

EFFICACY OF TETRACYCLINE AND ENROFLOXACIN AGAINST *WOLBACHIA* SPP. IN
MICROFILARIA OF *BRUGIA PAHANGI*

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ประสิทธิภาพของยาเตตราซัยคลินและเอนโรฟลอกซาซินต่อแบคทีเรียโวลบาเซียในไมโครฟิลาเรียของ
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Brugia pahangi is a major causative agent of animal lymphatic filariasis, one of the neglected tropical disease. Recent studies have also indicate that *B. pahangi* can cause clinical infection in human, with clinical appearances which are consistent with human lymphatic filariasis. Nowadays, the recognition of *Wolbachia* as new alternative drug choice for treatment and control of lymphatic filariasis is the most important in anti-filarial chemotherapy. Since, *Wolbachia* play important roles, including the development, viability, fertility and fecundity of filarial parasites. Depletion of *Wolbachia* results in sterilization, stunting and death of filarial worms. In this study, we investigated the effects of tetracycline and enrofloxacin on *B. pahangi* microfilarial motility and on *Wolbachia* depletion. Results showed that motility score of microfilaria treated with tetracycline were markedly reduced and high concentrations of enrofloxacin also can reduced worm motility. Quantitative real-time PCR indicated that the change of *Wolbachia* population in each treatment groups were decreased gradually. MEC of tetracycline was 128 and 32 µg/ml at 24 and 48 hours, respectively, while MEC of enrofloxacin was 512 µg/ml at 24 hours. Thus, tetracyclines should be considered as a drug of choice for treatment of animal lymphatic filariasis and suggest new antibiotic, enrofloxacin, as a substitute.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Brugia pahangi is a major causative agent of lymphatic filariasis particularly in cats, dogs and wild carnivores such as wild felids, civet cats and leaf monkeys. (Denham & McGreevy, 1977) Recently, some reports have demonstrated that *B. pahangi* can cause clinical infection in humans also. (Tan et al., 2011) Clinical appearances of *B. pahangi* infection in human are consistent with lymphatic filariasis (Denham & McGreevy, 1977) According to WHO reports, human lymphatic filariasis spreads in at least 80 countries around the world, including Thailand. Over 130 million people suffer from filarial infections. In South East Asia countries, there are over 60 million cases harboring microfilaremic infections which are account for a half of globally active lymphatic filariasis burden. (Ichimori et al., 2014) Mosquitoes in genus *Mansonia*, *Armigeres* and *Aedes* are in charge of lymphatic filariasis transmission. (Edeson, Wharton, & Laing, 1960) The tropical atmosphere is the most proper to these mosquito vectors to survive. The rise of global temperature is advantageous to mosquitoes not only to spread their offspring but also to enhance the effectiveness of pathogen transmission through the mosquito-borne diseases such as dengue, malaria and lymphatic filariasis. (Gubler et al., 2001) For example, there are some evidences indicated that prevalence of mosquito-borne disease in South America and South East Asia relates to El-Niño phenomenon. This relationship between El-Niño phenomenon and the expanding of these disease risk is because of the rising of global temperature. (M. Bouma et al., 1997; Menno Jan Bouma & Dye, 1997; Menno J Bouma & van der Kaay, 1996; Linthicum et al., 1999) To stop the spread of human lymphatic filariasis, WHO launched the Global Program to Eliminate

Lymphatic Filariasis (GPELF) in 2000. This program consists of strategies of preventive chemotherapy called mass drug administration (MDA) which has been used to treat the entire risk population. Diethylcarbamazine citrate, albendazole and ivermectin are used as recommendation drugs, targeting on adults and microfilariae of filarial nematodes. (Molyneux & Zagaria, 2003) *Brugia pahangi* is a closely related species to *Brugia malayi* which inhabit in the lymphatic system, similar to *B. malayi* in human. (Melrose, 2002) Several studies revealed that experimental infection with *B. pahangi* on human can result signs and symptoms of human lymphatic filariasis. (Mak, Pkf, Lim, & N, 1980; Palmieri et al., 1985) In the recent past, the first confirmed cases of lymphatic filariasis in human caused by natural infection with *B. pahangi* were reported in Malaysia. (Tan et al., 2011) Besides, there is a case report of zoonotic *B. pahangi* infection in the boy who lives in the southern part of Thailand in 2011 (Yokmek et al., 2012) *Wolbachia* is known as an endosymbiont bacteria resides in many species of filarial nematodes and arthropod. It can be divided into six major supergroups with *Wolbachia* of *Onchocerca* and *Dirofilaria* in supergroups C and those of *B. malayi*. *B. pahangi*, *W. bancrofti* and *Litomosoides sigmodontis* in supergroups D, based on 16S rDNA, *ftsZ*, *gltA*, *groEL* and *wsp* genes. (Bandi, Trees, & Brattig, 2001; Casiraghi et al., 2005) *Wolbachia* bacteria implicates in many roles, including the development, viability, fertility and fecundity of filarial parasites. (Stouthamer, Breeuwer, & Hurst, 1999) Depletion of *Wolbachia* results in sterilization, stunting and death of filarial worms (Slatko, Taylor, & Foster, 2010) Moreover, *Wolbachia* also plays important roles in the pathogenesis of lymphatic filariasis (Bandi et al., 2001) Nowadays, the recognition of *Wolbachia* as a new alternative drug choice for treatment and control of lymphatic filariasis is the most important in anti-

filarial chemotherapy. There are some *in vitro* and *in vivo* experiments have been demonstrated that treatment of antibiotic with chemotherapeutic efficacy against the bacterial group which *Wolbachia* belongs, (e.g., tetracycline) is effective. (Bandi et al., 1999) (Bosshardt et al., 1993) Unfortunately, the information of proper dosage and treatment protocol in anti-*Wolbachia* antibiotics in animals is insufficient. Therefore, the purpose of this experiment is to study the optimal dosage of tetracycline and enrofloxacin, one of the most common antibiotic used in veterinary field, against *Wolbachia* spp. in microfilaria of *Brugia pahangi*, *in vitro*.

1.2 Objective

To study the optimal dosage of tetracycline and enrofloxacin for treatment *Wolbachia* spp in microfilaria of *Brugia pahangi*, *in vitro*

CHAPTERS 2 LITERATURE REVIEW

2.1 Filariasis

Filariasis is an infectious vector-borne disease caused by parasites in a superfamily Filarioidea. The two families of worms most often associated with this disease are Filariidae and Onchocercidae. Lymphatic filariasis is one of the most debilitating neglected parasitic diseases affects global health. Infection with *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori* result in lymphatic filariasis in human which is a health problem in many countries all over the world such as South America, Asia, sub-Saharan African, and the Pacific Ocean. The World Health Organization (WHO) states that filarial parasites infect over 130 million people worldwide. (Ichimori et al., 2014) Clinical appearances in human are shown as

lymphoedema of the entire leg or arm and the formation of hydroceles in men, which also known as elephantiasis. Acute phases of local inflammation affect to the skin, lymphatic vessels and lymph nodes. These stages are caused by the innate immune reaction to parasites or the consequence of bacterial skin infections, which is relate to the partial loss of the body's normal defenses as a result of underlying lymphatic damages. (Denham & McGreevy, 1977) Another filarial species infecting humans including *Onchocerca volvulus* is the parasite that causes endemic onchocerciasis (river blindness) in Africa, Latin America and Yemen. *Onchocerca volvulus* infection shows clinical signs of skin disease and can cause blindness. Infection with *Loa loa* can cause repeated episodes of itchy swellings of the body, also known as subcutaneous Calabar, swelling and conjunctival irritation. This subcutaneous filariasis is found in West and Central Africa. *Dracunculus medinensis*, is generally known as a causative agent of guinea-worm disease, affects poor communities in backwood areas of Africa. (Ottesen, Hooper, Bradley, & Biswas, 2008) In mammal, *B. pahangi* is a major causative agent of lymphatic filariasis particularly in cats, dogs and wild carnivores such as civet cats, wild felids and leaf monkeys. Animals infected with *B. pahangi* are turned out to lymphangitis, lymphadenopathy, and limb edema which are similar to the clinical appearances reported in human. Histological finding shows fibrosis of the lymphatic ducts and lymph nodes. Lymph duct walls are thickened with a mononuclear inflammatory infiltrate. Dirofilariasis is caused by filarial nematodes in genus *Dirofilaria*. *Dirofilaria immitis* and *Dirofilaria repens* are the most commonly account for causing disease in mammals. Infection with *D. immitis* results in heart failure, severe lung disease and damage to other

organs while infection with *D. repens* shows clinical sign as subcutaneous nodules. (Melrose, 2002; Schacher, 1962)

2.2 Transmission

Lymphatic filariasis is transmitted by mosquitoes in genus *Culex*, *Mansonia*, *Armigeres Anopheles* and *Aedes* which are widespread across the world. (Edeson et al., 1960)

While feeding on infected host, mosquitoes are infected with microfilariae by ingesting blood. After that, microfilariae develop into infective third stage larvae within the mosquito. Third stage larvae can penetrate to another host body when infected mosquitoes feed another animals. Then, the larvae migrate to the lymphatic vessels where they mature into adult worms and produce their offspring called microfilaria to complete a cycle of transmission. (Christensen & Sutherland, 1984) (Esslinger, 1962)

