CHARACTERIZATION OF CATTLE FILARIAL PARASITE MORPHOLOGICALLY SIMILAR TO *WUCHERERIA BANCROFT!* (NEMATODA: FILAROIDAE) IN SOUTHERN GHANA

BY

HELENA BAIDOO (B.Sc. Hons.)

(10097418)

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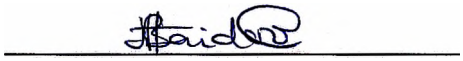
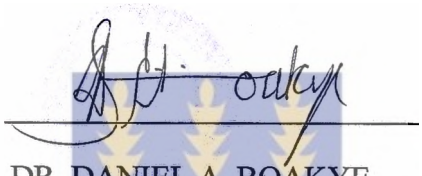
DECLARATION

This thesis is the result of research work undertaken by Helena Baidoo under the supervision of the following names listed below and all references stated have been duly acknowledged.



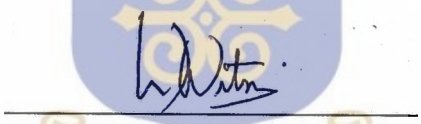
HELENA BAIDOO

(Student, 10097418)



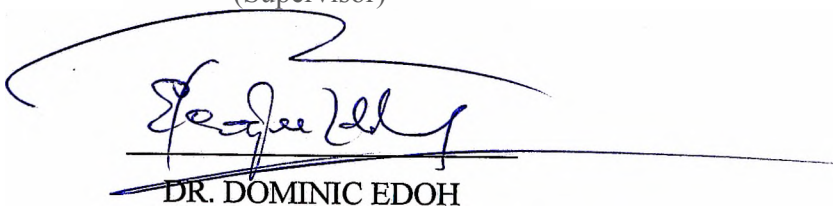
DR. DANIEL A. BOAKYE

(Supervisor)



PROF. M. D. WILSON

(Supervisor)



DR. DOMINIC EDOH

(Supervisor)

DEDICATION

TO ALL THE MEMBERS OF THE BAIDOO FAMILY

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LIST OF ABBREVIATIONS

CDC	Centre for Disease Control and Prevention, Atlanta, USA
DPD	Division of Parasitic Diseases
DEC	Diethylcarbamazine citrate
WHO	World Health Organization
ADL	Adenolymphangitis
ELISA	Enzyme-linked immunosorbent assay
ICT	Immunochromatographic whole blood test
PCR	Polymerase chain reaction
bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dTTP	deoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
EDTA	Disodium ethylene diamine tetraacetate.2H20
pH	Hydrogen-ion exponent
RNA	Ribonucleic acid
RNase	Ribonuclease
rDNA	Ribosomal DNA
rpm	Revolution per minute
$SddH_20$	Sterile double distilled water
S.S.	*sensu stricto*

TAE	Tris-acetate EDTA
TE	Tris EDTA
Tm	Melting temperature
Tris	2-amino-2-hydroxyl-1,3 -propanediol
TBE	Tris-Borate-EDTA Buffer
uL	Microlitre

ABSTRACT

The monitoring of the current global strategy for the elimination of lymphatic filariasis (LF) can be confounded if other parasites indistinguishable from *Wuchereria bancrofti* also occur in endemic areas. An incidental examination of cattle blood revealed microfilariae that were morphologically similar to *W. bancrofti.* This study was therefore conducted to characterize these cattle filarial parasite. A total of 284 cattle from Somanya, Winneba and Axim were screened randomly for filarial parasites using the traditional blood smear technique. Blood was collected from the positive cattle at 4-hour intervals over a 24-hour period to determine microfilarial intensity and periodicity, and for molecular studies.Six hundred and ninety mosquitoes were collected off the positive cattle from 00.00 - 04.00am by an aspirator into paper cups and sent to the laboratory. The mosquitoes were morphologically identified and dissected for filarial infections. DNA extracted from the microfilariae (mf) and infective stages (L3s) of the parasites were subjected to PCR analysis using *W. bancrofti* primers (NV1 and NV2). The prevalence of filarial infections were 3.5% (5/141) and 6% (2/33) at Winneba and Somanya respectively and negative for 110 cattle blood screened at Axim. An overall prevalence of filarial infections among cattle for the three study sites was found to be 2.4% (7/286). The geometric mean densities among infected cattle were found to vary between 4.6-20 microfilariae/1 OOul of blood and trend suggestive of subperiodicity. The two morphological types of microfilariae (sheathed and unsheathed) were observed and both were morphometrically similar, but different from *W. bancrofti* suggesting that they were not the latter species but rather *Setaria* species. One distinctive feature about the

microfilaria was the presence of a prominent inner korper, which is absent in *W. bancrofti.* Also both cattle filarial parasites and the infective larvae were negative by PCR. Thus indicating that they are not *W. bancrofti.* Among the 690 mosquitoes collected off cattie, 612 (88.7%) were *Culex,* 29 (4.93%), Anopheles, 43 (6.23%) *Mansonia and* 1 *(0.15%) Aedes.* Two infective stages of filarial worms (L3) were found in *Culex* mosquitoes. These results seem to indicate that the presence of these cattle filarial parasite in LF endemic areas may not confound the monitoring of LF intervention programmes.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Lymphatic filariasis also known as elephantiasis is a debilitating disease, which result from infection with vector-bome tissue dwelling nematodes called filariae. It is ai important public health and socio-economic problem worldwide. Both male and femali are equally susceptible to infection but due to different local, cultural, and social worl practices as well as exposure to insect vectors, one sex may be more exposed to infectioi than the other. All ages are susceptible and potentially microfilaremic as stated by Cool and Saunders, 1996. Microfilaremia rate increases with age through childhood and earl; adulthood, though clinical manifestations may be inapparent in endemic areas Manifestation of acute and chronic filariasis usually occurs after years of repeated anc intense exposure to infected vectors.

*

The disease is rarely fatal but the consequences of infection can cause significan personal and socio-economic hardships for those who are infected. Morbidity of humar filariasis is due mainly to the host reaction to microfilaria or to developing adult worms in different areas of the body (Evans *etal.,* 1993).

The World Health Organization (WHO) ranks lymphatic filariasis as the second leading cause of permanent disability worldwide. Currently 120 million people are infected worldwide, out of which 103 million (90%) of these infections are caused by infections with *Wuchereria bancrofti* and 13 million (about 10%) either by *Brugia malayi* or *Brugia timori.* (Michael *et al.,* 1996). More than 1.1 billion people (20%) of the world's population live in areas where they are at risk of infection.

About half of the world's burden of lymphatic filariasis is transmitted by *Cx. qumquefasiciatus* in India. This and other man-biting mosquitoes belonging to the *Culex pipiens* complex are responsible for most or all of the bancoftian filariasis transmission in Asian countries, Indonesia, Egypt, urban East Africa and the Americas (Subramanian *et al.,* 1997). In the Papuan sub-region and tropical Africa including Ghana, *Anopheles* mosquitoes transmit lymphatic filariasis (Appawu *et al.,* 2001).

Surveys in Ghana have indicated that bancroftian filariasis is present in most parts of the country, with considerable regional variations in prevalence (Dunyo *et al.*, 1996). Studies along the coast of Ghana have revealed endemic foci along the west coast of the country with an overall microfilaria (mf) prevalence of 9-25% and microfilarial intensities of 321-1172 mf/tnl of blood. Hydrocele affected 8.5-27% whilst elephantiasis of the limbs affected 8.5-27% of populations (Dunyo *et al.,* 1996). Hunter (1992) observed that a number of communities in Ghana with filariasis had water impoundment, such as small dams provided for agricultural purposes For example in Okyereko, an irrigation project community in the Central Region of Ghana where the prevalence of microfilaraemia was

26.4%, and hydrocele and elephantiasis were 13.8% and 1.4% respectively (Dzodzomenyo *etal.,* 1999).

Fortunately, several advancements in the drug therapy regimen for community-wide control programmes have been made. Several studies have shown 1hat simultaneous treatment with albendazole and the antifilarial drug ivermectin enhanced the suppression of microfilaiae (Ottesen *et al.,* 1990; Dreyer *et al.,* 1995; Bockarie *et al.,* 1997). It has also been reported that the disease can be eliminated worldwide with the use of mass chemotherapy of the affected communities (Kazura *et al.* 1993; Ottesen & Ramachandran 1995; Bockarie *et al.,* 1998). The co-administration of DEC with either ivermectin or albendazole, which is a single dose therapy, has been shown to reduce blood microfilaria levels effectively and to the same long-lasting degree as tihe previously recommended 12-day treatment course of DEC.

This strategy for controlling the disease has been adopted by the WHO for its programme on the global elimination of lymphatic filariasis. For it's implementation to be successful there will be the need for a very effective monitoring and evaluation system because many control programmes have failed as a result of premature cessation of control activities probably resulting from ineffective monitoring (Ottesen *etal.,* 1997). Effective monitoring depends on correct identification of parasites in human and insect hosts, (vectors) and also an in-depth knowledge of vector movement and distribution is essential. These entomological parameters need to be built up within any intervention

programme because information on these parameters gives indication of the situation at any point in time.

Determination of pre-intervention and post intervention entomological parameters of disease transmission have been used for monitoring intervention programmes against insect-bome diseases. A classical example is the monitoring of onchocerciasis control (Yameogo *et ah,* 1999). This approach however, could be misleading if there are other filarial parasites indistinguishable from those implicated as causative agent of the disease. This situation could become more confusing if the same vectors transmit the disease. An example is the occurrence of the cattle filarial *Onchocerca oche*ngi found in the members of the *Simulium damnosum* complex. This is a situation that could affect the accurate assessment and monitoring of oncho cerciasis control (Toe *et al,* 1998).

The programme to eliminate lymphatic filariasis is an initiative with a bold objective, which is to eliminate LF as a public health problem by the year 2020 (WHO, 2000). This elimination programme has been planned and has already started in many African countries including Ghana. The institution of a monitoring strategy based on vector transmission indices has been planned and a molecular methodology is being developed (Boakye *etal.,* 2001).

1.2 Rationale and Aims of the Study

An incidental examination of catde blood revealed some filarial parasites that on morphological examination showed some similarity to *W. bancrofti.* A polymerase chain reaction (PCR) conducted using the *W. bancrofti* Primers, NV1 and NV2 (Ramzy *et al.,* 1997) on the DNA of pooled parasite from the cattle also gave a product of the same size as *W. bancrofti* (M. D. Wilson, pers. Comm.). These observations indicated that the presence of these worms could pose a limitation to the use of entomological parameters for monitoring lymphatic filariasis intervention especially if the vectors are found to be members of the *Anopheles gambiae* complex and that these parasites are widely distributed in areas endemic for bancroftian filariasis. There is therefore the urgent need to properly characterize these worms and, determine their distribution and identify the possible vectors.

1.2.1 General objective

The project seeks to use both morphological and molecular methods to identify cattle filarial parasites morphologically similar to *Wuchereria bancrofti.* The vectors of these parasites will also be determined in order to establish how important they will be in the monitoring of lymphatic filariasis intervention in Ghana.

1.2.2 Specific objectives

The specific objectives of the study are as follows:

1. To examine cattle blood for the presence of filarial parasites
2. To determine if these parasites exhibited any form of periodicity as observed in *Wuchereria bancrofii*
3. To use morphological methods to identify these cattle parasites
4. To apply molecular methods developed for specific identification of *W bancrofii* on the cattle parasites
5. To determine the prevalence of blood filarial infection in cattle
6. To determine if these cattle parasites are also transmitted by members of *Anopheles gambiae* complex

CHAPTER TWO

LITERATURE REVIEW

2.1 Filarial Parasites

Filarial parasites (filaroids) are long hair-like tissue-dwelling nematodes. All except the Guinea worm *Dracunculus medinensis* (which uses a copepod) employ arthropods as intermediate hosts. All filaroids have a similar life cycle that includes an obligatory maturation stage in a blood-sucking insect or copepod, and a reproductive stage in the tissues or blood of a definitive host (McMahon and Simonsen, 1996).

Adult male and female worms live in the lymphatic, skin, or other tissues. Microfilariae (Fig 2.1) are produced by the female worm, which either circulate in the blood or invade the skin, and are ingested by the vector. Larval development but not multiplication occurs within the muscles of the vector. The infective stage L3 migrates to the proboscis and is transmitted to the new host during feeding. Unlike malaria, the infective stage is not directly injected into the skin of the new host. They are deposited onto the skin whilst the mosquito is feeding and find their own way through the skin, usually via the puncture made by the mosquito. Filaroids have been found in all classes of vertebrates except fish and are especially common in birds (McMahon and Simonsen, 1996). All but one of the species that cause human disease (the Guinea worm) belongs to the Family Onchocercidae.

2.1.1 Human filarial diseases

Of the hundreds of described filarial parasites, only 8 species cause natural infections in humans (Nissen, 2001). Human filarial parasites may be classified according to the habitat of the adult worms in the vertebral host. As such there are three groups of filarial parasites. These are the cutaneous group *(Loa loa, Onchocerca volvulus* and *Mansonella streptocerca*); lymphatic group (*Wuchereria bancrofii, Brugia malayi* and *Brugia timori*) and ihe body cavily group (*Mansonella perstans* and *Mansonella ozzardi).* Parasites of the cutaneous and lymphatic groups are of the most significant clinical interest (Nissen, 2001**).**

2.1.1.1 Lymphatic filariasis

Wuchereria bancrofii, Brugia malayi and *Brugia timori* are mosquito-bome and cause lymphatic filariasis. In many people's mind lymphatic filariasis is synonymous with "elephantiasis" characterized by the enlargement of scrotum or limbs (Figure 2.2). In most endemic areas elephantiasis only occurs in a small proportion of the people suffering from lymphatic filariasis. Bancroftian filariasis, caused by *W. bancrofii* is responsible for 90% of lymphatic filariasis cases and is found throughout the tropics and in some sub-tropical areas. *Brugia malayi* is confined to Southeast and Eastern Asia and is transmitted by mosquitoes including *Mansonia, Anopheles* and *Aedes* (WHO, 1997). *Brugia timori* is found only in Timor and its adjacent islands (McMahon & Simonsen 1996) and is transmitted by *Anopheles* mosquitoes (WHO, 1997). In West Africa the vectors of *W. bancrofii* are *An. gambiae* and *An. Junestus* whilst in east and Central Africa, it is *Culex* mosquitoes (Subramanian *et al.,* 1997).

2.1.1.2 Onchocerciasis

Onchocerciasis is a debilitating disease affecting 20-40 million people in 34 countries of Sub-Saharan Africa, the Arabian Peninsula and South America. It is transmitted by Simulid black fly. Microfilariae invade the skin and give rise to dermatitis, premature aging of the skin and skin atrophy. Development of the adult worm leads to nodule formation. Microfilariae invade the eye and cause an inflammatory reaction that can lead to blindness (Duke, 1990). In recent years, the prospects for its control have been improved with the development of community directed treatment with ivermectin, which is a safe effective and affordable drug. However encephalopathies with occasional fatal outcomes have been reported following its use to treat persons coinfected with *Onchocerca volvulus* and *Loa loa* (Boussinesq and Gardon, 1997; Chippaux *et at,* 1996; Gardon *et al.,* 1997). These adverse outcomes are an obstacle to the sustainability of large-scale ivermectin treatment for the control of onchocerciasis and lymphatic filariasis in areas of Africa where there is a potential for coendemicity with *L. loa* (Boussinesq and Gardon, 1997).

2.1.13 Loasis

Loasis occurs in the forested areas of West and Central Africa particularly in Nigeria and Cameroon. It is caused by *Loa loa* (sometimes called the African eye worm), which is transmitted to humans by the daytime-biting tabanid flies. Loasis very rarely causes serious complication and the adult worms generally go unnoticed unless they pass through the bridge of the nose or the conjunctiva of the eye. The disease is characterized by a range of clinical manifestation including "Calabar swellings", occurring commonly

in the hotter months, pruritis and eye inflammation (Pinder *et al.,* 1988). A localized, sometimes painful, swelling also occurs when the worm dies. It is the most common filaroid infecting travellers from non-endemic areas (Pinder *et al,* 1988).

