ASSESSMENT OF MICROFILARIAL LOAD IN THE BLOOD OF PATIENTS WITH *MANSONELLA PERSTANS* INFECTION AFTER ANTI-WOLBACHIAL TREATMENT WITH DOXYCYCLINE

BY

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DECLARATION

I hereby declare that this submission is my own work towards the M.Phil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

I dedicate this thesis to the Almighty God for His sustenance and protection during the sequence of events of this study.

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My foremost appreciation goes to the Almighty God for His countless love, grace and protection towards me in completing this thesis. My gratitude goes to my supervisor, Dr. Alexander Yaw Debrah and project coordinator, Dr. Mrs. Linda Batsa-Debrah for their guidance and support in making this thesis a success.

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ABSTRACT

Mansonella perstans is a vector-borne parasite; transmitted by tiny female bloodsucking insects of the genus *Culicoides* (biting midges). It is more prevalent in Sub-Saharan Africa. Most antifilarial drugs including ivermectin and DEC, and also benzimidazoles such as mebendazole, albendazole, levamisole, and thiabendazole, have proven ineffective against *M. perstans*. Therefore, this study was undertaken to assess the efficacy of doxycycline against *M. perstans* in the Asante Akim North District of Ghana. The study was an open randomized trial conducted in 8 communities of the Asante Akim North District. Out of a total of 1,229 individuals microscopically screened, 405 individuals were microfilaria-positive representing an overall prevalence of 33.0%. There was no significant difference (p=0.0568) between prevalence among males and females. Based on the findings from the initial blood samples, 102 eligible individuals were randomized to Early and Delayed groups. Individuals received 200mg of doxycycline daily for 6 weeks. The former group started medication at the initial stage while treatment was delayed for 6 months in the latter group. Five individuals who were not available during treatment, served as controls. Blood samples were taken and analyzed for microfilaria at 4 and 12 months of the study period. There was no significant difference (p=0.7655) between treatment groups at baseline level. Microfilarial load reduced marginally at 4 months even though there was no significant difference (p=0.5559) between the two treatment groups. However, at 12 months, the efficacy of doxycycline was evident. The Early group had a drastic reduction of microfilarial load resulting in significant difference between the two groups (p<0.0001). The distribution of microfilarial load among the controls throughout the study time points was relatively constant. A total of 10 individuals reported mild adverse events that resolved spontaneously. A well-designed specific real-time quantitative polymerase chain reaction (qPCR) was used to confirm *M. perstans* in the blood. Although PCR is arguably very sensitive, microscopy still remains reliable in the detection of *M. perstans*.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Mansonella perstans is a vector-borne parasite, transmitted by tiny female bloodsucking insects (biting midges) belonging to the genus *Culicoides* (Sharp, 1927). It is part of the nematode superfamily filaroidea, which is distributed in parts of South and Central America and Africa. It is prevalent in Sub-Saharan Africa with estimated 100 million people living with the infection (Simonsen *et al.,* 2011).The prevalence of this nematode is high in endemic localities with children also being affected (Stoll, 1947). Four species of filariasis-causing nematodes belonging to the genus *Mansonella* are responsible for human mansonellosis, *M. perstans*, *M. streptocerca*, *M. ozzardi*, and *M. rodhaini* (Bregani *et al.,* 2007a). Infections of *M. perstans* are considered asymptomatic with mild clinical features such as fever, headache, body itches, abdominal pains and eosinophilia (Bregani *et al.,* 2007a).

Sharp (1927) identified *Culicoides grahami* as a potential vector of the transmission of the infection. Studies conducted in Cameroon have described *C. grahami*, *C. austeni*, and *C. ornatipennis* as potential vectors (Sharp, 1927, 1928; Hopkins & Nicholas, 1952). Further studies were conducted in different countries to search for vectors of this parasite in endemic areas, including Congo (Noireau *et al.,* 1990), Nigeria (Agbolade *et al.,* 2006) and Zimbabwe (Clarke *et al.,* 1971). In East Africa, the taxonomy of *Culicoides* has been investigated by Khamala & Kettle in 1971). They were able to identify 61 species that had no local role in the transmission of *M. perstans*.

Humans become infected when bitten by insect vectors carrying the L3 infective larvae (CDC, 2016). In human filariae such as *Wuchereria bancrofti and Onchocerca volvulus*, there

is the possibility of the body's temperature triggering the larvae to leave the insect vector, thereby forcibly penetrating the skin (Simonsen *et al.*, 2011). In man, the time period for development of L3 larvae into adult stage is unknown. Adult *M. perstans* are difficult to be identified since they are located in serous cavities of the abdomen, lung and heart. They are sometimes recovered during surgery or autopsy.

Eberhard and Orihel (1984) and Baird *et al.* (1987) provided detailed morphological information of the adult worm. They are usually slender in shape. The size of the females ranges 50 80 mm 80 120um and the males 35 45 mm 50 60 um. Male and female adults nematodes are usually white, thread-like and cylindrical which reside in serous body cavities most commonly pleural and retroperitoneal and mesentery. Unlike other adult filarial nematodes such as *Onchocerca volvulus* which survives for 10 years (Habbema *et al*., 1990), the longevity of the *M. perstans* adults remains unknown. The females are viviparous producing about 3000 tiny larvae known as microfilariae (mf), which are unsheathed and possess blunt tails with nuclei extending to the tip of tail. Microfilariae circulating in the peripheral blood can live for approximately 4 months until ingested by vectors during blood meal (Asio *et al.*, 2009a). The parasite could be transferred to a new host after undergoing further growth in the insect vector.

Detection of microfilariae in the peripheral blood (Giemsa staining of thin blood smears) forms the basis for diagnosis of *M. perstans* infection in endemic regions (Simonsen *et al.*, 2011). This method is relatively time consuming and needs experienced personnel for examination, in order not to confuse diagnosis of mf of *M. perstans* with those of other filarial species.

M. perstans has been shown to destabilize immunity against Malaria (Metenou *et al.*, 2009). Wammes *et al.* (2010) observed that children with current helminth infestations respond poorly to vaccination (BCG) or malaria. And this was independent of the type of worm infection. It is therefore important to identify appropriate remedy against *M. perstans* infections in order to overcome the clinical consequences associated with it.

M. perstans remains one of the filarial infections difficult to manage. Clinical trials investigating the effect of antifilarial drugs such as diethylcarbamazine (DEC) and ivermectin, including other anthelmintics (albendazole, levamizole, mebendazole and thiabendazole) have been reported (Strohschneider, 1953; Richard-Lenoble *et al*., 1985; Bernberg *et al*.,1979; Van den Enden *et al*., 1992). Most of these trials involved a small number of participants, including expatriates and participants harbouring other filarial infections, and were performed within short follow-up periods without clear-detailed analysis of outcomes (Strohschneider, 1953; Adolph *et al.*, 1962). Hence, the outcomes are usually not credible. Effects of treatment have often been analyzed on blood microfilariae and in clinical cases, depletion or elimination of microfilaraemia was in association with amelioration of medical features of the infection in patients.

Diethylcarbamazine (DEC) was introduced as an efficient microfilaricide in onchocerciasis and lymphatic filariasis in 1940. However, investigation by Strohschneider (1953) on its activities on *M. perstans* revealed that it only brought about little and temporary loss of the microfilariae from patients' blood. On the contrary, Strohschneider (1953) and Adolph *et al*. (1962) who treated patients in Uganda and expatriates returning in the USA respectively noted that DEC eliminated microfilariae in large number of patients. Based on carefully performed study, DEC has been shown to reduce *M. perstans*

microfilariae intensities substantially (Bregani *et al*., 2006) but does not clear them all. In 1975, Maertens and Wery observed significant reduction of microfilariae intensities when combination of mebendazole and levamizole were administered in high doses for long periods to onchocerciasis patients in Congo. Similar outcomes have been documented by Bernberg *et al*. (1979) and Richard-Lenoble *et al*. (1985) in Gabon. Wahlgren (1982) and Wahlgren and Frolov (1983) also observed similar outcomes working with expatriates returning to Sweden and in Zimbabwe by Goldsmid and Rogers (1979).

Currently, intensive regimen of mebendazole is generally the preferred intervention against *M. perstans* infections (Bregani *et al*., 2006). However, the regimens are cumbersome to administer and not suitable for large-scale field use because of the many doses required. Administering DEC and mebendazole in combination treatment resulted in greater significant effect compared to the single use of either of the drugs (Bregani *et al*., 2006).

Albendazole was ineffective against *M. perstans* microfilariae assessment when given as few single doses (van den Enden *et al*., 1992; Asio *et al*., 2009b, 2009c), while higher regimens decreased and even eliminated microfilaraemia (Lipani *et al*., 1997; Duong *et al*., 1998). One or two dosages of thiabendazole were also active against the microfilariae of *M. perstans*, but prolonged usage of this drug was restricted by its relative toxicity (Bregani *et al*., 2003, 2006).

Ivermectin is one antifilarial drug that is efficient in eradicating a wide variety of nematodes that are of veterinary and medical importance, including the microfilariae of lymphatic filariasis and onchocerciasis (Bregani *et al*., 2006). However, treatment of *M. perstans* with single dose of ivermectin usually shows minor impact on microfilaraemias (Richard-Lenoble *et al*., 1988, 1989; Schulz-Key *et al*., 1993; van den Enden *et al*., 1993; Asio *et al*., 2009b, 2009c). Investigation by Gardon *et al.* (2002) has shown that treatment of patients

with ivermectin could result in severe complications if drug is repeatedly given for a longer period. Assessment of treatment with ivermectin for a long term among communities for the management of onchocerciasis has shown contradictory results in relation to its effect on concomitant *M. perstans* infections, by a reduction in microfilariae counts described in several places (Fischer *et al*., 1996; Kyelem *et al*., 2005).

Combination therapy involving albendazole (400 mg) and ivermectin (150-200ug / kg) is encouraged and currently being used in many communities by many programs against filariasis in several African countries (Asio *et al*., 2009b). Similar doses of drugs on *M. perstans* microfilaraemia have been extensively evaluated in Uganda in a double-blind study (Asio *et al*., 2009b, 2009c). No serious adverse events were noticed with marginal reduction in microfilariae levels that were too low and could not result in microfilariae elimination in the follow-up period of 1 year. The lack of adverse events side effects was consistent with previous study by Keiser *et al*.(2003), proposing that combining albendazole and ivermectin in therapy was fairly harmless for controlling lymphatic filariasis within areas co-endemic with *M. perstans* infection.

The activities of doxycycline in the life of worms that host the intracellualar endosymbiotic *Wolbachia* have been clearly identified. It interferes with the development, fertility and embryogenesis of worms (Hoerauf *et al*., 2003; Debrah *et al*., 2006). *Wolbachiae* are endosymbiotic bacterial of insects as well as various filarial nematodes including *Brugia malayi*, *Wuchereria bancrofti* and *Onchocerca volvulus* (Hoerauf & Pfarr, 2007). Although, endobacteria *Wolbachia* were not identified in *M. perstans'* microfilariae in many areas of Uganda (Büttner *et al*., 2003) and Gabon (Grobusch *et al*., 2003), the recent discovery of *Wolbachia* endosymbionts of *M. perstans* in Mali (Coulibaly *et al*., 2009) paved the way for

more extensive research to be conducted with regard to the use of doxycycline as a chemotherapeutic approach to *M. perstans* infections.

