ผลของยาด็อกซีไซคลินที่มีต่อไมโครฟิลาเรียของพยาธิโรคเท้าช้างชนิด *Brugia malayi*

นายศิวะพงษ์ สังข์ประดิษฐ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE EFFECTS OF DOXYCYCLINE ON *BRUGIA MALAYI* MICROFILARIAE

Mr. Sivapong Sungpradit

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By	Mr. Sivapong Sungpradit
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Thesis Advisor	Professor Surang Nuchprayoon, M.D., Ph.D., M.P.H.
Thesis Co-advisor	Tanittha Chatsuwan, Ph.D.

Accepted by Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

……Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

…….....Chairman
(Associate Professor Chintana Chirathaworn, Ph.D.)

…….....Thesis Advisor
(Professor Surang Nuchprayoon, M.D., Ph.D., M.P.H.)

…….....Thesis Co-advisor
(Tanittha Chatsuwan, Ph.D.)

…….....Examiner
(Associate Professor Padet Siriyasatien, M.D., Ph.D.)

…….....Examiner
(Assistant Professor Sonthaya Tiawsirisup, D.V.M., Ph.D.)

…….....External Examiner
(Associate Professor Pichart Uparanukraw, M.D., Ph.D.)

ศิวะพงษ์ สังข์ประดิษฐ์ : ผลของยาด็อกซีไซคลินที่มีต่อไมโครฟิลาเรียของพยาธิโรคเท้าช้างชนิด *Brugia malayi* (THE EFFECTS OF DOXYCYCLINE ON *BRUGIA MALAYI* MICROFILRIAE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.พญ.ดร.สุรางค์ นุชประยูร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร.ธนิษฐา ฉัตรสุวรรณ, 192 หน้า.

โรคเท้าช้างเป็นโรคที่มียุงเป็นพาหะ โรคนี้มีสาเหตุจากเชื้อ *Wuchereria bancrofti* และ *Brugia malayi* หลังจากผสมพันธุ์ พยาธิตัวเต็มวัยเพศเมียสามารถปล่อยตัวอ่อนระยะไมโครฟิลาเรียจำนวนมาก เข้าสู่กระแสเลือดของโฮสต์ ยาไอเวอร์เมกติน ไดเอทิลคาร์บามาซีน อัลเบนดาโซล รวมทั้งยาต้านแบคทีเรียกลุ่มริกเกตเซีย เช่น ด็อกซีไซคลิน ไรแฟมพิซิน และไซโพรฟลอกซาซิน ถูกนำมาใช้เพื่อลดจำนวนไมโครฟิลาเรียในคนและสัตว์รังโรค เพื่อป้องกันการแพร่กระจายของโรค ยาต้านแบคทีเรียกลุ่มริกเกตเซีย ยังมีผลต่อแบคทีเรียโวลบาเชียซึ่งเป็นแบคทีเรียชนิดแกรมลบ ที่อาศัยอยู่ในเซลล์พยาธิฟิลาเรียแบบพึ่งพากัน โดยยาจะมีผลกระทบต่อการเจริญของระยะเอมบริโอและตัวอ่อนของพยาธิ ความสมบูรณ์พันธุ์และการอยู่รอดของพยาธิเพศเมีย การศึกษานี้ได้ทำการศึกษาผลของยาด็อกซีไซคลิน ไรแฟมพิซิน และไซโพรฟลอกซาซิน ที่มีต่อการเคลื่อนที่ไมโครฟิลาเรียของพยาธิโรคเท้าช้างชนิด *B. malayi* โดยแสดงค่าความเข้มข้นของยาเป็นความเข้มข้นของยาที่น้อยที่สุด ที่สามารถหยุดการเคลื่อนที่ของไมโครฟิลาเรีย (minimum effective concentration; MEC) นอกจากนี้ แสดงค่าความเข้มข้นของยาที่น้อยที่สุด ที่สามารถยับยั้งการแบ่งตัวของแบคทีเรียโวลบาเชีย (minimum inhibitory concentration; MIC) โดยใช้วิธี real-time PCR เพื่อหาสัดส่วนยีนของแบคทีเรียโวลบาเชีย (*wsp*) ต่อยีนของไมโครฟิลาเรีย (*hsp70*) ยาด็อกซีไซคลินมีประสิทธิภาพดีที่สุด ในการยับยั้งการเคลื่อนที่ของไมโครฟิลาเรียอย่างสมบูรณ์ โดยมีค่า MEC เท่ากับ 128 ไมโครกรัม/มิลลิลิตร ยาไรแฟมพิซิน และไซโพรฟลอกซาซินมีประสิทธิภาพน้อยกว่า โดยมีค่า MEC มากกว่า 256 ไมโครกรัม/มิลลิลิตร ที่ 12 ชั่วโมงหลังทดสอบ ยาด็อกซีไซคลินมีค่า MIC เท่ากับ 128 ไมโครกรัม/มิลลิลิตร ยาไรแฟมพิซิน และไซโพรฟลอกซาซินมีค่า MIC มากกว่า 128 ไมโครกรัม/มิลลิลิตร ที่ 12 ชั่วโมงหลังทดสอบ ค่า MEC และ MIC สามารถนำมาใช้ประเมินยาต้านพยาธิโรคเท้าช้างและแบคทีเรียโวลบาเชีย ที่นำมาคัดเลือกในห้องปฏิบัติการ

เพื่อให้เกิดความเข้าใจในผลของยาด็อกซีไซคลินที่มีต่อไมโครฟิลาเรียในระดับโมเลกุล การศึกษานี้ได้ใช้วิธีไมโครอาเรย์ ในการศึกษาการแสดงออกของยีนของไมโครฟิลาเรียของพยาธิโรคเท้าช้างชนิด *B. malayi* หลังจากได้รับยาด็อกซีไซคลินขนาด 20 ไมโครกรัม/มิลลิลิตร เปรียบเทียบกับไมโครฟิลาเรียที่ไม่ได้รับยา ที่เวลา 61 ชั่วโมง ผลการศึกษาพบว่าไมโครฟิลาเรียมีการเปลี่ยนแปลงการแสดงออกของยีนอย่างมีนัยสำคัญทางสถิติหลังได้รับยาด็อกซีไซคลิน ยีนที่มีการเพิ่มการแสดงออก ได้แก่ ยีนที่เกี่ยวข้องกับการม้วนพับของโปรตีน เช่น heat shock protein 90 ในทางตรงกันข้าม ยีนที่เกี่ยวข้องกับการสร้างเอนไซม์ในกระบวนการขนส่งอิเลคตรอนในไมโตคอนเดรีย เช่น NADH dehydrogenase และ cytochrome oxidase มีการแสดงออกลดลง จากข้อมูลที่ได้พบว่ายาด็อกซีไซคลิน ปรับเปลี่ยนภาวะธำรงดุลโดยทางตรงต่อไมโครฟิลาเรียและโดยทางอ้อมต่อแบคทีเรียโวลบาเชีย

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4789684820 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS : LYMPHATIC FILARIASIS / DOXYCYCLINE / MICROFILARIA / *BRUGIA MALAYI* / *WOLBACHIA*

SIVAPONG SUNGPRADIT: THE EFFECTS OF DOXYCYCLINE ON *BRUGIA MALAYI* MICROFILARIAE. ADVISOR: PROF. SURANG NUCHPRAYOON, M.D., Ph.D., M.P.H. CO-ADVISOR: TANITTHA CHATSUWAN, Ph.D., 192 pp.

Lymphatic filariasis, is a mosquito-borne disease, caused by *Wuchereria bancrofti* and *Brugia malayi*. After mating, fertile adult female worms will release an abundance of offspring (microfilariae; mf) into the host blood circulatory system. Transmission-blocking agents such as ivermectin, diethylcarbamazine, albendazole, as well as anti-rickettsial agents (e.g. doxycycline, rifampicin, and ciprofloxacin) have been used to reduce microfilarial density in human and animal reservoir hosts, to prevent disease transmission. Anti-rickettsial drugs also have the effect on the obligate intracellular gram-negative bacteria, *Wolbachia*, the mutualistic endosymbiont that appears to exert influence on filarial nematode embryonic and larval development, adult female fertility, and filarial survival. We investigated the effects of doxycycline, rifampicin and ciprofloxacin on *B. malayi* microfilarial motility, by using the minimum effective concentration (MEC). The minimum inhibitory concentration (MIC), the concentration of the anti-rickettsial drugs that could inhibit *Wolbachia* growth in mf, derived from the single copy gene ratio of *Wolbachia* versus nematode (*wsp*/*hsp70*) using the quantitative polymerase chain reaction (qPCR), was also demonstrated. Doxycycline was showed as the best effective antimicrobial agent. Doxycycline at 128 μg/ml (MEC) inhibited microfilarial motility completely at 12 h. Rifampicin and ciprofloxacin were less effective, both with MECs of >256 μg/ml at 12 h. Doxycycline MIC was 128 μg/ml, whereas rifampicin and ciprofloxacin MICs were >128 μg/ml, at 12 h. The MEC and MIC could be used to evaluate anti-*Wolbachia* or antifilarial agents for *in vitro* screening.

To understand the molecular effect(s) of doxycycline on mf, we used microarray analysis to investigate temporal gene expression changes in *B. malayi* mf exposed *in vitro* to 20 μg/ml doxycycline as compared with non-treated control. By 61 h post-traetment, doxycycline-treated mf exhibited a significantly altered gene expression signature. We observed up-regulation of genes involved in protein folding such as, small heat shock protein and heat shock protein 90. In contrast, genes encoding for enzymes involved in the parasite mitochondrial electron transport chain, such as subunits of NADH dehydrogenase and cytochrome oxidase, were down-regulated. Our data suggest that doxycycline alters larval homeostasis either through a direct effect on the worm or through an indirect effect on the parasite's endosymbiont *Wolbachia*.

Field of Study : Biomedical Science Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

ABI	=	Applied Biosystems
ADL	=	adenolymphangitis
ADLA	=	acute dermatolymphangioadenitis
AFL	=	acute filarial lymphangitis
ALB	=	albendazole
BLAST	=	Basic Local Alignment Search Tool
bp	=	base pair
°C	=	degree Celsius
cDNA	=	complementary deoxyribonucleic acid
cm	=	centimeter
CO_2	=	carbon dioxide
DALYs	=	disability-adjusted life years
DEC	=	diethylcarbamazine
DNA	=	deoxyribonucleic acid
EDTA	=	ethylenediamine tetraacetic acid
ES antigen	=	excretory/secretory antigen
FBS	=	fetal bovine serum
g	=	gram (s)
g	=	gravitational constant
GABA	=	gamma-aminobutyric acid
gst	=	glutathione-S-transferase
GPELF	=	Global Program to Elimination Lymphatic Filariasis
HSP	=	heat shock protein

IFN-γ	=	interferon-gamma
IgG	=	immunoglobulin G
IL	=	interleukin
kDa	=	kilodalton
kg	=	kilogram
LB	=	Luria-Bertani media
LPS	=	lipopolysaccharide
M	=	molar
MDA	=	mass drug administration
mf	=	microfilariae
mg	=	milligram
mM	=	millimolar
mRNA	=	messenger RNA
MW	=	molecular weight
NCBI	=	National Center for Biotechnology Information
ng	=	nanogram
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
aRNA	=	amplified ribonucleic acid
rDNA	=	ribosomal deoxyribonucleic acid
RNA	=	ribonucleic acid
RNase	=	ribonuclease
RPMI	=	Roswell Park Memorial Institute
RT	=	reverse transcriptase
WHO	=	World Health Organization

WSP	=	*Wolbachia* surface protein
SD	=	standard deviation
TDR	=	Tropical Disease Research
TGF-β	=	transforming growth factor-beta
TLR	=	toll-like receptor
TNF-α	=	tumor necrosis factor alpha
TPE	=	tropical pulmonary eosinophilia
Tris	=	tris-(hydroxymethyl)-aminomethane
μg	=	microgram
μl	=	microliter
V	=	volts
VBDC	=	vector borne disease control center
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

1. Background and Rationale

Lymphatic filariasis is mainly caused by the filarial nematodes, *Wuchereria bancrofti, Brugia malayi,* and *B. timori* (Ottesen et al., 1997). The parasites are long, threadlike worms that lodge in the lymphatic vessels of their human hosts. Including Thailand, over 120 million people in 83 endemic countries are infected, with more than 40 million incapacitated or disfigured by the disease (Molyneux et al., 2003; Nuchprayoon et al., 2003; Triteeraprapab et al., 2001; World Health Organization, 2009). The majority of lymphatic filariasis is caused by *W. bancrofti* and *B. malayi* (confined mainly to southern India, with smaller foci in other parts of Asia). *B. timori* is focally important in areas of the Timor (Adinarayanan et al., 2007). Lymphatic filariasis is ranked by the World Health Organization (WHO) as the world's second leading cause of permanent and long-term disability, and targeted by the WHO to be eliminated as a public health problem by the year 2020 (Behbehani, 1998; World Health Organization, 1995). In terms of 'Disability Adjusted Life Years' (DALYs: the number of healthy years of life lost due to premature death and disability), lymphatic filariasis is responsible for 5.8 million DALYs lost annually, ranking third among the special program for research and training in tropical diseases (TDR), after malaria and tuberculosis (World Health Organization, 2004). One-third of people infected with lymphatic filariasis live in India, a third live in Africa, and the remainders live in the Americas, the Pacific

Islands, Papua New Guinea, and South-East Asia. Lymphatic filariasis and other neglected tropical diseases are recognized in the report on the Commission for Africa as contributing significantly to the overall African disease burden (Molyneux et al., 2005). In Thailand, the prevalence of lymphatic filariasis is 0.25 per 100,000 populations (Filariasis Division, 2009). Total of 158 people are infected, and 9 people of these have chronic pathology (6 people with lymphedema, and 3 people with elephantiasis). The active transmission area of brugian filariasis is limited to certain areas in Narathiwat province, located in Southern of Thailand, with the prevalence of 18.99 per 100,000 populations. Microfilariae are detected in 133 people, chronic pathology is found in 9 patients. Bancroftian filariasis is endemic in Tak, and Kanchanaburi provinces, located in Northwest Thailand, at the Thailand-Myanmar border. The prevalence of bancroftian filariasis in Tak province is 4.6 per 100,000 populations, while the prevalence in Kanchanaburi province is 0.12 per 100,000 populations. Chronic pathology is not reported in these areas.

Lymphatic filariasis is transmitted by mosquito vectors. Different mosquito species of the *Culex*, *Anopheles*, *Aedes*, *Ochlerotatus*, and *Mansonia* genera are vectors of the lymphatic filariasis, depending on geographical distribution (Bockarie et al., 2009; Zagaria and Vavioli, 2002). People become infected when bitten by mosquitoes carrying infective larvae, which migrate to the lymphatics where they mature into adult worms and cause damage. Once a pair of male and female worms mature and reproduce, they release an abundance of offspring called microfilariae (mf) into the host's blood circulation (estimate of 10,000 mf per adult female per day) (Fenn and Blaxter, 2004a). The mf matures within the mosquito to the stage infective to humans, completing the life cycle when they are taken a subsequent blood meal.

The range of clinical disease caused by lymphatic filariasis includes fever, inflammation of lymphatic vessels (lymphangitis), disease of the lymph nodes (lymphadenopathy), reversible and irreversible swelling (edema), elephantiasis of limbs, genitals, and breasts, filarial hydrocele (fluid in the scrotum), and a rarer tropical pulmonary eosinophilia syndrome characterized by wheezing and coughing at night, high counts of eosinophils, and lung nodules (Ottensen, 2006). The pathogenesis of lymphatic filariasis is thought to be caused by adult worms, host immune responses, and secondary bacterial, or fungal infection (McPherson et al., 2006; Ottesen, 1992). The pathology in examined lymphatic vessels consists of distinct histological features related to the existence of both alive and dead parasites (Jungmann et al., 1991; Jungmann et al., 1992). In asymptomatic patients living in endemic areas, lymphangiectasia (a term of pathology that describes dilation of the lymphatic vessels) is the most common change observed (Ahn et al., 2005; Dreyer et al., 2000). The lymphatic vessels are dilated with the presence of intact worms that do not provoke any inflammatory responses. In contrast, an inflammatory pathology is usually detected with the presence of dead worms from naturally occurring or anti-filarial drugs (diethylcarbamazine; DEC, or ivermectin) treatment reaction (Dreyer et al., 2000; Figueredo-Silva et al., 1996). The underlying pathology may be associated without any clinical symptoms, or with the acute lymphangitis. This acute manifestation is characterized by local inflammatory reactions around dead worms and by systemic febrile responses with significantly elevated levels of tumor necrosis factor alpha (TNF-α) (Das et al., 1996; Dreyer et al., 2000; Porksakorn et al., 2007). Therefore, lymphatic dysfunction can be caused by lymphangiectasia (may be congenital or acquired) and persistent attacks of acute lymphangitis. These factors, together with recurrent opportunistic bacterial infections, are major risk for

development of the chronic manifestations of lymphatic filariasis (Dreyer et al., 2000; McPherson et al., 2006; Ottesen, 1992).

The roles of mf in human filarial infections are involved in the immunomodulation, pathogenesis and induction of host cell apoptosis (Bennuru and Nutman, 2009; Harnett and Harnett, 2006; Harnett and Harnett, 2008; O'Connor et al., 2003; Semnani et al., 2003; Semnani et al., 2008). The mf of *B. malayi* affect human dendritic cells in at least three ways: 1) by interfering with their viability 2) by altering their function and 3) by causing caspase-dependent apoptotic cell death in human monocyte-derived dendritic cells (but not macrophage) (Semnani et al., 2003; Semnani et al., 2008). Moreover, the mf excretory-secretory (ES) components (proteins released from mf) are related to lymphatic dilatation in infected patients and can induce human lymphatic endothelial cell differentiation (lymphangiogenesis) *in vitro* (Bennuru and Nutman, 2009). The composition of products released from mf has been studied so far. Many mf ES products with predicted functions are associated with developmental processes and regulation of enzyme activity. Some may play a role in the immunology of the host-parasite relationship (Moreno and Geary, 2008; Specht and Hoerauf, 2009). The mf ES products, such as the mf stage-specific, serine proteinase inhibitors (serpins), can inhibit enzymatic activity of human neutrophil (Zang et al., 1999; Zang et al., 2000), and macrophage inhibitory factor (MIF) can alter human monocytes/macrophages activity (Pastrana et al., 1998). Moreover, the proteomic profile of mf ES products shows the abundant levels of proteins including collagens, ankyrins, cuticulins and cytoskeletal proteins such as myosin and kinesin-like molecules (Moreno and Geary 2008; Bennuru et al., 2009).

Mass treatment with antifilarial drugs is the mainstay of the Global Programme to Eliminate Lymphatic Filariasis (GPELF) (Molyneux and Zagaria, 2002). Mass treatment aims at reducing the mf load in the population, thereby reducing both mf uptakes by mosquitoes and transmission of infection. Three drugs are available for treatment of the lymphatic filariasis infection: diethylcarbamazine, ivermectin, and albendazole. DEC is the only useful drug for community filariasis control. It has since been joined by ivermectin, by albendazole-DEC, and by albendazole-ivermectin combinations, but DEC still retains its importance as a major element of filariasis control (Adinarayanan et al., 2007).

Transmission-blocking agents such as DEC, ivermectin, ivermectin combined with DEC, albendazole combinded with either ivermectin or DEC, and antimicrobial agents with anti-rickettsial activity, such as doxycycline, have been used to reduce the microfilaria density in human and animal reservoir hosts (Chansiri et al., 2005; Hoerauf et al., 2003; Ismail et al., 1996; Rao et al., 1990; Shenoy et al., 1999, Shenoy et al., 2000; Supali et al., 2002). In bancroftian and brugian filariasis, 3, 4, 6, and 8-week courses of treatment with 100 or 200 mg/day of doxycycline, alone or in combination with albendazole or ivermectin, result in macrofilaricidal effect, decrease of microfilaremic level, and diminish of adverse drug reactions (Debrah et al., 2007; Hoerauf et al., 2003a; Supali et al., 2008; Taylor et al., 2005; Turner et al., 2006a). Interestingly, single dose of doxycycline combined with the standard DEC regimen can increase the efficacy of standard treatment by reducing filarial antigen level as well as adverse drug reactions (Sanprasert et al., 2010).

It has been discovered that all stages of *W. bancrofti* and *B. malayi* harbor the intracellular bacteria *Wolbachia* (Kozek, 1977; Kozek and Manoquin, 1977; McLaren

et al., 1975). *Wolbachia* have generated substantial interest in recent years, primarily because of the effects they have on their arthropod hosts, which include induction of cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male-killing (Knight, 2001; Stouthamer et al., 1999; Werren et al., 2008). *Wolbachia* is a genus of the class Alphaproteobacteria and belongs to the order Rickettsiales, and family Anaplasmataceae (Dumler et al., 2001). These obligate intracellular gram-negative bacteria are widespread in arthropods and filarial nematodes, including *B. malayi*, *W. bancrofti*, and *Onchocerca volvulus*, the major human parasitic filarial nematodes, and *Dirofilaria immitis*, the pathogenic filarial nematode of canine and feline heartworm disease (Bandi et al., 1998; Henkle-Duhrsen et al., 1998; Sironi et al., 1995). Three supergroups of the genus *Wolbachia* are found in filarial nematodes: supergroup C *Wolbachia* in the lymphatic filarial parasites, *W. bancrofti* and *B. malayi;* supergroup D *Wolbachia* in most species of the genus *Onchocerca* and *Dirofilaria*, including *O. volvulus* and *D. immitis;* supergroup F *Wolbachia* in the nematode *Mansonella ozzardi*, that causes human mansonelliasis (Bandi et al., 1998; Casiraghi et al., 2001; Casiraghi et al., 2005; Lo et al., 2007). *Wolbachia* are found in all developmental stages of filarial nematodes in which the bacteria are restricted in the lateral hypodermal cords of filarial nematodes, and in the reproductive tissues of the females (e.g., in the oogonia, oocytes, embryos, and mf) (Kozek, 1977; Kozek and Marroquin, 1977; Fenn and Blaxter, 2004a; McGarry et al., 2004). This suggests that *Wolbachia* are vertically transmitted through the cytoplasm of the egg. While the arthropod *Wolbachia* can be characterized as parasitic, there is evidence to suggest that the association between *Wolbachia* and filarial nematodes is mutualistic (Fenn and Blaxter, 2004b). The phylogeny of the filarial nematode *Wolbachia* is parallel with that of their hosts. The evolutionary aspect suggests long-term co-evolution and

co-adaptation, which is usually seen in mutualistic relationships (Baumann et al., 1995; Bordenstein et al., 2009; Fenn and Blaxter, 2004b).

The effects of antimicrobial agents (e.g., tetracycline and doxycycline), which have anti-rickettsial activity on *Wolbachia*, result in blocking embryonic development, and inhibiting molting of third stage larvae (L3) to fourth-stage larvae (L4) in many filarial nematodes (e.g. *B. malayi* (Rajan, 2004), *D. immitis*, and *B. pahangi*) (Bandi et al., 1999; Smith and Rajan, 2000). Tetracycline and doxycycline can affect motility of *B. malayi* mf *in vitro* (Rao and Weil, 2002). Therefore, it has provided an alternative approach to the treatment and control of these filarial parasites with antimicrobial agents. Studies have shown that antibiotics with anti-rickettsial activity can eliminate the *Wolbachia* and result in worm growth retardation, infertility, and reduced viability (Chirgwin et al., 2003a; Chirgwin et al., 2003b; Hoerauf et al., 1999; Shakya et al., 2008; Townson et al., 2000). In contrast, nematodes not infected with *Wolbachia* are unaffected by antimicrobial agents treatment (Hoerauf et al., 1999). Interestingly, it has been shown that chemically modified tetracyclines (CMTs) col-3 and col-8, which have no anti-rickettsial activity, can block the L3 to L4 molting in *B. malayi* (Rajan, 2004). These derivatives might have other effects on the *Wolbachia* and worm protein synthesis directly and/or indirectly (Brazas and Hancock, 2005).

The murine filarial nematode gene, *Litosomoides sigmodontis* phosphate permease (*Ls-pp1*), is up-regulated after treating the filarial-infected mice with tetracycline (Heider et al., 2006). Furthermore, the *L. sigmodontis* mitochondria encoding subunit of respiratory chain complexes such as cytochrome *b*, cytochrome *c* oxidase subunit 1-3, NADH-dehydrogenase subunit 1-6, and ATPase subunit 6, are

also founded up-regulated. It is hypothesized that *L. sigmodonis* attempts to compensate the nucleotide and energy metabolisms caused by *Wolbachia* deprivation (Heider et al., 2006; Strübing et al., 2010). Moreover, *Wolbachia* depletion in onchocerciasis patients treated with 100 mg/day doxycycline for 6 weeks might have caused indirect effects, resulting in the up-regulation of worm mitochondrial *hsp60* gene. It is hypothesized that the increased expression of HSP60 in the absence of *Wolbachia* is due to a disruption of the homeostasis of the endosymbiosis (Pfarr et al., 2008).

Recent field trials of the oxytetracycline against onchocerciasis in cattle, caused by *O. ochengi* (Langworthy et al., 2000), and of doxycycline in human onchocerciasis, caused by *O. volvulus* (Hoerauf et al., 2000; Hoerauf et al., 2001; Hoerauf et al., 2003b), and lymphatic filariasis, caused by *W. bancrofti*, (Taylor et al., 2005; Hoerauf, 2003a), have demonstrated the validity of the antimicrobial agents. In all cases, the antimicrobialtreatments result in a pronounced reduction in microfilaremia/microfilaridermia and a prolonged inhibition of embryogenesis. Moreover, an eight-week course of doxycycline 200 mg daily also proved to be macrofilaricidal against *W. bancrofti* (Taylor et al., 2005). A study has shown that a three-week course of doxycycline can lead to a sustained amicrofilaremia, but does not result in macrofilaricidal activity (Turner et al., 2006b). Recently, *W. bancrofti* patients were treated with 200 mg doxycycline per day for 4 weeks and followed by a single dose of 150 µg/kg ivermectin. Four months after doxycycline treatment, cases had a significantly lower *Wolbachia* load than controls. Twenty-four months after treatment, microfilaraemia, antigenaemia, and frequency of filarial dance sign (FDS) were significantly lower in cases than controls. Most importantly, 4 weeks of

doxycycline killed 80% of mf, which is comparable with the results of a 6-week regimen (Debrah et al., 2007).

To date, no long-term *B. malayi* cell culture system exists for studying the mutualistic bacterial endosymbiont. Currently, *Wolbachia* that has been maintained in insect cell cultures are tested for susceptibility to 13 antimicrobial agents (Fenollar et al., 2003). An *in vitro* antimicrobial susceptibility study in *B. malayi* mf model showed that doxycycline, at the concentration of 80 and 40 μg/ml, caused >50% reduction in motility at 24 and 48 hours after exposure, respectively (Rao and Weil, 2002). However, the more experiments of mf antimicrobial susceptibilities should be performed, to evaluate anti-*Wolbachia* or antifilarial agents for *in vitro* screening. In addition, the *Wolbachia* antimicrobial susceptibilities, mf *Wolbachia*/nematode gene ratio, and gene expression profiles of mf and/or *Wolbachia* after antimicrobial agents' exposure have not yet been reported. Therefore, in order to understand the role and mechanism of these agents and to follow-up the lymphatic filariasis patients after treatment, a study to investigate the effect of the antimicrobial agents and that have the anti-rickettsial activity on mf should be considered.

In the first part of this study, we compared the effects of anti-rickettsial drugs such as doxycycline, rifampicin, and ciprofloxacin on the motility of *B. malayi* mf. We investigated the minimum effective concentrations (MECs), the standard concentration for antimicrobial test, at the concentration of 0.125-256 μg/ml, for antifilarial activity against *B. malayi* mf. The mf motility was observed and scored at the different time points, 12 h and 52 h, similar to previous study performed by Rao and Weil (2002). We expected that at 12 h after treatment, the antimicrobial agents could affect the mf motility. Furthermore, *Wolbachia* susceptibilities to antimicrobial

agents, including doxycycline, rifampicin and ciprofloxacin, were determined by quantitative PCR (qPCR). The results were presented as ratios of *Wolbachia* gene to mf gene (McGarry et al., 2004; Bazzocchi et al., 2008).

During the past few years, a new approach has emerged to access differences in gene expression between various cell types, or with in the same cells under different conditions. This technology, referred to as microarray technology or expression profiling, has the ability to rapidly, and reliably scan a large number of different mRNAs (Trevino et al., 2007). Recently, microarray analysis has provided the first broad gene expression view of the third (infective) stage larvae (Li et al., 2008), gender-regulated (Li et al., 2005; Li et al., 2011), and *B. malayi* adult treated with tetracycline (Ghedin et al., 2009). In addition, the microarray analysis has been used to investigate the effect of DEC on mf *in vivo*. *Meriones unguiculatus* (Mongolian gerbil), infected intraperitonelly with *B. malayi*, are given 10 mg/kg of DEC intraperitonelly for one hour and the mf are harvested. Many of the microfilarial genes that are significantly down-regulated with DEC treatment (such as 40S ribosomal protein, elongation factor 1α1, and ribosomal protein L10 and S15A) were shown to be involved in transcription/translation. Other down-regulated genes are involved in a variety of functions including intracellular iron storage and lipid metabolism. DEC up-regulated genes included hypothetical proteins and unknown transcripts (Weinkopff et al., 2008). However, no previous data about the molecular effect(s) of doxycycline on the blood-dwelling mf and *Wolbachia*, have been reported. Therefore, microarray technology should be used to study the set of gene expression profiles involved in doxycycline-treated mf.

In the second part of this study, microarray technology that permits high-throughput comparisons of gene expression was used to study the biology of mf cultured *in vitro* at 0, 23, and 48 h, similar to the previous study of mf motility performed by Rao and Weil (2002). Our data provide a transcriptional basis for understanding the mf biology, stage-specific focusing on ES proteins, and a suitable vaccine and pharmaceutical targets for controlling lymphatic filariasis using the Partek program (Downey, 2006) and gene ontology databases (Zdobnov and Apweiler, 2001). The microarray experiment also used to identify the mf genes after exposure to doxycycline *in vitro* at 13, 36, and 61 h, the concentration that caused the different mf motility phenotypes. Microarray datasets were also analyzed by using the Partek program and gene ontology databases.

2. Research questions

1) Do the anti-rickettsia drugs have the effects on the motility and *Wolbachia*/nematode gene copy number ratio of *B. malayi* mf?
2) Which are the groups of *B. malayi* mf genes that could up-regulate their expression in mf cultured *in vitro*?
3) Which are the groups of *B. malayi* mf genes that could up-regulate and down-regulate their expression following the treatment of mf by doxycycline *in vitro*?

3. Hypothesis

1) Anti-rickettsia drugs could have the effects on the motility and *Wolbachia*/nematode gene copy number ratio of *B. malayi* mf.

2) The groups of genes involved in the energy metabolism and excretory/secretory pathway of *B. malayi* mf could be up-regulated.

3) The groups of genes involved in the homeostasis of *B. malayi* mf and *Wolbachia* could up-regulate their expression.

4. Objectives

1) To study the effects of anti-rickettsia drugs on the motility and *Wolbachia*/nematode gene copy number ratio of *B. malayi* mf.

2) To characterize the groups of *B. malayi* mf genes that could up-regulate their expression in mf cultured *in vitro.*

3) To characterize the groups of *B. malayi* mf genes that could up-regulate and down-regulate their expression following the treatment of mf by doxycycline *in vitro*.

5. Keywords

Brugia malayi

Microfilaria

Doxycycline

Anti-rickettsial drugs

Wolbachia

Microarray

Gene expression

6. Expected benefits and applications

1) The study on the effects of anti-rickettsia drugs will be useful as an alternative drug regimen. The qPCR technique can be adapted to test the anti-*Wolbachia* agents for *in vitro* study and to follow-up the lymphatic filariasis patients after treatment.

2) This study will provide a transcriptional basis for understanding the mf biology, stage-specific focusing on ES proteins, and the suitable vaccine and pharmaceutical targets for controlling lymphatic filariasis.

3) The understanding of the *Wolbachia* and *B. malayi* mf relationship in gene expression should be valuable for finding the anti-filarial and anti-*Wolbachia* drug targets.

CHAPTER II

LITERATURE REVIEWS

1. Filarial nematodes

Filarial nematodes, or filariae, are thread-like parasites of humans and various animal species that are transmitted by blood-sucking vectors. They refer to nematodes of the order Spirurida that have been grouped into the superfamily Filarioidea (Anderson and Bain, 1976). This superfamily is consisted of two families: Filariidae and Onchocercidae. It is important to note that all the filarial nematodes that cause known diseases in humans are members of the subfamilies, Onchocercinae and Dirofilariinae, of the family Onchocercidae. The family also encompasses additional 6 subfamilies (Waltonellinae, Setariinae, Oswaldofilariinae, Icosiellinae, Splendidofilariinae, and Lemdaninae) (Bain and Chabaud, 1986). Taxonomy of the family Onchocercidae is shown in Figure 2.1.