2.1.1.4 Other filarial infections

Mansonella perstans which occurs in Africa, Central and South America, *Mansonella streptocerca* (Africa) and *Mansonella ozzardi* (Central and South America) are all transmitted by Culicoid midges. Most infections are asymptomatic but in the worse cases symptoms similar to *O. volvulus* may occur. This is especially relevant in Brazil where *M. ozzardi* and *O. volvulus* are sympatric in parts of the Amazonia oncherciasis focus. Also occasionally symptoms, which look very much like loasis, may occur (WHO, 1997). Attempts at controlling mansonelliasis have been few, but a recent report indicates that ivermectin may be effective as a microfilaricide. Other filariae also known to infect man includes *Wuchereria kalimantari, B. arbuta, B. beaveri, B. guyanensis* and *Dipetalonema sprenti.*

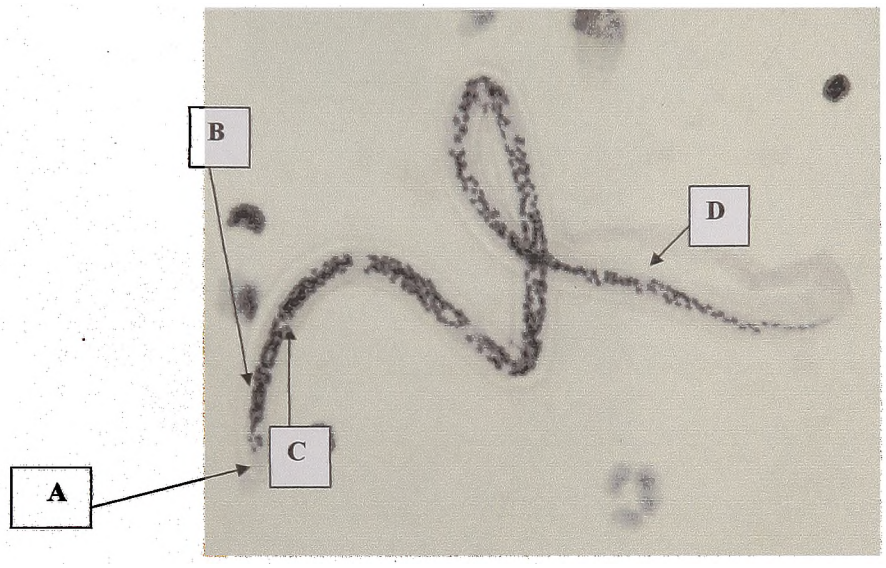

Fig 2.1: Microfilaria of *Wuchereria bancrofti,* A=Head space, B=Inner body, C=nerve ring and D= Sheath

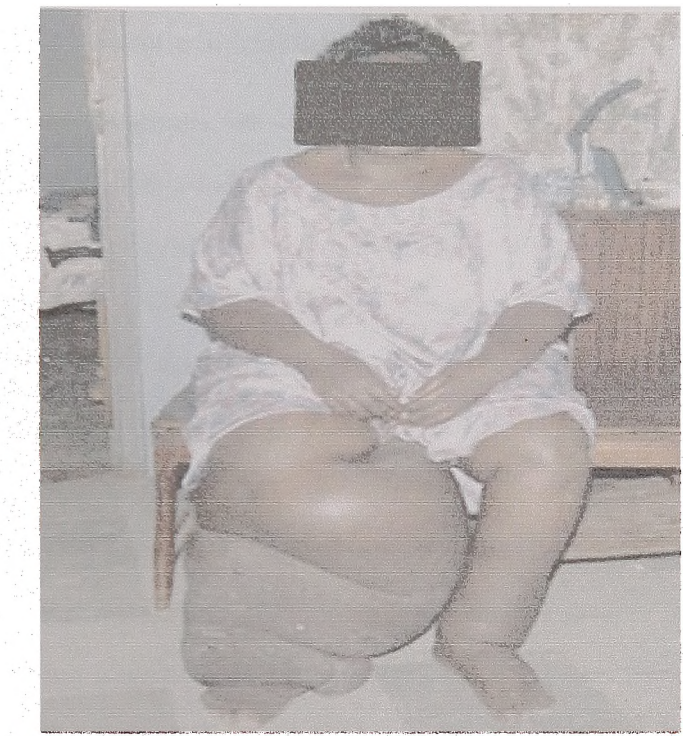

Fig *22:* Elephantiasis of the leg (WHO filariasis image library)

2.1.2 Zoonotic filarial infections

Human infections with various known and unknown animal filarial parasites have regularly been reported throughout the tropical and subtropical parts of the world including the southwestern United States of America (Nelson, 1965; Orihel and Eberhand, 1998). There could be a wide range of animal parasites that can infect man as well as their animal host. These include; *Dirofilaria immitis, D. repens, D. tenuis, D. spectans, D. striata, Setaria equina mdMeningonema peruzzi.*

Dirofilaria immitis is the most important filaroid parasite of domestic animals endemic in tropical and subtropical and warm temperate regions of the world (including the south western United States) which occasionally infects man (Rodrigues-Silva *et al.,* 1995). Adult worms reside in the right ventricle and pulmonary artery of the dog while the unsheathed microfilariae circulate in the blood. Once ingested by the appropriate mosquito vector, the microfilariae undergo development into infective larvae, which can be transmitted to both dogs and humans. However the worms never reach full maturity in humans. Most cases are asymptomatic and the worm becomes calcified in the lung resulting in a "coin lesion" which may be mistaken for carcinoma or tuberculosis. There are more than 200 cases of human pulmonary dirofilariasis reported throughout the world (Schneider *et al.*, 1986). Buckley (1958) demonstrated that man could suffer serious complications when inoculated with *Brugia* species from animals. Thereafter furnished experimental proof by Danaraj (1956) suggested that animal filariae were responsible for tropical pulmonary eosinophilia quite common on the Kenya coast.

Another dog filaroid, *Dirofilaria repens* can cause subcutaneous nodules, peri-orbital lesions, coin lesions in the lungs, and breast lumps in humans and rare eases have been reported throughout the world (Pampiglione *et al,* 1995). High levels of antifilarial IgG, IgE and IgM antibodies are present in patients with dirofilariasis (Simon *el al.,* 1997) but there can be high background level of dirofilaria-associated antibody in communities which are in close contact with infected dogs and the prevalence of *IX immitis* antibodies in humans is closely related to the number of infected dogs in the community (Welch and Dobson, 1974). At least six cases of human filarial dermatitis have been attributed to *Brugia beaveri* (Routh and Ebowmik, 1993), a filarial species whose natural host is the Raccoon and nine cases of lymph node infection by an unidentified *Brugia* species (Baird *et al,* 1986) possibly from the domestic cat. Animal filaroids seldom seen to develop and become sexually mature and produce a microfilaraemia in man but it does occur from time to time (Orihel and Eberhard, 1998).

Greene *etal.* (1978) found circulating microfilariae in a patient from Alabama who was suffering from Lupus erythematosis. Although the species could not be identified with certainly, the most likely species appeared to be *Mansonella interstitium,* which is a filaroid of squirrels. There has also been report of microfilaraemia and eosinophilia in a 70-year-old Greek man who had never been in an area endemic for human filarial parasites and was presumably infected with an animal species (Petrocheilou *et al.,* 1998).

2.1.2.1 *Setaria* species

Setaria species are widely distributed throughout the world and many species of the adult have been described. More than 200 species of filariae have been described in literature; the life cycle of only 38 have been studied. Nelson (1962) stated that 17 species have been found in Africa alone. Mosquitoes are suitable intermediate host of 23 out of the 38 species and these include filariae from lizards, frogs, birds, bats, carnivores, herbivores and primates. *Setaria* species are parasitic in the peritoneal cavity of sheep, cattle, the ox, deer and horses They are also known to be the major causative agents of epizootic cererobrospinal nematodiasis in sheep raised in Korea since 1939 (Kimura & Niimi, 1939,1940,1941).

Adult *Setaria digitata* is commonly found free with the abdominal cavity of several ungulates, which include cattle, sheep and horses in the Far East and Asia. In Korea, two species *S, digitata* and *S. marshalli* have been reported to parasitize cattle (Rhee *et al.,* 1994). Prevalence *of Setaria* spp among cattle raised in Korea ranges from 5 to seventy percent (Lshii *et al.,* 1953; Paick *etal.,* 1976; Rhee *etal.,* 1994 Moon and Kang, 2000; Mohanty *et al.,* 2000). In one particular study, a total of 110 cattle were examined in a bancroftian filariasis endemic area to determine the prevalence of infection of the bovine filarial parasite *Setaria digitata.* About 12.5% of cattle examined were found to harbour both adult worms in the peritoneum nd microfilariae (mf) in circulation. Seventy percent of the cattle were amicrofilaraemic but harboured adult worms. A third group of cattle (16.5%) were free of detectable mf and adult worms. The presence of adult worms and/or

mf did not influence the antibody levels of the 4-antigen preparation of *S. digitata* (Mohanty *etal,* 2000).

Although the adult worms of most *Setaria* species in the abdominal cavity are mostly harmless to cattle serious pathogenic results could occur in animals such as sheep, goats and horses in which larvae can migrate erratically into the central nervous system (Innes & Shoho, 1952, 1953). The ectopic parasitism of *S. digitata* has been reported in the eye of a horse (Jemelka, 1976). Except for one report on *S. digitata* in the cavity of a cow (Nair *et al,* 1993), heterotropic parasitism of cattle with *Setaria* species is rarely known. A case of a single-eyed blindness of two cattle in Korea and the isolation of a female *S. digitata* has been reported (Sung-Shik *et al.,* 2001). The worm removed was 5.6 cm long and was identified as female *S. digitata* after the light and electron microscopic morphological description of Rhee *et al.* (1994).

Setaria cervi (Setaria labiato-papillosa) is a cosmopolitan nematode parasite of cattle which is used to assess the efficacy of potential antifilarial agents, and bears close similarity to human filarial parasite *Wuchereria bancroft* (Singhal *et al.,* 1969, 1972). Work done by Heisch *et al.* (1959) revealed that out of 200 cows examined; 7 had microfilariae in blood films, 5 of which were infections due to *Setaria cervi*; the microfilariae were also similar to *Setaria equina.* Although in the survey only 5 cows were found with microfilariae in blood films, adult *Setaria cervi* were commonly seen in the peritoneal cavities of cattle slaughtered at the abattoir in Mombassa. Microfilarial densities have always been very low in the cattle examined in Kenya. This has made

feeding experiments with mosquitoes very difficult This problem has been overcome by implanting living adult setaria in the peritoneal cavities of monkeys, using technique developed by Williams (1955).

* Zoonotic *Setaria* infections in human is rare, however Panaitescu *et al.* (1999) have reported four cases of human filariasis due to *Setaria labiatopapillosa* in Bucharest, Romania. The vector insect however could not be specified. These are the first reported cases of human infection with *Setaria labiatopapillosa.*

2.2 Filarial Nocturnal Periodicity

Periodicity is a well known phenomenon which occurs with many filaroid worms, and various hypotheses put forward to explain periodicity have been comprehensively reviewed (Oishi, 1959; Hawking, 1967; Kamine, 1972; Masuya, 1976).

According to the periodic pattern of microfilariae (mf) in the human host's peripheral blood, lymphatic filariasis caused by *Wuchereria bancrofti* may be separated into three forms: (Aikat, 1977) a nocturnal periodic form, widely found in tropical and subtropical zones in Africa, Asia and Latin America, in which microfilarial densities peak close to midnight; (Dreyer *et al.,* 1996) a non-periodic or diurnal, sub-periodic form, prevalent in the islands of the South Pacific, in which maximum densities of mf occur around 16.30 hours; and (Gatika *et al.,* 1994) a nocturnal, sub-periodic form, with a focal distribution in western Thailand, which is characterized by a peak in microflarial density at around 20.30 hours (Harinasuta *et al.,* 1970 a,b; WHO, 1992).

A mathematical method for the analysis of microfilarial counts in peripheral blood during a 24-h cycle was developed by Sasa and Tanaka (1972), who believed that the circadian variation followed a simple harmonic wave. Aikat and Das (1977) devised another method, based on simple trigonometry, to calculate at which time densities of mf in the peripheral blood peaked and to calculate a periodicity index. The data available from surveys of periodicity among the mf of human parasites from various regions of the world were analyzed by Tanaka (1981), using both of these methods. Although the two

methods gave the same results, Tanaka (1981) considered that the trigonometric method of Aikat and Das (1977) was the easier to perform.

The method proposed by Aikat and Das (1977) have been used in various studies to establish the periodic pattern of *W. bancrofti* mf in the peripheral blood of subjects worldwide and also to determined appropriate time for sampling. For instance in the Brazilian city of Maceio, in Alagoas state, a study was conducted by Rocha and Fontes (1998) to establish the periodicity of *W. bancrofti.* In the study it was found that although all the subjects had a detectable microfilaraemia from 23:00 hours to 06.00 hours, no mf could be detected in most (71.4%) of the smears prepared from samples collected at 15.00 hours. Samples collected during 15.00 hours, contained 170 times fewer mf7nl than those collected at 01.00 hours when microfilaraemia were generally most intense. Therefore for diagnosis of bancroftian filariasis in this area, blood samples should be collected between 22.00 and 3.00 hours, when microfilarial counts will be at least 90% of the peak counts. This study confirmed other studies that have been done with regards to periodic patterns of *W. bancrofti* (Dreyer *et al,* 1996; Simonsen *etal.,* 1997).

Not much work has been done on animals in terms of periodicity but there have been a few reported studies on dogs, cattle and ungulates in general. *Dirofilaria immitis* is probably the best known filarial in the laboratory throughout the world. As such it has been used widely for the study of microfilaria periodicity in animals. It produces microfilariae that circulate in the peripheral bloodstream as well as the blood of all other parts of canine body. There is a tendency towards microfilarial periodicity in a day

(circadian rhythm) as well as seasonal periodicity showing a summit in summer throughout a year (Oishi, 1959). This appears to vary in different countries. Thus Tarplee and Bradley (1982) found maximal numbers at midnight in the USA; Euzeby and Laine (1951) found the lowest numbers at 08:00 hr and the greatest at 20:00 hr in France; Webber and Hawking (1955) found minimum parasitaemia at 06:00 hr and maximum at 18:00 hr in a Chineese strain of *D. immitis* in England. Moreover, several investigators in Japan have referred to the periodicity of *D. immitis* as being lowgrade nocturnal (Masuya, 1976), and a distinct nocturnal: maximal numbers were found at 24 hr and minimal numbers at 10:00 hr and the number of maximum was 6.5 times of minimal count from 28 dogs naturally infected with *D. immitis* (Oishi 1959).

On the contrary, Angus (1981) reported that there are both a distinct diurnal (16:00 hr) and lowgrade nocturnal (from 24:00 to 01: 00 hr) peal® in the periodicity of *D. immitis* microfiariae in cephalic venous blood of dogs in South Queensland. There were diurnal periodicity- maximum microfilarial counts of *D. immitis* were found at 11:00 hr and minimal at 22:00 hr in a dog from Tanzania (Matola, 1991). Moreover the microfilaraemia in a dog infected with *D. immitis* was diumally subperiodic with maximum microfilariae numbers between 12:00 and 16:00 hr (Grieve and Lamia, 1983) and Schnelle and Young (1944) observed minimum microfilaraemia at 11:00 hr and maximum at 16:30 hr in the USA A recent study by Rhee *et al.* (1998) in Korea determined the periodicity of *D. immitis* at two-hour intervals for 72 consecutive hours in 10 naturally infected war dogs aged 3-9 years. This study was done to facilitate harvesting of the microfilariae for possible use as an animal model and to elucidate

further periodicity of the microfilaria depending on geographic location. Although the periodicity had been observed as being low-grade nocturnal, maximal microfilaria, counts were found at 21:00 h and minimal at 11:00 hr, giving rise to an evident peak in fluctuation of the larval counts. Studies by Nelson *et al.* (1962) revealed that *Setaria* species just like *Mansonella* species are normally aperiodic and as such the microfilariae circulate in the peripheral blood throughout a 24-hr period without significant changes in their numbers.

2.3 The Vectors of Human Filariasis

A wide range of mosquitoes acts as vectors for filariasis. Depending on geographic region, human filariasis can be transmitted by mosquito species belonging to the genera *Culex, Aedes, Anopheles, Mansonia, Psorophora* and *Coquillettidia. Culex quinquefasciatus,* which is the most important species among the *Culex pipiens* complex, is the principal vector of Bancroftian filariasis in areas where *Wuchereria bancrofti* has nocturnal periodicity, but in other places, other species of mosquitoes may serve as insect host of the parasite (White 1989, WHO 1992). In Brazil, Cousey *et al.* (1945), Rachou *et al.* (1956) incriminated species of *Aedes* and *Anopheles* as hosts of secondary importance. In Maceio, capital state of Alagoas, northerwest Brazil, Deane *etal.* (1953) and Fontes *et al.* (1994) established that *Cx.* spp is the most important insect host of *W. bancrofti.* The *Cx. pipiens* complex consists of several named species including; *Cx. pipiens* s.s., *Cx quinquefasciatus, Cx. austratralicus* and *Cx. globocoxitus* (Savage & Miller, 1995). These species differ mainly in their behaviour, biology and morphologically, in minor details. The shape of the male genitalia, shape of larval siphon and number of the first siphonal hair are mostly used as distinguishing features. Within the *Cx. pipiens* complex, *Cx quinquefasciatus* is the most important vector species of *Wuchereria bancrofti* in Asia and parts of both urban and semi-urban East Africa (White, 1971, Subramanian *et al.,* 1997) but it is considered an unimportant LF vector in West Africa (Jayasekera *et ah,* 1980, Zielke and Chlebowsky, 1980, Dzozomenyo *etal.,* 1999, Appawu *etal.,* 2001).

Aedes egypti is frequently found in human habitations in Maceio and it was considered necessary to determine if this species plays a role in the transmission of *W. bancrofti.* *Aedes polynesiensis* and *Aedes samoanus* are the most important vectors in the pacific area. The former breeds in crab holes and tree holes making it a very difficult mosquito to control with conventional methods. They are day-biting mosquitoes thus accounting for the diurnal periodicity of filariasis in this region. *Aedes poecilius,* a night-biting mosquito is a major vector in the Philippines. *Mansonia* species are an important vector of *B. malayi,* and sometimes *W. bancrofti,* in areas where there are extensive areas of aquatic plants.

Anopheles species are important vectors of *W. bancrofti* in parts of Africa and Southern Asia and in Papua New Guinea. The *Anopheles gambiae* complex is considered as the most important LF vector in Ghana (Dzozomenyo *etal.,* 1999, Appawu *etal,* 2001). *An. gambiae si* is a group of six morphologically indistinguishable yet genetically and behaviorally distinct mosquito species that vary dramatically in their importance as vectors of various diseases including LF in Africa (Coluzzi *et al,* 1979). *Anopheles, gambiae sensu stricto* Giles, *An. arabiensis* Patton, and *An. quadriannulatus,* are freshwater species; saltwater species are *An. merus,* and *An. melas, An. bwambae,* is the only mineral water species (White, 1985). The species are morphologically indistinguishable.

