SUSCEPTIBILITY OF *WOLBACHIA*, AN ENDOSYMBIONT OF *BRUGIA MALAYI* MICROFILARIAE, TO DOXYCYCLINE DETERMINED BY QUANTITATIVE PCR ASSAY

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Abstract. Lymphatic filariasis, caused by filarial nematodes, is a mosquito-borne disease that affects over 120 million people in the tropics and subtropics. The disease is caused mainly by *Wuchereria bancrofti* and *Brugia malayi*. Fertile adult female worms release offsprings (microfilariae) into the host blood circulatory system. Transmission-blocking agents as well as antimicrobial agents have been used to reduce microfilarial density in human and animal reservoir hosts. Doxycycline and rifampicin have an effect on the obligate intracellular gram-negative bacteria, *Wolbachia*, which appears to exert an 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, expressed as minimum effective concentration (MEC), and on *Wolbachia* proliferation using quantitative PCR, expressed as the concentration of the drug to inhibit bacteria growth by 50% (IC_{50}). MEC of doxycycline was 128 and 32 µg/ml at 12 and 52 hours, respectively, but rifampicin and ciprofloxacin were ineffective (MEC >256 µg/ml). IC_{50} of doxycycline was 32 and 2 µg/ml at 12 and 52 hours, but this for rifampicin (8 µg/ml) and ciprofloxacin (32 µg/ml) were obtained only after 52 hour treatment. Thus, MEC and IC_{50} assay methods used in this study could be applied to screen other agents targeting filariae and their endosymbiont bacteria.

Keywords: *Wolbachia*, microfilaria, minimum effective concentration, IC_{50}, qPCR

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INTRODUCTION

Lymphatic filariasis, caused by lymphatic-dwelling nematode parasite, is transmitted through mosquito vectors (Guilbert, 2003). In Thailand, the majority of lymphatic filariasis is primarily caused by *Wuchereria bancrofti* (bancroftian filariasis) and *Brugia malayi* (brugian or malayan filariasis) (Triteeraprapab *et al*, 1999, 2000,

2001a,b; Nuchprayoon *et al*, 2001, 2003a,b, 2006). The adult worms reside in the lymphatic vessels, where they cause damage, and the females release an abundance of blood-stage microfilariae into the host's circulatory system (O'Connor *et al*, 2003). The microfilarial as well as adult stages are associated with the complicated disease pathology.

Transmission-blocking agents, such as ivermectin, diethylcarbamazine (DEC), ivermectin combined with DEC, albendazole combined with either ivermectin or DEC, and antimicrobial agents, such as doxycycline, have been used to reduce microfilarial density in human and animal reservoir hosts (Rao *et al*, 1990; Ismail *et al*, 1996; Shenoy *et al*, 1999, 2000; Supali *et al*, 2002; Hoerauf *et al*, 2003; Chansiri *et al*, 2005). In bancroftian and brugian filariasis, from 3- to 8-week course of treatment with doxycycline alone or in combination with albendazole or ivermectin, results in microfilaricidal effect, decreases microfilaremic level, and diminishes adverse drug reactions (Hoerauf *et al*, 2003; Taylor *et al*, 2005; Turner *et al*, 2006; Debrah *et al*, 2007; Supali *et al*, 2008). A single dose of doxycycline combined with the standard DEC regimen can increase the efficacy of standard treatments by reducing filarial antigen level as well as the adverse drug reactions (Sanprasert *et al*, 2010).

As microfilariae contribute to pathogenesis of lymphatic filariasis patients (Bennuru and Nutman, 2009), the microfilarial stage could provide a target to limit severity of disease pathology and interrupt disease transmission (Ottesen *et al*, 1997). The role of microfilariae in human filarial infection involves immunomodulation and induction of host cell apoptosis (O'Connor *et al*, 2003; Semnani *et al*, 2003; Harnett and Harnett, 2006, 2008; Semnani *et al*, 2008).

All stages of filarial nematodes, including *W. bancrofti* and *B. malayi*, contain the obligate intracellular gram-negative bacteria, the mutualistic endosymbiont *Wolbachia*, which is responsible for filarial nematode embryonic and larval growth and development, as well as adult female fertility and survival (Taylor and Hoerauf, 1999; Hoti *et al*, 2003; Fenn and Blaxter, 2004; McGarry *et al*, 2004; Taylor *et al*, 2005). In addition, *Wolbachia* released by living and/or dead microfilariae can induce an inflammatory response (unpublished data). *Wolbachia* proteins, such as *Wolbachia* surface protein (WSP) and heat-shock protein 60 (HSP60), are involved in stimulation of host inflammatory response (Simón *et al*, 2007). The reactions are associated with the increase in systemic proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 (Taylor, 2000, 2003; Porksakorn *et al*, 2007; Sanprasert *et al*, 2010) and with inflammatory mediators, such as *Wolbachia* lipoprotein (Turner *et al*, 2009).