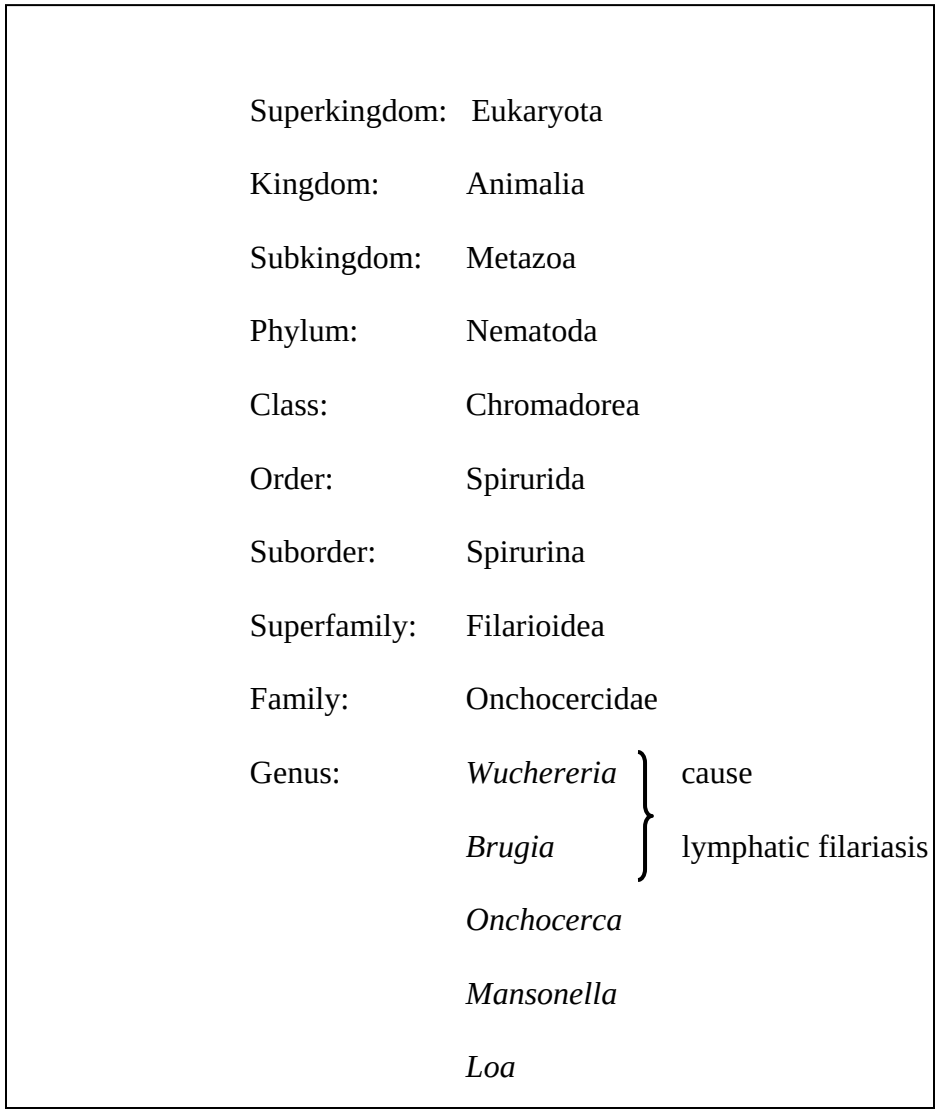


Figure 2.1 Taxonomy of the family Onchocercidae

Filarial diseases are a major health problem in many tropical and subtropical areas. The major parasites of humans are *W. bancrofti, B. malayi* and *Onchocerca volvulus* (Table 2.1). The adult worms inhabit specific tissues where they mate and produce microfilariae (mf), the characteristic tiny, thread-like larvae. The mf infects vector arthropods, in which they mature to the 3rd-stage infective larvae.

Table 2.1 Major pathogenic filarial parasites of humans (Garcia et al., 2001; Zagaria and Savioli, 2002)

Group	**Species**	Disease	**Adult locations**	**Mf locations**	**Major vectors**
Lymphatic filariasis	*Wuchereria bancrofti*	Lymphatic filariasis	Lymphatics	Blood	*Culex quinquefasciatus* *Aedes*/*Ochlerotatus* spp. *Anopheles* spp. *Mansonia* spp.
	Brugia malayi	Lymphatic filariasis	Lymphatics	Blood	*Mansonia* spp. *Anopheles* spp. *Aedes*/*Ochlerotatus* spp. *Coquillettidia crassipes*
	Brugia timori	Lymphatic filariasis	Lymphatics	Blood	*Anopheles barbirostris*
Subcutaneous filariasis	*Onchocerca volvulus*	Onchocerciasis, river blindness	Subcutaneous tissue	Skin	*Simulium* spp. (Black fly)
	Loa loa	Loaiasis	Subcutaneous tissue	Blood	*Chrysops silacea* *C. dimidiata*
	Mansonella streptocerca	Streptocerciasis	Subcutaneous tissue	Skin	*Culicoides* spp. (Biting midge)
Serous cavity filariasis	*Mansonella perstans*	Perstans filariasis	Body cavities	Blood	*Culicoides* spp.
	Mansonella ozzardi	Ozzardiasis	Subcutaneous tissue	Blood	*Culicoides* spp. *Simulium* spp.

2. Lymphatic filariasis (Elephantiasis)

Lymphatic filariasis, known as elephantiasis, is caused by the filarial parasites: *W. bancrofti*, *B. malayi*, and *B. timori* (Fischer et al., 2004; Ottesen et al., 1997). The majority of the infected cases are affected by *W. bancrofti* accounting for 90% of the cases, and the minority by *B. malayi* accounting for 10%, and 0.67% by *B. timori*. According to worldwide estimation, over 120 million people are infected in 83 countries, including Thailand, and 40 millions of them are seriously debilitated and disfigured by the disease (Triteeraprapab and Songtrus, 1999; Triteeraprapab et al., 2001; World Health Organization, 2003; World Health Organization, 2009). Lymphatic filariasis is ranked by the World Health Organization (WHO) as the world's second leading cause of permanent and long-term disability (Behbehani, 1998). On the other hand, it is the world's third of the most tropical diseases leading cause of long-term disability, with disease burden estimated at 5.6 million disability adjusted life-years (DALYs: the number of healthy years of life lost due to premature death and disability) (Morel, 2000). Among the pathogens causing lymphatic filariasis, *W. bancrofti* is prevalent in tropical areas worldwide, while *B. malayi* is limited to Asia, and *B. timori* is restricted to some islands of Indonesia

2.1. Life cycle

The infection is transmitted by biting of infected mosquitoes (Figure 2.2). The typical major vector for *B. malayi* filariasis are mosquito species from the genera *Mansonia*, *Anopheles*, *Ochlerotatus*, and *Coquillettidia* whereas the mosquito vector generas for *W. bancrofti* are *Culex, Anopheles, Aedes,* and *Ochlerotatus* (Garcia et al.,

2001; Zagaria and Savioli, 2002). During a blood meal, the infective larvae or third-stage larvae (L3) of lymphatic filarial parasites, penetrate into the bite wound, and pass to the lymphatic vessels and lymph nodes where they develop into an adult stage, mate, and ultimately produce mf. Adult lymphatic filarial parasites have a life span of 5-10 years, while mf can live long for 6-12 months (TDR, 2005; Vanamail et al., 1990).

Millions of the offsprings of the female adults are released into the host's blood circulation, and can infect a biting mosquito. After infection, these mf shed their sheath, penetrate the stomach wall, and migrate to the thoracic muscles. Then they undergo metamorphosis into first-stage larvae (L1), and subsequently the mature infective third stage larvae. The infective larvae migrate to the mosquito's proboscis, from which they pass to another human, and the life cycle is re-initiated via the mosquito bites (Garcia et al., 2001; Zagaria and Savioli, 2002).

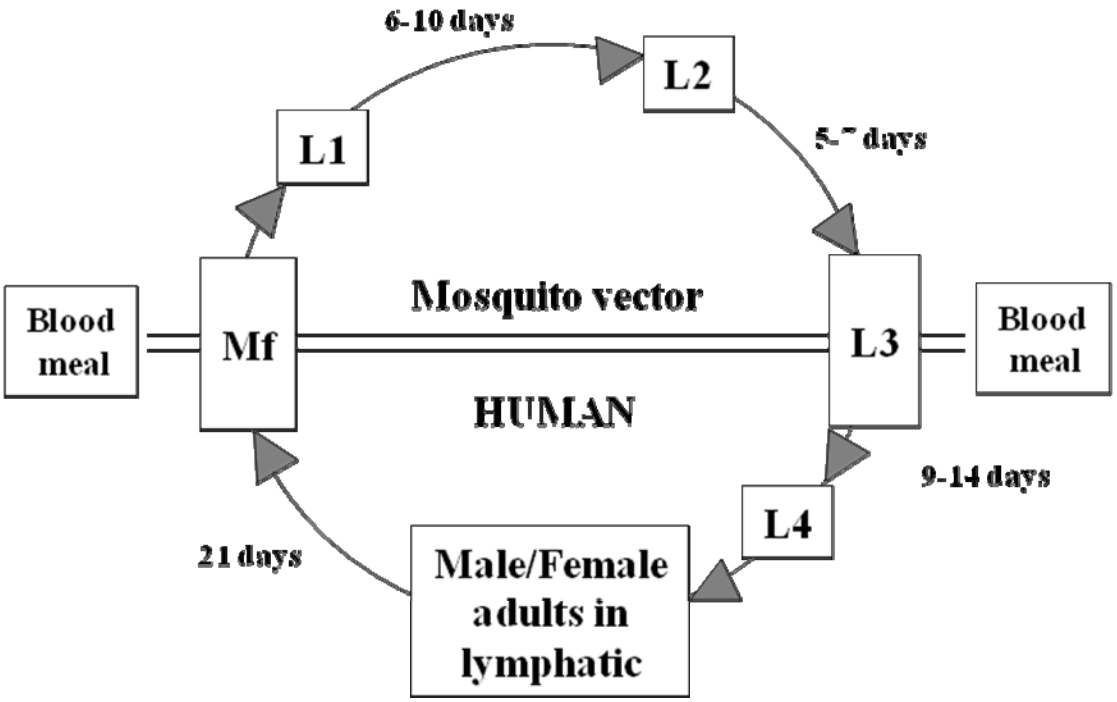


Figure 2.2 **Life cycle of lymphatic filarial parasites; Mf, microfilaria; L1, the first-stage larva; L2, the second-stage larva; L3, the third-stage larva (infective stage); L4, the fourth-stage larva**

The *W. bancrofti* female worms measure 80 to 100 millimeters (mm) in length by 0.24 to 0.3 mm in width, and males measure 40 mm in length by 0.1 mm in width. Adults produce sheathed mf, measuring 244 to 296 micrometers (µm) in length and 7.5 to 10 µm in width. The *B. malayi* female worms measure 43 to 55 mm in length by 0.13 to 0.17 mm in width, and males measure 13 to 23 mm in length by 0.07 to 0.08 mm in width. Adults produce sheathed mf, measuring 170 to 230 µm in length and 5 to 7 µm in width (Beaver, 1984).

2.2 Clinical manifestations of lymphatic filariasis

There is a wide range of clinical manifestations of longstanding infection with lymphatic filarial parasites (Figueredo-Silva et al., 2002). Generally, lymphatic filariasis consists of asymptomatic microfilaremia. Other patients who carry the adult worms may be amicrofilaremic and asymptomatic, or have acute lymphangitis and the chronic manifestations of the diseases (haematuria, hydrocele, chylocele, chyluria, lymphedema and elephantiasis). Additional manifestations of the filarial infection are tropical pulmonary eosinophilia (TPE) syndrome, and drug-induced adverse reactions (Dreyer et al., 1999)

2.2.1 Asymptomatic microfilaraemic state

In areas where lymphatic filariasis is endemic, the vast majority of infected individuals have a few overt clinical manifestations of filariasis, despite the presence of large numbers of circulating mf in the peripheral blood. It has now been clearly indicated that, although they may be clinically asymptomatic, almost all patients with *W. bancrofti* or *B. malayi* microfilaremia have some degree of subclinical disease. Subclinical forms of the disease recognized in microfilaremic individuals are haematuria and/or proteinuria that reflect low-grade renal damage (Dreyer et al., 1992). The renal abnormalities are found ~40% of the microfilaremic patients. Ultrasound has shown that approximately half of the men with asymptomatic microfilaremia have nests of motile adult worms in their scrotal lymphatics: the ‘filarial dance sign’ (FDS) (Amaral et al., 1994; Norões et al., 1996a; Shenoy et al., 2007a). Another form is from the observations on microfilaremic patients using lymphoscintigraphy to visualize the functional anatomy

of lymphatic vessels (Dissanayake et al., 1995; Freedman et al., 1994; Suresh et al., 1997). The lymphatics are abnormally dilated but there is no evidence of an inflammatory response (Amaral et al., 1994). Their lymphatics are markedly dilated with collateral channeling and increased lymph flow. By contrast, patients with elephantiasis show tortuosity, dermal back-flow, obstruction, stasis and poor regional node visualisation (Witte et al., 1993). However, these changes are not specific for lymphatic filariasis and can be found in residents of non-filarial-endemic areas as well (Marchetti et al., 1998). Although clinically asymptomatic, they have markedly abnormal, dilated and tortuous lymphatic vessels, and obviously atypical patterns of lymphatic flow (Dissanayake et al., 1995; Freedman et al., 1994; Shenoy et al., 2007b; Suresh et al., 1997).

2.2.2 Acute manifestations 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 *W. bancrofti* infection (bancroftian filariasis), in addition to the lymph nodes in the inguinal, axillaries and epitrochlear regions, the lymphatic system of the male genitalia is frequently affected, leading to funiculitis, epididymitis or orchitis, or to a combination of these (Pani et al., 1995). In brugian filariasis (*Brugia* infection), the affected lymph nodes are mostly positioned in the inguinal and axillaries, with inflammation along the course of the distal lymphatic vessels (Pani et al., 1990).

The acute clinical course of lymphatic filariasis may last for several days or up to 4-6 weeks with a fulminating episode, and may result in prolonged inability to work (Gyapong et al., 1996). The 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, two distinct types of acute ADL episodes are recognized: (a) ADL caused directly by the parasite infection itself; and (b) ADL secondary to bacterial or fungal infection. The former ADL is termed acute filarial lymphangitis (AFL). The most common presentation is that of a cord-like structure associated with retrograde lymphangitis in the lower or upper limps. Lymphangitis is frequently accompanied by mild fever, headache, and malaise. In the scrotal area or the breast it may present as a painful palpable nodule. Recurrence of these attacks at the same sites is common (Shenoy et al., 1995; Pani et al., 1995). The latter form of ADL is the most common form. It is usually recognized as a syndrome with a clinical picture that can include high fever, chills, myalgia and headache. Recent evidences have suggested that bacterial or fungal super infections of limbs with compromised lymphatic function play the primary role in triggering episodes of ADL (Montestruc et al., 1960; Olszewski et al., 1993), which themselves actually cause or exacerbate the chronic obstruction changes in the lymphatic vessels of affected patients. The acute process usually starts in the skin and then spreads along the lymphatic vessels to the lymph nodes (Olszewki et al., 1993). Based on the observations, this form of acute ADL is termed acute dermatolymphangioadenitis (ADLA) (Olszewki et al., 1993).

It has also been suggested that exposure to 3rd-stage filarial larvae causes lymphangitis and triggers the onset or progression of lymphoedema. A role for 3rd or 4th-stage larvae in lymphangitis or lymphoedema is supported by animal studies, experimental infections (Nutman, 1991) reports of disease in individual patients travelling from non-endemic areas (Moore et al., 1996) and epidemiologic observations that associate incidence of acute adenolymphangitis with filarial transmission intensity (Bockarie et al., 2002). However, a case definition has not been established for larva-associated lymphangitis that distinguishes it from AFL or ADLA; this makes epidemiological study difficult. Additional work is needed to clarify the incidence, possible mechanisms, and clinical expression of larva-associated filarial lymphangitis and to assess its public health importance in filariasis-endemic areas.

2.2.3 Chronic manifestations of lymphatic filariasis

The chronic signs of lymphatic filariasis rarely develop before the age of 15 years, and only a small proportion of the filarial-infected population is affected. However, immigrants from areas where filariasis is not endemic tend to develop elephantiasis more often, and much sooner (sometimes within 2-3 years) than do the local population of endemic areas (Partono, 1987). Out of 120 million cases of lymphatic filariasis, 16.02 million (13.3%) cases are of lymphoedema, and 26.79 million (22.3%) cases are of hydrocele (Michael et al., 1996). In bancroftian filariasis, the incidence of the major signs of chronic disease: hydrocele, chyluria, lymphoedema, 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 of frequency (Pani et al., 1990; Pani et al.1995;

Njenga et al., 2007). In brugian filariasis, the leg below the knee is characteristically affected, and sometimes the arm below the elbow. Genital involvement has not been reported, except in areas where brugian filariasis occurs together with *W. bancrofti*.

Lymphoscintigraphic studies have shown that lymphoedema is not always the results of occlusion of lymphatic channels, but can also happen when there is extensive collateralization. Skin changes such as skin fold thickening, hyperkeratosis, hypo- or hypertrichosis, pachydermia, pigmentary changes, chronic ulceration, epidermal and sub-epidermal nodules, may also be seen in chronic infection (Burri et al., 1996).

2.2.4 Tropical pulmonary eosinophilia (TPE)

TPE is a syndrome that results from an immunologic hyperresponsiveness to filarial parasites, *W. bancrofti* and *B. malayi*, and is characterized by cough, dyspnoea and nocturnal wheezing, diffuse reticulonodular infiltrates in chest radiographs and marked peripheral blood eosinophilia (Ong and Doyle, 1998; Ottesen and Nutman, 1992; Vijayan, 1996). Microfilaraemia are almost never present in the blood, but remnants of mf surrounded by aggregates of eosinophils are sometimes found in the liver, spleen, lymph nodes or lungs (Spry and Kumaraswami, 1982). Eosinophilia, an increased levels of IgE and of anti-filarial antibodies are commonly found (Ottesen and Nutman, 1992). The eosinophils found in bronchoalveolar lavage fluid of patients with TPE are degranulated and activated, and release abnormally high levels of toxic oxygen radicals (Pinkston et al., 1987; Rom et al., 1990). The eosinophilic granular protein, eosinophil-derived neurotoxin (EDN), associated with eosinophil trafficking is suggested to play the most important role in the pathogenesis of TPE (O'Bryan et al., 2003). In addition, a

major IgE-inducing antigen (Bm23-25) of the filarial parasite *B. malayi* has been identified from patients with TPE (Lobos et al., 1992). This Bm23-25 antigen, present in the infective L3 stage larvae of the filarial parasite, has been found to be the homolog of the enzyme gamma-glutaryl transpeptidase light chain subunit (Lobos et al., 1996). There is a molecular mimicry between the parasite gamma-glutaryl transpeptidase and the human gamma-glutaryl transpeptidase present on the surface of pulmonary epithelium (Gounni et al., 2001; Lobos et al., 2003; Vijayan, 2007).

2.3 Pathogenesis of lymphatic filariasis

In general, the microfilaremic patients can remain asymptomatic for undetermined period of time, or progress into the chronic disease. The pathogenesis of lymphatic filariasis results from a complex interplay of factors related to adult worms (pathogenic potential and worm burden), host immune responses (resistance or tolerance), and secondary bacterial and fungal infections (Dreyer et al., 2000; Freedman, 1998).

2.3.1 Subclinical lymphangiectasia caused by living adult lymphatic filarial parasites

The pathology in investigated lymphatic vessels consists of distinct histological features related to the presence of both live and dead parasites (Jungmann et al., 1991; Jungmann et al., 1992). In endemic areas, the most common change in patients who carry living adult worms is subclinical lymphangiectasia (Dreyer et al., 1999; Dreyer et al., 2002; Fox et al., 2005). In such pathology, lymphatic vessels that contain living adult worms are dilated, without any inflammatory responses in the wall. Lymphatic dilatation with none of inflammatory reactions can be observed in nude mice (a mutant

mouse strain that lacks a thymus gland and T lymphocytes) or severe combined immunodeficient mice (SCID mice: mice genetically engineered to lack T and B lymphocytes) infected with *Brugia* species (Nelson et al., 1991; Vincent et al., 1984), and can be reversed in nude mice by removing or killing the adult worms (Vickery et al., 1991). The data suggested that factors related to the parasites themselves, rather than being those immunologically mediated, contribute to lymphangiectasia. Since it can cause lymphatic dysfunction, lymphangiectasia is a major risk factor for development of chronic lymphatic disease.

2.3.2 Acute filarial lymphangitis (AFL) is triggered by the death of adult lymphatic filarial parasites

Acute filarial lymphangitis (AFL) is designated an acute condition that presents as a restricted inflammatory nodule or cord in a lymphatic or a lymph node of an extremity, a breast (in woman), or scrotum (in man). AFL is caused by death of adult lymphatic filarial parasites, either spontaneously or because of treatment with a macrofilaricidal drug (Dreyer et al., 1999a; Figueredo-Silva et al., 1996). It should be noted that not all natural or drug-triggered AFL episodes cause clinical illness. In many patients, granulomatous reaction develops around dead parasites in the lymphatics without any clinical outcome, and is only detected, incidentally, during physical examinations (Olszewski et al., 1993). However, in other patients, AFL accompanied by local pain, swelling, and tenderness develops corresponding to the granulomatous inflammatory reaction around dying or degenerating parasites.

The studies of human biopsy specimens suggest that AFL is an acute inflammatory process that is triggered by products released from dying or disintegrating parasites, and that neither living nor completely calcified dead parasites cause the acute inflammatory changes (Cooray, 1960; Galindo et al., 1962; Jungmann et al., 1991; Jungmann et al., 1992; Lichtenberg, 1957).

Ultrasonographic and histopathological studies have documented an episode of AFL triggered by treatment with diethylcarbamazine (DEC) that the acute attack in this case occurs in a body site, where previously living parasites were killed by the drug (Dreyer et al., 1995; Figueredo-Silva et al., 1996; Norões et al., 1997). Because of the high prevalence of living adult *W. bancrofti* in the lymphatics of the spermatic cord (Dreyer et al., 1996; Norões et al., 1996a; Norões et al., 1996b), treatment-triggered acute filariasis is particularly common in the scrotal area. The local reactions observed under histopathological investigations of biopsy samples from patients infected with *W. bancrofti* reveal mild infiltration of inflammatory cells in an early phase following parasite death by DEC treatment (Figueredo-Silva et al., 2002). In the later phase, the lymphatic vessel is blocked by granulomatous inflammatory reactions around dead parasites, with variable numbers of eosinophils, lymphocytes, plasma cells and large macrophages. Rarely, neutrophils may filtrate in the center of the granuloma. The systematic responses are characterized by significantly elevated levels of TNF-α levels, and a positive correlation between its levels and the severity of the AFL (Das et al., 1996).

The simultaneous death of many adult worms resulting AFL is a risk factor for development of some types of the chronic manifestations, such as hydrocele, chylocele and chyluria (Dreyer et al., 2000).

2.3.3 Acute dermatolymphangioadenitis (ADLA) by secondary bacterial and fungal infections

Dilatation of the lymphatic vessels induced by the presence of the adult parasite finally leads to lymphatic dysfunction, and accumulation of protein-rich fluid in the tissues. The lower limbs, in particular, become predisposed to recurrent bacterial infections. Trauma, interdigital fungal infections, and onchomycosis provide entry sites for bacteria, which multiply rapidly, and cause a reticular lymphangitis of the small collecting vessels, known as acute dermatolymphangioadenitis (ADLA) (Addiss and Brady, 2007; Jungmann et al., 1992). Bacteria that are generally regarded as commensal or saprophytic organisms have been isolated numerous times from the blood or tissue fluid during the acute attacks (Dreyer et al., 1999a; Dreyer et al., 2006; McPherson et al., 2006; Montestruc et al., 1960; Olszewski et al., 1997). In India, the bacteria most frequently associated with ADLA are Group A *Streptococcus*. Other bacteria are often found in cultures, including those that are usually regarded as non-pathogenic (Olszewski et al., 1997; Olszewski et al., 1999). Little has been published on the antimicrobial sensitivity of bacteria isolated from persons with ADLA in filariasis endemic areas. Available experience suggests that the organisms most commonly involved are sensitive to penicillin; thus, penicillin is usually recommended for treatment (World Health Organization, 2006). Recurrent bacterial infections are an important co-factor in the progression to lymphedema and elephantiasis (Dreyer et al., 2000; Esterre et al., 2000).

3. *Wolbachia* of arthropods and filarial nematodes

Wolbachia is a genus of the class Alphaproteobacteria belonging to the order Rickettsiales (Figure 2.3). These gram-negative intracellular bacteria are found widespread in arthropods as well as in filarial nematodes (Bandi et al., 1998; Werren, 1997). On the basis of *16S rDNA* gene and *groESL* operon sequence analysis, it is organized into the family Anaplasmataceae, which also includes all the species of the genera *Ehrlichia, Anaplasma, Cowdria*, and *Neorickettsia* (Dumler et al., 2001). In contrast to members of the family Rickettsiaceae, which grow in the cytoplasm or nucleus of their eukaryotic host cells, members of the Anaplasmataceae replicate while enclosed in a eukaryotic host cell membrane-derived vacuole.

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alphaproteobacteria
Order:	Rickettsiales
Family:	Anaplasmataceae
Genus:	*Wolbachia*

Figure 2.3 Taxonomy of the *Wolbachia* bacteria

Althought, purified *Wolbachia* bacteria from mosquito cell line were able to survive extracellularly for up to 1 week with no decrease in viability (confirmed by BacLight live-dead staining, PCR, and fluorescence in situ hybridization) (Rasgon et al., 2006), traditional methods for bacterial species and strain determination, which largely depend on pure culture of bacterial isolates, have not been used in the genus *Wolbachia*.

In the absence of a formal nomenclatural system, the *Wolbachia* community currently refers to the different lineages as supergroups (Bandi et al., 2001). In addition, the species name, *W. pipientis*, remains single until new data are generated in different research areas (e.g. comparative genomics, molecular phylogenetics, and screening for *Wolbachia* in new hosts). The DNA-sequence-based methods, including phylogenetic analysis based on *16S rDNA, dnaA, ftsZ, gltA, groEL* and *wsp* genes have been employed for taxonomic classification (Bandi et al., 1998; Zhou et al., 1998; Bordenstein and Rosengaus, 2005).

At present, 11 (named A-K) taxonomic supergroups are described for the genus *Wolbachia* by their places in molecular phylogenies (Figure 2.4). These 11 supergroups are labeled alphabetically, and include supergroup A and B found in various arthropods, supergroup C (*Onchocerca* sp., and *Dirofilaria* sp.) and supergroup D (*W. bancrofti, Brugia* sp., and *Litomosoides* sp.) restricted to filarial nematodes, supergroup E containing *Wolbachia* from springtails (*Folsomia candida*), supergroup F containing *Wolbachia* from termites (*Kalotermes flavicollis* and *Microcerotermes* spp.), weevils (Rhinocyllus conicus), and the filarial nematode *Mansonella ozzardi*, supergroup G and H found in *Wolbachia* from Australian spiders, and the Pacific dampwood termites (*Zootermopsis angusticollis* and *Z. nevadensis*), respectively (Werren et al., 1995; Bandi et al., 1998; Vandekerchove et al., 1999; Lo et al., 2002; Lo et al., 2007; Rowley et al., 2004; Bordenstein and Rosengaus, 2005). The more recently proposed supergroups I, J and K are comprised of cat flea (*Ctenocephalides felis*), filarial nematode (*Dipetalonema gracile*), and spider mite (*Bryobia* spp.) (Ros et al., 2009).

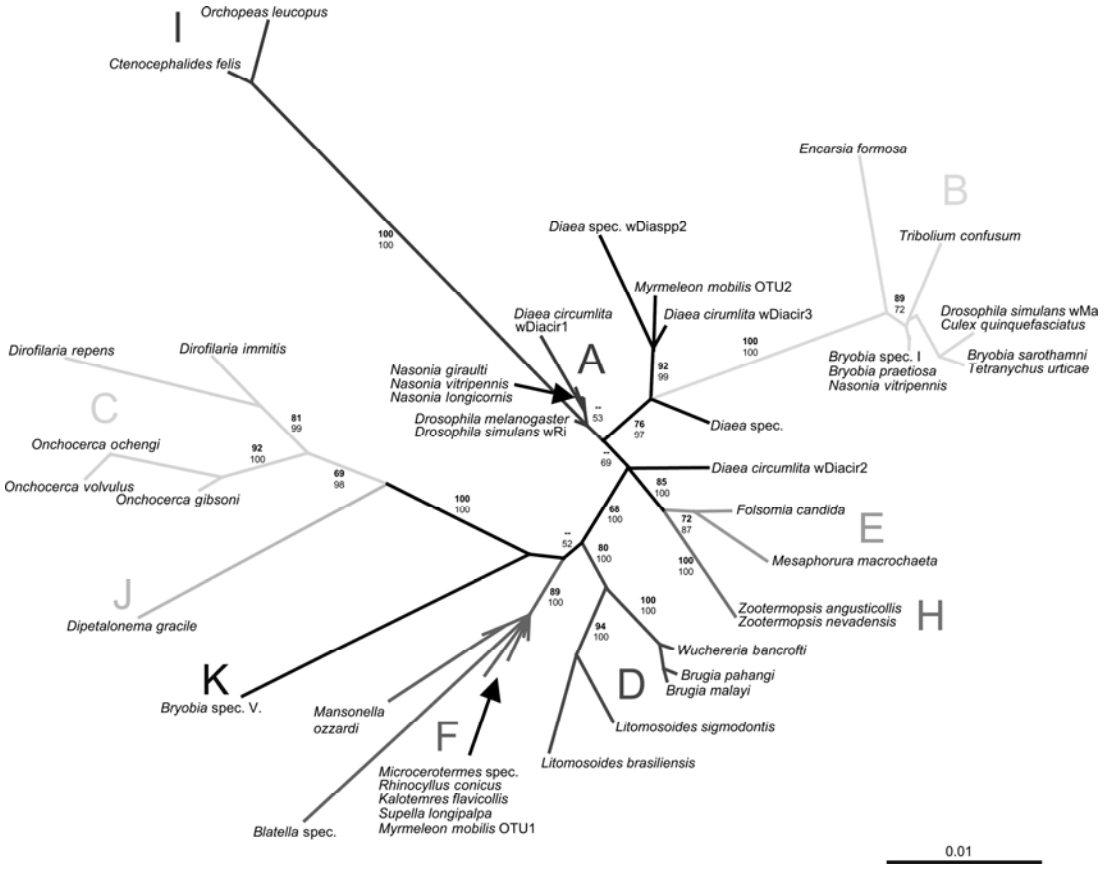


Figure 2.4 **Phylogeny of *Wolbachia* based on *16S rDNA* gene sequences** (Ros et al., 2009). Representatives of *Wolbachia* supergroup A–K are shown.

3.1 *Wolbachia* of arthropods

In 1924, intracellular bacteria were firstly reported, as rickettsia-like microorganisms, within the ovaries and testes of the mosquito *Culex pipiens* by Hertig and Wolbach. They were subsequently named *Wolbachia pipientis* (Werren et al., 1997; Stouthamer et al., 1999). Phylogenies based on 16S rDNA sequences have confirmed that morphological similarities to the Rickettsiae are based on phylogenetic relatedness (O'Neill et al., 1992; Rousset et al., 1992; Stouthamer et al., 1993). It has been estimated

that these bacteria infect at least 20% of all insect species (Haine and Cook, 2005). These estimates result from several *Wolbachia* screenings in which numerous species were tested for infection. However, tests were mostly performed on only one to two individuals per species. The actual percent of species infected will depend on the distribution of infection frequencies among species. A meta-analysis that estimates percentage of infected species based on data on the distribution of infection levels among species was presented (Hilgenboecker et al., 2008). They used a beta-binomial model that describes the distribution of infection frequencies of *Wolbachia*, shedding light on the overall infection rate as well as on the infection frequency within species. The main findings are that (1) the proportion of *Wolbachia*-infected species is estimated to be 66%, and that (2) within species the *Wolbachia* infection frequency within one species is typically either very high (>90%) or very low (<10%) (Hilgenboecker et al., 2008). *Wolbachia* have also been found commonly in isopods (Rousset et al., 1992) and mites (Jeyaprakash and Hoy, 2000). *Wolbachia* infecting the reproductive tissues of arthropods are transmitted maternally from infected females to their progeny via the egg cytoplasm, and have evolved to manipulate host reproduction.

Research interest in *Wolbachia* is initially sparked when it is discovered that they cause several kinds of reproductive changes in arthropod reproduction (Werren and O'Neill, 1997). These reproductive manipulations include (1) inducing embryonic lethality in insect embryos that result when uninfected females are mated to infected males (cytoplasmic incompatibility) (McGraw and O'Neill, 2004; Mercot and Charlat, 2004; Sinkins et al., 2004), (2) inducing parthenogenesis in infected insects (the ability of infected unfertilized insect eggs to successfully develop into functional female adults)

(Huigens et al., 2004; Stouthamer et al., 1993), and (3) overriding chromosomal sex determination in crustaceans to convert infected genetic males into functional phenotypic females (feminization of genetic males) (Cordaux et al., 2004; Moreau and Rigaud, 2003; Rigaud, 1997). Each of these reproductive effects enhances transmission of *Wolbachia* to the arthropod population that is not infected with *Wolbachia* (Werren and O'Neill, 1997).