2.3 *Brugia pahangi*

Brugia pahangi is a lymphatic filarial nematode of mammals, especially in cats, dogs and wild carnivores such as civet cat, raccoon, wild cat and monkey. (Fox & King, 2013; Schacher, 1962) Vectors of *Brugia pahangi* are mosquitos in genus *Aedes*, *Armigeres*, and *Mansonia*. (Ewert & Ho, 1967) Adult worms reside in the lymphatic vessels and lymph organs. However, immature and adult filarial worms can sometimes be found in subcutaneous tissues of infected host. (Nutman, 2017) The life cycle of *B. pahangi* starts when microfilariae in blood stream of infected animals are ingested by the mosquito vectors. Microfilaria migrate out of the mid-gut to the thoracic flight muscles of the mosquitoes, then they develop into infectious third stage larvae and migrate to the mouth parts of the mosquitoes. Infectious stage larvae are penetrated into another host during a subsequent blood meal. In mammal

hosts, L3 molt twice to mature into adult male and female worms which can produce their offspring circulating in blood circulation within 53 -94 day post infection. The adult males and females are 17.4 to 20 mm long and 38 to 63 mm long, respectively. (Denham, Ponnudurai, Nelson, Rogers, & Guy, 1972; Esslinger, 1962; Schacher, 1962) The pathogenesis and clinical sign of infected in cats has been studied for the purpose of using cat as model of human lymphatic filariasis infection. After 14 to 15 days post infection, some of the larvae allocate in the lymphatic sinus and lymph node whereas others migrate to the afferent lymphatic duct that distal to the popliteal lymph node. At this point of time, the dilation of lymphatic duct and the thickening of valves will occur. By 6 weeks post infection, the increasing of varicosity of the lymphatic duct will remarkably notice and pockets of worms will be found at the popliteal node area. After 16 weeks post infection, the lymphatic vessels will extreme and chronic inflammation and fibrosis with thrombo-lymphangitis. After 4 to 5 years post infection, cat can improve naturally and show less lymphatic dysfunction than cat infected for a year or so. In cats which are repeatedly infected with *B. pahangi* during long periods of time, the pathological changes are inconstant. Sometimes the popliteal lymph nodes are extremely enlarge and the nodes are hardly palpable. In the studies of repeat infection, many new or enlarged skin lymphatic vessels are observed. (Ah & Thompson, 1973; Ash, 1971, 1973; McCall, Malone, Ah, & Thompson, 1973; Schacher, 1962; Schacher & Sahyoun, 1967) Whereas, natural infection with *Brugia pahangi* in human manifests clinical sign with descending lymphangitis and cellulitis of the lower limb. (Wong & Guest, 1969)



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2.4 Geographic distribution

Brugia pahangi has been reported from mammals in Malaysia, Indonesia, Philippines and Thailand. Mak et al. (1980) reported that cats sampled in Peninsular Malaysia were positive for *B. pahangi* about 11%. In Thailand, Chungpivat and Sucharit (1993) demonstrated that up to 25% of blood samples from cats in Bangkok were found to have circulating microfilariae of *B. pahangi*. About 18.8% of cats in South Kalimantan, Indonesia were infected with *B. pahangi*. (Chungpivat & Sucharit, 1993; Mak et al., 1980; Palmieri et al., 1985) There are several studies reveal that experimental *B. pahangi* infection on human can produce clinical signs and symptoms of human lymphatic filariasis. Moreover, some volunteer were found to have microfilariae in their blood circulation. (Edeson et al., 1960) In the recent past, the first confirmed cases of human lymphatic filariasis caused by natural infection with *B. pahangi* were reported in 2010, in Malaysia. (Tan et al., 2011) Besides, there is a case report of zoonotic *B. pahangi* infection in the boy who lives in the southern part of Thailand in 2011. (Yokmek et al., 2012)

2.5 *Wolbachia* endosymbiotic bacteria

Wolbachia are *Rickettsia*-like, inherited intracellular, Gram-negative bacteria that infect many species of arthropod and filarial nematodes. (Bourtzis, 2008) They are found in all stages of filarial life cycle. In adult worms, *Wolbachia* are found throughout the hypodermis of the lateral cords. In female filarial worm, *Wolbachia* also locate in the oocytes, ovaries and in embryonic stages within the uterus. This implies that these bacteria are vertically transmitted through the egg. Studies on the dynamic population of the bacteria among each stages of the nematode have demonstrated that the numbers of bacteria remain stationary in microfilariae and the larval stages (L1, L2 and L3). (McGarry, Egerton, & Taylor, 2004) However, within the

7-10 days after invading into mammal host, number of bacteria increase markedly and the bacteria/nematode ratio reaches the highest of all life-cycle stages. The major period of bacterial development happens during the first month after infection in the mammalian host. (Bandi et al., 2001; Taylor, Bandi, & Hoerauf, 2005) The genus *Wolbachia* belongs to the order Rickettsiales (alphaproteobacteria). Recently, there is only one species in this genus, i.e. *Wolbachia pipientis*. Based on the analysis of 16S rDNA, *ftsZ*, *dnaA* and *wsp* genes, six lineages of *Wolbachia* have been indicated as supergroups A to F. Supergroups A and B include the *Wolbachia* infected in arthropods, while supergroups C and D include most of *Wolbachia* detected in filarial nematodes. The remaining supergroups E and F are lack of studies. Supergroup E encloses *Wolbachia* from collembolan whereas supergroup F includes *Wolbachia* found in *Mansonella ozzardi* and termites. (Casiraghi et al., 2005; Slatko et al., 2010; Stouthamer et al., 1999; Taylor et al., 2005)

2.6 Chemotherapy

WHO guidelines for lymphatic filariasis eradication recommend diethylcarbamazine, ivermectin and albendazole for treatment and control the disease and the additional treatment is using anti-*Wolbachia* antibiotics. (Ichimori et al., 2014)

Diethylcarbamazine is on the World Health Organization's List of Essential Medicines, widely used in the treatment of filariasis including lymphatic filariasis, onchocerciasis and loiasis. It acts as an inhibitor of arachidonic acid metabolism in filarial microfilaria. Diethylcarbamazine results in rapid disappearance of microfilariae from blood stream within 48-72 hours of administration. (Hawking, 1979) However, it has a little action on microfilariae in vitro because this drug makes microfilariae more



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susceptible to innate immune response, but does not completely kill microfilariae. In additions, diethylcarbamazine rarely affects in killing adult worm of *Wuchereria*, *Brugia*, *Onchocerca* and *Loa loa*. (S. Gunawardena, Ismail, Bradley, & Karunaweera, 2007)

Ivermectin is an antiparasitic drug which is effective against many types of parasites. For instance, it is used to treat scabies, intestinal nematode infections, onchocerciasis and lymphatic filariasis (Anselmi et al., 2015) The medicine binds to glutamate-gated chloride channels in the membranes of muscle and nerve cells, resulting in increased permeability to chloride ions, causing cellular hyperpolarization, paralysis and death, subsequently. (Moreno, Nabhan, Solomon, Mackenzie, & Geary, 2010) Ivermectin is an effective microfilaricidal drug, especially filariasis. Also, ivermectin treatment in cats naturally infected with *B. pahangi* results in amicrofilaremia within 9 weeks. (Taweethavonsawat & Chungpivat, 2013) Albendazole is a broad-spectrum anthelmintic agent that has efficacy for treatment many species of parasitic worm infections such as giardiasis, ascariasis, trichuriasis, neurocysticercosis, cystic hydatid disease, enterobiasis and filariasis. (Horton, 1989) (Liu & Weller, 1996) Albendazole is an effective macrofilaricidal drug which causes degenerative alteration in the parasites intestinal cells by binding to the tubulin colchicine-sensitive site, inhibiting cells polymerization or assembly into microtubules. Deteriorated uptake of glucose by the larval and adult stages occur due to the loss of cytoplasmic microtubules, then decreasing of ATP production that lead the parasites to immobilize and death. (Liu & Weller, 1996) Nonetheless, albendazole can't deplete microfilariae, thus the microfilarial count is not reduced immediately. (Molyneux & Zagaria, 2003)

Tetracycline is a broad-spectrum antibiotic which possess a wide range of antimicrobial activity against gram-positive and gram-negative bacteria. Tetracycline has recently been shown to have potential macrofilaricidal activity against filarial nematodes (Smith & Rajan, 2000) Tetracycline is primarily bacteriostatic. This drug inhibits protein synthesis in bacteria by binding to the 30S ribosomal subunit. The drugs in tetracycline class have closely similar antimicrobial activity. Moreover, tetracycline has anti-filarial activity due to the susceptibility of *Wolbachia* endosymbiont of filarial nematode. Effects of tetracycline have been considered as drug choice for lymphatic filariasis. (Bandi et al., 1999; Bosshardt et al., 1993) Recent studies state that tetracycline treatment of filarial infected animals can decreased microfilaremic levels and reduced adult worm burdens. (N. K. Gunawardena et al., 2005; Smith & Rajan, 2000). Besides, some studies support that tetracycline and doxycycline showed significant antifilarial effect while ciprofloxacin failed to against filariasis. These antibiotics might also have a direct effect to the filarial parasites (Mahajan et al., 2010b) Supali et al (2008) indicate that a 6-week treatment of doxycycline, either alone or combine with diethylcarbamazine or albendazole, causes a decrease in microfilariae in blood circulation and reduces adverse effects to antifilarial treatment in human infected with *B. malayi*. (Supali et al., 2008) Another evidence showed that the combination with doxycycline and ivermectin can remarkably reduce microfilaria in blood circulation following reductions in *Wolbachia* in filarial worm. (R. U. Rao, 2005)

Enrofloxacin belongs to fluoroquinolone family which is a subfamily of quinolone. Enrofloxacin is bactericidal antibacterial agents with broad-spectrum activity. It inhibits the enzyme topoisomerase II and Topoisomerase IV, DNA gyrases

that are necessary for the replication of bacteria. (Bandi et al., 1999) By inhibiting these enzymes, DNA replication and transcription is blocked. Enrofloxacin is effective in the treatment of local and systemic infections caused by Gram-positive and Gram-negative bacteria.(Drlica, Malik, Kerns, & Zhao, 2008) It also plays an important role in treatment of bacterial diseases that resistance to older antibacterial drugs (Cohn, Gary, Fales, & Madsen, 2003) Recently, fluoroquinolone drugs are used as a new class of antifilarial agent that is extremely potential, with efficacy against all the developmental stages of the filarial parasite *Setaria cervi*. (Mukherjee et al., 2018). Besides, there are studies indicate that enrofloxacin has efficacy against *Acanthoeilonema viteae*, *in vitro*. This drug also display activity in macrofilaricidal and microfilaricidal. (Srivastava, Chauhan, Bhaduri, Fatima, & Chatterjee, 2000)



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CHAPTERS 3

MATERIALS AND METHODS

3.1 Blood samples

Blood samples of naturally *Brugia* infected dogs were obtained from The Veterinary Teaching Hospital of Mahidol University and surrounding areas. Inclusion criteria for dogs enroll in this study including, not expose to any antibiotics at least 3 previous months and no history record in using any antifilarial drugs for 6 previous months with high level of parasitemia (>1000 microfilaria per ml). Samples from dog with inclusion criteria were previously screened by using the traditional Giemsa staining technique before undergoing molecular identification and parasite isolation. The animal use protocols were conducted under the approval of Chulalongkorn University Animal Care and Use Committee, Chulalongkorn University (Animal Use Protocol No. 1931004).

