UPTEC X 05 029
MAY 2005
ISSN 1401-2138

LINDA CATO

Identification of filarial vector mosquito, *Culex quinquefasciatus*, and infection using PCR assays

Master's degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 05 029	**Date of issue 2005-05**

Author

Linda Cato

Title (English)

Identification of filarial vector mosquito, *Culex quinquefasciatus*, and infection using PCR arrays

Title (Swedish)

Abstract

Culex quinquefasciatus is the main vector of the filarial parasite *Wuchereria bancrofti* which causes human lymphatic filariasis. In the present study a mass single dose treatment period with Diethylcarbamazine (DEC) and Albendazole was carried out in a filarial endemic area in Sri Lanka. Mosquitoes were collected and two detection methods were used; dissection and polymerase chain reaction. When the results from the two methods were compared the infection rate had decreased from 7.8% before treatment to 1.1% after the treatment period. The infectivity rate deceased from 1.4% to 0%. These results show that a single dose of a combination of DEC and Albendazole is an efficient program for eliminating human lymphatic filariasis.

Keywords

Culex quinquefasciatus, *Wuchereria bancrofti*, human lymphatic filariasis, Diethylcarbamazine, Albendazole, dissection, PCR

Supervisors

Professor Eric H. Karunanayake

Inst. of Biochemistry, Molecular Biology and Biotechnology, University of Colombo

Scientific reviewer

Assoc. Professor Lena Åslund

Dept. of Genetics and Pathology, Rudbeck Laboratory

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **29**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala

Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Identification of filarial vector mosquito, *Culex quinquefasciatus*, and infection using PCR assays

Linda Cato

Sammanfattning

Lymfatisk filariasis existerar i den tropiska och subtropiska delen av världen och orsakas av rundmaskarna *Wucheria bancrofti*, *Brugia malayi* och *Brugia timori*. Sjukdomen sprids via myggor och har både akuta och kroniska sjukdomstillstånd som drabbar kvinnor och män i alla åldrar. Idag är 128 miljoner människor infekterade i mer än 80 länder och 90% av de drabbade är infekterade av *W. bancrofti*. *Culex quinquefasciatus*-myggan är den huvudsakliga vektorn för *W. bancrofti*.

För att kontrollera spridningen av filaria parasiterna pågår flera storskaliga behandlingar av invånarna i de drabbade områderna. I denna studie har effekterna av en endos-behandling med medicinerna Diethylcarbamzine (DEC) och Albendazole i ett filariadrabbat område i Sri Lanka studerats. Myggor samlades in och analyserades genom dissektion och polymerase chain reaction (PCR). Den senare är en DNA-baserad metod som i denna studie används för att detektera myggarten *C. quinquefasciatus* och infektion av parasiten *W. bancrofti*. Infektions- och infektivitetsgraden hos myggorna innan och efter behandlingen studerades. Infektionsgraden är andelen infekterade myggor och infektivitetsgraden är andelen infekterade myggor där parasiten har nått det utvecklingsstadie då en ny värd kan infekteras. Innan behandlingsperioden påbörjades var infektionsgraden 7.8% och hade efter behandlingen sjunkit till 1.1%. Även infektivitetsgraden avtog från 1.4% till 0%. Dessa resultat tyder på att en endos-behandling med DEC och Albendazole har en inverkan på spridningen av lymfatisk filariasis.

Examensarbete 20 p i Molekylär bioteknikprogrammet
Uppsala universitet Maj 2005

SUMMARY

Culex quinquefasciatus is the main vector of the filarial parasite *Wuchereria bancrofti* which causes human lymphatic filariasis. The disease is a major cause of acute and chronic morbidity, affecting persons of all ages and sexes throughout the tropical and sub-tropical areas of the world. Nearly 1.2 billion people are at risk of developing lymphatic filariasis and 128 million are currently infected in more than 80 countries. Amongst the infected, 107 million (90%) are infected with *W. bancrofti* and 13 million with *Brugia malayi* and *Brugia timori*. For control of the filarial parasites, several mass treatment programs have been implemented. In particularly, treatment with a single dose Diethylcarbamazine (DEC) in combination with Ivermectin or Albendazole reduces microfilariemia effectively. To investigate new methods for identification of vector mosquito and detect infection it is necessary to follow up these large-scale control programs. Today, detection by microscopy is the most widely used method in the afflicted developing countries. The method becomes increasingly costly and laborious for large numbers of samples. A new more efficient method is being sought.

The aim of the present study is to develop new efficient tools for detection of filarial infection. The impact of a mass single dose treatment with a combination of DEC and Albendazole in a filarial endemic area in Sri Lanka was also investigated. The infection rate and infectivity rate amongst vector mosquitoes, collected in the area, were studied prior to and after the treatment period. Identification of filarial vector mosquito, *C. quinquefasciatus,* and infection by *W. bancrofti* amongst the collected mosquitoes was investigated by dissection and PCR assays. DNA was extracted from a single field collected mosquito and PCR with species-specific primers for *C. quinquefasciatus,* and *W. bancrofti* was run. When the results from the two methods were compared the infection rate had decreased from 7.8% before treatment to 1.1% after the treatment period. The infectivity rate decreased from 1.4% to 0%. These results show that a single dose of a combination of DEC and Albendazole is an efficient program for eliminating human lymphatic filariasis.

1. BACKGROUND

1.1. Prevalence of human lymphatic filariasis

1.1.1. Filariasis in the world

Lymphatic filariasis is considered one of only six potentially eradicable diseases by the World Health Organization (WHO). The disease is a result of infection by the nematode parasites *Wunchereria bancrofti* (Cobbold), *Brugia malayi* (Brug) and *Brugia timori*.[4]

Today lymphatic filariasis exists in greatest numbers in tropical regions (Central and South America, Asia, Africa and Pacific areas) and also in subtropical countries in the Middle East. Caribbean nations and coastal areas of Brazil are also endemic foci but with a lower grade of infection than those observed in Asian countries.[4, 11, 20, 15]

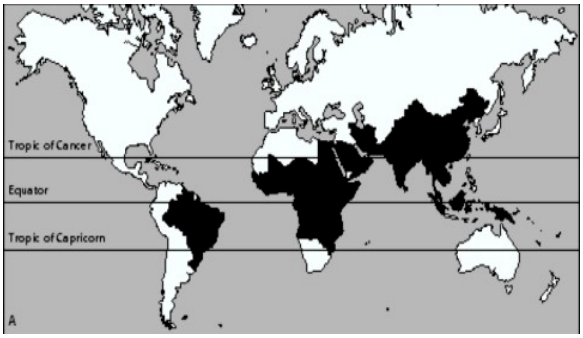

Figure 1. Prevalence of human lymphatic filariasis. Reproduced from *The Imaging of Tropical Diseases* (http://tmcr.usuhs.mil/tmcr/ chapter26/large26/26-03.jpg) with permission from Dr. M. Reeder, Tropical Medicine Central Resource.

Nearly 1.2 billion people are at risk of developing lymphatic filariasis. In additional 128 million estimated infected people in more than 80 countries the disease results in a leading cause of disability worldwide. Amongst the afflicted, 107 million (90%) are infected with *Wunchereria bancrofti* (Cobbold) and 13 million with *Brugia malayi* (Brug) and *Brugia timori*. Elephantiasis or disfiguring symptoms such as hydrocoele and lymphoedema appears amongst about 44 million people. The world-wide cost of Disability-adjusted life years (DALY) burden due to lymphatic filariasis is, in addition to the economic burden, 5.55 million of lost of productivity and medical costs.[9, 10, 11, 19, 20]

Increased unplanned urbanization and poor sanitation in developing countries in the tropics has resulted in polluted habitats that are the ideal breeding ground for mosquito species such as *Culex quinquefasciatus*, the major vector for *Wunchereria bancrofti*. Developing countries in Europe and North America have improved sanitation conditions and reduced breeding sites for mosquitoes. Transmission of human lymphatic filariasis is no longer persistent in these areas.[11, 20]

Figure 2. Ideal breeding ground for *Culex quinquefasciatus*. Private photo.

1.1.2. Filariasis parasites in Sri Lanka

Filariasis has been known in Sri Lanka for many years. As a cause of the Kalinga invasions during the 12th and 13th century, filariasis due to *B. malayi* was introduced. Bancroftian filariasis was brought to Sri Lanka by the Chinese army 200 years later.

The first reported cases of elephantiasis were detected in 1879 and cases of microfilaraemia in Matara hospital, 1892.[6, 7, 24]

Since the beginning of 20th century, several studies and assessments have been made. Dissanayake described from 1937 to 1939 two types of filariasis that were present in Sri Lanka (at that time Ceylon), the urban type due to *W. bancrofti* and the rural type due to *B. malayi*. The latter was more prevalent at the time.[6, 7]

Due to night blood screening and treatment of microfilaraemics, elimination of water plants by manual removal and residual spraying of DDT in mosquito breeding areas (mainly for elimination of Malaria mosquitoes) a dramatic decrease has been seen. No cases of *B. malayi* microfilaria have been reported after 1970.[6, 7]

In 1961, the filarial belt of Ceylon was reaching along the coast from Matara in the south to Negombo in the west. The occupied area was less than 400 square miles with a population of 1.5 million at risk of infection. In studies made in 1990 the belt had extended to about 90 km north from Negombo to Puttalam and about 120 km to the south-east of Matara to Kataragama. Today, 9.5 million are at risk of developing lymphatic filariasis in 8 districts in Sri Lanka.[7, 32]

A mosquito study was carried out in 1981-1982 of Sri Jayawardenepura, at the time the new capital of Sri Lanka was under rapid urbanization. A total of 45 species of mosquitoes were reported in the examined areas. Out of the observed species that were resting in human dwellings, about 95% was *C. quinquefasciatus.*[13]

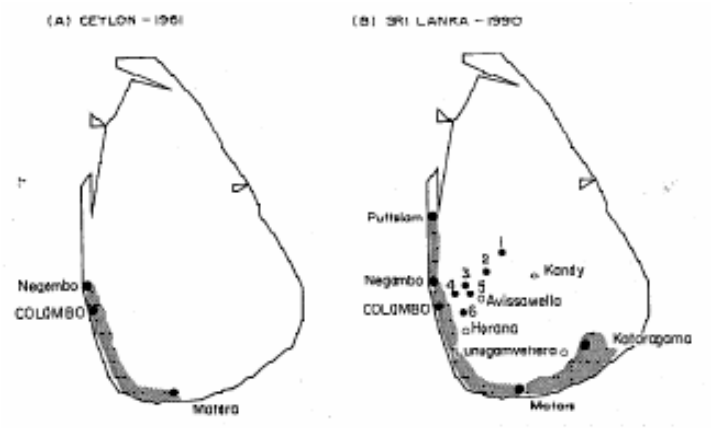

Figure 3. Prevalence of lymphatic filariasis in Sri Lanka 1961 (A) and 1990 (B). Reproduced from the Annals of Tropical Medicine and Parasitology, volume 85, page 124, with the permission of the Liverpool School of Tropical Medicine.

