THE ROLE OF DOXYCYCLINE IN THE AMELIORATION OF FILARIAL HYDROCELE

BY

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DEDICATION

I dedicate this work to the glory of God Almighty who has been a very present help in times of need.

ABSTRACT

There is an urgent need for a macrofilaricidal drug that can improve or halt progression of hydrocele pathology in lymphatic filariasis. In addition, new drugs with modes of action different to ivermectin must urgently be developed to serve as backup should ivermectin resistance develop. While diethylcarbamazine has partial macrofilaricidal effects, it does not ameliorate filarial hydrocele and cannot be used in Africa because of co-endemicity with onchocerciasis. The purpose of this study was to determine whether a six-week course of 200mg doxycycline could ameliorate filarial hydrocele and its associated clinical conditions. A randomized, double-blinded, placebo-controlled, clinical trial was conducted with 44 hydrocele patients. Twenty one (21) were treated with 200 mg of doxycycline for 6 weeks whilst 23 received placebo treatment for 6 weeks. Ninety one percent (91%) of the patients completed the full course of treatment. All study participants were given 150 µg/kg ivermectin plus 400mg albendazole 6 months after treatment in accordance with the nationwide mass drug administration programme. Ultrasound examinations of the scrotum were carried out for all patients at pre-treatment and at the 12-months and 24-month follow-ups. Patients were examined for dilation in scrotal lymphatic vessels, hydrocele stage, thickness of the scrotal skin and filaria dance signs. Parasitological examinations for circulating filaria antigens and microfilarial loads were also carried out. At the 12-month follow-up, there were no significant variations in the measured parameters between the doxycycline and placebo-treated patients. After 24 months of treatment, 94% of the doxycycline-treated patients had significant improvement and halt in progression of the dilations in the scrotal lymphatic vessels compared to 46% of placebo-treated patients (p=0.3188). Eighty two (82%) of doxycycline-treated patients had a significant improvement and halt in progression of hydrocele stage compared to 46% of placebo-treated patients (p=0.0349). The improvement was observed only in patients with early stage hydrocele. All the 6 worm nests in the doxycycline-treated patients had been lost after 24 months whilst thickening in the scrotal skin of 47% of doxycycline-treated patients were significantly improved as against 33% in placebo-treated patients (p=0.0120). Circulating filaria antigen levels did not vary significantly between the two groups (p=0.5726). Treatment with doxycycline was well tolerated as the incidence of adverse events was not significantly different between the treatment groups (p=0.3148). The study demonstrated that, in addition to its macrofilaricidal activity, a six-week course of 200mg doxycycline has ameliorative effect on filarial hydrocele. This effect occurred even in patients who had no active infection. Therefore, doxycycline can be used in hydrocele patients in whom the current antifilarial drugs do not provide amelioration.

CHAPTER ONE

1.0 INTRODUCTION

Lymphatic Filariasis is one of the oldest and most debilitating diseases known to humanity (WHO, 2010a). The disease is caused by the filarial worms *Wuchereria bancrofti*, *Brugia malayi* and *B. timori* and is transmitted by mosquitoes. It is endemic in more than 80 countries in tropical and sub-tropical areas of Africa, Asia, the Pacific, the Middle East and the Americas (Fernando *et al.*, 2011). More than one billion people – or one-fifth of the world's population – most of whom are the world's poorest, are at risk (WHO, 2010b). In Ghana, lymphatic filariasis is endemic in 8 out of the 10 regions (except for the Volta and Ashanti Regions), with an at-risk population of 9.9 million (Ghana Health Service, 2008).

Current WHO estimates put more than 120 million infections worldwide, with over 40 million individuals severely incapacitated and disfigured by the disease (WHO, 2010a). An estimated 27 million men have hydrocele and almost 16 million, the majority of whom are women, have lymphoedema or elephantiasis of the leg (WHO, 2004).

Filarial infection can cause a variety of clinical manifestations including recurrent acute attacks and chronic disease (Nutman, 2001). Acute attacks are extremely painful and are accompanied by fever and may result in temporary disability. Chronic manifestations of the disease include lymphoedema and hydrocele which leads to permanent disability. Hydrocele is the most common chronic manifestation of the disease (Eigege *et al.*, 2003) and it may be accompanied by thickening of the spermatic cord and changes in the scrotal skin and subcutaneous tissue that include edema, fibrosis, and oozing of lymph through the skin (WHO, 2002a).

About 69% of all hydroceles can be assigned to be of filarial origin (WHO, 2002b). However, because hydrocele is a pathological condition that can persist for a lifetime in the absence of surgery, whilst specific evidence of filariasis infection may not persist that long, it is difficult to exclude filariasis as an aetiological factor in all cases in endemic areas. The prevalence of other hydroceles in non-endemic areas is considerably low, so unless otherwise proven, all hydroceles in *W. bancrofti* endemic areas are to be considered as of filarial origin (WHO, 2002b). Infection is usually acquired in childhood, but the painful and profoundly disfiguring visible manifestations of the disease occur later in life (WHO, 2002b).

Hydrocele can have a profoundly detrimental effect on the quality of life of affected individuals (WHO, 2007). Besides the physical problems, hydrocele generates psychological, social and economic costs. The impact of the disease is more devastating among affected young men, who will have to live with this debilitating and disfiguring condition throughout their life (WHO, 2010a).

The disease has been considered to be potentially eradicable due to the inefficiency of transmission of the filarial parasites to humans. The goals of the current global lymphatic filariasis elimination program therefore are to (i) reduce microfilaraemia to a level that is too low to sustain transmission of filarial parasites to humans using filaricidal drugs and to (ii) reduce the morbidity associated with chronic filarial disease (WHO, 2010b).

1.1 Problem Statement

The antifilarial drugs used for the current WHO Mass Drug Administration programme, Diethylcarbamazine (DEC)- in Asia, ivermectin and albendazole in Africa, are predominantly active against microfilaria with DEC showing only a partial activity against adult worms (Ottesen *et al.*,

1997; Noroes *et al.*, 1997; Fernando *et al.*, 2011). In Africa, there is an additional drawback that DEC must not be used in many areas because of co-endemicity with onchocerciasis (Ottesen, 2000), due to severe adverse effects on eye-sight after killing *O. volvulus* MF in the eye.

In addition, it is realized that a disability alleviation strategy needs to be evolved for lymphatic filariasis patients who suffer from hydrocele, the burden of which is larger than that due to lymphoedema of the limbs (WHO, 2002b). Currently, the mainstay of hydrocele management is surgery as none of the MDA drugs is effective for treating the condition (Hise *et al.*, 2004).

Moreover, there are concerns over the possible development of resistance to ivermectin by *W. bancrofti*. Previous reports from some parts of Ghana show some evidence of sub-optimal response of *Onchocerca volvulus* parasites to ivermectin in communities receiving 6–18 rounds of treatment (Awadzie *et al.*, 2004a; 2004b; Osei-Atweneboana *et al.*, 2007; 2011). *Onchocerca volvulus* parasites from these communities show genetic changes associated with resistance to ivermectin in other nematodes. Although such resistance has not been reported in *Wuchereria bancrofti* (Fernando *et al.*, 2011), it increases concern over resistance to ivermectin developing in both lymphatic filariasis and Onchocerciasis (Bourguinat *et al.*, 2007; Prichard, 2007). This poses a great challenge for Africa in particular, where ivermectin is the main drug relied upon for the control of both diseases.

Therefore, there is the need to develop additional chemotherapeutic agents that will complement existing drugs towards the eradication of lymphatic filariasis (Molyneux *et al.*, 2003).

1.2 Rationale of the Study

Several attempts have been made to develop alternative therapies and one of the most successful is the novel approach targeting *Wolbachia* - a mutualistic, bacterial endosymbiont of filariae essential for worm development, fertility, survival and a component of inflammatory disease pathogenesis (Taylor *et al*., 2005a).

Hoerauf and colleagues (1998), in a study using *Litomosoides sigmodontis* discovered that tetracycline therapy eliminated *Wolbachia* and resulted in filarial growth retardation and infertility. Later, a pilot study with doxycycline (a tetracycline) administered at 200mg for 6 weeks resulted in depletion of *Wolbachia*, long-term amicrofilaremia, and 80% disappearance of worm nests from scrotal areas of infected men examined by ultrasonography (Hoerauf *et al.*, 2003a). Thus, anti-wolbachial chemotherapy with doxycycline appears to have a higher macrofilaricidal effect (80%) than DEC, which showed 30 - 40% worm nest disappearance, a finding that was interpreted as 30-40% macrofilaricidal effect (Dreyer *et al.*, 1998).

In addition to the anti-parasitic effects, a course of doxycycline treatment prior to anti-filarial treatment can reduce systemic inflammation (Turner *et al*., 2006), reduce markers of lymphangiogenesis and the severity of lymphoedema in lymphatic filariasis (Debrah *et al.*, 2006), a phenomenon potentially linked to the release of *Wolbachia* into the blood and the activation of innate inflammation (Cross *et al*., 2001).

Together, the results of these trials strongly suggest that treatment with doxycycline for a period sufficient to permanently deplete *Wolbachia,* results in long-term worm sterility and sustained reductions in microfilariae and/or the death of adult worms which may lead to the amelioration of lymphatic pathology. The study therefore sought to determine if a six-week treatment with

doxycycline will result in the amelioration of filarial hydrocele in patients with or without an active infection.

1.3 General objective

The aim of the study is to determine if doxycycline can ameliorate or halt the progression of filarial hydrocele.

1.4 Specific objectives

The specific objectives of the study are to:

- Assess dilation in scrotal lymphatic vessels for improvement in hydrocele
- Evaluate the stages of hydrocele in treated patients to monitor the impact of treatment
- Assess the effect of doxycycline on filarial dance signs
- Evaluate antigenemia in hydrocele patients
- Assess microfilaremia in hydrocele patients
- Assess adverse effects after treatment

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Burden of Lymphatic Filariasis

WHO reports that lymphatic filariasis (LF) is the second most common cause of long-term disability after mental illness accounting for the loss of 4.4 million disability adjusted life years (DALYs) in men and over 1.3 million DALYs in women annually (WHO, 2004; 2006). While not explicitly mentioned in the Millennium Development Goals, LF and other neglected tropical diseases are recognized in the report on the Commission for Africa as contributing significantly to the overall African disease burden. LF and other helminthic diseases make infected individuals, particularly women and children, more vulnerable to HIV/AIDS, tuberculosis and malaria (Ottesen *et al.,* 1997; WHO, 1999; Molyneux *et al.,* 2005; 2006).

The chronic manifestations of LF which includes filarial hydrocele can have significant, and often very negative, social impacts (Remme *et al.*, 1993; Bandyopadhyay, 1996; WHO, 2002a). The degree of social disability varies between cultural settings, but the degree of stigmatization appears to be directly correlated with the severity of visible disease (Evans *et al.,* 1993; Mujinja et *al.,* 1997). Gyapong *et al* (2000) suggest that the physical and psychological burden borne by men has a negative impact on their marriage and employment prospects. The extent of male sexual disability as a result of LF has not been extensively studied, but investigators believe that there is a significant "silent burden" (Dreyer *et al.,* 1997). Gyapong and colleagues (2000) found that hydrocele had a significant impact on young men, particularly at a time when they were struggling to establish their sexual identity and their capacity to be reliable economic providers.

However, unwillingness of men to admit to sexual dysfunction may shroud the real extent of this issue (WHO, 2007).

Additionally, affected individuals avoid seeking treatment for fear of drawing attention to their condition (Rauyajin *et al.*, 1995; Mujinja *et al.*, 1997). Meanwhile, failure to treat the disease results in recurrent acute febrile attacks and progressive damage to the lymphatic system. Without access to simple hygiene advice, sufferers are unable to prevent further progression of the outwardly visible complications of LF (Dreyer *et al.*, 1999). As the disease progresses, the individual's capacity to labour, both productively and reproductively, is increasingly hampered (WHO, 2007).

2.2 Epidemiology of Lymphatic Filariasis

Some studies have shown that 69% of all hydroceles could be assigned to be of filarial origin. However, since hydrocele is a pathological condition persisting for a lifetime in the absence of surgery, and since specific evidence of filariasis infection may not persist that long, it is difficult to exclude filariasis as an aetiological factor in all cases in endemic areas. Therefore, unless otherwise proven, all hydroceles in *W. bancrofti* endemic areas are to be considered as of filarial origin (WHO, 2002b).