The treatment of *M. perstans* infection still remains uncertain since an effective chemotherapeutic intervention is lacking. The optimal remedy of the infection is still being investigated by researchers.

1.2 Rationale

Infections caused by *M. perstans* could be considered as neglected tropical disease since it is one of the least addressed and discussed infections in the tropics. The lack of attention may be due to prevalence among poor rural populations and absence of distinct clinical representation. Information on it is usually derived as by-products from other parasitological studies including loiasis, lymphatic filariasis, and onchocerciasis in which individuals had *M. perstans* coinfection (Simonsen *et al*., 2011).

In Africa, more than 100 million people may be infected while large portions of SubSaharan Africa are known to be endemic (Simonsen *et al*., 2011). Surveys have been conducted in some parts of Africa including Cameroun, Senegal, Mali, Uganda, Nigeria and Gabon. It is therefore imperative for such research to be carried out in Ghana to ascertain the regions of endemicity as well as identifying possible vectors of transmission.

There are divergent reports in relation to the occurrence of *Wolbachia* endosymbionts in the microfilariae of *M. perstans*. According to Büttner *et al*. (2003) and Grobusch *et al*. (2003) there is no evidence of *Wolbachia* endobacteria in the microfilariae of *M. perstans* in Uganda and Gabon respectively, while microfilariae from Mali were identified with *Wolbachia* (Keiser *et al*., 2008; Coulibaly *et al*., 2009). Differences in methodology or the possibility of different strains of *M. perstans* occurring in parts of Africa could account for these conflicting reports.

Furthermore, an effective chemotherapeutic treatment is still lacking. Most antifilarial drugs including ivermectin and DEC, and also benzimidazoles such as albendazole, mebendazole, thiabendazole and levamisole, have proved ineffective against *M. perstans* (Bregani *et al*., 2007).The detection of *Wolbachia* endosymbiont in microfilariae in Mali (Coulibaly *et al*., 2009) provided a platform for further investigation into the remedy of *M. perstans* infection. Since doxycycline interferes with the development, fertility and embryogenesis of worms that harbor endobacteria *Wolbachia* (Taylor *et al*., 2005), it could be effective against endobacteria *Wolbachia* of *M. perstans* in Ghana.

In Ghana, *M. perstans* was first detected in the 1990s by Awazi as a by-product of onchocerciasis research (Awadzi *et al*., 1991). There is no further documentation on the prevalence of the infection. However, recent studies published by Phillips *et al*. (2014) who aimed at developing a vaccine against Buruli Ulcer Disease (BUD) detected *M. perstans* infections in BUD patients from the Agogo Sub-District of the Asante Akim North District in Ghana. Notably, *M. perstans* coinfections were detected in 23% of BUD patients whereas about 13% of healthy contacts of BUD patients were *M. perstans*-infected. Although the study groups in this approach were too small to reach sufficient statistical power, these data suggested that there might be differential BUD susceptibility due to *M. perstans* coinfections. Pfarr and Hoerauf from the Institute for Medical Microbiology, Immunology and Parasitology at Bonn University Hospital subsequently detected *Wolbachia* strains in *M. perstans* in the study area (Asante-Akim North District). This was a major milestone for this study as it renders *M. perstans*-infected individuals susceptible for deworming by antibiotics, i.e. doxycycline treatment.

1.3 Aim

The aim of the study was to analyze microfilarial load of *M. perstans* patients after anti-*Wolbachial* treatment with doxycycline.

1.3.1 Specific Objectives

To determine the prevalence of microfilariae (*Mansonella perstans)* infection among selected communities.

To further confirm *M. perstans* using real-time qPCR.

To quantify microfilarial load, before and after treatment with anti-*Wolbachia* drug, doxycycline.

CHAPTER TWO

LITERATURE REVIEW

2.1 Geographical Distribution of *M. perstans* in Africa

There is high prevalence of *M. perstans* in parts of Africa (Nelson, 1965). In SubSaharan Africa alone, it is estimated that about 33 countries representing a population of about 580 million individuals are infected (World Bank, 2008) as shown in Figure 2.1.There is no report of the infection in Southern Africa (Botswana, Lesotho, South Africa, Swaziland, Namibia), Northern Africa (Morocco, Mauritania, Tunisia, Algeria, Egypt, Libya), and Western Africa

(Ethiopia, Eritrea, Somalia, Djibouti) as well as many of the island countries (Seychelles, Comoros, Madagascar, Cape Verde, Mauritius).

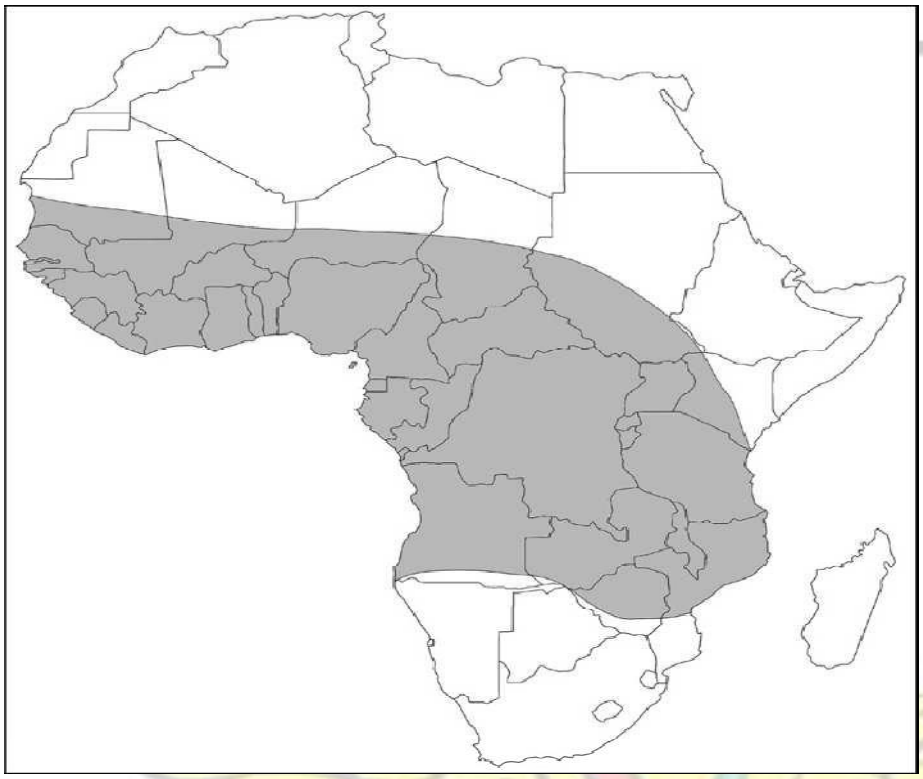

Figure 2. 1: An overview of the geographical distribution of *M. perstans* in Africa

(Simonsen *et al*. ActaTropica, 2011).

Furthermore, the prevalence of *M. perstans* discussed by Stoll (1947) reveals that in Africa; about 19 million individuals live with the infection. With an increase in the population of Africa about 6 times since 1947, it could mean that about 114 million people would be infected. Though the estimate is gross, in reality, it could be higher looking at the fact that more endemic sites are being identified regularly from diverse regions of Africa and reports are based on microfilariae detection only. Urban and peri-urban centres growth is not likely to have caused an increment in people living with *M. perstans* since the infection is essentially stationed at rural sites (Simonsen *et al*., 2011).

Countries such as Cameroon, Tanzania, Zimbabwe, Gabon, Gambia and Uganda have been able to conduct country-wide surveys at different periods to highlight the geographical distribution of infections due to *M. perstans* (Jordan, 1955a; Languillon, 1957; Clarke *et al*., 1971; Richard-Lenoble *et al*., 1980; Knight, 1980; Onapa *et al*., 2005). The general information from these surveys shows that there are significant variations among endemic countries, from infection-free areas to areas of high endemicity.

In most communities, microfilaraemia of *M. perstans* increases steadily with age and the adult population recording the highest levels of prevalence and intensity (Kershaw *et al*., 1953; Kershaw and Nicholas, 1954; Wijeyaratne *et al*., 1982; Gryseels *et al*., 1985; Arene and Atu, 1986; Noireau *et al*., 1989; Mommers *et al*., 1994; Asio *et al*., 2009c). Prevalence higher than 80% could be obtained in some endemic sites (Kershaw and Nicholas, 1954; Gryseels *et al*., 1985; Noireau *et al*., 1989; Mommers *et al*., 1994; Wanji *et al*., 2003; Onapa *et al*., 2005. Microfilaraemia in young children (even below 5 years) clearly differentiates the age trend of *M. perstans* from that of other filarial nematodes such as *W. bancrofti.* This could mean that the body's ability to develop resistance to *M. perstans* is not strong and that susceptibility to new infections still persists even at old age (Jordan, 1955a; Asio *et al*., 2009b). In the adult populations, microfilaraemia and intensities are higher in males than females (Kershaw *et al*., 1953; Kershaw and Nicholas, 1954; Jordan, 1955b; Gryseels *et al*., 1985; Arene and Atu, 1986; Noireau *et al*., 1989). This gender disparity is consistent in most filarial studies involving onchocerciasis and lymphatic filariasis and could be attributed to physiological or behavioural variations between the genders (Simonsen *et al.*, 2011).

2.2 Vectors and Transmission

Insects (Diptera: Ceratopogonidae) of the genus *Culicoides* commonly known as biting midges are the vectors involved in the transmission (Sharp, 1928). There are various forms of *Culicoides* from the Arctic to the Tropics. *Culicoides* possess proboscis which are short and vertical with wings folded around the abdomen. The wings are characterized by pattern of dark and light marks. They are about 1– 4 mm long. Like in many insects, blood meals are taken by females for egg maturation (Sharp, 1928).

There are few studies to ascertain the vector species of *M. perstans* in regions of endemicity. Also the taxonomy of the tropical *Culicoides* species has not been extensively studied. Studies on vectors of *M. perstans* have been performed in Cameroon, especially on *C. austeni, C. inornatipennis* and *C. grahami*, (Sharp, 1928; Hopkins, 1952; Hopkins and Nicholas, 1952; Kershaw *et al*., 1953; Nicholas *et al*., 1953; Nicholas, 1953a, 1953b; Nicholas and Kershaw, 1954; Duke, 1956). However, according to Linley *et al*. (1983), the identities of these species are debatable. Also, there have been attempts to question the vector species in Congo (Noireau *et al*., 1990), Nigeria (Agbolade and Akinboye, 2001) and Zimbabwe (Clarke *et al*., 1971).