The National Anti-filariasis campaign in Sri Lanka has during the last ten years been recording the development of filariasis in the endemic areas in Sri Lanka. In the program, investigation of the quantity of vector mosquitoes found in the areas such as infection rate and infectivity rate amongst the mosquitoes are recorded. The female *C. quinquefasciatus* mosquitoes and other possible vectors of filarial infection are counted and dissected. The calculated infection rate is the percentage of infected mosquitoes whereas the infectivity rate is the percentage of the infected mosquitoes reached the third larvae stage. When the parasite has reached this stage it is ready to infect a new human the next time the vector takes a blood meal.

Year	Infection rate	Infectivity rate
1995	0.63	0.06
1996	0.72	0.06
1997	0.55	0.05
1998	0.56	0.05
1999	0.49	0.04
2000	0.47	0.07
2001	0.46	0.03

Percentage of new cases among patients with clinical manifestations at clinics in the endemic areas in year 1995-2001 is presented in Table 1. The results show a pending in percentage of new cases over the seven-year study. A great increase in numbers was observed during the first three years. In 1998 an apparent decrease took place to be followed by a year of increasing numbers of new cases. A great drop was again manifested in year 2000 while during the last year of the study an increase was again recorded.

The vector infection and infectivity rates island wide, were also recorded during the same period and the results are presented in Table 2. As seen in Table 2, the infection rate over the years is fairly high. However, over the years the main pattern illustrates a reduced infection rate. The infectivity of the parasites is also rather high though a slight decrease is observed over the period of the study.

Year	Cases in endemic areas	Percentage (%)
1995	2838	18.3
1996	3474	23.7
1997	4108	27.5
1998	4743	15.0
1999	1974	17.7
2000	629	6.6
2001	1408	12.4

Table 1. New cases amongst patients with clinical manifestation at clinics in endemic areas in Sri Lanka. Data from the National Anti-filariasis Campaign.

In the Western province of Sri Lanka, where the filarial parasite is widely spread, a study was carried out during 2004. The data is presented in Table 3. The infection rate was more than twice as high this year compared to the island wide study in 2001 (see Table 2).

Table 2. Vector infection and infectivity rates in Sri Lanka. Data from the National Anti-filariasis Campaign.

In addition, the infectivity rate in the region is the double compared with the results from 2001 and the equivalent situation was found in Colombo only. A worrying result when the population density of Colombo is kept in mind.

Vector density	18.03 %
Infection rate	0.92
Infectivity rate	0.06
Microfilaria rate in Colombo	0.06

Table 3. Vector densities and microfilaria rate in the Western province of Sri Lanka in 2004. Data from the National Anti-filariasis Campaign.

1.2. Lymphatic filariasis due to Wuchereria bancrofti

1.2.1. The history of *W. bancrofti*

The origin of *W. bancrofti* is believed to be Southeast Asia. An existing monkey parasite in this area, *Wuchereria kalimantani*, is the closest relative of bancrofti. The disease was probably spread, beginning around 2,000 B.C., to the South Pacific by migrants. The parasite was likely carried from Southeast Asia via Madagascar to the African continent by another migration. Small statuettes from the Nok culture in West Africa around 500 A.D. portray the enlarged scrota characteristic for elephantiasis. By the 14th and 15th century, filariasis had reached through Central Africa into the Middle East. During the 17th and 18th century's slave trade the parasite was introduced to Latin America. Migration from South America introduced the parasite in the 19th century to Australia.[3, 30]

The first definitive reports of lymphatic filariasis began to appear in 16th century. Lymphatic is also known as "the curse of St. Thomas". In 1588-1592, the Dutch explorer Jan Huygen Linschoten visited Goa. He recorded that the descendants of those who killed St. Thomas were "all born

with one foot from the knee downwards as thick as an elephant's leg.[3]

The filaria bancrofti parasite was first discovered in Paris, 1863. Jean-Nicolas Demarquay described microfilaria in human hydrocele fluid from Havana, Cuba. A patient suffering from a cyst of the testicle returned one year later with a tumor in the scrotal sac. When tapping the tumor, a thick bluish-white fluid was collected that contained large numbers of actively motile worms. The absence of internal organs in these worms indicated to Demarquay that they were embryos. Independently, in urine, Otto Henry Wucherer made the same discovery in Bahia, Brazil, 1866.[3, 17]

During the next ten years, similar discoveries in examining urine and peripheral blood were made around the world. Dr. Timothy Lewis described the adult worm in Calcutta, India 1876, while the British helminthologist Cobbolt did the same in Australia, 1877.[3, 17, 30, 31]

In 1876 A. P. de Silva Araujo began to use the name of Wucherer in association with the microfilaria found in the blood. He then described the genus and named it *Wuchereria.*[17, 30]
Later in 1876, in a lymphatic abscess from arm, the first adult filarial worm was discovered by the Brisbane physician Joseph Bancroft. The next year the species was named *Filaria Bancrofti* in his honor by Cobbolt and in 1921 the worm got its name *Wuchereria Bancrofti.*[3, 17, 30]

In 1878, Patrick Manson, known as "the father of tropical medicine" was working in the city of Amoy (now called Xiamen) along the Chinese coast in Fujian Province. Manson demonstrated that mosquitoes were intermediate hosts for the lymphatic filariasis parasite. Manson also displayed the life cycle of *W. bancrofti* in 1878. However, Manson thought that the parasite escaped from the mosquito into water and that humans acquired infection by drinking contaminated water or via penetration of the skin. The actual mode of transmission was not established until a suggestion by the Australian parasitologist Thomas Lane Bancroft, son of Joseph Bancroft, were followed up by Manson's assistant George Carmichael Low, who demonstrated the presence of microfilaria in the mouthparts of mosquitoes in 1900.[3, 30, 31]

1.2.2. Classification and life cycle of *W. Bancrofti*

Wuchereria bancrofti belongs to the Phylum Aschelminthes, Class Nematoda, Order Filariidea, Family Dipetalnematidae, Subfamily Dipetalonemtidae. In the afflicted human the adult male and female worms of *W. bancrofti* species inhabit primarily the lumen of lymphaties. The sexually mature adult is the largest of all stages and the adult worms of lymphatic filariasis may live for 7-10 years. The length of adult male and female worms is 20-40 mm and 6-10 cm, respectively. After mating the adult female filarial worm, that is ovoviviparous, produces 10,000 or more of microfilaria or first stage larvae (L1). The microfilaria measures 270 µm by 10 µm and is encased in a sheath comprised of chitin. The sheath is possibly a remnant of an eggshell.[20, 24, 31]

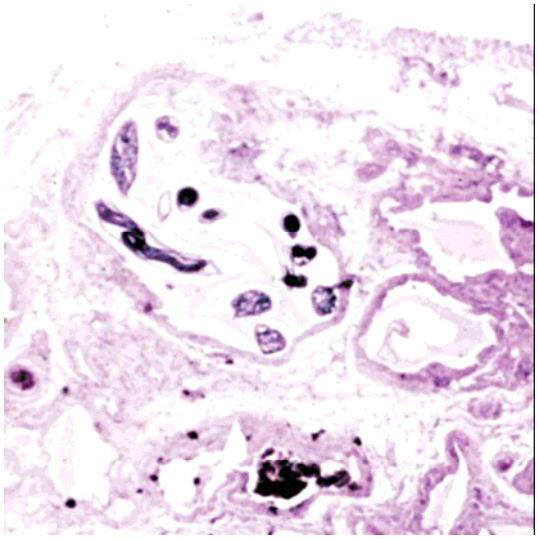

Figure 4. Adults of *W. bancrofti* in

lymphatic vessels. Reproduced from Filarial Journal (http://www.filariajournal.com/content/2/1/11) with permission from Dickson Despommier.