Lymphatic filariasis (LF) is caused by the filaria nematodes *W. bancrofti*, *B. malayi and B. timori* and transmitted by Mosquitoes. *W. bancrofti is* the most widely distributed filarial parasite among humans with distribution throughout the tropics and subtropics, including Asia and the Pacific Islands, Africa, areas of South America and the Caribbean basin (see Figure 2.0). *W. bancrofti* accounts for 90% of the estimated 120 million cases in more than 80 countries; and *B. malayi* (which has a distribution restricted to southeast Asia, with a minor

foci of the closely related *B. timori* in south eastern Indonesia) causes the remainder (The Carter Centre, 2002; Taylor *et al.*, 2010). Like brugian filariasis, there is both a periodic and sub-periodic form of the parasite. Generally, the sub-periodic form is found only in the Pacific Islands; elsewhere, *W. bancrofti* is nocturnally periodic. The natural vectors are *Culex* mosquitoes in urban settings and anopheline or aedean mosquitoes in rural areas (Nutman, 2001).

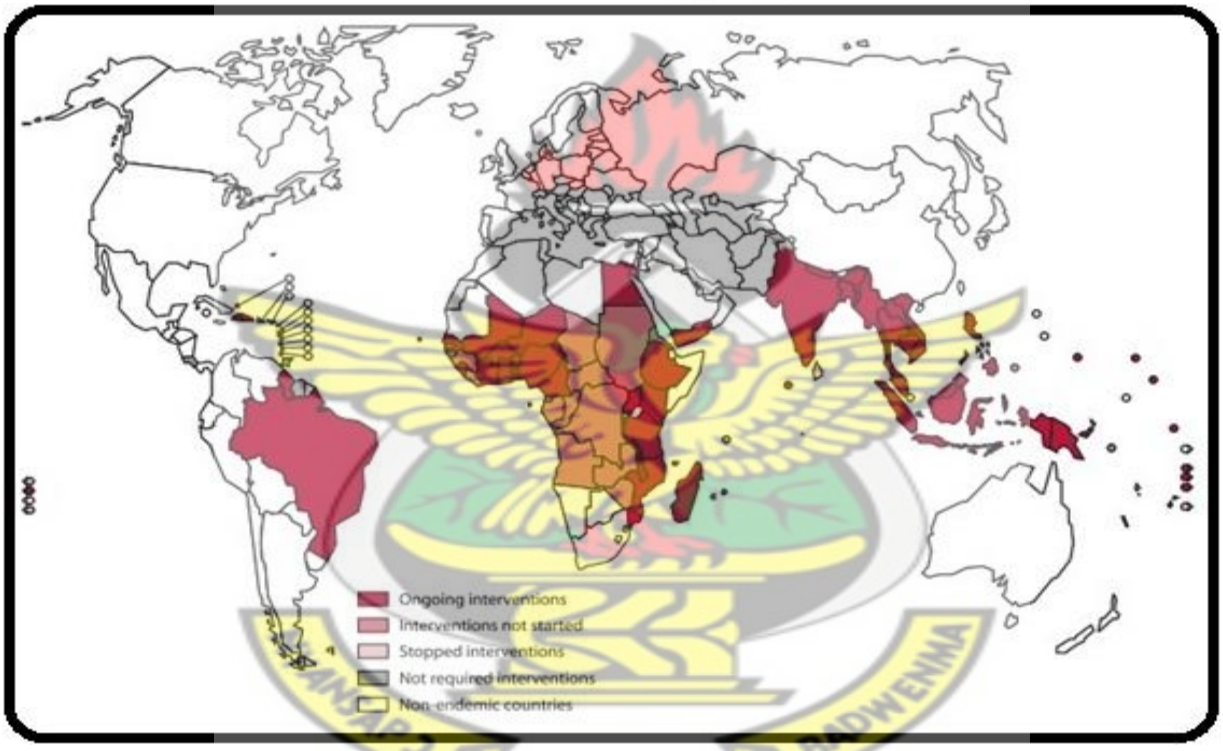


Figure 2.0: Global distribution of lymphatic filariasis and status of Mass Drug Administration (WHO, 2010a).

2.3 Description and Life cycle of *Wuchereria bancrofti*

The lymphatic filariae have similar life cycles in which larval development takes place in the mosquito (intermediate) host and larval and adult development takes place in the

human (definitive) host. Figure 2.1 shows the life cycle of *Wuchereria bancrofti*. Infection is initiated by the deposition of the third-stage larvae (L3) on the skin of the human host following a bite by an infective mosquito. The larvae enter the body through the skin and undergo an additional molt to the fourth larval stage (L4) as they mature into lymphatic-dwelling, thread-like adult male and female worms. Adult worms appear to remain in a constant location, in so-called 'worm nests' (Noroes *et al.,* 1996b) within lymphatic vessels in humans. Live adult worms exhibit a distinctive pattern of movement within the lymphatic vessels, termed the filaria dance sign (FDS) (Amaral et *al.,* 1994). Detection of FDS in the scrotum by ultrasonography is useful for diagnosis and monitoring of the success of antifilarial chemotherapy (Dreyer *et al.,* 1995b; Noroes *et al.,* 1997).

When lymphatic-dwelling adult filariae mate, fully-formed, sheathed, first-stage larvae (L1 or microfilariae) are released from the female. These microfilariae then enter the peripheral circulation of the human host, where they are available to be ingested by the vector during a blood meal (Nutman, 2001).

In the vector, the parasite penetrates the gut wall, migrates to the flight muscles, and molts to the second larval stage (L2). After several days, the parasite undergoes an additional molt to the L3 parasite. L3s migrate to the mouthparts of the vector, where they are positioned to be passed on to the vertebrate host during a subsequent blood meal. Different species of the following genera of mosquitoes serve as vectors of *W. bancrofti* filariasis depending on geographical distribution. They are *Culex*, *Anopheles*, *Aedes*, *Mansonia* and *Coquillettidia* (CDC, 2009).

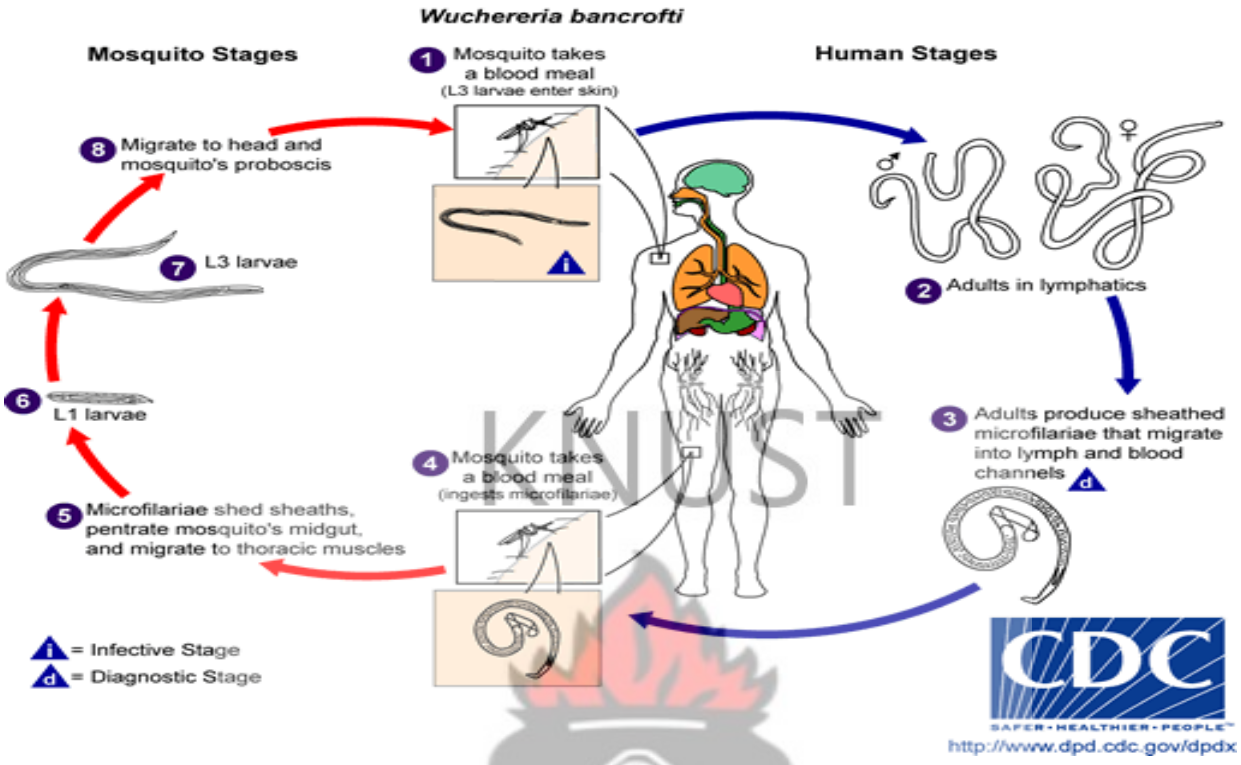


Figure 2.1: Life cycle of *Wuchereria bancrofti* (Source: CDC, 2009)

2.4 Clinical Manifestations of Lymphatic Filariasis

Lymphatic filariasis causes a wide spectrum of clinical and subclinical disease. The clinical manifestations of lymphatic filariasis vary from one endemic area to another and also differ, to some extent, according to the species of the parasite that is involved (Partono, 1987). In parts of Africa such as Tanzania, the most common clinical form of the disease is hydrocele, while lymphoedema and elephantiasis are much less common (Sasa, 1976). In other areas of the world such as India, both hydrocele and lymphoedema are seen with almost equivalent frequency (Pani *et al.*, 1991). Some forms of the disease, such as tropical pulmonary eosinophilia (TPE) and chyluria, are restricted in their distribution to certain parts of the world. For example, TPE has been reported most commonly from the Indian subcontinent, Brazil and Malaysia. A study by Brabin (1990) has shown that there is a slight preponderance of females

with chronic disease (when hydroceles are excluded) whereas other reports, especially from Ghana, indicate a male preponderance of the disease (Gyapong *et al.*, 1994; Nutman, 2001).

Differences in clinical presentation between bancroftian and brugian filariasis have also been reported (Sasa, 1976; Partono, 1987). It is recognized that inguinal lymphadenitis is more common in brugian filariasis than in bancroftian filariasis. Lymphangitis also occurs more frequently in brugian filariasis and in some individuals the inflamed lymphatics may stand out as cords. These are painful and prevent movement of the limbs. In the upper limbs, they can easily be palpated ('string sign'). Hydrocele and filarial scrotum is not seen in brugian filariasis (Nutman, 2001).

The manifestations of the disease have been described below as (i) Microfilaremic State (ii) Acute manifestations (iii) Chronic manifestations

2.4.1 The Microfilaremic State

In areas where *W. bancrofti* or *B. malayi* are endemic, the overwhelming majority of infected individuals have few overt clinical manifestations of filariasis, despite the presence of large numbers of circulating microfilariae in the peripheral blood. The prevalence of microfilaremia increases with age during childhood and usually reaches a plateau between 20 and 30 years of age. During the childbearing years, the prevalence tends to be higher among men than among women (Brabin, 1990).

A growing body of evidence indicates that, although they may be clinically asymptomatic, virtually all persons with *W. bancrofti* or *B. malayi* microfilaremia have some degree of subclinical disease. It was recognized that about 40% of these microfilaremic individuals have hematuria and/or proteinuria that reflect low-grade renal damage. The hematuria may

be macroscopic, but is most often microscopic and may be accidentally discovered during routine urine examination (Dreyer *et al.,* 1992). These renal abnormalities appear to be associated with the presence of microfilariae rather than adult worms, because clearing microfilariae from the blood results in complete reversal of these renal abnormalities (Dreyer *et al.,* 1992; Nutman, 2001).

Additionally, lymphoscintigraphic observations of the functional anatomy of microfilaremic individuals have demonstrated markedly abnormal, dilated and tortuous lymphatics as well as abnormal patterns of lymphatic flow (Freedman *et al.*, 1994; Dissanyake *et al.,* 1995; Suresh *et al.*, 1997; Nutman, 2001). In a parallel study from Brazil using ultrasound examination of scrotal lymphatics, microfilaria-positive, asymptomatic men demonstrated dilatation of lymph vessels (Noroes *et al.,* 1996a). Although the majority of individuals appear to remain asymptomatic for years, with relatively few progressing to the acute and chronic stages, it is clear that the 'asymptomatic microfilaremic state' is not as benign as initially believed (Nutman, 2001).

2.4.2 Acute Manifestation of Lymphatic Filariasis

The acute clinical manifestations of filariasis are characterized by recurrent attacks of fever associated with inflammation of the lymph nodes (adenitis) and/or lymph vessels (lymphangitis), termed adenolymphangitis (ADL). In bancroftian filariasis, recurrent attacks of fever associated with lymphadenitis are less frequently seen than in brugian filariasis (Partono,1987). In addition to the lymph nodes in the inguinal, axillary and epitrochlear regions, the lymphatic system of the male genitalia is frequently affected, leading to funiculitis, epididymitis or orchitis, or a combination of these (Pani *et al.*, 1995). In brugian filariasis, the affected lymph nodes are mostly situated in the inguinal and axillary regions, with inflammation along the course of the distal lymphatic vessels (Partono, 1987; Pani *et al.*, 1990).