3.2 Parasite identification

Infection were confirmed by morphological and molecular identification. Giemsa staining technique, Acid phosphatase activity and Polymerase chain reaction technique, using primers 5'-AGTGCGAATTGCAGACGCATTGAG-3' and 5'-AGCGGGTAATCACGACTGAGTTGA-3' were performed. The PCR procedures were executed according to Rishniw et al. (2006) The PCR products were run on a 2% agarose gel and visualized under ultraviolet light. (Rishniw et al., 2006)

To confirm the existing of *Wolbachia* DNA, forward 5'ATAACAGCAGGAATGGGTG GT-3' and reverse 5'-TCACGCACTCTA TTTGCTGCA-3' oligonucleotides were used for

amplification based on conserved sequences of *Wolbachia ftsZ* gene. Then, amplification products were purified using QIAquick® Gel Extraction Kit. DNA sequencing was performed to confirm that the amplified DNA represents DNA from *Wolbachia* bacteria. (Turba, Zambon, Zannoni, Russo, & Gentilini, 2012)

3.3 Separation of microfilaria by simple centrifugation method

Microfilariae were counted and total microfilaremic were calculated. EDTA-blood were spun at 8,500 RPM for 5 minutes at room temperature. Plasma content above the buffy coat layer were taken to individual tubes. 20 µl of microfilarial content from plasma layer was taken and quantitated. Total volume of this content were wash with RPMI-1640 for 3 times.

3.4 Separation of microfilaria by Percoll gradient method

The procedure for using Percoll gradient solution to separate microfilaria from whole blood were previously described. (Chandrashekar, Rao, Rajasekariah, & Subrahmanyam, 1984) Briefly, EDTA-blood were diluted with medium RPMI-1640 in the ratio of 1:1. Iso-osmotic Percoll (IOP) were prepared by mixing nine parts of Percoll with one part of 2.5 M sucrose. Dilutions of the IOP in 0.25 M sucrose were made to obtain 35 and 40% solution of density 1.068 and 1.074, respectively. For the isolation of microfilaria, 2 mL of 40% IOP solution were taken in a 15 mL conical centrifuge tube and were overlayed by the same volume of 35% IOP solution. Then, 0.5 ml of diluted blood were added upon the gradients. Tubes were spun at 2000 RPM for 30 minutes at room temperature. After centrifugation each fraction of plasma and motile microfilariae layers were transferred to individual tubes. 20 µl of microfilarial content from this layer was taken and quantitated. Total volume of microfilarial layer were wash with RPMI-1640 for 3 times.



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3.5 Drug treatment

Microfilariae were divided into one control and two drug treatment groups and arranged in duplicate experiments. Tetracycline and enrofloxacin at final concentrations between 0.125 to 512 µg/ml, prepared in a serial two-fold dilution were used (final concentration as 0.125, 0.5, 2, 8, 32, 128 and 512). Each of 100 microfilariae will were maintain in well plates with either culture media, or culture media with a dilution of antibiotics. Microfilariae were observed and their motility will be scored. In additions, they were collected daily for evaluation of the change of *Wolbachia* population.

3.6 Parasite viability assay

Microfilariae were transfered to each 96-well plates adding RPMI-1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 10% fetal calf serum, Streptomycin, AmphotericinB and Penicillin at 37 C in a 95% air-5% CO₂ atmosphere. Microfilaria motility and death were assessed visually by inverted microscope. The observations were conducted daily for 6 days and scored as 0-4 as described previously. (R. Rao & J Well, 2002)

Score	Criteria
0	Immotile or dead (No sign of motion for 10 sec)
1	Slightly active (Move but fail in forward progression)
2	Active and motile (Slow moving and forward progression)
3	Moderately active and motile (Regular moving and forward progression)
4	Highly active and motile (Vigorous moving, fast and forward progression)

3.7 Quantitative real-time PCR

Primers and probes according to Simoncini et al. (2001) and Turba et al. (2012) were used in this experiment. (Simoncini et al., 2002; Turba et al., 2012) The real-time PCRs were carried out with a mixture composed of 10 µl of PCR mix 2x (Luna® Universal Probe qPCR Master Mix, Massachusetts, USA), 900 nM concentrations (each) of forward and reverse primers, 300 nM TaqMan probe, 2 µl of template, and molecular-biology-grade water to reach a final volume of 20 µl. The real-time PCRs were carried out in a thermal cycling using a Bio-Rad CFX apparatus with a four-step protocol: for *Wolbachia* initial denaturation at 95°C for 10 min, followed by 45 cycles of 92.5°C for 15 s, 54°C for 15 s, and 54°C for 10 s with signal acquisition and finally at 72°C for 25s and for *B. pahangi* at 95 °C for 5 min followed by a 40-step amplification of 10 s at 95 °C, 10 s at 58 °C, and 10 s at 72 °C. Subsequently, the products were heated to 72 °C for 1 min. The calibration was carried out by

assessing each sample in triplicate. *Wolbachia/Brugia* DNA ratio of each groups were calculated.

Cycle Threshold (Ct) values were imported into a Microsoft Excel spreadsheet for data analysis. Difference in Ct (ΔCt) of *wolbachia* target gene (*ftsZ*) and the Ct of the filarial reference gene (ITS2) of the same sample, was calculated as follows:

$$\Delta\text{Ct} = \Delta\text{Ct}(ftsZ) - \Delta\text{Ct}(ITS2)$$

The change in ΔCt over time following treatment ($\Delta\Delta\text{Ct}$) was computed as:

$$\Delta\Delta\text{Ct}(n) = \Delta\text{Ct}(n) - \Delta\text{Ct}(0)$$

Where n is day after treatment and time 0 is defined as the pre-treatment time at day 0. The bacteria density in each sample relative to the calibrator sample (day 0) was then estimated using the formula $2^{-\Delta\Delta\text{Ct}}$.

3.8 Statistical Analysis

For parasite viability assay, data were analyzed by Mann-Whitney U test and differences were considered significant with P values <0.05

Two-way ANOVA were used to evaluate the differences in changing of *Wolbachia/Brugia* DNA ratio. Differences were considered significant when $p < 0.05$.

CHAPTERS 4

RESULTS

4.1 Patient description

3 years old, mixed breed dog presented with otitis externa were enrolled in this study. Physical exam and blood test for wellness exams, including blood parasite, were performed. No symptoms of lymphatic filarial infection were found. Patient's complete blood count and blood chemistry profile were in normal range. Microfilaria of *Brugia* spp. were found in thin blood smear. History taking for more information were fulfill. Dog has on experience of exposing any antibiotics and antifilarial drugs.

4.2 Identification of parasite

Thin blood films stained with 2.5% Giemsa revealed sheathed microfilaria of *Brugia* spp. with 2 nuclei extended to the tip of the tail, as shown in figure 1. While, acid phosphatase staining showed heavy and diffused acid phosphatase activity along the entire body of *B. pahangi* microfilariae, as shown in figure 2.

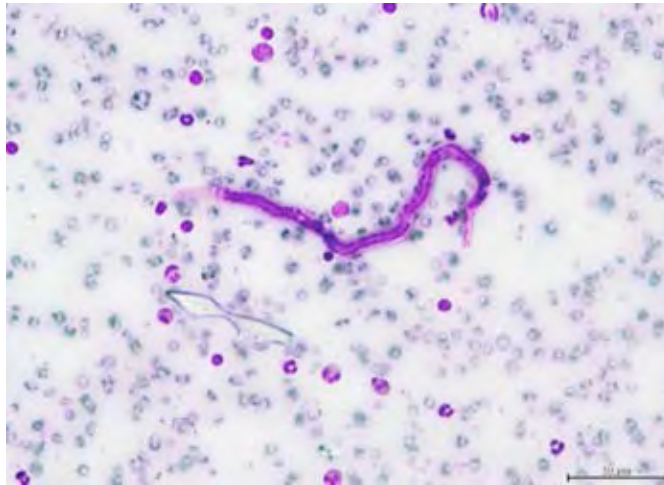


Figure 1 Characteristic morphology of *Brugia* spp. in 2.5% Giemsa staining



Figure 2 An acid phosphatase stain of *B. pahangi* showing acid phosphatase activity along the entire body length. The excretory and anal pores are recognizable.



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Then, molecular confirmation were conducted by using primers specific for the 5.8S-ITS2-28S gene of *B. pahangi* amplified DNA fragments of the anticipated product sizes of 665 bp. (figure 3)

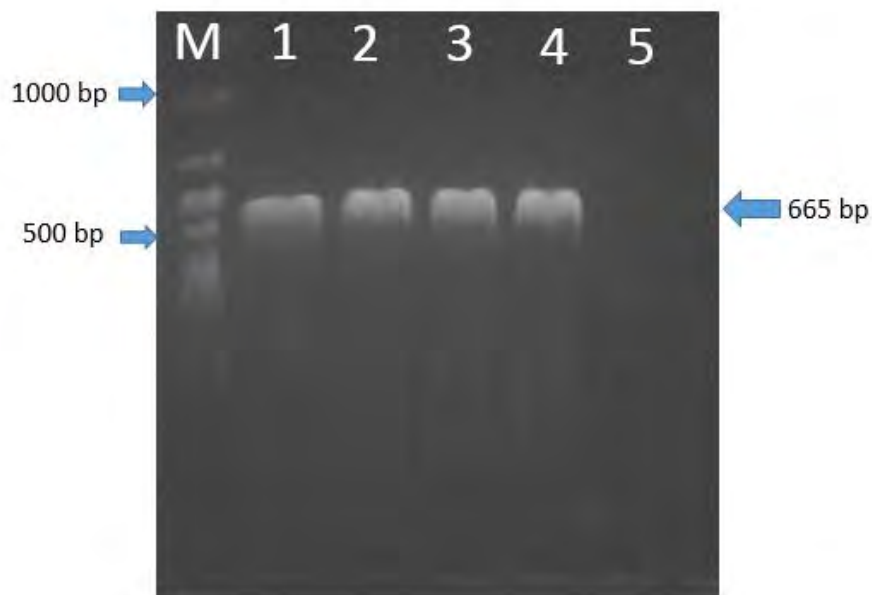


Figure 3 Agarose gel electrophoresis profile of PCR amplification of 5.8S-ITS2-28S gene from *B. pahangi*. Lane M and lane 5 represent DNA ladder marker and negative control, respectively. Lane 1; 2; 3 and 4 represent 665 bp PCR product of positive control and sample in triplicate, respectively

4.3 Confirmation of *Wolbachia* bacteria

The existing of endosymbiont bacteria, *Wolbachia* were confirm by using conventional PCR focusing on *ftsZ* gene. Amplified DNA fragments of the expected product sizes of 550 bp. Sequence analysis showed 99.0% sequence identity with *ftsZ* gene sequence of *Wolbachia pipentis* of *Brugia pahangi* as shown in figure 5.