In the female mosquito, during the first gonotrophic cycle postinfection, the microfilaria penetrates the midgut wall. They locate the thoracic flight muscles where they undergo three molts. The microfilaria shortens by metamorphosis into sausage-shaped bodies 240-250 µm in length. The tail portion atrophies and the intestinal tract of the helminthes become well defined. A second molt follows and the infective larvae at this third stage (L3) are slender and measure 1.4-2 mm in length. The period of time from ingestion of microfilaria to maturation of infective larvae in the insect muscle tissue is 10-14 days. In *C. quinquefasciatus* the majority of eggs laid by the females are produced during this cycle.[15, 20, 31]

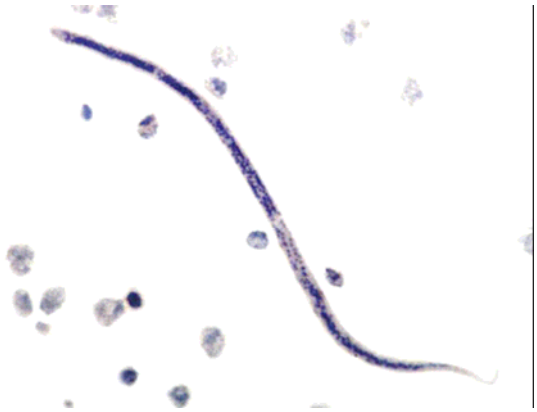

Figure 5. Microfilaria of *W. bancrofti*. 250µm. Reproduced from Filarial Journal (http://www.filariajournal.com/content/2/1/11) with permission from Dickson Despommier.

The third stage larvae locate to the biting mouth parts of the mosquito. The new host is infected when the microfilaria are deposited onto the skin adjacent to the bite wound during consumption of a subsequent blood meal. When the mosquito withdraws her mouth parts, larvae crawl into the open wound. The immature worms enter the skin and migrate through the subcutaneous tissues to the lymphatic vessels. There they molt to fourth-stage larvae and eventually develop into sexually adult worms.[20, 31]

The prepatent period (the time from infection to detection of microfilaria released by productive adult female worms) is approximately 1 year. Soon after copulation the worm begins shedding microfilaria. The longevity of adults, measured by the continuous production of microfilaria, is estimated at 5-8 years. Infections lasting 40 years have been reported.[20, 31]

1.2.3. Vector – parasite relationship

1.2.3.1. Vector of human infection

There are three forms of *W. bancrofti*, one of which is more widely distributed in the tropics, and is primarily spread by culicine mosquito vectors; the other two, spread mainly by anopheline mosquito vectors, are common in sub-Saharan Africa, a small focus in Egypt along the Nile River, South Asia (especially India, Sri Lanka and Bangladesh) extending east into Indochina, Indonesia, and Papua New Guinea, northern coastal regions of South America (Brazil, Guyana, Suriname) and parts of the Caribbean (Haiti, Dominican Republic, Trinidad and Tobago). These later forms are nocturnal, referring to the periodicity with which the microfilarias appear in the peripheral circulation. A diurnal *W. bancrofti* exists in the South Pacific area whose primary vectors are day-biting mosquitoes of the genus Aedes. There are no animal reservoir hosts for this parasite.[31]

Culex quinquefasciatus Say, the main vector of *W. Bancrofti*, feed nocturnally, with peaks landing on 22.00 and 02.00 hours. In general they feed on juices from plants. Only the females of *C. quinquefasciatus* Say feed on blood after mating and before they lay their eggs.[1]

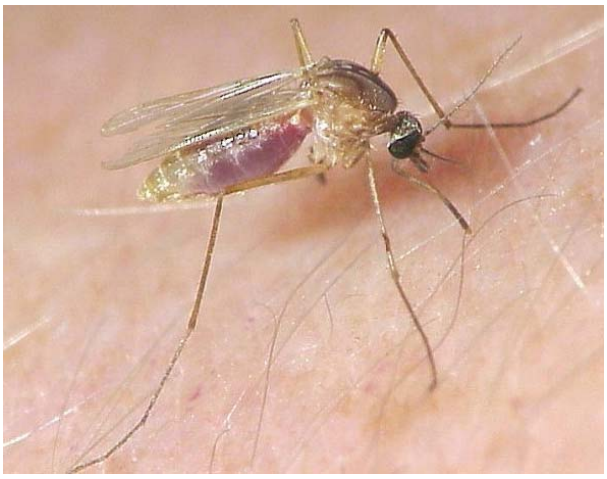

Figure 6. Adult female mosquito of *Culex quinquefasciatus*. Reproduced from NSW Arbovirus Surveillance & Vector Monitoring Program with permission from Richard Russell.

Figure 7. Adult male mosquito of *Culex quinquefasciatus*. Reproduced from NSW Arbovirus Surveillance & Vector Monitoring Program with permission from Richard Russell.

They release the excess fluid from their blood meals by diuresis. As the mosquito ingests blood, sensory information may inhibit movements in the mid gut allowing fluid to be moved back to the rectum for excretion. This process will continue while resting on walls, in cupboards, under beds and other dark areas. Within 6-8 hours after feeding the duiresis is completed and they leave their resting place.[1, 26]

The survival of the vector *C. quinquefasciatus* is an important determinant of the transmission dynamics of the human filarial parasite *W. Bancrofti*. For successful transmission of the parasite, the vector must survive longer than 12 days. This is the time frame for the parasite to develop to the stage of infective larva.[14]

1.2.3.2. Microfilarial periodicity

Microfilarias of *W. bancrofti* have unique circadian rhythms manifested as a variability of their number or concentration in the peripheral blood. They are classified as the nocturnally periodic strain and have peak concentrations from 22.00 to 02.00 hours.[20, 31]

During daytime hours no or few microfilaria are detected in the bloodstream. The periodicity of *W. bancrofti* is dependent primarily of the daily activities of the host and not alterations of day and night. When the activity of the host is increased the microfilaria migrate to the lungs and aggregate in the capillaries.[20, 31]

Nocturnal periodicity can be a result of the microfilaria's penchant for low oxygen tension, at which time they are found in the peripheral blood stream. Studies suggest that their periodicity is due to differences in oxygen tension between the arterial and venous blood in the lungs, or it may reflect slight pH changes in the pulmonary venous circulation during sleep. Experiments in which sleep habits of infected volunteers were reversed also reversed the periodicity of microfilaria.[20, 31]

The microfilarias of *W. bancrofti* have the same circadian rhythm as the feeding time of their vector. This coincidence suggests that the parasite has adapted their periodicity to the vector feeding behavior, possibly to facilitate their transmission. This relationship is reversed for the diurnally subperiodic form of *W. bancrofti* in the South Pacific. The form is transmitted by day-biting mosquitoes of the species *Aedes,* which is a poor vector of the nocturnally periodic strain.[20, 31]

1.3. Epidemiology

1.3.1. Intensity and efficiency of transmission

Definition of the prevalence and intensity of filarial infection is important for several reasons. The distribution of infection and disease of a population, in epidemiologic investigations, provide important information. This is necessary for rational allocation in areas of limited resources (i). The relationship between infection intensity and clinical sequels in addition to morbidity on a population can be assessed (ii). Epidemiology data are crucial in evaluating the impact of control measures such as vector eradication, chemotherapy or a vaccine (iii). The understanding of density dependence in transmission of the parasite is crucial for assessing the prospects of elimination (iv).[4, 20, 28]

World Health Organization (WHO) uses the terms "annual biting rate", "annual infective biting rate" and "annual transmission potential" for entomological parameters of infection. Important issues should be considered when using these parameters. Because the environmental conditions vary enormously among endemic areas, the disease epidemiology of *W. bancrofti* is depending on the local vector and the periodicity of the parasite. For instance, the transmission efficiency is known to be higher in Africa when compared with Asia. In most cases, *Anopheles* transmits disease much more efficiently than *Culex*.[4, 9, 20]

This dynamic of transmission efficiency can change significantly in endemic areas that implement mass treatment for disease control. Several studies, there of mathematical and statistical models, of this dynamic between parasite and vector have been made. These research results have great implications in the implementation of appropriate strategies of disease control. (See "Treatment and control" below).[4, 10, 14, 26, 28]

1.3.2. Prevalence of filarial infection

The mean prevalence of filarial infection is in general lower in females, when compared with men. This difference has been discussed weather it is a cause of lower exposure to the parasite amongst women or to immunological differences in the two sexes. Epidemiological studies show that the difference is great when compared with women in their reproductive years. This is also shown amongst children < 5 years. The transplacental immunity and breast-feeding may affect the prevalence and intensity of infection. A higher degree of resistance to infection, amongst offspring of previously infected mothers than those of uninfected mothers, is also detected.[4, 20]

In endemic areas, the prevalence of infection and intensity of microfilaremia decreases amongst people >50 years. A theory, that residents acquire protective immunity to filaria with repeated exposure, has been discussed. In addition to this, a higher incidence of pathology is detected in infected older people. This is presumably a cause of the death of the adult worms, which in some individuals disturb the fine balance between host and parasite.[5, 20]

1.4. Clinical aspects

1.4.1. Models of human filariasis infection

Two models of human filariasis have been reported: a statistic immunological viewpoint (i) and a dynamic model (ii). The statistic immunological model proposes that the outcome of infection could take two different routes. These are either a patent infection without overt pathology, or a form of inflammatory pathology without microfilaemia. Different immune responses lead to either infection

carrier or developing the disease. The dynamic model proposes that a patent infection, microfilaraemia and amicrofilaraemia, leads to development of the disease, by microfilaraemia and/or adult worms, in the host.[22]

1.4.2. Clinical manifestations

Human lymphatic filarasis due to *Wuchereria bancrofti* occurs in all ages and both sexes, but prevails in groups of low socioeconomic levels. This chronic disease is characterized by clinical manifestations such as lymphedema, elephantiasis and/or hydrocele. Most clinical manifestations are due to damaged lymphatic vessels, believed to be the result of several additive factors, including actions of the parasite itself, inflammatory response to the parasite, and parallel opportunistic infections. [8, 9, 25]

Amongst the afflicted, three groups are observed in the endemic areas. The first group incorporate patients that are often free of overt chronic disease manifestations, but with an active patent infection (i). Patients who display one or more of the disease manifestations such as hydrocele and/or lymphedema, with or without detectable filarial infections are distinctive for the second group (ii). The third group represents subjects who are free of patent infection and disease (iii). In addition to these physical manifestations of the infection, sub-clinical lymphatic and kidney damage occur among persons harboring adult worms and microfilaria, respectively.[9, 22]