The acute clinical course of filariasis may last for several days or up to 4–6 weeks with a fulminant episode. The diagnostic criteria for the identification of acute filarial attacks have been codified (WHO, 1992) and require pain, tenderness and local warmth, with either lymphadenitis, lymphangitis or cellulitis (Shenoy *et al.,* 1995) for lungs and breasts or epididymo-orchitis for the scrotum. The presence of scars at typical locations, e.g. over the inguinal and epitrochlear lymph nodes, supports the diagnosis of filariasis (Nutman, 2001).

In patients with filarial disease, acute attacks of ADL may involve the limb, breast or male external genitalia. These acute episodes are characterized by local pain, tenderness, warmth and lymphadenitis and/or lymphangitis. Other commonly associated findings include fever, edema, constitutional complaints and localized or ulcerated abscesses, especially in areas where *Brugia* is endemic. In endemic areas, there are two distinct types of acute ADL episodes: ADL secondary to bacterial or fungal infection and ADL caused directly by the parasite infection itself (Nutman, 2001).

2.4.3 Chronic Manifestations of Lymphatic Filariasis

The chronic signs of filariasis rarely develop before the age of 15 years, and only a small proportion of the filaria-infected population is affected; however, immigrants from areas where filariasis is not endemic tend to develop elephantiasis more often and much sooner (sometimes within 1–2 years) than do the indigenous populations of endemic areas (Partono, 1987).

In bancroftian filariasis, the occurrence of the major signs of chronic disease such as hydrocele, chyluria, lymphedema and elephantiasis —may differ from one area to another. The most common are hydrocele and swelling of the testis, followed by elephantiasis of the entire

lower limb, the scrotum, the entire arm, the vulva and the breast, in descending order of frequency (Shenoy *et al.*, 1995).

2.4.4 Development of Filarial Hydrocele

Figure 2.2 show examples of filarial hydrocele. Filarial hydroceles result as a consequence of the accumulation of fluid in the tunica vaginalis (WHO, 2002b). The fluid that accumulates is usually clear and the presence of microfilariae in the hydrocele fluid can occasionally be demonstrated. Hydroceles are the most common clinical manifestation in many endemic communities, with 40–50% of the males being affected. The prevalence of hydrocele in endemic communities is generally underestimated because small hydroceles can easily be missed with less sensitive techniques and inexperience on the part of the examiner (Nutman, 2001).

The mechanism of hydrocele development is still not fully known because a proportion of the patients do not have active infection, although prior infection is thought to induce these cases (Debrah *et al.*, 2009). Repeated episodes of ADL involving the testicular lymphatics may precede the development of hydrocele; however, hydroceles can also develop silently, and some ultrasound studies have shown that lymphangiectasia of the scrotal lymphatics is common in patients with hydroceles (Noroes *et al.*, 1996b ; Nutman , 2001).

Other urogenital conditions that are associated with filarial hydrocele include dilatation and tortuosity of the lymphatics of the spermatic cord, dilatation of the lymphatics of the scrotal skin which then rupture on the surface oozing lymphatic fluid, and chyluria or haematochyluria resulting from the rupture of the retroperitoneal lymphatics into the renal collecting system (WHO, 2002b).

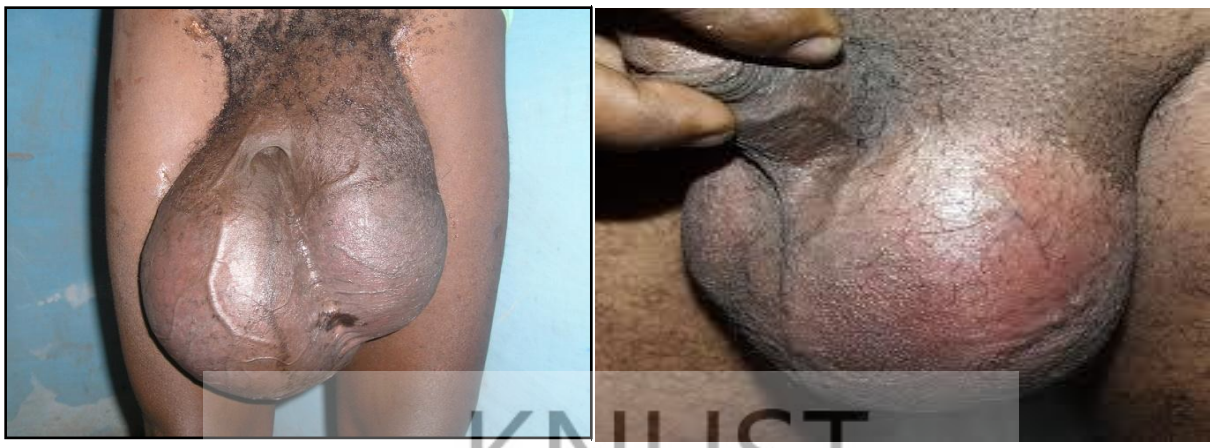


Figure 2.2: Filarial Hydroceles

2.5 Endosymbiotic Association of *Wolbachia* and Filarial Worms

Wolbachia bacteria (red spots in Figure 2.3) are essential symbionts of the major pathogenic filarial nematode parasites of humans, including *Wuchereria bancrofti, Brugia malayi and Onchocerca volvulus*. *Wolbachia* spp. is abundant in all developmental stages of filarial nematodes, including the hypodermis and reproductive tissue of adult parasites. In contrast to their relatives in arthropods, *Wolbachia* spp. in filarial nematodes appears to have evolved as an essential endosymbiont. Antibiotic therapy in humans and experimental filarial infection has shown that embryogenesis is completely dependent on the presence of *Wolbachia*. Furthermore, parasites recovered from tetracycline-treated animals are stunted with attenuated larval development (Andre' *et al*, 2002).

Previous studies of these symbiotic *Wolbachia* organisms suggest that they are important both as chemotherapeutic targets and disease-causing organisms (Taylor and Hoerauf, 1999). In a laboratory model of onchocercal keratitis, *Wolbachia* was shown to mediate neutrophil infiltration and stromal haze when a worm extract containing *Wolbachia* antigens was injected into the eyes of mice (Andre *et al.*, 2002; Taylor 2003; Hise *et al.*, 2003).

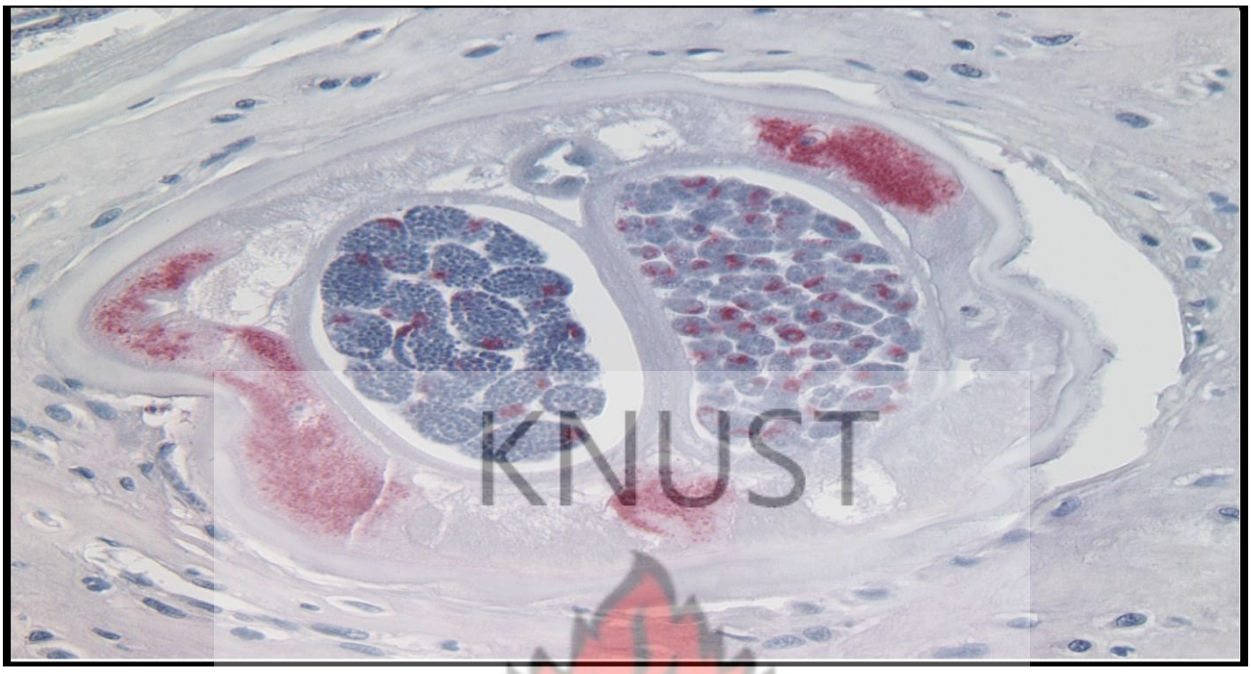


Figure 2.3: *Wolbachia* endosymbionts present in filarial worms including *Wuchereria bancrofti* (Hoerauf *et al.*, 2000).

2.6 Immunology of Lymphatic Pathology

The events that lead to the development of chronic pathology in lymphatic filariasis are not fully understood, but the immune responses of the human host to the parasites are believed to play a significant role in determining pathological manifestations such as hydrocele in infected individuals (Dreyer *et al.*, 1992; Addiss *et al.*,1995; Dreyer *et al.*,2000; Ravindran, 2003; Debrah *et al.*,2006).

The lymphatic vascular system plays a critical role in immune surveillance, tissue fluid homeostasis, and fat absorption (Witte *et al.*, 2001; Takahashi *et al.*, 2004). Perturbations in the maintenance and function of the lymphatic system can lead to a variety of pathological disorders, including lymphatic dilation and lymphoedema (Witte *et al.*, 2001; Saaristo *et al.*, 2002; Ruocco *et al.*, 2002).

Recent studies on the molecular mechanisms controlling the lymphatic vessels have shown that vascular endothelial growth factors C (VEGF-C) and VEGF-D specifically control lymphangiogenesis in humans (Achen *et al.,* 1998; Korpelainen and Alitalo , 1998). The expression of VEGF-C has also been shown to be up-regulated by proinflammatory cytokines like interleukin (IL)-1B and tumor necrosis factor (TNF), suggesting that proinflammatory cytokines could affect the lymphatic vessels via VEGF-C (Ristimaki *et al.*,1998; Debrah *et al.*,2006).

Taylor and colleagues (2000) using animal models have also shown that *Wolbachia*–derived molecules from *Brugia spp*. also induced proinflammatory cytokines, including TNF and IL-1B. Soluble extracts of *Brugia* and *Onchocerca volvulus* adult and microfilarial worms were also found to stimulate human peripheral mononuclear cells *in vitro*, resulting in the production of TNF, IL-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-10 (Raman *et al.,* 1999; Brattig *et al.,* 2000). This stimulation was not achieved using extracts from *Acanthocheilonema viteae*, a filarial species naturally devoid of *Wolbachia*, and, importantly, with *O. volvulus* extracts from patients that had been treated with doxycycline to deplete *Wolbachia* from the worms (Taylor *et al.,* 2005a).

Thus, it was concluded that in those filarial species that contain these endosymbionts, *Wolbachia* are the major stimulating principle for proinflammatory cytokines such as TNF. From this, it can be further hypothesized that exposure of host cells to *Wolbachia* from worms (either from dying adult worms or incoming L3/4 larvae, or from the proportion of degenerating embryos that are constantly released) may induce the production of lymphangiogenic factors such as VEGF-C by endothelial cells in LF patients (Debrah *et al.,* 2006).

Of importance for this study is the fact that *Wolbachia* depletion by doxycycline was associated with a reduction of pre-treatment elevated levels of proinflammatory cytokines such as TNF from plasma (Turner *et al.*, 2006). Together, these data suggest that targeting *Wolbachia* with doxycycline may ameliorate filarial pathology.

2.7 Control and Treatment of Filarial Hydrocele

Currently, the remedy available for filarial hydrocele is hydrocelectomy (WHO, 2002b). In Tanzania and coastal areas in Kenya in which this disease is endemic, hydrocelectomy accounts for 15–25% of all surgical cases (Wegesa *et al.*, 1979; Mwobobia *et al.*, 2000; DeVries *et al.*, 2002). Current MDA drugs such as diethylcarbamazine or ivermectin, usually given with albendazole—effectively kill the microfilariae (larval offspring of the parasite), but their effect on the macrofilariae (adult worms) is incomplete and none of them has an ameliorative effect on hydrocele pathology.