It has been proposed that the reproductive abnormalities induced by *Wolbachia* are of interest to applied biologists, who are looking for novel means to genetically manipulated populations of insect pests that are important for economic and health reasons (Beard et al., 1993). For instance, in control of transmission of vector-borne diseases, this approach aims to express foreign anti-parasitic or anti-viral gene products in *Wolbachia* harbored by insects. Parasitoids used in biological control of insects may be more effective when infected with parthenogenesis *Wolbachia* (Stouthamer, 1993). *Wolbachia* and its hosts also are ideal candidates for the study of mechanisms of host-parasite relationship, the evolution of infectious diseases, specifically host resistance, parasite virulence, and transmission dynamics (McGraw and O'Neill, 1999; Townson, 2002).

In a recent issue, two previous reports of the presence of *Wolbachia*-derived DNA sequences in a beetle (Kondo et al., 2002) and a filarial nematode (Fenn et al., 2006), was built on by screening whole-genome shotgun data from a wide range of nematodes and arthropods for signs of nuclear insertions of *Wolbachia* DNA (Hotopp et al., 2007). In the genomes of 23 insects, one tick, and two nematodes they found evidence for 22 insertions in five species. The insertions were verified by reamplification and sequencing from genomic DNA, and in the case of a large insertion in the fruit fly *Drosophila*

ananasse by *in situ* hybridization to the nuclear chromosome. All of the species in which insertions were found were known to be infected with *Wolbachia*. Six arthropods known to carry *Wolbachia* had no insertions. In addition, fragments of *Wolbachia* DNA were identified in introns of a previously sequenced gene from a filarial nematode.

Thus, infected animals can carry fragments, sometimes large fragments, of *Wolbachia* DNA in their nuclear genomes. An lateral gene transfer (LGT) with evolutionary significance would show two features: (1) longevity and (2) integration into the host's biology. These *Wolbachia* insertions do not display either feature. There is no evidence of maintenance over long evolutionary periods. The arthropod insertions all have high identity to extant *Wolbachia* genomes, suggesting very recent insertion. In contrast, the nematode insertions are different from extant *Wolbachia* but have accumulated many mutations rendering them nonfunctional (Fenn et al., 2006). Many of the arthropod junctional fragments derive from insertion of *Drosophila* transposons, a pattern also suggestive of nonfunctional DNA. In addition, if this process was important in the evolution of the hosts, they would expect to see *Wolbachia*-like genes in species that do not now have *Wolbachia* but did in the past, or insertions that are present in sister taxa following an insertion in an ancestral organism. Neither was observed. Integration into host biology remains to be demonstrated, in *Wolbachia*-cured but insertion-containing *D. ananasse*, identified RNA transcripts from 28 of 1208 genes in the *Wolbachia* genome (Hotopp et al., 2007), but whether these transcripts are translated and have biological meaning is unknown.

3.2 *Wolbachia* of filarial nematodes

3.2.1 Characteristics and distribution

At the beginning in 1970s, electron microscopy studies of various filarial nematodes, including *Dirofilaria immitis*, *B. pahangi*, *B. malayi*, and *O. volvulus*, have revealed the presence of the intracellular bacteria *Wolbachia* (Kozek, 1977; Kozek and Marroquin, 1977; Lee et al., 1975; McLaren et al., 1975). Although they occur in varying proportions between individual worms, and different developmental stages, they are found throughout all the life cycle stages of the filarial nematode hosts (Fenn and Blaxter, 2004b; Kozek, 1977; Kozek and Marroquin, 1977; McGarry et al., 2004). Within the body of filarial nematodes, the bacteria are restricted in the lateral chords of adults and the reproductive tissues of the females (i.e. in the oogonia, oocytes, embryos and mf) (Figure 2.5A, B and C). However, *Wolbachia* have not been demonstrated in the male reproductive system (Kozek, 2005; Sacchi et al., 2002). These suggest that the bacteria are vertically transmitted through the cytoplasm of the egg, and not through the sperm (Kozek, 1977; Kozek and Marroquin, 1977). Moreover, the following phylogenetic analysis reveals concomitant phylogeny of *Wolbachia* with that of the host filariae (Casiraghi et al., 2001). This provides indirect evidence that transmission has been at least mainly vertical.

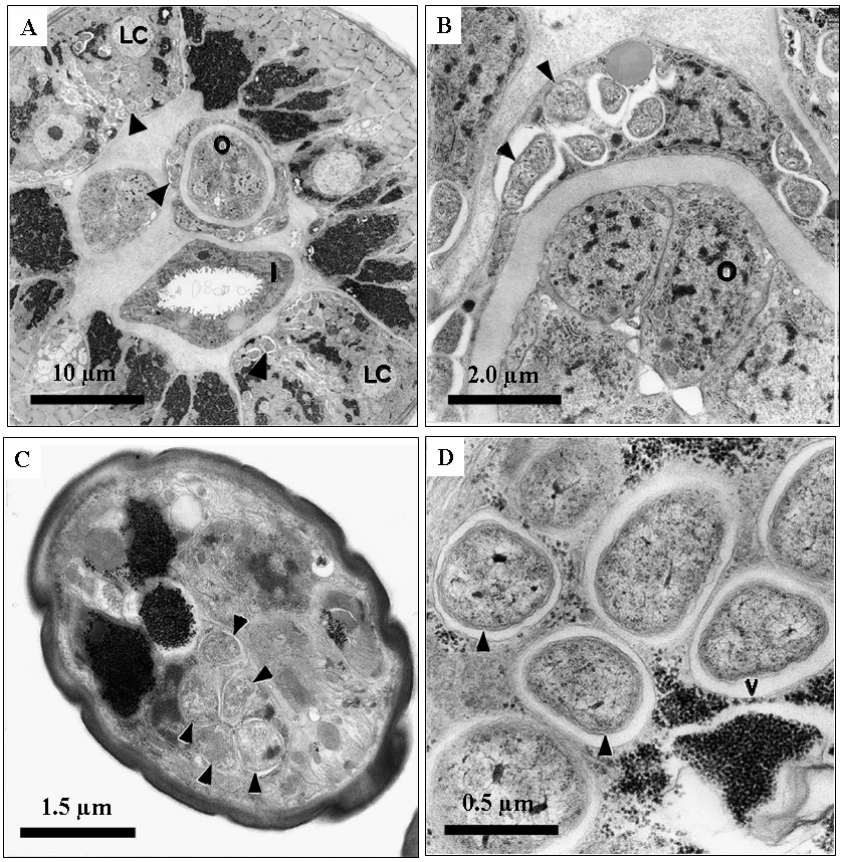


Figure 2.5 **Electron micrographs of *Wolbachia* of *Brugia malayi* and *Wuchereria bancrofti*** (Taylor and Hoerauf, 1999); A, *B. malayi* adult female, showing *Wolbachia* (arrows) in the lateral cords (LC), and the cell layer surrounding the oviduct (O) (I, intestine); B, *Wolbachia* (arrows) in the cell layer surrounding the oviduct (O) of adult female *B. malayi*; C, *Wuchereria bancrofti* mf, showing a cluster of five *Wolbachia* (arrows); D, *Wolbachia* within the lateral cord of adult female *B. malayi*, showing the characteristic double membrane (arrows) and host vacuole (V).

Their morphology are pleomorphic coccobacilli, appearing either as cocci (0.3-0.8 μm in diameter) or as short rods (up to 0.8 μm in diameter × 1.5 μm in length) (Kozek, 1977). Each *Wolbachia* cell lies in an individual vacuole enveloped by three layers of membranes. The outer layer is a host-derived membrane, followed by the outer cell wall of the bacteria; the innermost layer consists of the plasma membrane of the bacteria (Kozek and Marroquin, 1977; Taylor and Hoerauf, 1999) (Figure 2.5D). However, a few *Wolbachia* cells within a host-derived vacuole can be observed. *Wolbachia* may divide by binary fission, the most common mode of replication in bacteria, and possibly by a more complicated method which is similar to the developmental cycle of Chlamydia (Kozek, 2005). This process is accompanied by corresponding changes within the organism: there appears to be a condensation of cytosol (Figure 2.6A), formation of dense inclusions (Figure 2.6B) which may coalesce and form smaller entities within the parent organism (Figure 2.6C). The smaller entities will grow, become less electrondense, and develop into the bacterial forms (Figure 2.6D). Evidence of *Wolbachia* undergoing division is always reported in adult female of filarial nematodes, especially in the reproductive tissues (Kozek, 1977; Kozek, 2005).

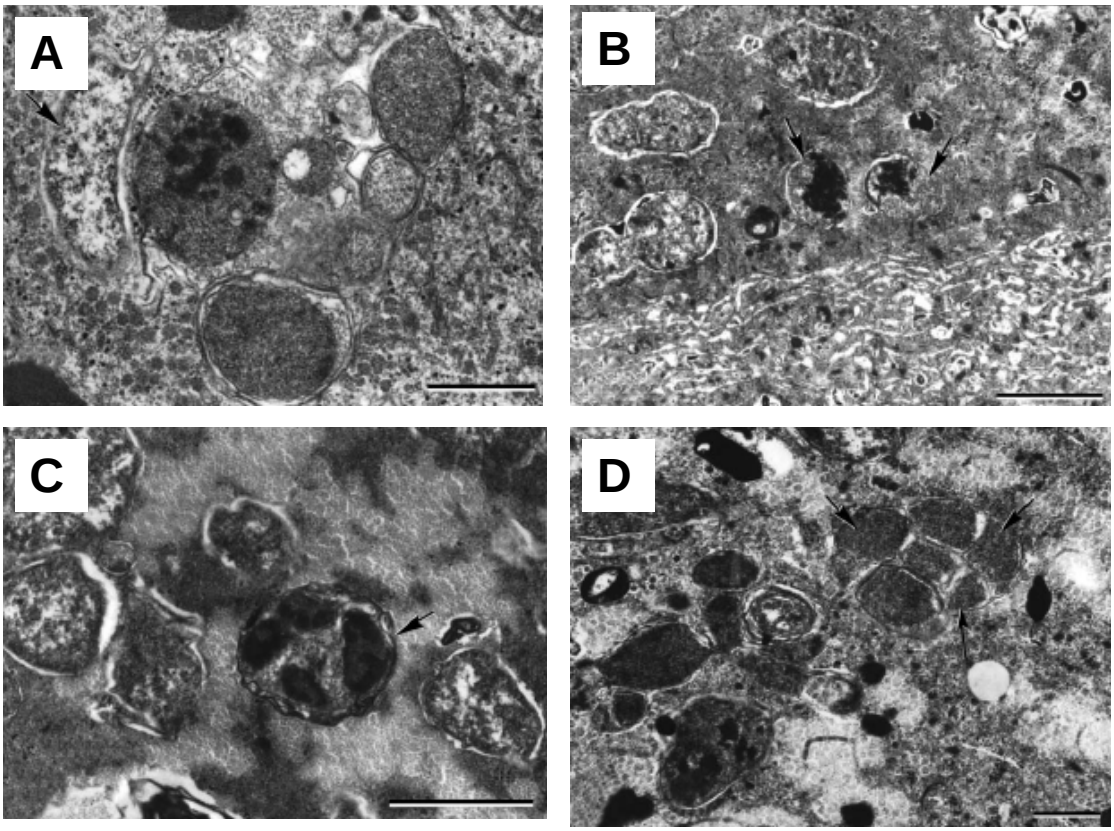


Figure 2.6 **Electron micrographs of *Wolbachia* of *Dirofilaria immitis*** (Kozek, 2005); A, Aggregations of material within a *Wolbachia* organism that is denser and larger than a typical *Wolbachia* (seen on the left, arrow); B, Aggregations of dense material in two *Wolbachia* organisms (arrow); C, Presumed formation of at least four new organisms within a *Wolbachia* (arrow) by Chlamydia-type reproductive cycle; D, Presumed developing forms of *Wolbachia* in the process of de-condensation (arrow). Scale bar of all figures = 0.5 μm.

Host exposure to *Wolbachia* can occur by any one, or a combination, of the following three mechanisms. *Wolbachia* are released when adults, or the larval stages, die as a result of host defense reaction, or after chemotherapy, and are degraded by the host. However, a slower but a more continuous release of *Wolbachia* from the uterus apparently occurs during the life span of the female when free *Wolbachia,* or those present in egg fragments are shed by the female worms when mf are released into the body of the vertebrate host. The third mechanism, perhaps more limited to the soluble Wolbachial products and the small Wolbachial forms (elementary body analogues), may occur through the canalicular network, present in the perienteric surface of the lateral chords, which may contact similar network on the lining of the excretory canals. Thus, the host can be continuously exposed to *Wolbachia* for the duration of the infection and develop an antibody response to *Wolbachia* and its products, e.g., the *Wolbachia* surface protein (WSP) antigen, as has been detected in cats (Bazzocchi et al., 2000), and in humans with pulmonary dirofilariasis (Simon et al., 2003).

In 1990s, two decades after the discover, based on DNA sequence data, the intracellular bacteria have been identified to be closely related to *Wolbachia*, a bacterial genus that encompasses microorganisms found in various arthropods (Sironi et al., 1995; Bandi et al., 1998). Besides an electron microscopy, the molecular techniques employed for surveys of *Wolbachia* are based on PCR followed by sequencing technique, and immunohistochemistry (Bandi et al., 2001). It is now known that *Wolbachia* are widespread in filarial nematodes. Out of the 14 genera so far examined, *Wolbachia* have been revealed in the 8 genera of the total of 19 filarial species (Table 2.2 and 2.3) (Bandi et al., 1998; Casiraghi et al., 2004; Egyed et al., 2002; Fischer et al., 2002; Grobusch et

al., 2003; Henkle-Dushrsen et al., 1998; Sironi et al., 1995). These include *B. malayi*, *W. bancrofti*, and *O. volvulus*, species of importance to human health, and *D. immitis* which causes dog heartworm disease (Bandi et al., 1998; Henkle-Duhrsen et al., 1998; Sironi et al., 1995).

Table 2.2 Detection of *Wolbachia* in the genera of filarial nematodes

Family	Subfamily	No. genera examined	Results for *Wolbachia*	
			Positive	Negative
Filariidae	Filarinae	1	-	1
Onchocercidae	Onchocercinae	8	5	3
	Dirofilariinae	3	1	2
	Waltonellinae	1	-	1
	Setarinae	1	-	1
	Oswaldofilariinae	0		
	Icosiellinae	0		
	Splendidofilariinae	0		
	Lemdaninae	0		

Table 2.3 Distribution of *Wolbachia* in filarial nematodes

Family	Subfamily	Genus	Presence	Absence
Filariidae	Filarinae	*Filaria*	-	*F. martis*
Onchocercidae	Onchocercinae	*Brugia*	*B. malayi* *B. pahangi* *B. timori*	-
		Wuchereria	*W. bancrofti*	-
		Litomosoides	*L. sigmodontis* *L. brasileiensis* *L. galizai* *L. hamletti*	*L. yutajensis*
		Dipetalonema	*D. gracile*	-
		Litomosa	*L. westi*	-
		Onchocerca	*O. volvulus* *O. ochengi* *O. gutturosa* *O. gibsoni* *O. lupi* *O. cervicalis*	*O. flexuosa*
		Mansonella	*M. ozzardi*	*M. perstans*
		Acanthocheilonema	-	*A. viteae* *A. reconditum*
	Dirofilariinae	*Dirofilaria*	*D. immitis* *D. repens*	-
		Foleyella	-	*F. furcata*
		Loa	-	*L. loa*
	Waltonellinae	*Ochoterenella*	-	*Ochoterenella sp.*
	Setarinae	*Setaria*	-	*S. equine* *S. labiatopapillosa* *S. tundra*

The presence of *Wolbachia* in filarial nematodes appears limited to the family Onchocercidae (Table 2.2). Within this family, the positive species are belonged to the subfamilies Onchocercinae and Dirofilariinae, while *Wolbachia* are found negative for the subfamilies Waltonellinae and Setarinae. However, there are both positive and negative species in the Onchocercinae and Dirofilariinae (Table 2.3). In these subfamilies, two filarial species pathogenic to humans, *Loa loa* and *Mansonella perstans*, the rodent filaria *Acanthocheilonema viteae*, the carnivore filaria *A. reconditum*, the bat filaria *Litomosoides yutajensis*, the deer filaria *O. flexuosa*, and the reptile filaria *Foleyella furcata* appear to be *Wolbachia* free (Bandi et al., 1998; Buttner et al., 2003; Casiraghi et al., 2001; Casiraghi et al., 2004; Grobusch et al., 2003; Henkle-Duhrsen et al., 1998; Plenge-Bonig et al., 1995). For the results of screening for *Wolbachia* in nematodes outside the order Spirurida, there is no evidence for the presence of *Wolbachia* (Bordenstein et al., 2003). It is consistent with the hypothesis that *Wolbachia* has entered the nematode phylum once, in an ancestral lineage of filarial nematodes.

Based on the distribution of *Wolbachia* that is placed on the taxonomy of filarial nematodes, hypotheses on their evolution could be: (1) *Wolbachia* could have been ancestrally absent from the lineages leading to *Filaria martis*, *Ochoterenella* spp., and *Setaria* spp.; (2) *Wolbachia* could have been acquired on the lineage once leading to the Onchocercinae/Dirofilariinae, and current negative species in these subfamilies are the results of secondary losses; (3) *Wolbachia* could have been acquired several times along various lineages of the Onchocercinae/Dirofilariinae; in this case, negative species in these subfamilies could represent either a primitive absence of the symbiosis or the effect of a secondary loss (Casiraghi et al., 2004; Taylor et al., 2005a).

At the population level, quantification of *Wolbachia* numbers in different developmental stages has been studied in *B. malayi* (McGarry et al., 2004; Fenn and Blaxter, 2004b). In microfilariae and the larval stages (L2 and L3) in the mosquito vectors, the numbers of *Wolbachia* remain static with the lowest ratios of *Wolbachia*/nematode DNA. However, within the first week of infection of the mammalian host, *Wolbachia* numbers increase dramatically and the *Wolbachia*/nematode ratio is the highest of all life-cycle stages. The rapid multiplication continues throughout L4 development. Microscopy confirms that there are few bacteria in mosquito-derived L3 but many, in large groups, in L4 collected 1–3 weeks after infection. It appears that the large clusters of bacteria observed throughout the hypodermal cord of adult worms originate from this rapid period of division, which thereafter is maintained at that level as demonstrated in adult male worms up to 15 months of age. In females, bacterial numbers increase further as the worms mature and as the ovary and embryonic larval stages become infected (McGarry et al., 2004). Further studies on the dynamics of population levels in other filarial species are going on and should serve to define the key features of the symbiotic association. Individual worms appear to vary widely in their bacterial load, which may reflect a dynamic change of population size over time or if constant the potential for a selective advantage in terms of longevity or fecundity in worms carrying more bacteria. A recent study comparing the different 'forest' and 'savanna' strains of *O. volvulus* of West Africa found a significantly greater ratio of *Wolbachia* DNA to nuclear DNA in the severe, ocular disease causing 'savanna' strain, supporting the role of the *Wolbachia* in the pathogenesis of ocular onchocerciasis (Higazi et al., 2005).

3.2.2 *Wolbachia*-nematode mutualistic relationships

In arthropods, *Wolbachia* act as a reproductive parasite in most of the known cases (Stouthamer et al., 1999; Werren, 1997). However, filarial nematode *Wolbachia* behave differently from arthropod *Wolbachia*. There are evolutionary aspects as well as experimental studies suggest that the association between *Wolbachia* and filarial nematodes is obligatory mutualistic (Bandi et al., 2001; Fenn and Blaxter, 2004b; Fenn and Blaxter, 2006). The term "obligatory mutualism" describes the association between species living together that neither species can survive under the natural condition without the other. The phylogeny of filarial nematode *Wolbachia* is in the main congruent with that of the filarial nematode hosts (Bandi et al., 1998). In another word, the bacterial phylogeny splits at the same time as the filarial nematode phylogeny. It is an evidence of a close relationship between filarial nematode *Wolbachia*, and their hosts with a stable and long association. There is also no evidence for multiple infections. In addition, in filarial species positive for *Wolbachia*, the prevalence of the infection appears 100% (Bandi et al., 2001). The phylogenetic pattern and the distribution of filarial nematode *Wolbachia* appear more comparable to those generally observed in obligatory bacteria (Taylor et al., 2005a). Nevertheless, as reviewed above, some of the species within the *Wolbachia*-positive genus or the *Wolbachia*-positive subfamilies lack the bacteria. For example, *O. flexuosa* has no *Wolbachia*, whereas all other *Onchocerca* species do. Similarly, *Litomosoides yutajensis* is found negative for the bacteria, whereas other members of genus *Litomosoides* harbor them. These observations indicate that the filarial nematodes might not be absolutely dependent on

their intracellular bacterial partners in a long-term phylogenetic sense (Fenn and Blaxter, 2004b).

The information available on the evolutionary aspects and distribution of *Wolbachia* are in general agreement that the relationship between filarial nematodes and filarial nematode *Wolbachia* are likely dependent. *Wolbachia* have not been cultured outside their host cells. In addition, there is an experimental study implying that their habitation is species specific. Filarial nematode *Wolbachia* can be transferred from a naturally infected species, *L. sigmodontis* to a naturally uninfected one, *A. viteae*. However, the level of *Wolbachia* in *A. viteae* reduces along the time that the filarial nematodes are cultured in the Mongolian gerbils (*Meriones unguiculatus*), and *Wolbachia* cannot transmit to the filarial progeny (Hartmann et al., 2003). The dependence indicates that *Wolbachia* should need some benefits from their filarial nematode hosts. On the other hands, *Wolbachia* could benefit their hosts some essentials. Investigation using antibiotics, such as tetracycline, which is known to be effective against *Rickettsiae*, have provided direct evidence for the existence of this dependence. However, the underlying molecular mechanism is largely unknown. The antibiotic showed detrimental effects on filarial nematodes which harbor *Wolbachia*, and no effects on filarial nematodes which do not have *Wolbachia* (e.g. *A. viteae*) (Hoerauf et al., 1999; Bandi et al., 1999).

The recent completion of genome sequencing and annotation of the metabolic pathways of *Wolbachia* from *B. malayi* have identified important candidates for the dependency of the symbiosis (Foster et al., 2005). In comparison with insect *Wolbachia* and related *Rickettsia*, the genome of *Wolbachia* from *B. malayi* is drastically

reduced in size, a feature common to the lifestyle of other endosymbiotic bacteria. However, *Wolbachia* contain more intact metabolic pathways, which may be important in contributing to the welfare and fecundity of its host. The ability to provide riboflavin, flavin adenine dinucleotide (FAD), haem and nucleotides are likely to be the bacterial contribution (Table 2.4), whereas the host nematode provides amino acids required for bacterial growth with the exception of the only amino acid synthesized by the bacteria, meso-diaminopimelate, a major component of peptidoglycan (Taylor et al., 2005a).

Table 2.4 *Wolbachia* metabolic pathways that might supply essential products to the host *Brugia malayi* (Fenn and Blaxter, 2006)

***Wolbachia* metabolic pathway**	**Physiological importance**
Riboflavin and flavin adeninedinucleotide biosynthesis	Essential coenzymes; biosynthesis genes are lacking from *B. malayi*
Haem biosynthesis	Prosthetic group of cytochromes that catalyses the biosynthesis of steroid hormones
Nucleotide biosynthesis	Might supplement host nucleotide pool
Glutathione biosynthesis	An essential metabolite for protection against oxidative stress; might supplement host defenses

The cell wall biosynthesis pathways are devoid of genes required for the biosynthesis of lipopolysaccharide (LPS) in common with the related *Wolbachia* from *Drosophila* (Wu et al., 2004) and *Ehrlichia* and *Anaplasma* sp. (Lin and Rikihisa, 2003). In addition, an unusual peptidoglycan structure is suggested with some possible similarities to peptidoglycan-derived bacterial cytotoxins. Other features include a common type IV secretion system and an abundance of ankyrin domain containing proteins, which could regulate host gene expression as suggested for *Ehrlichia phagocytophilia AnkA* (Park et al., 2004; Ijdo et al., 2007). Glutathione biosynthesis genes may be a source of glutathione for the protection of the host nematode from oxidative stress or immunological effector molecules. Heme from *Wolbachia* could be vital to worm embryogenesis as there is evidence that moulting and reproduction are controlled by ecdysteroid-like hormones (Warbrick et al., 1993), whose synthesis requires heme. Depletion of *Wolbachia* might therefore cease production of these hormones and block embryogenesis. Alternatively, or in addition, *Wolbachia* may be an essential source of nucleotides during embryogenesis. Thus, the completion of the *w*Bm genome offers a wealth of information, which may help to understand the molecular basis for the endosymbiosis between filarial nematodes and *Wolbachia*. Now the metabolites that might be provided by *Wolbachia* to the nematode and which are required by the endobacteria from the nematode, were known. This may open up the exciting possibility to find and test drugs already registered for use in humans, which might inhibit key biochemical pathways in the *Wolbachia* that could lead to sterility or killing of the adult worms (Taylor et al., 2005a; Ghedin et al., 2007).

4. Drugs used in lymphatic filariasis

The goals of treatment for lymphatic filariasis are (1) to prevent, halt or reverse progression of disease (individual treatment), and (2) to interrupt transmission of the parasites (mass treatment). In individual treatment, individuals who are found to be infected are treated. Selecting the appropriate therapy for the patients requires knowledge of clinical features and pathogenesis of disease (Addiss and Dreyer, 2000). The following three components of treatment should be considered: (1) antifilarial and anti-Wolbachial treatment, (2) supportive clinical care, and (3) patient education and counseling. In the past, treatment of lymphatic filariasis has focused primarily on antifilarial chemotherapy. However, it is now clear that supportive of clinical care are more important than antifilarial treatment (Addiss and Dreyer, 2000). In patients with chronic pathology, treatment which emphasize hygiene, prevention of secondary bacterial infections have gained wide acceptance for morbidity control. Chemotherapy in these patients should be reserved for individuals with evidence of active infection, because chemotherapy has been associated with clinical improvement. In contrast, during acute filarial lymphagitis, treatment with antifilarial drugs is not recommended because it may provoke additional adult worm death and exacerbate the inflammatory responses. After the acute attack has resolve, if patients remain mf or antigen positive, antifilarial drugs can be given to kill the remaining adult worms (Addiss and Dreyer, 2000). With the better definitions of clinical features and new tools to assess clinical status (e.g. ultrasound, lymphoscintigraphy, antigen assays, PCR), approaches to treatment based on infection status can be considered.

4.1 Antifilarial drugs

4.1.1 Diethylcarbamazine (DEC)

Since its discovery in 1947, diethylcarbamazine (DEC, Hetrazan, Banocide, and Notezine) has been, and still being most widely used drug for the treatment of lymphatic filariasis. With partial macrofilaricidal activity, DEC is a highly effective microfilaricidal drug. DEC has been associated with major adverse effects resulting from its action on the mf. These effects are related to the concentration of the drug in the plasma which, in turn, is influenced by urinary pH. The elimination half-life of DEC is prolonged and renal clearance reduced in alkaline urine. Under these conditions the microfilaricidal effect is enhanced, but the adverse reactions to treatment are more severe (Edwards and Breckenridge, 1988). The drug is absorbed from gastrointestinal tract and reaches peak levels in the blood 2-3 hours after intake (Bolla et al., 2002). The time required for the kidneys to excrete DEC increases with the pH of the urine, but even when the urine is alkaline, the half –life in the blood is only 11-14 hours (Bolla et al., 2002).

The standard DEC treatment regimen is 6 mg/kg per day for 12 days. This is suitable only for individual treatment but not for community-wide mass treatment programs. Current regimen in mass drug treatment with DEC is a single biannual or annual dose of 300 mg of DEC for adults and 150 mg for children (often combined with albendazole). Common cooking salt medicated with DEC in concentrations ranging from 0.1% to 0.6 % has also been effectively used in mass treatment programs. It can be utilized in most of the control programs, but cannot yet be recommended for conditions

where there is co-existing onchocerciasis or loiasis. The average dose of 6 mg/kg is associated with few side effects such as drowsiness, nausea and gastrointestinal upset and these are observed more frequently with increase in dosage of the drug. Adverse reactions that are triggered by DEC in persons with filarial infections can be either localized (associated with death of the adult worm) or systematic (associated with death of the mf). DEC also cause severe adverse reactions, including encephalitis, in patients with loiasis who have high levels of mf (Carme et al., 1991). For these reasons, *Loa loa* and *O. volvulus* infections should be excluded before treating patients for lymphatic filariasis.

The mechanism of action of DEC is still not well known. Several studies suggested that DEC does not have a direct effect on the mf surface, since exposure of mf to concentrations of drug that exceed the therapeutic dose leaves them undamaged (Hawking, 1979; Johnson et al., 1988). One of the most frequent findings is that DEC increases the microfilarial adherence to endothelial cells and granulocytes (Maizels and Denham, 1992). These studies led to the suggestion that DEC stimulates the innate arm of the immune system. Other studies suggested that nitric oxide (NO) might be involved in host defense against filarial parasites (Rajan et al., 1996; Taylor et al., 1996). However, DEC does not induce NO synthesis in mouse macrophages and rat endothelial cells (Rajan et al., 1998). In contrast, accumulated evidence indicates that a microfilaricidal effect of DEC is not dependent on a specific humoral response. DEC reduced mf levels by 95.8%, even when mf of *Litomosoides sigmodontis* released *in vitro* were transfused into a naive animal, suggesting that an adaptive immune response was not a sufficient condition for DEC effectiveness (Weiner and Soulsby, 1982).

DEC also interfere arachidonic acid metabolism (Maizels and Denham, 1992; Martin et al., 1997). The products of the arachidonic acid metabolic pathway, eicosanoids, have a number of biological effects, including inhibition of platelet aggregation; regulation of leukocyte activation and adherence; mediation of granulocyte chemotaxis and degranulation; and promotion of vasodilatation. It is well known that DEC inhibits enzymes of the 5-lipoxygenase pathway, leukotriene synthases (Bach and Brashler, 1986; Mathews and Murphy, 1982). Additionally, *in vitro*, DEC blocks endothelial cell production of the cyclooxygenase (COX) pathway products prostaglandin (PG) E_2, prostacyclin (PGI_2) and thromboxane A_2 but has no effect on platelet prostanoid production (Kanesa-thasan et al., 1991). In addition, the drug increases the rate and degree of mf adherence to granulocytes, with eosinophil adhesion in particular being augmented. The arachidonic acid pathway includes lipoxygenase and cyclooxygenase enzymes. The COX pathway has similarities with the nitric oxide pathway, since both have constitutive and inducible isoforms of their enzymes and are key regulators of inflammatory responses. The COX and NO pathways are known to interact with each other, with there being 'cross-talk' between NO/PGE_2 and iNOS/COX which is generally stimulatory but may also be inhibitory. DEC administered alone to *B. malayi* infected BALB/c mice resulted in a rapid and profound reduction in circulating mf within five minutes of treatment. Mf levels began to recover after 24 hours and returned to near pre-treatment levels two weeks later, suggesting that the sequestration of mf occurs independently of parasite killing. Treatment with DEC results in a reduction in the amount of COX-1 protein in peritoneal exudates cells (McGarry et al., 2005).

Recently, ultrastructural studies showed severe morphological damage to mf sheath of *W. bancrofti* after *in vitro* treatment with DEC, indicating a direct mode of action of this major anti-filarial drug (Peixoto et al., 2003). DEC promotes severe damage to mf cells, including the presence of several large vacuoles and completely lysis of the cytoplasm, with the destruction of all organelles in some cells and the absence of nucleolus and condensed chromatin, features which resemble an apoptotic process (Florencio and Peixoto, 2003; Peixoto et al., 2003; Peixoto et al., 2004).

4.1.2 Ivermectin

Ivermectin (MK-0933, 22,23-dihydroderivative of avermectin B1) is a synthetic derivative of a broad-spectrum antiparasitic class of macrocyclic lactones known as avermectins. Avermectin B was first isolated by fermentation of a soil microorganism, the actinomycete *Streptomyces avermitilis*. Ivermectin has a structure similar to that of macrolide antibiotics, but without antibacterial activity. It is used against a wide range of endoparasites (nematodes) and ectoparasites (insects, acarine) of animals and humans. The interest of dermatologists in ivermectin therapy grows when promising results were observed in the treatment of human ectoparasitoses such as scabies (Heukelbach et al., 2004; Mounsey et al., 2009). In humans, ivermectin was introduced against *Onchocerca volvolus* infestation in 1982 (Aziz et al., 1982). It has been used extensively in humans since 1987 to control endemic onchocerciasis in countries of Africa and Latin America (Soboslay et al., 1987; Walsh, 1987). The drug is highly effective for the treatment of loiasis and brancroftian filariasis, and other intestinal nematodes as strongyloidiasis and scabies.

Pharmacokinetic study of ivermectin shows good bioavailability. It is absorbed rapidly and given orally on an empty stomach, metabolized in the liver and excreted in the feces (98%) and urine (1%). Minimal concentration of this drug has been observed in human milk. Ivermectin reaches peak plasma levels 5 hours after oral administration and has a half-life of 36 hours (Baraka et al., 1996; Elkassaby, 1991). Peak plasma concentration of metabolites remains longer than that of the parent drug, suggesting enterohepatic recyculation (Baraka et al., 1996).

