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Molecular Diagnosis and Monitoring of Benzimidazole Susceptibility of Human Filariids

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1. Introduction

Lymphatic filarial nematode parasites, mainly *Wuchereria bancrofti* and *Brugia malayi,* are causing agents of lymphatic filariasis in humans, which can be effectively treated with antifilarial drugs including diethylcarbamazine (DEC) and ivermectin. Albendazole, an effective benzimidazole compound, acts as a board-spectrum anthelminthic drug, and when combined with either one of antifilarial drugs, it exerts synergistic effects on reduction of peripheral microfilaremia in lymphatic filariasis cases. However, the varying parasite infection levels in those treated with DEC or ivermectin alone or in combination with albendazole are due to differences in drug responses. The additional clearance of infection with albendazole relative to what is observed with DEC or ivermectin alone suggests that albendazole has different parasite target(s). The homologous *β-tubulin* gene of human and veterinary filariids that β-tubulin homologs have conserved domains structurally related to other orthologs among the nematodes, cestodes, trematodes and vertebrate hosts, is responsible for benzimidazole susceptibility. The genetic inheritance of resistance in nematode parasites can undergo under selection of benzimidazole compounds in a way that albendazole resistance mechanism involves one of two single amino acid substitutions from phenylalanine to tyrosine in parasite β-tubulin at position 167 or 200. This genetically-stable marker has shown promise for molecular diagnosis and monitoring of *W. bancrofti* infections that carry responsible genotypes associated with benzimidazole susceptibility or resistance. In particular, this approach can augment the surveillance and monitoring of mass treatment impacts on the parasite populations in target areas where long-running elimination programs for lymphatic filariasis are implemented at a large-scale by using a regionally-adopted combination therapy with antifilarial drugs, recommended by the World Health Organization.

2. Lymphatic filariasis towards elimination

2.1 Factors that favor elimination

Lymphatic filariasis (LF) is a mosquito-borne parasitic disease caused by three main species of thread-like filarial nematode parasites belonging to the superfamily Filarioidea, which are *Wuchereria bancrofti* and *Brugia malayi*, and lesser extent by *Brugia timori*. These endoparasitic nematode lifestyles have a highly conservative life-cycle development sequence in which they develop their metamorphosis in both human and mosquito (Fig. 1). The adult worms that cause clinical manifestations of the disease are dioecious. Male and female worms have separate reproductive system in their pseudocoelomatic body cavity and they live in the