1.4.2.2. Asymptotic infection

The majority of residents living in an endemic area do not manifest strong inflammatory responses to their filarial parasite load. They are noted to be asymptomatic even though they harbor circulating microfilaria. Resent studies of asymptomatic patients indicate slight pathology when examined more closely. For instance many of the patients considered asymptomatic do have impaired lymphatic drainage when they are imaged with sophisticated radionuclide studies. The asymptotic infection has two degrees of severity.[31]

Residents of endemic areas are amicrofilaremic and lack characteristic symptoms of lymphatic filariasis. Studies have manifested that the concept of chronic pathology is uniformly associated with amicrofilaremia. It is presumed that these subjects are exposed to infective larvae with a frequency similar to that of other persons residing in the same endemic region. Such individuals may be "partially" resistant to infection and exhibit high lymphocyte proliferation responses to filarial antigen.[4, 20]

The next degree of severity is asymptomatic microfilaremia. Studies of the immunopathogenesis of subjects in this category indicate presence of T-lymphocytes which are specifically unresponsive to filarial antigens prepared from worms solubilized in aqueous buffer.[20]

1.4.2.3. Acute manifestations

Some children with prolonged exposure to infected mosquitoes will go on to develop the next stage in the pathologic sequence of filarial infection. This occurs frequently during the adolescent years and is manifested with fevers and painful swellings over the lymph nodes.

Microfilaremic afflicted teenagers and adults can develop acute lymphadenitis. This typically occurs in the inguinal area. Episodes of painful swellings can last up to a week and recur five to eight times per year. Secondary bacterial infections may also result of this syndrome.[4, 20]

Some short-term travelers to endemic areas can also develop acute lymphadenitis. This may be due to hypersensitive immune reaction to developing or molting larvae. These individuals rarely have microfilaria detectable in the bloodstream. The phenomenon was described in the 1940s among troops returning from the Pacific theatre.[4, 20]

Continued exposure to infective larvae may eventually result in chronic manifestation. These symptoms are usually not evident until the age of >30 years.[20]

Filarial fever is an acute recurrent fever with headache, malaise and chills. Fever persists for 3-5 days. It is usually accompanied by symptomatic lymphangitis or other early signs of filariasis but may occur as fever alone.[16]

1.4.2.4. Chronic manifestations

A subset of patients with acute lymphadenitis and filarial fevers will go on to develop lymphoedema of the arms, legs, breasts and genitalia. These chronic manifestations of human lymphatic filaraisis appear most frequently later in life.[8, 20]

Genital manifestations are seen amongst subjects with inflammation of the lymphatics of the male genitalia such as epididymitis, thickening of the spermatic cords, transient hydrocele or testicular swelling.[20]

Bancroftian filariasis causes elephantiasis of the extremities and breasts as well as involvement of the male genitals, characterized by irreversible hydreoceles and thickening of the vas deferens. Patients with these severe disease manifestations are usually amicrofilaremic, although microfilaremia may persist in persons living in areas where transmission is intense.[20]

Lymphoedema can be classified as follows:

- *Grade I lymphoedema:* mostly pitting oedema; spontaneously reversible on elevation.
- *Grade II lymphoedema:* mostly non-pitting oedema; not spontaneously reversible on elevation.
- *Grade III lymphoedema (elephantiasis):* gross increase in volume in a grade II lymphoedema, with dermatosclerosis and papillomatous lesions.[16]

In maturity, the individual undergoes the consequences of filarial infection contracted early in life. After a period of quiet and apparently balanced host-parasite interplay, the disease begins to show its unpleasant face. In men, the gain and loss of filarial infection is the key factor leading to hydrocoele. In women, recurring bacterial skin infection is the main compounding factor that causes lymphoedema.[8]

The exact cause of elephantiasis is not jet fully understood. Studies confirm that patients with elephantiasis tend to have relatively high antimicrofilarial antibody titers and strong T-cell proliferation to antigens derived from adult stage parasites. The cause may be an immunopathological response to the dead or dying worms. In addition, the living microfilaria and worms suppress this reaction. Exactly how living worms and microfilaria suppress the host inflammatory response is unknown, except for the observations that microfilaria produce prostaglandin E2, a modulatory agent for leukocytes, and adult worms secrete anti-mitotic and immunosuppressive substances. It is also acknowledged that a secondary bacterial/fungal infection contributes to elephantiasis.[5, 20, 31]

Another chronic manifestation is chyluria. The symptom is defined as the excretion of chyle in the urinary tract. This is related to

the blockage of the lymph nodes which may permit flow of chyle into the urinary tract. The condition is usually painless and microfilaria may or may not be present in these patients.[16]

1.4.2.5. Tropical pulmonary eosinophilia (TPE)

TPE develops in some individuals with filarial infections. Hypersensitive responses predominate early during this course. The syndrome occurs frequently in restricted geographic regions such as southern India, particularly in young adult men. Extreme elevations of serum immunoglobulin E (IgE), high levels of IgE and IgG anti-microfilarial anibody titers and amicrofilaremia are characteristic for TPE. Other symptoms are nocturnal asthma, fatigue, weight loss and eosinophilia.[20, 31]

Administration of Diethylcarbamazine (DEC) is a highly effective of patients with TPE symptoms. If the disease remains untreated, chronic restrictive pulmonary disease may develop. The pathogenesis of this syndrome is likely secondary to local immune reactivity to microfilaria in the pulmonary vasculature. Eosinophil accumulation in the lung and the release of potentially toxic molecules such as eosinophil cationic protein and reduced oxygen products cause tissue damage with following fibrosis.[20, 31]

1.5. Immunology

Human lymphatic filariasis is clinically a spectral disease. The pathogenesis of lymphatic filariasis is, because of the complex interaction of the parasite with the host immune system, one of the least understood aspects of the infection. Its characteristic damage is thought to involve three components: mechanism damage by motile worms; bacterial super-infection in previously damage vessels and local immunological response to circulating filarial antigen (CFA). The most consistent finding of studies conducted in several endemic areas is manifested suppression of cell-mediated immunity to mitogens and filarial antigens.[4, 5, 20]

As the only multicellular parasite to reside in the lymphatics, the adult filariae worm evolved a multiplicity of anti-inflammatory strategies that enhance its survival. The infection is initiated by L3 deposited in the skin, itself an immunological organ containing Langerhans cells and keratinocytes. Both components have the capacity to modulate local inflammatory responses.

Distinct antigens are displayed on the surface of the parasite and excreted as metabolic products. Some antigens can be functional enzymes. For example, microfilarial stage-specific chitinase antigen appears as microfilaria mature and becomes able to infect and develop in a susceptible mosquito vector.[5, 20]

The high levels of microfilaria that the adult female worm produce is a strategy to contribute to the antigen load in infected individuals. Access of the parasites excretory-secretory products to immune cells allows them to interfere directly with the immune responses such as T-cell activation, antigen presentation and cytokine production.[5]

The immune response phenotypes such as filarial specific T-cell proliferation, IFN-γ, IL-5 and IL-10 production, levels of antibodies to filarial carbohydrates, level of filarial IgG1, IgG2, IgG4 and presence of anti-sheath antibodies are significant different between the clinical poles of individuals. The immunopathogenesis of the two groups of patients, asymptotic microfilaremia (i) and chronic pathology (ii), have been studied. Individuals of the first group manifest immunologic hyporesponsiveness to filarial antigen

during *in vitro* test. In comparison, individuals of the latter group have increased filarial antigen-specific lymphocyte blastrogenesis, T- and B-lymphocyte precursor frequency and serum IgG levels.[4, 22]

The surface and excretory-secretory antigenic molecules elicit high titers of immunoglobulin M, IgG and IgE antibodies. Resent studies show that specific subclasses of IgG antibody may be involved in determining the outcome of infection. IgG3 antibody responses to filarial antigens are made almost exclusively by patients who develop lymphatic pathology, whereas production of IgG4 antibodies were present in significantly higher titers in asymptotic microfilaremic subjects than in symptomatic amicrofilaremic persons with elephantiasis.[20]

1.6. Treatment and control

1.6.1. Detection and treatment of human lymphatic filariasis

The most widely used diagnostic method has been the detection of microfilaria in peripheral blood by microscopy. The examination of thick smear is relatively simply and can be performed at low cost. However, due to the nocturnal periodicity of the nematodes in most parts of the world, late night examinations are required and low levels of microfilaria are difficult to detect. A more sensitive technique is membrane filtration of venous blood. However, this is also an inconvenience to communities where the disease has a nocturnal periodicity. In addition, the membrane filtration requires well-trained personnel to perform the filtration and read the results.[4]

The specificity of DNA probes has increased as more repetitive sequence information becomes available. Specific oligonucleotides have been synthesized from the conserved regions that are species-specific and used as either hybridization probes or as primers for polymerase chain reaction (PCR). These give more sensitive and cost effective diagnostic tests for detection of filarial parasites in humans.[4, 18]

Diethylcarbamazine (DEC) has been the standard chemotherapy for all forms of human filariasis. The veterinary medicine ivermectine may have some advantages over DEC in the treatment of lymphatic filariasis. DEC acts primary as a microfilaricidal drug and may be a reason to the host immune reactivity to microfilaria being modified after administration. Ivermectin has an advantage over Diethylcarbamazine in that only one dose is required to have a comparable therapeutic effect. However, DEC is still considered the treatment of choice for lymphatic filariasis.[20, 31]