Anopheles funestus Giles is the second most important LF vector in Ghana (Dunyo *et al,* 1996; Dzodzomenyo *et al.,* 1999; Appawu *et al,* 2001). *Anopheles, funestus* is also a member of a species complex comprising at least nine members, the adults of which are

not easily distinguished on the basis of morphological characteristics (Gillies & De Meillon 1968; Gillies & Coetzee, 1987) although some species may be distinguished using larval characteristics. The members of this complex are *An. funestus* s.s., *An. vaneedeni* Gillies and Coetzee, *An. parensis* Gillies, *An. arum* Sobti, *An. conjusus* Evans and Leeson, *An. rivulomm* Leeson, *An. juscivenosus* Leeson, *An. leesoni* Evans, and *An. brucei* Service. Traditionally, the only other method for distinguishing members of the *An.Junestus* group has been by chromosomal inversion karyotypes (Green, 1982). More recently, however, single-strand conformation polymorphism (SSCP) analysis has been used to identify four member of this group (Koekemoer *et al.,* 1999). Of the nine species in the complex, *An. funestus* s.s. has the widest distribution. This mosquito is also highly anthropophilic (Gillies and De Meillon, 1968).

2.3.1 Identification of *Culexpipiens* complex

Adults of the *Cx. pipiens* complex are light brown mosquitoes that lack distinctive markings on the proboscis and legs, and are not rapidly separated from other *Culex (Culex)* mosquitoes. Adult females of the complex are usually identified by the presence of distinctive, basal, pale abdominal bands. Abdominal bands are broadly rounded medially and distinctly constricted sublaterally before joining large, lateral scale patches. Male adults of *Cx. pipiens* and *Cx. quinquefasciatus,* and with less precision *Cx. pipiens-quinquefasciatus* hybrids, can be identified by use of the DV/D ratio of the genitalia (Savage and Miller, 1995). Larvae of the *Cx. pipiens* complex can be identified by the presence of a moderately long siphon that has 6-13 pecten teeth locate on the basal 1/3, and 4-branched siphonal tufts, one of which is laterally and out-of-alignment with the

other three; and double-branched lateral setae on abdominal segments IE-IV (Savage & Miller, 1995). The shape of the siphon and number of branches on setae I on abdominal segment 3-4 can be used to characterised *Cx. pipeins* and *Cx. quinquefasciatus.* However, no reliable means of specific larval identification is available in areas where hybrids may occur.

Recent molecular studies have led to the development of species specific polymerase chain reaction (PCR) primers that can be used in a PCR "cocktail" to identify *Cx. salinarius, Cx. restuans,* and the *Cx. pipiens* complex (Crabtree *et al.,* 1995). Unfortunately, variation between *Cx. pipiens* s.s. and *Cx. quinquefasciatus* was insufficient to develop diagnostic primers for these taxa. Later, Crabtree *et al.* (1997) used genomic subtractive hybridization to identify a region of nucleic acid heterology between the genomes of the two latter species.

2.3.2 Identification of *the An. gambiae* and *An.funestus* complex

After the discovery of the existence of the sibling species in *An. gambiae* group in 1956 by crossing experiments (White, 1974), several methods have been exploited to try and develop a consistent and easy to use identification method. At first, hybridisation experiments were the only possibility, but naturally this is far too laborious and moreover inconvenient because live mosquitoes are needed. The morphology of the species was studied, but this can only distinguish between some of the species and even then there is a lot of overlap (White, 1974). Gillies and De Meillon, (1968) and Gillies and Coetzee, (1987) provides established identification techniques, by using morphological characters,

for the anopheline mosquitoes. *An. gambiae* adult females are identified by their smooth palps with 3 pale bands on the 3rd 4th and 5* segments; the wing field is pale with yellowish or creamy markings and has pale fairly long costal spots. The femora, tibia and 1st tarsal segment speckled to a variable degree. The abdomen is pale brown and hairy with scales on the 8th tergite and scales on the cerci. Adult female *Anopheles funestus* are identified by the observation of three pale bands on the 2nd, 3rd and 5th segments of the palps. The dark wings also bears characteristic pale scales, with the costa having four pale spots usually shorter than the intervening dark areas. The abdomen is dark brown and lack scales including the cerci, while the legs are usually dark with a small apical white spot on the tibia.

Identification by cytotaxonomy was and perhaps still the most reliable method to use. Species are identified on the basis of polytene chromosome banding patterns. The X chromosome differentiates between *An. gambiae, An. arabiensis* and *An. quadricmnulatus;* the two autosomal chromosomes more subtly distinguish the other species (White, 1974).

The use of isoenzymes of the enzyme octanol dehydrogenase for species identification (Miles, 1978, 1979) has the advantage that it is not stage or sex-specific. It can however only be applied to freshly caught or frozen specimens. Moreover, there are no single or sets of isoenzymes that are uniquely associated with the different species of complex; there is always some overlap (Collins *etal.,* 1988). Other investigative techniques that

were brought to practice at a large scale include the use of cuticular hydrocarbons (Carlson & Service, 1980) and of sex chromosome heterochromatin (Gatti *et al.,* 1982).

In 1987 two DNA probe-based identification methods were independently developed. A major advantage of these techniques is that they can be applied to frozen, dried, alcohol and isopropanol preserved specimens, because of the stability of DNA molecules. There have been several improvements on these techniques (Hill *et al.,* 1991a, 1991b). The latest identification method that was developed makes use of a technique called the polymerase chain reaction (PCR). PCR involves the amplification of unique sequences from a mixture of sequences via the use of two primers (Saiki *et al,* 1985, 1988). Each primer anneals to a complementary piece of DNS, which triggers the binding of a DNA polymerase that then copies the segment. By this procedure, it is possible to synthesize many copies of a chosen piece of DNA in the presence of vast excesses of other DNA sequences (Kocher *et ah,* 1989). The PCR-based assay also differentiates between all complex members except *An. bwambae* (Paskewitz and Collins, 1990; Collins *et ah,* 1990; Scott e/ *ah,* 1993).

2.4 Trasmission Dynamics in Filariasis

Several scientists have extensively reviewed the host-parasite relationship and the transmission dynamics of filariasis. Two modelling frameworks lymfasim (Plaisier *etal.,* 1998) and Epifil (Chan *et al.,* 1998) are based on the life history of the parasite, its transmission, the unmuno-regulatory role of the host immune system, the development of disease and the impact of control measures.

In general, the mosquito infection rate, that is the number of mosquitoes that contain microfilariae after a blood meal and the number of microfilariae ingested per mosquito, increases with increasing parasitaemia. Although there is no multiplication in the vector, the number of infective larvae is around six times greater than that expected by microfilariae density (Brito *et al.,* 1998). Bockarie *et al.* (1997) has shown that the annual infective biting rate and the annual transmission potential show a positive correlation with microfilariae rate, microfilariae density and prevalence of leg oedema suggesting that transmission intensity is a major determinant of patent infection and morbidity. Kazura *et al.* (1997) studied the relationship between annual transmission potential (ATP) and disease status in 1666 individuals from five similar but distinct *W. bancrojti-inkcted* communities from a highly endemic area of Papua New Guinea. Annual transmission potentials and microfilariae prevalence (MF) in these villages were: 2,344 (MF 94%); 1,338 (MF 82%); 279 (MF 56%); 179 (MF 66%) and 31 (MF 52%) respectively. In all villages the prevalence of leg odema was highly positively related to the ATP (correlation coefficient r=0.89 and probability p=0.04). The incidence of acute

filarial attacks is also related to the transmission intensity. Gyapong *et al.* (1996) found that the incidence of acute filarial attacks were reduced in the dry season when the transmission potential was at its lowest.

The concept of "facilitation" - that the proportion of microfilariae that develop increases as the number of microfilariae ingested increases (WHO, 1992) is an important consideration when the possibility of eradication of filariasis is being considered. In theory, it should be possible to reach a point where there are insufficient circulating microfilariae in the population to support transmission (WHO, 1992). It is important to note however, that persons with microfilariae densities as low as 3/ ml can still infect mosquitoes and that residual low-density microfilaraemics after mass treatment programs have the potential to cause rapid resurgence of filariasis (Lowrie *etal,* 1989; Southgate, 1992). Beckett (1973) found that a heavy uptake of microfilariae from the blood can cause life-threatening damage to the internal organs of the mosquito during larval development but this finding is not supported by the recent work of Brito *et al.* (1998).

The feeding of mosquitoes on individuals with medium or low microfilaria] densities may therefore enhance transmission potential especially if the main vector is a culicine rather than an anopheline (Webber, 1981). The reasons for this difference, termed "limitation" is discussed by Bryan *et al.* (1990), Webber and Southgate (1981); Southgate and Bryan (1992) who suggest, that in contrast to culicines, anopheline mosquitoes have a well developed pharyngeal armature which damages microfilariae when they are ingested. If the number of ingested microfilariae is low there may be insufficient viable microfilariae

to infect the mosquito. Loss of microfilariae also occurs in fluid expelled from the anus of anopheline but not culicines (Bryan & Southgate, 1988a,b).

The concepts of facilitation and limitation and their role in filariasis transmission has been critically reviewed by Wada *et al.* (1995) who concluded that "there was no clear evidence to support the existence of facilitation and limitation-based unstable equilibrium in relation to microfilariae prevalence and density below which filariasis would spontaneously disappear even when the vector was *Anopheles.* Instead, the existence of a critical level of man/mosquito contacts for the disappearance of filariasis was suggested".

The microfilariae density in animals varies considerably from one place to another. Unfortunately much work has not been done on animals to determine prevalence of filarial infections in Ghana. However, prevalence of *Setaria species* among cattle raised in Korea ranges from 5 to 70% (Moon and Kang, 2000). Work done at the coastal part of Kenya revealed that out of the 200 cows examined; 7 had microfilariae in blood films. 5 of these infections were with *Setaria labiato-papillosa (,Setaria cervi);* the microfilariae were similar also to *S. equina.* Although in the survey only 5 cows were found with microfilariae in blood films, adult *S. labiato-papillosa* were commonly seen in the peritoneal cavities of cattle slaughtered at the abattoir in Mombassa. Microfilarial densities have always been very low in the cattle examined in Kenya. This has made feeding experiments with mosquitoes very difficult. This problem has been overcome by implanting living adult *Setaria* in the peritoneal cavities of monkeys, using the technique

described by Williams (1955). Adequate microfilarial densities have been maintained for several weeks and complete development of *S. labiato-papiHosa* have been seen in *Aedes aegypti* fed on the infected monkeys.

2.4.1 Factors that influence microfilaria prevalence and density

A spontaneous decrease in microfilaraemia prevalence can occur in the absence of vector control or mass chemotherapy. In a study in Benin the prevalence of microfilaraemia decreased from 9.4% to 0.48% over a 10-year period. The prevalence of people with chronic pathology remained the same. The changes could not be explained by environmental or sociological changes in the region or even by changes in the demographics of the study population (Myung *etal.,* 1998). Microfilariae density is also subject to considerable daily variation but this is unrelated to lunar phase lactation, or the menstrual cycle (Nathan *et al.,* 1982). Fasting during the month of Ramadan has been shown to reverse nocturnal periodicity (Nathan *etal.,* 1982). The fecund life span of *W. bancrofti* has been calculated by various means and methods and has been variously estimated from 5 to 15 years. A recent study by Vanamail *et al.* (1996) suggests that the life span is at the lower end of previous estimates - around five years.

2.5 The Global Burden of Lymphatic Filariasis

Current global estimates suggest that around 80 countries are endemic for lymphatic filariasis. Of the three parasites causing LF, *Wuchereria bancrofti* accounts for over 90% of the global burden. *Brugia malayi* is limited in distribution to Asia, and *Brugia timori* to a few islands in Indonesia. It has been estimated that 1.1 billion people living in areas endemic for this disease are exposed to the risk of infection, and that there are about 120 million cases with either disease or infection (microfilaria carriers). Almost half (49.2%) of the 120 million estimated cases are in the (WHO, 2001) South-East Asia Region (India alone accounts for about 40% of the global cases) and another 34.1% of cases are in the African region; the rest are in the western Pacific (16.1%), eastern Mediterranean (0.3%) and Americas (0.3%).

The 120 million cases of LF include 83.63 million cases of microfilaria carriers, 16.02 million cases of lymphoedema, and 26.79 million cases of hydrocele; this clearly shows that the burden of genital manifestations in filariasis in terms of hydrocele is greater than that due to lymphoedema. Of the 120 million LF cases in the world, although Asia accounts for the majorily of infection (because of the large populationsliving in endemic areas), the prevalence of *W. bancrofti* is higher in Africa (8.97%) than in Asia (2.25%). However, this is expected to be an underestimate as the proportion of endemic population who are amicrofilaraemic but have evidence of infection in terms of a positive antigen test (as many as an additional 18%of endemic populations)was not included, so the actual number of infected or diseased people could be much higher.

2.6 Socio-economic Impact of Filariasis

It is necessary to understand the social and economic impact of diseases in order to set priorities within the health system. This requires information on the types of symptom that are experienced; their prevalence, frequency and duration; and the nature of the associated social and economic costs. Much of this information is not available for lymphatic filariasis, and in its absence it is not surprising that the disease is not given higher priority in much of the endemic world (Evans *et al,* 1993)

According to Gyapong *etal.* (1996a) a major hurdle for procuring funding for control and research has been the lack of information regarding the social and economic impact of the disease. The estimated socio-economic impact of filariasis varies widely from study to study and documentary evidence of loss of production and income is difficult to find. The current global estimate of the disability associated with lymphatic filariasis is 850,000 disability-adjusted life years lost (Gyapong *et al,* 1996). This is probably a serious under-estimate given the lack of recent prevalence information for many of the endemic countries.

There is very strong evidence that late stage chronic disease with its accompanying disability reduces productive capacity as suggested by Evans *et al.* (1993), but the economic impact is reduced somewhat by the fact that most people with advanced chronic pathology are beyond their most productive years (Wijers, 1977). The impact on some communities can be severe. Gyapong *et al.* (1996) found that because of chronic filariasis 4.1% of the productive female labour force and 20% of the productive male labour force were disabled by between 10 and 60% and 20% people with advanced

2.7 Diagnosis of lymphatic filariasis

Efficient diagnosis of *W. bancrofti* infection is especially important as control programmes move towards the new strategy of community treatment and repeated, annual mass therapy with single-dose DEC or combination regimens (Ottesen and Ramachandran, 1995). Several methods are available for diagnosis of lymphatic filariasis infections. These include; direct and concentrated techniques, Antigen detection (ELISA) microfilaria membrane filtration, DNA detection (PCR), antibody serology, and, ICT card test (WHO, 1994).

2.7.1 Direct detection of microfilariae

In areas where microfilariae exhibit nocturnal periodicity blood should be taken within two hours either side of midnight when the highest density of microfilariae is expected to occur. Where they show diurnal periodicity blood should be taken two hours either side of midday. Simonsen *et al.* (1997) have devised a method to adjust for the effect of sampling time on microfilariae density. Using their technique it is possible to predict what the microfilariae density would be at midnight in blood collected at probably, 2200 hours.

The simplest technique for direct diagnosis of microfilaraemia is a Giemsa-stained thick blood film of capillary blood collected by finger-prick (Schultz, 1988). If a measured amount of blood is used (such as 40- 60 (J.1, the number of microfilariae per ml can be

calculated (Moulia-Pelat *et al,* 1992). A modification (Southgate 1974), which allows easier counting of microfilariae, is to expel 60jj,l of blood onto the slide so it forms three even linear strips. The dried films are de-haemoglobinised in buffered water before staining with Giemsa stain.

The disadvantage of thick films, like other direct methods, is that they underestimate the prevalence of microfilaraemia if microfilariae densities are low because theoretical detection limit for such procedures is between 15 and 50 microfilariae per ml (Panicker *et al.,* 1991). Even if 60pl of blood is used subjects with less than 60 microfilariae per ml will not be reliably detected. Another problem is loss of microfilariae from the film during processing especially if anticoagulated blood is used. Southgate (1973) observed a loss of up to 51% and Denham *et al* (1971) a loss of between 10% and 40%. Loss of microfilariae is minimised if un-anticoagulated blood is used and the films are dried over night at room temperature (Partono and Idris, 1977). Youssef *et al* (1995) have shown that applying a thin film of agar to the thick film before staining greatly reduces the loss of microfilariae. The thick film does lack sensitivity when microfilariae density is low but is still a useful and cheap technique for survey work where other more sensitive techniques are too expensive (Moulia-Pelat *etal.,* 1992) or when it is difficult for cultural or other reasons to obtain venous blood.

It has also been suggested that more microfilariae are present in capillary blood than venous blood and that the use of capillary blood may be an advantage when microfilariae densities are low (Eberhard *et al,* 1988). There is no question, however, that direct

techniques often fail to identify patients with low parasite densities and concentration techniques should be used wherever possible (Weller *et al.,* 1982). Acridine orange staining and fluorescence microscopy has been used as an alternative to Giemsa staining and various combinations of stains can be used to demonstrate the internal structure of microfilariae (Lowrence and Simpson, 1969). Counting chamber techniques using various diluents have been used for counting microfilariae by several investigators (Denham, 1979; Southgate, 1973) and, although not as sensitive as concentration techniques, are a reasonable compromise if 0.1ml of blood is used (McMahon *et al.,* 1979).

2.7.2 Concentration methods for microfilariae

The most widely used is the method developed by Knott (1935). For this method one ml of blood is added to 9ml of a 1% formalin solution in normal saline. After red cell lysis is complete the mixture is centrifuged and the deposit examined for microfilariae. Because the formalin preserves the microfilariae, Knott's tests can be set up in the field and processed later in the laboratory. The theoretical detection limit is one microfilaria per ml. The accuracy of the Knott's test and its ease of use is compromised when blood is processed from individuals with excessive amounts of plasma gamma globulins, a common finding in tropical populations. The formalin precipitates the protein and makes the examination of the deposit very difficult. The method has been improved by Melrose *et al.* (2000) who added a small Triton X-100 to the diluent, which dissolved most of the proteinaceous deposit thus enhancing the visibility of the microfilariae. If a citrate-

microfilariae and other blood parasites in diluted, saponin-lysed blood and it is claimed to be 7.5 times more sensitive than a thick film (Petithory *et al,* 1997).