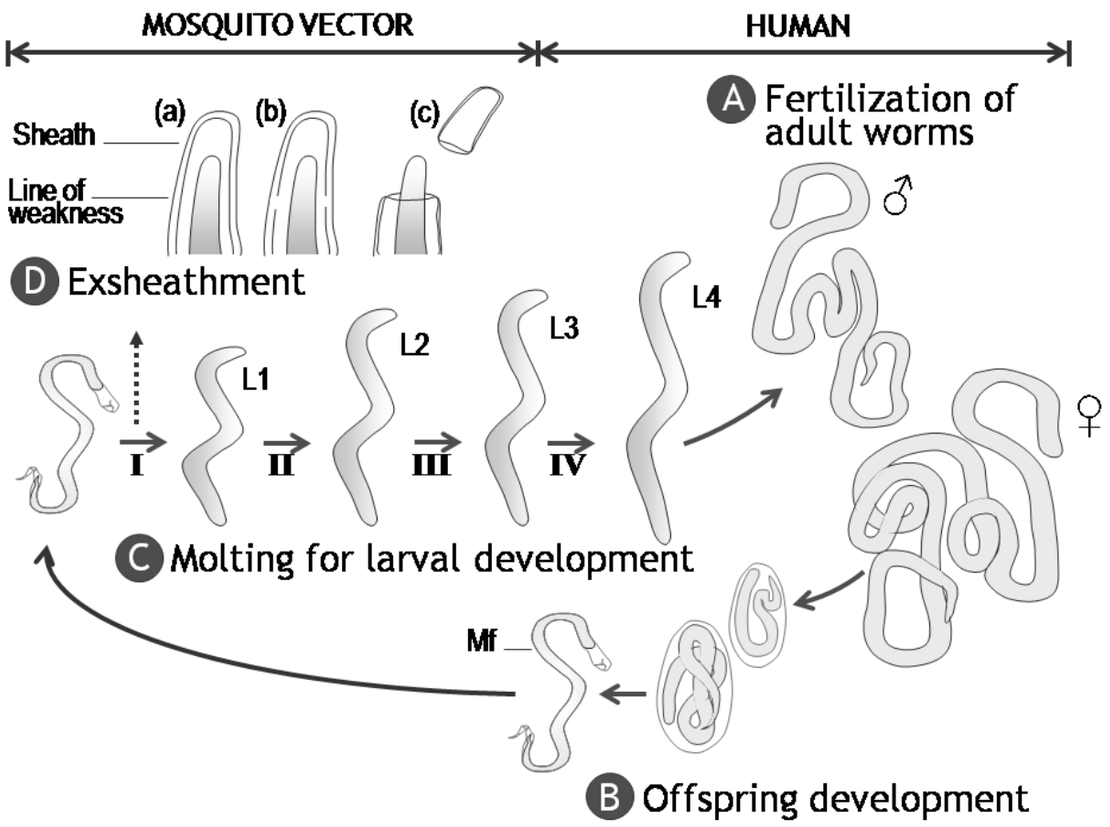

Fig. 1. A development sequence of filarial nematode parasite in human and mosquito (A-D). After the induction of L3 infection in susceptible human host, the mature filarial female worm (A) that possesses fecundicity and fertilization can produce microfilariae (Mf). These offsprings develop from fertilized eggs (B) in the uterus of female worm. They are ingested during the bite of mosquito vector, and consequently, a 5-stage molting progression (B) initially starts after exsheatment of the Mf in mosquito's midgut (C). In this regard, the probable mechanisms involve: (a) proposed anterior of line of weakness, (b) internal digestion of the sheath, and (c) exsheatment of anterior end. Third stage larvae (L3) possess post-infective stage development in mosquito thoracic muscle and then migrate to the proboscis. They are transmitted by infective mosquito during a blood meal, and that they become the L4 (or L5) and mature adult worms in the lymphatic system in human.

human lymphatic system, i.e., lymphatic vessels, with 5-15 years of life expectancy (Table 1). Only when its fecundic lifespan is capable of mating does the lymphatic-dwelling female worm produce advanced stage of sheathed larvae called "microfilariae". These short-living offsprings then penetrate the blood circulation. For a complete life cycle, the microfilariae are ingested by susceptible female mosquito during a blood meal in which they can develop further into larval stages L1-L2 to infective L3 stage. Transmission occurs when the infected mosquito transmits this L3 stage to susceptible persons during other blood meals. The naturally acquired transmission is associated with both intrinsic and extrinsic factors that can regulate the parasitic worm burdens in an endemic population (Fig. 2).