Adverse effects during treatment are common. Toxicity from DEC is of two general types. Direct toxicity is dose related, manifested as nausea and vomiting, and associated to central nervous system effects of DEC. This form of toxicity occurs in both infected and uninfected individuals. The other major form of toxicity occurs in infected persons only and appears to be secondary to death of microfilaria. The symptoms that are related to death of the microfilaria include fever, nausea, vomiting, depression, and headache and, in the most severely affected individuals, asthmatic attacks. Other symptoms are painful scrotal nodules associated with the killing of the adult worms.[9, 20]

For years the recommended treatment for lymphatic filariasis has been 12 days of DEC, a course of therapy that is not open to mass treatment campaigns. DEC is an effective microfilariacide and particularly

effective macrofilariacide, although the treatment often comes with adverse side effects. In addition, at least as effective is a single dose of DEC with Albendazole that results in dramatic and sustained reduction in blood microfilaria (MF) counts.[9, 23]

Long-standing research to develop a macrofilaricide with qualities compatible with public health use has, to date, failed. Today, the Human Genome Project gives new options when providing the potential for identification of susceptibility/resistant genes for infection. An aspect to be considered within the control program is to investigate the genetic background on the success of individual treatment.[5, 19]

The genomic sequences of parasites are rapidly becoming available. For identification of new drug targets, the challenge to use this information efficiently will require functional genomics tools such as bioinformatics, microarrays, proteomics and chemical genomics to allow development of optimized lead compounds.[2]

Over recent years, the *Wolbachia* endosymbionts of filaria has been in focus as potential drug targets. Studies were antibiotics are being used to eliminate the bacterial endosymbiont *Wolbachia* have led to a new approach for treating filarial nematodes. Doxycycline is effective at reducing *Wolbachia* from *W. bancrofti*. A daily dose of 200 mg doxycycline for six weeks resulted in a significant reduction of *Wolbachia* DNA measured by PCR. Today, research is focused on evaluating the microfilaricidal effect of doxycycline treatment of bancroftian filariasis. Also, to investigate the loss of bacterial endosymbionts on the development pathology and adverse reactions to current drugs.[19]

The Parasite Genome Project has the potential to identify novel targets for vaccine design, for drug development and for diagnosis. However, these advances in basic research will not be translated into the field without the continuing commitment of both national and international funding agencies.

1.6.2. Mass drug treatment

Human lymphatic filariasis control strategies currently rely for operational purposes on drugs that have microfilaricidal activity. Since 1988, Merck and Co Inc. donated ivermectin for treatment of onchocerciasis for as long as needed; a treatment that the elimination program of lymphatic filariasis also benefit from. Another generous drug donation was made by GlaxoSmithKline (GSK) for control of lymphatic filariasis. The drug Albendazole was provided in 1998.[19]

In the Dano district in the Bougouriba Basin, Burkina Faso is fascinating. In between 40-75% of the children under 15 years lymphatic filariasis was found with prevalence of circulating filarial antigen (CFA). A biannual treatment with ivermectin started in 1996. One group (i) in the area was treated twice a year with ivermectin and another control group (ii) without any ivermectin treatment covered 65% of the population during six years. Blood-film examinations and CFA tests indicated that treatment with ivermectin alone reduced the prevalence and intensity of *W. bancrofti*, but the transmission still continued, due to the CFA levels in the younger age groups. These results manifest that ivermectin decreases the levels of microfilaria, but cannot alone eliminate lymphatic filariasis during a six-year treatment.[19]

Several studies have shown that mass treatment with a single dose of a DEC, ivermectin, Albendazole or a combination of these drugs for 5-8 years leads to strong reduction in the prevalence and intensity of microfilaria. Although the results of

community-based trials are promising, the number of treatment rounds in these studies is usually limited.[9, 27]

In countries were onchocerciasis and lymphatic filariasis are co-endemic (Africa and Yemen) ivermectin cannot be provided because of the contraindications of the use of DEC in patients with onchocerciasis. When Albendazole is not registered as treatment for lymphatic filariasis, an expansion of the lymphatic filaraisis program in all countries have been made. A period of safety monitoring of two-drug combination: (i) ivermectin and Albendazole (in Africa and Yemen) and (ii) DEC and Albendazole (in other endemic regions). The results showed that two-drug treatment was significantly more effective than treatment with either drug alone, yielding up to 99% clearance of microfilaraemia up to one year after treatment.[19, 21]

Following studies manifested that: (i) Albendazole co-administrated with ivermectin was equally effective as DEC in combination with ivermectin against *W. bancrofti* infection (a two-drug regime that could be used safety in Africa and Yemen) and (ii) Albendazole could also be safely used in combination with DEC to treat lymphatic filariasis elsewhere in the world.[21]

The frequent and severity of reactions (similar to those seen in individuals after DEC treatment) in long-term, high-dose Albendazole-treated individuals discouraged further study of this treatment regime, even though the effectiviness of Albendazole against *W. bancrofti* infections had been clearly established. However, a single dose of Albendazole (600 mg), especially in combination with ivermectin or DEC, showed long-term effectiveness and safety in decreasing levels of microfilaria in *W. bancrofti* infections.[21]

For successful effect and elimination of filarasis a few constrains should be kept in mind regarded annual treatment. It is difficult to develop an infrastructure capable of distributing the drug (i), the need of high coverage levels (ii) and reduction of fulfillment because of adverse reactions (iii). A combination of prepared salt and DEC has shown that the lower dose of DEC in these studies was better tolerated and the adverse effects rarely seen. Detecting severe adverse reactions that are probably associated with therapy during the treatment of very large populations is challenging, particularly because hospitalization following treatment is an indicator of a severe reaction.[9, 19]

2. INTRODUCTION TO THE PRESENT STUDY

The high productivity of *Culex quinquefasciatus*, together with the increase in number of breeding places due to uncontrolled urbanization, may have contributed to the recent increase in prevalence rates of *W. bancrofti* from 9.7% up to 14.9%. Mass treatment aims at reducing the microfilaria load in the population, thereby reducing both microfilaria uptake by mosquitoes and transmission of infection.[15, 27]

Lymphatic filaraisis is today targeted for a global elimination program. Combined drug treatment regimens using annual treatment with microfilaricidal drugs are the method of choice for combating filarial infection. Elimination programs using this strategy have commenced in more than 30 countries.[8, 10]

In 1947 the Government established the National Anti-Filariasis Campaign (AFC) in Sri Lanka (at that time Ceylon) and is under the control of the Deputy Director of Health Services. The head quarter is located in Colombo with nearly 20 field

stations in the endemic areas. The current program of AFC is (i) vector control, (ii) parasite control and (iii) health education. An important issue is to educate citizens about breeding places of filaria vectors.[6, 12]

The Anti-Filariasis Campaign (AFC) introduced in August 2003 a mass single dose treatment with Diethylcarbamazine (DEC) and Albendazole as a prophylactic measure (Prof Eric Karunanayake, personal communication, July 2004). This is carried out once every six months in the filariasis endemic areas. The dosage of DEC used for children 2-12 years is 150 mg and for people above 12 years the dosage is 300 mg with 400 mg of Albendazole used in both groups.

The access of tools to observe the presence or absence of parasites in the vector, both efficiently and effectively, is an essential requirement. Due to the lack of volunteer humans to submit to regular blood examinations, an assessment of infection in vectors has been applied. This gives advantages for the monitoring of infection after a mass treatment program,.[1, 10]

Two methods of detecting infection in vector populations that are now being widely used are dissection and PCR. Dissection is the traditional tool for measuring infection levels in mosquitoes. However, dissection becomes increasingly costly and laborious in areas where mosquito infection prevalence drops below 1%.[10]

Preliminary studies demonstrate that PCR technique is a more sensitive tool for detecting filarial parasites in mosquitoes than conventional dissection and microscopy. The ability to adapt PCR techniques to detect microfilaria in pools of mosquitoes suggests that current PCR methods should be adequate for testing large numbers of mosquitoes in the context of filariasis elimination programs. Although a PCR-based method for the detection of filarial larvae in mosquitoes was successfully developed several years ago, the technique is not yet widely used as a practical tool for routine control of filarial infection.[1, 10]

Several studies of different diagnostic tools for monitoring the progress of lymphatic filariasis elimination programs have been made. PCR assays for detection of microfilaria DNA in blood have been developed[10]. Other research groups have compared dissection with PCR of extracted DNA from pools of collected mosquitoes, in relation to mass drug treatment programs. The results manifest the PCR as a more sensitive tool for detection of filarial infection.[24, 29]

The aim of this study is to investigate the impact of a single dose treatment with a combination of DEC and Albendazole in a filarial endemic area in Sri Lanka. With this study, a new tool for identification of infection in a single filarial vector mosquito was also introduced and tested. DNA is extracted from a single field collected mosquito and the traditional dissection method is compared with PCR assays with species-specific primers for *C. quinquefasciatus,* and *W. bancrofti*.

3. MATERIAL AND METHODS

3.1 Adult house resting mosquito collection

In September 2004 a mass single dose treatment of DEC and Albendazole was carried out in the filariasis endemic area Piliyandala, Bokundara in Colombo, Sri Lanka. A dosage of 150mg DEC and 400 mg Albendazole for children 2-12 years was given. For people above 12 years, a dose of 300 mg DEC together with 400 mg Albendazole was used.

Figure 8. Piliyandala, Bokundara in Colombo, Sri Lanka; endemic area of bancroftian filariasis. Private photo.

During August and November 2004, the National Anti-filariasis campaign in Sri Lanka collected mosquitoes from human dwellings in the filariasis endemic area. The collection was carried out during daytime when the mosquitoes usually rest on surfaces close to their blood meal source after feeding. The resting mosquitoes were collected using mouth aspirators. The mosquitoes were made unconscious with a small amount of chloroform on cotton buds that were put into the tube.