Biting midges being a holometabolous dipteran as shown in Figure 2.2, develop progressively from the egg, larva, pupa and to adult stage. Depending on environmental conditions, it takes 2-6 weeks to complete developmental cycle. The females usually take blood meal at dusk and dawn (Hopkins, 1952; Nicholas, 1953a). The females could also bite in the day or at night. At every oviposition, between 70–180 eggs are released after 3- 4 days of blood meal (Nicholas, 1953a). The role of water is important for the survival of the insect as well as egg and larvae development (Sharp, 1928). Based on preference of *Culiciodes* species, eggs are laid in heaps at various locations. These areas include salt marshes, dung, rubbish pits, swamps, semi-rotting plants, tree holes, mud and riverbanks (Sharp, 1928). In Cameroon, vector species of *Culicoides*

laying eggs within banana stumps have been reported (Hopkins, 1952; Hopkins and Nicholas, 1952; Nicholas *et al*., 1953).

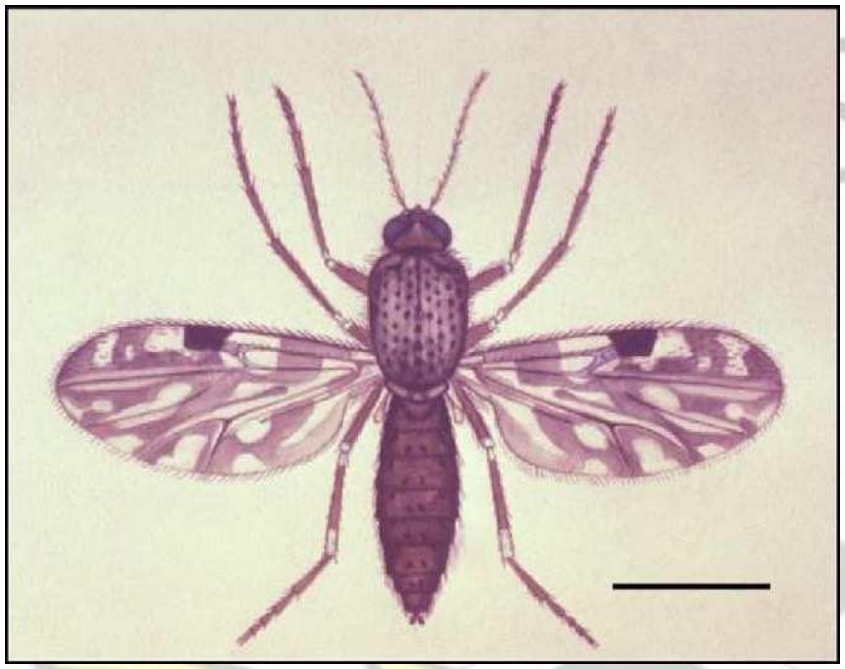

Figure 2.2: Biting midge; *Culicoides sp.* (Source: CDC Public Health Image Library)

Hatching of eggs after being laid occurs about 3 days (Nicholas, 1953a). There are four larval instars, which feed on rotting vegetable materials. The complete life cycle takes about 25–28 days (larval stage: 19-22 days; pupal: 2-3 days) (Nicholas, 1953a). Adult *Culicoides* could live for many weeks. They could fly for about hundred metres away from their larval habitations but may sometimes be dispersed by wind current (Nicholas, 1953b).

There is the need for more research to be conducted in the categorization of species of vector as well as bionomics and behaviour, at various endemic regions (Simonsen *et al*., 2011).

2.3 Life Cycle of *M. perstans*

An infected female midge introduces the third-stage infective larvae (L3 larvae) onto the site of wound during blood meal (CDC, 2016). It then undergoes further development into an adult

residing in the cavities of the body, specifically the pleural, peritoneal and the pericardial cavities. The female adult worm measures 70 to 80 mm in length and 120 µm in width while the male are usually 45 mm by 60 µm. Microfilariae produced by adult worms are characteristically unsheathed and subperiodic, measuring 200 by 4.5 µm reaching the blood stream (Eberhard and Orihel, 1984; Baird *et al*., 1987). During a blood meal, microfilariae are ingested by a midge. Microfilariae then penetrate midge's midgut and migrate through the hemocoel to the thoracic muscles. At this stage, the microfilariae develop into first-stage larvae and subsequently into third-stage infective larvae. The L3 larvae then migrate to the head and midge's proboscis ready to infect another human when blood meal is taken by the midge (CDC, 2016). The life cycle of *M. perstans* has been illustrated in Figure 2.3.

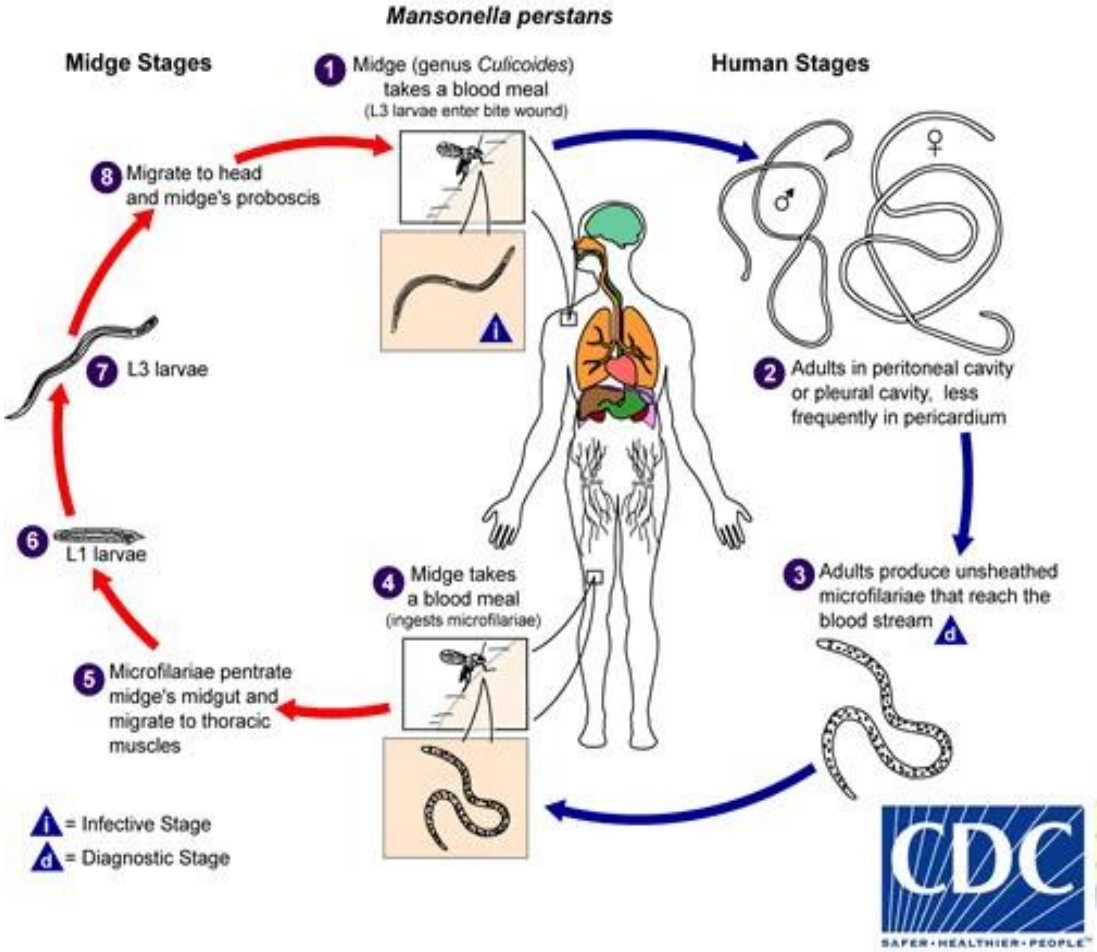

Figure 2.3: Life cycle of *Mansonella perstans* (http://www.cdc.gov/dpdx/mansonellosis/index.html)

2.4 Diagnosis of Infection in Humans

M. perstans are more difficult to locate because of their presence internally in the mesentery. Therefore it is customary to base routine identification on the larval form, the microfilaria (mf) in humans (Asio *et al*., 2009a). Microfilariae are morphologically distinguishable by their position within the body of the constituent nuclei (Sasa, 1976; Post *et al*., 2003). Thick or thin blood smears stained with Giemsa can be used to identify microfilariae

when available in high numbers in the blood of patients often living in endemic regions (Simonsen *et al*., 2011). The stained microfilariae are characteristically thin and short without sheath, and possess a bluntly rounded tail with nuclei extending to the tip (Figure 2.4). In most field surveys, the stained thick blood smear approach is often employed. According to Fischer *et al*. (1996); Adolph *et al*. (1962) and Brown *et al*. (2004), the Knott's concentration technique which is accompanied by staining of the material with Giemsa, have proved valuable. In recovering microfilariae of *M. perstans*, the membrane filtration techniques could be helpful (Bell, 1967).

Adult worms and microfilariae may however sometimes spread to parts of body organs, even the bone marrow (Molina *et al*., 1999; Bregani *et al*., 2007a), and adult *M. perstans* could be isolated during autopsy and surgery (Baird *et al*., 1987).

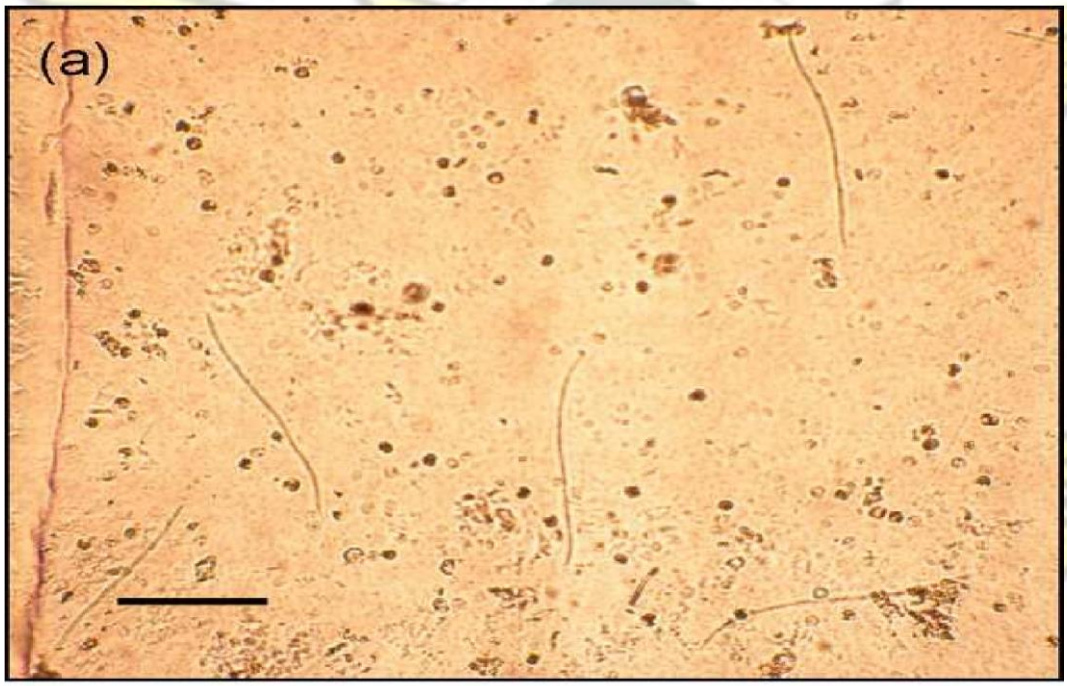

Figure 2.4: Microfilariae of *M. perstans* on counting chamber (Simonsen *et al*., 2011).