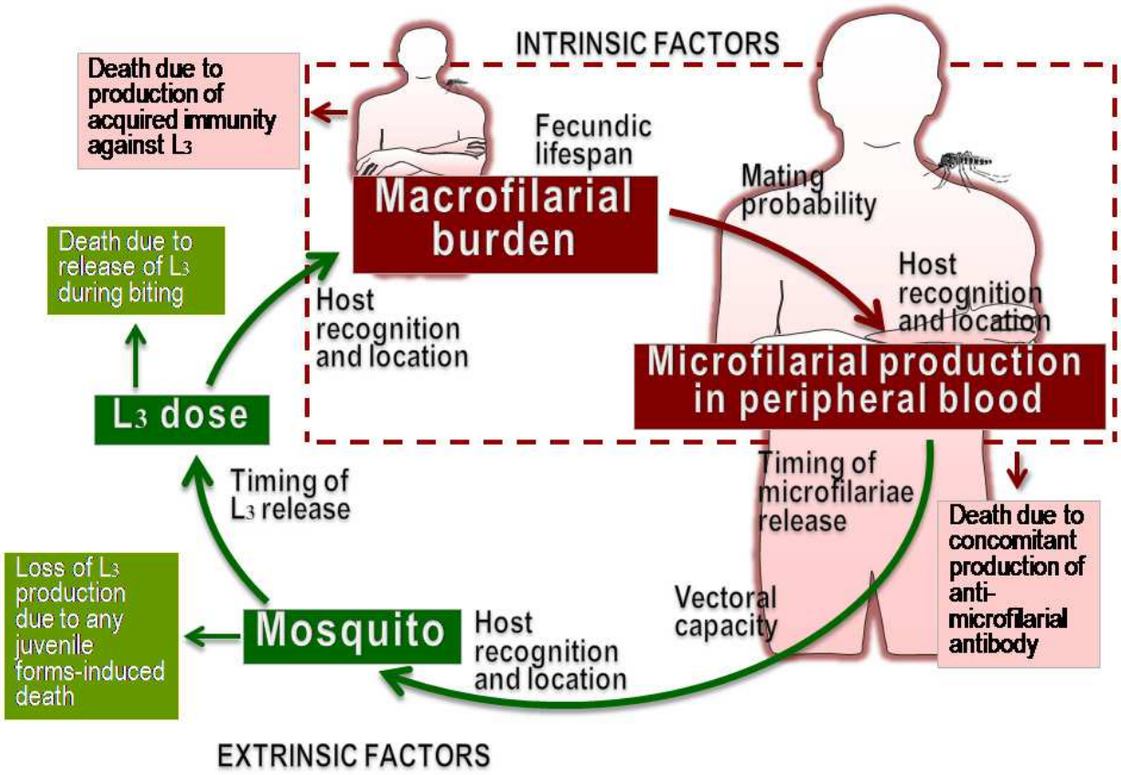

Fig. 2. An explanatory model of human-parasite-vector interactions and favorable factors that influence its adaptation in hosts. The lymphatic filarial infections in humans depend on extrinsic factors of incoming L3 inocula and host recognition and location, and a proportion of adult worms with fecundicity and mating probability. Transmission is influenced by host-vector combination (host location and recognition of mosquito feeding habits), microfilarial loads in blood with timing of microfilariae release, and vectoral capacity (longevity and low refractoriness). Long lifespan of the mosquito and timing of L3 release are favorable factors of transmission.

As such, understanding of how these filarial nematode parasites can be removed from the human hemisphere is to understand basically the biology of their life cycle. How do the parasite taxa succeed their complete life cycle in the certain conditions under which they evoke host exploitation strategies? The biology of the parasitic symbionts can disclosure the parasite diversity and fitness shaped by hosts and environmental constraints that the

parasites can evolve the adaptation, i.e., the ability to control the physiology and behavior of their host for their own benefit. It has been known so far their distribution among hosts and strategies of host exploitation are restricted in number of host species. The *W. bancrofti*, which is highly host-specific parasite, is sessile to human while other zoonotic *Brugia* taxa exploit other non-human reservoir hosts. This host-specificity variation does not account for the origin of the parasitism as they succeed their parasitism and share common transmission pattern and complex life cycle in vertebrate hosts and arthropods. The geographical variation, on the other hand, can be restrained by selective pressures from the hosts, or the physio-chemical environments such as therapeutic agents and insecticides, or due to phylogenetic constraints. Such this selection can be explained by the experimental infections, mainly using *Brugia* species in rodents (e.g., jirds, hamsters, rats and mice), dogs, ferrets, cats and monkeys. All of which were proposed not just for understanding vertebrate immunology, pathology and chemotherapy, but also for exploring the interactions of the parasite and host. Among these host-parasite systems, *B. pahangi* that infects naturally cats in the Southeast Asia is experimentally appropriate for studies of infection and disease dynamics (Table 1).