Several studies have demonstrated that ivermectin has no macrofilaricidal effect, although there are indications that it reduces fertility of the adult worms (Dreyer *et al.*, 1995b; Plaisier *et al.*, 1999; Richard-Lenoble *et al.*, 2003). Ivermectin is thus usually regarded as a pure microfilaricide, killing nearly all microfilariae. Some macrofilaricidal effect might occur, though, if ivermectin is combined with the broad-spectrum albendazole (Dreyer *et al.*, 1998). A single dose of diethylcarbamazine has good microfilaricidal effect and is thought to kill about 50% of adult worms (Noroes,1997; Ottesen,1999; Kshirsagar,2004; Fernando *et al.*, 2011). Only the combination of diethylcarbamazine and albendazole has macrofilaricidal effects comparable to doxycycline (56–87%) (El Setouhy *et al.*, 2004; Kshirsagar *et al.*, 2004).However, no ameliorative ability has been demonstrated for this combination (Fernando *et al.*, 2011).

The search for macrofilaricides which can ameliorate filarial hydrocele therefore remains a top research priority. One of the most promising leads in treating lymphatic filariasis is targeting *Wolbachia*, the intracellular bacterial symbionts of filarial parasites (Taylor, 2000).

Taylor and colleagues (2005b) demonstrated that an 8-week course of doxycycline kills the adult worm by depleting *Wolbachia*. In their study, they observed a strong reduction in the number of worm nests in the scrotum and levels of filarial antigens in the blood with an almost complete clearance of microfilariae 14 months after treatment. There were no serious side effects associated with this treatment. A subsequent study with a six-week regimen of 200mg doxycycline showed similar effects (Debrah *et al.*, 2006). The macrofilaricidal effects of doxycycline observed in these studies is high compared with that of the currently used antifilarial drugs (Ivermectin and albendazole in Africa and DEC in Asia).

2.8 Diagnosing Filarial Hydrocele

The diagnosis of filarial diseases can be problematic, because these infections require parasitological techniques to demonstrate the offending organisms. In addition, satisfactory methods for the definitive diagnosis in amicrofilaremic states can be difficult because a definitive diagnosis of filariasis can only be made by the demonstration of the parasites (Nutman, 2001) and hydrocele patients may not have an active infection.

Microfilariae may be found in the blood, hydrocele fluid or, occasionally, in another body fluid. These fluids can be examined microscopically, either directly or, for greater sensitivity, after concentration of the parasites by the passage of fluid through a polycarbonate cylindrical filter (pore size, 5µm) or by the centrifugation of fluid fixed in 2% formalin (Knott's concentration technique) or 2% formalin/10% Teepol (Dickerson *et al.*, 1990).

Ultrasonography, ELISA and PCR can also be used to diagnose filarial hydrocele (Nutman, 2001).

2.8.1 Detection of Circulating Filaria Antigens

Assays for circulating filaria antigens of *W. bancrofti* permit the diagnosis of microfilaremic and amicrofilaremic infection (More and Copeman, 1990; Chanteau *et al.*, 1994; Lammie *et al.*, 1994; Rocha *et al.*, 1996; Weil *et al.*, 1997).There are currently two commercially available tests, one in an ELISA fomat (Trop-Ag *W. bancrofti*, JCU Tropical Biotechnology Pty. Ltd, Townsville, Queensland, Australia) and the other a rapid-format card test (Binax, Portland, ME, USA). Both assays have reported sensitivities in the range of 96–100% and specificities that approach 100% (More and Copeman, 1990; Chanteau *et al.*, 1994; Lammie *et al.*, 1994; Rocha *et al.*, 1996; Weil *et al.*, 1997).

2.8.2 Serodiagnosis Using Parasite Extract

The development of serodiagnostic assays of sufficient sensitivity and specificity for routine use has proved problematic (Ambroise-Thomas, 1974; Speiser, 1980; Voller and deSavigny, 1981), primarily because of their poor specificity. Extensive cross-reactivity is found in the sera of individuals infected with closely related helminth parasites and even certain protozoal parasites (Maizels *et al.*, 1985; Lal and Ottesen, 1988).

Further, as is the case for serodiagnosis of most infectious diseases, it is difficult to differentiate previous infection or exposure to the parasite (aborted infection) from current active infection. In fact, most residents of filariasis-endemic regions are antibody-positive (Ottesen *et al.*, 1982). Nevertheless, such serologic assays have a definite place in diagnosis, as a negative assay result effectively excludes past or present infection. The prominent role of

antifilarial antibodies of the IgG4 subclass in active filarial infection (Ottesen *et al.*, 1985) has led to the development of sero-diagnostic assays based on antibodies of this subclass. Antifilarial IgG4 antibodies have improved specificity, but positive assays may still be seen in uninfected individuals living in endemic areas (Chanteau *et al.*, 1994) and in those infected with other filarial species (e.g. onchocerciasis, loiasis, mansonelliasis) (Nutman, 2001).

2.8.3 Molecular Diagnostics

PCR-based assays for DNA of *W. bancrofti* and *B. malayi* in blood have also been developed. In a number of studies evaluating PCR-based diagnosis, the method is of equivalent or greater sensitivity compared with parasitological methods, detecting patent infection in almost all infected subjects. In addition, the technique is able to detect cryptic infection (amicrofilaremic, circulating antigen-positive infection) in some subjects (Lizotte *et al.*, 1994; Abbasi *et al.*, 1996; Williams *et al.*, 1996), and parasite DNA can be detected in the saliva of microfilaremic individuals (Abbasi *et al.*, 1996). The technique is, in addition, useful for the speciation of parasite material removed at surgery, especially when morphologic diagnosis is not possible (Nutman, 2001).

2.8.4 Ultrasonography

In cases of suspected filarial hydrocele, examination of the scrotum using high-frequency ultrasound in conjunction with Doppler techniques may result in the identification of motile adult worms within dilated lymphatics. Worms may be visualized in the lymphatics of the spermatic cord in up to 80% of infected men (Dreyer *et al.*, 1995a; Noroes *et al.*, 1996b; Mand *et al.*, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Areas

The study was carried out in 10 communities in the Nzema East and the Ahanta West Districts in the Western Region of Ghana. The communities are Ampatano, Asemkow, Butre, Busua, and Miamia, in the Ahanta West District and Agyamra, Salman, Kangbunli, Sanwoma and Ampain, in the Nzema East District (see Figure 3.0).

These communities have been identified as endemic for lymphatic filariasis with prevalence ranging from 5 to 20% (Dunyo *et al*., 1996). The community populations range from 40-700 with the main building being mud houses with roofing made from palm or coconut fronds. The main occupations in the study communities are fishing and farming.

Habits such as sleeping along the coasts and without mosquito nets increase the exposure of inhabitants to the bites of mosquitoes. Moreover, most of these communities do not have health facilities and thus do not seek medical attention unless in the case of emergencies, where they go to the nearest health facilities in Aiyinasi, Eikwe, Nkroful, Essiama, Asemasa, Axim, Agyamra and Discove. Because of this, acute attacks that disappear after a few days mostly pass unattended to. Moreover, the superstitious nature of the village folks see them resorting to herbalists and 'witch doctors' to treat chronic manifestations of the disease such as hydrocele.

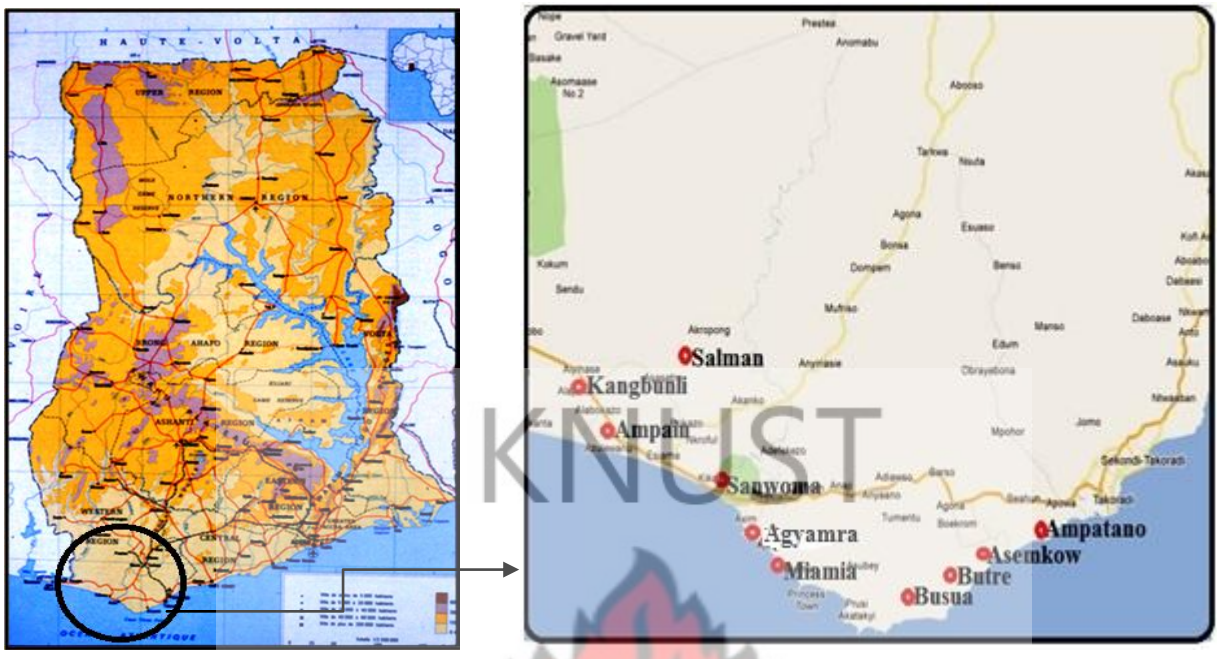


Figure 3.0: Map of Ghana showing study areas. Study communities are labelled with red spots. A common characteristic of most of the study communities is the proximity to rivers, lagoons and mangroves which serve as breeding sites of the Anopheline vectors.

3.2 Ethical Approval

Ethical clearance for this clinical trial was obtained from the Committee on Human Research, Publications and Ethics (CHRPE), School of Medical Sciences, Kwame Nkrumah University of Science and Technology, and Komfo Anokye Teaching Hospital (KATH) in Kumasi, Ghana.

3.3 Recruitment of Study Participants: Inclusion and Exclusion criteria

Individuals recruited for the study were males between the ages of 18 and 60 years (body weight >40kg) with good general health without any clinical condition requiring long-term medication. They were positive for hydrocele as determined by ultrasonography with normal renal profile (creatinine [53-126 µmol/L]), normal hepatic profile (alanine aminotransferase, ALT [0-45 IU/L]; gamma glutamyl transpeptidase, ɣ -GT [>60 IU/L] and aspartate aminotransferase [0-40

IU/L]) measured by Reflotron® dipstick chemistry, and normal white blood cell count (2.6-8.3 x 10^9 /L).

Individuals excluded from the study included those with history of severe allergic reactions, severe alcohol abuse, anti-filarial therapy within the last 10 months, mental disorders, and laboratory evidence of liver disease (AST and/or ALT and/or ɣ -GT greater than 1.25 times the upper limit of the reference ranges stated above).

During a meeting with the village elders and interested villagers, the study was explained in English and then in the local languages 'Twi' and 'Nzema'. The participants were requested to ask questions and these were answered. During surveys, persons identified as eligible to participate in the study were informed individually in English, 'Twi' and 'Nzema' according to Good Clinical Practice. Informed, signed/thumb printed, or witnessed consent was then obtained from all participants.

3.4 Study Design

The study was a randomized, placebo-controlled, double-blinded clinical trial with 44 patients placed into one of two arms namely the doxycycline arm and placebo arm. Participants in the doxycycline arm received 2 capsules of 100mg doxycycline for 6 weeks (Vibromycin, Pfizer). The remaining participants received doxycycline-matching placebo (supplied by the manufacturer) for 6 weeks.

The drugs were given on a daily observed treatment (DOT) basis. Study participants were given ivermectin (150µg/kg) and albendazole (400mg oral dose) after 6 months of treatment in line with the Mass Drug Administration program of the communities. Occurrences of adverse effects after drug administration were closely monitored during the treatment.

3.5 Follow-up Examinations

Follow-up of study participants were carried out at 12 and 24 months after treatment during which antigenemia (CFA) and microfilaraemia were assessed. Ultrasonography of the scrotum was performed to measure dilation of lymphatic vessels, scrotal skin thickness and hydrocele sizes and to test for filarial dance signs. These parameters were used to monitor improvement, halt or worsening of hydrocele pathology in the trial patients at the various time points.