In additions, *Wolbachia* DNA from blood sample of dogs and cats that were joined in this study but not match to the inclusion criteria were extracted. DNA sequencing and phylogenetic analysis was performed. Phylogenetic analysis on *ftsZ* gene using the Neighbour-Joining (NJ) algorithm revealed that filarial wolbachiae in this study can be divided into three lineages based on the genus of their filarial hosts as shown in figure 6.



Figure 4 Agarose gel electrophoresis profile of PCR amplification of *ftsZ* gene from *Wolbachia* bacteria
Lane M and lane 1 represent DNA ladder marker and negative control, respectively.
Lane 2 and 3 represent 550 bp PCR product of positive control and sample, respectively



Figure 5 Alignment of Wolbachia ftsZ gene

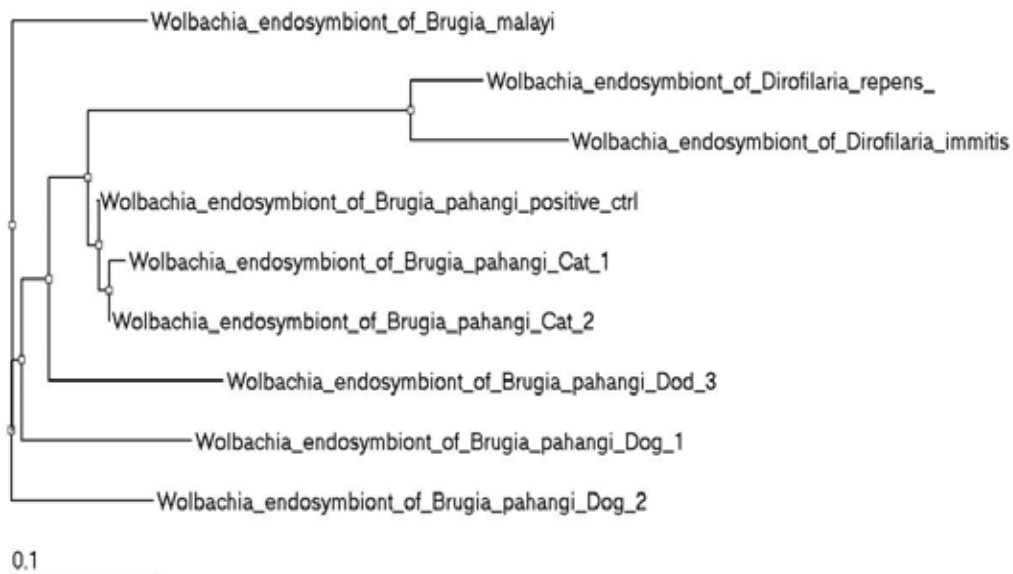


Figure 6 Phylogenetic tree shows the relationships of filarial Wolbachia, based on *ftsZ* gene sequences which are related to their filarial hosts

4.4 Separation of microfilaria

Recovery rate of microfilaria

After centrifuged by simple centrifugation method, three layers of plasma layer, buffy coat layers and packed red blood cell layer were shown. Plasma layer which interfaced buffy coat layer were taken separately. Comparing with Percoll gradient solution method, four layers consisting of plasma with 35% and 40% layers, motile microfilariae layer and cellular sediment were separated immediately (figure 7). 20 μ l of each layers were taken onto a microscopic slide and microfilariae were counted. A number of microfilariae were respectively calculated. The per cent recoveries of the parasite from each methods are presented in the Table1. Approximately 90 per cent of microfilariae were distributed in the motile microfilariae layer from Percoll gradient solution

method. Each 20 μ l of filarial content approximately distributed 35-40 microfilariae. Whereas, calculated recovery rate of microfilaria from simple centrifugation method was only 43%.

Table 1 Percentage recovery of microfilariae by gradient separation and simple centrifugation

Methods	Simple centrifugation	Percoll gradients solution
% Recovery of Microfilaria	43%	90%

Microfilaria from 2 separation methods were cultured with completed RPMI culture media. The scoring of motility was compared between two groups. Microfilariae separated by simple centrifugation method, motility score in this group were markedly reduced and reached score 0 on day 4 while microfilariae separated by using Percoll, showed the highest active motility as score 4 until day 9.

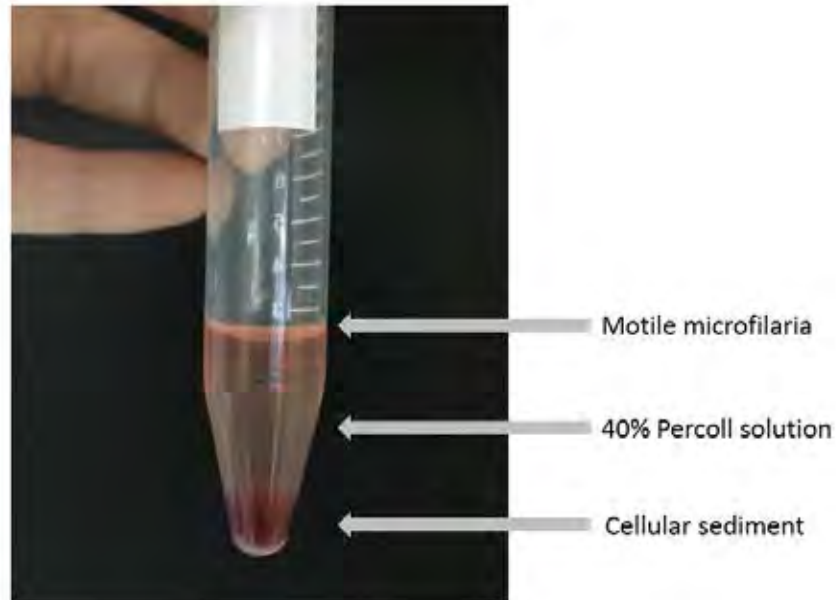


Figure 7 Separation of the viable microfilaria after Percoll gradient centrifugation



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4.5 Effects of anti-Wolbachia antibiotics on microfilaria motility

Microfilariae were divided into 3 groups as followed, 1) untreated control 2) treatment with tetracycline at concentration of 0.25- 512 $\mu\text{g/ml}$ 3) treatment with enrofloxacin at concentration of 0.25- 512 $\mu\text{g/ml}$. All microfilariae were cultured with completed RPMI culture media at 37 C in a 95% air-5% CO_2 atmosphere. Motility was assessed visually and the observations were scored as 0-4 to estimate the survival rate. The scoring of motility was compared between three groups. Effects of anti-Wolbachia agents on microfilaria motility were measured as MECs. Group 1 showed the highest active motility as score 4 until day 7. Treatment with tetracycline at 128 $\mu\text{g/ml}$ and 32 $\mu\text{g/ml}$ inhibited microfilarial motility at 24 and 48 hours, respectively. Whereas, enrofloxacin showed less efficacy with the MECs of 512 $\mu\text{g/ml}$ at 24 and 48 hours. These decreases in motility of tetracycline at 32,128 and 512 $\mu\text{g/ml}$ and enrofloxacin at 128 and 512 $\mu\text{g/ml}$ compare to control were statistically significant ($P < 0.05$).

Table 2 Motility score of microfilaria after treatment with tetracycline at concentration of 0.25- 512 $\mu\text{g/ml}$ (day 0 to day 6)

Conc.	0.125 $\mu\text{g/ml}$	0.5 $\mu\text{g/ml}$	2 $\mu\text{g/ml}$	8 $\mu\text{g/ml}$	32 $\mu\text{g/ml}$	128 $\mu\text{g/ml}$	512 $\mu\text{g/ml}$	Control
Day 0	4	4	4	4	4	4	4	4
Day 1	4	4	4	3	2	0	0	4
Day 2	4	3	3	3	0	0	0	4
Day 3	4	3	3	2	0	0	0	4
Day 4	4	3	2	2	0	0	0	4
Day 5	3	2	2	0	0	0	0	4
Day 6	3	1	1	0	0	0	0	4

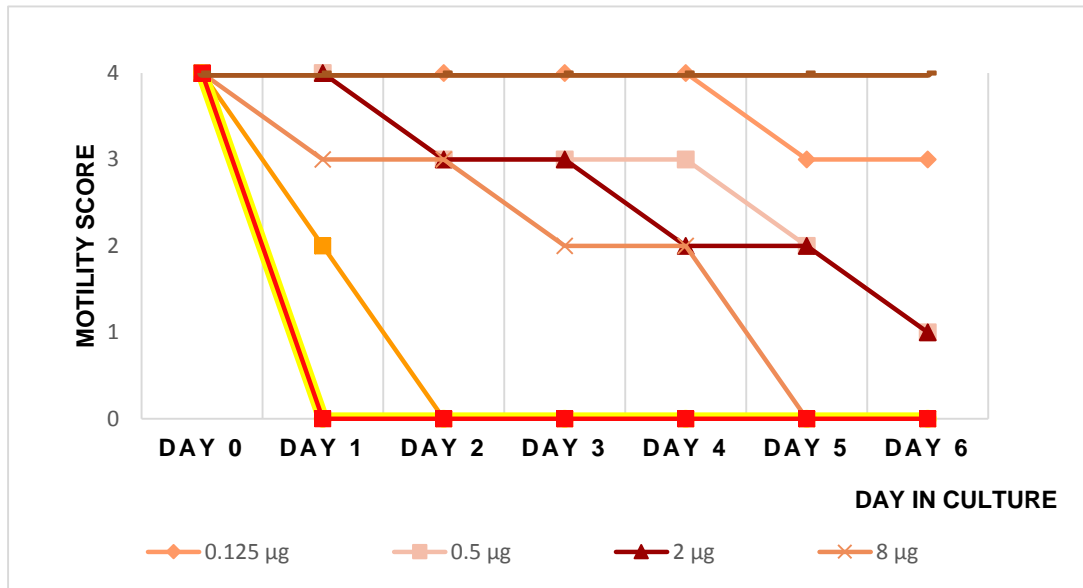


Figure 8 Effect of tetracycline on inhibition of microfilarial motility using MECs assay at Day 0 to Day 6