Ivermectin acts by binding selectively to specific neurotransmitter receptors that function in the peripheral motor synapses of parasites (Bowman et al., 1991). It has an endectocidal effect (simultaneously against endo and ectoparasites) causing paralysis of nematodes, arthropods and insects by suppressing the conduction of nervous impulses in the interneuronic (intermediary neurons) synapses of nematodes and the nerve-muscle synapses of the arthropods and insects (Wang and Pong, 1982). Specifically, ivermectin blocks chemical transmission across the nerve synapses that use glutamate-gated anion channels or γ-aminobutyric acid gated chloride channels. Stimulation of γ-aminobutiric acid (GABA) release from presynaptic nerve endings and enhancement of the binding to the postsynaptic receptors accomplishes this (Feng et al., 2002). In this way nerve impulse conduction cease and paralysis and death of the parasites (Brownlee et al., 1997). Ivermectin is well tolerated by mammals because glutamate-gated anion channels or GABA-gated chloride channels are localized only in the central nervous system; the drug cannot penetrate through the hematoencephalic barrier. Thus, ivermectin selectively paralyzes the invertebrates.

Ivermectin is a potent microfilaricidal agent. A single dose of 200-400 ug/kg profoundly suppresses the levels of *W. bancrofti* and *B. malayi* mf in peripheral blood for 6-24 months (Dreyer et al., 1995a; Eberhard et al., 1992). A single annual dose of 400 ug/kg, either alone or in combination with 6 mg/kg of DEC, has been proved to be very effective in producing long-term suppression of microfilaraemia in lymphatic filariasis in a number of countries (Pani et al., 2004). Although macrofilaricidal effect of ivermectin has been proposed but it is clear now from recent ultrasound studies that the adult worms are not killed even at total doses of 4800 µg/kg over a period of 6 months (Dreyer et al., 1996; Dreyer et al., 1995b). Like DEC, it is also associated with several adverse effects. Serious side effects, mainly *Loa loa* encephalopathy, following ivermectin treatment for control of onchocerciasis in areas, co-endemic for loaisis have been reported (Gardon et al., 1997; Twum-Danso, 2003).

4.1.3 Albendazole

Albendazole (ALB) is a benzimidazole drug that is widely used against human intestinal helminthes because of its broad spectrum and low toxicity (Raccurt et al., 1990). The drug is absorbed and reaches peak levels in the blood 1-2 hours after intake (Amsden et al., 2007). The half-life in the blood is only 9-12 hours (Amsden et al., 2007; Jung et al., 1992). The detailed mechanism of action of ALB is unclear and experimental evidence with several intestinal helminthes shows that ALB, like other benzimidazoles, acts to inhibit β-tubulin polymerase, causing disruption of cytoplasmic microtubule formation. Moreover, albendazole treatment result in altered distribution pattern of the alkaline phosphatase (ALP), adenosine triphosphatases (ATPase), succinate dehydrogenase (SDH), and glutamate dehydrogenase (GDH); while lactate

dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G-6-PD), and non-specific esterases exhibited slightly enhanced activity in the epithelium (Kaur and Sood, 1990). It has been reported that repeat high-dose albendazole may have a macrofilaricidal effect against *W. bancrofti* (Jayakody et al., 1993). Single dose of albendazole appear to have no significant short-term effect on microfilaremia (Addiss et al., 1997), but result in long-term reduction of microfilaremia (Ismail et al., 1998). Only few adverse reactions that are triggered by albendazole have been reported, including dizziness, nausea, vomiting, and diarrhea (Wen et al., 2008).

Albendazole has a potential role in lymphatic filariasis control program. Because DEC is only partially effective against the adult worms, WHO currently recommends co-administration of albendazole with DEC or with ivermectin (in countries where onchocerciasis or loiasis is co-endemic) (World Health Organization, 2009).

In Thailand, bi-annual mass drug administration (MDA) were carried out in the endemic areas as part of the national lymphatic filariasis elimination program. All villagers (except children aged <2 years and pregnant mothers) were targeted for the coverage. Each MDA program was taken up after the campaign by information, education and communication; which include posters, pamphlets, and video message, formal and informal meetings with various local villager volunteers and through local school students. The drugs were provided by the staff of the Vector Borne Disease Control Center, and the local village volunteers between 6 PM and 9 PM at several distribution points in the village.

Although the dosage of DEC is based on body weight (6 mg/kg), owing to the cumbersomeness of weighing individuals, DEC tablets were administered depending on the age criteria (one tablet of 300 mg for ≥ 12 years old and half tablet for 2-12 years old). The dosage of albendazole is kept uniform, i.e. one tablet of 400 mg for all age groups in the co-administration, with directly observed ingestion of the drugs.

4.2 Anti-Wolbachial drugs

4.2.1 Tetracycline derivative

The tetracyclines were first discovered in the 1940s as a product of *Streptomyces aureofaciens*. Tetracyclines are a family of antimicrobial agents that inhibit protein synthesis by binding to the bacterial 30S ribosomal subunit and prevent aminoacyl-tRNA binding to the ribosomal A site, thus preventing addition of amino acids to the growing polypeptide chain. Tetracyclines bind more specifically to the bacterial ribosome compared with eukaryotic ribosomes. This class of compounds also has a number of additional properties. They have a number of secondary binding sites on the bacterial ribosome, the significance of this mechanism is no completely understood. They also have anti-inflammatory and immunomodulatory activity (Sapadin and Fleischmajer, 2006; Webster and Del Rosso, 2007) and have been used in the treatment of various dermatologic and nondermatologic diseases. In addition, the tetracycline analogues (doxycycline and chemically modified tetracycline; COL-3) can induce caspase-dependent and -independent apoptosis in human colon cancer cells (Onoda et al., 2006), but minocycline, a tetracycline derivative, inhibit caspase expression in mammalian cells and in animals (Chen et al., 2000).

Chlortetracycline and oxytetracycline, both discovered in the late 1940s, were the first members of the tetracycline group. These molecules were products of *Streptomyces aureofaciens* and *S. rimosus*, respectively. Other tetracyclines were identified later, either as naturally occurring molecules, e.g., tetracycline from *S. aureofaciens*, *S. rimosus*, and *S. viridofaciens* and demethylchlortetracycline from *S. aureofaciens*, or as products of semisynthetic approaches, e.g., methacycline, doxycycline, and minocycline. The most recently discovered tetracyclines are the semisynthetic group referred to as glycylcyclines, e.g., 9-*(N,N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline, 9-(*N,N*-dimethylglycylamido)-minocycline, and 9-*t*-(butylglycylamido)-minocycline (Chopra and Roberts, 2001). Currently, tigecycline is the first drug to be approved from the glycylcyclines. This agent has been developed to overcome bacterial mechanisms of tetracycline resistance such as ribosomal protection and efflux pumps (Noskin, 2005). Tigecycline binds 5-fold more effectively to this ribosomal site compared with the tetracyclines (Bergeron et al., 1996). Tigecycline (Wyeth Pharmaceuticals), a tetracycline derivative that inhibits bacterial protein synthesis and cell growth, which is under clinical trials, can be tested against experimental filarial infections (Muralidharan et al., 2005).

Tetracyclines across the outer membrane of gram negative bacteria via the OmpF and OmpC porin channels (Thanassi et al., 1995). Uptake of tetracyclines across the cytoplasmic membrane is energy dependent and driven by the ΔpH component of the proton motive force (Nikaido and Thanassi, 1993; Schnappinger and Hillen, 1996). Within the cytoplasm, tetracycline molecules are likely to become chelated since the internal pH and divalent metal ion concentrations are higher than those outside the cell

(Schnappinger and Hillen, 1996). At present, tetracycline resistance in bacteria can occur by acquisition of ≥1 of the 36 different genes, by mutations to host efflux pumps or in their 16S rRNA sequences, or by alteration in the permeability of the cell (Roberts 2003).

The tetracycline molecules bind reversibly with the prokaryotic 30S ribosomal subunit, thus, stopping protein synthesis. Diluting out the antimicrobial can reverse the effect, and protein synthesis will begin in the cell. Tetracycline binds with the 70S ribosomes found in mitochondria and can inhibit protein synthesis in mitochondria. Tetracycline interacts weakly with the 80S ribosome of eukaryotic cells, causing a relatively weak inhibition of protein synthesis, which is the reason for the selective antimicrobial properties of the tetracyclines, with limited side effects in humans (Roberts 2003).

It has been recognized that the tetracyclines have the effects on various protozoan parasites such as *Plasmodium falciparum, Entamoeba histolytica, Giardia lamblia, Leishmania major, Trichomonas vaginalis,* and *Toxoplasma gondii* (Chopra and Roberts, 2001). Tetracyclines are also used as supplements to quinine in the treatment of malaria caused by *P. falciparum* when resistance to quinine has been reported (World Health Organization, 1995). Malarial parasites have two extrachromosomal DNAs with prokaryotic organelle-like characteristics, apicoplast and mitochondrial DNA. These organelles are likely the target of numberous antimicrobial agents. Tetracyclines are suggested to specifically inhibit expression of the apicoplast genes, leading to the distribution of nonfunctional apicoplasts into daughter merozoites and a subsequent block in parasite development. The site of action of teracyclines is the apicoplast but that loss

of apicoplast function is not clear until late in the cycle following treatment, explaining the slow action of these agents (Dahl et al., 2006; Dahl and Resenthal, 2008).

More recently, tetracycline treatment of filarial nematode–infected animals has reduced levels of adult worms and mf, suggesting that tetracycline may be beneficial for treatment of humans infected with filarial nematodes (Smith and Rajan, 2000). In *Brugia* infected animals, tetracycline is prophylactic and affects the molting of infective larvae (Bosshardt et al., 1993; Casiraghi et al., 2002; McCall et al., 1999), and causes distortion of male/female sex-ratios (Arumugam et al., 2008; Casiraghi et al., 2002). The members of tetracycline family (tetracycline, oxytetracycline, doxycycline, and minocycline) were found to be effective against worms. These antibiotics also affect *Wolbachia* after treatment (Bandi et al., 1999; Hoerauf et al., 2000; Langworthy et al., 2000; Townson et al., 2000). Modes of action of these antibiotics are generally on bacterial RNA polymerases, protein synthesis, and other processes, and these agents may affect similar pathways in both worms and their *Wolbachia*. In several nematode worm infections, these antimicrobial agents have multiple effects on worm growth and development; worm fertility (particularly female worm embryogenesis) and worm survival (Langworthy et al., 2000; Hoerauf et al., 1999). Moreover, when microfilaraemic animals were treated, their mf numbers were reduced in the circulation (Hoerauf et al., 1999). In contrast, in animals infected with *A. viteae* worms, which do not carry these bacteria, similar long-term treatment has no effect on worm biology and development (Hoerauf et al., 1999), suggesting that these bacteria play a very important role in the growth and reproduction of the filarial worms that harbour them.

Interestingly, in addition to anti-*Wolbachia* properties, tetracyclines markedly affected the normal embryogenesis profiles by causing damage and degeneration of intrauterine embryos (Bandi et al., 1999; Hoerauf et al., 1999; Hoerauf et al., 2000; Langworthy et al., 2000; Sacchi et al., 2003; Townson et al., 2000) Polymerase chain reaction (PCR) assay also confirmed the clearance of *Wolbachia* DNA after prolonged therapy (Bandi et al., 1999; Hoerauf et al., 2000). The reduction or clearance of bacterial specific hsp60 and *Wolbachia* surface protein (WSP) as determined by immunohistochemical staining indicated the absence or clearance of *Wolbachia* in treated worms (Hoerauf et al., 2000; Kramer et al., 2003).

The filarial *Wolbachia* genome sequencing has been recently completed (Foster et al., 2005) and several new targets necessary for the bacteria are being identified. These might lead to investigate a new class of anti-*Wolbachia* drugs that benefit filarial chemotherapy research. More potent prophylactic antibiotic drugs or antibacterial agents in eliminating *Wolbachia* followed by parasites may be identified within pharmaceutical research platforms.

The success of antimicrobial treatment against animal filariae has been extended to human filarial infections. Trials of doxycycline have been completed for populations infected with *O. volvulus* and *W. bancrofti*. For both species, the level of larvae were zero or near zero after treatment (Hoerauf et al., 2001; Hoerauf et al., 2003a). Examination of worms from treated nodules of onchocerciasis patients showed that depletion of *Wolbachia* led to a block in embryogenesis that appears to be permanent (Hoerauf et al., 2003b). Most recently, there has been evidence for the killing of adult *W. bancrofti* by doxycycline therapy (Taylor et al., 2005b).

Recently, doxycycline has been confirmed its effective in *B. malayi* (Supali et al., 2008). There was a long-term absence of mf in the blood when administered doxycycline at 100 mg/day for 6 weeks, probably due to either a sterilizing activity on the female worms, or a macrofilaricidal effect. Unfortunately, in *B. malayi*, there is currently no test to measure adult worm activity, because worm nests cannot be located in brugian filariasis, and circulating filarial antigen cannot be detected (Mand et al., 2006).

Another advantage of doxycycline in treating lymphatic filariasis is its ameliorating effect on disease. The scrotal lymph vessels' diameter, a marker for lymphangiogenesis induced by filarial worms, is reduced after doxycycline treatment (Debrah et al., 2006), an effect not detected with DEC (Dreyer et al., 2002). A previous study has showed that treated patients also display a reduction in the plasma levels of VEGFs (Debrah et al., 2006), which are essential for angiogenesis (VEGF-A) as well as lymphangiogenesis (both VEGF-A and VEGF-C (Adams and Alitalo, 2007; Alitalo et al., 2005; Halin et al., 2007), and produced by cells of the immune system in response to bacterial stimuli. *Wolbachia* are released in the lymphatic system either upon adult worm death or constantly with debris from female worms such as degenerated embryos. This has agreed to the concept that *Wolbachia* are important inducers of VEGFs, and their depletion following doxycycline treatment leads to decreased angiogenic stimuli, thus leading to reversion of lymph dilation. Similar mechanisms may underlie the reduction in size of small to medium hydroceles after doxycycline, and a single nucleotide polymorphism (SNP) in the promoter region of VEGF-A predisposes for hydrocele (Debrah et al., 2007).

Doxycycline cannot replace the MDA, because cost–benefit ratios and the practicable, so that short regimens (ideally once or twice a year) are essential. Doxycycline is also not to be recommended if patients remain in endemic areas where they have any chance to acquire new infection and thus would have to take doxycycline in yearly intervals (Hoerauf, 2008).

4.2.2 Rifampicin

Rifampicin is a semi-synthetic derivative of rifamycin B, a fermentation product of *Streptomyces mediterranei.* The mechanism of this bacteriocidal agent involves inhibition of bacterial DNA-dependent RNA polymerase, thereby interfering with protein synthesis (Campbell et al., 2001). Rifampicin has the effect on both coagulase-positive and -negative staphylococci as well as other gram-positive cocci, such as *Streptococcus pyogenes,* penicillin-sensitive *S pneumoniae,* enterococci, and *Peptostreptococcus* sp. Among gram-negative bacteria, *Neisseria meningitides, N gonorrhoeae,* and *Haemophilus influenzae* are the most sensitive. Rifampicin also is active against a closely related *Wolbachia*, *Chlamydia* spp. (Siewert et al., 2005). Rifampin diffuses freely into tissues, living cells, and bacteria, making it effective against intracellular pathogens like *Mycobacterium tuberculosis*.

Resistance to rifampicin develops rapidly when it is used alone. Most strains of *M. tuberculosis* are susceptible to rifampicin and isoniazid, but resistant strains have been isolated (Banerjee et al., 2008). The susceptibility of other mycobacteria is variable. *M. leprae* is killed faster by rifampin than by sulfones and *M. fortuitum* and *M. chelonei* are resistant (Alsayyed, 2004). Resistance to rifampicin develops rapidly when

it is used alone; it never should be used as monotherapy for any serious infection. Rifampicin plays an important role in the treatment of patients who have tuberculosis, both as part of a multidrug regimen for active disease and as prophylaxis for latent infection. As prophylaxis, rifampicin is appropriate only when resistance to isoniazid is suspected or when isoniazid is contraindicated. The bactericidal activity of rifampicin is to from its high-affinity binding to, and inhibition of, the bacterial DNA-dependent RNA polymerase (RNAP) (Villain-Guillot et al., 2007). The essential catalytic core RNAP of bacteria (subunit composition $\alpha_2\beta\ \beta'\omega$) has a molecular mass of around 400 kDa and is evolutionarily conserved among all cellular organisms (Archambault and Friesen, 1993).

Treatment with rifampicin specifically inhibits apicoplast transcription (Lin et al., 2002; McConkey et al., 1997) and causes immediate death in *P. falciparum*. A rifampicin-insensitive phage type RNA polymerase likely blocks mitochondrial transcription in *P. falciparum,* so this drug is assumed not to disturb mitochondria directly (Li et al., 2001).

Studies of the effect of anti-rickettsials such as tetracycline and rifampicin in animals infected with filarial nematodes have shown, using immunohistochemistry, that these drugs deplete the *Wolbachia* from the worms. After the *Wolbachia* are depleted, the worms develop a distinct phenotype. Monitoring the mf levels in the blood showed that the number of mf in the treated animals was lower than the number in the control groups, and that the number of mf in the treated group neared zero with time. Examination of the adult worms showed that embryogenesis was blocked and the uteri contained degenerated embryos (Molyneux et al., 2003). A study done in cattle infected with *O. ochengi* even showed killing of adult worms (Langworthy et al., 2000). In

addition, *Litosomoides sigmodontis* infected BALB/c mice were treated with rifampicin 50 mg/kg daily orally or the combination of rifampicin plus doxycycline 25 mg/kg daily orally over a shorter period of 14–21 days initiated with infection, *Wolbachia* were depleted from the worm tissue as evidenced up to 63 days post infection using immunohistology and semiquantitative PCR (Volkmann et al., 2003).

4.2.3 Quinolones

Ciprofloxacin, norfloxacin, and ofloxacin are the most commonly used quinolones. They inhibit type 2 topoisomerase, a DNA gyrase involved in the supertwisting of bacterial DNA molecules, replication, and repair. These enzymes are present in mammalian organisms, but the quinolones selectively inhibit bacteria. They are bactericidal, and bacteriostatic under anaerobic conditions. Quinolones are effective against *P. aeruginosa*, staphylococci, streptococci, and the respiratory pathogens including *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, and *L. pneumophilia*. Quinolones are also effective against rickettsial diseases and enteric pathogens. Quinolones demonstrate synergy when used with β-lactams, aminoglycosides, and rifampin. Ciprofloxacin may cause fever, rash, GI complaints, and in one to four percent of people, mild neurological complaints such as insomnia, anxiety, and depression. Animal studies have suggested that long-term therapy with ciprofloxacin may cause phototoxicity, arthritis, and cataract formation (Eliopoulos, 2004). Rifampicin and ciprofloxacin have previously been shown to be effective against *Wolbachia,* determined by methods: an immunofluorescent-antibody test and a real-time quantitative PCR assay (Fenollar et al., 2003).

Quinolones have been discovered as a new class of antifilarial agents. This has led to the design, synthesis, and antifilarial evaluation of a number of N-substituted quinol-4(1*H*)-one-3-carboxamide derivatives 4-6. The macrofilaricidal activity of the target compounds was initially evaluated in vivo against *Acanthoeilonema viteae* by oral administration of 200 mg/kg × 5 days. Among all the synthesized compounds, 13 displayed activity, with the most potent compound (4a) exhibiting 100% macrofilaricidal and 90% microfilaricidal activities. Compound 4e elicited significant macrofilaricidal (80%) response while compound 5c showed 100% sterilization of female worms. Finally, the two most potent macrofilaricidal compounds, namely 4a and 4e, have been screened for their potency against DNA topoisomerase II, and it has been observed that both have the capability to interfere with this enzyme at 10 μmol/mL concentration

5. Gene expression analysis

Evaluating gene expression has greatly extended our understanding of behavior and function of cells and tissues under varying conditions. A diversity of technologies has been developed to assess levels of gene expression, ranging from analysis of single genes (i.e., Northern blot and PCR) to thousands of genes simultaneously [i.e., serial analysis of gene expression (SAGE) and microarray] (Table 2.5). Based on experimental approach, a distinction can be made between "closed" and "open" profiling methods. Closed approaches rely on hybridization of genes of interest to complementary nucleic acids, and, therefore, genome knowledge is a prerequisite. Examples of closed approaches are Northern blot (Alwine et al., 1977) and DNA microarrays (Schena et al., 1995). In contrast, open approaches do not depend on genome knowledge since they are

based on sequencing of poly(A)$^+$ mRNA molecules expressed in the cell or tissue of interest. Examples of open approaches are expressed sequence tag (EST) sequencing (Adams et al., 1993), SAGE (Velculescu el al., 1995), and massively parallel signature sequencing (MPSS) (Brenner et al., 2000). The identity of transcripts is determined by matching the experimental sequence to available genomic data.

Table 2.5 Overview of gene expression analysis technologies (Vos et al., 2007)

Technology	Approach	No. of gene analyzed	Advantages	Limitations
Northern blot	Closed	1	Hightly specific, detection of low abundant transcripts	Low throughput, less sensitive than RT-PCR
Subtractive hybridization	Open	10-100	Positive selection of differentially expressed genes, gene discovery	Low throughput, Low detection limit
Polymerase chain reaction	Closed	1	Fast, highly sensitive	Low throughput
Differential display	Open	>100	Fast, gene discovery	High rate of false positives
Expressed sequence tag sequencing	Open	>100-1,000	Highly specific, gene discovery	Costly, time consuming, limited number of samples
Serial analysis of gene expression	Open	>1,000	Highly specific, gene discovery, efficient sequencing strategy	Limited sample nember, technically demanding bioinformatics
DNA microarray	Closed	>1,000	Ease of use, large number of samples	Bioinformatics

5.1 Serial analysis of gene expression (SAGE)

SAGE is an open high-throughput expression profiling technique that allows unbiased assessment of virtually all polyadenylated transcripts in a single sample (Velculescu el al., 1995, Velculescu el al., 1997). The outline of the technology is presented in Figure 2.7. Shortly, double-stranded cDNA is synthesized from mRNA molecules that are biochemically purified by the poly(A)$^+$ tail. In a series of two endonuclease reactions, representative short nucleotide sequences of 10–14 bp are isolated. In the first endonuclease step, the restriction enzyme *Nla*III is used to digest double-stranded cDNAs at every four-base CATG sequence. Since each cDNA is immobilized at the poly(A)$^+$ tail, only the most 3' fragments are captured for further processing. Before the second endonuclease step, a linker sequence containing the recognition site for the second restriction enzyme, *Bsm*FI, is attached to the generated four-base CATG overhang. The type IIs endonuclease *Bsm*FI specifically cleaves at 10–14 bp distance from its recognition site, thereby releasing the 10- to 14-bp fragments from the 3'-end immobilized cDNA fragments. Although relatively short (10–14 bp), these so-called SAGE tags contain sufficient genetic information to uniquely identify individual transcripts since they are derived from a defined position within each individual transcript: directly adjacent to the most 3' recognition site of *Nla*III. Serial ligation of multiple SAGE tags into long multimers not only allows qualitative and quantitative determination of SAGE tags but also drastically increases sequencing efficiency by limiting the number of required sequencing reactions. The identity of SAGE tags representing known genes is then established by comparing its nucleotide sequence to available gene sequences deposited in genetic databases. Since SAGE libraries are generated by random sampling of transcripts, expression profiles

will become more accurate when analyzing larger numbers of tags. When sufficient SAGE tags are analyzed, the sensitivity of SAGE appears to be comparable to DNA microarrays in estimating expression levels of, in particular, medium to highly abundant genes (Evans et al., 2002; Ibrahim et al., 2005; Ishii et al., 2000).

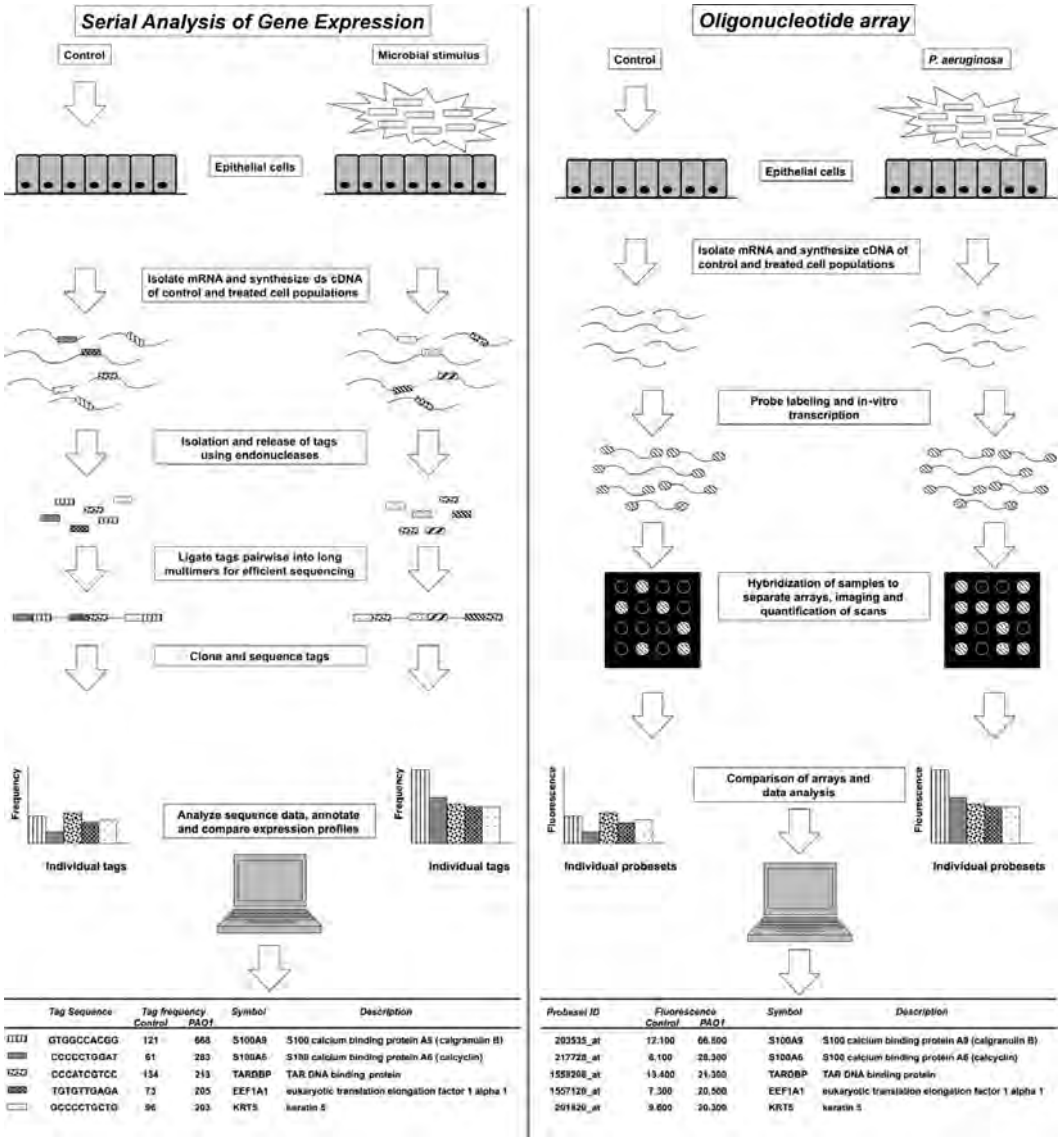


Figure 2.7 **Outline of the serial analysis of gene expression (SAGE) and microarray technology. A flow chart of the SAGE and microarray technologies is presented from top to bottom. In the first step, cultured cells are exposed to a pathogen. Isolated RNA is processed as is required for SAGE or microarray technology.**

Collected data reveals the expression profiles in the cells of interest under the studied conditions as illustrated (*bottom*) (Vos et al., 2007).

5.2 DNA microarrays

The microarray technology is a closed high-throughput method that enables the measurement of a large, predetermined set of known genes or sequences (Schena et al., 1995). In microarray technology, DNA molecules representing specific transcripts are fixed onto a solid support, ranging from oligonucleotides (25–70 mer) to complete cDNAs. Inherent to closed approaches like microarray, a finite collection of arrayed sequences can be analyzed. However, microarrays are available that ~45,000 probe sets covering all known human genes as well as thousands of undefined ESTs.

To visualize gene expression on microarrays, samples are labeled with a fluorescent dye before hybridization, and fluorescence intensity is quantified as a measure for gene expression in the original sample. The outline of the labeling and hybridization steps for the single-color microarray technology is described in Figure. 2.7. For the single-color microarray, each sample is hybridized to a separate microarray. Before hybridization, samples are biotin labeled and stained with a streptavidin-bound fluorophore (i.e., phycoerythrin) and visualized by confocal laser microscopy. The single-color approach is common to commercial oligonucleotide arrays. Advantages of oligonucleotide microarrays are that 1) repetitive sequences within the genome can be circumvented because of the use of short and uniquely designed probes; 2) probes have more uniform hybridization efficiencies; and 3) standardized protocols and equipment are used providing consistent and reproducible

data generation (Sievertzon et al., 2006). Because of unique probe design and uniform hybridization efficiencies, oligonucleotide arrays have a larger dynamic range of detecting gene expression (Barrett and Kawasaki, 2003). A disadvantage of oligonucleotide arrays is that the production procedure is costly and relatively inflexible.

An alternative approach for single-color microarray is the two-color microarray. The two-color approach is mostly used for cDNA microarrays but can also be applied to oligonucleotide arrays. For cDNA arrays, long and double-stranded cDNA probes are fixed onto this type of microarray. For hybridizing cDNA microarrays, samples are each labeled with a distinct fluorescent dye (i.e., Cy3 and Cy5) and cohybridized to the same microarray. Binding of transcripts from both samples is detected using confocal laser microscopy by scanning the chip for the two fluorescent channels separately. Expression levels of genes within the two cohybridized samples can be directly compared. An advantage of this approach is that two samples can be applied at once to a single microarray. However, a disadvantage of the two-color approach is the need to perform dye-swap experiments and mathematical signal normalization strategies to control for varying emission intensities of the Cy3 and Cy5 dyes (Barrett and Kawasaki, 2003).

Since a substantial number of academic organizations invested in their own arraying facilities, the cDNA microarray is frequently used. When equipment and probe collections are available, "inhouse-made" cDNA are more flexible and cost effective compared with the commercially available oligonucleotide microarrays. However, cDNA microarrays have a smaller dynamic range because of a less-efficient signal-to-noise ratio (Barrett and Kawasaki, 2003). This less-efficient signal-to-noise

ratio is partly because the arrayed cDNA probes are lengthy and double stranded, increasing the likelihood of nonspecific and cross hybridization to related sequences (Petersen et al., 2005). In addition, the density of arrayed cDNA probes is generally lower compared with oligonucleotide microarrays. Despite these differences, cDNA and oligonucleotide microarrays perform equally with respect to detection of abundantly expressed genes (Petersen et al., 2005).

A major advantage of DNA microarrays is the commercialization of the most labor-intensive parts of the methodology: collecting sequences (synthesized oligonucleotides or cDNA clones) and array fabrication. Commercial manufacturers of microarrays provide extensive and well-documented annotation of probe sets, which eases data mining and interpretation, whereas annotation of SAGE tags is not straightforward. Today, ready-to-use microarrays are available for many different organisms, which makes the use of this technology possible without the need of having microarray fabrication equipment and owning cDNA clone collections. Collecting data by using prefabricated microarrays typically takes less than one week. Therefore, this high-throughput profiling technology is often the preferred choice of many scientists. However, in contrast to SAGE, comparing DNA microarray results between experiments and between laboratories is hampered by differences in the type of array used (single vs. dual color; oligo vs. cDNA; homemade vs. commercial), the spotted probe sequences, and the lack of standardized experimental procedures. Not all fluorescent labels perform equally, and different probe sequences used by the various manufacturers representing the same gene may give rise to varying hybridization efficiencies. To facilitate comparative analysis on microarray data, the Microarray Gene Expression Data Group (MGED; http://www.mged.org/) proposed a uniform annotation format for microarray experiments (MIAME) (Brazma et al.,

2001). Although complying with the same standardized annotation format, complex computational normalization methods are still required to conduct comparative research based on microarray experiments.

The global analysis of gene expression using microarray is established in *C. elegans* (Kim et al., 2001; Murphy et al., 2003). A recent paper described the global analysis of dauer gene expression in *C. elegans* and identified 1984 genes that show significant expression changes during the transition from the dauer to the non-dauer state (Wang and Kim, 2003). Genes identified included those encoding transcription factors, components of signaling pathways and those involved in metabolic pathways important for dauer survival and longevity. This analysis has clear relevance for understanding the mechanisms involved in larval arrest associated with several parasitic nematode infections.

Recently, microarray analysis was provided the study of gender-regulated gene expression in *B. malayi*. The slide array (comprised of 65-mer oligos representing 3,569 EST clusters) was spotted with sequences selected from the extensive *B. malayi* EST database. Arrays were hybridized with Cy dye labeled male and female cDNA. The experimental design included both biological and technical (dye-flip) replicates. The data were normalized for background and probe intensity, and the relative abundance of hybridized cDNA for each spot was determined. Genes showing two-fold or greater differences with $P<0.05$ were considered gender-regulated candidates. One thousand one hundred and seventy of 2,443 clusters (48%) with signals above threshold in at least one sex were considered as gender-regulated gene candidates. This included 520 and 650 clusters up-regulated in male and female worms, respectively. Fifty of 53 (94%) gender-regulated candidate genes identified

by microarray analysis were confirmed by real-time RT-PCR. Approximately 61% of gender-regulated genes had significant similarity to known genes in other organisms such as *C. elegans*. Many *C. elegans* homologues of these genes have been reported to have reproductive phenotypes (sterility or abnormal embryo development) by RNA interference (Li et al., 2005; Li et al., 2011). In addition, several high-throughput gene expression analyses were performed in Schistosomes using microarrays to study gender differences in gene expression of *S. mansoni* and *Schistosoma japonicum*, as well as stage specific and species specific differences between *S. mansoni* and *S. japonicum* (Dillon et al., 2006; Fitzpatrick et al., 2004; Gobert et al., 2006; Hoffmann et al., 2002; Vermeire et al., 2006; Waisberg et al., 2008). Microarrays were also used to investigate the vertebrate hepatic host response to infection with *S. mansoni* (Hoffmann et al., 2001) and the effects of sexual pairing on gene expression (Fitzpatrick and Hoffmann, 2006). The present findings for *B. malayi* and Schistosomes provide a basis for a detailed exploration of differentially regulated molecules and might assist in the search for novel drug or vaccine target in parasitic nematodes.