2.7.3 The Diethylcarbamazine (DEC) Provocation Test

If 1.5 to 2 mg of DEC per kg of body weight is given during the daytime, microfilariae are "provoked" into leaving the lungs and entering the peripheral circulation where they can be detected by any of the techniques above (WHO, 1987). The use of this test should be discouraged as it has a lower sensitivity than night blood collection and mns the risk of also provoking a severe reaction (see below) especially in areas where *W. bancrofti* occurs with *O. volvulus* or *L. loa* (WHO, 1987). The other antifilarial drugs ivermectin and albendazole do not induce microfilariae to enter the blood during the day (Dunyo *et al,* 1999).

2.7.4 Detection of filarial antigen

Filarial antigenaemia is associated with active filarial infection and several assays for filarial antigen using both polyclonal and monoclonal antibodies raised against various antigens have been developed (Harinath, 1986; Lai *et al.,* 1987; Weil and Liftis, 1987; Weil *et al,* 1987; Zheng *et al,* 1987; Cheirmaraj *et al,* 1990). The first commercial assay Trop Bio Og4C3 Antigen Test (Trop Bio Pty Ltd, Townsville, Australia) is based on the assay developed by More and Copeman (1990; 1991). The monoclonal antibody is raised against *Onchocerca gibsoni* antigen and shows very strong specificity for *W. bancrofti* antigen. Since it detects antigen from both adult worms and microfilariae the

Og4C3 assay will detect amicrofilaraemic and microfilaraemic infections (More and Copeman, 1990) and is a very good marker of active filarial infection with adult worms (Chanteau *et al.,* 1994a,b). Unlike microfilaraemia, antigen levels show no significant nocturnal or diurnal variation and blood can be taken at any time of day or night (Moulia-Pelat *etal.,* 1993).

Blood samples taken onto filter paper strips can be used for the Og4C3 assay and Lalitha *et al.* (1998) and Itoh *et al.* (1998) found that the capillary blood collected on filter paper and serum gave comparable results. By contrast, Gyapong *et al.* (1998) found that the sensitivity of the filter paper test to be significantly inferior with only 50.3% positive with both tests. More and Copeman (1990) showed no cross-reaction in the Og4C3 test with *Brugia* species or other helminths but there is a recent report by Rocha *et al.* (1996) of a person from a "non endemic" filarial area who tested positive with the Og4C3 test. This individual was found to be parasitized by *Hymenolepsis nana.* Whether this represents a true cross-reaction or not is debatable since the same individual also showed a positive immunoblot with crude *B. malayi* antigen. The reported diagnostic sensitivity of the Og4C3 assay for *W. bancrofti* varies from 73% (Chanteau *et al.,* 1994a) to 100% (Lammie *et al.,* 1994). The variation in sensitivity might be partially explained by the variation in the amount of blood used for the detection of microfilariae, which varied from 20(j.l to 1ml. A more recent study by Rocha *et al.* (1996) suggests that the sensitivity of the Og4C3 assay may be reduced when microfilariae density is very low. At microfilarial densities of <1, 1 to 30, and >30 the sensitivity was 72.2, 97.6 and 100%

respectively and the log OD of the assay was strongly associated with microfilariae density.

The ICT antigen test (ICT Diagnostics, Sydney, Australia) is rapid immuno-chromatographic technique using specific monoclonal and polyclonal antibodies. It utilises capillary or venous blood and is simple enough for field use by technicians and requires a minimum training (Weil *etal.,* 1997). The efficacy of filarial antigen tests has been reviewed by Nguyen *etal.* (1999) and Phantana *etal.* (1999). Phantana *etal.* (1999) also compared the ICT to thick blood films and membrane filtration and obtained the following results; Sensitivity 100%, specificity 96.3%, predictive value positive 70.7% and predictive value negative 100%. Pani *et al.* (2000) however, found it less sensitive than the filtration test for detecting low-level microfilaraemia citing an 88.3% positive predictive value when compared with the latter test.

2.7.5 Filarial antibody assay

Lack of readily obtainable adult worms has made it very difficult to prepare *W. bancrofii* antigen for immuno-diagnosis but the recent introduction of ultrasonic detect (Amaral *et al.,* 1994; Suresh *et al.,* 1997) should make the task easier. The only animals that can be infected with *W. bancrofii* are the leaf monkeys *Presbytis cristata* (Palmieri *etal.,* 1982; Rajasekariah *et al.,* 1986) and *P. melalophos* (Sucharit *et al.,* 1982) and these animals are expensive and difficult to maintain in captivity (Tranke *et al.,* 1987). *Wuchereria bancrofii* microfilariae can be maintained in culture or isolated from blood (El Bassiouny *et al.,* 1993) and used to prepare antigen for antibody studies. The viability of separated

or cultured microfilariae can be ascertained by means of a tetrazolium formazan assay (Mukheijee era/., 1997).

Fortunately, there is marked cross reactivity between filaroid species (Tandon *et al.,* 1981) and wide range of other crude filarial parasites antigens have been utilised for filarial antibody detection: *Dirofilaria immitis* (Ata *et al,* 1993); *Setaria cervi* (Almeida *etal,* 1990); *Setaria digitata* (Dissanayake and Ismail, 1980); *B. malayi* (Ottesen *etal,* 1985). There is a down side to this cross reactivity - antifilarial antibodies cannot be used to distinguish between filaroid species (Rajasekariah *etal.,* 1986).

Various methods can be used to detect filarial antibody: Complement fixation, indirect haemagglutination, gel diffusion, immunoelectrophoresis, counter current immunoelectrophoresis, indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA) have all be used (Ambroise-Thomas, 1980). Currently, almost all filarial antibody studies use ELISA. Cross reactivity limits the usefulness of IgG antibody in filariasis diagnosis but they may be of some value in communities where parasites other than *W. bancrofti* are absent or rare (Chanteau *et al,* 1991) and have been used to diagnose occult filariasis in Indian children (Chaturvedi *et al,* 1995). Humans are not able to synthesize anti-phosphocholine or anti-carbohydrate antigen IgG4 (Maizels *et al,* 1987; Lai *et al.,* 1991) therefore the filarial IgG4 antibodies assay greatly increases specificity and enhances the diagnostic ability of the test (Lai and Ottesen, 1988). Chanteau *et al* (1995); Terhell *et al.* (1996) and Mahanty *et al.* (1994) have shown that antifilarial IgG4 is a good index of the intensity and duration of filarial exposure in

endemic populations, and (Maizels *et al.,* 1995) found that the level of IgG4 antibody correlates with microfilariae counts. Filter paper collection techniques can be used to detect filarial IgG4 antibody (Chanteau *etal.,* 1991; Terhill *etal.,* 1996). IgG4 antibodies are extremely useful for the detection of *Brugia* species because they will not be detected by the current antigen tests since they are specific for *W. bancrofti* (Rahmah *et al.,* 1998). There appears to be very little literature on the usefulness of anti-filarial IgM antibody for filariasis diagnosis but Ata *et al.* (1993) found it to be more specific than IgG. It should be borne in mind that sero-positivity does not always indicate active filarial infection. The antibody may have been raised by exposure to infective larvae without an adult worm being present or the antibody may persist after parasites have been cleared and although the sensitivity of antibody tests is high, the specificity and hence the predicative value, is low (Chanteau *et al.,* 1991).

2.7.6 PCR assays for filarial detection in vectors

Molecular detection of filarial DNA in humans or mosquitoes can best be done with polymerase chain reaction (PCR) assays to detect specific DNA sequences. A repeat sequence (188 bp) designated *Sspl,* identified from a *W. bancrofti* genomic library was found to characterize *Wuchereria* (Zhong *et al.,* 1996) as distinct from *Brugia* and other filariae. A PCR assay developed to amplify this *Sspl* family of repeated DNA elements using specific primers (NV-1 and NV-2). This assay was shown to be sensitive enough to detect 0.1 picogram of *W. bancrofti* genomic DNA, representing less than 1% of one infective larval DNA within its mosquito host (Chanteau *etal,* 1994).

Recently, several research groups in African countries started using this sensitive PCR mosquito-pool screening approach in preference to the classical method of dissecting mosquitoes to determine their filarial infection and transmission potential. Also in India, scientist at VCRC have devised simpler, quicker and less expensive PCR assay procedure for screening mosquitoes to detect *W. bancrofii* (VCRC unpublished data; Ottesen *etal.,* 1997).

The PCR approach to detect *W. bancrofii* can be used for diagnosis of infection in the human host and more importantly, detection of filarial DNA in the mosquito vector (Farid *et al.,* 2001). A reliable, sensitive, specific and faster immunochromatographic (ICT) card format for rapid diagnosis of *W. bancrofii* active infection in humans is also available (Weil *etal.,* 1997), but is more costly ($1.50/test). Thus, compared to ICT card tests or mosquito dissection, the PCR technique is economical, can be applied to humans and has more practical value for xenomonitoring by detection of filarial DNA in mass screening of mosquito vectors. The PCR method has advantage of detecting a single worm in a pool of wild-caught mosquitoes (Ramzy *et al.,* 1997), being cost effective for field application. Thus, screening of pools for wild caught mosquitoes by PCR, a non-invasive means, could be used for identifying endemic regions, but would be particularly useful for monitoring transmission in areas where mosquito infection rates are very low, especially during active control programmes.

In applying this assay to field collected samples, experiences of the DNA diagnostics laboratory of the Onchocerciasis Control Programme (OCP) in West Africa may prove to

be a helpful model in overcoming this obstacle. For the past nine years, the OCP's laboratory has been using a PCR assay based on the amplification of a 150bp repeat sequence family to monitor infection in the *Simulium damnosum* complex vectors of African onchocerciasis. Initially the OCP laboratory's efforts were primarily directed toward identifying individual parasites dissected by the OCP's field teams, but the laboratory has been successfully applying a pool screening approach to monitor infection in the vector population (Laurent *et al,* 1999). Similarly, the Onchocerciasis Elimination Programme of the Americas (OEPA) has been applying this technology to monitor transmission since 1999 (Toe *et al,* 1999).

PCR assays are not quantitative therefore it is impossible to determine if a positive pool contains one or more than one infected mosquito. However, it is possible to state that pools that produce negative results do not contain any infected mosquitoes. This observation has been used to develop a method to calculate the prevalence of infection in the vector population, based upon the simple computer program called *Poolscreen* (Katholi *et al,* 1995). In addition to the development of practical ways to employ the PCR detection techniques in specific field situations, additional research is needed to develop and apply similar PCR assays specific for *B. malayi* and *B. timori* (without cross-reactions to other enzootic *Brugia* species) for xenomonitoring prevalence via vector. Also, for measuring transmission potential and monitoring the impact of transmission control, specific PCR assays that detect only L3 infective larvae would be useful.

2.8 Control of lymphatic filariasis

Rapid population growth and inadequate sanitation in urban and rural areas have resulted in enhancement of transmission of *W. bancrofti* and expansion of its distribution. On the other hand, due to the long-term control efforts by some countries, there is evidence of reduction in the prevalence of the disease. The control strategy based on vector control and treatment of microfilaria carriers, practiced in the region till recently had a limited effect since it lacked the necessary political commitment and financial support. Recently there have been significant developments in regards to filariasis control based primarily on mass chemotherapy supplement by case management and vector control. These measures constitute the substance of the revised strategy for control of lymphatic filariasis (Rafei, 1999-2000).

2.8.1 Vector control

A variety of means for controlling the vectors of filariasis are available today. These include *Bacillus sphaericus,* a toxin-producing bacterium: polystyrene beads, which help to limit the breeding of mosquitoes in certain situations; insecticide-impregnated bednets and curtains, which limit host/vector contact; indoor spraying of long-lasting pyrethroid insecticide, especially effective for adult *Mansonia* and *Culex;* and community participation in integrated vector management. All these methods help to decrease vector numbers and transmission, but exactly how and when the tools are cost-effective and useful in large scale-scale control programmes for lymphatic filariasis have not yet been clearly defined (Webber, 1991).

Vector control have a part to play and can be very successful in situations where malaria and filariasis have common vectors. In the Solomon Islands for instance, where both malaria and filariasis are transmitted by *Anopheles farauti* and *Anopheles koliensis,* the prevalence of microfilaraemia was reduced from around 15% to <2% by vector control earned out during a malaria campaign (Webber, 1977, 1991). Vector control however does take a long time to become effective. For instance, Schuurkamp *et al.* (1987) estimated that it would take 11 years to reduce the prevalence of microfilaraemia to <2% in the Tabubil area of the Western Province of Papua New Guinea using vector control alone. Such prolonged campaigns are very labour intensive and costly and the development of insecticide resistance makes them become less and less effective. Data collected during a 5-year vector control program in Pondicherry, India, found that vector control alone might have little impact on the overall age-prevalence of infection even when sustained for long periods (Srividya *etal.,* 1996).

Toilet pits and the like are prime breeding grounds for *Culex quinquefasiattus* (Nwoke *et al.,* 1993) and the use of polystyrene beads can be a very effective control mechanism for this and other *Culex* species. Using this approach Maxwell *et al.* (1989) reduced the numbers of mosquitoes entering houses by about 97%. Maxwell *et al.* (1999) found that although the treatment of excess pools and pit toilets with polystyrene beads could reduce mosquito populations in homes, the cost of achieving this could not be justified by its impact on filariasis alone, but the reduction in nuisance biting might increase the community support for other control programs.

2.8.1.2 Insecticide treated/untreated bed-nets

The impact of bednets on the transmission of the parasites causing lymphatic filariasis has received less attention than their impact on the transmission of malaria parasites. Burkot *et al.* (1990) showed that use of untreated bednets reduced transmission of *W. bancrofii* by *Anopheles punctulatus* in Papua New Guinea by 70%. Although the nets had no apparent effect on the size of the vector population, the human blood index was reduced from 96% to 90% for indoor resting mosquitoes, and from 84% to 46% for outdoor resting, while the proportion of blood meals taken in dogs increased. Recently, Bockarie *et al.* (2002) demonstrated that long-term use of untreated bednets significantly reduced the prevalence of both human infections with *W. bancrofii* and symptomatic bancroftian filariasis, in an area of Papuan New Guinea where *An. farauti* was the vector. The prevalence of microfilaraemia, antigenaemia and hydrocoele among the bednet users were 27% and 70% lower, respectively, than those observed in the individual who were not using bednets.

The probable impact of insecticide-treated materials on lymphatic filariasis has mainly to be inferred, indirectly from knowledge on their effects on the various mosquito species that act as vectors of the filarial. It is known that most human filariasis is caused by *W. bancrofii* transmitted by *Anopheles* spp and *Culex quinquefasciatus* (White, 1989; WHO, 1994). Insecticide-treated material generally has a substantial killing effect on anopheline mosquitoes as documented in the studies on malaria. In contrast, Curtis *et al.* (1996) demonstrated that pyretheroid-impregnated bednets kill very few *Cx. quinquefasciatus,* although they do reduce this species' success in feeding (to a greater extent than

impregnated eave and wall curtains). Repellency could be part of the explanation for the low mortality of *Cx. quinquefasciatus,* but Curtis *et al.* (1996) noted that: 'Presumably this mainly associated with the general tolerance of *Cx. quinquefasciatus* to tarsal contact with insecticides. It is possible that *Culex* are more readily irritated than *Anopheles* and inclined to leave the pyrethroid-treated surfaces.

Bogh *et al.* (1998) studied the impact of pyrethrum-impregnated bednets on the resting and feeding behaviour of filarial vectors on the Kenya coast. The introduction of the impregnated bednets reduced the numbers of indoor-resting *An. gambiae* si and *An. fimestus* by 95.6% and 98.1% respectively but as expected caused no significant change in the number of *Cx. quinquefasciatus* collected indoors. Despite the feet that pyrethrim-impregnated bdnets do not kill *Cx. quinquefasciatus,* these results indicate that the impregnated bednets are likely to reduce *W. bancrofti* transmission by *An. gambiae, An. fimestus* Mid *Cx. quinquefasciatus* efficiently. In a more recent investigation Qinones *et al.* (2000) also detected changes in mosquito feeding behaviour a consequence of the introduction of pyrethrim-impregnated bednets. The effect of pyrethrum-impregnated bednets on the transmission of *W. bancrofti* was investigated directly for the first time by Pedersen and Mukoko (2000). In the study it was observed that the overall mosquito density in the village with pyrethrin-impregnated bednets, reduced by 22.6% in the post-intervention year. The transmission of *W. bancrofti* was drastically reduced in the studied villages.

2.8.2 Chemotherapy

There are essentially two regimes used to control filarial infections using chemotherapy: selective and mass treatment. In selective therapy individuals are examined for the presence of disease and those found to be infected are treated. There are major problems associated with that approach. If microfilaraemia is used as the indicator of infection many infected people will be overlooked since not all those who have the disease are microfilaraemic. People with very low microfilarial densities may not be detected and it has been shown that such people are capable of infecting mosquitoes and causing a resurgence of disease (Lowrie *et al.,* 1989). This approach has however worked in many countries including Japan. The use of antigen testing will overcome these problems but there is a problem with the logistics involved with screening all members of the community. If selective chemotherapy is used the lost infections are balanced by new infections to produce a dynamic equilibrium and there needs to be continual reassessment of the filarial status of the community to identify the newly infected.

Woolhouse *etal.* (1997) by studying the transmission potential of a number of diseases, including helminth parasites, found that, typically, 20% of the infected host population contributes at least 80% of the net transmission potential and that control programs which fail to cover all of this core group, a problem with selective treatment programs, will be less effective in reducing levels of infection in the group as a whole. Using mass chemotherapy j-egardless of parasite status therefore overcome problems associated with selective therapy.

Mass treatment aims to treat all members of the endemic community at the same time and will therefore treat pre-patent as well as patent infections. In other words the community becomes the focus of the control program rather than the individual. Originally antimony and arsenic based drugs and naphthalene sulfonic acid (Suramin) were used to treat filariasis but with limited success. These compounds had a moderate amount of anti-filarial activity and were very toxic. Suramin has been shown to be moderately active against adult *W bancrofii* and *Brugia phahangi* (Howells *et al.,* 1983) but it has no effect on microfilariae and will therefore not interrupt the transmission cycle in the short term. It is also more toxic than other antifilarial drugs and is not widely used today. Levamisole has been shown to have limited activity against filarial parasites (Rogers and Denham 1976; McMahon 1979).