Figure 9. Mosquito collection. A mouth aspirator and lightener were used for collection of resting mosquitoes in dwellings of endemic areas in Colombo, Sri Lanka. Private photo.

The mosquitoes were counted and put in tubes and the house number, time of collection and date of collection were recorded. The national Anti-filariasis campaign will study half of the collection by microscopy for identification of mosquito species and detection of infective larval stages of the *W. bancrofti.*

Figure 10. Data collection. House number and number of collected mosquitoes were recorded. Collection equipment presented on the table are sample tubes, scope, pipette, cotton buds and bottle of chloroform (white). Private photo.

The other part of collected mosquitoes will be used for extraction of DNA and PCR studies with species-specific primers for *C. quinquefasciatus* and *W. bancrofti.* The mosquitoes were transported to the insectary at the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo. The mosquitoes were held in cages of 30 cm^3 for 72 h to digest possible blood. They were fed on 10% glucose solution. The mosquitoes were then collected in tubes and stored at -80°C.

3.2. DNA extraction

DNA was extracted from a total of 404 mosquitoes from the pre-treatment and post-treatment collection. The numbers of samples were 203 and 201, respectively.

A single mosquito in an Eppendorf tube was homogenized with a pestle. Lysis buffer (100 mM Tris HCl (pH 8), 50 mM EDTA, 50 mM NaCl), 1% SDS and 0.3 mg/ml proteinase K were added to a final volume of 300 µl. The mixture was

incubated at 56˚C for two hours or over night at 50˚C. The longer incubation time was mainly used for samples from the pre-treatment collection, while, for the post-treatment collection, the shorter incubation time was in general used.

After the incubation, 300 µl of (Phenol 25: Chloroform 24: Isoamylalcohol (IAA) 1) was added. The samples were mixed for 20 min and then centrifuged at 10 000 rpm for 10 min in R.T. The aqueous layer was pipette and transferred to a new tube. A mixture of (24 Chloroform: 1 IAA) was added to a final volume of 600 µl. The samples were mixed and centrifuged as above. The aqueous layer was pipetted to a new tube. The DNA was precipitated with 1.2 M NH_4Ac and 60% ethanol to a final volume of 840 µl. The samples were mixed gently and stored at -70˚C for 2 h followed by centrifugation for 30 min at 12 000 rpm in R.T.

The supernatant was removed and the pellet was washed with 200 µl of 70% ethanol for 15 min at 13 000 rpm in R.T. The pellet was resuspended in 20 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA).

2 µl of mosquito DNA and 1 µl of loading buffer were loaded on a thin 2% agarose gel. The mosquito DNA concentration was roughly estimated comparing λ-DNA of the concentrations of 10 ng/ µl and 25 ng/ µl.

3.3. PCR

For identification of requested vector mosquito, *C. quinquefasciatus*, oligonucleotide primers according to Hettiarachi *et al.* were used. The PCR primers have been derived from a cloned repetitive cloned sequence from the genome of *C. quinquefasciatus* and amplify a fragment of approximately 500 bp.

Forward primer FCQ: 5' – GGT CGT ATC AAC AAA GTC CA – 3'
Reverse primer RCQ: 5' – ATG GAT ACG GAC TAC ACT GG – 3'

The PCR assay was carried out in 20 µl reaction mixtures containing 1xPCR buffer, 0.2 mM of each dNTPs, 0.25 mM of each primer, 3 mM $MgCl_2$ and 2.5 units of Taq polymerase. The amount of template DNA used in individual experiments is indicated in the result part of this paper.

PCR was performed using a PTC-100™ Programmable Thermal Controller (Peltier-Effect Cycling, MJ RESEARCH, INC.) with the following PCR cycle conditions: 95˚C/5 min, 30 cycles at 94˚C/30 s, 60˚C/40 s, 72˚C/30 s, followed by 15 min at 72˚C.
To investigate possible filarial infection of the identified *C. quinquefasciatus*, PCR was carried out with *W. bancrofti*-specific primers. Oligonucleotide primers, based on the nucleotide sequence of the previously reported *W. bancrofti*-specific DNA probe pWb 12, were used. The primers amplify a fragment of a repeated DNA sequence in the genome of *W. bancrofti*.[24]

Forward primer WbF_3: 5' – CTG AGT GAA ATC AAT GAA CTCG – 3'
Reverse primer WbR_2: 5' – GTC CAT CCG ATG AAG TTC CACC – 3'

Each amplification reaction was carried out in 20 µl reaction mixtures containing 1xPCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each oligonucleotide primer, 0.1 mg/ml of gelatin and 0.5 units of Taq polymerase. The amount of mosquito DNA used was 2, 4 and 8 ng.

PCR program according to Siridewa *et al.* A number of twenty amplification cycles with template denaturation at 94˚C/30 s, primer annealing at 55˚C/30 s, primer

extension at 72˚C/30 s and in the final cycle an extra 30-sec extension was used.

The presence of an amplified product was detected by electrophoresis through a 1% agarose gel. The percentage of filarial vector mosquito, *C. quinquefasciatus,* infective rate and infectivity rate were calculated.

3.4. Microscopy and dissection

The additional part of the study included identification of filarial vector mosquito by microscopy. The National Anti-filariasis campaign in Colombo, Sri Lanka studied 394 mosquitoes by microscopy. Of the pre-treatment and post-treatment collection, 194 and 200 samples were obtained, respectively. The different species and male *C. quinquefasciatus*-mosquitoes were recorded. The identified female *C. quinquefasciatus*-mosquitoes were then dissected and studies by microscopy for investigation of possible bancrofti infection. The number of infected female mosquitoes and the worm load was recorded. From the infected mosquitoes, the infective mosquitoes and worm load were counted. Other possible filarial vectors were also dissected to investigate potential bancrofti infection. The worm load of infected and infective mosquitoes was documented. Infection and infectivity rate of the collections before treatment and after treatment were then calculated and compared.

4. RESULTS

In order to investigate filarial infected mosquitoes in relation to single-dose mass treatment in filariasis endemic areas, PCR with primers specific for the vector mosquito, *C. quinquefasciatus*, and the filarial parasite, *W. bancrofti*, in addition to dissection by microscopy were used.

4.1. DNA extraction

DNA was extracted from a single mosquito and genomic DNA concentrations were obtained by electrophoresis and determined by comparing with λ-DNA of known concentrations.

The numbers of extracted mosquitoes were 203 and 201 from pre-treatment collection and post-treatment collection, respectively. No RNAse treatment of the samples was performed. By comparing the genomic DNA from the mosquitoes with known concentrations of λ-DNA, the quality of the DNA could be investigated. Figure 11 shows an example of a set of loaded genomic DNA samples. The mosquito-DNA samples were compared with λ-DNA of mixture concentrations of 10 ng/µl and 25 ng/µl. Of each mosquito-DNA sample, 2 µl was loaded and was compared with 1-2 µl of loaded λ-DNA.

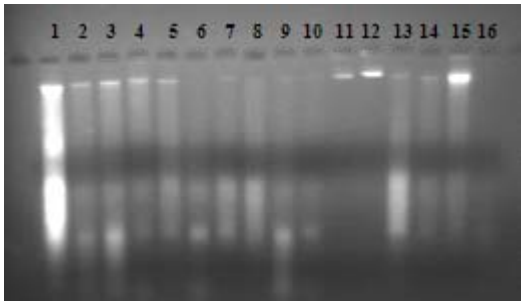

Figure 11. Mosquito genomic DNA. Lanes 1-10, 15-16 mosquito genomic DNA. Lane 11, λ-DNA 20 ng and lane 12, 50 ng.

As seen in Figure 11, most of the genomic DNA was degraded. No RNAse treatment of the samples was performed. This means that the actual mosquito-DNA concentrations most likely were lower than presented in Figure 11.

From the pre-treatment collection, genomic DNA from each of 203 mosquitoes was extracted. Amongst the collected samples, the obtained DNA concentrations differed within and between batches. The obtained DNA concentrations varied from approximately <2 ng/µl-8 ng/µl.

Genomic DNA was extracted from each of 201 mosquitoes collected after the treatment period. Like in the first collection, the DNA concentrations fluctuated amongst the different batches. However, in general more DNA was obtained from mosquitoes collected during the post-treatment collection. The DNA concentrations varied from approximately 2.5 ng/µl-15 ng/µl.

4.2. PCR

3.2.1. Culex quiquefasciatus

The PCR protocol was optimized using DNA template extracted from fresh *C. quinquefasciatus* isolated from colonies maintained in the insectary of the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo.

To identify the *C. quinquefasciatus* amongst the collected mosquito samples, the PCR fragments were run by electrophoresis. A 1 kb-ladder was also loaded to determine size of the fragments. The *C. quinquefasciatus*-specific primers, FCQ and RCQ, amplified a fragment of approximately 500 bp. The assay gave no amplifications when using undiluted DNA. A serial dilution of the DNA samples was performed to further optimize the PCR conditions. Positive PCR results were obtained from variable DNA dilutions. From the diluted samples the best amplification was obtained from reactions of 0.4-0.7 ng of *C. quinquefasciatus* genomic DNA.

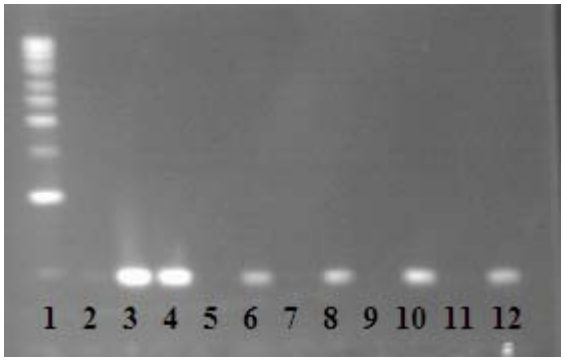

Figure 12. Polymerase chain reaction amplification of mosquito genomic DNA with primers FCQ and RCQ. Lane 1, 1-kb ladder. The lowest fragment represents a size of approximately 500 bp. Lane 2, positive control, lane 5, negative control. Lane 3, 4, 6-12 shows PCR product of mosquito genomic DNA samples.