To date, no long-term *B. malayi* cell culture system exists with which to study the mutualistic mechanism of the bacterial endosymbiont and related filarial parasites. Antimicrobial susceptibility of *Wolbachia* and related species (*viz*, *Chlamydia trachomatis*, *Rickettsia* spp, *Ehrlichia* spp, *Anaplasma phagocytophilum*, and *Coxiella burnetii*) has been studied using conventional (*viz*, microscopic counting and immunological assay) and molecular biology [*viz*, quantitative polymerase chain reaction (qPCR)] assays (Hermans *et al*, 2001; 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; Makepeace *et al*, 2006). *Wolbachia* maintained in insect cell cultures have been tested for

susceptibility to 13 antimicrobial agents (Fenollar *et al*, 2003), with doxycycline and rifampicin showing high effectiveness as anti-*Wolbachia* agents. An *in vitro* antimicrobial susceptibility study in *B. malayi* microfilariae has shown that doxycycline causes >50% reduction in microfilarial motility after 24 and 48 hours after exposure to 80 and 40 µg/ml, respectively (Rao and Weil, 2002).

As *Wolbachia* antimicrobial susceptibility in a filarial model has not yet been developed, we used a *B. malayi* culture system (Rao and Weil, 2002) to study the effects of antimicrobial agents, focusing on blood stage microfilaria. We measured minimum effective concentration (MEC), which is defined as the minimum concentration of an antimicrobial agent that can inhibit *B. malayi* microfilarial motility as determined by a motility-scoring test, at different time points. In addition, IC_{50} value, defined as the concentration of an antimicrobial agent concentration that inhibits 50% *Wolbachia* growth in microfilariae, was determined by qPCR.

MATERIALS AND METHODS

Parasite

B. malayi microfilariae (TRS strain) were isolated from peritoneal cavity of infected male jirds (*Meriones unguiculatus*) at >120 days post-infection. Jirds were obtained from 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). Microfilariae were washed 3 times with RPMI-1640 (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). Microfilarial concentration was determined by dilution counting under light microscope.

Preparation of antimicrobial agents

Antimicrobials used (0.125 to 256 µg/ml) were doxycycline (Sigma-Aldrich, St Louis, MO), rifampicin (Sigma-Aldrich) and ciprofloxacin (Fluka BioChemika, Buchs, Switzerland). Stock solutions were prepared as described by the manufacturer and stored at -20°C. Doxycycline and ciprofloxacin were dissolved in sterile distilled water and rifampicin in chloroform. Final antimicrobial solutions were made up fresh before use by diluting concentrated stock solutions in culture medium (RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/ml streptomycin, 100 mg/ml penicillin, 0.25 mg/ml amphotericin B, and 10% fetal calf serum). The culture medium has been proven to have no effect on microfilarial motility (Rao and Weil, 2002).

Microfilarial motility assay

Three cultures containing 1,000 microfilariae in duplicate were set up in 24-well multiwell plates (Becton Dickinson, Franklin Lakes, NJ) containing 1 ml of culture medium. Microfilariae were cultured overnight in either culture medium alone (control) or culture medium containing various concentrations of antimicrobial agents under 5% CO_2 atmosphere at 37°C. Microfilariae were observed under a stereomicroscope (Nikon SMZ-1500, Tokyo, Japan) after 12 hours and 52 hours by two independent investigators and motility was scored (0-4) as described previously (Rao and Weil, 2002). MEC was the lowest antimicrobial concentration required to inhibit microfilarial motility (score 0) compared with control at the two time points.

Antimicrobial susceptibility of *Wolbachia* assay using qPCR

B. malayi microfilariae [1,000, cultured in either culture medium or culture medium with antimicrobials (0.125, 0.5, 2, 8, 32, and 128 µg/ml)] were separated from culture medium by centrifugation at 1,400*g* for 10 minutes, and genomic DNA isolated using lysis buffer and phenol-chloroform extraction method as previously reported by McGarry *et al* (2003). Concentration of each microfilaria DNA sample was measured with a nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) according to the manufacturer's protocol. DNA samples then were stored at -20ºC until used.