2.8.2.1 Mass drug treatment with DEC

Diethylcarbamazine (DEC) has been available for treatment of filarial disease for almost 50 years and is till used today. It destroys larval worms and even some of the adult worms but has drawbacks if used in heavy infections when the destruction of large numbers of worms may cause side effects. Current practice is a single annual dose of 300 mg of DEC for adults and 150 mg for children (often combined with Ivermectin, and sometimes Albendazole). This simplifies the treatment, increases compliance and can be easily accommodated into existing primary health care networks without over-burdening them (Wijers, 1984). Low dose DEC has been shown to significantly reduce the prevalence and density of microfilariae in treated communities and reduce the prevalence of chronic pathology (Partono *etal.,* 1984, 1989). Panicker *etal.* (1991) found that there

was a reduction in microfilariae prevalence of 74.9% in the annual treatments and *90%* in the biannual treatments. The authors also found that attacks of filarial fever and incidence of recent oedema cases were also significantly reduced after DEC treatment. A trial in Tanzania achieved microfilariae clearance rates of 92% (Meyrowitsch *et al.,* 1996). Meyrowitsch and Simonsen (1998) have shown that the beneficial effects of DEC treatment persist for at least 4 years and although microfilaraemia does recur in some patients, especially those who had high microfilaria densities before treatment, the level of microfilaraemia does not reach the pretreatment levels. Khan *et al.* (1998) found that 5 years after a dose of 72 mg/kg of DEC given over a 21 day period, 51% of subjects were stil amicrofilaraemic, 36.4% had densities less than pre-treatment levels and 11.8% had increased microfilariae counts. In Samoa, 3 annual treatments of DEC 6 mg/kg, achieved an estimated 80% reduction of microfilaria in the population and lowered the annual transmission potential from 2.18 to 0.67 (Kimura *et al.,* 1992). Mataiki *et al.* (1998) obtained similar results with a five-year annual treatment program in Fiji.

2.8.2.2 Use of Ivermectin

Ivermectin has been used very successfully for the treatment of onchocerciasis for a number of years (Goa *et al.,* 1991; Townson *etal.,* 1994) and a single annual dose of 400 Hg/kg, either alone or in combination with Diethylcarbamazine, has proved to be very effective producing long-term suppression of microfilaraemia in Bancroftian lymphatic filariasis in a number of countries (Zheng *et al.,* 1991a,b; Cartel *et al.,* 1992) and is equally effective against brugian filariasis (Shenoy *et al.,* 1993). Nquyen *et al.* (1996) found that twice yearly doses of 100 fig/kg of Ivermectin did not reduce the prevalence of

microfilaraemia, but when the dose was increased to 400 ng/kg the prevalence dropped from 21% to 7% and the microfilariae density to 0.5% of its initial value. Plaisier *et al.* (1999) found that Ivermectin is very effective at reducing the microfilariae load and at a does of 400 jig/kg a single treatment irreversibly reduces the microfilariae of the adult parasite by at least 65%. Zheng *etal.* (1991) found that Ivermectin was especially useful in the treatment of filarial relapses after DEC treatment Like DEC, Ivermectin treatment does give rise to adverse side effects. Cao *etal.* (1997) describes them as mild and "flu like", generally mild, and well tolerated by patients. Mild reactions were also noted by Zheng *etal.* (1991) who also found that local flare-up of acute filariasis was less likely with Ivermectin than with DEC. The severity of the reaction was strongly associated with the pre-treatment microfilariae density but independent of the dose (Cao *et al.,* 1997). Weil *et al.* (1991) stated that local adverse reactions such as nodule formation, lymphangitis and epididymitis are not seen with Ivermectin therapy because these reactions are caused by the death of adult filarial worms and Ivermectin is not a macrofilaricide.

As with DEC, the macrofilariacidal action of Ivermectin is debatable. Ismail *etal.* (1996) found that multiple, high dose Ivermectin treatment (12 fortnightly doses of 400 jig/kg) does have macrofilaricidal activity but the results are neither predictable nor consistent. Eberhard *et al.* (1997) reported that although filarial antigen fell after treatment, in no case did it fall to zero, even in individuals who remained amicrofilaraemic for several years after treatment suggesting that some adult worms survived. By contrast however, Dreyer *et al.* (1995b; 1996) could find no evidence of macrofilariacidal activity. In their

study 15 men who had living, adult W. bancrofti detected by ultrasound were treated with 400-|ig/kg-body weight of Ivermectin at 2-week intervals for 6 months (total dose 4.8 mg/kg). Microfilaraemia was rapidly suppressed but no changes in the motility or location of the adult worm were detected. In another study Dreyer *et al.* (1995b) removed live adult worms from several patients 8 months after being treated with 400 Hg/kg of Ivermectin.

2.8.23 Albendazole

Albendazole has been used for the treatment of intestinal helminths for a number of years but it has only recently been tried as an anti-filarial. Addiss *et al.* (1997) have shown that at a stated dose of 400 mg per person it is an effective microfilaricide. It is even more effective when combined with DEC or Ivermectin. The problem of adverse reactions with Albendazole is no better or worse than with DEC or Ivermectin. The potential for Albendazole to be used as a macrofilaricide for the treatment of individual patients is regarded by Ottesen *et al.* (1999) as one of the most important questions in filarial research.

2.8.2.4 Combination therapy with DEC and Ivermectin

A combination of DEC and Ivermectin has proven to be very effective in providing rapid and long-term clearance of microfilariae. This is the current global strategy for controlling the disease. Nicolas *et al.* (1997) found DEC and Ivermectin to be more effective than either drug alone for clearing circulating filarial antigen in both amicrofilaraemic and microfilaraemic subjects. Addiss *et al.* (1997) also showed that a

combination of 200-400 pg/kg of Ivermectin plus 400 mg of albendazole is more effective in clearing microfilariae than either drug alone. Ismail *et al.* (1998) studied the effects of Albendazole, DEC, and Ivermectin alone and in combination and showed that although all were well tolerated and effective macrofilaricide, a single dose of a combination of 600 mg of Albendazole and 400 (ig/kg of Ivermectin was the most effective. Decreasing levels of filarial antigens after treatment suggested that all four regimes have significant macrofilaricidal activity. The most effective, however, was a combination of 600 mg of Albendazole with 6 mg/kg of DEC. With this combination, filarial antigen levels decreased by 77% fifteen months after therapy.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

The study was conducted in two districts that are known from a 1994 national survey to be endemic for filariasis and one non-endemic district (Gyapong *et al,* 1994). These are namely Winneba, Axim and Yilo Krobo districts all located in the southern sector of Ghana (Fig 3.1). Depending on the number of cattle Kraal that were found in the districts, villages were randomly selected at random within each one for cattle blood sampling and examination. Mosquitoes were also collected off the microfilaraemic cattle screened.

Winneba is located at the southern sector of Ghana, about three kilometres off the main Accra-Cape Coast road. The vegetation is coastal savanna with stretches of mangrove swamps. This area receives the least amount of rain in Ghana (Dickson and Benneh, 1988). The annual rainfall is between 74 and 89 centimetres. Relative humidity is however, high throughout the year and thus compensates for the scanty annual rainfall. The inhabitants are mainly fishermen and farmers and grow crops like cassava, plantain and other vegetables. There are 6 kraals in the area and the number of cattle per site ranges from 40-150. Three kraals located at Muni lagoon, animal husbandly and Mpotah were chosen randomly for screening and collection of mosquitoes.

Axim is also located in the south-western part of Ghana. This is the wettest climatic district in Ghana. Mean annual rainfall is above 190 centimetres and, and on the average there is no month that receives less than 2.5 centimetres of rain. The highest mean monthly temperature of about 30°C occurs between March and April and the lowest of about 26°C in August. Average monthly relative humidity ranges between 75-80% during the two rainy seasons. Six sampling sites namely Asaasetre, Teleko Bokazo, Nkroful Secondary School, Kanbule, Arokpogwe and Police Quarters with varying number of cattle were chosen because of scarcity of cattle in Axim.

Yilo-krobo District of Ghana is located at the southern part of Somanya District. This site, which is non-endemic for filariasis, was included in the study because an incidental examination of catde blood revealed some filarial parasites that were morphologically similar to *Wuchereria.* Yilo-krobo district has a mean annual temperature of about 26oC, Rainfall in the area is seasonal with two peaks occurring in June and September. The area has wide stretches of savanna or grassland which cattle owners use as grazing fields. The vegetation type is that of savanna woodland.

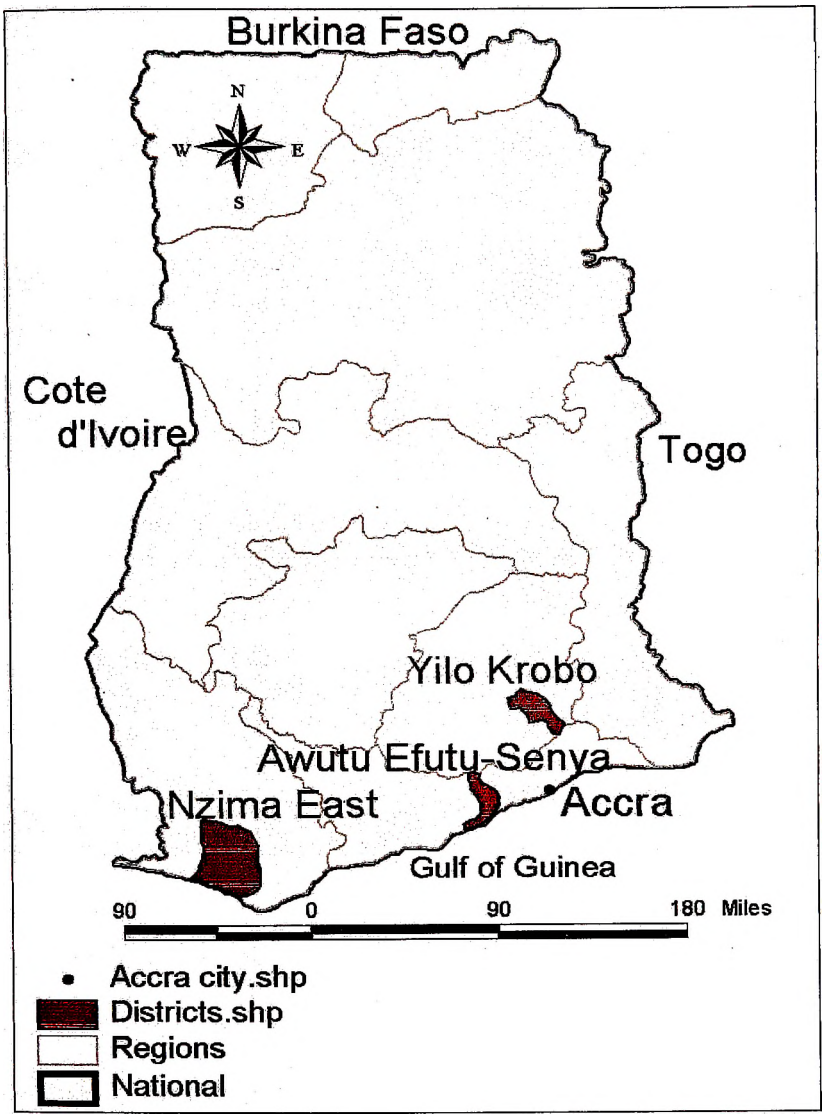

Fig 3.1: A map of Ghana showing the study sites, Yilo-Krobo, Winneba (Awutu Efutu District), Axim (Nzema East) and Accra, the Capital City.

3.2 Parasitological Studies

These studies were undertaken to help identity and classify the filarial parasites that were found in the cattle blood. The cattle were screened using traditional staining techniques for filarial parasites. Microfilariaemic density was determined at regular intervals of time over a 24 hr period. Blood was also collected into heparinized tubes for molecular studies. Moiphometric measurements were taken and the values obtained were compared to those of *W. bancrofti* and *Setaria* species.

3.2.1 Screening of cattle for filarial infections

Screening was carried out between the hours of 06-08 hrs in the morning and 1700-1900 hrs in the evening at ihe three study sites. The cattle were randomly selected after which they were tagged and information on age, sex and type of breed were recorded.

The ear lobe of each cattle was cleaned with a cotton wool ball soaked in alcohol. It was then pricked with a lancet and the blood was allowed to ooze freely. The blood was then drawn into a sterile calibrated capillary tube and later transferred onto a microscope slide and smeared uniformly avoiding bubble formation. The slides were air-dried after which they were immersed in tap water until the haemoglobin leached out of the smear. This took about 3-5 minutes. The slides were placed horizontally and the smear allowed to dry at room temperature. After this the blood films were stained for 25 minutes in a 1:20 dilution of Giemsa stain of pH of 6.8-7.2. The films were then washed for 3-5 minutes under running tap water. They were then dried in a vertical position. The stained blood films were then observed under low and high power to detect the presence of microfilaria.

3.2.1.1 Collection of cattle blood for molecular studies

About 10ml of blood was obtained from the positive animals by bleeding from the jugular vein using 10ml syringes and needles. This was done on a four hourly interval for 24 hours at the various sites where infections were detected. The blood was stored in heparinized tubes. They were then kept cold in an icebox in the filed and transported to the laboratory where they were preserved at 4°C until ready to be used. lOfxl of blood was also collected using capillary tube into tubes containing 1ml of 3% acetic acids.

3.2.2 Determination of microfilaraemic density

The counting chamber technique for determining microfilariae concentration (mf) in the blood was used. A 3% acetic acid was prepared and 990|il aliquoted into tubes. lOOjxl capillary tubes were then used to draw blood from the ear lope and then drained into the tubes containing the 3% acetic acid and thoroughly mixed. The content of each tube was transferred to a clean counting chamber and examined under the microscope at high power xlOO magnification. The microfilariae were counted and the density expressed as mf7100|xl of blood. This was repeated over a 24-hour period at 4 hour intervals to investigate the variations in blood microfilarae density. The total counts of microfilariae in each tube were used to calculate the geometric mean using formular given in appendix

3.2.3 Morphometric studies of cattle blood microfilariae

The thick blood films prepared in the field were used for morphometricc studies. Microfilariae, at lOOx magnification were drawn onto A4 paper using camera lucida. One centimetre on a ruler was first traced on the paper. Then the outline of each microfilaria was drawn using a pencil. The total length, width, the length of the inner korper and headspace of each microfilaria were measured from the paper using a thread. In all about 40 sheathed microfilaria and 5 of the unsheathed were drawn. The values obtained which were in centimetres were converted into micrometers by dividing by 10^3 taking into account the magnification at which the drawings were made. Comparative statistical analyses were also carried out.

3.3 Entomological Studies

Mosquitoes were collected as part of the study to determine the vectors of the cattle filarial parasites. Specially made mosquito nets were erected at the various sampling sites. They were sorted out in the laboratory and dissected for all stages of filarial parasites.

3.3.1 Mosquito surveys

The cattle that were infected were isolated and confined in a mosquito net, which had an opening at one side to allow entry of mosquitoes (Fig 3.2). Resting mosquitoes on nets or those coming to take blood meal were then collected by aspiration between the hours of 00-04hr. Mosquitoes were also obtained from rooms of microfilaraemic individuals living close to the sampling site using pyrethrum spray catch. The mosquitoes were kept in paper cups and sent to the laboratory where they were sorted out according to species using the criteria of Grilles and de Meillon (1968).

3.3.2 Dissection of mosquitoes for filarial infections

The legs and the wings of each female mosquito were first removed and then placed in a drop of normal saline on a slide. The head, thorax and the abdomen were separated under approximately 20X magnification, and transferred to separate drops of saline. Using fine needle, the labium was separated from the other parts of the proboscis. The infective filarial worms if present normally emerge from the labium into the saline. The remainder

of the head, thorax and the abdomen were dissected separately at XI0 of the dissecting microscope and examined for infections with all filarial stages (LI, L2 and L3). Any filarial-like parasites were recorded and the positive slides as well as a few negatives were placed in racks and kept at - 4°C for the subsequent molecular studies.

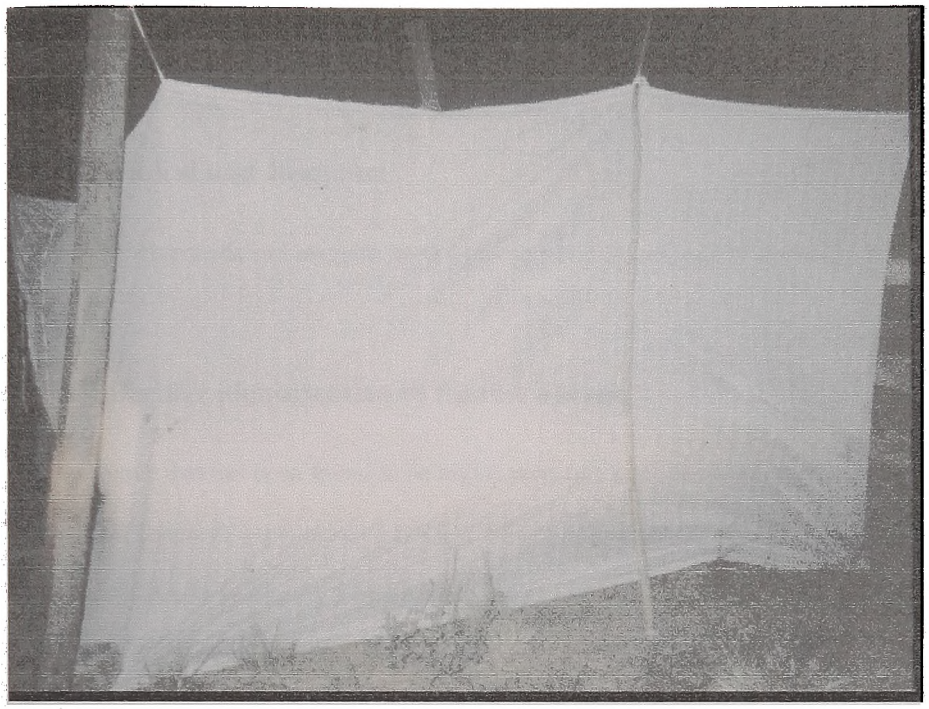

Fig 3.2: The frout (top) and rear (bottom) view of the mosquito net erected over cattle, with flap open for mosquito entry

3.4 Molecular Studies

3.4.1 Chemical and Reagents

The various chemicals and reagents used were prepared as outlined in Appendix I

3.4.2 Molecular identification of filarial worms

One technique that has been found to be highly sensitive and specific in identifying *Wuchereria bancrofti* using parasite DNA is the polymerase chain reaction (PCR) which has been developed to amplify a family of repeated DNA element, the 188bp *SspJ* repeat, specific for *Wuchereria* genus. The blood that was obtained from the jugular vein was washed thoroughly and used for DNA extraction followed by PCR using the appropriate primers.