Table 3 Motility score of microfilaria after treatment with enrofloxacin at concentration of 0.25- 512 µg/ml

Conc.	0.125 µg/ml	0.5 µg/ml	2 µg/ml	8 µg/ml	32 µg/ml	128 µg/ml	512 µg/ml	Control
Day 0	4	4	4	4	4	4	4	4
Day 1	4	4	4	3	3	1	0	4
Day 2	4	4	3	3	3	0	0	4
Day 3	4	3	3	2	2	0	0	4
Day 4	4	3	2	1	2	0	0	4
Day 5	3	2	2	1	0	0	0	4
Day 6	3	2	1	1	0	0	0	4

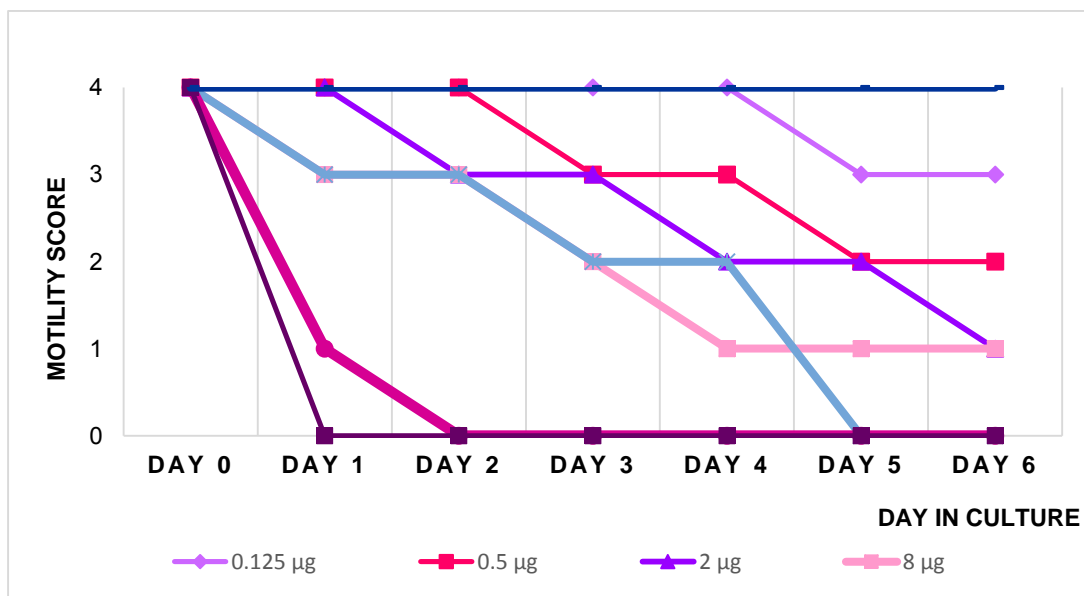


Figure 9 Effect of enrofloxacin on inhibition of microfilarial motility using MECs assay at Day 0 to Day 6 after treatment

4.6 Effects of anti-Wolbachia antibiotics on Wolbachia population

In order to verify their specificity, primers for the amplification of a 98nt fragment of *ftsZ* gene and ITS2 gene were tested in a single PCR and confirmed by agarose gel electrophoresis. Susceptibility of *Wolbachia* to anti-*Wolbachia* antibiotic was measured by qPCR. Amplification of both *ftsZ* gene and ITS2 gene was optimized for efficient probes detection for each target in same samples. The number of cycles of *B. pahangi* gene amplification required to cross the cycle threshold value, Ct, remained stable throughout day 6, while *Wolbachia* gene showed a daily decrease after treatment with antibiotics.

Difference in Ct (ΔCt) of *wolbachia* target gene (*ftsZ*) and the Ct of the filarial reference gene (ITS2) of the same sample, was calculated as follows:

$$\Delta\text{Ct} = \Delta\text{Ct}(\textit{ftsZ}) - \Delta\text{Ct}(\text{ITS2})$$

The change in ΔCt over time following treatment ($\Delta\Delta\text{Ct}$) was computed as:

$$\Delta\Delta\text{Ct}(n) = \Delta\text{Ct}(n) - \Delta\text{Ct}(0)$$

Where n is day after treatment and time 0 is defined as the pre-treatment time at day 0. The bacteria density in each sample relative to the calibrator sample (day 0) was then estimated using the formula $2^{-\Delta\Delta\text{Ct}}$. The relative bacteria abundance were plotted over time (fig. 10.1-10.8 and 11.1-11.8). The qPCR plots are similar to those based on microscopy data. The natural log of relative bacteria abundance against time were plotted for each treatment groups and fitted well with a simple log linear model as showed in figure 10.1-10.8H and 11.1-11.8. Then, two-way ANOVA were



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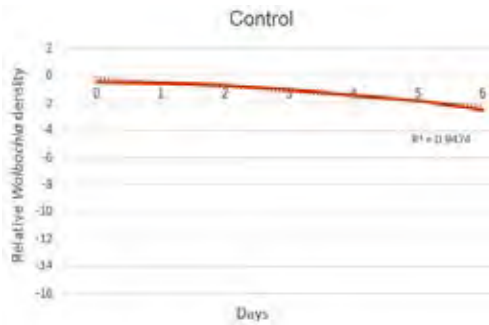
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used to evaluate the differences in changing of *Wolbachia/Brugia* population. Statistical analysis demonstrated that the depletion of *Wolbachia* population after treatment with tetracycline 32,128 and 512µg/ml and enrofloxacin at 128 and 512 µg/ml compare to control were statistically significant ($P < 0.05$). There are good agreement between the motility score of microfilaria and the depletion of *Wolbachia* population parameter values estimated directly from microscopy and qPCR.

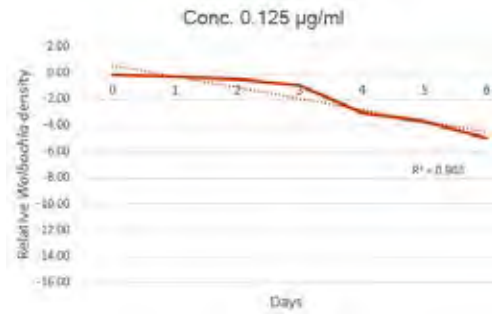


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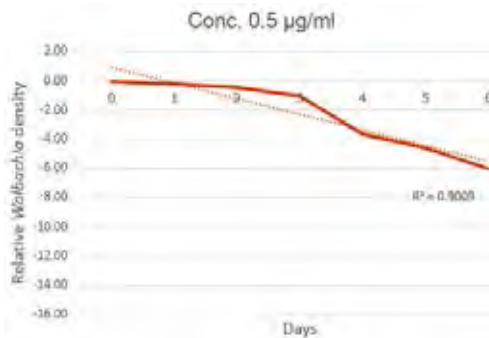
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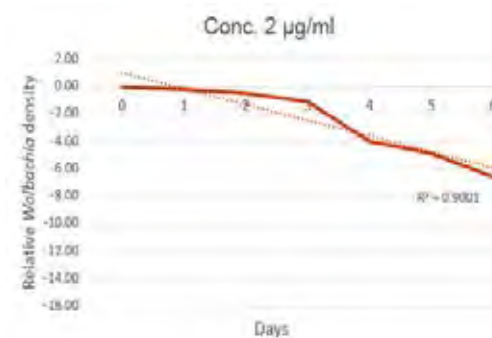
10.1 Decrease in relative density of *Wolbachia* DNA in control group



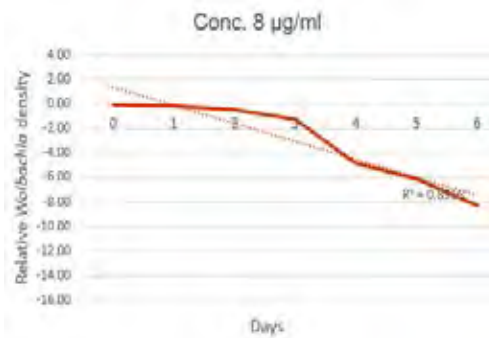
10.2 Decrease in relative density of *Wolbachia* DNA in group treated with 0.125 µg/ml tetracycline



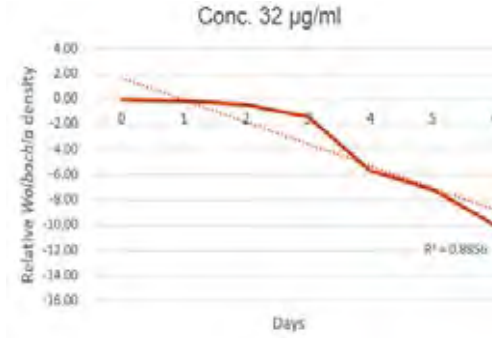
10.3 Decrease in relative density of *Wolbachia* DNA in group treated with 0.5 µg/ml tetracycline



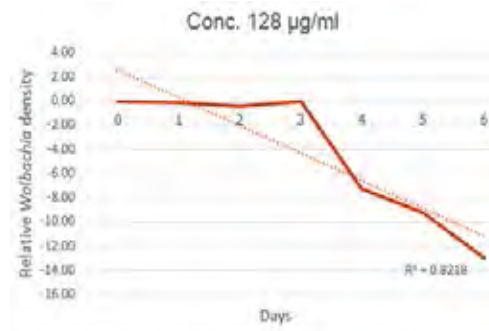
10.4 Decrease in relative density of *Wolbachia* DNA in group treated with 2 µg/ml tetracycline



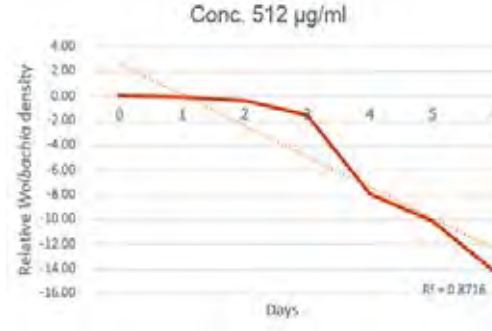
10.5 Decrease in relative density of *Wolbachia* DNA in group treated with 8 µg/ml tetracycline