Primers specific for single copy gene of *Wolbachia* (*wsp*) and of *B. malayi* (*hsp70*) were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA), to amplify an 87-bp *wsp* and 70-bp *hsp70* amplicon, respectively (GenBank accession no. AJ252061 and XM_001900162, respectively). The sequences of *wsp* and *hsp70* forward and reverse primers (purchased from Applied Biosystems) are shown in Table 1. SYBR green I dye qPCR assay was used to determine copy number of *wsp* relative to that of *hsp70*. Reaction mixtures were prepared in a 96-well optical reaction plate (Applied Biosystems), according to the manufacturer's protocol. In brief, qPCR mixture consisted of 12.5 µl of 2X SYBR green PCR master mix, 0.45 µl of each 50 µM primer, 5 µl of microfilaria DNA, and water to make up to a final volume of 25 µl. After centrifugation at 126*g* for 2 minutes, the reaction plate was placed in the ABI Prism 7500 instrument (Applied Biosystems). Cycle threshold (C_t) values were determined employing a thermocycling program consisting of 2 minutes at 50ºC; 10 minutes at 95ºC; and cycling at 95ºC for 15 seconds and at 60ºC for 60 seconds. PCR was carried out in duplicate. Analysis was performed using ABI 7500 system SDS software package (Applied Biosystems) and the results were evaluated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmitten, 2001). IC_{50} value for antimicrobial inhibition of *Wolbachia* growth was the concentration of antimicrobial that caused *wsp*/*hsp70* copy number (a_x) in the presence of drug to be 0.5 of *wsp*/*hsp70* copy number (a_o) in the absence of the antimicrobial.

RESULTS

Antimicrobial susceptibility of *B. malayi* microfilariae

The effects of three antimicrobial agents on microfilarial motility, measured as MEC, demonstrated that treatment by doxycycline for 12 and 52 hours inhibited microfilarial motility at 128 and 32 µg/ml, respectively (Table 2). Rifampicin and ciprofloxacin were less effective, with MEC >256 µg/ml at 12 and 52 hours.

Antimicrobial susceptibility of *Wolbachia*

The susceptibility of *Wolbachia* to antimicrobial agents was evaluated based on IC_{50} values obtained by qPCR using a comparative C_t method. Doxycycline was the most effective compound, with IC_{50} value of 32 and 2 µg/ml at 12 and 52 hours, respectively (Table 2). Rifampicin and ciprofloxacin did not inhibit *Wolbachia* growth at concentration up to 128 µg/ml at 12 hours, but at 52 hours IC_{50} value of 8 and 32 µg/ml was obtained for rifampicin and ciprofloxacin, respectively.

DISCUSSION

We investigated the effects of antimicrobial agents (doxycycline, rifampicin and ciprofloxacin) on the motility of *B.*

Table 1
Primers used in qPCR analysis of *Wolbachia* and *B. malayi*.

Gene	Primer	Sequence (5´⟶3´)	Amplicon length (bp)
Wolbachia wsp	Forward	GGT GTT GGT CTT GGT GTA GCA TAT	87
	Reverse	GTA AGC AAA ACC AAA CCC ATG TT	
B. malayi hsp70	Forward	CAG AAG AGA TTT CGT CGA TGG TT	70
	Reverse	ACC GCG TGA CCC AGA AAA	

Table 2
B. malayi MEC and *Wolbachia* IC_{50} values for doxycycline, rifampicin and ciprofloxacin.

	12 hour treatment			52 hour treatment		
	Doxycycline	Rifampicin	Ciprofloxacin	Doxycycline	Rifampicin	Ciprofloxacin
MEC (µg/ml)	128	>256	>256	32	>256	>256
IC_{50} (µg/ml)	32	>128	>128	2	8	32

malayi microfilariae in comparison with their ability to inhibit *Wolbachia* growth.

MECs of the antimicrobial agents reflect microfilarial viability, and were used to compare efficacies of the antimicrobial agents. Our findings support earlier studies demonstrating that doxycycline is the most effective antimicrobial agent compared with rifampicin and ciprofloxacin (Rao and Weil, 2002; Mahajan *et al*, 2010). Bacteriostatic drugs, such as tetracycline, also can inhibit eukaryotes by inhibiting mitochondrial protein translation (Zhang *et al*, 2005). The insensitivity of *B. malayi* microfilariae to rifampicin and ciprofloxacin is not surprising as the compounds inhibit bacterial DNA-dependent RNA polymerase (Calvori *et al*, 1965) and DNA gyrase, respectively (Gore *et al*, 2006). In assessing the status of microfilarial viability, we did not use the standard MTT assay, which reflects mitochondrial succinate dehydrogenase activity, as *Wolbachia* also contains succinate dehydrogenase and such MTT results obtained from antimicrobial-treated microfilariae that contain *Wolbachia* would lead to a misinterpretation of the parasite viability (Townson *et al*, 2006).