3.4.2.1 Extraction of filarial DNA

Aliquots of 100-200^1 of blood were transferred into 1.5ml eppendorf tubes. Approximately 1ml sterile distilled water was added, vortexed and centrifuged at 14,000 rpm for 10 minutes and the supernatant decanted. This washing process was repeated several times until the haem component of the blood was completely washed off. The worms (which are pelleted) were then stored in iso-propanol at -20°C until ready to be used for DNA extraction. The parasite's genomic DNA was extracted using the DNeasy Tissue Kit (QIAGEN Inc., USA). The modified protocol for extraction of DNA from animal tissue was followed (see Appendix II) for details.

3A.2.2 PCR identification of cattle filariae

The PCR method using the previously published oligonucleotide primer sequences NV-1 (5 ' -CGT GATGGC ATC AAAGT AGCG-3 ') and NV-2 (5'-CCCTCACTTACCATAAGACAAC-3') for identification of *W. bancrofti* was used (Ramzy *et al.,* 1997). The composition of the PCR reaction mix is shown in Table 3.1 of appendix VII. The contents of each tube was mixed thoroughly, centrifuged briefly and overlaid with 25µl of mineral oil to minimize evaporation and refluxing. A positive control containing known *W. bancrofti* DNA and a negative control without DNA were also included for each reaction. The PCR cycling conditions used were an initial denaturation at 94oC for 3 minutes, followed by 35 cycles of denaturation at 94oC for 1 minute, annealing at 55oC for 1 minute and extension at 72oC for 2 minutes and a final cycle of 94oC for 1 minute, annealing at 55oC for 1 minute and extension at 72oC for 10 minutes.

3.4.3 Molecular identification of *Anopheles gambiae* s.l

The *Anopheles gambiae* were first morphologically identified. The genomic DNA from carcass of dissected *An. gambiae* s.l. mosquitoes was used as template for the PCR method for the identification of the member species (Scott *et al.,* 1993).

3.4.3.1 DNA extraction of mosquitoes using Bender buffer protocol

Genomic DNA was extracted from *Anopheles* mosquitoes using bender buffer (0.1 M NaCl, 0.2M Sucrose, 0.1M Tris-HCl, 0.05M EDTA pH 8.0 and 0.5% SDS) extraction protocol proposed by Collins *et al.* (1987). Each mosquito was triturated in a 100(0.1

Bender buffer in a 1.5ml eppendorf tube and incubated at 650C for 30 minutes. 125|j,l of phenol was added to homogenate. The mixture was then vortexed, and spun at 14,000 rpm for 10 minutes. The supernatant was transferred into a fresh tube and 250^1 of equal volume of phenol/chloroform was then added, vortexed and spun at 14,000rpm for another 10 minutes. This process was repeated depending on how impure the mixture is. The supernatant was then transferred into a new tube and 250^.1 of pre-chilled absolute ethanol and 1 Ojal of 8M-potassium acetate was added to the mixture and kept at or at -40oC for just one hour or overnight. The mixture was spun at 10,000rpm for 10 minutes after which the supernatant was poured off. The pellet was then rinsed with 200nl of 70% ethanol, spun at 100,000rpm for 5 minutes and the supernatant was poured off. The pellet was allowed to dry for about an hour and redissolved in 25|il of TE RNase and kept on ice for 1 hour. Two microlitres was used for PCR.

3.4.3.2 Species identification of *Anopheles gambiae* si using PCR

PCR method for the identification of mosquitoes of the *An. gambiae* complex published by Scott *et al.* (1993) was used. The sequence details of the named oligonucleotide primers are as follows; UN (GTG TGC CCC TTC CTC GAT GT) GA (CTG GTT TGG TCG GCA CGT TT), ME (TGA CCA ACC CAC TCC CTT GA), AR (CTG GTT TGG TCG GCA CGT TT) and QD (CAG ACC AAG ATG GTT AGT AT). The UN anneals to the same position on the rDNA sequences of all five species and GA anneals specifically to *An. gambiae s.s.,* ME to both *An. merus* and *melas,* AR to *An. arabiensis,* and QD to *An. quadriannulalus.* The expected sizes of the PCR products are 390, 315, 153 and 464/466 respectively. The amplification was carried out using a PTC 100 thermal cycler

(MJ Research Inc., USA) using the cycling conditions: 94oC for 2 minutes, 30 cycles of (94oC for 30 s, 50oC for 30 s, 72oC for 30 s). A typical mix for a 25 pi reaction is given in Table 3.2 of Appendix VI.

3.4.4 PCR identification of *Anopheles funestus* group

This reaction was performed using protocol developed by Koekemoer *etal.* (2002) which is a species-specific PCR assay able to rapidly identify five of the most commonly found members of the *An. funestus* group: *An. funestus, An. rivulorum, An. leesoni, An. parensis* and*An. vaneedeni.* Two sets of primers, 3DA and 3DB were used and their sequences are GAC CCG TCT TGA AAC ACG GA and TCG GAA GGA ACC AGC TAC TA respectively. The following PCR cycling conditions were used, initial denaturation at 94oC for 3minutes, 30 cycles of (94oC at 30s, 400C for 30s, 72s), and 1 cycle of 94oC for 30s, 40oC for 30s and 72oC for lmin. Details of a 25 pi reactions mix for the reaction are found in Table 3 .3 of Appendix VI.

3.4.5 Analysis of PCR product (Agarose gel electrophoresis)

Electrophoresis buffer (lxTAE) was prepared, (see appendix I). Agarose was first weighed into beaker and dissolved in specific volume of the buffer already prepared making a *2%* gel. The slurry was heated in a microwave oven until the agarose was completely dissolved. The solution was cooled under tap water and ethidium bromide (0.5|ig/ml) was added and poured into the gel tray with the well-forming comb. This was allowed to cool and set. The comb was carefully removed and the gel was transferred and

positioned in the chamber with just enough electrophoresis buffer solution to submerge it. The PCR products, mixed with orange G loading buffer were carefully loaded A DNA molecular weight ladder (Sigma, USA) was run along side to enable the estimation of the size of the PCR products. The gel was run at a voltage of 100-120V until the orange G tracking dye had migrated to give distance.

3.4.6 Estimation of size of PCR product

The gels were visualized under UV using a UV transilluminator (UPA, USA), at short wavelength and photographed using a Polaroid model IBI 46400 (Polaroid Inc., USA) fitted with an orange filter and a Polaroid type 667 film. The size of the PCR products was estimated by comparison with the mobility of marker of known DNA molecular sizes. The expected length for *W bancrofti* is 188bp. The expected lengths of *Anopheles gambiae and Anopheles funestus* are 390bp and 400bp respectively.

CHAPTER FOUR

RESULTS

4.1 Study Population

A total of 284 cattle 33 from Somanya, 141 from Winneba and 110 from Axim were studied. The mean ages of cattle were 3.8 years (range 2-25 years) for Somanya, 5.6 years (ranges 3 months-9 years) for Winneba and 6.3 years (range= 2-25 years) for Axim. The age distribution is shown in Table 4.1 and it shows that most (63%) of the cattle at Winneba were between 6-10 years, 59.4% were between 2-5 years at Axim and were about equal of the age groups of the cattle screened at Somanya. Females formed 79.4% of tile cattle from Winneba, 84.7% at Axim and 75 .8% at Somanya. West African Short Horn formed majority of the breed types (66% for Winneba; 87.4% for Axim and 100% for Somanya). Details of demographic characteristics of the cattle are given in Appendix V

4.2 Microfilaraemic density and observed frequencies

The prevalence rates of filarial infections were 3.5% (5/141) and 6% (2/33) at Winneba and Somanya respectively, and zero for 110 cattle blood screened at Axim. The cattle found to be positive were of the West African Short Horn breed type and were above 10 years of age. The geometric mean densities among infected cattle were found to vary

between 4.6-20 microfilariae/100(il of blood (Table 4.2 of appendix VI). Maximal microfilarial densities of 26 microfilariae/100jj.1 of blood at 2:00 pm and minimal of 6 microfilariae/1 00p.l of blood at 2:00 am on the average were recorded. The curve is suggestive of a diurnal subperiodic. This feature distinguishes the cattle filariae from *W. bancrofti* which exhibits a nocturnal form of periodicity.

Table 4.1: The sex and age distributions of cattle populations at the three study sites

Site	• #	**Sex**		**Age Categories**			
			#F	<iy	l-5y	6-10y	>10y
Winneba	141	29 (20.6%)	112 (79.4%)	7 (4.9%)	45 (32%)	89 (63.1%)	0
Somanya	33	8 (24.2%)	25 (75.8%)	10 (30.3%)	12 (36.3%)	11 (33.3%)	0
Axim	110	16 (14.4%)	94 (84.7%)	0	66 (59.4%)	34 (30.6%)	10 (9.9%)
Total	284	53	231	17	123	134	10

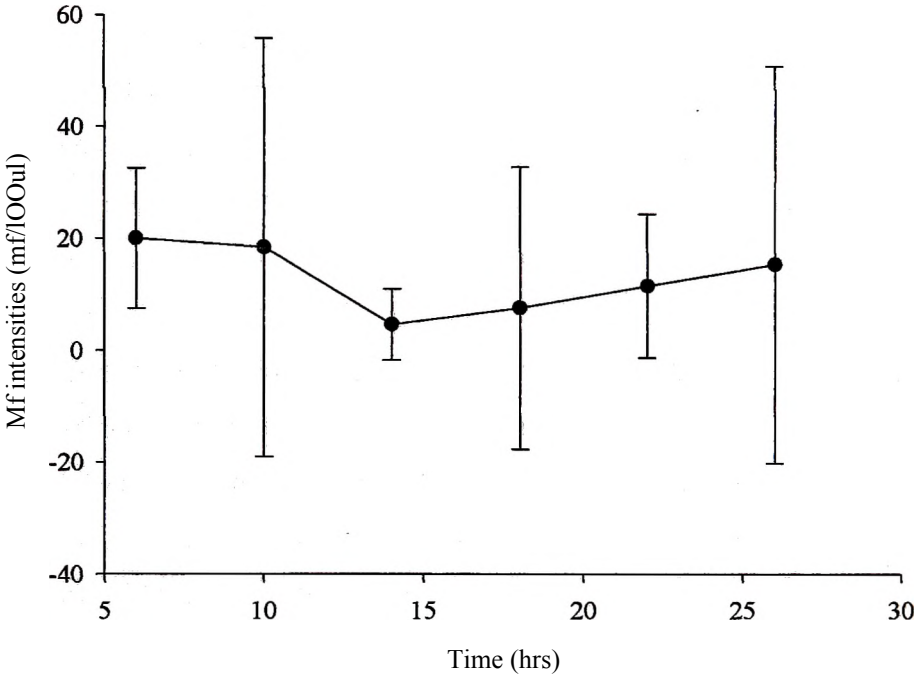

Figure 4.1: The microfilaria densities of the five cattle studied at Winneba

4.3 Morphometric/Morphological Description of Cattle Filariae

4.3.1 Sheathed microfilaria

A total of five sheathed microfilariae were studied. Their sheaths stained pink in Giemsa (Fig 4.3). The length and width measured ranged between 152-182fim (mean=162 ± 12.2) and 6-7jun (mean=7.4 ± 0.9) respectively. The headspace measured between 6-8[m (mean=6.8 ± 0.8) whilst the inner korper was between 25-38p,m (mean=32.4 ± 5.3). The nucleus is large and located at the end of the column. The column of the nucleus is very compact and it starts out multiple and also stains deeply in Giemsa. The tail is anucleated and tapered to a round tip. The inner body is very conspicuous and stains pink in Giemsa.

4.3.2 Unsheathed microfilaria

A total of 40 unsheathed microfilariae were studied. These were morphologically similar to the sheathed ones except that they lacked sheath (Fig 4.2). The morphometric measurements such as width, headspace and inner korper were quite similar to those of the sheathed except for the length, which was found to be between 128-200p,m (mean=164 ± 15.6). The column of the nucleus is also compact just like the sheathed microfilariae and start out multiple and stain intensely in Giemsa. There is also a large nucleus at the end of the column. The tail also tapers to a round tip and is anucleated. The inner body stained with Giemsa and was veiy conspicuous.

4.3.3 Comparison of cattle filariae with *Wuchereria banerofti*

The two cattle filarial worms were compared with published characteristics of *W. banerofti* (WHO, 1997). There were similarities as well as differences in their morphology. Differences were very evident in their lengths, which is much longer in *W. banerofti,* (244-296pm) than the cattle filarial (128-200pm). The sheath of *W. banerofti* does not stain in Giemsa at all but that of the cattle filariae stained pink in Giemsa. The nucleus of the cattle filarial is very compact and stained intensely in Giemsa whilst that of *W bancrofii* is dispersed and does not stain in Giemsa. The presence of a prominent inner koiper in the cattle filariae however distinguishes it from *W. bancrofii,* which does not possess an inner koiper. Similarities were found in features such as the presence of sheath, a short headspace and length of width. Also the tails in both filariae are anucleated tapering to a rounded posterior end.

4.3.4 Comparison with *Setaria* species

The cattle filarial worms were compared to the general description published for *Setaria* worms (Nelson *et al.,* 1962; Omri *et al.,* 1978). Morphometries of the cattle microfilaria especially of the length of the microfilaria (128-200pm) falls within the range of *Setaria* species (122-365[xm). The width of the cattle filarial however was slightly wider (4-8(xm) than *Setaria* species (4-6jxm), but the difference was not significant. There was also striking similarity in terms of features such as sheath and inner body, which stained intensely in both cases as well as the tapering of the tail.

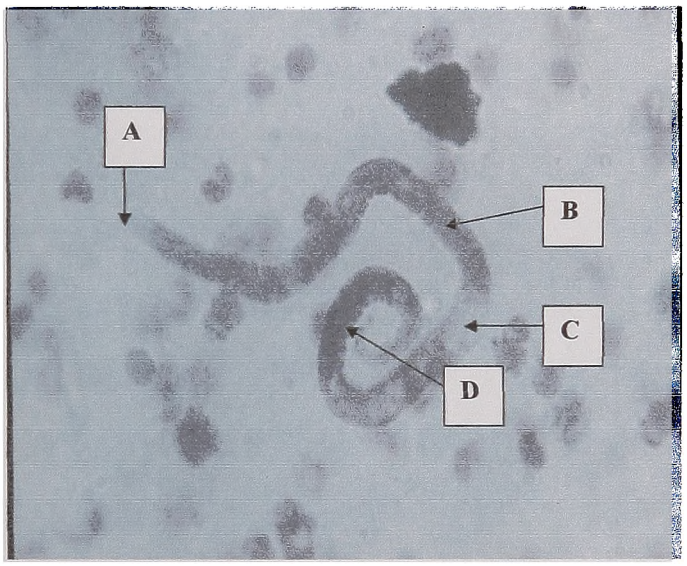

Fig 4.2: Unsheathed microfilaria (mf) observed in cattle blood after staining with Giemsa (X1000). A = Head Space, B = Compact nuclei, C = Inner korper D= tail end.

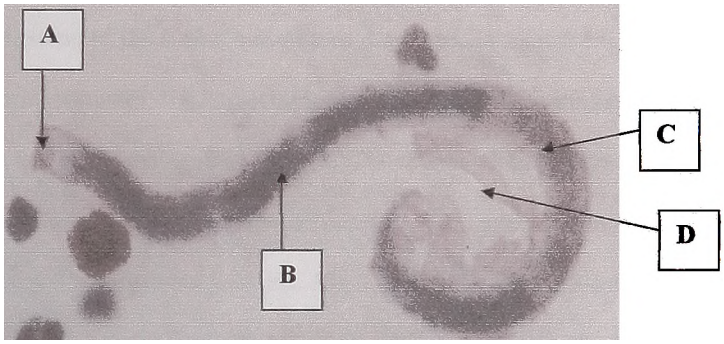

Fig 4.3: Sheathed microfilaria (mf) from cattle blood after staining with Giemsa (XlOOO). A = Head space, B=Compact nuclei, C = Inner korper and D = Sheath

4.4 Mosquito Surveys

A total number of 690 mosquitoes were collected off cattle at Winneba and *Culex* species was the most abundant species forming 88.7% (612/690). *Mansonia* species was 6.2% (43/690), *Anopheles* species was 4.9% (29/690) and the least was *Aedes,* which was 0.15% (1/690) [Fig 4.3], Two infective stages of filarial worms (L3) were found and both occurred in *Culex* mosquitoes thus an infective rate of 0.3% (2/612). There were some 4 microfilariae (mfs) in some of the *Culex* mosquitoes dissected. In total there were 3 Lis (first stage of the filarial parasite), 1 L2s (second stage) and 2 L3s (third/infective stages) thus giving infection rate in the mosquitoes as 0.9%. There were 42 mosquitoes caught by pyrethrum spray catch and these were mostly of the *Anopheles* species comprising of 7 *Anopheles gambiae* and 35 *Anopheles funestus.* None of the *Anopheles* mosquitoes was found to have the infective stage of the *W. bancrofti.* However there were 2L2's and 1 microfilaria in one of the *Anopheles gambiae* (Fig 4.8).