2.5 Morbidity and Public Health Importance

Although infections due to *M. perstans* are without distinct clinical manifestation as seen in other infections such as lymphatic filariasis, buruli ulcer etc, there are clinical symptoms suspected to be result from *M. perstans* infection. Infections with *M. perstans*, though usually asymptomatic, could cause abdominal pains, fever, eosinophilia, headaches, subcutaneous swellings and body itches (Bregani *et al*., 2007a). According to Adolph *et al*. (1962), *M. perstans* could induce these manifestations:

- Skin itching with or without rash or ulceration.
- Throbbing in the liver region.
- Acute swelling in hands, forearms or face often recurring, similar to Calabar swellings.
- Pain in bursae and/or joint synovia.
- Psychic and neurological symptoms.
- Pain in serous cavities.
- Extreme exhaustion

Studies which emphasize on the potential relationship between filarial infections (including *M. perstans*) and malaria, HIV and tuberculosis (Brown *et al*., 2003, 2004, 2006; Hillier *et al*., 2008), and their connection to anaemia (Muhangi *et al*., 2007) and atopic sensitization (Mpairwe *et al*., 2008), are imperative direction in providing reasonable information of the public health importance as well as giving a much reasonable picture of the clinical significance of *M. perstans* infections.

2.6 Management and Treatment of *Mansonella perstans* Infection

The treatment of *M. perstans* infection remains uncertain since an effective chemotherapeutic intervention is still lacking (Bregani *et al*., 2006). Clinical trials investigating

antifilarial drugs including ivermectin and DEC, and also benzimidazoles such as thiabendazole, albendazole, mebendazole and levamisole have been performed. According to Bregani *et al*. (2006), the use of drugs such as ivermectin, praziquantel and DEC are generally ineffective while mebendazole and thiabendazole show greater reduction in microfilariae.

Reports by some researchers indicate albendazole had no remarkable impact on *M. perstans* microfilariae appraisal when given as few single measurements (Van Enden *et al*., 1992; Asio *et al.*, 2009b, 2009c), while higher regimens diminished and even dispensed with microfilaraemia (Lipani *et al*., 1997; Duong *et al.*, 1998). A couple of dosages of thiabendazole were additionally active against the microfilariae of *M. perstans*, but extensive use of this medication is limited by its relative dangers (Bregani *et al*., 2003, 2006).

Ivermectin is viewed as a first-line specialist for the treatment of numerous filarial diseases (particularly onchocerciasis). It has indicated next to zero adequacy against M. perstans at a dosage of 200 µg/kg body weight or at a measurement of 600 µg/kg body weight (Bregani *et al*., 2006). A study in Uganda that assessed the impacts of ivermectin, albendazole, and a joined regimen of both medications on *M. perstans*-positive individuals showed that, single measurements of ivermectin alone had no stamped impact on *M. perstans* microfilaraemias in the 12 months after medicines, with the tallies staying to pretreatment values. This is reliable with the discoveries of past studies which have proposed ivermectin, when utilized alone, has almost no impact on *M. perstans* microfilaraemias. A decrease of microfilariae in patients has been noted, however it takes quite a while to accomplish (more than 3 years of administration of ivermectin), and is in this way not helpful in the treatment of symptomatic patients for a short term (Bregani *et al*., 2006)

Diethylcarbamazine (DEC) is the antifilarial drug with suboptimal effect against *M.*

perstans infection. Strohschneider (1953) and Adolph *et al*. (1962) who treated patients in Uganda and expatriates returning in the USA respectively noted that DEC eliminated microfilariae in large number of patients. Furthermore, based on carefully performed study, DEC has been shown to reduce *M. perstans* microfilariae intensities substantially but does not clear them all (Bregani *et al*., 2006). In a 2005 study of 160 patients with symptomatic *M. perstans* infection in South Chad, DEC was administered in 200mg doses, twice daily for 21 days with a gradual dosage increase in the first three days (Bregani *et al*., 2006). The single course of DEC lowered microfilariae in 80% of subjects, but did not get rid of the infection or related symptoms. A second course was therefore administered, and was successful in eliminating the microfilarial burden in most cases. No persistent effect of DEC on microfilariae was noted on long-term follow up. These outcomes correctly represent the overall efficacy of DEC in treating *M. perstans* (Bregani *et al*., 2006).

Mebendazole, another conceivable treatment for *M. perstans* filariasis, has appeared to be powerful in essentially diminishing microfilariae levels. It has been more successful than both ivermectin and DEC with a more prominent number of responders (Bregani *et al*., 2006).

However, trial involving the use of doxycycline has shown significant outcomes which calls for further evaluation in order to produce a consensus optimal approach for the infection (Coulibaly *et al*., 2009).

2.7 *Wolbachia* Endosymbiont in Filariae

Wolbachia is a symbiotic bacteria (order Rickettsiales) present in most arthropods. Most insect species (20-80%) are estimated to be infected with these Gram-negative alphaproteobacteria (Hilgenboecker *et al*., 2008). It can also be found in terrestrial crustaceans

(Bouchon *et al*., 1998) and in many chelicerates (Goodacre *et al*., 2006; Baldo *et al*., 2007). Furthermore, there are many reports on the detection of the bacteria in filariae nematodes (McLaren *et al*., 1975; Kozek & Marroquin, 1977; Kozek, 1977). These were subsequently recognized as *Wolbachia* (Sironi *et al*., 1995; Bandi *et al*., 1998; Casiraghi *et al*., 2004).

Filarial nematodes are mostly infected with an intracellular bacterium, *Wolbachia* (Taylor *et al*., 1999a). In the human filarial nematode *Brugia malayi*, these bacteria have been detected in all investigated life-cycle stages, individual adult worms and isolates from different endemic regions (Kozek, 1977; Taylor *et al*., 1999b; Taylor *et al*., 2000). The female reproductive apparatus of filariae nematodes contain *Wolbachia* but not in that of males (Hoerauf *et al.*, 2003; Casiraghi *et al.*, 2004; Bain *et al.*, 2008). The bacteria reside intracellularly, within host-derived vacuoles, throughout the syncytial hypodermal cord cells. They are transmitted vertically by adult females and can be detected in the ovarian tissues, oogonia, oocytes and developing embryos within the uterus (Kozek, 1977; Taylor *et al*., 1999b).

Antibiotic clearance of *Wolbachia* from all infected filarial nematodes dramatically inhibits or blocks the development of larval and embryonic stages with longer-term effects on adult worm fertility and viability (Taylor *et al*., 2001; Debrah *et al*., 2006). In *Brugia* species, antibiotic treatment affects larval and adult worm growth, development, motility, moulting and viability, and in adult females blocks embryogenesis and causes embryotoxicity, with subsequent reductions in microfilaraemia (Chirgwin *et al*., 2003). This suggests that the nematodes have evolved to become dependent on the bacteria for a diverse range of biological processes. The evidence for a mutualistic association is supported by phylogenetic studies, which show a long and congruent evolution of bacterium and nematode (Casiraghi *et al*., 2001), and a genome one third smaller than that of insect *Wolbachia* (Sun *et al*., 2001).

How *Wolbachia* contribute to the biological processes of the nematode is an important area of research, both to understand the fundamental nature of the symbiotic relationship and to identify key processes as targets for therapeutic intervention. *Wolbachia* are also an important stimulus of innate inflammation and immune responses associated with disease pathogenesis and adverse reactions to filarial treatment (Cross *et al*., 2001; Punkosdy *et al*., 2003).

2.8 Antibiotic (Doxycycline) Treatment Targeting *Wolbachia* Endosymbiont in Filariae

Some researchers have highlighted the connection between *Wolbachiae* and filariae and have recommended that the *Wolbachia* bacteria may possibly present a novel target for antibiotic-based treatment or a novel anti-symbiotic chemotherapy (Hoerauf *et al*., 2000; McGarry *et al*., 2004; Martin & Gavotte, 2010).

Tetracyclines, including doxycycline, are identified to be effective against Rickettsiae. It inhibits bacterial protein synthesis. Treating *Wolbachia* with tetracyclines has biological effects on filariae: the bacterial depletion obstructs early embryogenesis and female worm development (Hoerauf *et al.*, 2000); it also impedes the last moulting process from larval stage to adults (Casiraghi *et al.*, 2002) and it impairs microfilariae development into L3 (Arumugam *et al.*, 2008). Moreover, antibiotic treatment hinders microfilarial production in *W. bancrofti and O. volvulus* as well as having macrofilaricidal effects (Hoerauf *et al.*, 2001; Hoerauf *et al.*, 2003; Hoerauf *et al.*, 2008; Specht *et al.*, 2008; Mand *et al.*, 2008; Mand *et al.*, 2009). In effect, filarial management with antibiotics results in infertility of worms and impairs larval development and adult worm viability. In filariae that do not contain *Wolbachia*, treatment does not yield any effect (Brouqui *et al.*, 2001). This outcome underscores the relevance of symbiosis on filarial survival.

The main challenges in relation to antibiotic treatment are treatment duration (daily drug administration for 4-6 weeks) and the precautions necessary for their use: these drugs are contraindicated in people with hepatic and renal impairment, systematic lupus erythematosus (SLE), pregnant women, lactating mothers and children under eight years of age (Goe *et al*., 2004). Such precautions make doxycycline unsuitable for mass drug administration (MDA). However, its use is of particular interest in individual treatment (IDA) for loiasis where onchocerciasisis also endemic (Hoerauf, 2008), because the MDA of antifilarial drugs such as ivermectin for onchocerciasis is associated with serious adverse events in co-endemic area with loiasis (WHO, 2009; Boussinesq, 2008).

2.9 Adverse Events of Doxycycline

Doxycycline side effects usually include: vomiting, nausea, upset stomach, mild diarrhea, skin rash or itching (Goe *et al*., 2004). Patients are usually advised to take in food before ingesting doxycycline, in order to curtail most of these effects.

In most clinical trial involving the use of doxycycline, adverse reactions are transient and do not involve life threatening situations (Hoerauf *et al*., 2001, 2008 and 2009). Coulibaly *et al*. (2009), in their randomized clinical trial with doxycycline observed mild adverse events such as headache, diarrhea and respiratory symptoms in the doxycycline group. Adverse events resolved spontaneously in all subjects without any serious effects. Hoerauf *et al*. (2009) made similar observations during 5-weeks treatment of patients with doxycycline against adult *Onchocerca volvulus.*

2.10 Mode of Action of Doxycycline

Doxycycline (tetracycline) being bacteriostatic, enter gram-negative bacteria via the porin channels using the process of passive diffusion while for gram-positive bacteria, energy-dependent active transport process is required (Goe *et al.,* 2004). It is intracellularly concentrated in vulnerable cells. Doxycycline binds reversibly to 30S ribosomal subunit of bacteria after entering the cell (Goe *et al.,* 2004). This bond prevents the attachment of aminoacyl-tRNA to the acceptor site on the ribosomal-mRNA complex. This inhibits the process of amino acids addition to the emerging peptide chain.