In Figure 12, the results from one set of PCR are presented. The gel photo shows 9 reactions of field collected samples together with positive and negative controls. To roughly estimate the size of amplified fragment, the samples were compared with 1-kb ladder.

From the pre-treatment collection, 196 out of 203 samples were positive for the *C. quinquefasciatus*-specific primers. The prevalence of *C. quinquefasciatus* before the treatment period was hence 196/203=0.9655. A total of 188 out of 201 samples from the post-treatment collection were positive for the *C. quinquefasciatus*-specific primers. The prevalence of *C. quinquefasciatus* after treatment was hence 188/201=0.9350.

3.2.2. *Wuchereria bancrofti*

To investigate the filarial infection rate amongst the female *C. quinquefasciatus*, the samples were run by PCR with *W. bancrofti*-specific primers. To optimize the PCR protocol, PCR was run with DNA template extracted from pools of 30 third stage larvae (L3). In each reaction, 0.8-10 pg of template DNA was used. Amplified fragments were seen as faint bands when run on electrophoresis. Other positive controls, such as female *C. quinquefasciatus* infected by feeding on

filarial patients, were not available. From the PCR, run with DNA template from the field collected female *C. quinquefasciatus* mosquitoes, no samples were positive for the *W. bancrofti*-specific primers. Of this, no infection rate could therefore be estimated under these conditions.

4.3. Microscopy and dissection

3.3.1. *Culex quinquefasciatus*

The results of the adult mosquito dissection carried out by the national Anti-filariasis campaign in Colombo, Sri Lanka are presented in Table 4. A total of 194 mosquitoes were received from the pre-treatment collection for dissection. Of these, 136 were identified as female *C. quinquefasciatus.* The male *C. quinquefasciatus* were recognized as other species together with the 58 remaining mosquitoes. The rate of female *C. quinquefasciatus* was manifested to 136/194=0.701.

	Pre-treatment batch	**Post-treatment batch**
Total no. received	194	200
No. of dissected *C. quinquefasciatus*	136	178
No. infected	11	2
Worm load	10;7;13;3;8;18;3;10;2	3;1
No. infective	2	0

Table 4. Results from the adult mosquito dissection carried out by the national Anti-filariasis campaign (AFC) in Colombo, Sri Lanka.

Amongst the post-treatment batch, 178 out of 200 mosquitoes were identified as female *C. quinquefasciatus.* Other species recognized were *Armigeres* (3), *Aedes* (4) and *C. gelidus* (2). Neither of these are vectors of filarial infection. The total number of *C. quinquefasciatus* of the collection was 191. The rate of female *C. quinquefasciatus* was manifested to 178/200=0.890 and the rate of *C. quinquefasciatus* (males and females) was calculated to 191/200=0.955.

3.3.2. *Wuchereria bancrofti*

Amongst the number of 136 *C. quinquefasciatus* female mosquitoes that were dissected, 9 mosquitoes were identified with *W. bancrofti* larvae. Only one of these was detected with larvae of infective stage and the worm load was 1 larva. The worm load of the other infected females was 10, 7, 13, 3, 8, 18, 3 and 10, respectively. In addition, 6 mosquitoes of other species than *C. quinquefasciatus* were dissected. A number of two mosquitoes were identified infected with worm loads of 2 and 1. Only the second one was infected with *W. bancrofti* larvae of infective stage. The infection rate was calculated to (9+2)/(136+6)=0.0775 and the infectivity rate to 2/(136+6)=0.0141.

Of the 178 *C. quinquefasciatus* female mosquitoes that were dissected, 2 mosquitoes were identified with *W. bancrofti* larvae with worm loads of 3 and 1, but neither of these larvae were of infective stage. The infection rate was hence calculated to 2/178=0.0112 and the infectivity rate to 0/178=0.

5. DISCUSSION

The rapid urbanization in the tropics has resulted in polluted still water. This is the ideal breeding ground for mosquitoes of the *Culex quinquefasciatus*-species, the main vector for *Wuchereria bancrofti*. One way to prevent the rapidly spread of these parasites is to build better drainage in the cities to prevent mosquitoes to breed in densely populated areas. However, this is a major program as the majority of the afflicted countries are tropical development countries. The cost of

rebuilding the drainage systems is high and usually not first priority for these countries.

Human lymphatic filariasis is not only a health problem but also a social problem for the afflicted individual. Severe lymphoedema results in immobilization which makes the individual unable to work. In developing countries that lack social welfare, the situation can lead to increased poverty amongst the afflicted persons. In addition, young people with lymphoedema may have difficulties to find a partner, though the condition is not contagious.

Mass treatment programs have shown that elimination of the filarial parasite is possible. However, continuous treatment programs are necessary. Constraints such as drug distribution to the endemic areas and assessment of the range of taken drugs by the individuals are common. Adverse effects of the drugs may result in interrupted treatment.

The World Health Organization (WHO) has recommended a single dose treatment of the population in endemic countries. This dosage consists of 150mg Diethylcarbamazine and 400 mg Albendazole for children 2-12 years and 300 mg Diethylcarbamazine together with 400 mg Albendazole for people above 12 years. In the present study, a single dose treatment was carried out in September 2004 in Piliyandala, Bokundara in Colombo, Sri Lanka. Mosquitoes were collected in the area before and after the treatment period. The mosquitoes were divided into two batches. Microscopy and PCR-based detection assay were performed on the two batches, separately.

The extracted DNA from each single mosquito was investigated by electrophoresis. The results show that most DNA was degraded. Due to high costs, no following RNAse treatment of the mosquito samples was performed. This signifies the actual DNA concentration to be lower than estimated. In a separate study, amounts of obtained extracted DNA from single mosquitoes were investigated. The results showed that more DNA was obtained when extracting DNA from fresh mosquitoes compared with mosquitoes kept in -80˚C storage. These results suggest that some DNA may have been degraded during storage. This can be one factor for the low concentrations obtained of mosquito-DNA. Another factor can be the protocol used for the DNA extraction. Proteinase K used in the first step of the extraction was 0.3 mg/ml. This is relatively low when compared with other DNA extraction protocols.

To achieve an efficient extraction with Proteinase K, repeated incubation after adding more Proteinase K is preferred. From the DNA extraction of the post-treatment collected mosquitoes, higher concentrations of DNA were obtained. The incubation temperature was, for these samples, increased to 56˚C and the incubation time was only 2 hours. This temperature is optimal activity for Proteinase K and may be the reason for higher obtained amounts of DNA. In addition, most standard protocols for DNA extraction include precipitation of DNA with sodium acetate. In the present study, ammonium acetate was used.

Amongst the mosquitoes, the results from microscopy and PCR generate a percentage of *Culex quinquefasciatus*. When comparing the results from the microscopy study and PCR method, the PCR showed a higher percentage of the *C. quinquefasciatus* species. Detection of mosquito species by PCR does not separate the female and male mosquitoes. The prevalence of *C. quinquefasciatus* in the PCR study showed hence both sexes of the species. During the dissection, done by the National Anti-filariasis campaign, only the females of *C. quinquefasciatus* were counted. The males of *C. quinquefasciatus*

were in the first batch recognized as other species. This makes the presented results from the different detection methods incomparable. To avoid this problem, a PCR assay can be developed to separate the different sexes of *C. quinquefasciatus* by the X- and Y-chromosome.

Apart from the variation in performance, the differences in prevalence of *C. quinquefasciatus* may be a result of the specificity in the more reliable PCR method with species-specific primers. Microscopic studies demand an experienced eye and inconsistent results may be a consequence of human errors. This can also give contradictory results amongst the two detection methods and the quantity of *C. quinquefasciatus* before and after the treatment period. In the PCR study the percentage *C. quinquefasciatus* in the first collection is higher when compared to the after the treatment collection. The situation is the opposite in the microscopy method, where the percentage *C. quinquefasciatus* in the first collection is quite low compared to other studies[13]. These differences can be avoided by using higher numbers of collected mosquitoes in the study. However, the PCR technique, presented in this study, requires fewer numbers of mosquitoes as investigated one by one, in comparison to PCR assays of pools of mosquitoes.

In the present study the treatment by the combination of DEC and Albendazole confirms a distinct decrease in infective and infectivity rate amongst the mosquitoes in the area. These results suggest that a single round of mass treatment can have an impact on transmission of human lymphatic filariasis. Conversely, the PCR with *W. bancrofti*-specific primers gave no positive results with the obtained DNA template from the mosquito extraction. This may be a result of the degraded DNA that disturbed the polymerase chain reaction. The PCR with *C. quinquefasciatus*-specific primers required highly diluted DNA samples and several PCR tests for successful amplification. The case may be the same for the PCR with *W. bancrofti*-specific primers. However, the amount parasitic DNA is much lower, than the mosquito DNA, and will unlikely be enough for amplification after highly diluted samples. The most likely reason from inhibition of the PCR is thus the degradation of DNA during the extraction and with no following RNAse treatment of the samples was performed. RNA in the DNA samples may have inhibited the PCR. This was probably the reason for difficulties in the detection of *C. quinquefasciatus*.

In earlier studies, very low concentrations of *W. bancrofti* DNA have been amplified. Siridewa *et al.* (1996) manage to amplify a single infective larva when using the same primers as in the present study. However, the larva in that case was fresh when starting the DNA extraction and not stored in -80˚C as the mosquitoes here.