3.6 Biochemical Tests to Assess Kidney and Liver Functions

In order to assess the kidney and liver functions of patients, biochemistry tests were done using stick- technology by the Reflotron® system (Boehringer Mannheim, Germany, Roche). Blood samples were collected and centrifuged to separate plasma from blood cells. About 500µl of each patient's plasma was pipetted into 1.8ml eppendorf tubes labeled with the unique number assigned to each patient. About 100µl of plasma was then pipetted to the various Reflotron® test strips using a Reflotron® pipette following the protocol of the manufacturer. Parameters measured by the stick technology were glutamate-pyruvate –transaminase (GPT), gamma-glutamyl- transpeptidase (GGT) and creatinine (CREA).

3.7 Assessing Microfilaraemia in Hydrocele Patients

In order to assess the microfilaria levels of study participants, 10 ml of venous blood was collected between 10:00pm and 12:00pm from the patients. About 100µl of the collected blood sample was diluted in 900µl of 3% acetic acid. The mixture was poured into a Sedgwick counting chamber and examined microscopically for microfilaria. The microfilaria load was expressed as Mf/ml of blood. To further confirm the results from the Sedgwick counts, 100µl (in the case of high Sedgwick counts) or 1000µl (for very low Sedgwick counts) of blood was

filtered through 5µm Millipore filters (Whatman's nucleopore, Kent, UK), stained with Giemsa and microfilaria counted (see Figure 3.1).

Figure 3.1: Parasitological examinations in the laboratory

3.8 Assessment of Circulating Filaria Antigens in Hydrocele Patients

The presence of Circulating Filaria Antigens (CFA) in the blood of the hydrocele patients was assessed quantitatively using the Og4C3 Enzyme-Linked Immunosorbent Assay (More and Copeman, 1990).

Following the manufacturers instruction, 100µl of each patient's plasma sample was diluted in 300µl of sample diluents containing EDTA. The samples were then boiled to liberate the heat-stable CFA that is detected in positive samples. After centrifugation, 50µl of the supernatant were added in duplicates to the wells of polystyrene micro-titer plates pre-coated with the monoclonal anti-filarial antibody (Og4C3). Antigen standards with known concentration of CFA were added to some of the wells on the micro-titer plates as described by the manufacturer to prepare a standard curve for the determination of antigen concentration in unknown test samples. The samples were incubated for 2 hours and subsequently washed 3 times, after which rabbit

anti-CFA antibody was added. After incubation for an hour and subsequent washing 3 times as before, anti-rabbit horse-radish-peroxidase-conjugate was added to the samples and again incubated for another hour and washed 3 times. Finally, 100µl of chromogen was added to each well and incubated for an hour and washed as before. The optical density was measured at 405 nm using an ELISA reader (Spectra MAX; California, USA).

The test is set up for semi-quantitative analysis, where the color intensity for a given serum or plasma sample (diluted 1:4) is compared to standard values pre-diluted by the manufacturer (levels: 32,000, 8,192, 2,048, 512, 128, 32, <10 antigen units, corresponding to titer groups 7, 6, 5, 4, 3, 2 1, respectively). Titer group 3 is considered equivocal, groups 2 and 1 are considered antigen negative whereas titre group 4 upwards are considered antigen positive.

3.9 Ultrasound Examinations of the Scrotum in Hydrocele Patients

Participants were examined with a hand carried Sonosite 180 Plus ultrasound system (Sonosite ® Inc: Washington, USA) equipped with an L 38 mm, 5–10 MHz linear transducer plus Pulse Wave- and Colour Doppler device. Ultrasound examinations were carried out in rural communities in darkened rooms, such as school buildings, community halls or tents. The volunteers were examined in a supine position, legs crossed to support the scrotum and the penis covered with paper towels held by the patient's hand. The examination was done with patients in a supine position in order to reduce interference by movements of the patients themselves.

The scrotum was then scanned in transverse sections of the right and the left sides, followed by longitudinal sections of both sides and a transverse scan of the backside. The transducer was positioned in panorama mode at each section to provide optimal information about the testis, epididymis, layers of the tunica vaginalis, lymphatic- and blood vessels and the spermatic cord.

3.9.1 Determination of Dilation in Scrotal Lymphatic Vessels

The degree of lymphatic dilation caused by filarial worms is considered an indirect measurement of altered lymphatic function (Pollitt *et al.,* 2005). In this study, the dilation in the scrotal lymphatic vessels was determined by measuring the largest diameter detectable in the two-dimensional, b-mode of the ultrasound machine. The grading system used to determine the degree of lymphatic dilation was developed by Debrah and colleagues (2006) as seen in Figure 3.2:

(A) **Category 1:** patients with minimal lymphatic dilation of up to 0.2 cm

(B) **Category 2:** patients with mild dilation from 0.21–0.50 cm

(C) **Category 3:** patients with moderate dilation from 0.51–1.0 cm

(D) **Category 4:** patients with severe dilation of above 1.0 cm.

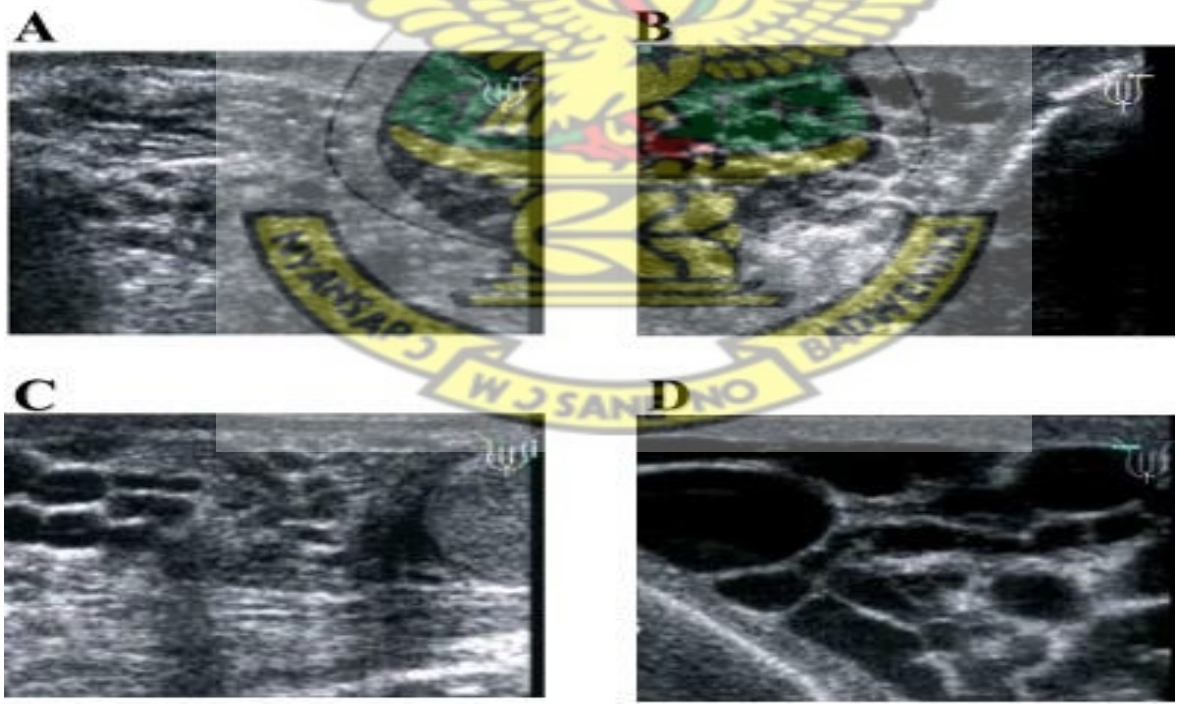


Figure 3.2: Grading of dilations in scrotal lymphatic vessel in filarial hydrocele patients as displayed by ultrasonography (Debrah *et al.*, 2006).

3.9.2 Determination of Hydrocele Stages

Hydrocele was defined as fluid surrounding the testis (>0.2 cm in the 2-dimensional b-mode) in the area of the upper or lower pole of the testes or mid-testicular. The four-stage system of Debrah *et al.* (2007) was used to grade the amount of fluid. According to this, stage 1 (minimal fluid collection around the testis, greater than 0.2 cm at the upper and lower pole) is subclinical, whereas stage 2 (the maximal longitudinal and transverse diameters of the hydrocele do not exceed 1.9 and 1.6 cm, respectively), stage 3 (the maximal longitudinal and transverse diameters of the hydrocele do not exceed 3.8 and 3.2 cm, respectively) and stage 4 (the maximal longitudinal and transverse diameters of the hydrocele are greater than 3.8 and 3.2 cm, respectively) are clinical stages.

3.9.3 Determination of Scrotal Skin Thickness

Scrotal skin examinations were carried at both follow-up time points to assess the thickness of the scrotal skin after treatment. The diameter of epidermal, dermal and subcutaneous layers was measured in b-mode from the surface of the skin to the parietal layer of the tunica vaginalis. The normal thickness of the scrotal skin is 0.21 cm.

3.9.4 Detection of Filaria Dance Signs

Patients were also scanned for the presence or absence of the filaria dance signs characteristic of adult worms. Adult filariae in scrotal lymphatic vessels were verified by reflection of their typical movements seen in the 2-dimensional b-mode of the ultrasound machine and confirmed using a Pulse Wave Doppler mode to display the Filaria Dance Signs in the form of unequivocal, irregular peaks (Faris *et al.,* 1998; Mand *et al.,* 2003; Hussein *et al.,* 2004; Mand *et al.,* 2010).

3.10 Statistical Analysis

All statistical analyses were performed using Graph pad Prism® software. Analyses were done using descriptive statistics for frequency distribution, Chi-square analysis, non-parametric tests for paired (Wilcoxon signed test), unpaired (Mann-Whitney-U test and unpaired t test) and Kriskal-Wallis test for paired samples from more than 2 time points. A two-tailed *p*-value lower than 0.05 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Adherence to Treatment and Drop Outs

The flow chart (Figure 4.0) below summarizes the adherence to treatment and dropouts at the various time points. Of the 44 eligible volunteers recruited for this study, 40 completed the full course of treatment. At the 12 month follow-up time point, 16 patients were absent whilst 10 patients were absent at the 24 months time points.

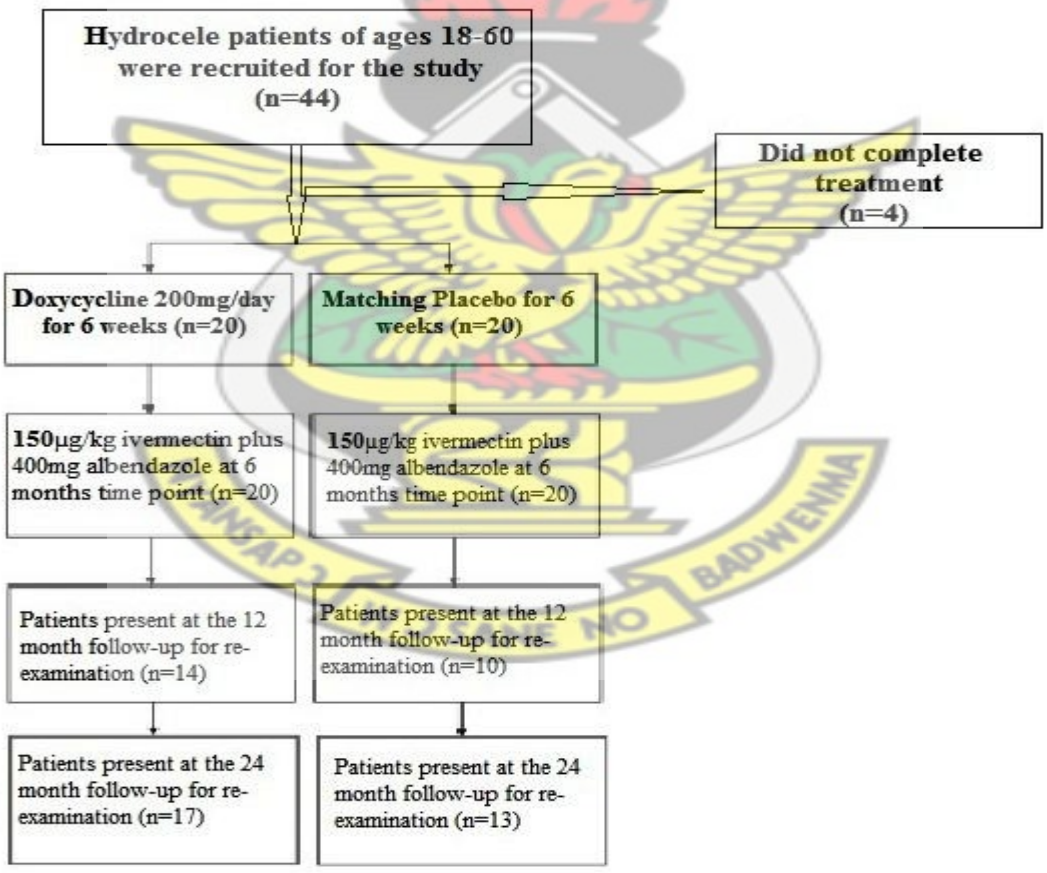


Figure 4.0: Flow chart of adherence to treatment and drop-outs at the various time points.