In the mammalian cells, doxycycline do not bind to the 60S or 30S ribosomes and also the carrier molecules required for active transport of doxycycline is not present. Hence, these are the two reasons that account for the selective toxicity of doxycycline to the microbes (Goe *et al.,* 2004).

CHAPTER THREE

METHODOLOGY

3.1 Study area

The study was conducted in selected communities of the Asante Akim North District of Ghana namely: Afrisere, Dukusen, Nhyieso, Bebuso, Anokye-Beemu, Ananekrom, Serebouso and Abutantri. These communities were selected based on the initial observation by Richard Phillips *et al*., 2014 during Buruli ulcer research. Microfilariae of *M. perstans* were identified from the blood of Buruli ulcer patients hailing from these communities. The district lies in the tropical rain forest although there are some transitional zones due to farming and logging activities. The climate is tropical; temperatures vary from 20 °C to 36 °C with monthly rainfall varying from 2.0 mm in February to 400 mm in July. There are two rainy seasons; a major one extending from April to August and a minor one from October to November. The local economy is based on cash crops like cocoa, coffee and oil palm, although subsistence farming is the main

occupation. This district is divided into four sub-districts and has a population of about 126,000 people.

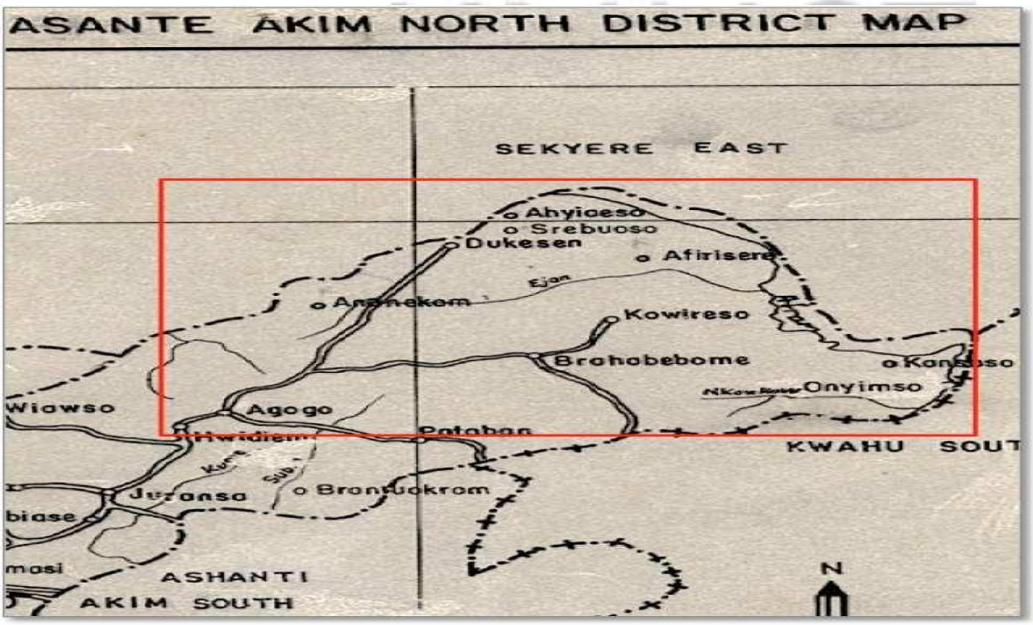

Figure 3. 1: A map showing the study communities in red outline.

3.2 Ethical Approval

The study was granted permit by the Committee on Human Research, Publication, and Ethics of the School of Medical Sciences (SMS) of the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi (Ref: CHRPE/AP/433/13).

Additional permission was sought from the District Health Directorate of the district. Chiefs, opinion leaders and inhabitants of participating communities were also consulted.

3.3 Study Design

The study was an open randomized control pilot trial. Through computerized randomization, eligible individuals were assigned to either Early or Delayed treatment group. However, participants who were not available during treatment served as controls. At the onset (January, 2015), the Early Group contacts received 200mg of doxycycline daily for 6 weeks while the Delayed Group received 100mg of Vitamin C (placebo) daily for 30 days. After 6 months (July, 2015), the Delayed Group were given 200mg of doxycycline daily for 6 weeks. Medication was administered by Daily Observed Treatment (DOT). The research team at the nearest health centre and the trial clinician carried out the daily distribution of drugs during the treatment periods. All medical complaints were recorded on Case Report Form and addressed appropriately. For the women, pregnancy test was carried out before treatment and every 14 days during treatment. Blood samples were taken from study participants at pretreatment, 4 months and 12 months during the study period.

3.3.1 Inclusion criteria for selecting participants for the trial.

Participants of both sexes with the following characteristics were considered eligible;

- *M. perstans* mf-positive status.
- Good general health without any clinical condition requiring long-term medication.
- Body weight > 40 kg.
- Age > 12 years.
- Normal renal laboratory profiles (creatinine and urea).
- Normal hepatic laboratory profiles namely: Gamma glutamyl transferase(GGT), Alanine aminotransferase (ALT), and Aspartate aminotransferase (AST).
- Willingness to participate in the study by signing the ICF.

3.3.2 Exclusion Criteria

- Known intolerance to doxycycline.
- Pregnancy (pregnancy tests were carried out at pre-treatment and on 14 days after treatment onset).
- History of severe allergic reaction or anaphylaxis.
- Alcohol or drug abuse.
- Evidence of clinically significant neurological, cardiac, pulmonary, hepatic, metabolic, rheumatologic or renal disease as far as can be assessed by history of participants, physical examination, and/or laboratory examinations.
- Behavioural, cognitive or psychiatric disease that in the opinion of the trial clinician affects the ability of the participant to understand and cooperate with the study protocol.
- Severe asthma (emergency room visit or hospitalization).
- Participation in other drug trials while this study was ongoing.
- Laboratory evidence of liver disease (ALT, µGT greater than 1.5 times the upper limit of normal results as stated by the manufacturer of dipstick tests, CHEMISTRY;

  ALT: (0 – 75 U/L), µGT: (♀: 0 – 69.5 U/L; ♂: 0 – 80.7 U/ L)
- Laboratory evidence of renal disease (serum creatinine greater than 1.2 times the upper limit of normal results as stated by the manufacturer of dipstick tests, CHEM7â; Creatinine: (0 – 1.8 mg/dL)).

- Any other condition that, in the opinion of the investigator (trial clinician), would risk the safety or rights of a participant in the trial or would render the subject unable to comply with the protocol.

3.3.3 Subject withdrawal criteria

A subject was withdrawn from the study if any of the following was observed:

- Appearance of serious or unexpected adverse events such as bloody diarrhoea, where the continuation of medication poses a serious risk to the participant, as assessed by the trial clinician.
- Repeated non-compliance: (e.g. due to travelling) for more than 3 consecutive days. When a participant was absent from daily treatment of the study drug, an extra dose at the end of treatment was given.
- At the request of the patient (withdrawal of consent for any reason), sponsor or ethics committee.

3.4 Study Procedure

3.4.1 Screening of Participants

Participants were informed in detail about the study as shown in Plate 1, following the informed consent procedures according to Good Clinical Practice (GCP). Individuals willing to participate after providing written informed consent (sign or thumb print on the Informed Consent Form (ICF)) were clinically examined (Plate 2). Participants meeting inclusion criteria were enrolled into the study. Referrals for follow up examinations were provided.

Plate 1: Details of the study being explained to participants

Plate 2: Informed Consent being explained to a participant.

3.4.2 Screening Techniques

3.4.2.1 Finger prick test

In the communities, the middle finger of the patient's left hand was wiped and massaged simultaneously to ensure adequate blood flow to the tip of the finger. Using skin disinfectant, the tip of the finger was cleaned and pricked with a sterile lancet. About 20µl of blood was pipetted and applied on clean-labelled microscope slide. Cover slip was placed over the blood on the slide and observed under X10 objective lens of the light microscope. The presence of microfilariae confirms patient's positivity.

About 10ml of venous blood was then taken from all mf-positive participants to the laboratory.

3.4.2.2 Sedgewick Technique

In the laboratory, about 50µl of blood was pipetted into 1.5ml Eppendorf® tube containing 950µl of 3% acetic acid and mixed thoroughly. The solution was poured into a Sedgewick counting chamber ensuring no air bubbles in the chamber. The fluid was then observed under light microscope using the X10 objective lens as shown in Plate 3. The microfilariae were counted and expressed as mf/ml of blood.

Plate 3: Examination of microfilariae using light Microscope

3.5 Determination of Microfilarial load

Using syringe, 1ml of blood was filtered through 3.0µm pore-size membrane placed on a holder. After washing with 4ml of distilled water, 3ml of methanol was applied to ensure fixation. The membrane was removed and placed on a labeled slide and allowed to dry before staining with 1:20 dilution of Giemsa. The Giemsa stained slide was then examined under X20 objective lens of the light microscope. The microfilariae numbers were recorded with a tally counter and expressed as mf/ml of blood.

3.6 Assessment of Renal and Hepatic Profiles of Study Participants

Clinical biochemistry tests were performed to assess volunteers' kidney and liver functions. About 10mls of blood were collected from each volunteer and then centrifuged to separate plasma from blood cells. About 1ml of each volunteer's plasma was pipetted into a 1.8 ml Eppendorf® tube bearing the volunteers identification numbers, which were used to determine kidney and liver enzymes levels of study volunteers. Liver transaminases aspartate aminotransferase (AST), alanine aminotransferase(ALT), glutamyltransferase (GT) and creatinine were the enzymes that were checked to monitor the liver and kidney functions of study volunteers.

3.7 Molecular identification of *Mansonella perstans* using duplex qPCR

DNA from blood was extracted using QIAamp® DNA kit. About 0.2ml of blood was added to 1.5ml microcentrifuge containing 20ul of protease. Then 0.2ml of buffer AL was added and mixed thoroughly. It was incubated at 56^{o}C for 10 minutes, after which 0.2ml of absolute ethanol was added to the mixture and vortexed for 15 seconds. About

0.65ml of the mixture was transferred into a separate tube and spun. Then 0.6ml each of buffer AW1 and AW2 was added and spun sequentially with filtrates discarded. Afterwards, about 50ul of buffer AE was added to elute DNA from the sample.

The primers used for the PCR were Mp ITS1(GenBank: KJ631373). This was designed to be specific for *Mansonella perstans* and consisted of a forward primer: 5′GGTGATATTCGTTGGTGTCTAT-3′ , a reverse primer, 5′ AGCTATCGCTTTATCTTCATCA-3′, and a probe, FAM TCCAAATTATCGCCTAAACCGTCGA-BHQ. Murine IFN-gamma, inhibition control (GenBank: AC_000032.1), forward primer (5′TCAAGTGGCATAGATCTGGAAGAA3′), a reverse primer (5′-TGGCTCTGCAGGATTTTCATG3′) and a probe (5'HEXTCACCATCCTTTTGCCAGTTCCTCCAG3'BHQ1). Detailed constituent of the master-mix preparation has been shown in Table 3.1.