10.6 Decrease in relative density of *Wolbachia* DNA in group treated with 32 µg/ml tetracycline

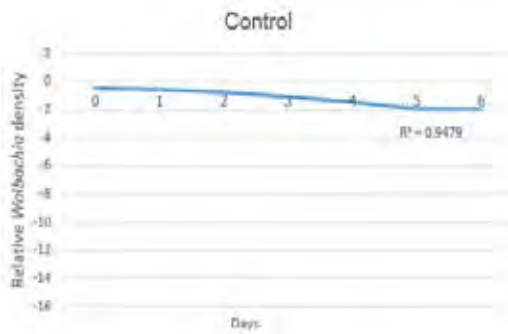


10.7 Decrease in relative density of *Wolbachia* DNA in group treated with 8 µg/ml tetracycline

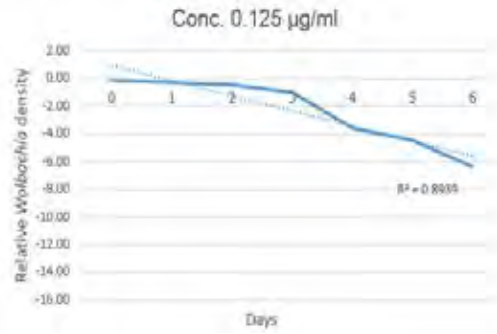


10.8 Decrease in relative density of *Wolbachia* DNA in group treated with 32 µg/ml tetracycline

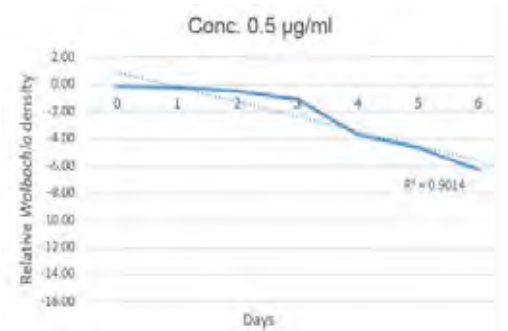
Figure 10.1-10.8 Illustrates qPCR data, analysis using the $\Delta\Delta Ct$ calculation method. Relative density of *Wolbachia* population was rapidly decrease after treatment with tetracycline.



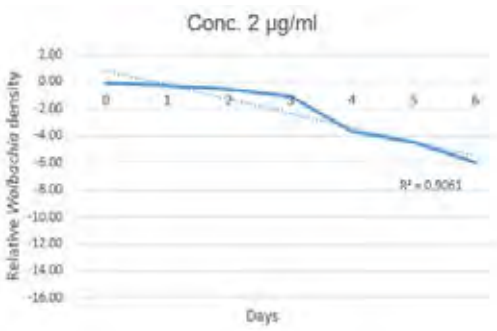
11.1 Decrease in relative density of *Wolbachia* DNA in control group



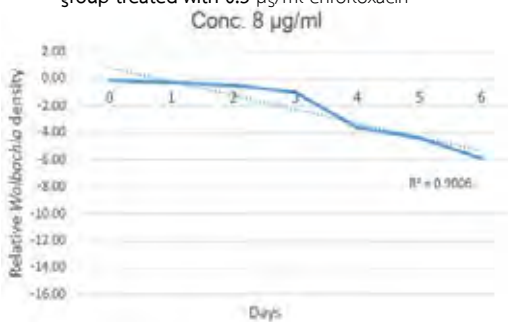
11.2 Decrease in relative density of *Wolbachia* DNA in group treated with 0.125 µg/ml enrofloxacin



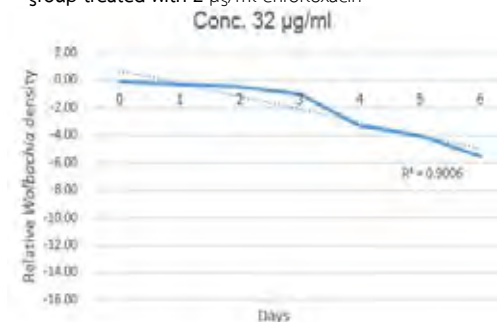
11.3 Decrease in relative density of *Wolbachia* DNA in group treated with 0.5 µg/ml enrofloxacin



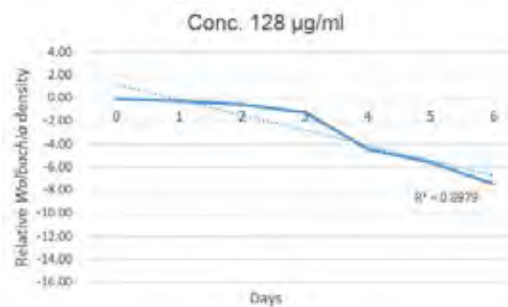
11.4 Decrease in relative density of *Wolbachia* DNA in group treated with 2 µg/ml enrofloxacin



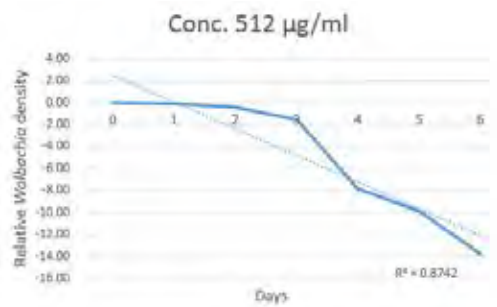
11.5 Decrease in relative density of *Wolbachia* DNA in group treated with 8 µg/ml enrofloxacin



11.6 Decrease in relative density of *Wolbachia* DNA in group treated with 32 µg/ml enrofloxacin



11.7 Decrease in relative density of *Wolbachia* DNA in group treated with 128 µg/ml enrofloxacin



11.8 Decrease in relative density of *Wolbachia* DNA in group treated with 512 µg/ml enrofloxacin

Figure 11.1-11.8 illustrates qPCR data, analysis using the $\Delta\Delta C_t$ calculation method. Relative density of *Wolbachia* population was rapidly decrease after treatment with tetracycline

CHAPTERS 5

DISCUSSION AND CONCLUSION

5.1 Discussion

The current study presents the validation of the effectiveness of Percoll as a tool for microfilarial separation from whole blood sample. Since its recognition in 1977, Percoll has become the density gradient medium of choice for many researchers worldwide. It was used in separating cells and other microorganisms. (Wiser & Lanners, 1992) Results showed that separation with Percoll gradient method, comparing with simple centrifugation method, provided better recovery rate of microfilariae and maintained their motility score as the highest active score until day 6 in untreated control group. Similar to Chandrashekar et al. (1984), percent recovery of *B. pahangi* microfilaria by Percoll gradient separation yielded 92% of microfilarial recovery. There are some possible explanations including, Percoll has low viscosity, allowing more rapid sedimentation and the use of lower centrifugal forces that less damaging to microfilariae. Whereas, to isolate microfilariae, simple centrifugation required higher centrifugal force. (Obeck, 1973) In additions, using Percoll solution microfilaria were free from cellular component, especially white blood cell that can disturb the viability of microfilaria. (Sneller & Weinstein, 1982)

To investigate the effects of anti-*Wolbachia* antibiotics on motility of *B. pahangi* microfilaria and its correlation of *Wolbachia* susceptibility, the MECs of antibiotics were used to evaluate the efficacies of two anti-*Wolbachia* antibiotics. Our findings support earlier studies that doxycycline and tetracycline are the most effective anti-*Wolbachia* antibiotic. (Mahajan et al., 2010a; R. Rao & J Well, 2002)

The *Wolbachia/Brugia* DNA which obtained from qPCR, can be used to indicate the presence of *Wolbachia* in microfilarai of filarial nematodes. The single copy genes of *Wolbachia* and *B. pahangi* were used to study the dynamic of *Wolbachia* population. This study also revealed the use of a new qPCR assay to measure parasite clearance after anti-*Wolbachia* antibiotic treatment. To overcome problems with absolute amount of parasite, the delta-delta CT ($\Delta\Delta$ CT) method were used for calculation of relative parasite density in each drugs regimens. (Livak & Schmittgen, 2001) This method that was first established at the UK reference laboratory for parasitology (Hospital for Tropical Diseases, London), is commonly used to compare treatment response among patients treated with different drug regimen or concentration. The benefit of this assay including its highly sensitive, rapid and easy to apply without measuring blood sample volumes. In this study, results of calculated $\Delta\Delta$ CT *Wolbachia/Brugia* gene correlation revealed the same marking in decrease of *Wolbachia* with the motility score assay. Recent studies which investigate the insect *Wolbachia* drug susceptibility by using immunofluorescence antibody and qPCR methods, indicate that after *Wolbachia*-infected cells were treated with 13 antimicrobial drugs for 6 days, both assays showed similar results; doxycycline and rifampicin are the most effective drugs, with MICs of 0.125 and 0.06-0.125 $\mu\text{g/ml}$ after 6 days of treatment, respectively. (Fenollar, Maurin, & Raoult, 2003) Our study demonstrated that tetracycline, the same group of antibiotic as doxycycline, had the efficacy at 128 $\mu\text{g/ml}$ at 24 hour after treatment.

However, studies of susceptibility of anti-*Wolbachia* antibiotic in filarial *Wolbachia* have some limitation because the lack of long-term filarial culture system



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available. (Higazi, Shu, & Unnasch, 2004) Motility of microfilaria in untreated control group could retain highly active as score 4 until day 9 of experiment.

In this study, the hypothesized that both tetracycline and enrofloxacin treatments could deplete *Wolbachia* spp. in microfilaria of *Brugia pahangi*, *in vitro*. Our results revealed that antimicrobial agents directly affect the motility of microfilaria phenotypes. Bacteriostatic drugs that are inhibitors of eukaryotic and bacterial mitochondrial translation, such as tetracycline, can directly reduce energy generation through the mitochondrial respiratory chain and lead to mobility defect in *Caenorhabditis elegans*, a free-living nematode. (Mallo et al., 2002) *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. (Hoerauf et al., 2008) Enrofloxacin has been shown effective for treating *Rickettsia* infections in cell line, also in dogs and cats. Recent studies demonstrated that drug in fluoroquinolone group can use as a new class of antifilarial agent with potential efficacy against all the developmental stages of the filarial parasite. (Mukherjee et al., 2018). This drug also show the efficacy in macrofilaricidal and microfilaricidal activities. (Srivastava et al., 2000) In the other hand, some studies demonstrated that ciprofloxacin, metabolite of enrofloxacin, failed to inhibit *Wolbachia* growth. (Sungpradit, Chatsuwana, & Nuchprayoon, 2012) In this study, enrofloxacin was used as an anti-*Wolbachia* drug. Our results indicated that efficacy of enrofloxacin was less than tetracycline but can deplete *Wolbachia* population. Despite of the better efficacy of tetracycline and doxycycline comparing to enrofloxacin, the use of tetracycline and doxycycline has some consideration when use in animal with liver disease or digestive problem. (Papich, 2016) Thus,

enrofloxacin might be introduced as an alternative drug choice for treatment filariasis in animal.