Parameter	**Cat**	**Human**
Parasite	*Brugia pahangi*	*Wuchereria bancrofti* *B. malayi, B. timori*
Longevity (years)	7-8	5-15
Mature female worm length (cm)	3-7	5-10
Location of :		
Adult	Lymphatics	Lymphatics
Microfilariae	Blood	Blood
Vector	Mosquito	Mosquito
Typical infection/disease sequlae[a]	Infection – Loss of infection – Pathology	Infection – Loss of infection – Pathology

[a]For both host-parasite systems, infection patterns encapsulate a spectrum ranging from asymptomatic persistent microfilaremia to symptomatic amicrofilaremia or complete refractoriness to infection. Adapted from Grenfell et al (1991).

Table 1. Correlation between feline and human lymphatic filariasis

Because of their conservation of complex life-cycle development between feline and human lymphatic filarial parasites, it induces the infection and clinical sequelae in susceptible cats, and hence, resembles that of *Brugia* and *Wuchereria* in humans. However, the parasite does not always succeed their population diversity by increasing its fitness in the hosts (Fig. 2). Its population dynamic is primarily influenced by naturally acquired immunity (i.e., a type of concomitant immunity), which plays a significant role in host selection pressure to restrict a parasitic worm burden (Bundy et al, 1991; Grenfell et al, 1991; Mitchell, 1991; Grenfell and Micheal, 1992). This immunity against incoming L3 cannot remove them from the infection in humans but restrict a number of L3 by a production of concomitant immunity. Variability of adult worm burden results in a proportion of microfilaremic and amicrofilaremic persons in the population. The circulating microfilariae have short life cycle in human blood circulation; a proportion of the microfilariae can be removed by anti-microfilarial antibody

(Simonsen et al, 1996; 2008; Ravindran et al, 2003). They develop two molts (L1 and L2) and induce the infection in susceptible mosquito vectors while the infected mosquitoes can regulate melanization involved in innate immune defense and wound healing to the penetration of filarial nematodes (Zou et al, 2010; Castillo et al, 2011). In blood-engorged mosquitoes that harbor high microfilarial density, they can induce death due to the vector intolerance against development of juvenile forms, and consequently, this leads to loss of L3. Furthermore, release of the L3 does not always permit a passage at an equal number during other blood meal taken by the infective mosquito due to the vector tolerance. Thus, they are naturally killed by host immune, vector barrier, and physical environment; these contributing factors can favor the LF elimination in humans.

As was, LF becomes one of six potentially eradicable diseases of which the criteria for assessing their eradicability (CDC, 1993) are based not only on the scientific feasibility of understanding the biological information mentioned earlier and practical use of the public health interventions and other methods to be applied or used in existing national control program, but also on the political will or popular support of executing the implementation of LF control strategies. First, the scientific feasibility depends basically on the disease vulnerability. Unlike the *W. bancrofti* that exists only in humans, the *B. malayi* spends an enzoonotic life-cycle in which the parasite is transmitted by the potent mosquito and thrive in domestic cats as non-human reservoir, and a *per se* epizoonotic cycle in which the parasite is transmitted and thrive through which the mosquito vector takes blood meal from infected cats, and subsequently, transmit the infective stage to humans during other blood meal. Controversially, the humans carrying *B. malayi* infection can serve as a source for the infection to permit a passage to the animal reservoir through bites of the mosquito vector. Nonetheless, both diseases do not always ease their spread into the population as a result of the naturally-induced immunity and duration of microfilarial production in susceptible individuals or communities (Maizels and Lawrence, 1991; Ottesen, 1992) (Fig. 2). More important, the infections can be both easily diagnosed using advanced tools and effectively treated with the antifilarial drugs that are safe, inexpensive and easily deployed. The feasibility of elimination has been shown that the operation of the pragmatic diagnostic methods or interventions is demonstrable at a large scale in the target populations. Last, the political will/popular support is pivotal for the program manager to capture information required to analyze situations of the perceived burden of LF that is figured out by a large number of suffering and disabled persons worldwide (WHO, 2002; 2008; 2010): the details are available at the websites of the Global Program to Eliminate Lymphatic Filariasis (GPELF) (http://www.who.int/lymphatic_filariasis/disease/en/) and Global Alliance to Eliminate Lymphatic Filariasis (http://www.filariasis.org). Also, the National Program to Eliminate Lymphatic Filariasis (PELF) by which the resource mobilization and funding structure are administered must be allied, by adopting the GPELF's strategies, to practical considerations of interventions, methods, logistic supplies (medical and field-work), delivery processes, expenditures (unit costs and cost-effectiveness), integration of control activities into existing health systems services (or other health development programs), socio-economic impacts on gaining health benefits, community awareness and acceptance, and ecological disturbances to target disease, non-target disease and the environment. For the resource-limited countries as allied nations, a national budget plan that notifies outsources and fund raising needs the subsidy of the internationally collaborative program.