CHAPTER III

MATERIALS AND METHODS

<u>Part I</u>: The effects of anti-rickettsial drugs on *B. malayi* mf determined by the minimum effective concentration (MEC) and on *Wolbachia* determined by the minimum inhibitory concentration (MIC)

We expected that the treatment of *B. malayi* mf with anti-rickettsial drugs (doxycycline, rifampicin, and ciprofloxacin), might reduce *Wolbachia* within mf leading to decrease or stop the mf motility. Our primary aim was to use the *B. malayi in vitro* culture system to study the effects of antimicrobial agents, focused on the blood-stage mf. We investigated the minimum effective concentrations (MECs), the concentration of the antimicrobial agents that could inhibit *B. malayi* mf motility, as determined by the motility scoring. For secondary aim, we investigated the minimum inhibitory concentrations (MICs), the concentration of the antimicrobial agents that could inhibit *Wolbachia* growth in mf, as determined by DNA copy number ratio, were analyzed using quantitative PCR (qPCR), a molecular technique that can determine the number of *Wolbachia* after treatment. The results were showed as single copy gene ratio, *wsp*/*hsp70* (*Wolbachia*/*B. malayi*), at different antimicrobial concentrations.

1. Parasite materials

B. malayi mf (TRS strain) were isolated from the peritoneal cavity of infected male jirds (*Meriones unguiculatus*) at 120 days post-infection. Jirds were shipped by the National Institute of Health/National Institute of Allergy and Infectious Disease (NIH/NIAID) Filariasis Research Reagent Repository Center (FR3) (University of Georgia, Athens, GA). Mf were washed 3 times with RPMI-1640 media (Gibco BRL, Grand Island, NY) to eliminate host cell contamination, and then washed 5 times with RPMI-1640 containing 100 μg/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO) (Rao and Weil 2002). Mf concentrations were determined by dilution counting under microscope.

2. Source and preparation of antimicrobial agents

The antimicrobials assayed were doxycycline (Sigma-Aldrich), rifampicin (Sigma-Aldrich), and ciprofloxacin (Fluka BioChemika, Buchs, Swithzerland). The concentration ranges used in this study were 0.125 to 256 μg/ml. Stock solutions were prepared as described by the manufacturer and stored at -20°C. Doxycycline and ciprofloxacin were dissolved in sterile distilled water. Rifampicin was dissolved in chloroform. Final antimicrobial solutions were made up fresh before use by diluting concentrated stock solutions in culture media (RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin, 0.25 μg/ml of amphotericin B, and 10% fetal calf serum). The culture media was proved to have no effect on mf motility as control.

3. Antimicrobial assay and microfilarial motility

Three 1,000-mf cultures arranged in duplicate experiments were performed in 24-well multiwell plates (Becton Dickinson, Franklin Lakes, NJ) containing 1 ml of RPMI-1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 μg/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml amphotericin B, and 10% fetal calf serum. Mf were cultured in either culture media, or culture media with a serial two-fold dilution of antimicrobial agents at final concentrations between 0.125 to 256 μg/ml. Cultured mf were incubated in 5% CO_2 at 37°C overnight. Mf were observed by two independent technicians and their motility were scored (0-4) as described previously by Rao and Weil (2002) (Table 3.1) with a Nikon SMZ-1500 stereo microscope (Nikon, Tokyo, Japan) at 12 h and 52 h. The MEC was defined as the lowest antimicrobial concentration required to stop the mf motility (score 0) compared with the control (without antimicrobial agents) at the different time points.

Table 3.1 Microfilarial motility scoring criteria

Score	Criteria
0	immotile or dead
1	slightly active
2	active and motile
3	moderately active and motile
4	highly active and motile

4. DNA extraction of *B. malayi* microfilariae

Genomic DNAs from *B. malayi* mf, cultured in either culture media or culture media with a dilution of antimicrobials at final concentrations of 0.125, 0.5, 2, 8, 32, and 128 μg/ml, were isolated from 1,000 mf, using lysis buffer and phenol-chloroform extraction methods. Mf were separated from culture media by centrifugation at 5,000 rpm for 10 min. The extraction method was performed as previously reported by McGarry (2003) with a slight modification. Mf containing *Wolbachia* pellets were digested in 1.5 ml eppendorf tubes containing 200 μl lysis buffer (50 mM Tris-HCl, pH 8.0 20 mM EDTA, pH 8.0, 2% SDS) and subsequently digested by addition of 10 μl proteinase K (20 mg/ml). Samples were incubated in a 65°C-water bath for 3 h, and enzyme activity was inactivated by incubation at 90°C for 10 min. DNA was extracted with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Invitrogen), mixed, and centrifuged at 12,000 rpm for 5 min. The aqueous phase (upper phase) was transferred to a fresh tube and sequentially extracted by one volume of chloroform. After centrifugation, the DNA was precipitated with 2.5 volume of ice-cold absolute ethanol, and 0.1 volume of 3 M sodium acetate pH 5.2 at -70°C for 30 min. The precipitate was centrifuged 14,000 rpm at 4°C for 30 min. The pellet was washed with cold 70% ethanol, allowed to dry, and resuspended in 100 μl nuclease-free water. Each mf DNA sample was measured with a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) according to the manufacturer's protocol. DNAs were then stored at -20°C until use.

5. Synthetic oligonucleotides (or primers)

The oligonucleotides for PCR and real-time PCR amplification of *B. malayi* and *Wolbachia* were synthesized, and purchased from Invitrogen and Applied Biosystems (Foster City, CA). The details of oligonucleotide sequences and lengths of the PCR products were showed in Table 3.2.

6. PCR amplification

PCR was performed using *wsp* and *hsp70* primers (Table 3.2). The PCR reaction was performed in 50 µl volumes under the following final conditions: 1X *Pfx* amplification buffer, 1 mM $MgSO_4$, 0.3 mM of each dNTP, 0.3 µM each of forward and reverse primers, and 1 unit of Platinum *Pfx* DNA polymerase (Invitrogen). The PCR amplification was performed by 1 cycle of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 15s, primer annealing at 50°C for 30s, and primer extension at 68°C for 50s for *wsp* and 30s for *hsp70*, followed by a final extension step at 68°C for 30 min. After PCR, the amplified DNA fragments were purified by QIAquick® Gel Extraction Kit (Qiagen Inc, Valencia, CA) in order to remove impurities such as small RNA, proteins, unincorporated nucleotides or primers.

Table 3.2 Primers and annealing temperatures used for PCR and real-time PCR analysis for genes of interest

Gene	Primer	Sequence (5´→3´)	Amplicon length (bp)	Use
Wolbachia_wsp	Forward Reverse	CAC CAT GCA TTA TAA AAA GTT TTT TTC AGC A CTA TTA GAA ATT AAA CGC TAT TCC AGC TT	723	plasmid
Wolbachia_wsp	Forward Reverse	GGT GTT GGT CTT GGT GTA GCA TAT GTA AGC AAA ACC AAA CCC ATG TT	87	qPCR
Bm_hsp70	Forward Reverse	CAC CAC GCT AAM* CGA CTG ATT GGT CAG CA CTA CCA AM*C Y*AW* AAG CAA TAG	361	plasmid
Bm_hsp70	Forward Reverse	CAG AAG AGA TTT CGT CGA TGG TT ACC GCG TGA CCC AGA AAA	70	qPCR

qPCR : Quantitative PCR

*** M = A+C, Y = C+T, W= A+T**

7. Cloning of *wsp* gene from *B. malayi Wolbachia* and *hsp70* gene from *Brugia malayi*

Plasmids containing inserts of the amplified *wsp* and *hsp70* genes sequences were prepared for use as standards in the quantitative real-time PCR.

7.1 Ligation of PCR product into the pCR®-Blunt II-TOPO® vector

PCR products of *wsp* and *hsp70* were cloned into pCR®-Blunt II-TOPO® Vector (Invitrogen). The map and diagrammatic features of the vector are showed in Figure 3.1. The plasmid vector (pCR®-Blunt II-TOPO®) is supplied linearized with *Vaccinia* virus DNA topoisomerase I covalently bound to the 3´ end of each DNA strand (referred to as "TOPO®-activated" vector). The TOPO® Cloning Reaction can be transformed into chemically competent cells or electroporated directly into electrocompetent cells. In addition, pCR®-Blunt II-TOPO® allows direct selection of recombinants via disruption of the lethal *E. coli* gene, *ccd*B (Bernard et al., 1994). The vector contains the *ccd*B gene fused to the C-terminus of the LacZα fragment. Ligation of a blunt-end PCR product disrupts expression of the *lac*Zα-*ccd*B gene fusion permitting growth of only positive recombinants upon transformation. Cells that contain non-recombinant vector are killed upon plating. Therefore, blue/white screening is not required.

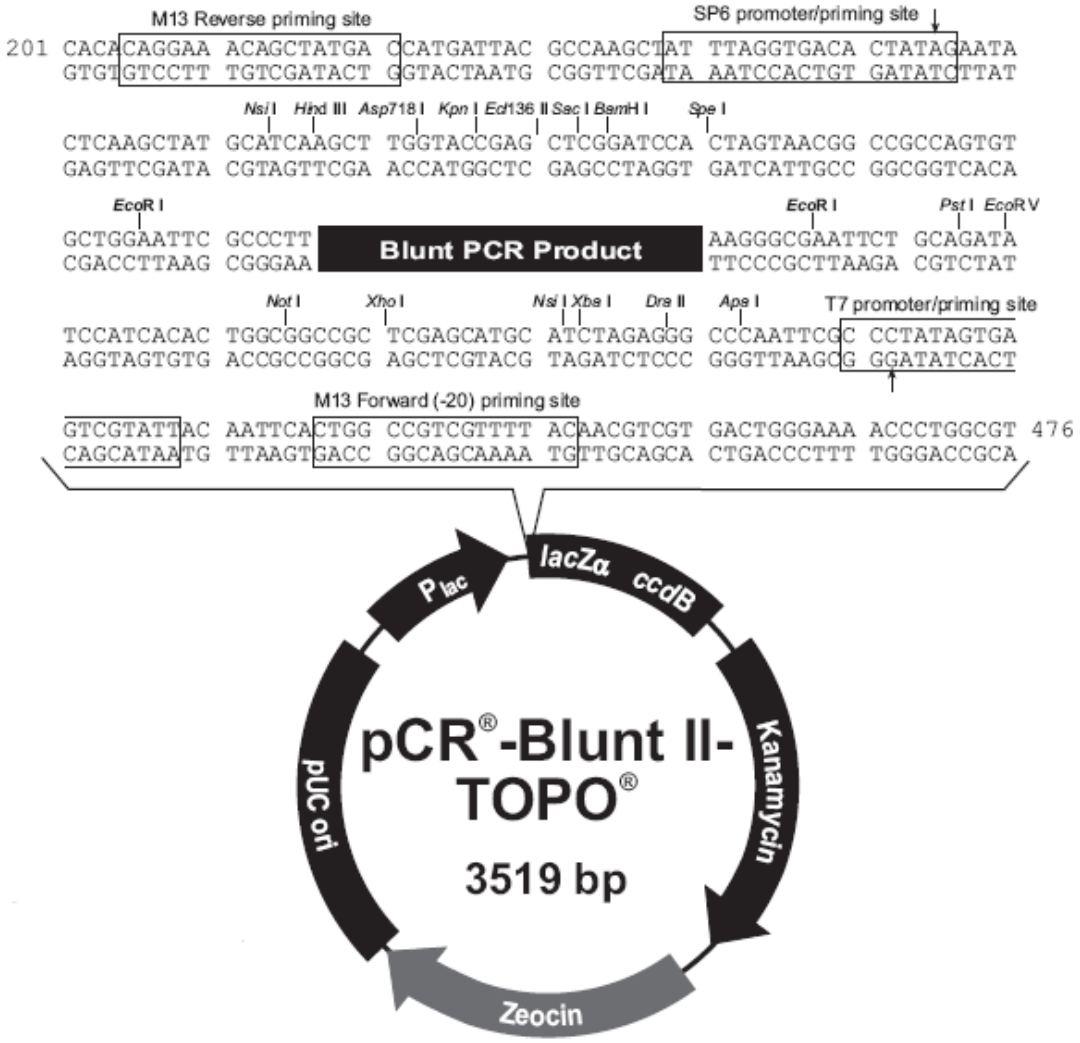


Figure 3.1 Map and features of the pCR®-Blunt II-TOPO® vector

The blunt end PCR fragments (4 μl) were added into the mixture containing 10 ng of pCR®-Blunt II-TOPO® Vectors, and 1μl of salt solution. The ligation reaction was stored at room temperature for 5 min.

7.2 Transformation of ligation product into DH5-α *Escherichia coli*

The ligation products were used to transform DH5-α *E. coli* competent cells prepared by $CaCl_2$ method. Genotype of DH5-α *E. coli* is:

Genotype: F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1λ^-

This *E. coli* strain carries the partially deletion of the *lac*Z gene (*lac*ZΔM15) of this *E. coli* allows α complementation of the β-galactosidase gene required for blue/white selection on X-gal agar plates. The mutated *end*A1 is for improved plasmid DNA preparations, and better results in downstream applications due to the elimination of non-specific digestion by Endonuclease I. For reduced occurrence of non-specific recombination in cloned DNA, the *rec*A1 is mutated.

E. coli DH5- α competent cells were mixed with 2 μl of ligation products and immediately placed on ice for 30 min. The cells were subjected to heat-shock at 42°C for 30 seconds and placed on ice for an additional 3 minutes. The transformed cells were mixed with 250 μl of SOC media and incubated at 37°C for 1 hour with shaking at 200 rpm. Finally, 100 μl of the transformed culture was spread on an antibiotic agar plate containing 50 μg/ml ampicillin. The plate was incubated at 37°C overnight. The colonies were picked and grown in LB medium supplemented with 50 μg/ml ampicillin. The recombinant plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen) and cut with *EcoR* I to confirm the presence of recombinant plasmids. The plasmids were sequenced in both directions to confirm that the *wsp* and *hsp70* genes represented correct orientation.

8. Preparation of quantitative PCR standard curve

The size of the *wsp* and *hsp70* plasmids were 4,686 and 4,324 bp, including the 723-bp and 361-bp *wsp* and *hsp70* fragments, respectively. The concentration of

the plasmids containing inserts of the amplified single copy gene were measured using a Nanodrop spectrophotometer (ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies). Sequence-specific standard curves were generated using 10-fold serial dilutions of *wsp* and *hsp70* encoded plasmid template. The DNA copy numbers were calculated as described by the manufacturer (Applied Biosystems). Plasmids were prepared at the concentration ranging from 0.1 $fg\mu l^{-1}$ to 1 $pg\mu l^{-1}$ (equivalent to about $3x10^1$ to $3x10^5$ for *wsp* and *hsp70* plasmids).

The number of copies/ml was calculated using the following formula:

$$\text{Copies/ml} = \frac{6.023 \times 10^{23} \times C \times OD_{260}}{MWt}$$

C = conversion factor = 5×10^{-5} g/ml for DNA

MW = molecular weight = base pairs x 6.58×10^2 g

9. Quantitative PCR assay

The SYBR green I dye qPCR assay was used in this study. Reaction mixtures were prepared in a 96-well optical reaction plate (Applied Biosystems), according to the manufacturer's protocol. In brief, the real-time PCR mixture consisted of 12.5 µl of 2X SYBR green PCR master mix, 0.45 µl of each primer at 50 µM, and 5 µl of mf DNA, and water was added up to a final volume of 25 µl. After centrifugation at 1,500 rpm for 2 min, the reaction plate was placed in the ABI Prism 7500 instrument (Applied Biosystems). Cycle threshold (C_t) values were determined using cycles 3 through 15 as baseline measurements. The amplification program included 2 min of

incubation at 50°C, a denaturation step for 1 cycle at 95°C for 10 min, and 40 cycles of denaturation at 15s at 95°C and 60s at 60°C for combined annealing and extension. The specificity of amplification was confirmed by melting curve analysis. All PCRs were carried out in duplicate. The number of copies of each sample transcript was then calculated and analyzed using the ABI 7500 system SDS software package (Applied Biosystems). Results were showed as single copy gene *wsp*/*hsp70* (*Wolbachia*/*B. malayi*) ratio. The MIC was defined as the lowest antimicrobial agent concentration allowing statistically significant inhibition of *Wolbachia* growth compared to the no-drug control at the different time points.

10. Data analysis

Data were recorded and analyzed by using Microsoft Excel 6.0 program paired Student *t*-test was used to compare *Wolbachia/Brugia* gene ratios and drug concentrations between groups with a $p < 0.05$ being taken as significant.

Part II: Characterization of the groups of *B. malayi* mf genes that could change their expression from non-treated and doxycycline-treated mf cultured *in vitro*

In the second part of this study, microarray technology was used to study the gene expression of mf cultured *in vitro* at 0, 23, and 48 h. The microarray experiment also used to identify the mf genes after exposure to 20 μg/ml doxycycline (the concentration that caused the different mf motility phenotypes) *in vitro* at 13, 36, and 61 h.

1. Parasite materials and culture system

B. malayi mf (TRS strain) were isolated from the peritoneal cavity of infected male jirds (*Meriones unguiculatus*) provided and shipped by the National Institute of Health/National Institute of Allergy and Infectious Disease (NIH/NIAID) Filariasis Research Reagent Repository Center (FR3) (University of Georgia, Athens, GA). Mf were washed 3 times with RPMI-1640 (GIBCO, Green Island, NY) to eliminate host cell contamination, and then washed 5 times with RPMI-1640 containing 100 μg/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml amphotericin B (Sigma, St. Louis, MO) (Rao and Weil 2002). Triplicated 300,000 Mf and 3 time points (0, 23, and 48 h for non-treated and 13, 36, and 61 h for treated-mf) were cultured in a 50-ml screw up conical tube (Sarstedt Inc., Newton, NC) containing 40 ml of RPMI-1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 μg/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml amphotericin B, and 10% fetal calf serum, with a

continuous shaking rocker (model RP-50) (Elmeco Laboratory Instruments, Rockville, MD). Cultured mf were incubated at 37°C in a 95% air-5% CO_2 and the motility were scored (0-4) as described previously (Rao and Weil 2002) with a Nikon SMZ-1500 stereo microscope (Nikon, Tokyo, Japan), and then harvested at 0, 23, and 48 h (13, 36, and 61 h for treated-mf). Mf were performed a snap frozen and stored at -80°C for further RNA extraction.

2. Total RNA extraction method from filarial mf

Treated and non-treated mf (300,000 mf per batch) were washed in 1X PBS-DEPC treated and centrifuged 3,500 *g* at 25°C for 5 min. The mf pellet was resuspended with 1 ml Trizol reagent (Invitrogen), incubated for 5 min at 25°C, and then pipetted to the 2 ml tube containing 1.4 mm ceramic sphere beads; Lysing Matrix D (MP Biomedicals, Solon, OH). Column was agitated for 15 s using the FastPrep FP120 homogenizer (Savant Instrument, Holbrook, NY, USA) in order to break the mf cuticle and homogenization, incubated the column at room temperature for 10 min, added chloroform 200 µl, shook by hand for 15 s, and incubated at room temperature for 10 min. Subsequently, the column was centrifuged 15,300 *g* at 4 °C for 15 min and removed RNA-containing clear aqueous phase to the 1.5 ml eppendorf tube. Ten micrograms RNase-free glycogen (Ambion Inc., Austin, TX) and 500-µl isopropyl alcohol were added and incubated -20°C overnight to precipitate RNA. Next day, the sample were centrifuged 15,300 *g* at 4°C for 15 min, removed the supernatant, and washed the pellet with 1 ml 75% ethanol. Subsequently, the eppendorf tube was vortexed briefly, centrifuged 5,900 *g* at 4 °C for 15 min, removed ethanol, and dried

the pellet for 10 min in the fume hood. RNA was dissolved in 50-µl RNase-free water and incubated in the water bath 50°C for 10 min. One microliter of RNA was determined the yield and quality by Nanodrop instrument (Nanodrop technologies) and RNA Nano LabChip analysis on a model 2100 Bioanalyzer (Agilent, Palo Alto, CA).

3. Microfilaria RNA amplification

One microgram of treated and non-treated mf RNA was used to performed the reverse transcription, first strand cDNA synthesis. RNA from an individual sample was primed with 1 µl T7 oligo-dT primer (KREATECH Biotechnology, Amsterdam, The Netherlands) and nuclease-free water was added to a final volume of 12 µl. The reaction was vortexed briefly to mix and then centrifuged to collect the mixture at the bottom of the tube. The mixture was incubated 70°C for 10 min in a thermal cycler (Perkin Elmer, Oak Brook, IL), centrifuged briefly, and placed on ice. The mixture was added with 8 µl of the reverse transcription master mix (2 µl 10X first strand buffer, 4 µl dNTP mix, 1 µl RNase inhibitor (KREATECH Biotechnology), and 1 µl ArrayScript) (KREATECH Biotechnology), mixed by gently vortexing, centrifuged briefly, and incubated in a 42°C incubator for 2 h. After the incubation, the samples were centrifuged briefly, placed on ice, and immediately proceeded to second strand cDNA synthesis.

For the second strand cDNA synthesis, the samples were mixed by gently vortexing, centrifuged briefly (5 s), and place on ice. The mixture was added with 80 µl of second strand master mix (10 µl 10X second strand buffer, 4 µl dNTP mix, 2 µl DNA polymerase, 1 µl RNase H (KREATECH Biotechnology), and 63 µl nuclease-

free-water) to each sample. The reactions were mixed by pipetting up and down 2-3 times, flicked the tube 3-4 times, centrifuged briefly, and then incubated in a 16°C thermal cycler (Perkin Elmer) for 2 h. After the incubation, the reactions were placed on ice, and proceed to cDNA purification or immediately freezed reactions at -20°C.

The cDNAs purification was performed by adding 250 µl of cDNA binding buffer to each sample, mixed by pipetting up and down 2-3 times, flicked the tube 3-4 times, and followed up with a quick spun to collect the reaction in the bottom of the tube. The mixture was pipetted onto the center of the cDNA filter cartridge, centrifuged at 10,000 *g* for 1 min, discarded the flow-through, and replaced the cDNA filter cartridge in the wash tube. Five hundreds microliter of wash buffer was applied to each cDNA filter cartridge, centrifuged at 10,000 *g* for 1 min, discarded the flow-through, and spun the cDNA filter cartridge for an additional minute to remove trace amounts of wash buffer. The cDNA filter cartridge was transferred to a cDNA elution tube and 10 µl of nuclease-free water (preheated to 50-55°C) was applied to the center of the filter in the cDNA filter cartridge. The mixture was left at room temperature for 2 min, centrifuged at 10,000 *g* for 1.5 min, and the second 10 µl of nuclease-free water was applied. The purified cDNAs were proceeded directly to *in vitro* transcription (IVT) to synthesized the amplified RNA (aRNA) or placed at -20°C.

The 40-µl IVT reaction (16-µl double-strandes cDNA in nuclease-free water, 16 µl 75 mM T7 rNTP mix, 4 µl 10X reaction buffer, and 4 µl enzyme mix) was mixed by pipetting up and down 2-3 times, flicked the tube 3-4 times, and centrifuged briefly. The tubes were incubated in a hybridization oven (Affymetrix Inc., Santa Clara, CA at 37°C for 6 h. The reaction was stopped by adding nuclease-free water to

each aRNA sample, to bring the final volume to 100 µl, mixed thoroughly by gentle vortexing, and proceeded to the aRNA purification step or store at -20°C.

The aRNAs purification was performed to remove enzymes, salts, and unincorporated nucleotides from the aRNA. The aRNA was added with 350 µl of aRNA binding buffer and 250 µl of analytical grade 100% ethanol to each sample, and the reaction was mixed by pipetting up and down 3 times. The mixture was pipetted onto the center of the aRNA filter cartridge, centrifuged at 10,000 *g* for 1 min, discarded the flow-through, and replaced the cDNA filter cartridge bachk into the aRNA collection tube. Six hundreds fifty microliter of wash buffer was applied to each aRNA filter cartridge, centrifuged at 10,000 *g* for 1 min, discarded the flow-through, replaced the aRNA filter cartridge back into the aRNA collection tube. The mixture was added with 650 µl of 80% ethanol to each aRNA filter cartridge, centrifuged at 10,000 *g* for 1 min, discarded the flow-through, and spun the aRNA filter cartridge for an additional 1 min to remove trace amounts of ethanol and wash buffer. The filter cartridge was transferred to a fresh aRNA collection tube and 100 µl of nuclease-free water (preheated to 50-60°C) was applied to the center of the filter. The mixture was left at room temperature for 2 min, centrifuged at 10,000 *g* for 1.5 min. The purified aRNA was determined the yield and quality by RNA Nano LabChip analysis on a model 2100 Bioanalyzer (Agilent). The aRNA can be stored at -80 °C for up to 1 year.

4. The universal linkage system (ULS) labeling procedure

The 20-µl labeling reaction was composed of 4 µg purified aRNA, 2 µl Cy3-ULS dye (KREATECH Biotechnology), and 2 µl 10X labeling solution

(KREATECH Biotechnology). RNase-free water was added to a final volume of 20 µl, mixed by pipetting, incubated in a thermal cycler at 85°C for 15 min, placed on ice, and then spun down to collect contents of tube before proceeding with purification.

The excess Cy3-ULS dye was removed by using KREApure column (KREATECH Biotechnology). The column material was resuspended by vortexing, placed in a 2 ml collection tube, spun the column at 20,800 *g* for 1 min, discarded flow through, and re-used collection tube. The column was washed with 300 µl of RNase free water, spun column at 20,800 g for 1 min, discarded flow through, and put column in a new (RNase free) 1.5 ml micro centrifuge tube. The ULS-labeled aRNA was applied on to column bed, spun column at 20,800 *g* for 1 min. The flow through was purified labeled aRNA and the degree of labeling (DOL) was measured by following formula:

$$\% \text{ Labeling} = \frac{340 \times \text{pmoldye} \times 100}{\text{ng nucleic acid} \times 1000}$$

For the labeled aRNA defragmentation, the labeled aRNA was transferred to a microfuge tube and 1/10 volume of 10X fragmentation buffer (Ambion Inc.) was added to decrease the fragment size to 60-200 bases. The reaction was incubated at 70°C for 15 min, spun the tube briefly, added 1 µl of stop solution (Ambion Inc.), mixed by pipetting, and placed on ice until further used.

5. Microarray experiment

The *B. malayi* V2 filarial array slides comprising 18,104 oligonucleotides in duplicate that represent expressed genes and predicted ORFs from *B. malayi*, *Onchocerca volvulus*, *Wuchereria bancrofti*, and *Wolbachia* (Table 3.3) were used for the mf gene expression analysis. The *B. malayi* elements cover ~85% (18,104) of the genes in this species.

5.1 Pre-hybridization

The prehybridization solution consisting of 5X SSC (Invitrogen), 0.1 µg/ml BSA, 0.1% SDS, and Milli-Q water was prepared, and warmed the solution in the hybridization oven (Affymetrix Inc.) at 42°C for 1 h. The slides were placed flat in the glass staining dish containing the solution, with the elements up and made sure the slides were not stacked, and covered by the solution. The container containing slides was incubated in the hybridization at 42°C for 1 h. After the incubation was completed, the slides were washed with Milli Q water 3-4 times to got off any excess pre-hybridization solution. The slides were spun immediately at 1,600 *g* for 2 min in a microfuged equipped with a slide adaptor and proceeded for hybridization.

Table 3.3 V2 *B. malayi* array oligonucleotide information

Oligonucleotide name	Oligonucleotide source	Examples
BMX1-BMX9241	TIGR *B. malayi* genomic gene models	15559.m00008
BMX9242-BMX11130	TIGR gene indices *B. malayi* EST clusters TIGR gene indices *B. malayi* EST singletons	TC7731-TC9779 AA007707, AI919741, CB289772, N41098, W59868 (not TCXXXXX)
BMX11131-BMX11884	John Macpherson V2 Oligos	BMB_gene_32.412, BMBC_gene_65.1287, BM889300, BMW00282.660
BMX11885-BMX12756	*W. bancrofti* EST clusters-Nutman Lab	WB-contig_1000
BMX12757-BMX13772	TIGR gene indices *O. volvulus* EST clusters	TC2740-TC3840
BMX13773-BMX14576	*Wolbachia* gene models-NEB	Wbm0804
Bm.00001-bm.03569	*Brugia* Chip V1	BMC05846

5.2 Hybridization

The hybridization solution, consisting of 5X SSC, 25% formamide (Inc.), 0.1% SDS, 0.1 mg/ml Herring sperm DNA (Invitrogen), 25% KREA*block* (KREATECH Biotechnology), was added to the fragmented aRNA sample in the 45-µl reaction. Subsequently, the 0.5 ml tube was vortexed, flicked, spun down, and incubated at 95°C for 5 min in the thermal cycler. The reaction was centrifuged at 20,800 *g* for 2 min and applied the sample along the middle of the V2 filarial array slide carefully, by avoided touching the array with the pipette tip and creating air bubbles. The slides were covered with the cover glass (washed with 95% ethanol, milli Q water, and allowed to air-dry) and fixed in the hybridization chamber. The chamber containing array slides was incubated in the hybridization oven at 42°C for 16 h.

5.3 Post-hybridization

After disassembled the hybridization chambers, the array slides were applied in the slide rack with handle and immersed in the glass staining dish containing 2X SSC and 0.1% SDS at 42°C, until the cover glass moved freely away from the slides. The slides were transferred to 2X SSC and 0.1% SDS at 42°C for 5 min with stirring, and washed twice in 1X SSC and 0.1% SDS at 42°C for 2 min. Subsequently, washed twice in 0.1X SSC at room temperature for 1 min. The slide were dried by centrifugation at 1,600 *g* for 2 min and then proceeded to scan using Axon GenePix 4200AL scanner using a 10 µm pixel size (Axon Instruments, Union City, CA). Laser power was set to 100%, and the photomultiplier tube voltage was adjusted to maximize effective dynamic range and minimize pixel saturation. The spot size, location, and quality were determined using GenePix software Pro 6.0 algorithms, and

potential misidentifications of spot locations and quality were corrected manually. Scan images were analyzed, and Cy3 signal and ratio values were obtained using Genepix software. The minimum signal intensity was set to 200 fluorescent units, and the signal-to-background ratio cutoff was set to 2.0 Cy3 channel. Two or three biological replicates were performed for each experimental set. The background-subtracted median fluorescent values for good spots (no bad, missing, absent, or not-found flags) were normalized according to a LOWESS normalization method.

5.4 Image acquisition and data analysis

Microarray slides were scanned on an Axon Genepix 4200AL Scanner using a 10-μm pixel size (Axon Instruments, Union City, CA) and the images were analyzed with the GenePix software Pro 6.0. The intensities of the hybridized targets and associated background were estimated. Laser power was set to 50%, and the photomultiplier tube voltage was adjusted to maximize effective dynamic range and minimize pixel saturation. The spot size, location, and quality were determined using GenePix software Pro 6.0 algorithms, and potential misidentifications of spot locations and quality were corrected manually. Scanned images were analyzed, and Cy3 signal to noise ratio values were obtained using Genepix software. The minimum signal intensity was set to 200 fluorescent units, and the signal-to-background ratio cutoff was set to 2.0 Cy3 channel. The background-subtracted median fluorescent values for good spots (no bad, missing, absent, or not-found flags) were normalized according to a LOWESS normalization method. Identification of genomic and EST sequences, and assignment of putative gene functions were performed according to Nembase database version 3 (http://www.nematodes.org/nembase3/index.shtml) (Parkinson et al. 2004), TIGR

database http://www.tigr.org/tdb/e2k1/bma1/intro.shtml), TIGR *B. malayi* gene indices release 5.1 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=b_malayi) and *O. volvulus* gene indice release 4.1 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=o_volvulus) (Quackenbush et al. 2000) and WormBase (http://www.wormbase.org/) (Rogers et al. 2008).

Raw expression values from the V2 filarial arrays were analyzed using Partek software (Partek Inc., St. Louis, MO). Raw intensity values were imported by setting up robust multiarray analysis background correction and normalization. Principal components analysis (PCA) was performed as implemented in Partek as it is an excellent method for visualizing high-dimensional data. False discovery rate (FDR) was determined for the *p*-value in order to correct for multiple testing. Analysis of variance (ANOVA) was determined for treatment vs. control as a categorical factor. ANOVA *p*-values were filtered using the FDR cutoff of 0.1. To be included in the final gene list, values were analyzed by ANOVA, Significance Analysis if Microarrays (SAM) (FDR≤0.1%), student's *t*-test ($P \leq 0.05$), signal above background, call consistency ≥ 90%, and a 2-fold or higher (2X) minimum threshold filter was applied (Downey, 2006).