4.2 Pre-Treatment Findings

Individuals in doxycycline and placebo groups were similar in age (18-60 years), body weight (greater than 40kg) and blood microfilaria counts. Pre-treatment findings for dilation of scrotal lymphatic vessels, hydrocele stages, scrotal skin thickness, filaria dance signs, circulating filaria antigen levels and microfilaraemia, did not significantly differ between treatment groups (*p*-values = 0.2159, 0.1374, 0.0945 [unpaired t test] and 0.6285, 0.8351, 0.7895 [Mann-Whitney U test] respectively).

4.3 Occurrence of Adverse Events during Treatment

Table 4.0 summarizes the adverse events recorded after treatment. Individuals were asked to report any signs and symptoms that were not experienced prior to treatment. All symptoms were documented in volunteers' case report forms and medication was provided where necessary during the six-week DOT treatment. Adverse events were recorded in 8 out of the 20 volunteers who received 200mg doxycycline whilst 4 placebo-treated patients experienced adverse effects.

Table 4.0: A summary of adverse events (AEs) following doxycycline and placebo treatment of hydrocele patients.

Treatment	Total No. of patients with AEs	No. of pat. with diarrhoea	No. of pat. with stomach pain	No. of pat. with vomiting	No. of pat. with joint pains	No. of pat. with other AEs	*p*-Value
Doxycycline	8	1	2	1	1	3	**0.3148**
Placebo	4	3	0	0	0	1	

The incidence of adverse events did not significantly vary between the two treatment groups (*p*=0.3148, Chi square test). Symptoms in both groups were mild and included diarrhoea, stomach pain, vomiting, joint pains and others (scrotal pain, chest pain and abdominal pains) lasting between 1 and 5 days for the doxycycline-treated patients and 1-3 days for the placebo

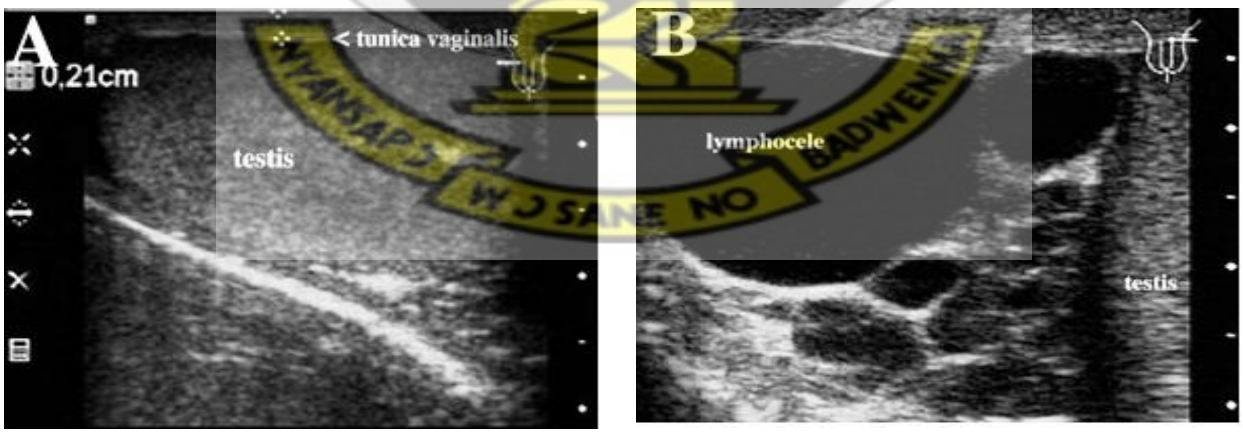
group. No patient reported any adverse events after 5 days and no evidence or complaint of symptoms consistent with doxycycline-associated photosensitivity was observed. There was no instance where treatment was discontinued because of adverse reactions.

4.4 Examinations for Improvement in Hydrocele Patients after Treatment

Ultrasonography (USG) was used to detect filarial dance sign, measure the dilation of scrotal lymphatic vessels, hydrocele sizes, and scrotal skin thickness in order to monitor improvement, halting or deterioration of hydrocele condition.

4.4.1 Evaluation of the Dilations in the Lymphatic Vessels of the Scrotum after Treatment

Before treatment, USG showed that 40 patients (20 doxycycline- and 20 placebo-treated) had dilations in their scrotal lymphatic vessels. Figure 4.1 and 4.2 and Table 4.1 illustrate the state of dilation in lymph vessels at pre-treatment and also at 12 and 24 months follow-ups. Reduction in the stage of lymphatic vessel dilation by one or more in the left, right or both scrotums was regarded as an improvement, whereas an increase by one or more stages was recorded as a worsening in the dilation of scrotal lymphatic vessels.



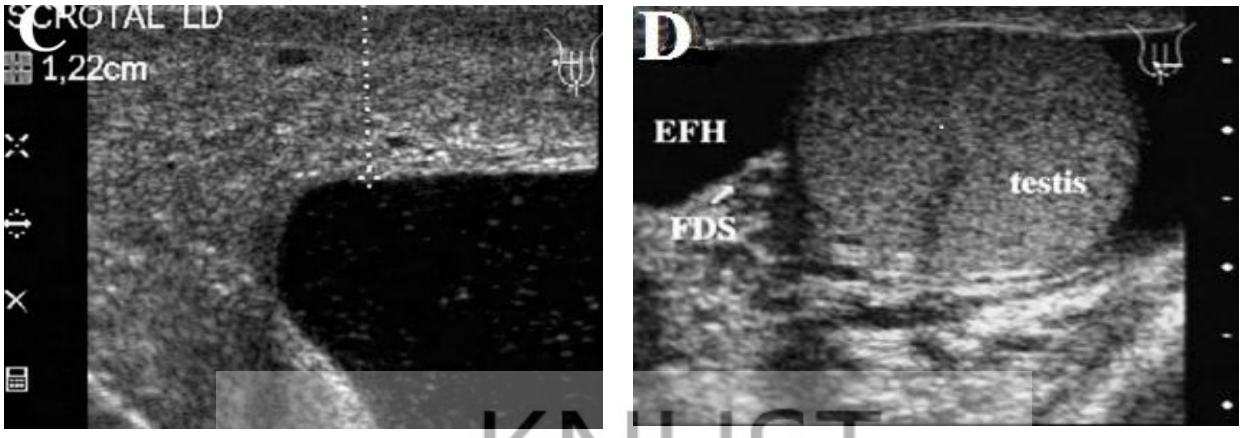


Figure 4.1: USG scans of the scrotum (A) Transverse scan of the right scrotum showing a normal testis. The normal layer thickness of the scrotal skin is 0.21 cm (marker) measured from the parietal layer of the tunica vaginalis to the surface of the dermis. (B) Transverse scan of the left scrotum. Separated fluid collection in an extremely dilated lymphatic vessel in the upper pole (spermatic cord) of the scrotum is shown, surrounded by dilated lymphatic vessels (stage 4). Part of the testis is seen at the right side of the image. (C) Transverse scan of the left scrotum: the scrotal skin is thickened (1.22 cm from surface of the scrotal skin up to the parietal layer of the tunica vaginalis), representing a lymph scrotum. (D) Adult worms were detected by their typical movements (Filaria Dance Sign) in a dilated lymphatic vessel in the area of the epididymis (arrow).

At the 12 months follow-up time point, the state of dilation in scrotal lymphatic vessels did not vary significantly (p=0.7907, unpaired t test) between the two treatment arms. At the 24-month time point, 10 out of 17 (59%) doxycycline-treated patients showed a reduction in the dilation of their scrotal lymphatic vessels in comparison to 3 out of 13 (23%) patients in the placebo group as shown in Table 4.1. The dilations of the lymphatic vessels were significantly improved in the doxycycline- treated patients compared to placebo-treated patients (p=0.0318, unpaired t test). Together, 94% (59% + 35%) of doxycycline-treated patients had either an improvement or a halt in the dilations of the scrotal lymphatic vessels.

Table 4.1: State of lymphatic vessel dilation (LD) of patients 24 Months after treatment.

Treatment Group	No. of Patients at 24 months	No. of Pat. with improved LD	No. of Pat. with same condition	No. of Pat. with deteriorated LD	*p-Value
Doxycycline	**17**	**10/17(59%)**	**6/17(35%)**	**1/17(6%)**	**0.0212**
Placebo	**13**	**3/13(23%)**	**3/13(23%)**	**7/13(54%)**	

*Difference in improvement between doxycycline- and placebo-treated patients was statistically significant using the Chi Square test.

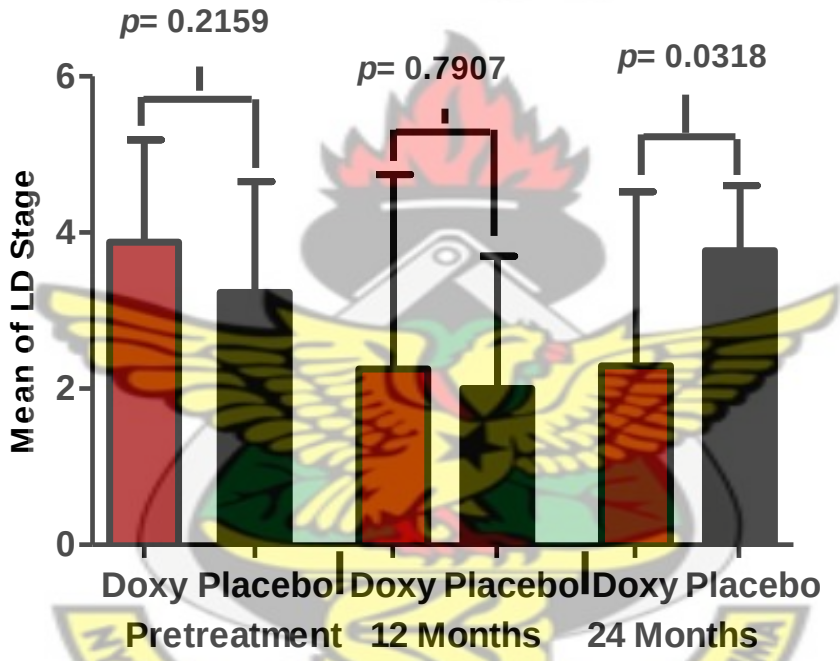


Figure 4.2: The effect of doxycycline treatment on the dilation of scrotal lymphatic vessels at various study time points. The scrotal lymphatic vessel dilation was determined before treatment and 12 and 24 months thereafter, using USG. The mean scrotal lymphatic vessel dilation of doxycycline-treated (Doxy) patients improved significantly (p=0.0140, Wilcoxon signed rank test) at 24 months compared to pre-treatment values, in contrast to the placebo group (p=0.9153, Wilcoxon signed rank test). Error bars represent the standard deviation.

4.4.2 Evaluation of Hydrocele Sizes in Study Patients after Treatment

Table 4.2 and Figure 4.3 illustrate the state of hydrocele sizes at the various time points. The sum of the hydrocele stages of both the left and right testes was used to determine improvement, halting, or worsening of hydrocele in the study patients. Reduction of hydrocele stage by one or more stage(s) in the left, right or both scrotums was regarded as an improvement, whereas an increase by one or more stages was recorded as a worsening in hydrocele size.

Table 4.2: State of hydrocele (hyd) in patients 24 months after treatment.

Treatment Group	No. of Patients at 24 months	No. of Pat. with improved hyd. condition	No. of Pat. with same hyd. condition	No. of Pat. with deteriorated hyd. condition	*p*-Value
Doxycycline	17	8/17(47%)	6/17(35%)	3/17(18%)	**0.0349**
Placebo	13	3/13(23%)	3/13(23%)	7/13(54%)	

At 12 months, the hydrocele condition of the doxycycline-treated patients had improved significantly (p=0.0292, Mann-Whitney U test) whereas there was no significant improvement seen in the placebo- treated patients (p=0.2586, Mann-Whitney U test) from the pre-treatment conditions. The inter-group variation in hydrocele stages was however not significant (p=0.1681, unpaired t test). At the 24-month follow-up period, the hypothesis that doxycycline can ameliorate filarial hydrocele was supported as 82% (47% +35%) in the doxycycline group either had a significant improvement or a halt in the progression of hydrocele stage compared to the 46% (23%+23%) in the placebo group. The placebo group further recorded a worsening of hydrocele condition in 54% of treated patients as against 18% in the doxycycline group.