2.2 Mass chemotherapy as elimination strategy

Ideally, the success of controlling the disease depends definitely on the objectives and ultimate goals of disease control spectrum (CDC, 1993); with this regard, the rational management of LF elimination differs with control (Table 2). As recommended by the World Health Organization (WHO), two pillars of global elimination strategies emphasize interruption of transmission and elimination of the infection in humans, and the other large-scale morbidity control to prevent disease and disability (WHO, 1999a; 2002; 2008; 2010). Principle outcomes of the GPELF reduce numbers of microfilaremic persons and disease cases as preventing new infection introduced among the population at risk of, or affected with, the infection. To meet this criteria, the GPELF proposes a mainstay of elimination strategy effectively available for mass drug administration (MDA) in target population; a combined treatment with diethylcarbamzine citrate (DEC) 6 mg/kg plus 400 mg albendazole (in *W. bancrofti* transmssion areas where *Onchocerca volvulus* is not coendemic), or with 200 μg ivermectin plus 400 mg albendazole (in *W. bancrofti* transmssion areas where *O. volvulus* is coendemic) (Ottesen et al, 1997; 1999; Ottesen, 2000; WHO, 1999a; 2000; 2001; 2002). An annual MDA with coverage of 60-80% for 4-5 years is considered to be effective enough to interrupt transmission in control areas in the absence of vector control (Gyapong et al, 2005). Also, new options for mass treatment of at risk population are effectively available: DEC-fortified salt for 1 year; and a combination of single annual dose of albendazole plus DEC, followed by DEC-fortified salt (Weaver et al, 2011). The ample supply and distribution of DEC-fortified salt can be administered in some countries.

2.3 Surveillance and monitoring systems

As for presenting dynamics of the infection and disease in nature (Grenfell et al, 1991; Srividya et al, 1991; Ottesen, 1992; Meyrowitsch et al, 1995), the parasites cause a wide spectrum of clinical manifestations in the affected population that are characterized by asymptomatic microfilaremia, acute lymphatic inflammation and chronic lymphatic pathology (WHO, 1992a; 1992b; 1994). Susceptible persons develop clinically LF as a result of prolonged exposure to multiple infective bites of potent mosquitoes from several months to years. On the other hand, the people living for at least 6 months are at the greatest risk for the infection. The chronic filariasis cases represent a tip of the iceberg, as microfilaremic carriers are the reservoir of the infection to others. The prevalence and intensity of the infection in humans depends mainly upon a number of microfilaremic persons and a geometric mean of microfilarial loads in the affected population. These proximate measures are indicative of the degrees of endemicity. The prevalence and geographical distribution of the disease are important not just for determination of its potential transmission in mosquito, but also for diagnosis and surveillance in different endemic settings. Therefore, the recognition of what is the filarial origin of the disease and where the affected individuals or communities are is fundamental for public health importance to identify the solution and control rationale. Diagnostic approaches that emphasize the detection and specific identification of microfilaremic infection in the individual by using most standard microscopic methods are important component of LF control program, which is aimed to reduce number of microfilaremic persons. The suitable blood collection and microscopic diagnosis in health settings can be effectively available to identify anyone infected, and subsequently, treated with antifilarial drug regimens. However, this specific objective of control is less important as the elimination is desired not just to interrupt transmission and clear human infection on a large-scale, but also to reduce the morbidity attributed to the

disease and hence improve both personal-focused hygiene and health care to all beneficiaries in target population at risk (Table 2).