Table: 3.1 *M. perstans* PCR reaction Mixture

Reagent	Volume per sample(µl)	Final conc.
2X QuantiNova Buffer	10	
MpITS1 FW (10 µM)	1	500 nM
MpITS1 RV (10 µM)	1	500 nM
mIFN-g FW (10 µM)	0.8	400 nM
mIFN-g RV (10 µM)	0.8	400 nM
10 µM MpITS1 Probe (green)	0.1	50 nM
10 µM mIFN-g Probe (yellow)	0.2	100 nM
mIFN plasmid DNA 10E-6	1	10E-6
PCR water	3.1	
DNA	2	
Total volume	20	

Amplifications were performed using thermal cycler Corbett Rotor-Gene 6000 realtime PCR machine connected to a computer. The PCR consisted of an initial heating phase at 95°C for 5 min, followed by 35 cycles of denaturation (95°C for 10 seconds) and annealing (62°C for 10 seconds) as shown in Table 3.2. All amplifications were performed in a total reaction volume of 20uL containing 18uL master mix and 2uL DNA template. A negative control was used to detect possible contamination while inhibition control was used to ensure that the amplification conditions were appropriate.

Table: 3.2 PCR Cycling Condition

Cycling Program	Condition
Step1: Taq activation	95 °C for 5 min
Step2: 35 cycles	95 °C for 10 sec 62 °C for 10 sec

3.8 Data Analyses

The raw data were entered onto spreadsheet Microsoft Excel® 2007. Statistical analyses were conducted with Microsoft Excel® 2007 and Graphpad Prizm® 6.01. Descriptive statistics were used to obtain general descriptive information such as the mean and standard deviation from the data. The microfilarial loads were expressed in geometric means. Paired *t*-test was used to compare two qualitative proportions or groups. For nonparametric data set, comparisons of two population means were done using Mann-Whitney U test. P-values of less than 0.05 ($p<0.05$) were considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Characteristics of Study Participants

A total of 1,229 individuals from eight villages were screened for this study. From Table 4.1 below, 49.2% were males with mean age of 27.8 years while 50.8% were females with a mean age of 27.8 years. There was no significant difference between the ages for both sexes (p=0.401). The overall mean age was 27.8 years.

Table 4.1: Characteristics of study participants

Study participants	Number of participants (%)	Age (mean±SD)/ years
Male	605 (49.2)	27.8 ± 14.62
Female	624 (50.8)	27.8 ± 14.61
Total	1229	27.8 ± 14.62

4.2 Microfilaria Prevalence of Study Communities

From the eight communities, Anokye-Beemu recorded the highest prevalence (71.4%) with more than half of its members living with the microfilaria. The second highest was the Abutantri community with prevalence of 53.0%. Dukusen recorded 39.3%, while Afrisere and Bebuso recorded 37.8% and 14.0% respectively. Ananekrom being a periurban centre had the least prevalence of 1.7%. The overall prevalence for all the communities was 33.0% as shown in Table 4.2.

Table 4.2: Microfilariae Prevalence of the study Communities

Community	Total number examined	Number of mf Positive	Prevalence (%)
Abutantri	132	70	53.0
Afrisere	164	62	37.8
Ananekrom	119	2	1.7
Bebuso	93	13	14.0
Anokye Beemu	210	150	71.4
Dukusen	117	46	39.3
Nhyiaeso	195	24	12.3
Serebuoso	199	38	19.1
Total	**1229**	**405**	**33.0**

4.3 Prevalence of Microfilariae according to Age group and Gender

As shown in Table: 4.3 below, age group 18-30years, comprising 52.5% males and 47.5% females, recorded the highest prevalence (29.1%) while 61years and above group had 3.21%, the least prevalence, with 76.9% males and 23.1% females. Age group 31-45years recorded 28.4%. Children (9-17years), had prevalence of 26.4%. A prevalence of 12.8% was recorded in age group 46-60 years comprising 63.5% males and 36.5% females (Table 4.3). Although the infection was higher in males (57.0%) than in females (43.0%), the difference was not statistically significant (p=0.0568).

Table 4.3: Prevalence of Microfilariae according to Age group and Gender

Age Group	Total	Male (%)	Female (%)	Overall Prevalence (%)
9 – 17	107	67 (62.6)	40 (37.4)	26.4
18 – 30	118	62 (52.5)	56 (47.5)	29.1
31 – 45	115	59 (51.3)	56 (48.7)	28.4
46 – 60	52	33 (63.5)	19 (36.5)	12.8
61 and Above	13	10 (76.9)	3 (23.1)	3.2
Total	**405**	**231 (57.0)**	**174 (43.0)**	**100**

(Paired t-test for males against females, $p = 0.0568$)

4.4 Trial Profile of Participants

Out of 1229 individuals screened for this study, 102 individuals from 5 communities namely: Afrisere, Nhyiaeso, Serebouso, Abutantri, and Dukusen were enrolld on the trial. Based on their initial microfilarial load, they were randomized into either Early (50) or Delayed (47) treatment groups. However, five participants who were not available during treatment served as controls. Blood samples were taken during the follow-up periods. Only individuals who were available at all the study time points were included in the analysis as shown in Figure 4.4.

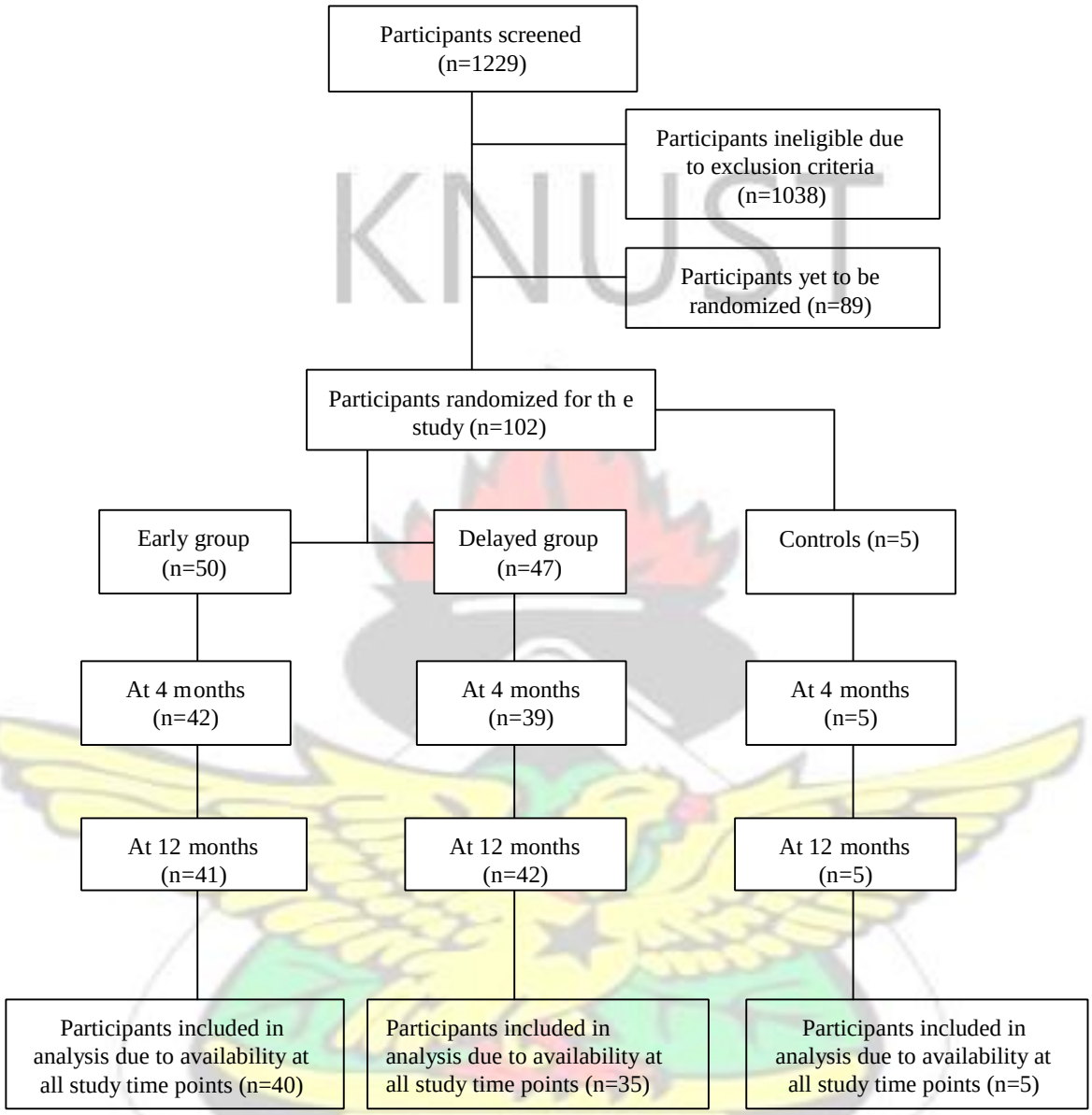

Figure 4.4: Trial Profile of Participants

4.5 Assessment of Microfilarial load among Treatment (Early and Delayed) Group at various time points

At pretreatment level, microfilarial load of 221mf/ml and 159mf/ml were observed among the Early and Delayed Groups respectively, and the difference was not statistically significant

(p=0.7655). At the 4th month, the microfilarial load among the Early and Delayed Group had reduced to 76 and 85 mf/ml respectively. However, there was no significant difference (p=0.5559) between them at the 4th month follow up time point. At 12th month, a significant difference (p<0.0001) was observed between the two groups. Microfilarial load for the Early Group was 2mf/ml while 39mf/ml was recorded for Delayed Group as shown in Figure 4.5.