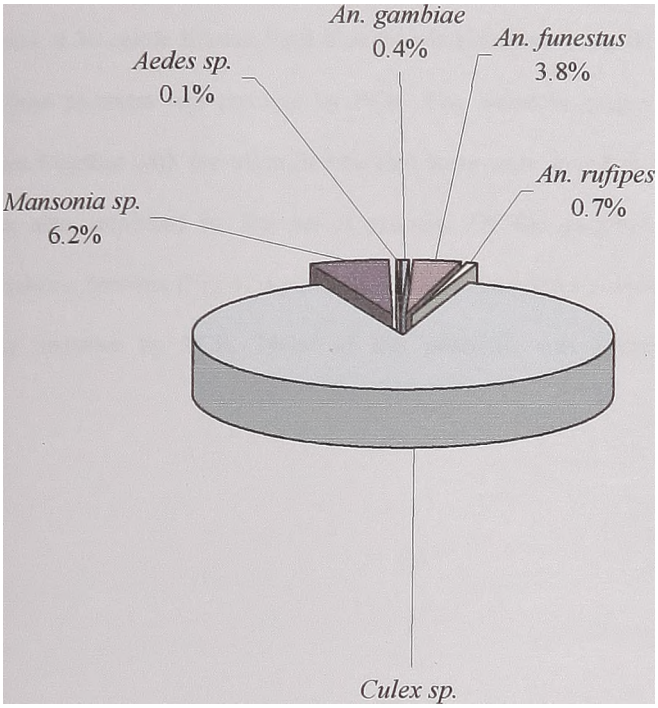

Fig 4.4: Proportion of various species of mosquitoes collected off cattle at Winneba (n=690)

4.5 Molecular Identification of Cattle Filariae and *Anopheles* Mosquitoes

A total of 30 catttle filariae from blood were amplified using *W. bancrofti* primers. None of these parasites was positive by PCR. Two infective stages (Fig 4.8) and the other stages together with ihe microfilariae that were were found in the dissected mosquitoes were also amplified by the set of primers. Of the *Anopheles* species there were 7 *Anopheles funestus* (Fig 4.5) confirmed and 3 *Anopheles gambiae* (Fig 4.6) and the rest were negative by PCR. None of the parasites was positive by PCR (Fig 4.7).

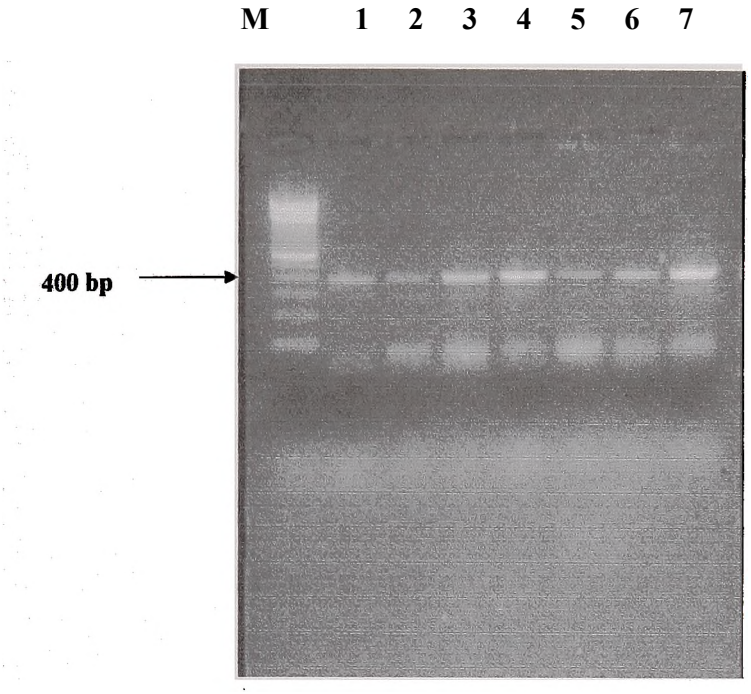

Fig 4.5: Agarose gel electrophoregram (2%) of the amplified *Anopheles funestus s.s.* DNA fragment of band size 400bp observed under UV light after staining with ethidiom bromide. Lane 1= lOObp DNA molecular marker, lanes 1-7 — *Anopheles funestus* (Mosquitoes collected off cattle).

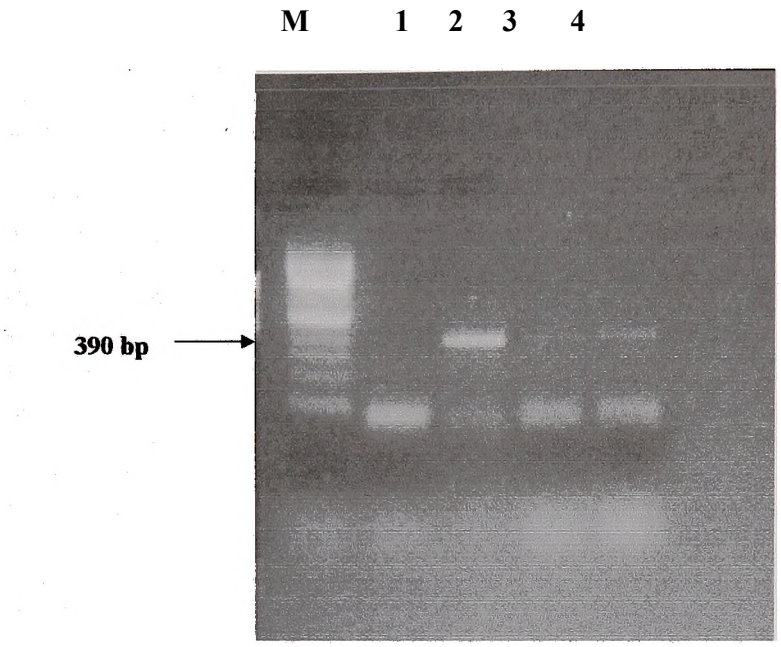

Fig 4.6: Agarose gel electrophoregram (2%) of the amplified *Anopheles gambiae s. s.* DNA fragment of band size 390bp observed under UV light after staining with ethidium bromide. (Mosquitoes collected of cattle). Lane l=100bp marker, lane 2 = negative control and Lanes 3-5 = *Anopheles gambiae s.s.*

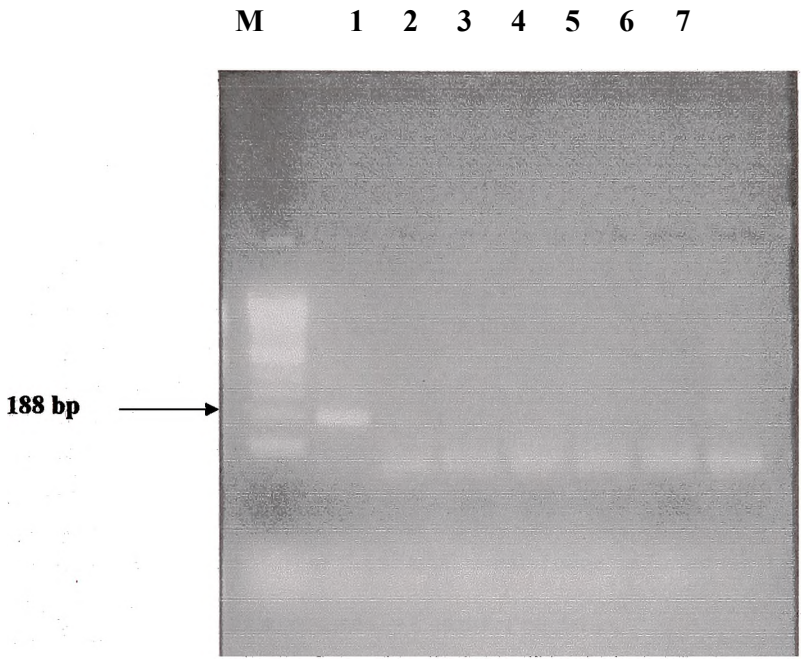

Fig 4.7: Agarose gel electrophoregram (2%) of PCR products amplified rDNA products in *Wuchereria bancrofti* microfilariae, cattie filarial parasites and mosquitoes. The reactions were performed using oligonucleotide primers NV-1 and NV-2 designed to amplify a family of the DNA elements, the 188 base pair (bp) *Sspl* repeat, specific for the genus *Wuchereria.* Lane M = 100 base pair marker, lane 1 = Positive control, lane 2 = Negative control and lane 3-7 = Filarial worm from catde

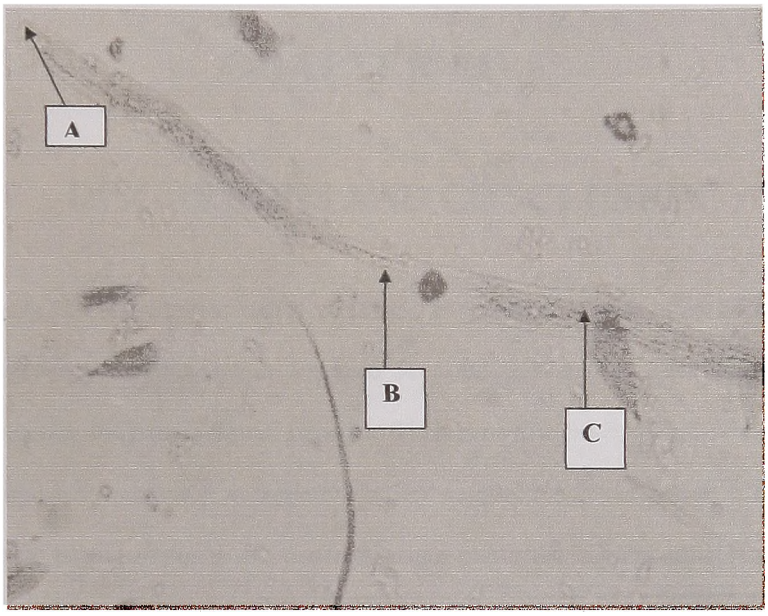

Fig 4.8: Infective stage (L3) of the filarial worm found in *Culex* mosquito (xlO). A= Head space, B= Inner korper and C= Compact nuclei.

CHAPTER FIVE

DISCUSSIONS AND CONCLUSION

The ultimate aim of the present study was to use morphological and molecular techniques to characterize a filarial parasite reported in cattle that shared some morphological similarities with the human filarial parasite, *Wuchereria bancrofti.* Morphometries of the cattle filarial worms made it possible to classify it under the genus *Setaria.* Comparison of the cattle filarial worm to *W. bancrofti* using published identification keys (WHO, 1997) revealed some differences as well as similarities. One distinctive feature about the cattle filarial worm is the presence of a very prominent inner korper in 70% of the worms identified. This feature is not very distinct in *W. bancrofti.* Also the compact large nuclei distinguished it from *Wuchereria* which has dispersed nuclei. The differences in morphometric measurements of the sheathed and unsheathed microfilariae were found not to be significant which suggest that they probably belong to the same Genus if not the same species. Even though *Setaria* species are sheathed, only 5 of the cattle filariae were sheathed. This could have been due to loses during slide preservation and preparation since some lose sheath were found on slides.

The results supports what has been reported earlier by Nelson *etal.* (1962) that *Setaria* spp from cattle, sheep, the ox, deer and horses, stain intensely with Giemsa stain same as *W. bancrofti* and *B. malayi.* The similarity is evident in features such as the short headspace, tail that is devoid of nucleus and lengths. It is therefore not out of place to

find that there was no amplification using microfilariae and L3 DNA since the primers were specifically designed for *W. bancrofti* DNA sequences. The PCR results confirm that the cattle filarial worms were not *W. bancrofti.*

The periodicity of a given species or geographic variant is especially useful in determining the best time of day to collect blood samples for examination. Several studies have confirmed the nocturnal periodicity of *W. bancrofti* worldwide (Sasa, 1976; WHO, 1992; Dreyer *et al,* 1996; Simonsen *et al.,* 1997). Normally, in Africa, where night-biting mosquitoes transmit nocturnal forms of the parasite, the highest intensities in bancroftian filariasis, the mf periodicity in the peripheral blood occur at night and few or none during the day. In several foci of Southeast Asia and tie Pacific but not in Africa, *W. bancrofti* and *B. malayi* are known to be subperiodic meaning that the microfilariae are detectable in the peripheral blood at any time. So far studies have established *Setaria* species to be aperiodic (Nelson *et al* 1962; Omri *et al,* 1978). The present study however does not support these findings which seem to indicate subperiodicity. However more studies are needed before any firm conclusion can be drawn, because of the small sample size and limited period of this study.

About half of the world's burden of lymphatic filariasis is transmitted by *Cx. quinquefasciatus* in India. This and other man-biting forms of *Culexpipiens* complex are responsible for most or all of the bancroftian filariasis transmission in Asia countries, Indonesia, Egypt, urban East Africa and the Americas. Globally, the majority of *W. bancrofti* is transmitted by *Cx. quinquefasciatus. Anopheles* mosquitoes are known to

transmit lymphatic filariasis in rural areas of tropical Africa and the Papua sub-regions. In Ghana, members of the *An. gambiae* complex and *An. funestus* are the known vectors of the disease (Gyapong *et al.,* 1994; Dzozomenyo *et al.,* 1999; Appawu *et al.,* 2001). From the present study *Culex* species was found to be the most dominant species (89%), with an infective rate of 0.3%. This species has however been found to be unimportant in the transmission of lymphatic filariasis in Ghana. It is therefore difficult to understand why the same mosquito species is a possible vector of cattle filariae and not of *W. bancrofti.* The *An. gambiae* and *An. junestus* caught off cattle did not harbour any of the infective stages of the parasites and most importantly formed the minority. This implies that there could not be cross infections from human to cattle since different vectors are responsible for transmission.

It can be concluded that the cattle filarial cannot be *Wuchereria bancrofti.* This is based on the morphological, molecular and periodicity studies conducted. The two forms of the cattle filariae were found to be similar morphologioally and the absence of sheath could have resulted from loss during processing. Features of the cattle filarial agree with general features of *Setaria* species. *Culex* species has been implicated by the present study as the vector of the cattle filariae. In Ghana it is known to be refractory to *W. bancrofti.* These findings suggest that entomological parameters can still be used for monitoring LF intervention programmes without any problem most especially in our study area. However further studies need to be conducted especially in other LF endemic areas for confirmation.

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APPENDICES

APPENDIX I

CHEMICALS AND REAGENTS

Preparation of Giemsa solution

Preparation of500ml of stock solution

Reagents Needed: Giemsa Powder 3.8g

Glycerol 250ml

Methanol 250ml

Procedure: The methanol was measured and added to a measured amount of Giemsa powder in a dry brown bottle. Glycerol was measured out and added to the stain preparation. The mixture was heated to 50°C for up to two hours, and mixed at intervals.

Preparation of 2% Giemsa solution

49ml of buffered water, pH 7.1 was added to 1ml of Giemsa stain and mixed gently.

Preparation of Electrophoresis buffer

Electrophoresis buffer, Tris-acetate (TAE), concentrated stock solution; 242g of Tris base was dissolved in 27.1ml of glacial acetic acid and 100ml of0.5M ofEDTA (pH 8.0), and the volume was made up to 1000ml with distilled water. The 50x solution was dispersed into aliquots and stored at room temperature.

Preparation of Ethidium bromide

Ethidiim bromide, lOmg/ml: O.lg of ethidium bromide was added to 10ml of water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The container was wrapped with an aluminium foil and stored at room temperature. Gloves were worn when working with the solution that contained this dye because it is carcinogenic.

Preparation of Orange G

5x orange G: 20% (w/v) Ficol, 25mM EDTA, 2.5% (w/v) orange G: 2.5g of Ficol and 0.25g of orange G were dissolved in a few ml of sterile distilled water, then 500ul of 5M EDTA was added and the volume made up to 10ml with distilled water.

- lOObp ladder molecular weight marker (Sigma-P1473): the marker contains 10 bands ranging from lOOOObp in exact lOObp increments. This marker was prepared according to manufacturer recommendations.

APPENDIX II

DNeasy Protocol for DNA purification from animal tissues

1. Cut up to 25mg tissue (up to 1 Omg spleen) into small pieces, place in a 1. 5ml microcentrifuge tube, and add 180ul Buffer ATL.
2. Add 20ul proteinase k, mix by vortexing, and incubate at 55°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

   Optional: RNase treatment of the sample. Add 4ul of RNase A (lOOmg/ml), mix by vortexing, and incubate fir 2minutes at room temperature.
3. Vortex for 1 Ssec. Add lOOul Buffer AL to the sample, mix thoroughly by vortexing, and incubate at 75°C for lOminutes.
4. Add 200ul ethanol (96-100%) to the sample, and mix thoroughly by vortexing.
5. Pipet the mixture from step 4 into the DNeasy mini column sitting in a 2-ml collection tube (provided). Centrifuge at 8000rpm for 1 minute. Discard flow-through and collection tube.
6. Place the DNeasy mini column in a new 2-ml collection tube (provided), add 500p.l buffer AW1 and centrifuge for 1 minute at 8000rpm. Discard flow-through and collection tube.
7. Place the DNeasy mini column in a 2ml collection tube (provided), add 500ul buffer AW2 and centrifuge for 3 minutes at full speed to dry the DNeasy membrane. Discard flow through and collection tube.

8. Place the DNeasy mini column in a clean 1. 5ml or 2ml micro centrifuge tube (not provided), and pipette 200ul Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 minute, and then centrifuge for 1 minute at 8000rpm to elute.
9. Repeat elution as described in STEP 8.