Interestingly, cases of macrocyclic lactone failure in prevention and treatment animal filariasis increase gradually. In additions, cases of macrocyclic lactone adverse reaction are widely reported. Nowadays, the use of either anti-*Wolbachia* antibiotic or macrocyclic lactone combine with anti-*Wolbachia* antibiotic are more recommended for treatment in animal filariasis. (Kramer et al., 2018)

For the further study, there are some studies indicate the principles of dosage conversion between *in vitro* and *in vivo* experiments (Nair and Jacob, 2016; Checkley et al, 2015). Evaluation of the accurate dosage use in animal should be perform to set a standard protocol for the treatment of *B. pahangi* infection in animals.

5.2 Conclusion

In conclusion, this study revealed that tetracycline was the most effective anti-*Wolbachia* agent for inhibiting microfilaria motility and was effective against the *Wolbachia* endosymbiont organism. Tetracycline and enrofloxacin can alter *B. panangi* microfilaria homeostasis through an indirect effect on the parasite's endosymbiont. For the further study, the accurate dosage use in animal should be determine by *in vivo* experiment.

REFERENCES

- Ah, H.-S., & Thompson, P. E. (1973). *Brugia pahangi*: infections and their effect on the lymphatic system of Mongolian jirds (*Meriones unguiculatus*). *Experimental parasitology*, 34(3), 393-411.
- Anselmi, M., Buonfrate, D., Espinoza, A. G., Prandi, R., Marquez, M., Gobbo, M., . . . Moreira, J. M. (2015). Mass administration of ivermectin for the elimination of onchocerciasis significantly reduced and maintained low the prevalence of *Strongyloides stercoralis* in Esmeraldas, Ecuador. *PLoS neglected tropical diseases*, 9(11), e0004150.
- Ash, L. R. (1971). Preferential susceptibility of male jirds (*Meriones unguiculatus*) to infection with *Brugia pahangi*. *The Journal of parasitology*, 777-780.
- Ash, L. R. (1973). Chronic *Brugia pahangi* and *Brugia malayi* infections in *Meriones unguiculatus*. *The Journal of parasitology*, 442-447.
- Bandi, C., McCall, J. W., Genchi, C., Corona, S., Venco, L., & Sacchi, L. (1999). Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. *International journal for parasitology*, 29(2), 357-364.
- Bandi, C., Trees, A. J., & Brattig, N. W. (2001). *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. *Veterinary parasitology*, 98(1-3), 215-238.
- Bosshardt, S. C., McCall, J. W., Coleman, S. U., Jones, K. L., Petit, T. A., & Klei, T. R. (1993). Prophylactic activity of tetracycline against *Brugia pahangi* infection in jirds (*Meriones unguiculatus*). *The Journal of parasitology*, 79(5), 775-777.
- Bouma, M., Poveda, G., Rojas, W., Chavasse, D., Quinones, M., Cox, J., & Patz, J. (1997). Predicting high-risk years for malaria in Colombia using parameters of El Niño Southern Oscillation. *Tropical Medicine & International Health*, 2(12), 1122-1127.
- Bouma, M. J., & Dye, C. (1997). Cycles of malaria associated with El Niño in Venezuela. *Jama*, 278(21), 1772-1774.

- Bouma, M. J., & van der Kaay, H. J. (1996). The El Niño Southern Oscillation and the historic malaria epidemics on the Indian subcontinent and Sri Lanka: an early warning system for future epidemics? *Tropical Medicine & International Health*, 1(1), 86-96.
- Bourtzis, K. (2008). Wolbachia-based technologies for insect pest population control *Transgenesis and the management of vector-borne disease* (pp. 104-113): Springer.
- Casiraghi, M., Bordenstein, S., Baldo, L., Lo, N., Beninati, T., Wernegreen, J., . . . Bandi, C. (2005). Phylogeny of Wolbachia pipientis based on gltA, groEL and ftsZ gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the Wolbachia tree. *Microbiology*, 151(12), 4015-4022.
- Chandrashekar, R., Rao, U., Rajasekariah, G., & Subrahmanyam, D. (1984). Separation of viable microfilariae free of blood cells on Percoll gradients. *Journal of Helminthology*, 58(1), 69-70.
- Christensen, B. M., & Sutherland, D. R. (1984). Brugia pahangi: exsheathment and midgut penetration in Aedes aegypti. *Transactions of the American Microscopical Society*, 423-433.
- Chungpivat, S., & Sucharit, S. (1993). Microfilariae in cats in Bangkok. *Wetchasan Sattawaphaet*.
- Cohn, L. A., Gary, A. T., Fales, W. H., & Madsen, R. W. (2003). Trends in fluoroquinolone resistance of bacteria isolated from canine urinary tracts. *Journal of Veterinary Diagnostic Investigation*, 15(4), 338-343.
- Denham, D., & McGreevy, P. (1977). Brugian filariasis: epidemiological and experimental studies *Advances in parasitology* (Vol. 15, pp. 243-309): Elsevier.
- Denham, D., Ponnudurai, T., Nelson, G., Rogers, R., & Guy, F. (1972). Studies with Brugia pahangi—II. The effect of repeated infection on parasite levels in cats. *International journal for parasitology*, 2(4), 401-407.
- Drlica, K., Malik, M., Kerns, R. J., & Zhao, X. (2008). Quinolone-mediated bacterial death. *Antimicrobial agents and chemotherapy*, 52(2), 385-392.
- Edeson, J., Wharton, R., & Laing, A. (1960). A preliminary account of the transmission,

- maintenance and laboratory vectors of *Brugia pahangi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 54(5), 439-449.
- Esslinger, J. (1962). Behavior of microfilariae of *Brugia pahangi* in *Anopheles quadrimaculatus*. *The American Journal of Tropical Medicine and Hygiene*, 11(6), 749-758.
- Ewert, A., & Ho, B. C. (1967). The fate of *Brugia pahangi* larvae immediately after feeding by infective vector mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 61(5), 659-662.
- Fenollar, F., Maurin, M., & Raoult, D. (2003). *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. *Antimicrobial agents and chemotherapy*, 47(5), 1665-1671.
- Fox, L. M., & King, C. L. (2013). 110 - Lymphatic Filariasis. In A. J. Magill, D. R. Hill, T. Solomon, & E. T. Ryan (Eds.), *Hunter's Tropical Medicine and Emerging Infectious Disease (Ninth Edition)* (pp. 815-822). London: W.B. Saunders.
- Gubler, D. J., Reiter, P., Ebi, K. L., Yap, W., Nasci, R., & Patz, J. A. (2001). Climate variability and change in the United States: potential impacts on vector- and rodent-borne diseases. *Environmental Health Perspectives*, 109(suppl 2), 223-233. doi:10.1289/ehp.109-1240669
- Gunawardena, N. K., Fujimaki, Y., Aoki, Y., Mishima, N., Ezaki, T., Uni, S., & Kimura, E. (2005). Differential effects of diethylcarbamazine, tetracycline and the combination on *Brugia pahangi* adult females in vitro. *Parasitology International*, 54(4), 253-259. doi:<https://doi.org/10.1016/j.parint.2005.06.005>
- Gunawardena, S., Ismail, M., Bradley, M., & Karunaweera, N. (2007). Factors influencing drug compliance in the mass drug administration programme against filariasis in the Western province of Sri Lanka. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 101, 445-453. doi:10.1016/j.trstmh.2006.09.002
- Hawking, F. (1979). Diethylcarbamazine and New Compounds for the Treatment of Filariasis. In S. Garattini, A. Goldin, F. Hawking, I. J. Kopin, & R. J. Schnitzer (Eds.), *Advances in Pharmacology* (Vol. 16, pp. 129-194): Academic Press.
- Higazi, T. B., Shu, L., & Unnasch, T. R. (2004). Development and transfection of short-term primary cell cultures from *Brugia malayi*. *Molecular and biochemical*

parasitology, 137(2), 345.

- Hoerauf, A., Specht, S., Büttner, M., Pfarr, K., Mand, S., Fimmers, R., . . . Büttner, D. W. (2008). Wolbachia endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study. *Medical Microbiology and Immunology*, 197(3), 295-311. doi:10.1007/s00430-007-0062-1
- Horton, R. J. (1989). Chemotherapy of Echinococcus infection in man with albendazole. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 83(1), 97-102. doi:10.1016/0035-9203(89)90724-4
- Ichimori, K., King, J. D., Engels, D., Yajima, A., Mikhailov, A., Lammie, P., & Ottesen, E. A. (2014). Global programme to eliminate lymphatic filariasis: the processes underlying programme success. *PLoS neglected tropical diseases*, 8(12), e3328-e3328. doi:10.1371/journal.pntd.0003328
- Kramer, L., Crosara, S., Gnudi, G., Genchi, M., Mangia, C., Viglietti, A., & Quintavalla, C. (2018). Wolbachia, doxycycline and macrocyclic lactones: New prospects in the treatment of canine heartworm disease. *Veterinary parasitology*, 254, 95-97.
- Linthicum, K. J., Anyamba, A., Tucker, C. J., Kelley, P. W., Myers, M. F., & Peters, C. J. (1999). Climate and Satellite Indicators to Forecast Rift Valley Fever Epidemics in Kenya. *Science*, 285(5426), 397-400. doi:10.1126/science.285.5426.397
- Liu, L. X., & Weller, P. F. (1996). Antiparasitic Drugs. *New England Journal of Medicine*, 334(18), 1178-1184. doi:10.1056/nejm199605023341808
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods*, 25(4), 402-408.
- Mahajan, R. S., Veerpathran, A., Dakshinamoorthy, G., Sharma, R. D., Goswami, K., & Reddy, M. V. (2010a). Effect of certain antibiotics against filarial parasite *Brugia malayi* in vitro: possible role of oxidative stress. *Indian Journal of Clinical Biochemistry*, 25(4), 362-366.
- Mahajan, R. S., Veerpathran, A., Dakshinamoorthy, G., Sharma, R. D., Goswami, K., & Reddy, M. V. (2010b). Effect of Certain Antibiotics Against Filarial Parasite *Brugia malayi* In Vitro: Possible Role of Oxidative Stress. *Indian journal of clinical*