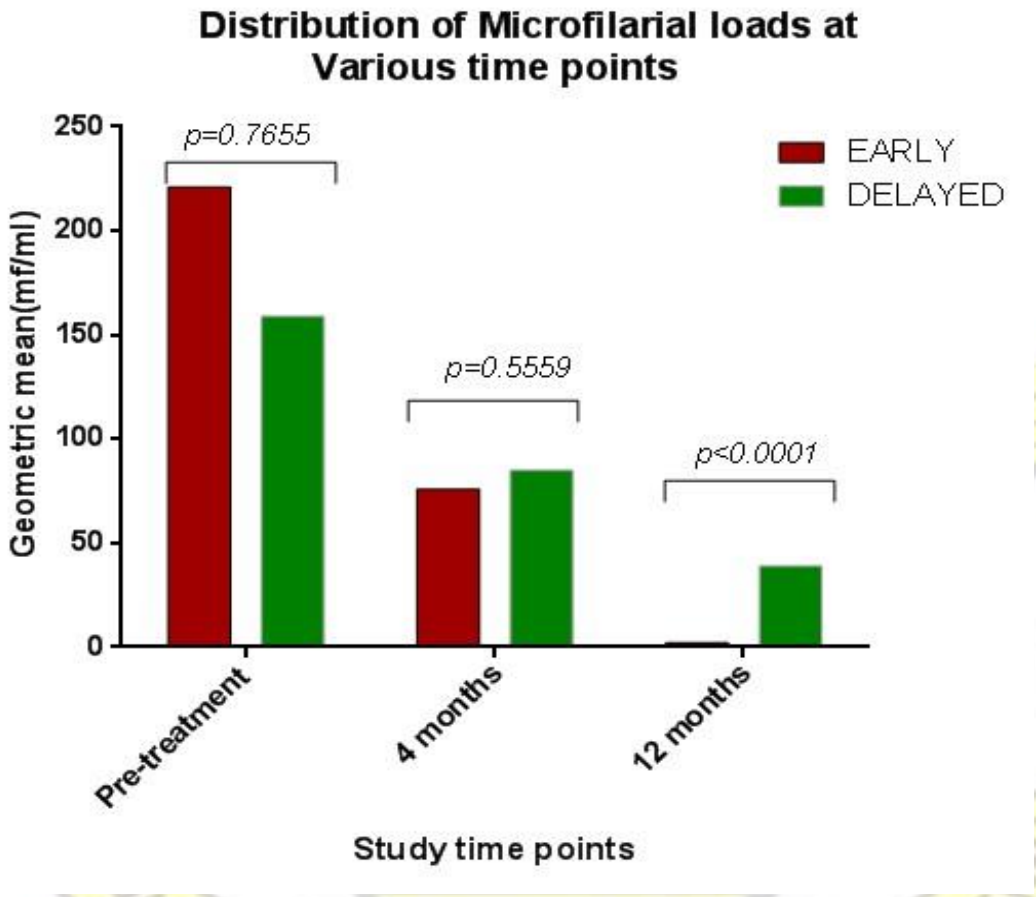

Figure 4.5: Microfilarial load among Early and Delayed Group at different time points

4.6 Comparison of Microfilarial load among Early and Control Groups at various time points

Although there was difference between the geometric means of microfilarial load for the Early and Controls at the baseline level, it was not statistically significant (p=0.2693). At the 4thmonth, no difference was seen between the two populations (p=0.6154). However, significant difference (p=0.0001) was observed at the 12th month as can be seen in Figure 4.6.

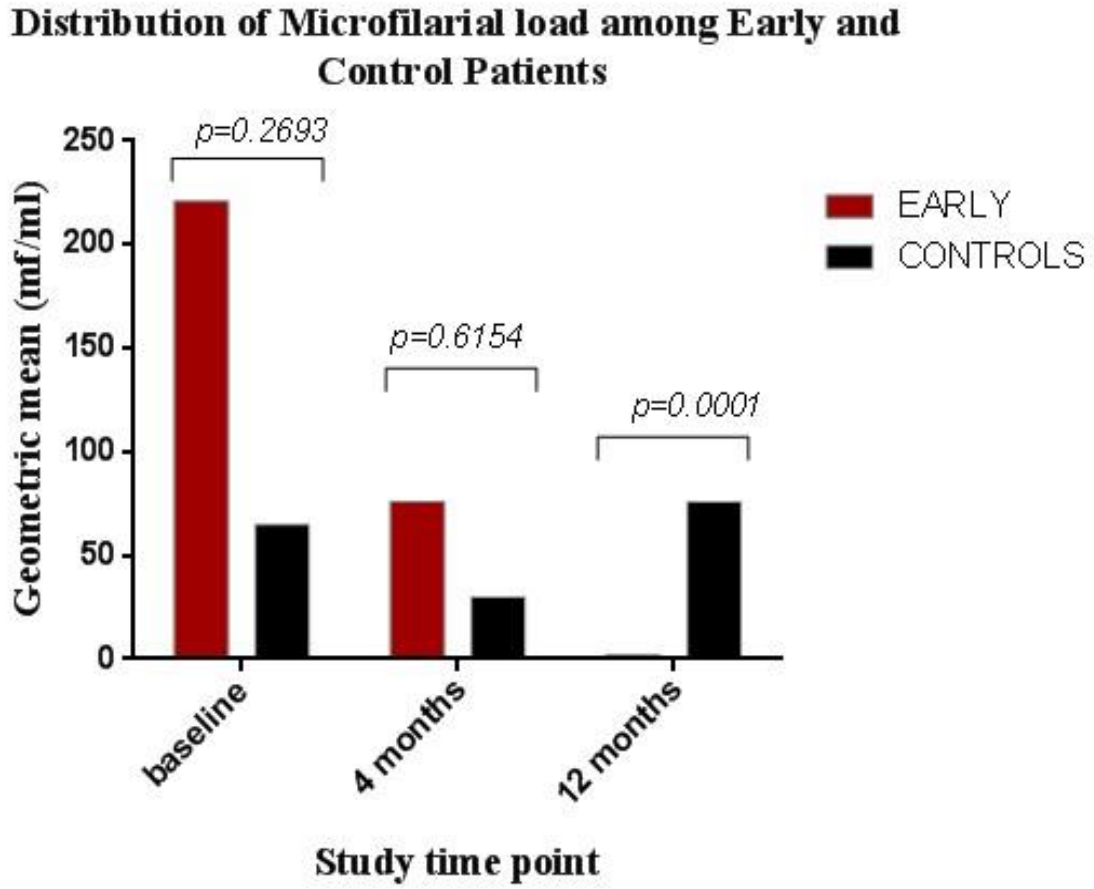

Figure 4.6: Distribution of Microfilarial load among Early and Controls at different time points

4.7 Comparison of Microfilarial load between Delayed and Control Groups at various time points

The difference between the microfilarial load of the Delayed and Controls at the baseline level, was not statistically significant (p=0.4106). At the 4th month, no difference was seen between the two populations (p=0.5193). Furthermore, there was no significant difference at 12th month (p=0.5378) as shown in Figure 4.7.

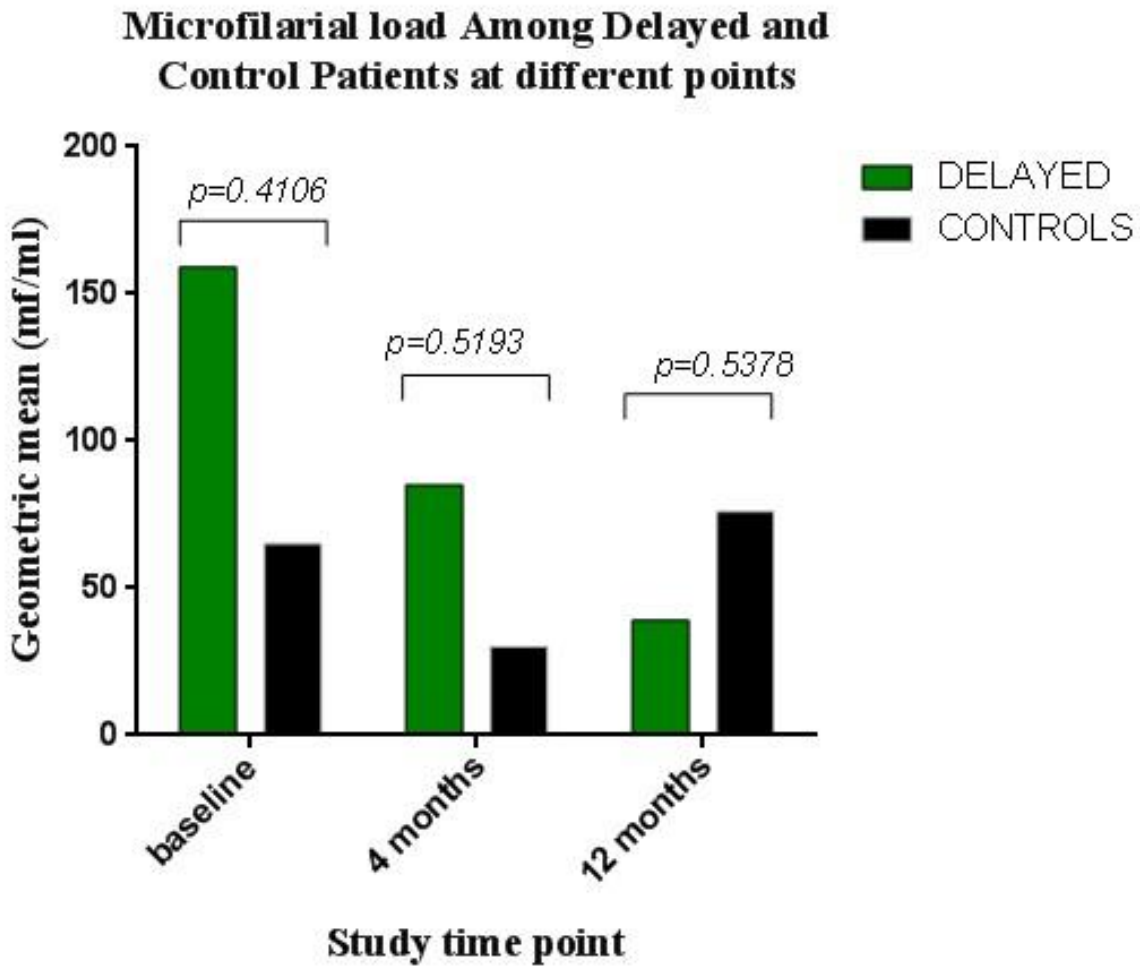

Figure 4.7: Distribution of Microfilarial load among Delayed and Controls at different time points.

4.8 Adverse Events Reported by the Participants

Few participants from both treatment groups upon receiving doxycycline reported the following symptoms: dizziness, nausea, headache, diarrhea, abdominal pain and general body

weakness as shown in Table 4.8. These are common experiences found in most trials involving doxycycline**.** However, when participants were reminded again to eat before taking the drug, these minor adverse events stopped.

Table 4.8: Adverse Events of Reported by the Participants

Events	Frequency (n)
Dizziness	2
Nausea	2
Headache	1
General Body Weakness	2
Diarrhea	2
Abdominal Pain	1
Total	10

4.9 Prevalence of Other Tissue Parasites

As part of this study, co-infection with other tissue parasites were also studied (Table 4.9). All the 102 trial participants were examined for other blood parasites namely; *Wuchereria bancrofti* and *Plasmodium sp*. *W. bancrofti* recorded prevalence of 5.9% while *Plasmodium sp* recorded 16.7% prevalence.

Table 4.9: Coinfection of other Tissue Parasites among Study Participants

Community	Number Examined	*Wuchereria bancrofti*	*Plasmodium sp*
Afrisere	15	0	4
Nhyiaeso	11	0	0
Serebuoso	19	0	2
Abutantri	35	4	9
Dukusen	22	2	2
Total	**102**	**6 (5.9%)**	**17 (16.7%)**

4.10 Confirmation of *M. perstans* using real-time Polymerase Chain Reaction

Thirty-nine blood samples which were microscopically positive for *M. perstans* were randomly selected for qPCR analysis. They were all positive for the qPCR analysis.

Furthermore, 3 microscopically negative blood samples also confirmed negative with qPCR.