To generate Gene Ontology (GO) associations for differentially expressed genes detected in the microarray experiments, we identified enriched GO terms. A list of GO terms relevant for *B. malayi* was then generated using the InterProScan tool (Zdobnov and Apweiler, 2001). Multiple genes were identified within the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway maps http://www.genome.jp/kegg/ (Ogata et al. 1999). The stage-specific mf genes were

analyzed and compared with mf ES proteins (Bennuru et al. 2009a). The potential drug targets for *B. malayi* mf were identified using *Brugia* homologs in *C. elegans* that have been listed for knockout phenotypes using RNA interference (RNAi).

CHAPTER IV

RESULTS

<u>Part I</u>: The effects of anti-rickettsial drugs on *B. malayi* mf determined by the minimum effective concentration (MEC) and on *Wolbachia* determined by the minimum inhibitory concentration (MIC)

1. Effects of anti-rickettsial drugs on mf motility determined by minimum effective concentration (MEC) assay

The anti-rickettsial agents, doxycycline, rifampicin, and ciprofloxacin at the concentration of 0.125-256 μg/ml, the standard concentration for antimicrobial test, were studied for antifilarial activity against *B. malayi* mf. The effects of anti-rickettsial agents on mf motility, measured as MECs, demonstrated that treatment of doxycycline at 128 and 32 μg/ml inhibited mf motility at 12 and 52 h, respectively, as showed in Figure 4.1. Rifampicin and ciprofloxacin were less effective, with the MEC of >256 μg/ml at 12 and 52 h. The untreated mf showed highly active and motile (score 4) at these time points.

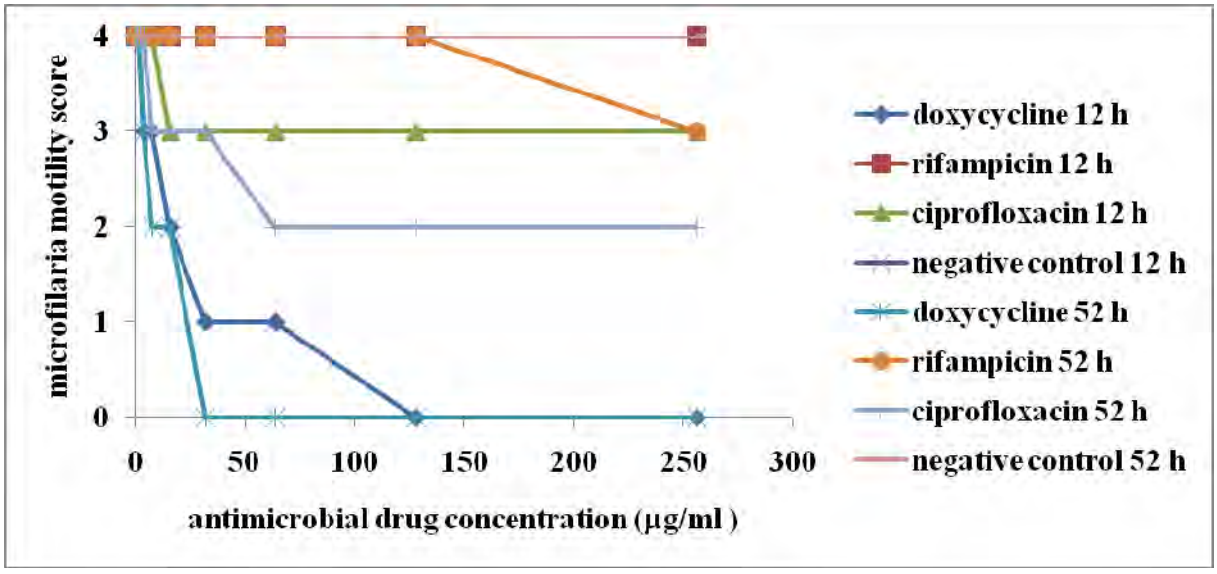


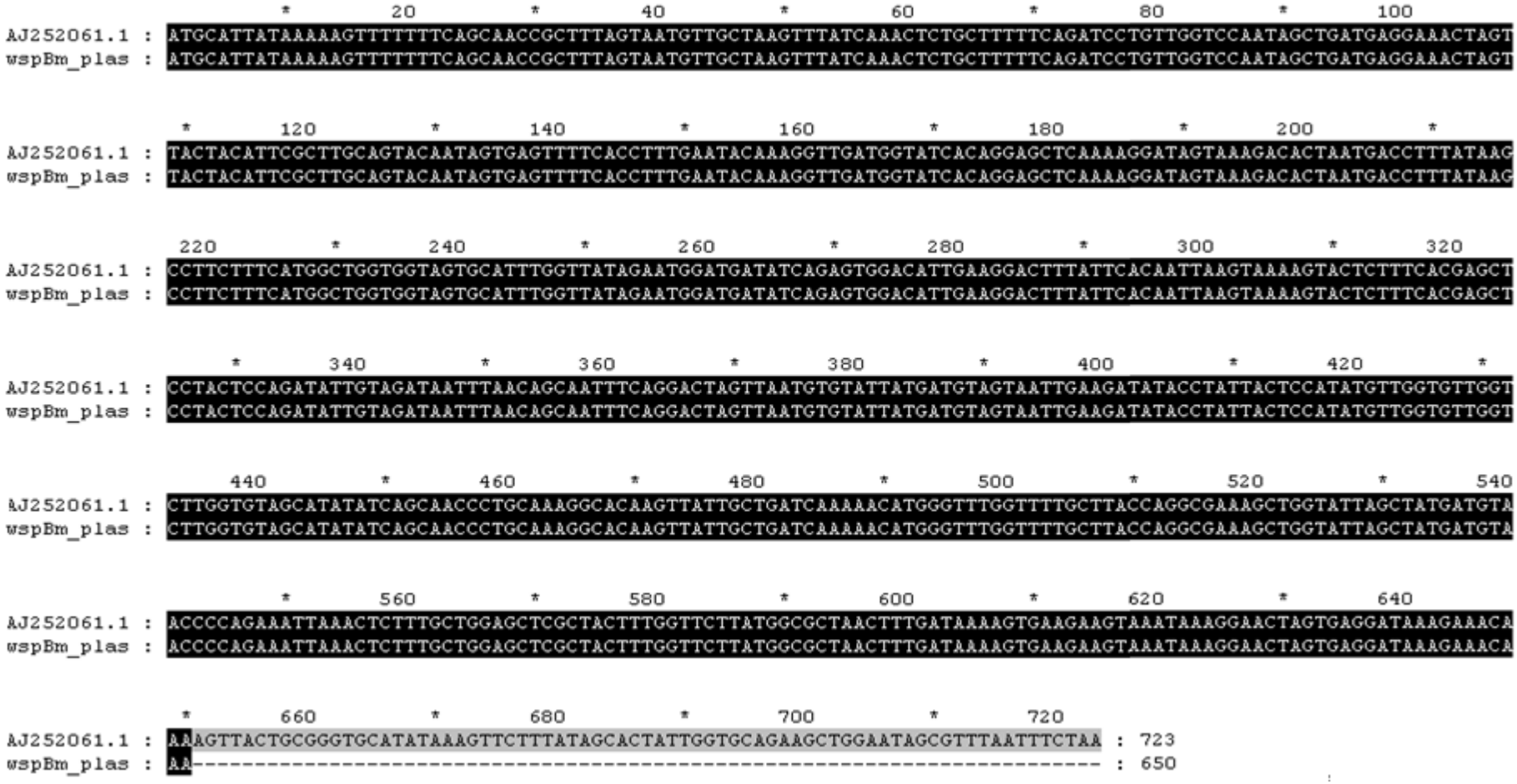
Figure 4.1 Effect of the antimicrobial agents on inhibition of mf motility using MEC assay at 12 and 52 h

2. Specificity of the quantitative PCR assay

Plasmid encoding *Wolbachia* surface protein and *B. malayi hsp70* genes were sequenced and showed in Figure 4.2. The specificity of *wsp* and *hsp70* PCR products were confirmed by agarose gel electrophoresis (Figure 4.3). Tenfold serial dilution of purified plasmid carrying *wsp* and *hsp70* genes were used to construct standard curve, from $3x10^5$ copies at the start to $3x10^1$ copies (Figure 4.4). The detection limit of the assay was determined to be at least 30 copies of plasmid per reaction. Although the Taqman qPCR can detect as low as one copy number of DNA, the SYBR green qPCR cost used in this study is cheaper than the Taqman qPCR. The standard curves were generated from the C_ts of the amplification plots with ABI Sequence Detection software. The slopes of the *wsp* and *hsp70* standard curves were -3.404 and -3.567, respectively. The results showed that the efficiency of the PCR was approximately

96.79% and 90.70%, respectively, according to the equation $E = 10^{-1/s} - 1$, where E is the run efficiency and s is the slope of the generated standard curve (Fink et al. 1998).

A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wspBm_plas : ATGCATTATAAAAAGTTTTTTTTCAGCAACCGCTTTAGTAATGTTGCTAAGTTTATCAAACTCTGCTTTTTCAGATCCTGTTGGTCCAATAGCTGATGAGGAAACTAGT
* 120 * 140 * 160 * 180 * 200 *
AJ252061.1 : TACTACATTCGCTTGCAGTACAATAGTGAGTTTTCACCTTTGAATACAAAGGTTGATGGTATCACAGGAGCTCAAAAGGATAGTAAAGACACTAATGACCTTTATAAG
wspBm_plas : TACTACATTCGCTTGCAGTACAATAGTGAGTTTTCACCTTTGAATACAAAGGTTGATGGTATCACAGGAGCTCAAAAGGATAGTAAAGACACTAATGACCTTTATAAG
220 * 240 * 260 * 280 * 300 * 320
AJ252061.1 : CCTTCTTTCATGGCTGGTGGTAGTGCATTTGGTTATAGAATGGATGATATCAGAGTGGACATTGAAGGACTTTATTCACAATTAAGTAAAAGTACTCTTTCACGAGCT
wspBm_plas : CCTTCTTTCATGGCTGGTGGTAGTGCATTTGGTTATAGAATGGATGATATCAGAGTGGACATTGAAGGACTTTATTCACAATTAAGTAAAAGTACTCTTTCACGAGCT
* 340 * 360 * 380 * 400 * 420 *
AJ252061.1 : CCTACTCCAGATATTGTAGATAATTTAACAGCAATTTCAGGACTAGTTAATGTGTATTATGATGTAGTAATTGAAGATATACCTATTACTCCATATGTTGGTGTTGGT
wspBm_plas : CCTACTCCAGATATTGTAGATAATTTAACAGCAATTTCAGGACTAGTTAATGTGTATTATGATGTAGTAATTGAAGATATACCTATTACTCCATATGTTGGTGTTGGT
440 * 460 * 480 * 500 * 520 * 540
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* 660 * 680 * 700 * 720
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wspBm_plas : AA---------- : 650

B

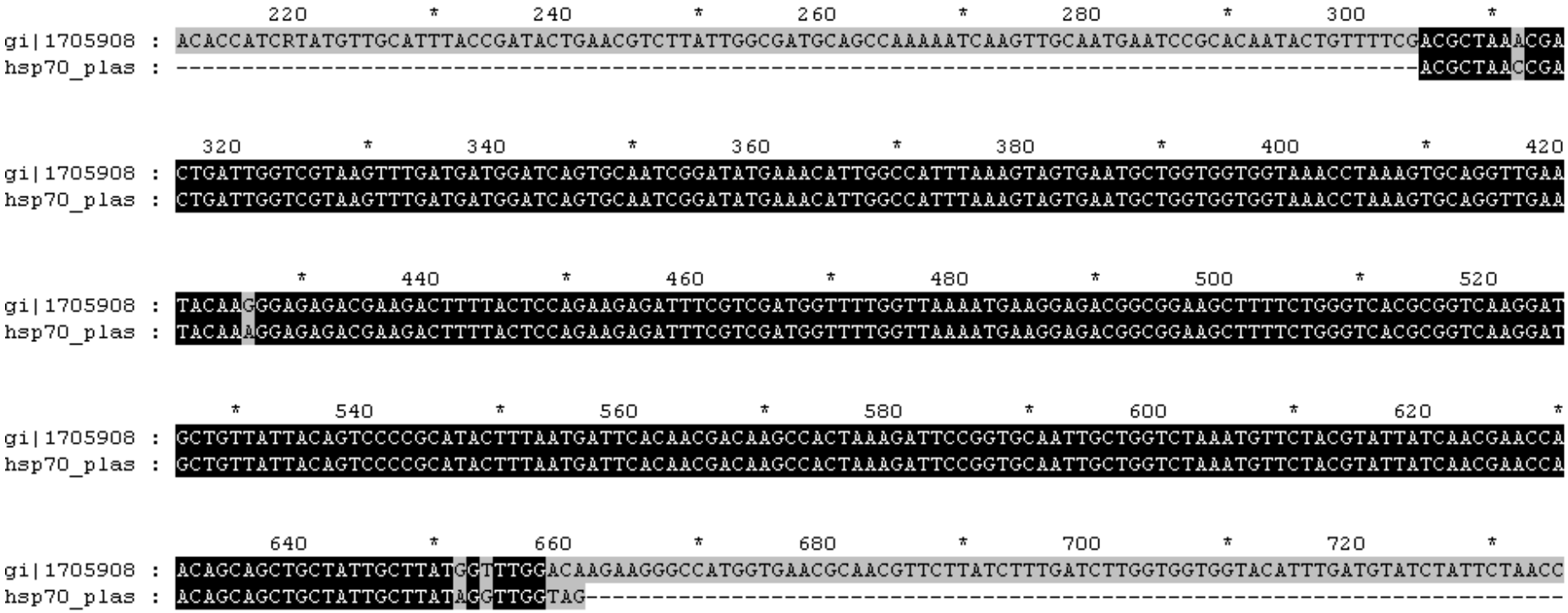


Figure 4.2 **Alignment of *Wolbachia wsp* and *B. malayi hsp70* with Genbank database.** Plasmid encoding *wsp* gene was analysed with *Wolbachia wsp* from Genbank database (accession number AJ252061.1) (A). Plasmid encoding *hsp70* gene was analysed with mf *hsp70* from Genbank database (accession number gi1705908) with plasmid encoding *hsp70* gene (B).

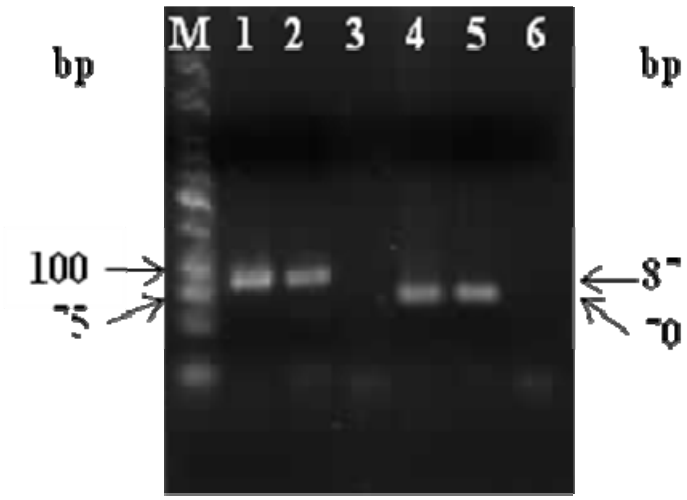


Figure 4.3 **Ethidium bromide staining patterns of the PCR products on a 2.5% agarose gel.** Lane 1, PCR products obtained from positive control *wsp* plasmid; Lanes 2 and 5, PCR products of *wsp* and *hsp70*, respectively, obtained from *B. malayi* mf; Lanes 3 and 6, negative control containing no DNA; Lane 4, PCR products obtained from positive control *hsp70* plasmid; Lane M, low molecular weight DNA ladder (New England Biolabs, Ipswich, MA)

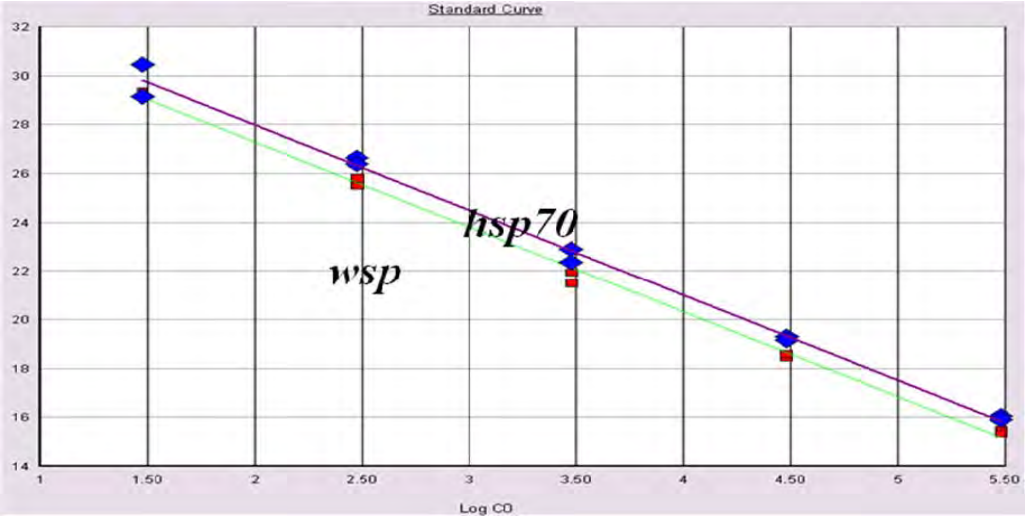


Figure 4.4 Establishment of the *wsp* and *hsp70* standard curve for *Wolbachia*/nematode ratio quantification. Plasmid copy numbers are (from left to right) $3x10^1$, $3x10^2$, $3x10^3$, $3x10^4$, and $3x10^5$. For each dilution, the log plasmid copy numbers are plotted against the PCR cycle number. Standard curve generated from the C_t values of the amplification plots with ABI Sequence Detection software.

3. Antimicrobial susceptibility of *Wolbachia* using qPCR and minimum inhibitory concentration (MIC) assays

The susceptibility of *Wolbachia* to anti-rickettsial agents was measured by MICs, as showed in Table 4.1, 4.2 and Figure 4.5. From *wsp/hsp70* single copy gene ratio analysis, doxycycline and rifampicin showed the effciency with MICs of 128 μg/ml with DNA ratio = 0.26 ($P = 0.02$) and >128 μg/ml with DNA ratio = 0.39 ($P > 0.05$), respectively at 12 h. In contrast, the *wsp/hsp70* gene ratios obtained from ciprofloxacin-treated mf at the concentration of 2, 32 and 128 μg/ml increased (ratio from 0.52 to 0.59 and 0.60, respectively) ($P < 0.01$), when compared with untreated microfilariae (ratio from 0.37 to 0.17). Doxycycline showed the best effective of MIC compared with rifampicin and ciprofloxacin. At 52 h, there were no statistical differences in MICs among drug-treated mf and untreated control ($P > 0.05$).

Table 4.1 Gene copy number and *wsp/hsp70* gene ratio of untreated and drug-treated mf at 12 h

Anti-rickettsial drugs	Concentrations (μg/ml)	gene copy number		*wsp/hsp70* ratio
		wsp	*hsp70*	
control	-	1.26×10^4	3.39×10^4	0.37
doxycycline	0.125	1.67×10^4	5.58×10^4	0.30
	0.5	2.12×10^4	7.41×10^4	0.29
	2	1.65×10^4	4.24×10^4	0.39
	8	1.23×10^4	3.36×10^4	0.37
	32	5.70×10^3	2.83×10^4	0.20
	128	1.42×10^4	5.51×10^4	0.26*
rifampicin	0.125	1.91×10^4	4.54×10^4	0.42
	0.5	1.10×10^4	2.90×10^4	0.38
	2	1.01×10^4	2.59×10^4	0.39
	8	1.13×10^4	4.00×10^4	0.28
	32	1.03×10^4	2.74×10^4	0.38
	128	1.38×10^4	3.55×10^4	0.39

Anti-rickettsial drugs	Concentrations (μg/ml)	gene copy number		*wsp/hsp70* ratio
		wsp	*hsp70*	
ciprofloxacin	0.125	$1.94x10^4$	$6.49x10^4$	0.30
	0.5	$1.80x10^4$	$4.44x10^4$	0.41
	2	$1.94x10^4$	$3.73x10^4$	0.52**
	8	$2.36x10^4$	$5.13x10^4$	0.46
	32	$1.57x10^4$	$2.66x10^4$	0.59**
	128	$2.44x10^4$	$4.09x10^4$	0.60**

* $P = 0.02$ (decreased gene ratio, drug treated versus non-drug treated)

** $P < 0.01$ (increased gene ratio, drug treated versus non-drug treated)

Table 4.2 Gene copy number and *wsp/hsp70* gene ratio of untreated and drug-treated mf at 52 h

Anti-rickettsial drugs	Concentrations (μg/ml)	gene copy number		*wsp/hsp70* ratio
		wsp	*hsp70*	
control	-	$9.27x10^{1}$	$5.55x10^{2}$	0.17
doxycycline	0.125	$1.46x10^{4}$	$8.75x10^{4}$	0.17
	0.5	$1.76x10^{4}$	$7.17x10^{4}$	0.25
	2	$6.33x10^{3}$	$3.99x10^{4}$	0.16
	8	$1.27x10^{4}$	$6.19x10^{4}$	0.20
	32	$1.57x10^{3}$	$1.41x10^{4}$	0.11
	128	$2.91x10^{4}$	$1.35x10^{5}$	0.22
rifampicin	0.125	$6.18x10^{3}$	$3.51x10^{4}$	0.18
	0.5	$1.05x10^{4}$	$4.71x10^{4}$	0.22
	2	$9.63x10^{3}$	$4.58x10^{4}$	0.21
	8	$3.81x10^{3}$	$3.20x10^{4}$	0.12
	32	$1.58x10^{4}$	$9.87x10^{4}$	0.16
	128	$6.97x10^{3}$	$3.39x10^{4}$	0.21

Anti-rickettsial drugs	Concentrations (μg/ml)	gene copy number		*wsp/hsp70* ratio
		wsp	*hsp70*	
ciprofloxacin	0.125	$6.31x10^{3}$	$3.50x10^{4}$	0.18
	0.5	$1.56x10^{3}$	$4.89x10^{3}$	0.32
	2	$3.35x10^{3}$	$2.47x10^{4}$	0.14
	8	$1.38x10^{4}$	$5.63x10^{4}$	0.25
	32	$1.15x10^{3}$	$1.48x10^{4}$	0.08
	128	$1.68x10^{3}$	$4.28x10^{3}$	0.39

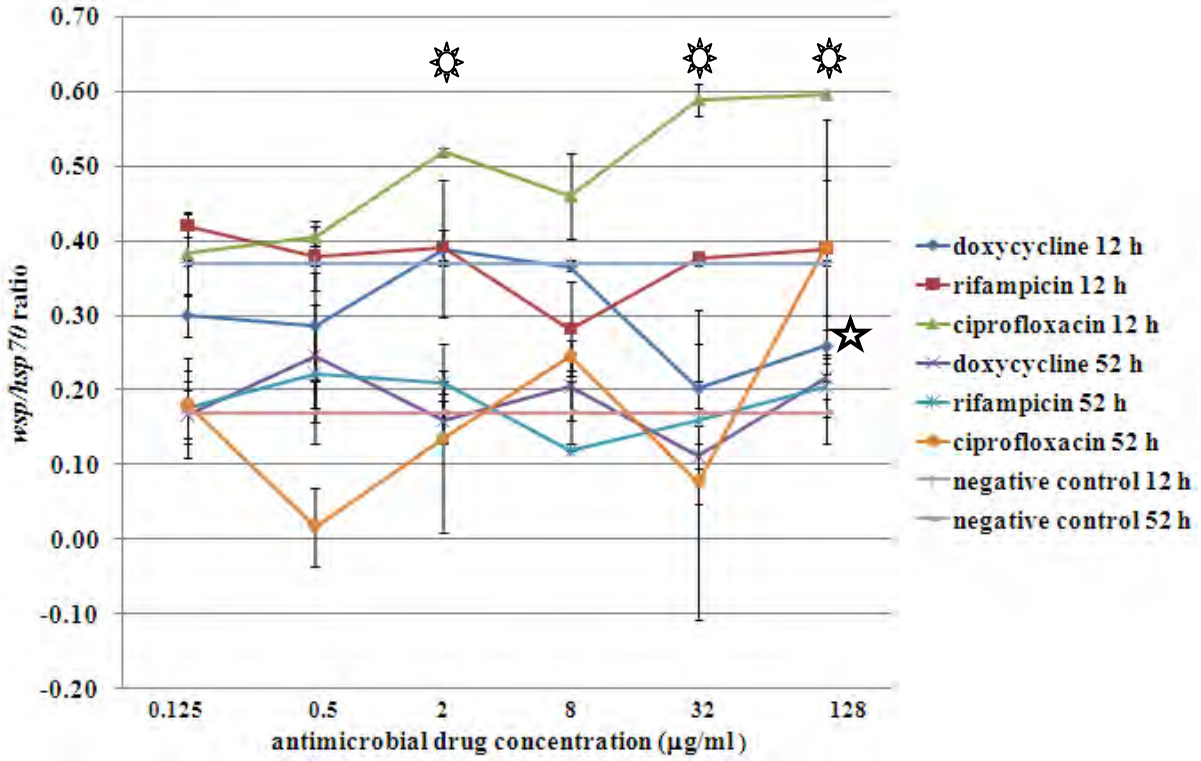


Figure 4.5 **qPCR for *Wolbachia*/nematode DNA, expressed as *wsp/hsp70* gene ratio.** The means of the copy number ratios were calculated, and statistical significance was determined using a two-tailed paired Student *t* test.

☆ $P = 0.02$ (decreased gene ratio, drug treated versus non-drug treated)

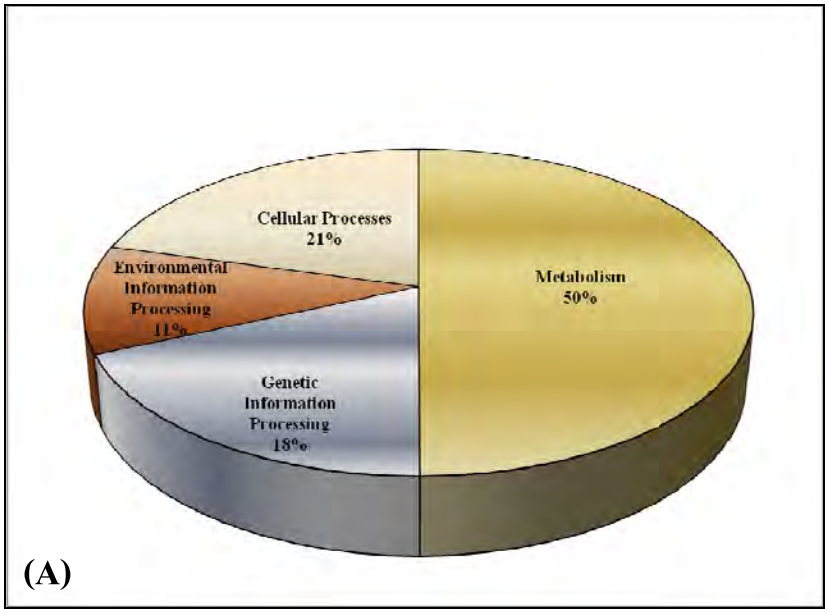
✲ $P < 0.01$ (increased gene ratio, drug treated versus non-drug treated)

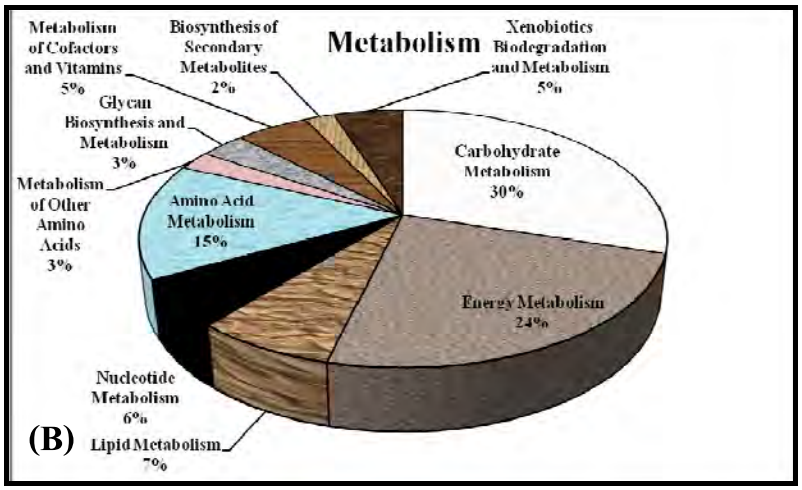
<u>Part II</u>: Characterization of the groups of *B. malayi* mf genes that could change their expression from untreated mf compared with doxycycline-treated mf cultured *in vitro*

1. Characterization of the groups of *B. malayi* mf genes that could change their expression from untreated microfilariae

1.1 Selected gene expression profile from untreated mf

The mf motility at 0, 23, and 48 h was scored as 4 (highly active and motile). At 23 h, no significant different of up- or down-regulated *B. malayi* mf gene was observed. At 48 h, 2,200 genes (12.12%) of *B. malayi* mf were up-regulated while 2 genes (0.01%), protein involved in Snf1 protein kinase complex and hypothetical protein, were down-regulated. The degree of differential expression ranged from 336.72-fold increases to 11.47-fold decreases. The percentages of these up-regulated genes that were classified according to their metabolic pathways were expressed as pie charts (Figure 4.6). The main pathways of *B. malayi* mf include metabolism (carbohydrate metabolism, energy metabolism, and amino acid metabolism), genetic information processing (translation and folding sorting and degradation), environmental information processing (signal transduction and signal molecule and interaction), and cellular processing (cell communication, immune system, and cell motility).


Cellular Processes
21%
Environmental
Information
Processing
11%
Genetic
Information
Processing
18%
Metabolism
50%
(A)


Metabolism
Metabolism
of Cofactors
and Vitamins
5%
Biosynthesis of
Secondary
Metabolites
2%
Xenobiotics
Biodegradation
and Metabolism
5%
Glycan
Biosynthesis and
Metabolism
3%
Metabolism
of Other
Amino
Acids
3%
Amino Acid
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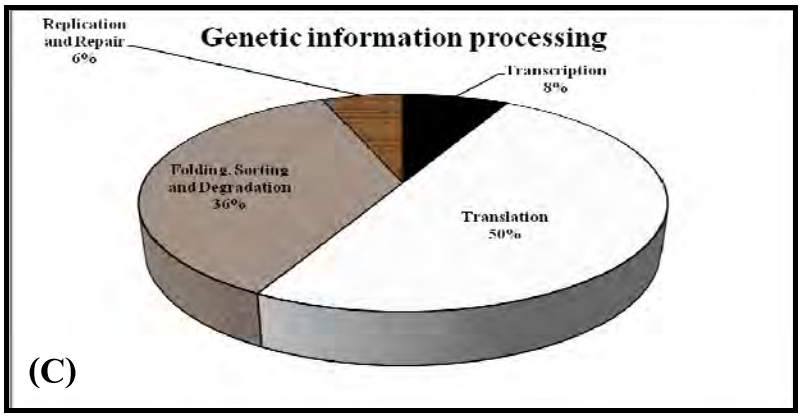
24%
Nucleotide
Metabolism
6%
Lipid Metabolism
7%
(B)


Genetic information processing
Replication
and Repair
6%
Transcription
8%
Folding, Sorting
and Degradation
36%
Translation
50%
(C)

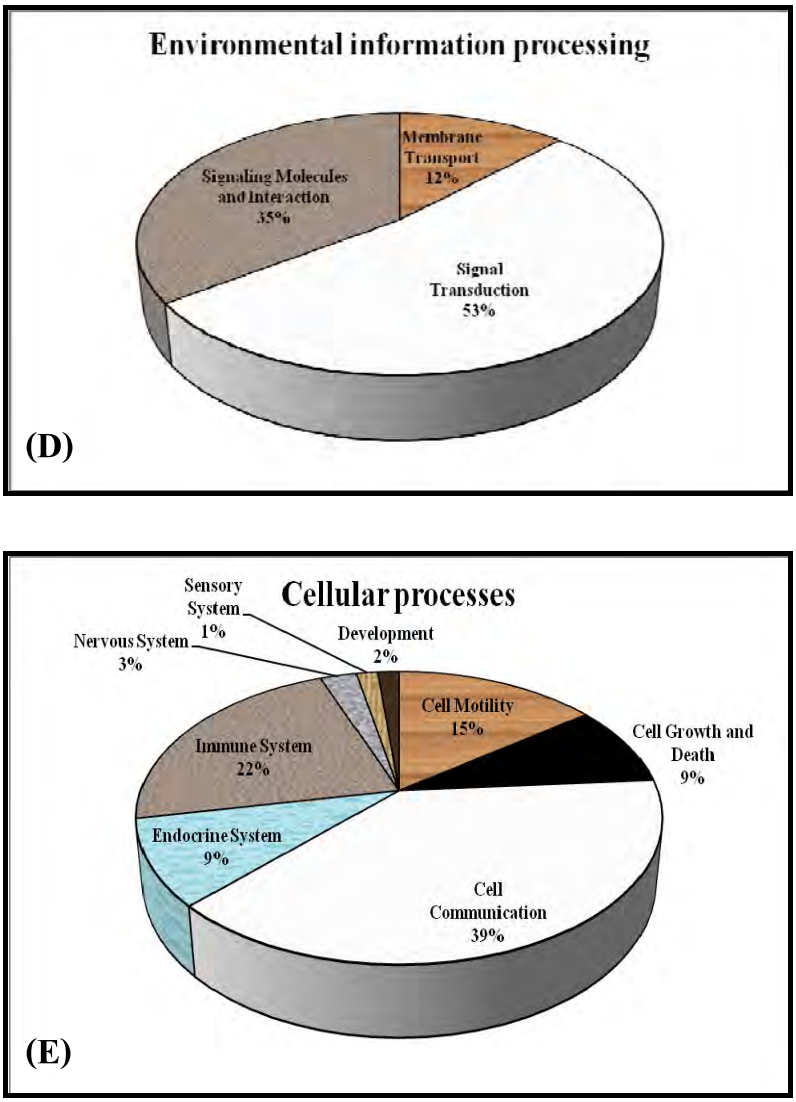


Figure 4.6 Distribution of *B. malayi* mf metabolic pathways (A) including metabolism (B), genetic information processing (C), environmental information processing (D), and cellular processes (E)

1.2 The activities of the glycolytic and tricarboxylic acid cycle (TCA) enzymes in *B. malayi* microfilariae

B. malayi mf were found to have enzymes important for both glycolysis and tricarboxylic acid (TCA) cycles (Tables 4.3). The high expression of TCA cycle

including succinate dehydrogenase (14968.m01521; 122.40 fold) and malate dehydrogenase (13478.m00071; 73.97 fold) enzyme genes of TCA cycle was obtained.