Wolbachia DNA content, obtained from qPCR assay, was used to investigate the presence and proliferation of the bacteria in filarial nematodes. The single copy genes of *Wolbachia* (*wsp* and *ftsz*) and of *B. malayi* (*gst*, *tub-1* and *ama-1*) have been used to study the population dynamics of *Wolbachia* during all stages of *B. malayi* life cycle (Fenn and Blaxter, 2004; McGarry *et al*, 2004). *Wolbachia*/*B. malayi* gene ratios obtained from such studies produce the same pattern irrespective of the gene pair employed. Furthermore, *Wolbachia*/nematode DNA ratios have been used to study infected adult *Dirofilaria immitis* treated with a combination of ivermectin and doxycycline, and in the differentiation of virulent and mild strains of infected *Onchocerca volvulus* (Higazi *et al*, 2004; Bazzocchi *et al*, 2008). Thus, the bacterium/nematode DNA

ratios provide a convenient indicator of changes in *Wolbachia* population within the parasite under various conditions. In this study, we could not obtain MIC values of antimicrobials against *Wolbachia* located in blood-stage microfilariae because the bacteria divided slowly compared with *Wolbachia* in an insect cell culture system (Fenollar *et al*, 2003) or in filarial infective (L3) stage (McGarry *et al*, 2004). Although qPCR is useful for the detection of small amounts of bacterial DNA and/or RNA, it has not proven to be suitable for determining filarial *Wolbachia* MIC in this study. Recently, the expression of *Wolbachia wsp* following treatment of *B. malayi* microfilariae with ivermectin or moxidectin has been investigated using qRT-PCR (Tompkins *et al*, 2010). However, measurement of only *wsp* expression does not indicate the possible drug's effect on microfilariae.

Previous studies of antimicrobial susceptibility of filarial *Wolbachia* have been limited because no long-term filarial cell culture system has been developed. Recently, insect *Wolbachia* drug susceptibility has been investigated using immunofluorescense and qPCR techniques, in which *Wolbachia*-infected insect cells were incubated with 13 different antimicrobial agents for 6 days (Fenollar *et al*, 2003). Both assays showed similar results, namely, doxycycline and rifampicin were the most effective drugs, with MIC of 0.125 and 0.06-0.125 µg/ml, respectively. In this study, we showed that, among rifampicin, ciprofloxacin and doxycycline, the latter was the most efficacious against filarial *Wolbachia* residing in *B. malayi* microfilariae. Previous studies have shown that tetracycline could not clear *Wolbachia* DNA from *B. malayi* L3 larvae, cultured in antibiotic-containing medium for 8 days (Smith and Rajan, 2000; Rajan, 2004). Tetracycline inhibits *Wolbachia* protein synthesis but the *Wolbachia* remnants in adult *B. malayi* uterine microfilariae still are present even after 5 days after treatment (Ghedin *et al*, 2009). There was a concordance in IC_{50} and MEC values determined at two time points (12 and 52 hours), but the decrease in MEC for *B. malayi* at the longer exposure was not as large as that observed for IC_{50} value of *Wolbachia*, possibly due to the existence of a plethora of repair mechanisms in eukaryote against xenobiotics. Rifampicin and ciprofloxacin did not manifest any anti-Rickettsia property at the short time exposure, and required a longer exposure to show bactericidal properties, which may be due to intrinsic insensitivity of *Wolbachia* to these two drugs and/or limitation of access of the drugs (including doxycycline) to the target bacteria located in the microfilariae hypodermal cells than cells in culture (Rao and Weil, 2002). Nevertheless, these results lend support to the mutualistic relation between this bacterium and its nematode host, in which *Wolbachia* provides essential products such as riboflavin, flavin, heme and nucleotide to microfilariae (Fenn and Blaxter, 2006).

In summary, we showed that doxycycline is the most effective antimicrobial agent for inhibiting *B. malayi* microfilarial motility and is effective against endosymbiont *Wolbachia*. The MEC and IC_{50} assays used in this study can be adapted to test other anti-*Wolbachia* agents.

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