites per month in 2014 and 2015 respectively for most communities. These MBR levels were substantially higher than the WHO tolerable levels. However, the vector infection levels were even lower than that of the WHO threshold of 0.5 L3 per 1000 parous flies. Therefore the risk of infection during the study period was very low, but the community members were still exposed to considerable vector biting nuisance which could be counter-productive and eventually could impact on income generating activities and the local economies, especially where most of the people are farmers (WHO, 1979; Okoye & Onwuliri 2007; Duerr & Eichner 2009).

The molecular component of the study was set out to develop a more simplistic PCR based assay comprising of PCR amplicons from mitochondrial DNA sequence diagnostic of two or more *Onchocerca* spp. with some diagnostic restriction sites for specific *Onchocerca* species. Though this goal was not achieved in this study, we have been able to validate a PCR assay using the *12S* mitochondrial RNA gene sequences for the identification of *Onchocerca* spp., (Keddie *et al.,* 1998). The validation was done using known adult *Onchocerca volvulus* worms and *O.ochengi*. DNA from known adult worms. The *12S* mitochondrial RNA gene sequence was amplified for all developmental stages of *O. volvulus* (i.e. adult, microfilaria and infective larvae) when compared with the *16S* and *ND5* gene. Using the *12S* mitochondrial RNA gene sequences, we were able to confirm the accession that *O. ochengi* is a sister species of *O. volvulus* (Bain, 1981; 2002; Morales-Hojas and Cheke 2006), moreover, both species induce the formation of nodules around the adult worms.

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Amplification of the *12S* mitochondrial RNA gene sequence to identify the *O. volvulus* at the infective stages in the black fly's head was successful. Also amplification of the gene of interest to identify *O. ochengi* microfilariae to confirm the presence of animal filariae (*O. ochengi*) in our study areas in skin snips taken from the cattle was successful.

The next stage of the study will be to develop a simple tool that can discriminate between *O. ochengi* and *O. volvulus* infection in the same pool, especially in the case of mixed infection in vectors. The use of a molecular assay to detect infective larvae is a very useful adjunct to the dissection method because it may be capable of providing evidence of infections even at very low infection levels. The presence of *Onchocerca ochengi* in a mixed infection may seem of less clinical importance because it does not cause human blindness, however, it does exaggerate entomological indices used in assessing elimination efforts as established in Northern Cameroon (Wahl *et al*., 1994).

Yameogo *et al* (1999) found that, the prevalence of infection determined by dissection differed insignificantly from that determined by PCR assay. In contrast to Yameogo's finding, Boatin *et al* (1998) did evaluate three new diagnostic tests for onchocerciasis under development: an immunological assay, a PCR-based assay and diethylcarbamazine (DEC) patch test, and found that the PCR assay was significantly more sensitive and specific when compared to the DEC patch. The results suggest that the PCR assay may be a useful tool for epidemiologic surveillance for *O. volvulus* when infection levels are very low.

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Obtaining an efficient and cost effective DNA extraction method is beneficial to molecular biology research in developing countries with limited resources. The comparison between the Qiagen extraction kit and the Direct lyses buffer extraction techniques was mainly based on the quest to obtain a cheaper but effective DNA extraction method to ensure that limited funds are capable of doing substantial volume of work. Though the Direct lyses buffer was about ten times cheaper than Qiagen extraction kit, it provided adequate DNA yield for just 15% of samples processed. This DNA extraction process needs to be optimized to produce a high yield DNA.

5.2 Conclusion

The findings of this study confirm that though onchocerciasis still remain a public health problem in Ghana, especially during the rainy season (Lumberton et al 2014), transmission of the disease is significantly minimized during the dry season. The low level of transmission in the study areas during the dry season is quite promising and effective control strategy must be put in place to ensure that there is no significant rise in transmission during the rainy season. This research was able to identify the sister species of *O. volvulus* known as *O. ochengi* for the first time in Ghana. The presence of *O. ochengi* exaggerates entomological indices. This means the Ghana Health service would have to review its estimation of transmission indices by factoring in its transmission determinant models that recognizes the presence of *O. ochengi* as we have now confirmed *O. ochengi* in our study communities, which may exaggerate infectivity rates in vector population.

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We conclude that transmission is still ongoing in our study areas and therefore the IVM-treatment has to be keenly observed and also the Ghana Health Service has to invest in sensitization of endemic population to keenly comply with the ongoing MDA.

The molecular assay validated can be used to identify *Onchocerca* spp. both in the cattle and in the vector. However the limitation with the proposed assay is its inability to discriminate between the different *Onchocerca* spp. in the infective fly heads, the way forward is to carry out more investigation to develop assays that can discriminate between the various *Onchocerca* parasite species in the vector population.

5.3 Recommendation

- There is a need for extensive molecular research to develop simple PCR-Assays that can be used to discriminate between the various *Onchocerca* spp. in vector populations, so as to enable accurate assessment of transmission indices in these endemic communities.
- There should be ivermectin distribution monitoring, coupled with sensitization of the at-risk population on the essence of compliance with treatment regime.
- It would be important to carry out an extensive epidemiological study on animal onchocerciasis in Ghana to map out areas of mix infections of both animal and human *Onchocerca* spp. This will help delineate transmission zones so that areas where molecular diagnosis is required to confirm vector transmission are clearly defined.

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APPENDIX I

1.5% agarose gel

1.5g agarose powder

100 ml of 1x TAE buffer or distilled water

2.5 µl ethidium bromide

Tank buffer (1000 ml)

1000 ml 1x TAE buffer

25 µl ethidium bromide (2.5 µl /100ml)

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