Table 4.3 The activities of the glycolysis and tricarboxylic acid cycle enzymes in *B. malayi* microfilariae

Enzymes	Fold change
Glycolysis	
Pyruvate kinase	37.52
Glucose phosphate isomerase	18.49
Alcohol dehydrogenase (NAD/NADP)	14.48
Glyceraldehyde-3-phosphate dehydrogenase	12.82
Aldolase	5.79
Cytoplasmic glycerol-3-phosphate dehydrogenase	5.73
Triosephosphate isomerase	3.78
Lactate dehydrogenase	3.64
Fructose-1, 6-bisphosphatase	3.06
Phosphoglycerate kinase	2.77
Phosphoglucomutase	2.67
Phosphoenolpyruvate carboxykinase	2.38
Phosphofructokinase	2.30
Tricarboxylic acid (TCA) cycle	
Succinate dehydrogenase	122.40
Malate dehydrogenase	73.97
Isocitrate dehydrogenase (NADP)	6.65
Pyruvate dehydrogenase	3.23

3. Major up-regulated genes for *B. malayi* mf ES proteins and genes as potential drug targets

The up-regulated *B. malayi* mf genes obtained from this study were analyzed with the stage-specific mf ES proteins obtained from the proteomic analysis (Moreno and Geary 2008; Bennuru et al. 2009a). The degree of differential up-regulated expressions ranged from 2.22 fold to 56.07 fold (Table 4.4). For example, the exonuclease family protein (14992.m11178; 56.07 fold), homeobox-domain containing protein (14975.m04498; 14.93 fold), phosphatidylethanolamine-binding protein (14956.m00481; 13.83 fold), actin (14258.m00140; 7.41 fold), serpin (14777.m00108; 6.08 fold), and endochitinase (14932.m00512; 5.41 fold). Moreover, many of the genes up-regulated in mf have *C. elegans* homologues with RNAi phenotypes, and some have been previously suggested by Kumar et al. (2007) as potential drug targets because of their significant differences in biochemical properties and phylogenetic distance between human and nematode including ribosomal proteins, NADH-ubiquinone oxidoreductase, and mitochondrial ATP synthase (Table 4.5).

Table 4.4 Major up-regulated genes for *B. malayi* mf ES proteins and RNAi phenotypes of *C. elegans* homologues

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	Fold change	RNAi phenotype in *C. elegans**
14992.m11178	exonuclease family protein	C05C8.5	56.07	not_Emb
12524.m00106	conserved hypothetical protein	B0035.12	55.77	Clr, Egl, Lva, Unc, Emb, Gro
14975.m04498	Homeobox-domain containing protein	F31E8.3	14.93	WT
14968.m01463	hypothetical protein	C39E9.8b	14.32	WT
14956.m00481	Phosphatidylethanolamine-binding protein	Y69E1A.5	13.83	WT
14080.m00070	ATP-dependent helicase DDX48	F33D11.10	9.58	Ste, Lva, Led Larval
14094.m00132	hypothetical protein	F23H11.2	9.72	Ste, Sma, not_Emb, Stp
14979.m04397	PAN domain containing protein	B0361.9	7.58	WT
14258.m00140	actin	T04C12.5	7.41	Emb Unclassified, Lvl, Cyk, Sck, Lva, Pvl, Ste
14972.m07327	conserved hypothetical protein	Y73F8A.6	6.65	WT
13275.m00188	Pao retrotransposon peptidase family protein	F15D4.7	6.44	WT

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	Fold change	RNAi phenotype in *C. elegans**
14777.m00108	serpin	C05E4.1	6.08	WT
14932.m00512	Endochitinase	C04F6.3	5.41	Emb
14961.m05180	Utp21 specific WD40 associated domain containing protein	Y45F10D.7	5.40	Emb Larval
14990.m07851	Brix domain containing protein	K09H9.6	5.17	Emb, Lvl, Unclassified Lpd, Ste, Sck Larval
14992.m11170	Serine/threonine protein phosphatase 2A, 56 kDa regulatory subunit,epsilon isoform	W08G11.4	5.14	aldicarb_resistant
14971.m02831	COG5 protein	C43E11.11	4.94	Emb Unclassified Gro
13133.m00072	prediction-related	T04A8.4	4.84	WT
14981.m02382	Gaba	C32D5.9	4.21	Emb
14961.m05094	sf3a2-prov protein	F11A10.2	4.20	Lva, Emb
15190.m00018	conserved hypothetical protein	none	4.15	none
14294.m00134	Hypothetical 55_2 kDa Trp-Asp repeats containing protein T10F2_4 inchromosome III,	T10F2.4	3.88	Unclassified Ste, Rup, Bli, Pvl, Emb

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	Fold change	RNAi phenotype in *C. elegans**
13253.m00070	WD-repeat protein BING4 related	F28D1.1	3.71	Larval
14979.m04495	HT014	ZK353.9	3.67	WT
14972.m07888	WD-repeat protein 26 related	Y39H10A.6	3.46	Emb, Ste
12721.m00014	Hypothetical 45_4 kDa protein in thiaminase I 5' region-related	C13C4.4	3.23	WT
14980.m02753	conserved hypothetical protein	D2007.4	3.06	Emb, Lva Larval
14956.m00512	hypothetical protein	Y67A10A.7	2.75	WT
15261.m00022	RIKEN cDNA 2810439K08-related	K02G10.3	2.65	WT
12950.m00042	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial precursor	F56D12.1a	2.48	Slu, Unc, Stp, Gro
13717.m00172	hypothetical protein	F56H1.5	2.46	WT
13843.m00008	Hypothetical protein	Y9C9A.16	2.33	WT
14972.m07280	initiation factor 2-associated protein	Y116A8A.9	2.27	Emb Unclassified, Stp, Gro, Fgc, Gon

Table 4.5 Genes as potential drug targets in microfilarial gene set

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	RNAi phenotype in *C. elegans**
14972.m07061	hypothetical protein	C01B10.11	Dpy, Unc
14972.m07044	SD01790p-related	Y41E3.1	Sck, Ste, WT
14979.m04655	WH2 motif family protein	C34E10.11	Emb, WT
14981.m02425	hypothetical protein	C52A11.2	Dpy, Lva, Egl, Emb, Unc, Rup, WT
14972.m07319	hypothetical protein	W04G3.2	Bmd, Lva, Lvl, Unc, WT
14972.m07555	GH05862p-related	F42G8.10	Lva, Emb, Unclassified, WT
13156.m00091	hypothetical protein	F37C4.4	Emb, WT, Stp
14992.m11309	Lipase family protein	T08B1.4	Aldicarb resistant, WT
14058.m00576	Mitochondrial ATP synthase coupling factor 6 family protein	*atp-4*	General Pace of Development, abnormal, Emb, Lvl, Ste, Adl, Gro
13322.m00194	GM16138p-related	F49C12.12	Emb, Sterile F0/Fertility Problems, Lvl
15081.m00161	NADH-ubiquinone oxidoreductase subunit B14.5b.-related	Y71H2AM.4	Lva, Emb, WT, Gro
14992.m10983	hypothetical protein	*ril-1*	Lva, General Pace of Development abnormal, Emb, Gro
14980.m02739	hypothetical protein	F57B10.14	Osmotic Integrity defective, Emb, WT

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	RNAi phenotype in *C. elegans**
15443.m00042	50S ribosomal protein L10.-related	K01C8.6	Lva, Emb, Larval Arrest-Early (L1/L2), Unclassified, Sma, WT, Gro
15135.m00169	zgc:101038 protein-related	Y69A2AR.21	Emb, WT
15304.m00109	sulfakinin receptor protein, putative	T23B3.4	Emb, Unclassified, WT
14980.m02754	Chain A, Structure Of A Brca2-Dss1 Complex., putative	Y119D3B.15	Sck, Ste, WT
14971.m02896	TspO/MBR family protein	C41G7.3	Him, General Pace of Development abnormal, WT
14932.m00524	hypothetical protein	ZK1236.5	Clr, Gro, WT
14715.m01248	ATP synthase e chain, mitochondrial, putative	R04F11.2	Lva, Emb, Unclassified, WT, Gro
14704.m00455	TolA protein.-related (similar to sut-1, involved in trans-splicing)	T13B5.8	Emb, WT
13398.m00096	Mediator protein 4-related	*mdt-4*	Egl, Pch, Emb, Rup, WT
12701.m00057	40S ribosomal protein S12.-related	*rps-12*	Sck, WT
15131.m00094	RH01479p-related	Y95D11A.1	Unclassified, Larval Arrest-Late (L3/L4), WT, Stp, Gro
15213.m00007	Troponin T, putative	*tnt-2*	Slu, Ste, Unc, Stp, Gro, WT
14977.m04949	hypothetical 36.5 kDa protein C56G2.3 in chromosome III.-related	C56G2.3	Stp, Gro, WT
14396.m00009	conserved hypothetical protein	*pqn-22*	Emb, WT

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	RNAi phenotype in *C. elegans**
14318.m00072	vacuolar ATP synthase subunit H, putative	*vha-17*	Osmotic Integrity defective, Lva, Sck, Emb, Lvl, Ste
14973.m02604	conserved hypothetical protein	*pqn-38*	abnormal, Emb, Unc, Rup, Gro, WT
14992.m11195	hypothetical protein	pbs-5	Lva, Ocs, Emb, Passage Through Meiosis defective, Unc, Ste
13066.m00231	hypothetical protein	H35B03.2	Lva, Unclassified, Larval Arrest-Late (L3/L4), Stp, Gro
14972.m07569	conserved hypothetical protein	B0310.1	Unclassified, WT
14972.m07157	hypothetical protein	*hmg-5*	WT, Stp
14961.m04944	conserved hypothetical protein	C15C7.5	Clr, Unclassified, Unc, WT, Gro
14094.m00132	hypothetical protein	F23H11.2	Ste, Sma, WT, Stp
15304.m00111	major sperm protein, putative	*ssp-16*, *ssp-19*	Unclassified, WT
15076.m00116	hypothetical protein	F09E5.11	Prz, Clr, Lvl, Unc, Lva, Larval Arrest-Early (L1/L2), Gro, WT
14924.m00113	hypothetical protein	C26B9.3	Egl, Pvl, Unc, WT
14961.m05319	hypothetical protein	K12H4.5	Lva, Emb, WT, Larval Lethal-Early (L1/L2), Gro
14972.m06948	Resistance to inhibitors of cholinesterase protein 3-related, putative	*ric-3*	Lva, Emb, Unclassified, WT
14972.m07421	conserved hypothetical protein	*tnt-4*	Larval Arrest-Early (L1/L2), WT
14980.m02723	F-box domain containing protein	K03H1.11	Emb, WT

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	RNAi phenotype in *C. elegans**
14961.m05112	hypothetical protein	Y57A10A.27	Pch, Unclassified, Lvl, Lva, Sck, Stp, Gro, WT
14972.m07478	Mitochondrial ATP synthase g subunit family protein	*asg-1*	Bmd, General Pace of Development abnormal, Emb, Etv, WT, Stp, Gro
14975.m04488	Conserve Hypothetical protein	Y54E10A.7	Lva, Emb, WT, Gro
15560.m00010	conserved hypothetical protein	ZK930.3	Egl, Pvl, Unc, WT
15295.m00028	NADH-ubiquinone oxidoreductase B12 subunit.-related	C18E9.4	Lva, Emb, WT, Gro
15081.m00162	cytochrome-c oxidase, putative	Y71H2AM.5	Lva, Clr, Emb, WT, Stp, Gro
14971.m02895	conserved hypothetical protein	*smn-1*	Emb, Unclassified, Lvl, Unc, Lva, Sck, WT, Stp, Gro
13322.m00190	Peroxin-3 family protein	C15H9.10	General Pace of Development abnormal, Larval Arrest-Early (L1/L2), WT
14961.m05035	hypothetical protein	C29H12.6	Unclassified, WT
14230.m00222	hypothetical protein	F01F1.3	Emb, WT
14975.m04471	hypothetical protein	*mut-7*	Lva, WT
14990.m08152	hypothetical UPF0172 protein CG3501.-related	F25H2.4	Clr, Emb, Unclassified, Unc, Etv, Complex Phenotype, Lva, Gro, Stp, WT
14968.m01521	Succinate dehydrogenase, putative	F33A8.5	Lva, Clr, Emb, WT, Gro
14961.m05325	RE35789p-related	F33D4.7	Lva, Emb, Unclassified, WT
14961.m05095	LAMP family protein lmp-1 precursor.-related	*lmp-1*	Clr, WT

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	RNAi phenotype in *C. elegans**
14961.m05267	Ubiquinol-cytochrome C chaperone family protein	C35D10.5	Emb, Larval Lethal-Early (L1/L2), WT, Gro
14643.m00073	hypothetical protein	*snr-5*	Lva, Sck, Emb, Lvl, Ste, WT, Larval Lethal-Early (L1/L2)
14944.m00552	Cytochrome c oxidase polypeptide Vb, mitochondrial precursor.-related	*cco-1*	Age, Clr, Emb, Unclassified, Lpd, Ste, Lva, Gro, WT
14974.m00848	NADH-ubiquinone oxidoreductase 18 kDa subunit, mitochondrial precursor.-related	*lpd-5*	Lva, Emb, WT
14972.m07819	conserved hypothetical protein	C39E9.11	Emb, Unc, WT
14975.m04466	DNA segment, Chr 7, Wayne State University 180, expressed, putative	*crn-5*	Lva, Pch, Larval Arrest-Early, (L1/L2), WT, Gro
14990.m08061	zgc:101594-related	Y47G6A.9	Lva, Clr, Pvl, Rup, WT, Stp, Gro
14097.m00079	Ubiquinol-cytochrome C reductase hinge protein	T27E9.2	Gro, WT
14981.m02398	protein T01B7.5, putative	T01B7.5	Egl, Pch, Ste, Gon, Lva, Gro, Stp, WT
14980.m02753	Conserved hypothetical protein	D2007.4	Lva, Emb, Larval Lethal-Early (L1/L2), WT, Gro
14972.m07286	hypothetical protein	ZK792.5	Unclassified, Stp, WT
14990.m07875	putative transcription factor	*tag-313*	Unclassified, WT, Gro
12698.m00331	Bm1_02520, 1300013D05Rik protein-related	F52C9.7	Dpy, Sle, Emb, WT
14954.m01709	Zinc finger, C2H2 type family protein	*lir-1*	Emb, Mlt, Lvl, Unc, WT

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	RNAi phenotype in *C. elegans**
14832.m00025	UPF0279 protein C14orf129 homolog.-related	T24H7.3	Emb, WT
14961.m05175	CG32584-PB.-related	ZK1248.11	Unclassified, WT
12823.m00025	LD15209p, putative	C13F10.7	Emb, WT
14250.m00299	conserved hypothetical protein	F37C12.2	Lva, Emb, WT
14977.m04900	membrane-associated RING-CH protein III, putative	C17E4.3	Emb, WT
13066.m00250	DNA polymerase epsilon p17 subunit, putative	T26A5.8	Pnm, Emb, WT

***Abbreviations of RNAi phenotypes (alphabetical):** Adl: adult lethal; Age: life span abnormal; aldicarb_resistant: aldicarb response abnormal; Bli: blistered; Bmd: body morphology defect; Cyk: cytokinesis defect; Emb: embrionic lethal; Clr: clear; Dpy: dumpy; Egl: egg laying defective; Emb: embrionic lethal; Etv: embrionic terminal arrest; Fgc: fewer germ cells; Gon: abnormality in gonad arms; Gro: slow growth; Him: high incidence male progeny; Led: late embryo defect; Lva: larval arrest; Lvl: larval lethal; Mlt: moult defect; Ocs: one-cell arrest; Pch: patchy coloration; Pnm: pronuclear migration alteration in early embryo; Prz: paralyzed; Rup: ruptured; Sck: sick; Sle: slow embrionic development; Slu: sluggish; Sma: small; Ste: sterile; Stp: sterile progeny; Unc: uncoordinated; WT: wild type.

2. Characterization of the groups of *B. malayi* mf genes that could change their expression from doxycycline-treated microfilariae

2.1 Selected gene expression profile from doxycycline-treated microfilariae

Doxycycline at the concentration of 20 ug/ml had the effect on mf motility at 13 h after treatment. The motility showed active and motile (score 2). One gene, alt-1 protein (BMC11647), was significantly up-regulated (7 fold, P = 0.000038) at this time point. In contrast, mf motility at 36 h after treatment showed slightly active (score 1). One gene, WD-repeat protein BING4 (13253.m00071), was significantly down-regulated (4.50 fold, P = 0.0000014). Doxycycline at this concentration completely inhibited mf motility (score 0) at 61 h. At this time point, 57 (0.31%) *B. malayi* mf genes were up-regulated whereas 2,164 (11.92%) genes were down-regulated compared with untreated control (Figure 4.7). The degree of differential expression ranged from 2.21-22.16 fold increases and 2.01-298.50 fold decreases. A group of heat shock proteins (*hsp*) genes such as small *hsp*, *hsp*20, *hsp*70 and *hsp*90 were up-regulated and a group of genes responsible for the oxidative phosphorylation pathway such as cytochrome b, cytochrome c oxidase (subunit I, II and III), NADH dehydrogenase (subunit 1-6), and ATPase subunit 6 were down-regulated (Table 4.6).

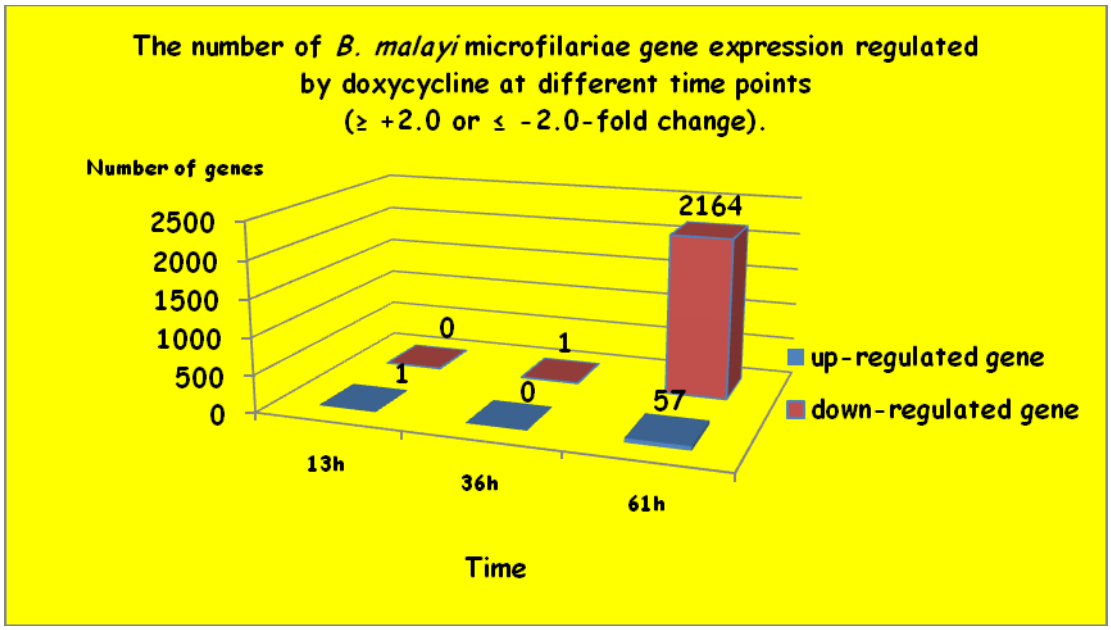


Figure 4.7 **The number of genes expression at different time points for *B. malayi* mf**

Table 4.6 Genes with significant differences ($P < 0.05$ and ≥2–fold change) at 61 h post treatment

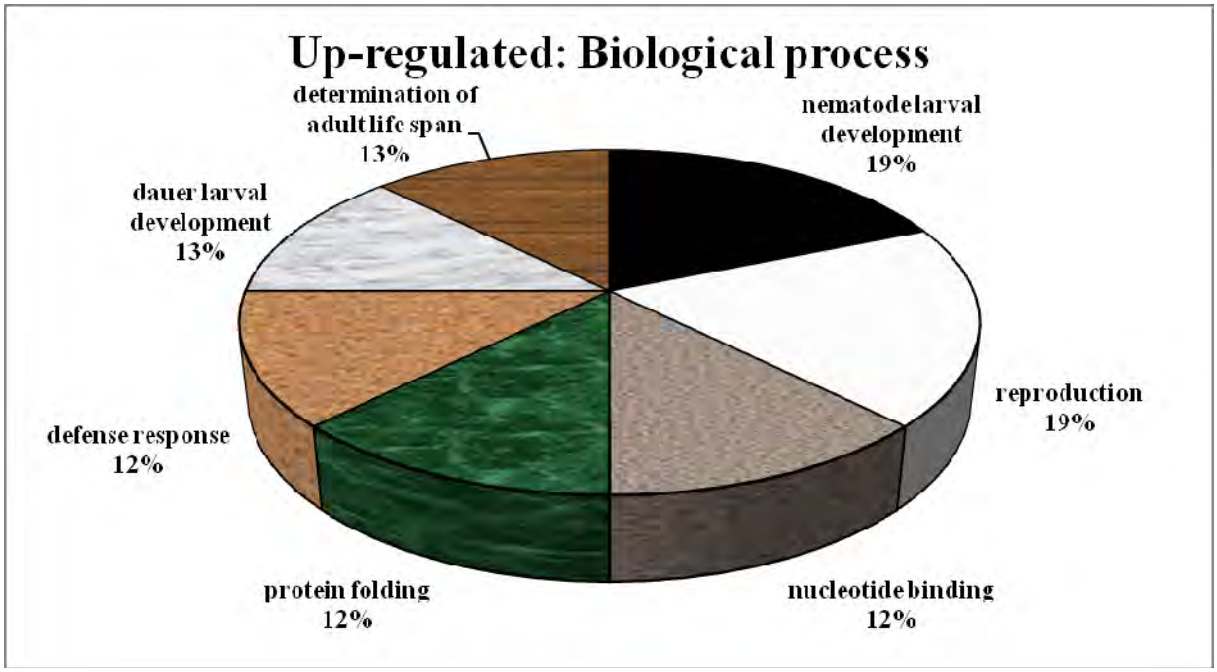
Oligo ID	Product (s)	Fold change
Protein folding		
15334.m00009	Small heat shock protein	17.24
13293.m00125	Hsp20/alpha crystallin family protein	13.98
BMC03771	Small heat shock protein	12.84
BMC05057	Heat shock protein 90	6.79
BMC04926	Heat shock protein 90	5.72
BMC12102	Heat shock protein 70	5.11
BMC00470	Heat shock protein 70	4.49
WB-contig_1184	Heat shock protein 70	2.97
BMC04991	Chaperone protein	2.92
Protein synthesis		
TC2753	28S ribosomal RNA	6.35
BMC00161	Ribosomal protein S18	-2.12
TC3380	Eukaryotic translation initiation factor 3 subunit 4	-2.16
BMC00179	60S ribosomal protein L35	-2.34
BMC11648	40S ribosomal protein S13	-2.39
14981.m02412	40S ribosomal protein S23	-2.42
TC3032	40S ribosomal protein S23	-2.73
14039.m00115	60S ribosomal protein L3	-2.99
14947.m01136	Ribosomal protein L14p/L23e	-4.07
WB-contig_251	Mitochondrial 40s ribosomal protein	-4.70
BMC12216	40S ribosomal protein S19	-4.81
BMC12245	Ribosomal protein L32	-4.93
14972.m07725	40S ribosomal protein S2	-4.94
BMC08806	Mitochondrial ribosomal protein L14	-4.95
15178.m00023	Ribosomal protein L36	-5.20

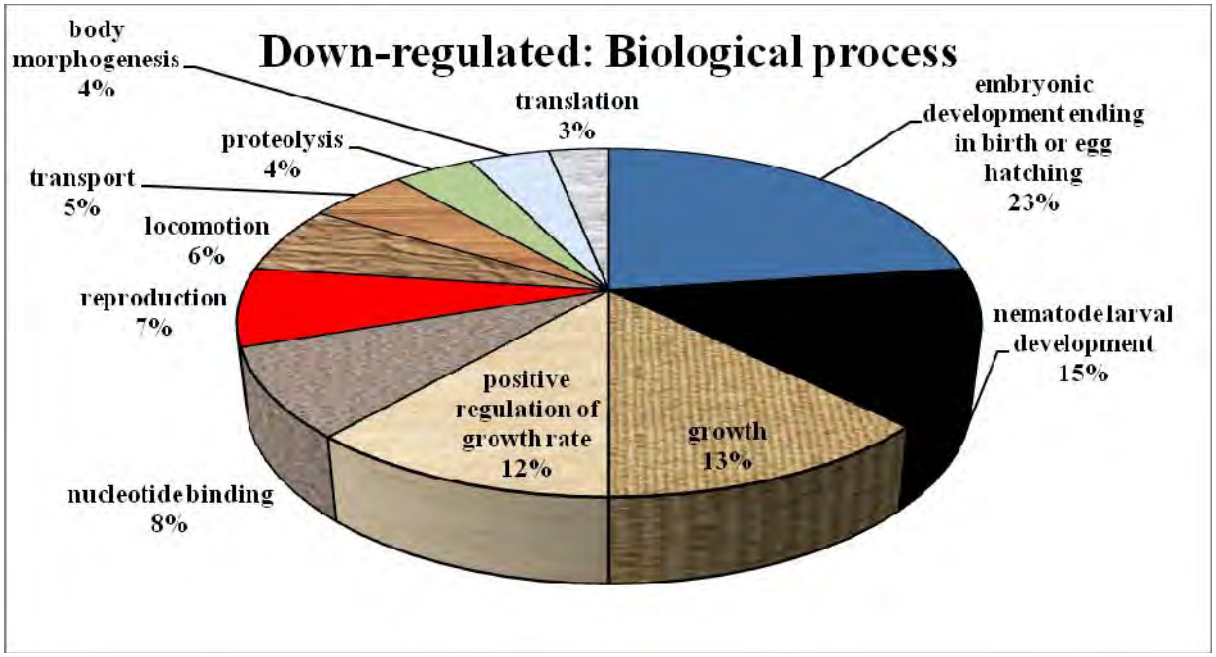
BMC02342	Ribosomal protein L36	-6.06
BMC01157	60S ribosomal protein L28	-6.32
15443.m00042	50S ribosomal protein L10	-6.46
TC3633	18S small subunit ribosomal RNA	-6.82
TC3733	60S ribosomal protein L3	-7.05
14961.m05081	Mitochondrial ribosomal protein L23	-7.15
WB-contig_499	60S ribosomal protein L31	-7.36
BMC06468	Ribosomal protein L4	-7.58
BMC03821	39S ribosomal protein L35	-8.45
14920.m00434	39S ribosomal protein L35	-9.81
BMC12161	60S ribosomal protein L10	-10.65
BMC03199	60S ribosomal protein L27	-17.84
WB-contig_491	40S ribosomal protein S13	-52.59
Oxidative phosphorylation		
BMC06066	Cytochrome c oxidase	-6.20
BMC06398	Cytochrome c oxidase	-6.74
BMC00097	Cytochrome c oxidase subunit I	-29.72
BMC00071	Cytochrome c oxidase subunit II	-35.75
BMC12116	Cytochrome c oxidase subunit II	-37.37
BMC04259	NADH dehydrogenase subunit 4L	-37.57
BMC01168	NADH dehydrogenase subunit 5	-38.65
BMC00340	Cytochrome c oxidase subunit I	-40.55
BMC12112	Cytochrome c oxidase subunit I	-44.86
BMC12343	NADH dehydrogenase subunit 1	-56.79
BMC11631	Cytochrome b oxidase	-57.65
WB-contig_383	NADH dehydrogenase subunit 4	-59.57
BMC11624	Cytochrome b oxidase	-60.15
BMC06165	Cytochrome c oxidase subunit I	-60.52
BMC12169	Cytochrome c oxidase subunit 1	-62.85
BMC00030	Cytochrome c oxidase subunit II	-66.82
BMC03215	NADH dehydrogenase subunit 5	-70.97
TC2857	NADH dehydrogenase subunit 4	-73.26

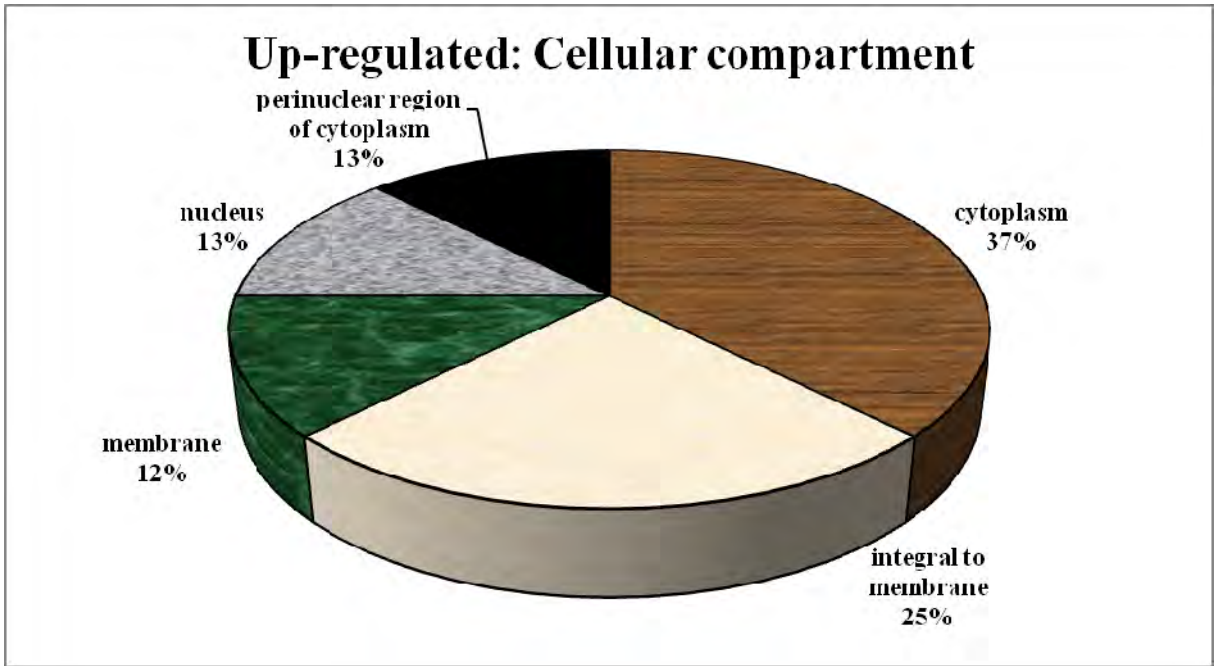
BMC05926	NADH dehydrogenase subunit 5	-74.87
BMC11474	Cytochrome c oxidase subunit 1	-76.05
BMC03890	ATPase subunit 6	-77.76
BMC12111	Cytochrome c oxidase subunit I	-79.38
BMC11037	NADH dehydrogenase subunit 3	-80.98
BMC05255	NADH dehydrogenase subunit 6	-86.98
BMC12392	NADH dehydrogenase subunit 5	-88.36
WB-contig_275	NADH dehydrogenase subunit 3	-93.20
BMC01714	NADH dehydrogenase subunit 4	-95.70
TC2809	ATPase subunit 6	-98.68
BMC00796	NADH dehydrogenase subunit 3	-101.90
BMC12081	NADH dehydrogenase subunit 6	-114.86
BMC12077	ATPase subunit 6	-115.33
BMC06774	NADH dehydrogenase subunit 1	-126.24
BMC12349	NADH dehydrogenase subunit 3	-138.84
BMC02280	NADH dehydrogenase subunit 1	-180.55
BMC00745	NADH dehydrogenase subunit 1	-181.02
BMC08859	NADH dehydrogenase subunit 1	-209.02
BMC12082	NADH dehydrogenase subunit 6	-216.30
BMC00485	NADH dehydrogenase subunit 2	-232.23