CHAPTER FIVE

DISCUSSION

5.1 Level of Endemicity of *Mansonella perstans* infection among Study Communities

According to surveys conducted, *Mansonella perstans* infection is widely distributed across Sub-Saharan Africa, of which Ghana is no exception (Simonsen *et al*., 2011). Knowledge of prevalence is one of the important approaches towards understanding the epidemiology of the infections. In view of this, the criterion for categorizing endemicity according to the African Programme for Onchocerciasis Control (APOC, 2010) was employed. Based on microfilaria prevalence, a community can be termed hypoendemic, mesoendemic or hyperendemic with ≤ 20%, 21%-59% and ≥ 60% prevalence respectively. The hypoendemic communities identified in this study were Serebuoso, Nhyiaeso, Bebuso and Ananekrom, having prevalence of 19.1%, 12.3%, 14.0% and 1.7% respectively. Most of the individuals from these communities were engaged in tree planting projects in which the use of personal protective equipment such as nose mask, safety clothes and boots was a prerequisite. Low exposure to the biting midges (*Culicoides spp*) might have contributed to the low prevalence recorded. Ananekrom being a peri-urban centre recorded the least prevalence of 1.7%. This supports the claim that the infection is more localized in the rural centres than urbans (Oyerinde *et al*., 1988; Anosike *et al.*, 2005). Communities identified to be mesoendemic were Dukusen, Afrisere and Abutantri. Crop cultivation being the main occupation coupled with presence of the Afram River may be the main risk factors leading to the transmission of the infection. Where there is extensive cultivation of banana resulting in decomposing of banana stems, some vector species breed in large numbers (Linley *et al*., 1983). The swampy environment created by the Afram River also served as breeding and development site for some vectors. The only hyperendemic community identified was Anokye-Beemu which lies in the interior part of the district. This is a densely forested site. Farming and hunting of animals were the source of livelihood. Their regular interaction with the environment predisposed them to the bites of the vectors transmitting the infection. High prevalence often occurs in densely forested areas (Anosike *et al*., 1992). An

overall prevalence of 33.0% for all the study communities (Table 4.2) was indicative of a zone of mesoendemicity.

5.2 Distribution of Microfilaria according to Gender and Age-Group level

In this study, 57.0% and 43.0% of male and female volunteers, respectively, had microfilaria. The result showed that the prevalence of microfilaria was higher in males than in females but there was no significant difference between both genders (Table 4.3). Patterns of the infection have been shown to occur more frequently in males than females (Kershaw *et al.*, 1953; Kershaw and Nicholas, 1954; Jordan, 1955a; Gryseels *et al.*, 1985; Arene and Atu, 1986; Noireau *et al*., 1989). Almost all the male participants were continually engaged in farming irrespective of their age and may explain for the higher prevalence.

The reason for the lack of significant difference between males and females in the study was that, both genders are equally involved in farming and hence the levels of exposure to the *Culicoide*s bites were similar.

Microfilaria prevalence levels among age groups 9-17, 18-30 and 31-45 years were not significantly different (Table 4.3). These are the youthful and active group with high energy level and ability to work, and hence, exposure to vector bites. There was a sharp decline from age-group 46-60 years to 61 and above. Although these groups possess little energy, males were dominant. The reason for this observation was that males continually engaged in active farming activity whiles in the females there was a reduction in active farming. Furthermore, immunological studies by Sahu *et al.* (2008) suggest IgA which seems to be more produced in females compared to male plays a protective role in filarial infections.

5.3 Effect of Doxycycline on *Mansonella perstans*

Chemotherapeutic interventions against *M. perstans* have proven futile. The use of anti-helminthic drugs such as ivermectin, praziquantel, DEC and mebendazole, could not yield any reliable results (Bregani *et al*., 2006).

The success story behind application of doxycycline in the fight against most filarial infections such as onchocerciasis, lymphatic filariasis and loiasis has paved the way for further studies on other filarial worms. *Wolbachia* endosymbiotic relationship with nematodes is the principal factor determining the success in most trials involving doxycycline treatment.

5.3.1 Assessment of Microfilarial load at baseline level

Determination of microfilarial load before treatment is imperative in trials since it exposes any variations after the introduction of an intervention. The study recorded the highest quantity of microfilariae among participants at this level, due to their active involvement in daily duties predisposing them to the bites of insect vectors. Moreover, this could be attributed to the fact that none of the participants at this stage had received any medical intervention. This was confirmed from the knowledge of general medical history gathered from the participants. There was no significant difference (p=0.7655) in microfilarial load for the Early and the Delayed groups (Figure 4.5). Similar trend was observed in the comparison between the Early and Controls as well as Delayed and Controls in Figure 4.6 and Figure 4.7 respectively.

5.3.2 Assessment of Microfilarial load at 4 months

It has been documented that complete depletion of *Wolbachiae* endosymboints which was the basis of the doxycycline treatment for this study, occurs at 4 months after doxycycline treatment in onchocerciasis (Hoerauf *et al*., 2001, 2008) and lymphatic filariasis (Taylor *et al.,* 2005; Debrah *et al*., 2007), which consequently lead to microfilariae reduction. At the 4th month after treatment for the Early Group, there was no significant difference (p=0.5559) between the microfilariae load of the Early and the Delayed groups (Figure 4.5). Similar trend was observed in the comparison between the Early and the Controls (Figure 4.6). This was due to the fact that doxycycline has been shown to have long-term effect on filarial nematodes (Debrah *et al*., 2006). No significant difference (p=0.5193) between the Delayed and the Controls in Figure 4.7 was observed because of lack of medical intervention.

There were some reductions in the means of microfilariae among the Early and Delayed Group at the 4th month (Figure 4.5). The following reasons could account for the reduction: microfilariae of *M. perstans* circulating in peripheral blood, can survive for approximately 4 months if not ingested by insect vectors during blood meal (Asio *et al*., 2009a). This could mean that some of the microfilariae that had not been ingested by insect vectors to undergo further development into L3 larvae had died by 4 months. Also, the body's immune system could elicit responses to the infection. Antibodies and white blood cells such as eosinophils and neutrophils play protective role against filarial antigens (Sahu *et al.,* 2008). However, the effect of doxycycline on the Early Group could also be influential on the microfilariae level. Participants in the Delayed Group might have experienced the placebo effect after taking vitamin C. In some clinical trials, amelioration due to placebo has been linked to psychological and neurobiological mechanisms (Benson and Freidman, 1996; Goebel *et al*., 2002; Benedetti, 2009).

5.3.3 Assessment of Microfilarial load at 12 months

In most doxycycline trials, the depletion of microfilariae is seen at 12 months after treatment (Debrah *et al*., 2006; Hoerauf *et al*., 2001, 2008). This is directly in line with the outcome of this study. At the 12th month, the mean microfilarial load of the Early Group had significantly dropped to 2mf/ml. Furthermore; there was significant difference ($p<0.0001$) between the Early and Delayed Groups (Figure 4.5). Also comparison between the Early and Controls recorded significant difference ($p=0.001$) in Figure 4.6. The activity of doxycycline includes; retardation of embryogenesis of worms, permanent sterility of worms and filaricidal effect on adult worms (Hoerauf *et al.*, 2003; Hoerauf *et al.*, 2008; Mand *et al.*, 2009; Specht *et al.*, 2008). In 2009, Hoerauf and his colleagues on administering 100mg of doxycycline daily to onchocerciasis patients for 5 weeks resulted in depletion of *Wolbachia* and a complete interruption of embryogenesis in all worms that were assumed to have been present during treatment (Hoerauf *et al*., 2009). This is also consistent with results obtained by Coulibaly *et al*., (2009) who discovered that at 12 months, 67 of 69 subjects representing 97% who had received treatment with 200mg doxycycline daily for 6 weeks had no detectable *M. perstans* microfilariae in blood. Similarly, 100mg of doxycycline daily for 6 weeks in *Brugia malayi* infection showed 77% reduction at 12 months (Supali *et al*., 2008).

Unlike the Early Group, the 12th month of the study time point represents 6 months after treatment for the Delayed Group. Six months after doxycycline treatment usually does not lead to significant microfilariae depletion and hence there was no significant difference ($p=0.5378$) between the Delayed and the Controls as shown in Figure 4.7.

The microfilarial load among the controls was relatively constant throughout the study time points with no significant differences. This could be attributed to regular bites from the insect vectors.

5.4 Adverse Event of Doxycycline

Doxycycline was well-tolerated among study participants. In most trials involving the use of doxycycline, adverse reactions are transient and do not involve life threatening situations. This study observed mild adverse events such as headache, dizziness, nausea, abdominal pain, general body weakness and diarrhea (Table 4.8). Most of these cases evolved from lack of food ingestion before taking drug. Adverse events resolved spontaneously in all subjects, when they were advised to eat before taking the drug. No serious adverse events were reported. Similar observation was made by Hoerauf *et al*. (2009), during 5-weeks treatment of patients with doxycycline against adult *Onchocerca volvulus.*

5.5 Prevalence of Coinfections

This study recorded 16.7% *Plasmodium sp* and 5.9% *Wuchereria bancrofti* infections (Table 4.9) in the studied population. Infections by both *W. bancrofti* and *P. falciparum* could result in significant morbidity and mortality, making them priorities for elimination and control programmes (Zagaria & Savioli, 2002; WHO, 2005).

M. perstans coinfection with these parasites could be detrimental to the health of the host, since *M. perstans* has been shown to polarize immunity against Malaria (Metenou *et al*., 2009). In 2010, Wammes *et al*, found that children with current helminth infestations responded poorly to vaccination (BCG) independent of the type of worm infection.

5.6 Confirmation of *M. perstans* using real-time qPCR

The result of the real-time PCR, confirmed that microfilariae from blood of participants were that of *M. perstans.* Furthermore, 3 blood samples that did not have microfilariae during microscopic examinations also could not reveal the presence of *Mansonella* genes by qPCR. Even though PCR is arguably more sensitive in diagnostics, the use of microscopy in the detection of *M. perstans* is also very effective.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Mansonella perstans infection is mesoendemic (33.0% prevalence) in the rural communities of the Asante Akim-North District. Doxycycline given at 200 mg daily for 6 weeks was well-tolerated and effective in depleting *M. perstans* microfilariae in patients providing further evidence that *M. perstans* in the rural communities might harbor endosymbiont *Wolbachia*.

6.2 Recommendations

Based on the outcome of this study, it is recommended that:

I. Further epidemiological surveys should be conducted in various regions to provide information on the prevalence of *M. perstans* infection in Ghana.

II. Public education should be given to the inhabitants of these communities to sensitize them on the need to protect themselves from bites of the insect vectors.

III. Proper placebo-control trial should be done to provide more information on the efficacy of doxycycline on *M. perstans*.

6.3 Limitations of the Study

The study encountered the following limitations;

I. During the early stages of the study, membrane filter of 1.0um pore size was ineffective in filtering the microfilariae and was changed to 3.0um to ensure maximum recovery of microfilariae.

II. Some of the participants were reluctant in allowing blood taking during the follow up periods. This resulted in a reduction in the number of participants during the follow up periods.

III. Relocation of settlement by some participants resulted in a decline in the number participants during the follow ups.

IV. Conflict between Commuters and Fulani herdsmen, which led to loss of lives and properties, posed a dangerous risk which brought the study to a halt shortly and was continued after events subsided.

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