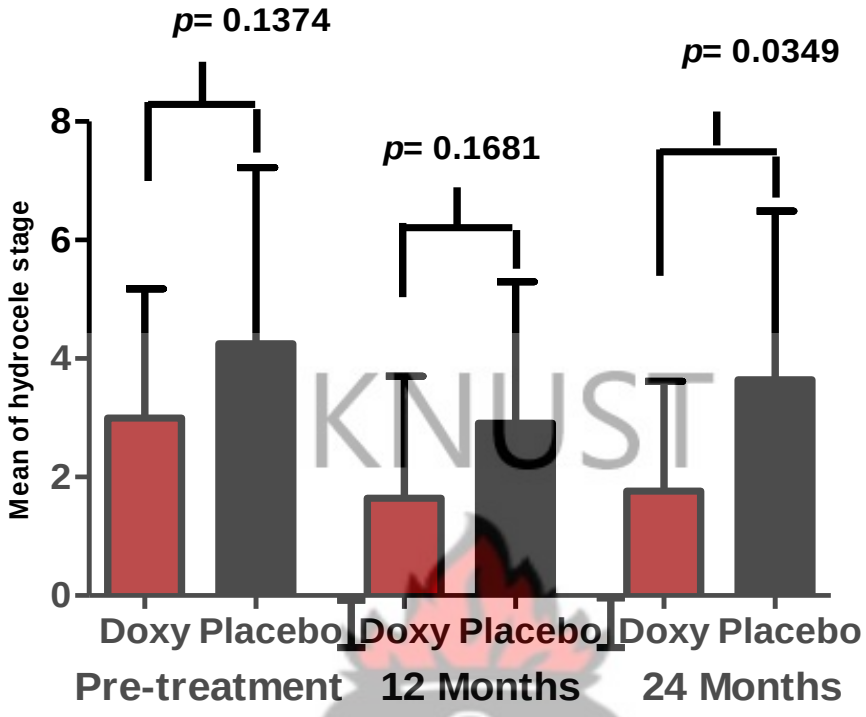


Figure 4.3: The effect of doxycycline on hydrocele stages at various follow-up time points. The mean (mean +SD) of the sum of left and right hydrocele stage were compared at all time points. Pre-treatment variations between the groups was not significant (*p*=0.1374, using the unpaired t test). At 24 months, the doxycycline group showed a significant improvement (*p*=0.0349) in hydrocele stage compared to placebo treated patients.

Improvement in hydrocele condition occurred even in doxycycline-treated patients who had no adult worm nests detectable at pre-treatment.

4.4.3 Evaluation of Scrotal Skin Thickness after Treatment

The thickness of the scrotal skin in the hydrocele patients was also used to monitor treatment. The average of the skin thickness of both the left and right scrotums was computed and used to assess hydrocele condition. At pre-treatment, there was no significant difference in the thickening of the scrotal skin of both doxycycline- and placebo-treated patients (*p*=0.2775, Mann-Whitney U test). There was no significant difference in the state of scrotal skin thickening

between doxycycline-patients and placebo-treated patients at the 12 months follow-up (p=0.7679, Mann-Whitney U test).

Twenty four (24) months after treatment however, 47% of doxycycline-treated patients had improvement in the thickness of their scotal skins against 33% in the placebo group. The inter-group variation in the improvement of the thickening of the scrotal skin was significant (p=0.0120, Mann-Whitney U test).

4.4.4 Determination of Filarial Dance Signs after Treatment

Table 4.3 summarizes the number of worm nests at the various time points. At pre-treatment, 3 out of 20 doxycycline and 2 out of 20 placebo-treated patients had detectable filaria dance signs. Together, the doxycycline group had a total of 6 worm nests whilst the placebo group had 2 worm nests. Notably, the doxycycline-treated patients had more worm nests detectable by ultrasound than the placebo-treated patients. Twelve (12) months after treatment, 2 of the 3 doxycycline- and 1 of the 2 placebo-treated patients had lost their worm nests detectable by USG. Two patients (one each from both groups) were absent for USG examinations.

At the 24 months time point, all 3 doxycycline-treated patients had lost all 6 worms nests (present at pre-treatment) confirming the macrofilaricidal activity of doxycycline. One patient from the placebo group (with only 1 worm nest at pre-treatment) had also lost the worm nest. The other placebo-treated patient was absent at 12 and 24 months and so could not be included in the outcome analysis.

Table 4.3: Summary of worm nests at the various time points

Treatment	Worm nests at Pre-treatment	Worm nests at 12 months	Worm nests at 24 months
Doxycycline	**6**	**2**	**0**
Placebo	**2**	**1**	**0**

4.5 Assessment of Circulating Filaria Antigen Levels in Hydrocele Patients

At pre-treatment, 25% of the doxycycline-treated patients and 20% of the placebo-treated patients had detectable circulating filaria antigens (CFA) in their blood samples (see Table 4.4). The inter-group variation in CFA levels was not significant at pre-treatment (p= 0.8351, Mann Whitney U test).

Table 4.4: A summary of the number of patients positive for circulating filaria antigens at the various follow-up time points.

Treatment	No. of CFA+ patients at pre-treatment	No. of CFA+ patients at 12 months	No. of CFA+ patients at 24 months
Doxycycline	5	4 (1 new CFA+, 1 absent)	3 (2 absent)
Placebo	4	3 (1 new CFA+, 2 absent)	1 (3 absent)

Twelve (12) months after treatment, 1 out of the 5 doxycycline-treated patients showed a complete clearance of circulating filaria antigens whilst all placebo-treated patients that were present still had detectable CFA in their blood. Both groups recorded one new antigenemic patient. At the 24 months time point, CFA levels were significantly reduced from pre-treatment values in both the doxycycline- and placebo-treated patients (p=0.0017 and 0.0038 respectively using the Wilcoxon signed rank test) and the inter-group variation in CFA levels was not statistically significant (p= 0.5726 , Mann-Whitney U test).

4.6 Assessment of Microfilaremia in Hydrocele Patients

Only one patient from the placebo arm was microfilaremic (with 180 mf /ml) at pre-treatment. However, the patient was absent at the 12 and 24 month follow-up time points and thus could not be included in the outcome analysis. However, all patients present at 12 and 24 months had no microfilaria in their blood.

CHAPTER FIVE

5.0 DISCUSSION

There is an urgent need for a long-term sterilizing and macrofilaricidal drug which has the additional ability to ameliorate hydrocele pathology in lymphatic filariasis to complement ivermectin and Diethylcarbamazine. In addition, new drugs with modes of action different to ivermectin must urgently be developed to serve as backup in case ivermectin resistance develops (Borsboom *et al.*,2003), a scenario that may not be too far in the future.

The purpose of this study was to investigate whether a six-week course of 200mg doxycycline significantly improved filarial hydrocele. The dilation of lymphatic vessels of the scrotum, hydrocele sizes, thickness of scrotal skin and filaria dance signs were used to assess amelioration, halting or worsening of hydrocele pathology. The study also monitored circulating filaria antigen levels and microfilaremia in patients before and after treatment.

5.1 Improvement in the Dilations of Lymphatic Vessels of the Scrotum after Doxycycline Treatment

In this study, a six-week course of 200mg doxycycline resulted in sustained improvement and halt in the dilations of the lymphatic vessels of the scrotum in 94% of treated patients in contrast to 46% of placebo patients at the 24 months follow-up. However, there were no significant improvement and halt in lymphatic vessel dilations at the 12 months follow-up from the pre-treatment condition in both groups.

The finding in this study is consistent with an earlier study by Taylor *et al* (2005b) in which they did not observe significant reductions in lymphatic vessel dilation 12 months after a six-week

treatment with 200mg doxycycline. However, between 18-22 months after treatment, they observed significant reductions in lymphatic vessel dilation in the doxycycline-treated patients. The macrofilaricidal activity of doxycycline is gradual and indirect and it is best seen 1 year after treatment (Hoerauf, 2008). This is in contrast to the fast but relatively short-term microfilaricidal activity of ivermectin which wanes after 6-9 months of treatment and yearly administration must be sustained in order to achieve an interruption in transmission (Nicolas *et al.,* 1997). Although studies with DEC have indicated microfilaricidal activity coupled with partial activity against adult worms after 1 year (Andrade *et al.,* 1995; Weerasooriya *et al.,* 1998; Freedman *et al.,* 2001; Beuria *et al.,* 2002; Ramzy *et al.,* 2002; Fernando *et al.,* 2011), the unpleasant adverse effects that accompany its administration in areas co-endemic with onchocerciasis excludes its use in Africa (WHO, 2002b) and it has no ameliorative effects on hydrocele pathology (Fernando *et al.,* 2011).

The improvement in the dilation of the scrotal lymphatic vessels of doxycycline-treated patients who had no adult worm nests detectable by ultrasonography at pre-treatment supports the hypothesis that doxycycline has an additional mode of action apart from *Wolbachia* depletion. Earlier studies on the molecular mechanisms controlling the lymphatic vessels have established that the vascular endothelial growth factors (VEGF) C and D specifically control lymphangiogenesis in humans (Achen *et al.,* 1998; Korpelainen and Alitalo, 1998) by activating the VEGF receptor-3 (VEGFR-3) (Jeltsch *et al.,* 1997; Cao *et al.,* 1998; Veikkola *et al.,* 2001; Baldwin *et al.,* 2001), which is principally restricted to the lymphatic endothelium in adult humans (Kaipainen *et al.,* 1995; Kukk *et al.,* 1996). Jeltsch *et al* (1997), using animal models, demonstrated that the over-expression of VEGF-C in the skin of transgenic mice results in the

proliferation of lymphatic endothelium and the dilation of lymph vessels similar to lymphatics infected with filarial parasites (Taylor *et al.,* 2001).

According to Debrah *et al* (2006), doxycycline reduces plasma VEGF-C and soluble VEGFR-3, thereby inhibiting angiogenesis in lymphoedema patients. More recent insights from the work of Fainaru and colleagues (2008) further demonstrates that doxycycline prevents VEGF-induced vascular permeability resulting in less fluid accumulation in body organs such as the testes. Thus, in addition to its macrofilaricidal effects (Taylor *et al.*, 2005b), doxycycline acts as a regulator of the factors responsible for proliferation of lymphatic endothelium and dilation of lymph vessels (Fainaru *et al.*, 2008). This could explain the observed amelioration and halt in progression in the dilation of lymphatic vessels of the scrotum even in patients without adult worms in their lymphatic vessels in this study. However, more research is needed in this area.

The observed reduction and halt in progression in the dilation of scrotal lymphatic vessels in 46% of placebo-treated patients were unexpected. Notably, only 1(7%) of these placebo patients had worm nests at pre-treatment. The presence of adult worms in the scrotal lymphatic vessels could lead to perturbations in the maintenance and function of the lymphatic system resulting in a variety of pathological disorders including dilation in the scrotal lymphatic vessels. Thus, in the absence of the adult worms, the previously obstructed lymphatics vessels (Witte *et al.,* 2001; Ruocco *et al.,* 2002; Saaristo *et al.,* 2002; Takahashi *et al.,* 2004) could return to their normal condition leading to reversion of lymphatic vessel dilation.

Moreover, all study patients were given ivermectin plus albendazole at 6 months and although no ameliorative effects has been reported for this combination (Fernando *et al.*, 2011), there is the need to conduct more studies to investigate the effect of this combination on filarial hydrocele.

However, it is evident from the present study that when doxycycline is combined with ivermectin and albendazole, there is a greater effect on lymphatic vessel dilation than with ivermectin and albendazole alone. In spite of this, assuming that the amelioration and halt observed in the 46% placebo-treated patients were due to the activity of ivermectin plus albendazole, then that of 48% (94%-46%) of doxycycline-treated patients could be attributed to the action of doxycycline alone.

5.2 Improvement in Hydrocele Sizes after Treatment with Doxycycline

It is realized that a disability treatment strategy needs to be evolved for lymphatic filariasis patients who suffer from hydrocele, as currently the mainstay of hydrocele management is hydrocelectomy (WHO, 2002b).

A major outcome of this study is that doxycycline treatment resulted in a remarkable improvement and halt in progression of the sizes of hydroceles in 82% (47% improvement, 35% halt) of treated patients in contrast to 46% (23% improvement, 23% halt) of placebo-treated patients. The reduction in hydrocele sizes by one or more stages was observed only in patients with early stage hydrocele (stage 1 and 2). This finding confirms a report by Debrah and colleagues (2009) that a six-week treatment with 200mg doxycycline improved early stage hydrocele in patients with circulating filaria antigens. An additional observation from this study however is that, even patients who had no detectable circulating filaria antigens, adult worm nests and microfilaria had significant improvement in their hydrocele condition after doxycycline treatment. This further supports the hypothesis that doxycycline has another mode of action other than depletion of *Wolbachia*.

The ability of doxycycline to reduce hydrocele sizes as demonstrated in this study can be attributed to its ability to reduce vascular hyper-permeability via down regulation of VEGF C and D (Debrah *et al.*, 2006), resulting in less fluid accumulation (Fainaru *et al.*, 2008) in the tunica vaginalis of the testes and the gradual killing of the adult worms (Taylor *et al.*, 2010) to prevent further damage.