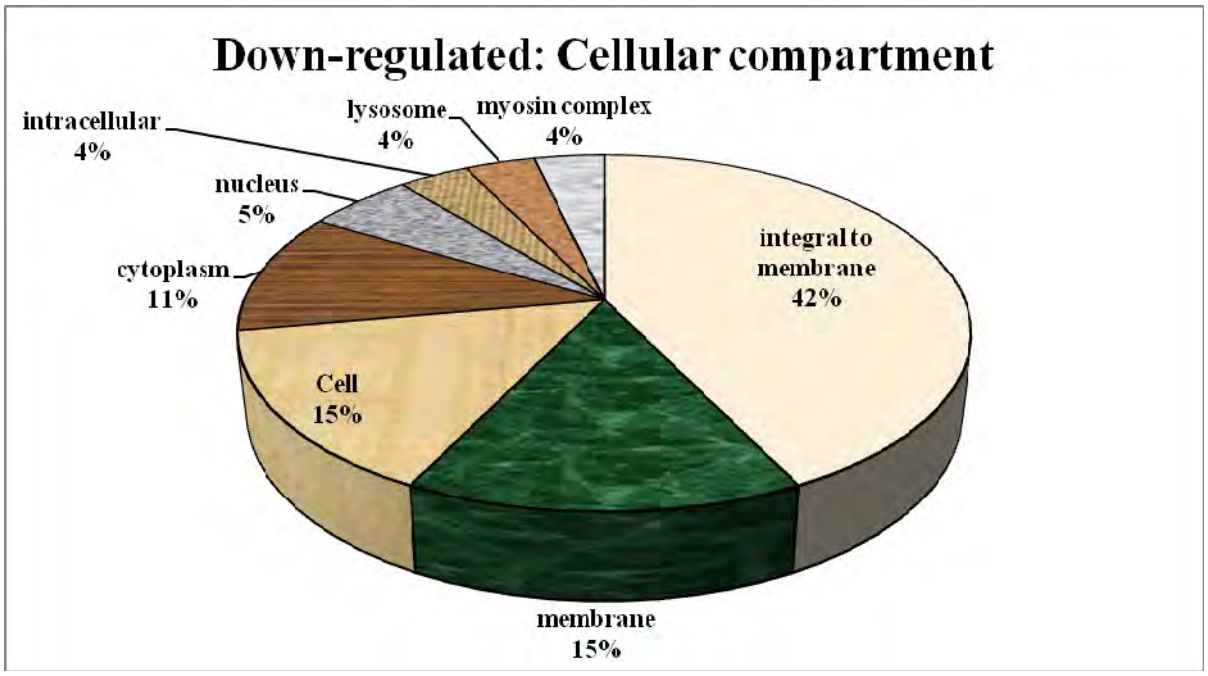
2.2 Gene ontology of up- and down-regulated doxycycline-treated microfilariae

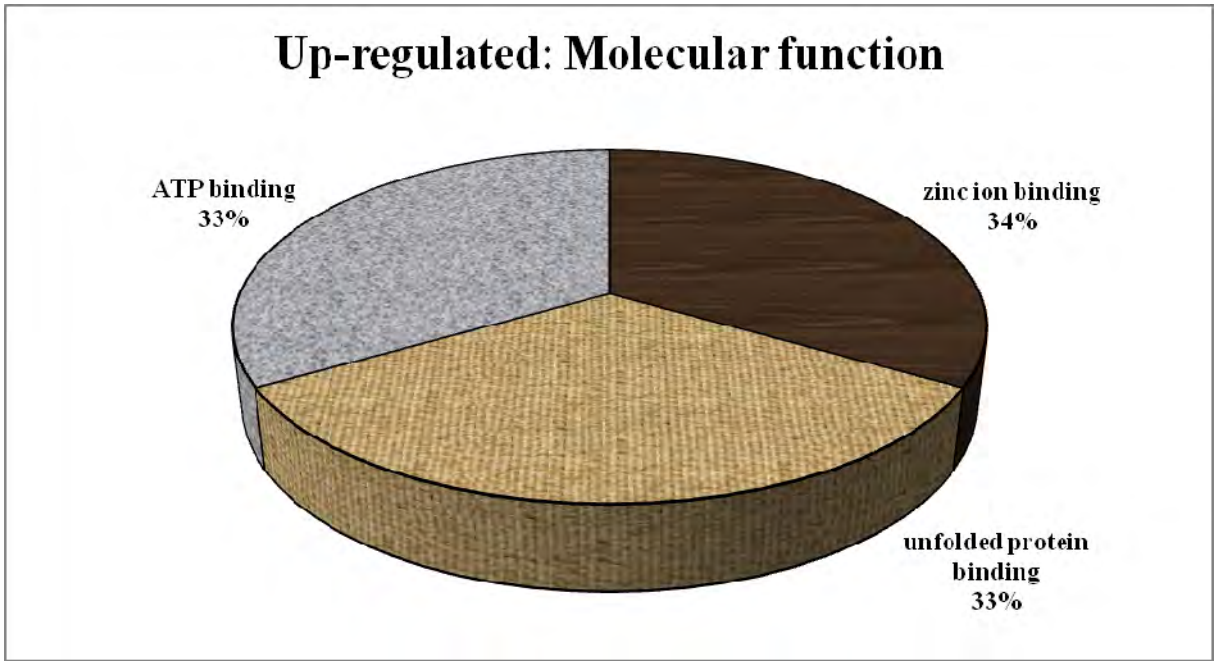
The percentages of these up-regulated genes at 61 h that were classified according to the gene ontology are expressed as pie charts in Figure 4.8. The main biological processes of doxycycline-treated *B. malayi* mf include nematode larval development and reproduction whereas the main molecular function of drug-treated mf include unfold protein binding, zinc ion binding, and ATP binding.


Up-regulated: Biological process
determination of adult life span 13%
nematode larval development 19%
dauer larval development 13%
reproduction 19%
defense response 12%
protein folding 12%
nucleotide binding 12%


Down-regulated: Biological process
body morphogenesis 4%
translation 3%
embryonic development ending in birth or egg hatching 23%
proteolysis 4%
transport 5%
locomotion 6%
reproduction 7%
nematode larval development 15%
positive regulation of growth rate 12%
growth 13%
nucleotide binding 8%


Up-regulated: Cellular compartment
perinuclear region of cytoplasm 13%
nucleus 13%
cytoplasm 37%
membrane 12%
integral to membrane 25%


Down-regulated: Cellular compartment
intracellular 4%
lysosome 4%
myosin complex 4%
nucleus 5%
cytoplasm 11%
integral to membrane 42%
Cell 15%
membrane 15%



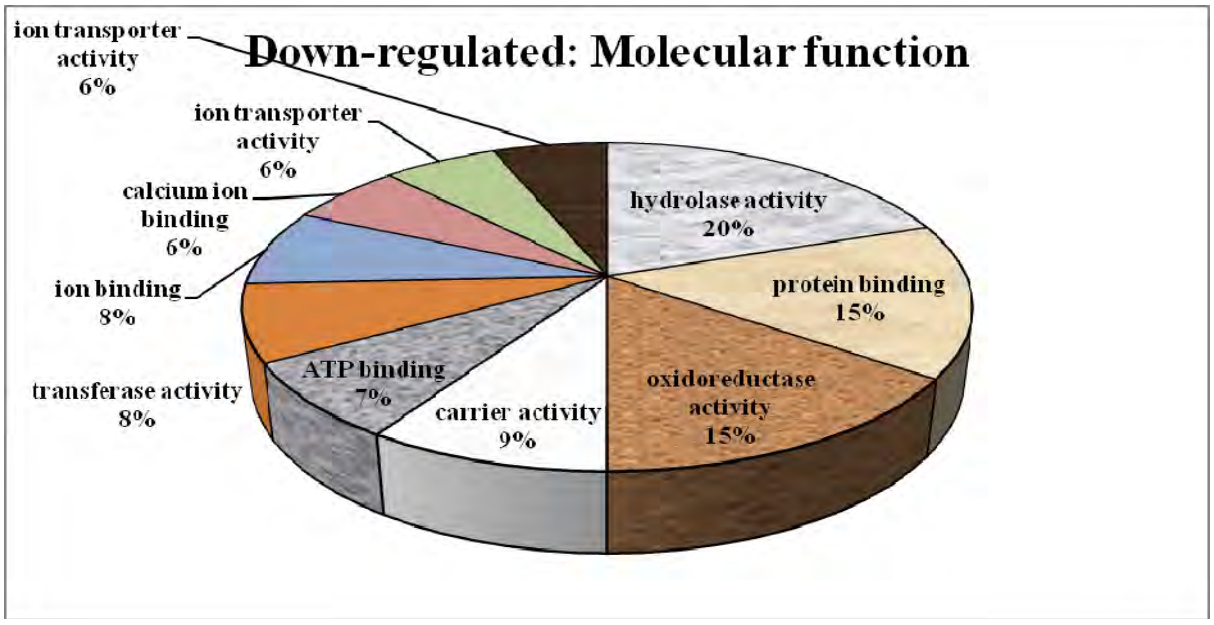


Figure 4.8 **Gene ontology including biological process (A and B), cellular compartment (C and D, and molecular function (E and F), of *B. malayi* mf genes regulated upon doxycycline exposure**

2.3 Major down-regulated genes for doxycycline treated *B. malayi* mf ES proteins

The up- and down-regulated *B. malayi* mf genes obtained from this study were also analyzed with the stage-specific mf ES proteins lists published by Moreno and Geary (2008) and Bennuru et al. (2009). The degree of differential down-regulated expressions ranged from 2.30 fold to 34.57 fold (Table 4.7). For example, the exonuclease family protein (14992.m11178; 2.59 fold), endochitinase (14932.m00512; 5.26 fold, 13269.m00308; 6.57 fold, 14932.m00514; 6.74 fold), neurotransmitter-gated ion-channel transmembrane region family protein (14880.m00012; 7.55 fold), macrophage migration inhibitory factor 2 (14250.m00294; 8.25 fold), serpin (14777.m00108; 19.36 fold), and phosphatidylethanolamine-binding protein (14956.m00481; 34.57 fold).

Table 4.7 Major down-regulated genes for doxycycline treated *B. malayi* mf ES proteins and RNAi phenotypes of *C. elegans* homologues

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	Fold change	RNAi phenotype in *C. elegans**
13717.m00172	Hypothetical protein	F56H1.5	- 2.30	WT
14979.m04495	HT014	ZK353.9	- 2.35	WT
14981.m02382	Gaba	C32D5.9	- 2.39	Emb
14961.m05094	sf3a2-prov protein	F11A10.2	- 2.39	Lva, Emb
15261.m00022	RIKEN cDNA 2810439K08-related	K02G10.3	- 2.40	WT
12950.m00042	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial precursor	F56D12.1a	- 2.56	Slu, Unc, Stp, Gro
14956.m00512	Hypothetical protein	Y67A10A.7	- 2.58	WT
14992.m11178	Exonuclease family protein	C05C8.5	- 2.59	not_Emb
14961.m05180	Utp21 specific WD40 associated domain containing protein	Y45F10D.7	- 2.65	Emb Larval
14294.m00134	Hypothetical 55.2 kDa Trp-Asp repeats containing protein T10F2.4 in chromosome III	T10F2.4	- 2.96	Unclassified Ste, Rup, Bli, Pvl, Emb
15080.m00105	Met-10+ like-protein	C53A5.2	- 3.19	WT

Oligo ID	*B. malayi* gene description	*C. elegans* homolog	Fold change	RNAi phenotype in *C. elegans**
13133.m00072	Hypothetical protein	T04A8.4	- 3.25	WT
14972.m07280	Initiation factor 2-associated protein	Y116A8A.9	- 3.41	Emb, Unclassified Stp, Gro, Fgc, Gon
14968.m01463	Hypothetical protein	C39E9.8b	- 4.33	WT
14979.m04397	PAN domain containing protein	B0361.9	- 4.53	WT
14932.m00512	Endochitinase	C04F6.3	- 5.26	Emb
14963.m01803	Kinesin motor domain containing protein	K12F2.2a	- 5.31	WT
13843.m00008	Hypothetical protein	Y9C9A.16	- 5.87	WT
13269.m00308	Endochitinase	C04F6.3	- 6.57	Emb
14932.m00514	Endochitinase	C04F6.3	- 6.74	Emb
14880.m00012	Neurotransmitter-gated ion-channel transmembrane region family protein	F09G2.5	- 7.55	WT
14250.m00294	macrophage migration inhibitory factor 2	C52E4.2	- 8.25	WT
14972.m07327	Hypothetical protein	Y73F8A.6	- 19.04	WT
14777.m00108	Serpin, putative	C05E4.1	- 19.36	WT
14956.m00481	Phosphatidylethanolamine-binding protein	Y69E1A.5	- 34.57	WT

[*]**Abbreviations of RNAi phenotypes (alphabetical):** Adl: adult lethal; Age: life span abnormal; aldicarb_resistant: aldicarb response abnormal; Bli: blistered; Bmd: body morphology defect; Cyk: cytokinesis defect; Emb: embrionic lethal; Clr: clear; Dpy: dumpy; Egl: egg laying defective; Emb: embrionic lethal; Etv: embrionic terminal arrest; Fgc: fewer germ cells; Gon: abnormality in gonad arms; Gro: slow growth; Him: high incidence male progeny; Led: late embryo defect; Lva: larval arrest; Lvl: larval lethal; Mlt: moult defect; Ocs: one-cell arrest; Pch: patchy coloration; Pnm: pronuclear migration alteration in early embryo; Prz: paralyzed; Rup: ruptured; Sck: sick; Sle: slow embrionic development; Slu: sluggish; Sma: small; Ste: sterile; Stp: sterile progeny; Unc: uncoordinated; WT: wild type.

CHAPTER V

DISCUSSION AND CONCLUSION

Part I: The effects of anti-rickettsial drugs on *B. malayi* mf determined by the minimum effective concentration (MEC) and on *Wolbachia* determined by the minimum inhibitory concentration (MIC)

Wolbachia endosymbiont of *Brugia malayi* microfilarial susceptibility to doxycycline

We investigated the effects of antimicrobial agents on the motility of *B. malayi* mf and its correlation to *Wolbachia* susceptibility. The MEC of antimicrobial agents, which is neccessary to disrupt microfilarial motility which represent for mf viability, was used to compare the efficacies of different anti-rickettsial agents. Our findings support earlier studies in that doxycycline is the most effective anti-rickettsial agent compared with rifampicin and ciprofloxacin (Rao and Weil 2002; Mahajan et al. 2010).

The *Wolbachia*/mf DNA, obtained from qPCR, can be used to investigate the presence of *Wolbachia* in filarial nematodes. The single copy genes of *Wolbachia* (*wsp* and *ftsz*) and *B. malayi* (*gst*, *tub-1* and *ama-1*) are used to study the population dynamic of *Wolbachia* in all stages of *B. malayi* life cycle. The entire *Wolbachia*/*B.*

malayi gene ratios obtained from these studies represented the similar pattern (Fenn and Blaxter 2004b; McGarry et al. 2004). Furthermore, the *Wolbachia*/nematode ratios are used in the study of adult *Dirofilaria immitis* treated with the combination of ivermectin and doxycycline, and the differentiation in severe and mild strains of *Onchocerca volvulus* (Bazzocchi et al. 2008; Higazi et al. 2005). The ratio is a beneficial indicator of changes in *Wolbachia* population following drug exposure. Recently, the expression of *Wolbachia wsp* following treatment of *B. malayi* mf with ivermectin or moxidectin has been investigated using qRT-PCR (Tompkins et al. 2009). The only expression of *wsp* might not represent the drug's effect on mf. Here, we first investigated the *Wolbachia* MIC using *Wolbachia wsp* and *B. malayi* mf *hsp70* single copy genes by a SYBR green qPCR technique.

Untreated *B. malayi* mf gene (*Wolbachia*/nematode, *wsp*/*hsp70*) ratios obtained from this study (0.37, at 12 h), were in agreement with the previous *B. malayi* mf DNA ratio report (0.19-1.03) (McGarry et al. 2004). Our results showed that the non-drug treated mf gene ratio decreased slowly from DNA ratio 0.37 to 0.17, at 12 to 52 h, respectively. *Wolbachia* may be released from mf when certain untreated mf die in the culture system, and/or *Wolbachia* may pass through the excretory canal located at the mf lateral chord (Kozek 2005). Our experiments demonstrated that *Wolbachia* DNA was not detected in the culture supernatant, presumably due to the very low amount of DNA (data not shown) and the degradation of *Wolbachia* DNA in the culture media. This phenomenon is also observed when 0.25×10^6 mf were cultured and *Wolbachia* gene could not be amplified from the culture media (Bennuru et al. 2009a).

The success of qPCR methods for susceptibility testing has previously been reported in *Wolbachia* in insect cell culture, dog heartworm (*D. immitis*), and intracellular-related *Wolbachia* (e.g., *Chlamydia trachomatis*, *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma phagocytophilum*, and *Coxiella burnetii*) (Rolain et al. 2002; Brennan and Samuel 2003; Fenollar et al. 2003; Boulos et al. 2004; Branger et al. 2004; Hunfeld et al. 2004; Storm et al. 2005; Bazzocchi et al. 2008). Recently, *wsp* gene expression has been investigated after *B. malayi* mf and adults were treated with ivermectin or moxidectin using quantitative RT-PCR. (Tompkins et al. 2009). In this study, with a limited number of mf, we demonstrated a DNA copy number ratio using a SYBR green qPCR assay, to determine the susceptibility of *Wolbachia* to antimicrobial agents.

Studies of antimicrobial susceptibility in filarial *Wolbachia* have been limited because there is no long-term filarial cell culture system available (Higazi et al. 2004). Recently, insect *Wolbachia* drug susceptibility has been investigated, by using immunofluorescense antibody and qPCR methods, after *Wolbachia*-infected cells were incubated with 13 antimicrobial agents for 6 days. Both assays show similar results; doxycycline and rifampicin are the most effective drugs, with MICs of 0.125 and 0.06-0.125 μg/ml after 6 days of treatment, respectively (Fenollar et al. 2003). We showed that doxycycline had the efficacy with the MIC of 128 μg/ml at 12 h. Antimicrobial drugs may pass through *Wolbachia*-infected cell culture more readily than *Wolbachia* located in the mf hypodermal cell (Rao and Weil 2002). In addition, we could not maintain the mf until day 6 because the presence of no-drug treated *Wolbachia*/nematode DNA ratio decreased to less than 0.19 when the mf were cultured for 52 h. Moreover, our study demonstrated that the DNA ratio obtained from ciprofloxacin-treated mf increased at 12 h. Ciprofloxacin might fail to inhibit

Wolbachia growth, which supported the previous study of this antimicrobial agent on *B. malayi* mf and third-stage larvae (Smith and Rajan 2000; Mahajan et al. 2010).

Antimicrobial agents affect the motility of mf phenotypes directly or indirectly. Bacteriostatic drugs that are inhibitors of bacterial and eukaryotic mitochondrial translation, such as doxycycline and chloramphenicol, can directly impair energy generation through the mitochondrial respiratory chain and lead to a *Caenorhabditis elegans*, a free- living nematode, mobility defect (Tsang et al. 2001). *Wolbachia* depletion in onchocerciasis patients treated with 100 mg/day doxycycline for 6 weeks might have caused indirect effects, on *Onchocerca volvulus*, resulting in the up-regulation of worm mitochondrial *hsp60* gene (Pfarr et al. 2008). In accessing the status of microfilarial viability, we did not use the MTT assay that correlated with the mitochondrial succinate dehydrogenase enzyme. Since *Wolbachia* also contains succinate dehydrogenase gene, the MTT results obtained from antimicrobial agents-treated mf containing *Wolbachia* would lead to a misinterpretation of its viability (Townson et al. 2006).

Part II: Characterization of the groups of *B. malayi* mf genes that could change their expression from untreated mf compared with doxycycline-treated mf cultured *in vitro*

Microfilarial-stage specific gene expression profile of *Brugia malayi*

To study the expression of gene, especially more than thousand of genes simultaneously, the high throughput molecular techniques such as serial analysis of gene expression (SAGE) and microarray have been developed (Velculescu et al. 1995; Schena et al. 1995). The one-color microarray analysis used in this study is simplicity and adaptability (Patterson et al. 2006). Here, we used the *B. malayi* V2 filarial array slides containing 18,104 oligonucleotides in duplicate that cover ~85% (15,412) of the genes in this species for studying the mf biological pathways *in vitro*. About 15% of the remainder oligonucleotides contain expressed genes and predicted ORFs from *Onchocerca volvulus*, *Wuchereria bancrofti*, and *Wolbachia*.

Carbohydrate, energy generation, and cell motility metabolisms are the major metabolic pathways in *B. malayi* mf. In this study, succinate dehydrogenase (14968.m01521) and malate dehydrogenase (13478.m00071) enzymes that are responsible for TCA cycle, showed significantly higher expression (122.40 and 73.97 fold, respectively), compared with glycolysis enzymes such as pyruvate kinase (BMC03213; 37.52 fold). Our findings were in agreement with the previous observations in *B. pahangi* mf that was cultured in an *in vitro* anaerobic atmosphere. By this condition, the mf survival is depending on the homolactate fermentation (Rew and Saz 1977; Srivastava et al. 1988). The mf of *B. pahangi* use aerobic energy generation for their motility whereas in anaerobic fermentation, mf decrease glucose utilization leading to decrease the motility (Rew and Saz 1977). In addition, the

motility mf of *B. malayi*, cultured in the anaerobic condition (Sungpradit et al. unpublished data), were reduced in the following week.

The gene expressions of mf-specific ES proteins obtained from this study were also observed in the previous data (Bennuru et al. 2009), for example, the exonuclease family genes, homeobox domain containing proteins, and phosphatidylethanolamine-binding proteins. Exonucleases are responsible for biological processes, such as replication, recombination, repair, turnover, processing, and stability (Moser et al. 1997). Exonuclease family proteins (13480.m00174, 13480.m00174, 14975.m04472, 15443.m00045, 13156.m00092, 14208.m00923, 14977.m04896, and 14992.m11178) are identified from the adult *B. malayi*, (Ghedin et al. 2007) and one of the exonuclease family genes (14992.m11178, 56.07 fold) is significantly expressed. In *C. elegans*, the *eri-1* gene encodes a 3′-to-5′ exonuclease that is proved to be a negative regulator of RNA interference (RNAi) (Kennedy et al. 2004). The role of exonuclease family proteins in *B. malayi* mf may also be responsible for the 3′-to-5′ exonuclease activity.

Thirty homeobox-domains containing proteins are identified in the adult *B. malayi* (Ghedin et al. 2007). In our study, the expression of a homeobox-domain containing protein gene (14975.m04498; 14.93 fold) was obtained. Homeobox-containing genes are responsible for the regulation of developmental processes (e.g., regulation of cell proliferation, differentiation, adhesion, and migration) (Derelle et al. 2007). Three homeobox genes are isolated from the nematode *C. elegans*. The homeobox codes for a homeodomain, which is supposed to bind specifically DNA sequences through an a-helix-turn-a- helix motif (Schaller et al. 1990). Our finding was in agreement with the previous observation of the orthologs of *C. elegans*

homeobox genes that are also identified in six species from the Nematoda. For instance, *Bm-ant-1* and *Bm-egl-5* mRNAs are detected using quantitative RT-PCR in mf before entry into the mosquito vector *in vitro* (Aboobaker and Blaxter 2003). The role of these genes (*Bm-ant-1* and *Bm-egl-5*) in *B. malayi* mf may be involved in the regulation of transcription.

Phosphatidylethanolamine-binding proteins (PEBP) (14973.m02599, 14956.m00481, and 14990.m07662) are identified from the adult *B. malayi* (Ghedin et al. 2007). In the present study, we obtained the increase of the PEBP gene expression (14956.m00481, 13.83 fold). This protein is also found in the previous observation of the adult *B. malayi* and mf ES products (Hewitson et al. 2008; Hewitson et al. 2009). PEBP is a greatly conserved group of multifunctional proteins and it functions as a serine protease inhibitor (Hengst et al. 2001). PEBP homologues have been also identified in *Onchocerca volvulus* (Ov-16 Ovd1, and Ovd2), *Toxocara canis* (TES-26), Drosophila, *Plasmodium falciparum*, and *P. vivax* (Lobos 1990; Erttmann and Gallin 1996; Gems et al. 1995; Rautureau et al. 2009; Trottein and Cowman 1995; Arakaki et al. 2007).

Recently, *B. malayi* and *B. pahangi* mf gene expression have been studied *in vivo* (Kariuki et al. 2010). Interestingly, sixteen zinc fingers (C_2H_2 type family protein), responsible for the interaction of proteins with DNA, RNA, and other proteins of *B. malayi* mf, are identified, whereas in our *in vitro* study, these genes did not show a significant up-regulation. The low expression of this protein is also found in *B. pahangi* mf harvested from jird peritoneal wash (Kariuki et al. 2010). The differences between mf species and culture conditions (*in vivo* vs. *in vitro*) on the binding properties of C_2H_2 type family protein in mf should be considered.

Differential gene expression in *Brugia malayi* microfilaria following the treatment with doxycycline

The previous studies have shown that doxycycline at the concentration of 100 and 128 μg/ml could stop mf motility at 12 and 48 h post-treatment respectively (Rao and Weil, 2002). In our study, doxycycline at the concentration of 128 μg/ml reduced the *Wolbachia*/mf gene ratio significantly. Furthermore, the ultrastructural finding is shown that adult *B. malayi* exposed to 40 μg/ml tetracycline for three days (72 h) could reduce the endosymbiont, *Wolbachia*, from adult *B. malayi* hypodermis (Ghedin et al., 2009). Here, we used 20 μg/ml doxycycline, the concentration that caused different mf motility phenotype at 13 h (score 2), 36 h (score 1), and 61 h (score 0). This concentration should also reduce the *Wolbachia* bacteria located in the mf lateral chord.

In our study, a group of heat shock proteins (*hsp*) genes, responsible for protein folding and stress response, such as small *hsp*, *hsp*20, *hsp*70 and *hsp*90 were up-regulated. The previous report has shown that *Wolbachia* depletion in onchocerciasis patients treated with 100 mg/day doxycycline for 6 weeks might have caused indirect effects, by disrupting the homeostasis of *Wolbachia* and filarial worm, resulting in the up-regulation of worm mitochondrial *hsp60* gene (Pfarr et al., 2008). Moreover, doxycycline has no effect on the intracellular bacteria, *Tropheryma whipplei*, stress response when *T. whipplei* are treated with 0.5 and 5 mg/L of doxycycline *in vitro* (La et. al, 2007). Doxycycline used in this study may not directly cause the mf stress response.

The previous study has shown that *B. malayi* adult treated with tetracycline *in vivo*, cause up-regulated of genes involved in translational process such as ribosomal

proteins (40S ribosomal proteins S4 and S23 and 60S ribosomal proteins L3, L4, L5, L10, L14, L22, L24), the eukaryotic translation initiation factor eIF5A-2, and the alpha and gamma subunits of the translation elongation factor 1 (EF-1) (Ghedin et al., 2009). In contrast, our results showed that a number of 40S and 60S ribosomal proteins (except 28S ribosomal protein) and eukaryotic translation initiation factor 3 were down-regulated. In our experiment, mf were exposed to the higher doxycycline concentration (20 μg/ml) and directly *in vitro* compared with adult worm harvested from tetracycline-treated jirds (2.5 mg/ml in drinking water). Doxycycline may pass through mf cell easier than adult worm (Rao and Weil 2002). In addition, various derivatives of antibiotic drug, drug concentration, drug exposure time, and mf culture condition (*in vivo* vs. *in vitro*) may cause the different in gene expression profile.

The recent *in vivo* study has shown that tetracycline-treated *Litomosoides sigmodontis* mitochondria encoding subunit of respiratory chain complexes such as cytochrome *b*, cytochrome *c* oxidase subunit 1-3, NADH-dehydrogenase subunit 1-6, and ATPase subunit 6, are found to be up-regulated. It is hypothesized that *L. sigmodonis* attempts to compensate the nucleotide and energy metabolisms caused by *Wolbachia* deprivation (Strübing et al., 2010). In contrast, our results showed that a number of genes responsible for the oxidative phosphorylation pathway such as cytochrome *b*, cytochrome *c* oxidase (subunit I, II and III), NADH dehydrogenase (subunit 1-6), and ATPase subunit 6 were down-regulated. In our experiment, *B. malayi* mf were exposed to the higher doxycycline concentration (20 μg/ml) and directly *in vitro* for 61 hours compared with *L. sigmodonis* harvested from tetracycline-treated mice (50 mg/kg intraperitoneally). The short time and higher dose of doxycycline exposure may cause the different in mitochondrial gene expression profile.

In conclusion, we showed that doxycycline was the most effective anti-rickettsial agent for inhibiting mf motility and was effective against the *Wolbachia* endosymbiont organism. The MEC and MIC assays can be adapted to test the antifilarial and anti-*Wolbachia* agents for *in vitro* screening and to follow-up the lymphatic filariasis patients after treatment using qPCR assay.

Microarray analysis is a useful screening tool to determine stage-regulated gene expressions in *B. malayi* mf and related metabolic pathways. The mf-stage specific ES gene expressions, such as exonuclease family protein, homeodomain-containing protein, phosphatidylethanolamine-binding protein, endochitinase, serpin, and actin, support the previous *B. malayi* mf secretome data. The genes discovered in our study should assist research toward a better understanding of the basic molecular biology of *B. malayi* mf. The roles of these genes as a target for developing novel antifilarial drugs or vaccines should be verified.

Doxycycline alters *B. malayi* mf homeostasis through either a direct effect on the worm or an indirect effect on the parasite's endosymbiont. Important candidate genes related to protein folding, translational process, and mitochondrial oxidative phosphorylation have been proposed as possible future antifilarial drug targets. For the further study, the accurate fold change of *B. malayi* mf gene expression should be confirmed using qPCR.

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APPENDICES

APPENDIX A

RESEARCH INSTRUMENTS

Automatic adjustable micropipette (Eppendorf, Germany)

Balance (Precisa, Switzerland)

Beaker (Pyrex, USA)

DNA Thermal cycler 2400 (Perkin Elmer, Cetus USA)

Electrophoresis Chamber set (BIO-RAD, USA)

Flask (Pyrex, USA)

Genepix 4200AL Scanner (Axon Instruments, USA)

Microcentrifuge (Eppendorf, USA)

Stereo microscope (Nikon, Japan)

Multiwell plates (Becton Dickinson, USA)

Parafilm (American National Can, USA)

Pipette boy (Tecnomara, Switzerland)

Pipette tip (Axygen, USA)

Polypropylene conical tube (Elkay, USA)

pH meter (Eutech Cybernataics)

Reagent bottles (Duran, Germany)

Real-time PCR machine, ABI 7500 system (Applied Biosystems, USA)

RNA Nano LabChip (Agilent, USA)

Spectrophotometer (NanoDrop Technologies, USA)

Vortex (scientific Industry, USA)

APPENDIX B

RESEARCH REAGENTS

Absolute ethanol (Merck)

Acetic acid (Merck)

Agarose (USB, Spain)

Amphotericin B (Sigma-Aldrich, USA)

Chloroform (Merck)

Ciprofloxacin (Fluka BioChemika, Swithzerland)

Deoxynucleotide triphosphates (dNTPs) (Invitrogen, USA)

Disodium ethylenediamine tetraacetic acid: EDTA (Merck)

Doxycycline (Sigma-Aldrich, USA)

Ethidium bromide (Sigma-Aldrich, USA)

Glucose (Merck)

Glycerol (Phamacia Amersham)

Hydrochloric acid (Merck)

Lysing Matrix D (MP Biomedicals, USA)

Oligonucleotide primer (Invitrogen, USA)

Penicillin/streptomycin (Sigma-Aldrich, USA)

Phenol-chloroform-isoamyl alcohol (Invitrogen, USA)

Proteinase K (Phamacia Amersham)

Rifampicin (Sigma-Aldrich, USA)

RNA ampUSLe: amplification and labeling Kit (KREATECH Biotechnology, The Netherlands)

RPMI-1640 (Gibco BRL)

Sodium acetate (Merck)

Sodium hydroxide (Merck)

SYBR green I PCR master mix (Applied Biosystems, USA)

Taq DNA polymerase (Invitrogen, USA)

Trizol (Invitrogen, USA)

100 bp DNA ladder (NEB, USA)

BIOGRAPHY

Name: Sivapong Sungpradit **Gender:** Male

Date of Birth: November 6, 1975 **Place of Birth:** Bangkok, Thailand

Address: Department of Pre-clinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University

Education and professional training:

Year	Degree/ Position	Institution
2004-2010	Doctor of Philosophy (Biomedical Science)	Chulalongkorn University, Thailand
2007-2008	Guest Researcher	The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University
2000-2003	Master Degree in Immunology	Mahidol University, Thailand
1992-1997	Bachelor Degree in Doctor of Veterinary Medicine (Second Class Honors)	Kasetsart University, Thailand

Awards and Scholarships:

2009	Keystone Symposia Travel grant Awards
2009	American Society of Tropical Medicine and Hygiene Graduate Students Travel grant Awards
2000 and 2004	University Development Commission (UDC) grant

Publications:

- **Sungpradit S**, Nuchprayoon S, Chatsuwan T. 2010. Cost-effectiveness analysis of alkaline lysis, MagNA Pure, and phenol-chloroform DNA extraction methods followed by measurement of single gene copy number using quantitative real-time PCR for *Dirofilaria immitis* microfilaria. Chulalongkorn Medical Journal. 54(6): 549-561.
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