APPENDIX III

MORPHOMETIC MEASUREMENTS & ANALYSIS

UNSHEATHED MICROFILARIAL

(MORPHOMETRICS)

#	Length (fim)	Width (|um)	Head space(jim)	Inner cupa(jj.m)
1	167	8	8	26
2	157	7	7	35
3	167	7	8	35
4	146	8	6	22
5	164	8	7	27
6	151	6	7.5	30
7	144	6	8	33
8	128	7	7	32
9	159	6	6	33
10	144	6	6	35
11	159	7	7	33
12	158	7	7.5	31

151	5	8	29
141	6	6.5	29
156	6	8	32
152	7	7	28
149	6	6	32
155	5	7	28
178	8.7	8.5	27
172	8.7	8	35
168	8.7	6.8	25
168	8.4	7.5	25
196	7	8	30
149	8.7	7	25
172	7.4	6	22
173	8.7	8	23
169	7.4	6	24
166	8	8	30
155	8	7	22
180	8.7	8.5	25
173	8	7	39

32	200	7	8	36
33	185	8	7.5	37
34	160	8	8	38
35	190	7	7.5	33
36	183	8	7.3	28
37	170	8	6	35
38	188	8	7	33
39	168	8	6	34
40	160	6	5	32
Mean	164.275	7.31	7.1525	30.2
St Dev	15.62212025	1.05946285	0.85033553	4.686040371

SHEATHED MICROFILARIAL (Morphometries)

#	Length (jim)	Width (|im)	Head space(jim)	Inner cupa(jxm)
1	160	6	7	32
2	152	8	7	30
3	153	8	8	25
4	165	8	6	38
5	182	7	6	37
Mean	162.4	7.4	6.8	32.4
St Dev	12.17784874	0.89442719	0.836660027	5.319774431

APPENDIX IV

RESULTS OF MICROSCOPIC EXAMINATION AND DEMOGRAPHIC FEATURES (FIELD DATA)

Screening At Somanya

ID # Age Sex Breed Result

1	**5m**	**F**	**WASH**	**NEG**
2	**5m**	**M**	**WASH**	**NEG**
3	**7m**	**F**	**WASH**	**NEG**
4	**7m**	**F**	**WASH**	**NEG**
5	**iy**	**F**	**WASH**	**NEG**
6	**3y**	**F**	**WASH**	**NEG**
7	**ly**	**F**	**WASH**	**NEG**
8	**7m**	**M**	**WASH**	**NEG**
9	**6m**	**F**	**WASH**	**NEG**
10	**3m**	**F**	**WASH**	**NEG**
11	**ly**	**M**	**WASH**	**NEG**
12	**ly**	**M**	**WASH**	**NEG**
13	**7y**	**M**	**WASH**	**NEG**
14	**7y**	**F**	**WASH**	**NEG**

15	3m	F	WASH	NEG
16	3m	F	WASH	NEG
17	3y	F	WASH	NEG
18	7m	F	WASH	NEG
19	3y	M	WASH	POS
20	2y	F	WASH	NEG
21	3y	M	WASH	NEG
22	3y	M	WASH	NEG
23	10y	F	WASH	NEG
24	lOy	F	WASH	NEG
25	6y	F	WASH	NEG
26	10Y	F	WASH	NEG
27	lOy	F	WASH	NEG
28	lOy	F	WASH	NEG
29	lOy	F	WASH	NEG
30	3y	F	WASH	NEG
31	lOy	F	WASH	NEG
32	32y	F	WASH	NEG
33	vy	F	WASH	POS

Screening at Winneba (Muni lagoon				
ID#	Age	Sex	Breed	Result
139	**6y**	**M**	**ZEBU**	**NEG**
165	**5y**	F	**ZEBU**	**NEG**
101	**6y**	F	**WASH**	**NEG**
105	**5y**	F	**WASH**	**NEG**
129	**4y**	F	**WASH**	**NEG**
136	**6y**	F	**ZEBU**	**NEG**
159	**7y**	F	**WASH**	**NEG**
102	**8y**	F	**WASH**	**NEG**
122	**7y**	F	**ZEBU**	**NEG**
147	**6y**	F	**WASH**	**NEG**
112	**8y**	F	**ZEBU**	**NEG**
164	**7y**	**M**	**ZEBU**	**NEG**
107	**3y**	F	**ZEBU**	**NEG**
135	**6y**	F	**WASH**	**NEG**
154	**8y**	F	**WASH**	**NEG**
103	**8y**	F	**WASH**	**NEG**
145	**8y**	**M**	**WASH**	**NEG**
178	iy	F	**WASH**	**NEG**

182	8y	**F**	**WASH**	**NEG**
163	By	**F**	**WASH**	**NEG**
115	**9y**	**F**	**ZEBU**	**NEG**
142	8y	**F**	**WASH**	**NEG**
169	lOy	**F**	**WASH**	**POS**
148	**9y**	**F**	**ZEBU**	**NEG**
167	6y	**F**	**WASH**	**NEG**
179	8y	**F**	**WASH**	**NEG**
18	**5y**	**F**	**ZEBU**	**NEG**
151	**7y**	**M**	**WASH**	**NEG**
6	**2y**	**F**	**WASH**	**NEG**
8	**2y**	**F**	**ZEBU**	**NEG**
166	**5y**	**F**	**WASH**	**NEG**
150	**7y**	**F**	**ZEBU**	**NEG**
118	**7y**	**F**	**WASH**	**NEG**
10	**2y**	**M**	**ZEBU**	**NEG**
5	**3y**	**F**	**WASH**	**NEG**
114	8y	**F**	**ZEBU**	**NEG**
102	6y	**F**	**WASH**	**NEG**
168	**3y**	**M**	**WASH**	**NEG**
103	**4y**	**M**	**WASH**	**NEG**
21	**3y**	**M**	**WASH**	**NEG**

9	**3y**	**M**	**ZEBU**	**NEG**
17	3y	M	ZEBU	NEG
171	6y	F	ZEBU	NEG

Screening at animal husbandary

2131	4y	F	**WASH**	**NEG**
2132	**6y**	F	**WASH**	**POS**
2133	5y	F	**WASH**	**NEG**
2135	6y	F	**WASH**	**NEG**
2136	**7y**	F	**WASH**	**NEG**
2137	5y	M	**WASH**	**NEG**
2138	**8y**	F	**WASH**	**NEG**
2139	**7y**	F	**WASH**	**NEG**
2140	8y	F	**WASH**	**NEG**
2121	5y	F	**WASH**	**NEG**
2122	**8y**	F	**WASH**	**NEG**
2123	6m	F	**WASH**	**NEG**

2124	8y	F	WASH	NEG
2125	8y	F	WASH	NEG
314	9y	F	WASH	NEG
2126	6y	F	WASH	NEG
2127	2y	M	WASH	NEG
2128	3y	M	WASH	NEG
2129	7y	F	WASH	NEG
2130	6y	F	WASH	NEG
2111	8y	F	WASH	NEG
2112	3m	F	WASH	NEG
2113	8y .	F	WASH	NEG
2114	6y	F	WASH	NEG
2115	3y	M	WASH	NEG
2116	4y	M	WASH	NEG
2117	4y	F	WASH	NEG
2118	3y	F	WASH	NEG
2119	3y	M	WASH	NEG
2120	6y	M	WASH	NEG
2142	5y	F	WASH	POS
2143	3y	M	WASH	NEG
2144	2y	M	WASH	NEG
2145	9m	F	WASH	NEG

2146	**iy**	**M**	**WASH**	**NEG**
2147	**ly**	**M**	**WASH**	**NEG**
2148	**6m**	**F**	**WASH**	**NEG**
2150	**6y**	**F**	**WASH**	**NEG**
2110	**3y**	**F**	**WASH**	**NEG**
2109	**2m**	**M**	**WASH**	**NEG**
2108	**3m**	**M**	**WASH**	**NEG**
2107	**3m**	**F**	**WASH**	**NEG**
1681	**4y**	**F**	**ZEBU**	**NEG**
1682	**6y**	**F**	**ZEBU**	**NEG**
1683	**6y**	**F**	**ZEBU**	**NEG**
1684	**6y**	**F**	**ZEBU**	**NEG**
1685	**1 5y**	**M**	**WASH**	**NEG**
1686	**6y**	**F**	**WASH**	**NEG**
1687	**3y**	**F**	**WASH**	**NEG**
1688	6y	**F**	**ZEBU**	**NEG**
1689	**6y**	**F**	**WASH**	**NEG**
1690	5y	**M**	**ZEBU**	**NEG**
1671	**4y**	**F**	**ZEBU**	**NEG**
1672	**4y**	**M**	**ZEBU**	**NEG**
1673	8y	**F**	**ZEBU**	**NEG**
1674	1.5y	**M**	**WASH**	**NEG**

1675	5y	M	WASH	NEG

Screening at Mpotah

1641	**8y**	**F**	**ZEBU**	**NEG**
1642	**8y**	**F**	**ZEBU**	**NEG**
1643	8y	**F**	**WASH**	**NEG**
1644	**7y**	**F**	**WASH**	**NEG**
1645	**4y**	**F**	**WASH**	**POS**
1646	**7y**	**F**	**WASH**	**NEG**
1647	**8y**	**F**	**ZEBU**	**NEG**
1648	**7y**	**F**	**WASH**	**NEG**
1649	**8y**	**F**	**ZEBU**	**NEG**
1650	**8y**	**F**	**ZEBU**	**NEG**
1651	**8y**	**F**	**ZEBU**	**POS**
1652	**5y**	**F**	**ZEBU**	**NEG**
1653	10y	**F**	**ZEBU**	**NEG**
1654	**5y**	**F**	**WASH**	**NEG**
1655	**8y**	**F**	**ZEBU**	**NEG**
1656	8y	**F**	**ZEBU**	**NEG**
' 1657	**7y**	**F**	**WASH**	**NEG**
1658	**4y**	**M**	**ZEBU**	**NEG**
1659	**8y**	**F**	**ZEBU**	**NEG**

1660	**8y**	F	**ZEBU**	**NEG**
1661	**7y**	F	**WASH**	**NEG**
1662	**8y**	F	**ZEBU**	**NEG**
1663	8y	F	**ZEBU**	**NEG**
1664	**8y**	F	**WASH**	**NEG**
1665	**8y**	F	**WASH**	**NEG**
1666	**7y**	F	**WASH**	**NEG**
1667	**6y**	F	**ZEBU**	**NEG**
1668	**8y**	F	**ZEBU**	**NEG**
1670	**6y**	F	**ZEBU**	**NEG**
1676	**By**	F	**WASH**	**NEG**
1677	**8y**	F	**ZEBU**	**NEG**
1678	**6y**	F	**WASH**	**NEG**
1679	**8y**	F	**WASH**	**NEG**
1680	**8y**	F	**WASH**	**NEG**
2101	8y	F	**WASH**	**NEG**
2102	**8y**	F	**ZEBU**	**NEG**
2104	**7y**	F	**WASH**	**NEG**
201	**8y**	F	**ZEBU**	**NEG**
202	**7y**	F	**ZEBU**	**NEG**
203	**6y**	F	**WASH**	**NEG**

Screening at Axim (Asaasetre)

ID#	Age Sex Breed	Result
204	lOy F WASH	NEG
205	4y F WASH	NEG
206	5y F WASH	NEG
207	5y F WASH	NEG
208	4y F WASH	NEG
209	6y F WASH	NEG
210	12y F WASH	NEG
231	20y F WASH	NEG
232	3y F WASH	NEG
233	2y F WASH	NEG
234	3y F WASH	NEG
235	5y F WASH	NEG
236	6y F WASH	NEG
237	4y F WASH	NEG
238	25y F WASH	NEG
239	23 y F WASH	NEG
240	24y F WASH	NEG
211	3y F WASH	NEG
212	8y F WASH	NEG

213	24y F WASH	NEG
214	15y F WASH	NEG
215	17y F WASH	NEG
216	30y F WASH	NEG
111	14y F WASH	NEG
218	4y M WASH	NEG
219	8y F WASH	NEG
220	3y F WASH	NEG
221	2y F WASH	NEG
222	ly F WASH	NEG
223	3y F WASH	NEG
224	3y F WASH	NEG
225	8y F WASH	NEG
226	5y F WASH	NEG
227	2y F WASH	NEG
228	2y F WASH	NEG
229	ly F WASH	NEG
230	2y M WASH	NEG

Screening at Teleko Bokazo

241	9y F WASH	NEG
242	8y F WASH	NEG
243	7y F WASH	NEG
244	7.5y F WASH	NEG
245	6y F WASH	NEG
246	6y F WASH	NEG
247	8y F WASH	NEG
248	*ly* F WASH	NEG
249	3y F WASH	NEG
250	4y F WASH	NEG
191	3y F WASH	NEG
192	3y F WASH	NEG
193	lOy F WASH	NEG
194	*ly* F WASH	NEG
195	3y F WASH	NEG
107	4y F WASH	NEG
108	6.5y F WASH	NEG
109	6y F WASH	NEG
110	5y F WASH	NEG
130	3y F WASH	NEG
121	4y M WASH	NEG

Screening at Nkroful Sec Sch

200	7y M WASH	NEG
199	5y F WASH	NEG
198	4y F WASH	NEG
197	5y M WASH	NEG
196	6y F WASH	NEG
101	7y F WASH	NEG
241	9y M WASH	NEG
102	8y F WASH	NEG
103	7y M WASH	NEG
104	6y F WASH	NEG
105	5y F WASH	NEG
106	2y M WASH	NEG

Screening at Kanbule

122	4y F WASH	NEG
123	4y F WASH	NEG
124	3y M WASH	NEG
125	3y M WASH	NEG
126	3y F WASH	NEG
127	5y M WASH	NEG
128	5y F WASH	NEG

129	**4y**	**F**	**WASH**	**NEG**
131	**6y**	**F**	**WASH**	**NEG**
132	**5y**	**F**	**WASH**	**NEG**
133	**8y**	**F**	**WASH**	**NEG**
134	**8y**	**F**	**WASH**	**NEG**
135	**5y**	**F**	**WASH**	**NEG**

Screening at Arokpogwe

181	**5y**	**M**	**WASH**	**NEG**
182	**8y**	**M**	**WASH**	**NEG**
190	**8y**	**M**	**WASH**	**NEG**
183	**5y**	**F**	**WASH**	**NEG**
184	**5y**	**F**	**WASH**	**NEG**
185	**5y**	**F**	**WASH**	**NEG**
186	**4y**	**F**	**WASH**	**NEG**
187	**6y**	**F**	**WASH**	**NEG**
188	**5y**	**F**	**WASH**	**NEG**
189	**8y**	**F**	**WASH**	**NEG**
141	**6y**	**F**	**WASH**	**NEG**
142	**3y**	**F**	**WASH**	**NEG**
143	**5y**	**F**	**WASH**	**NEG**
144	**3y**	**F**	**WASH**	**NEG**

145	3y	F	WASH	NEG

Screening at Police Quarters

111	3y	F	WASH	NEG
112	7y	F	WASH	NEG
113	3y	F	WASH	NEG
114	4y	F	WASH	NEG
115	3y	F	WASH	NEG
116	4y	M	WASH	NEG
117	5y	F	WASH	NEG
118	4y	F	WASH	NEG
119	3y	F	WASH	NEG
120	4y	F	WASH	NEG
171	2y	F	WASH	NEG
172	6y	M	WASH	NEG
173	4y	M	WASH	NEG

APPENDIX V

FORMULAS

1. Geometric Mean:

Geometric Mean = antilog {X log X+l} - 1

2. Prevalence: $\frac{\text{Number of cattle infected}}{\text{Total Number screened}} \times 100$

3. Infective rate: $\frac{\text{Number of cattle infected}}{\text{Total cattle dissected}} \times 100$

APPENDIX VI

Constituents of a 25JJ,1 PCR reaction mix

Table 3.1: Constituents of PCR reaction mix for *W. banerofti*

Reagent	Volume	Final concentration
Sterile distilled water	To make up 25 (xl	
lOx PCR buffer		lx
50mM MgCb		2.5mM
10|JM of each of dATP, dCTP, dGTP, dTTP	0.5^1 each	200[jM each
20JIMNV-1 primer	0.5 (0.1	200 nM
20nM NV-2 primer	**0.5 (j.1**	200 nM
5U Taq polymerase	0.125)0.1	0.025U/|JL1
DNA template	**5 JJIl**	
Final Volume	25(0.1	

Table *32:* **Typical reaction components of a 25pJ PCR reaction for** *An. gambiae* **identification**

H20	To complete final volume of25JJ.1	Finalconc
10X PCR reaction buffer	2.5p;l	IX
dNTPs	2.510.1	20mM
UN Primer	l.Op.1	12.5ng
GA, AR, ME, QD	1.0^1 each	20|JM
0.6 U *Taq* polymerase (5U/p.l)	O.lul	0.625
2-2.5 mM each dATP, dCTP, dGTP, dTTP		

Table 33: Typical reaction components for a 25pl reaction for identification *An. funestus*

H20	To complete final volume of 25jil	FinalConc
10X PCR reaction buffer containing	2.5|j.l	**IX**
25 mM MgCl	l.Ojal	
dNIP solution	2.5|xl	
Primers, D3 **A,** D3B	l.Ofxl	**20^iM**
0.6 U Taq polymerase (5U/JJ.1)	0.1 nl	0.625
2-2.5 mMeach dATP, dCTP, dGTP, dTTP	20mM	

Table 42: Microfilaria intensities during the 24-hour collection period

ID#	Age	Sex	Mf intensities (mf/lOOpl) at the hours of examination					
			6:00am	10:00am	2:00pm	6:00pm	10:00pm	2:00am
169	lOyrs	F	22	18	16	7	13	16
2142	5yrs	M	29	11	7	11	12	4
2132	6yrs	F	21	92	0	60	34	88
1651	8yrs	F	4	5	5	0	2	22
1645	4yrs	F	0	3	1	0	3	1
Geometric Mean			**20**	18.4	4.6	7.6	11.6	15.5