biochemistry : IJCB, 25(4), 362-366. doi:10.1007/s12291-010-0068-0

- Mak, J. W., Pkf, Y., Lim, P., & N, R. (1980). Zoonotic implications of cats and dogs in filarial transmission in Peninsular Malaysia. *Tropical and geographical medicine*, 32, 259-264.
- Mallo, G. V., Kurz, C. L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y., & Ewbank, J. J. (2002). Inducible antibacterial defense system in *C. elegans*. *Current Biology*, 12(14), 1209-1214.
- McCall, J. W., Malone, J. B., Ah, H.-S., & Thompson, P. E. (1973). Mongolian jirds (*Merlans unguiculatus*) infected with *Brugia pahangi* by the intra-peritoneal route: a rich source of developing larvae, adult filariae, and microfilariae. *Journal of Parasitology*, 59(3).
- McGarry, H. F., Egerton, G. L., & Taylor, M. J. (2004). Population dynamics of *Wolbachia* bacterial endosymbionts in *Brugia malayi*. *Molecular and biochemical parasitology*, 135(1), 57-67.
- Melrose, W. D. (2002). Lymphatic filariasis: new insights into an old disease. *International journal for parasitology*, 32(8), 947-960. doi:[https://doi.org/10.1016/S0020-7519\(02\)00062-0](https://doi.org/10.1016/S0020-7519(02)00062-0)
- Molyneux, D., & Zagaria, N. (2003). Lymphatic filariasis elimination: Progress in global programme development. *Annals of tropical medicine and parasitology*, 96 Suppl 2, S15-40. doi:10.1179/000349802125002374
- Moreno, Y., Nabhan, J. F., Solomon, J., Mackenzie, C. D., & Geary, T. G. (2010). Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*. *Proceedings of the National Academy of Sciences of the United States of America*, 107(46), 20120-20125. doi:10.1073/pnas.1011983107
- Mukherjee, S., Joardar, N., Mondal, S., Schiefer, A., Hoerauf, A., Pfarr, K., & Babu, S. P. S. (2018). Quinolone-fused cyclic sulfonamide as a novel benign antifilarial agent. *Sci Rep*, 8(1), 12073. doi:10.1038/s41598-018-30610-7
- Nutman, T. B. (2017). 121 - Filarial Infections. In J. Cohen, W. G. Powderly, & S. M. Opal (Eds.), *Infectious Diseases (Fourth Edition)* (pp. 1046-1052.e1041): Elsevier.
- Obeck, D. K. (1973). Blood microfilariae: new and existing techniques for isolation.

Journal of Parasitology, 59(1), 220-222.

- Ottesen, E. A., Hooper, P. J., Bradley, M., & Biswas, G. (2008). The Global Programme to Eliminate Lymphatic Filariasis: Health Impact after 8 Years. *PLoS neglected tropical diseases*, 2(10), e317. doi:10.1371/journal.pntd.0000317
- Palmieri, J. R., Ratiwayanto, S., Masbar, S., Tirtokusumo, S., Rusch, J., & Marwoto, H. A. (1985). Evidence of possible natural infections of man with *Brugia pahangi* in South Kalimantan (Borneo), Indonesia. *Tropical and geographical medicine*, 37(3), 239-244.
- Papich, M. G. (2016). Enrofloxacin. In M. G. Papich (Ed.), *Saunders Handbook of Veterinary Drugs (Fourth Edition)* (pp. 287-289). St. Louis: W.B. Saunders.
- Rao, R., & J Well, G. (2002). In vitro Effects of Antibiotics on *Brugia malayi* Worm Survival and Reproduction. *The Journal of parasitology*, 88, 605-611. doi:10.1645/0022-3395(2002)088[0605:IVEOAO]2.0.CO;2
- Rao, R. U. (2005). Endosymbiotic *Wolbachia* of parasitic filarial nematodes as drug targets. *Indian Journal of Medical Research*, 122(3), 199.
- Rishniw, M., Barr, S. C., Simpson, K. W., Frongillo, M. F., Franz, M., & Dominguez Alpizar, J. L. (2006). Discrimination between six species of canine microfilariae by a single polymerase chain reaction. *Veterinary parasitology*, 135(3), 303-314. doi:<https://doi.org/10.1016/j.vetpar.2005.10.013>
- Schacher, J. F. (1962). Developmental stages of *Brugia pahangi* in the final host. *The Journal of parasitology*, 693-706.
- Schacher, J. F., & Sahyoun, P. F. (1967). A chronological study of the histopathology of filarial disease in cats and dogs caused by *Brugia pahangi* (Buckley and Edeson, 1956). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 61(2), 234-243.
- Simoncini, L., Casiraghi, M., Bazzocchi, C., Sacchi, L., Bandi, C., & Genchi, C. (2002). Real-time PCR for quantification of the bacterial endosymbionts (*Wolbachia*) of filarial nematodes. *Parassitologia*, 43, 173-178.
- Slatko, B. E., Taylor, M. J., & Foster, J. M. (2010). The *Wolbachia* endosymbiont as an anti-filarial nematode target. *Symbiosis*, 51(1), 55-65. doi:10.1007/s13199-010-0067-1

- Smith, H. L., & Rajan, T. V. (2000). Tetracycline Inhibits Development of the Infective-Stage Larvae of Filarial Nematodes in Vitro. *Experimental parasitology*, 95(4), 265-270. doi:<https://doi.org/10.1006/expr.2000.4525>
- Sneller, V.-P., & Weinstein, P. P. (1982). In vitro development of *Dirofilaria immitis* microfilariae: selection of culture media and serum levels. *International journal for parasitology*, 12(2-3), 233-238.
- Srivastava, S. K., Chauhan, P. M. S., Bhaduri, A. P., Fatima, N., & Chatterjee, R. K. (2000). Quinolones: Novel Probes in Antifilarial Chemotherapy. *Journal of Medicinal Chemistry*, 43(11), 2275-2279. doi:10.1021/jm990438d
- Stouthamer, R., Breeuwer, J. A., & Hurst, G. D. (1999). *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annual Reviews in Microbiology*, 53(1), 71-102.
- Sungpradit, S., Chatsuwan, T., & Nuchprayoon, S. (2012). Susceptibility of *Wolbachia*, an endosymbiont of *Brugia malayi* microfilariae, to doxycycline determined by quantitative PCR assay. *Southeast Asian Journal of Tropical Medicine & Public Health*, 43(4), 841-850.
- Supali, T., Djuardi, Y., Pfarr, K. M., Wibowo, H., Taylor, M. J., Hoerauf, A., . . . Sartono, E. (2008). Doxycycline treatment of *Brugia malayi*—infected persons reduces microfilaremia and adverse reactions after diethylcarbamazine and albendazole treatment. *Clinical infectious diseases*, 46(9), 1385-1393.
- Tan, L. H., Fong, M. Y., Mahmud, R., Muslim, A., Lau, Y. L., & Kamarulzaman, A. (2011). Zoonotic *Brugia pahangi* filariasis in a suburbia of Kuala Lumpur City, Malaysia. *Parasitology International*, 60(1), 111-113. doi:<https://doi.org/10.1016/j.parint.2010.09.010>
- Taweethavonsawat, P., & Chungpivat, S. (2013). Successful treatment of *Brugia pahangi* in naturally infected cats with ivermectin. *The Korean journal of parasitology*, 51(6), 759-761. doi:10.3347/kjp.2013.51.6.759
- Taylor, M. J., Bandi, C., & Hoerauf, A. (2005). *Wolbachia*. Bacterial Endosymbionts of Filarial Nematodes. In J. R. Baker, R. Muller, & D. Rollinson (Eds.), *Advances in Parasitology* (Vol. 60, pp. 245-284): Academic Press.

- Turba, M. E., Zambon, E., Zannoni, A., Russo, S., & Gentilini, F. (2012). Detection of Wolbachia DNA in blood for diagnosing filaria-associated syndromes in cats. *Journal of clinical microbiology*, *50*(8), 2624-2630. doi:10.1128/JCM.00528-12
- Wiser, M. F., & Lanners, H. N. (1992). Rapid transport of the acidic phosphoproteins of *Plasmodium berghei* and *P. chabaudi* from the intraerythrocytic parasite to the host membrane using a miniaturized fractionation procedure. *Parasitology research*, *78*(3), 193-200.
- Wong, M. M., & Guest, M. F. (1969). Filarial antibodies and eosinophilia in human subjects in an endemic area. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *63*(6), 796-800.
- Yokmek, S., Warunyuwong, W., Rojanapanus, S., Jiraamornimit, C., Boitano, J., & Wongkamchai, S. (2012). A case report of Brugian filariasis outside an endemic area in Thailand. *Journal of Helminthology*, *87*, 1-5. doi:10.1017/S0022149X12000533

APPENDIX

Appendix A

Preparation of reagents

- 1. 35% Percoll gradient solution**
 - 1.1 Percoll 18 ml
 - 1.2 2.5 M sucrose 2 ml
 - 1.3 sterile water 6.5 ml

- 2. 40% Percoll gradient solution**
 - 2.1 Percoll 18 ml
 - 2.2 2.5 M sucrose 2 ml
 - 2.3 sterile water 6 ml

- 3. RPMI complete media**
 - 3.1 Fetal bovine serum (FBS) 50 ml
 - 3.2 100x L-glutamine 5 ml
 - 3.3 100x antibiotic/antimycotic 5 ml
 - 3.4 HEPES 12.5 ml
 - 3.5 Advance RPMI-1640 to 50 ml



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Appendix B

Protocol for parasite culture

1. Add 50 μ l of RPMI complete media to each wells of 96-well culture plate
2. Prepare a two-fold dilution of drugs until reach 1/512 dilution
3. Add 50 μ l of microfilarial content in the serial dilution of drugs
4. Incubate at 37 °C for 7 days in a humidified atmosphere containing 5% CO₂
5. Observe daily under inverted microscope for 7 days and scored as 0-4



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LIST OF ABBREVIATIONS

Bp	base pair
Ct	cycle Threshold
FBS	fetal bovine serum
G	gram (s)
HEPES	hydroxyethyl piperazineethanesulfonic acid
IFN	interferon
IL	interleukin
Kb	kilobase pair
MECs	minimum effective concentration
ml	millilitre (s)
mM	millimolar
mm	millimetre (s)
qPCR	quantitative polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
μ l	microliter



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