The only control available during the study was DNA extracted from pools of 30 third stage larvae. The samples had been stored in -20˚C since 1997 and the quality was not the desired. In addition, the samples available contained more DNA than estimated in the field collected mosquitoes and a comparison is difficult. The poor results from the *W. bancrofti*-PCR would likely have been prevented with the presence of positive controls from infected individuals. Government policy, poor administration and the lack of volunteers make night-blood collections difficult in Sri Lanka. For the present study the optimal case would be blood fed mosquitoes of infected patient following by storage of mosquitoes in -80˚C. The extracted DNA would have the same history as the field collected samples and could be used to streamline the optimization of the PCR-program.

Developing a PCR-based method for identifying *C. quinquefasciatus* mosquitoes and infection by *W. bancrofti* will provide an efficient and necessary tool for further research of the filariasis elimination program. The aim of this study was to develop efficient PCR assays for identification of filarial vector mosquito, *C. quinquefasciatus,* and detection of the *W. bancrofti* parasite. Limited resources during this study prevented further analysis of the PCR assays.

The optimal technique, for time saving identification, would be a PCR assay that manages to amplify the DNA of the vector mosquito species and the filarial parasite in the same reaction. The result can be seen immediately by electrophoresis. With reliable DNA extraction method and PCR-programs the National Anti-filariasis campaign can effectively detect the mosquito species and infection by using electrophoresis. This research methodology may be a major step into a future without human lymphatic filariasis.

6. ACKNOWLEDGMENTS

My thanks are due to Nalini Jayasekera for providing field collected mosquitoes. I would also like to thank her for the contact with the National Anti-filariasis campaign and good supervising. I am grateful to Y.M.N.Y. Bandara for technical assistance with the mosquito samples. I would also like to thank my supervisor, Eric H. Karunanayake and Lena Åslund for reading the manuscript.

7. REFERENCES

1. Chadee, D.D., Williams, S.A. & Ottesen, E.A. (2002). Xenomonitoring of *Culex quinquefasciatus* mosquitoea as a guide for detecting the presence or absence of lymphatic filariasis: a preliminary protocol for mosquito sampling. *Annuals of tropical medicine & parasitology*. **96** (2), 47-53.

2. Cowman, A.F. & Crabb, B.S. (2003). Functional genomics: identifying drug targets for parasitic diseases. *TRENDS in Parasitology.* **19** (11), 538-543.

3. Cox, F.E.G. (2002), History of Human Parasitology. *Clinical Microbiology Reviews.* October **15** (4), 595-612.

4. De Almeida, A.B., Freedman, D.O. (1999). Epidemiology and immunopathology of bancroftian filariasis. *Microbes and infection.* **1**, 1015-1022.

5. Devaney, E. & Yazdanbakhsh, M. (2001). Prospects and challenges in lymphatic filariasis. *Parasite Immunology*. **23**, 323-325.

6. Dissanaike, A.S. (1968), Control of filariasis in Ceylon. Proceedings of seminar on filariasis and immunology of parasitic infections and laboratory meeting. Unpublished.

7. Dissanaike, A.S. (1991), Filariasis in Ceylon then (1961) and in Sri Lanka now (1990-30 years on). *Annals of Tropical Medicine and Parasitology*. **85** (1), 123-129.

8. Dreyer, G., Norões, J.& Figueredo-Silva, J. (2000). Elimination of lymphatic filariasis as a public health problem: New insights into the natural history and pathology of bancroftian filariasis: implications for clinical management and filariasis control programmes. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **94**, 594-596.

9. Freeman, A.R., Lammie, P.J., Houston, R., LaPointe, M.D., Streit, T.G., Jooste, P.L., Brissau, J.M., LaFontant, J.G. & Addiss, D.G. (2001), A community-based trial for the control of Lymphatic filasiasis and iodine deficiency using salt fortified with diethylcarbamazine and iodine. *Am. J. Top. Med. Hyg.* **65**(6), 865-871.

10. Goodman, D.S., Orelus, J-N., Roberts, J.M., Lammie, P.J. & Streit, T.G. (2003), PCR and mosquito dissection as tool to monitor filarial infection levels following mass treatment. *Filaria Journal.* **2** (11), 1-9.

11. Hettiaratchi, U.P.K., Munasingha, D.H.N., Chandrasekharan, N.V., Karunanayake, E.H. & Jayasekera, N. (2000) A polymerase chain reaction based method for the detection of

Culex quinquefasciatus (Diptera: Culicidae). *Bulletin of entomological research*. **90**, 63-68.

12. Ismail, M. (1993), Filariasis Control in Sri Lanka-Current Status and Future Strategies. Faculty of Medicine, University of Colombo, Sri Lanka.

13. Jayasekera, N. Chelliha, R.V., Jansen, C.G. & Pathmanathan, S. (1986) Mosquito studies in Sri Jayawardenepura – the new capital of Sri Lanka. *Mosquito-Borne Dis Bull* Vol.2, **4**, 87-93.

14. Krishnamoorthy, K., Subramanian, S., Van Oortmarssen, G.J., Habbema, J.D.F. & Das, P.K. (2003). Vector survival and parasite infection: the effect of *Wuchereria bancrofti* on its vector *Culex quinquefasciatus*. *Parasitology*. **129**, 43-50.

15. Lima, C.A., Almeida, W.R., Hurd, H. & Albuquerque C.MR. (2003). Reproductive aspects of the mosquito *Culex quinquefasciatus* (Diptera: Culicidae) infected with *Wuchereria bancrofti* (Spirurida: Onchocercidae). *Mem. Inst. Oswaldo Cruz, Rio de Janeiro*. Vol. **98**(2), 217-222.

16. *Lymphatic filariasis: the disease and its control. Fifth report of the WHO Expert Committee on Filariasis.* Geneva, World Health Organization, 1992 (WHO technical report series, No. 821)

17. Manson-Bahr, P. (1958) The Story of Filaria Bancrofti. *The journal of tropical medicine and hygiene*. March, 53-61.

18. McReynold, L.A., Poole, C.B. & Williams, S.A. (1991). Filarial DNA Probes in Jakarta. *Parasitology Today.* Vol. 7, **4**, 65-67.

19. Molyneux, D.H., Bradley, M., Hoeraul, A., Kyelem, D. & Taylor, M.J. (2003), Mass drug treatment for lymphatic filariasis and onchocerciasis. *TRENDS in parasitology.* Vol.19, **11**, 516-522.

20. Nanduri J. & Kazura J.W. (1989). Clinical and Laboratory Aspects of Filariasis. *Clinical Microbiology Reviews.* Vol. 2, **1**, 39-50.

21. Ottesen, E.A., Ismail, M.M. & Horton, J. (1999). The Role of Albendazole in Programmes to Eliminate Lymphatic filariasis. *Parasitology Today.* Vol. 15, **9**, 382-386.

22. Ravindran, B. (2003). Aping Jane Goodall: insight into human lymphatic filariasis. *TRENDS in Parasitology.* Vol. 19, **3**, 105-109.

23. Setouhy, M.E., Ramzy, R.M., Ahmed, E.S., Kandil, A.M., Hussain, O., Farid, H.A., Helmy, H. & Weil, G.J. (2004). A randomized clinical trial comparing single- and multi-dose combination therapy with diethylcarbamazine and albendazole for treatment of bancroftian filariasis. *Am. J. Trop. Med. Hyg.* **70**(2), 191-196.

24. Siridewa, K., Karunanayake, E.H. & Chandrasekharan, N.V. (1996). Polymerase chain rection-based technique for the detection of *Wuchereria bancrofti* in human blood samples, hydrocele fluid and, mosquito vectors. *Am. J. Top. Med. Hyg.* **54**(1), 72-76.

25. Smith, H.L. (2000). Investigating development of infective stage larvae of filarial nematodes. *Frontiers in Bioscience.* **5**, September, 95-102

26. Stobbart, RH. (1977), The control of the diuresis following a blood meal of the yellow fever mosquito *Aedes aegypti* (L). *Journal of Experimental Biology.* Aug:**69**:53-85.

27. Stolk, W.A., Swaminathan, S., van Oortmartssen, G.J., Das, P.K. & Habbema, J.D.F. (2003). Prospects for elimination of Bancrofti Filariasis by Mass Drug Treatment in Pondicherry, India: A Simulation Study. *The Journal of Infections Diseases.* **188**, 1371-81.

28. Stolk, W.A., Van Oortmarssen, G.J., Subramanian, S., Das, P.K., Borsboom, G.J.J.M., Habbema, J.D.F. & de Vlas S.J. (2004). Assessing density dependence in the transmission of lymphatic filariasis: uptake and development of *Wuchereria bancrofti* microfilariae in the vector mosquitoes. *Medical and Veterinary Entomology.* **18**, 57-60.

29. Williams, S.A. et al. (1996). A polymerase chain reaction assay for the detection of *Wuchereria bancrofti* in blood samples from French Polynesia. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **90**, 384-387.

30. Stanford University: http://www.stanford.edu/class/humbio103/ParaSites2004/Filariasis/history.html (12 May 2005).

31. Medical ecology: http://www.medicalecology.org/pdf/filariasis.pdf (12 May 2005).

32. Worlds Health Organization: http://www.who.int/docstore/wer/pdf/2002/wer7716.pdf (12 May 2005).

33. Filarial Journal: http://www.filariajournal.com/content/2/1/11 (12 May 2005).

34. Tropical Medicine Central Resource: tmcr.usuhs.mil/tmcr/chapter26/geography2.htm (12 May 2005).

35. NSW Arbovirus Surveillance & Vector Monitoring Program http://www.arbovirus.health.nsw.gov.au/areas/arbovirus/mosquit/photos/mosquitophotos_culex.htm#quinq (12 May 2005).