The observed improvement and halt in progression in hydrocele sizes in 46% of placebo-treated patients were not expected. All study patients were given ivermectin and albendazole six months after treatment. Although, this combination has demonstrated partial macrofilaricidal activity in some studies (Dreyer *et al.*, 1998; Ismael *et al.*, 1998; 2001), no study has demonstrated any ameliorative effect on hydrocele with the combination (Fernando *et al.*, 2011). It is clear from the present study that when doxycycline is combined with ivermectin and albendazole, there is a greater effect on hydrocele sizes than with ivermectin and albendazole alone. Nevertheless, if it is assumed that the observed improvement and halt in hydrocele sizes in the 46% placebo-treated patient could be due to the activity of ivermectin plus albendazole, then still that of 36% (82%-46%) of doxycycline-treated patients can be attributed to the activity of doxycycline alone. However, more research is needed to test for the effects of ivermectin plus albendazole on hydrocele pathology.

5.3 Improvement in the Thickness of Scrotal Skin after Treatment with Doxycycline

The study demonstrated a significant improvement in the thickening of the scrotal skin of doxycycline-treated patients (p=0.0120) when compared with the placebo-treated patients after 24 months. Forty seven (47) % of doxycycline-treated patients had improvement in the thickness of their scrotal skins as against 33% in the placebo group.

The use of scrotal skin thickness as an indicator of urogenital pathology in lymphatic filariasis was developed by Mand *et al* (2010), after they consistently observed a thickening in the scrotal skin that was dependent on the stage of hydrocele. They concluded that the thickness of the scrotal skin was an indicator for the risk to develop a lymph scrotum (Mand *et al.*, 2010). Thus, improvement in the thickness of the scrotal skin due to the activity of doxycycline translates into a lesser risk of developing complications associated with filarial hydrocele in the treated patients. However, the exact mechanism by which doxycycline reduces scrotal skin thickness is not known and more research is needed in the area.

5.4 Macrofilaricidal Activity of Doxycycline

Before treatment, the patients in the doxycycline arm had a total of 6 worm nests compared to just 2 worm nests for the placebo group. Twelve (12) months after treatment, 4 of the 6 (67%) worm nests in doxycycline-treated patients had been cleared. By the 24 months follow-up however, all 6 worm nests in the doxycycline-treated patients had been cleared whilst only one worm nest was cleared in the placebo group. Thus, the study confirmed the adulticidal activity of doxycycline which has been previously reported by other investigators (Hoerauf *et al.*, 2001; 2003a; Taylor *et al.*, 2005b; Debrah *et al.*, 2006).

The findings in this study is also consistent with that of a previous placebo-controlled study which showed that treatment with a daily dose of 200 mg doxycycline for 6 weeks led to a macrofilaricidal effect of 92% , observed 24 months after treatment (Debrah *et al.*, 2006). They also observed death of adult worms in 27% of placebo-treated patients. The death of the adult worms in placebo-treated patients could be a result of natural death as the worm ages since a similar rate of adult worm death has been observed in onchocercomas in untreated onchocerciasis patients (Hoerauf, 2008). It could also be the due to the effect of ivermectin plus

albendazole that were given to all patients 6 months after treatment since some macrofilaricidal effect has been reported with this combination (Dreyer *et al*., 1998; Ismael *et al*., 1998; 2001).

The confirmed macrofilaricidal effect of doxycycline is especially important in lymphatic filariasis as the pathology of the disease is associated with obstruction of the lymphatic vessels by the adult worms (Nutman, 2001), and the release of *Wolbachia* after worm death (Taylor *et al.,* 2001; 2003; Hoerauf, 2008).

The observed macrofilaricidal effect of doxycycline does not appear within days as seen with DEC (Hoerauf, 2008). In fact, a previous study by Taylor *et al* (2005b) did not find any significant macrofilaricidal activity after 12 months of treatment with a six-week course of 200mg doxycycline. However, follow-up of these patients between 18-22 months after treatment demonstrated a significant loss of filarial dance signs. This gradual, indirect killing of the adult worms by doxycycline is especially advantageous as it eliminates the severe adverse events such as pain, abscess formation or both, associated with rapid killing of the adult worms by DEC (Dreyer *et al.,* 2000; Noroes *et al.,* 2003; Babu *et al.,* 2006) and this can increase compliance to treatment.

5.5 Circulating Filaria Antigens in Filarial Hydrocele Patients

In this study, the prevalence of circulating filaria antigens (CFA) among hydrocele patients at pre-treatment was 22.5%. This finding is not surprising as several independent investigators have consistently demonstrated low CFA prevalence in hydrocele patients.

A study by Addiss *et al* (1995) showed a CFA prevalence of 43% in hydrocele patients. Later, Mand *et al* (2010) in a cross-sectional study observed that of the 1004 patients infected with lymphatic filariasis that were examined, 54% had detectable CFA. They further demonstrated

that patients with hydrocele had significantly lower CFA levels than those without hydrocele (Mand *et al*., 2010).

According to Nicolas *et al* (1997), circulating filaria antigen is a marker of adult *W. bancrofti* infection. Although adult worms of *W. bancrofti* can live for about 5 years in an infected patient, hydrocele pathology can persist for life in the absence of surgery (WHO, 2002b). Thus, in an endemic community, the absence of CFA does not necessarily exclude *W. bancrofti* as the aetiological agent of hydrocele (WHO, 2002b).

In the current study, two patients each from the doxycycline and placebo groups had reductions in their CFA levels after 24 months. Although, CFA levels were significantly reduced from pre-treatment levels in both the doxycycline and placebo-treated patients (p=0.0041 and 0.0001 respectively), no generalizations can be made because of the small numbers of patients with CFA at pre-treatment and the high dropout at the follow-ups. Albeit, previous investigators have documented the ability of a six-week course of 200mg doxycycline to clear CFA in infected patients after 12-27 months (Taylor *et al*., 2005b; Debrah *et al*., 2006).

5.6 Microfilaremia in Filarial Hydrocele Patients

Of the 44 patients recruited for this study, only one had microfilaria at pre-treatment. This finding is not surprising as previous studies have consistently reported very low prevalence of microfilaremia in patients with chronic manifestations of lymphatic filariasis such as hydrocele (Michael *et al*., 1994; Addiss *et al.*, 1995; Mand *et al.*, 2010).

A standard tenet in the epidemiology of lymphatic filariasis is that patent infection is negatively related to chronic disease such as hydrocele (Michael *et al.*, 1994). Epidemiologic models support the concept that individuals pass sequentially from microfilaremic to amicrofilaremic

states and then go on to develop lymphatic pathology such as hydrocele and lymphoedema (Lammie *et al.,* 1993). To this end, a study by Mand *et al* (2010) demonstrated that as hydrocele progressed from stage 1 through to stage 4, the microfilaria prevalence reduced (15%, 10%, 7% and 14% for stage 1, 2, 3 and 4 respectively). Another study by Addiss and colleagues (1995) in Haiti also yielded a microfilaria prevalence of 39% in 57 hydrocele patients examined. They further observed that men with hydrocele but without microfilaria had significantly larger size and longer duration of hydroceles than microfilaremic hydrocele men. This generally low microfilaria prevalence observed in hydrocele patients could be due to the absence of adult worms through natural death or the presence of adult worms that have lost their reproductive ability with age.

Although the present study could not assess the microfilaricidal activity of doxycycline due to insufficient microfilaremic patients, the ability of doxycycline to clear microfilaria has been demonstrated by previous investigators (Hoerauf *et al.*, 2000; 2001; 2003a; 2003b; Debrah *et al*., 2006; 2007; 2009; Taylor *et al.,* 2010). They conclude that although doxycycline does not kill microfilaria directly, it blocks embryogenesis and subsequent microfilaria production by the female adult worms; the already produced microfilariae die off after a year (Hoerauf, 2011).

5.7 Safety of Doxycycline

In this study, the adverse effects associated with doxycycline treatment did not vary significantly from those in the placebo group (p=0.3148), indicating that treatment with doxycycline is relatively safe.

Doxycycline is a relatively well-tolerated drug in the tetracycline class (Leggat, 2009). It is already registered and is widely used in clinical practice as a broad spectrum antibiotic. It also

remains one of the major "weapons" for the treatment of a variety of atypical infections. It has an important application as a malaria chemoprophylaxis in a number of countries (Leggat, 2009; Holmes and Charles, 2009; Sagar, 2010).

In more than 1000 volunteers treated with doxycycline so far, there have not been any severe adverse effects (Hoerauf, 2008) and there are a variety of strategies that can be used to reduce the incidence of some of the common side-effects of doxycycline (Leggat, 2009; Holmes and Charles, 2009; Sagar, 2010). Doxycycline is however contraindicated in those with allergy or sensitivity to the drug, pregnant and lactating women and children below 8 years.

CHAPTER SIX

6.0 CONCLUSION

Antiwolbachial chemotherapy of lymphatic filariasis is still being investigated as an alternative method of treatment for individuals living with lymphatic pathology in endemic communities (Debrah *et al.,* 2006; 2009).

This study has demonstrated that doxycycline significantly reduces and halts the progression of dilation of scrotal lymphatic vessels, hydrocele sizes and thickening in the scrotal skin 24 months after treatment. Additionally, doxycycline cleared all 6 worm nests present at pre-treatment in contrast to the loss of only one worm nest in the placebo group, confirming the previously reported adulticidal effect of doxycycline. Thus, the present study has shown that in addition to its macrofilaricidal activity, doxycycline has an ameliorative effect on filarial hydrocele and its associated conditions such as scrotal skin thickening and dilations in scrotal lymphatic vessels. By reducing scrotal skin thickness, doxycycline also reduces the risk of developing lymph scrotum.

The study has also demonstrated that although a successful treatment with 200mg doxycycline requires 6 weeks of treatment, it has limited side effects and more importantly does not elicit the adverse reactions of drugs that kill adult filariae too rapidly, which could result in pain, abscess formation or both (Dreyer *et al.,* 2000; Noroes *et al.,* 2003; Taylor *et al.,* 2005b; Babu *et al.,* 2006) and this could increase compliance of patients to treatment.

Although the present study could not determine the impact of doxycycline on circulating filaria antigens and microfilaremia in hydrocele patients because of the low prevalence at pre-treatment and high dropout rate at the follow-ups, the ability of doxycycline to clear circulating filaria antigens and microfilaremia has been documented by previous investigators (Hoerauf *et al.*, 2003a; 2003b; Debrah *et al*., 2006; 2007; 2009; Taylor *et al.*, 2010).

Together, the data from this study provide evidence that doxycycline can be used for the successful treatment of filarial hydrocele as it leads to amelioration in early stage hydrocele and a halt in progression in late stage hydrocele and is relatively safe. Meanwhile, doxycycline can be administered at the individual level at the health centers in endemic communities although the current duration of treatment is not applicable for MDA programmes. It can also be given to individual patients who have left a transmission area as it can achieve a strong reduction of the adult worm load in the absence of any re-infection.

6.1 Recommendations

It is recommended that doxycycline should be incorporated into mainstream treatment of filarial hydrocele in our health centers as it has demonstrated significant ameliorative ability on the condition and it is generally safe.

Long-term follow-up of the study patients is also recommended so that the extent of doxycycline activity can be assessed. This will aid in the design of treatment programmes involving the use of doxycycline. Additionally, a placebo-controlled study to assess a six-week course of doxycycline alone without ivermectin and albendazole should be carried out to see exactly what percentage of amelioration of hydrocele can be attributed to doxycycline.

The health systems in endemic communities must be developed to aid in early diagnosis of filarial hydrocele which can then be treated with doxycycline. Late stages of hydrocele should be referred for surgery but doxycycline can be administered to clear all adult worms, microfilaria and circulating filaria antigens to prevent relapse after surgery. When the community health systems are properly developed, even a six-week treatment with doxycycline can be effectively transferred to the responsibility of a village through community-directed treatment methods as demonstrated by Wanji *et al* (2009) in Cameroon.

Moreover, the potential use of antibiotics for treating lymphatic filariasis is far from exploited. Research should now focus on identification of treatment, based on doxycycline or other antibiotics that are practical for use in mass treatment and have strong ameliorative and macrofilaricidal effects similar to doxycycline. Combination of two or more antibiotics can be investigated in preclinical studies and subsequently in human trials in an effort to reduce the treatment duration from the current six-week regimen.

Finally, it is recommended that more research into the biology of *Wolbachia* and the exact nature of their relationship with filarial worms be carried out. This probe could lead to the discovery of more targets for other antibiotics to serve as back-up for doxycycline and the current antifilarial drugs. More knowledge about *Wolbachia* will also help to further understand the mechanisms at play in the development of pathology in lymphatic filariasis.