	Control	Elimination
1. Definition	- Reduction of microfilaremia prevalence	- Reduction of microfilaremia prevalence as arbitrarily qualitative or quantitative level of control as no longer a public health problem
2. Outcome indicators as options for monitoring tools	- Annual infection prevalence; microfilaremia rate (a microfilaremic number surveyed in endemic population) - Disease rate (a number of disease cases surveyed in endemic population) - Annual transmission potential (ATP) (i.e., a number of infective bite per annum which acquire the infection) as mosquito infection rate is not useful indicator	- Degrees to which transmission is interrupted; microfilaremia rate mosquito infection rate and antigenemia rate - Disease rate is not useful indicator
2.1 Objective	- **To assess whether the control program achieves its goals or desired outcomes** (e.g., reduction in microfilaremia and/or disease prevalence)	- **To assess whether the elimination program achieves its goals or desired outcomes†** (e.g., reduction in microfilaremia prevalence, antigenemia prevalence)
3. Process indicators as options for monitoring and evaluation tools	- Coverage of selective treatment and follow-up, as mosquito net utilization, insecticide spraying and reservoir control can be useful indicators	- Mass treatment coverage, drug compliance coverage (e.g., adverse drug reactions prevalence and severe adverse event reports), KAP survey
3.1 Objective	- **To assess how well the various components of the control program are functioning** (e.g., the number of newly and follow-up microfilaremic persons treated with antifilarial drugs)	- **To assess how well the various components of the elimination program are functioning‡** (e.g., the number of new infections, the number of drug tablets distributed)

Adapted from the GPELF (http://www.who.int/lymphatic_filariasis/disease/en/).
†,‡Outcome indicators can be optionally used for monitoring systems; longitudinal surveillance of populations in sentinel sites, cross-sectional "spot checks" in other sites, and auxiliary "background" surveillance.
KAP - Knowledge, attitudes and practices.

Table 2. Rational approaches to lymphatic filariasis control and elimination

Tools	Advantages for the applications	Chief obstacles when applied or used in demonstrated areas	References
ICT Filariasis	• Simple-to-use rapid diagnostic test kit commercially available for use in qualitative detection of *W. bancrofti* adult worm circulating antigens present in whole blood/serum/plasma samples; stronger positive test line equivalent to higher antigen levels; • Sensitive and specific for anytime-of-day determination of active infection (antigenemic) whether microfilaremia is present, or the treatment is given; • Highly reproducible and practical when lots of large-scale blood samples (100 µl each) are analyzed either under the field conditions by not-well-trained field workers or in laboratory settings (with 100 µl each of serum/plasma samples freshly prepared or frozen) by laboratory personnel in order to assess the human infection rates in areas known as endemic for *W. bancrofti* or respective areas of emergence/reemergence; • Suitable for rapid assessment survey to detect the early infection in endemic carriers including migrants, refugees, any persons who work in endemic areas (mine in rainforest and border) for years, or visitors to the areas; • Suitable to monitor and evaluate the infection in humans inhabiting in risk areas in initial surveillance before MDA; drug responses during MDA; and the new infection in post-MDA areas whether they are certified as eliminated areas	• Costly; • Indicate, but not quantify, the level of the circulating antigens; somehow, provides false-negative identifications with the infections harboring very low antigen titers; • Cannot differentiate the status between infection and disease, occurrence and recurrence, or sensitivity and resistance • Primarily requires standardization and quality control of lots of samples (finger-prick blood) that are collected and analyzed	Weil & Liftis, 1987 Weil et al, 1987; 1996; 1997 Freedman et al, 1997 Ramzy et al, 1999 Bhumiratana et al, 1999; 2002; 2004; 2005 Nguyen et al, 1999 Phantana et al, 1999 Simonsen et al, 1999 Omar et al, 2000 Pani et al, 2000; 2004 Sunish et al, 2002; 2003 Braga et al, 2003 Engelbrecht et al, 2003 Koyadun et al, 2003 Nuchprayoon et al, 2003 Siriaut et al, 2005 Ruberanziza et al, 2009 Foo et al, 2011

Tools	Advantages for the applications	Chief obstacles when applied or used in demonstrated areas	References
Og4C3 ELISA	• Commercially available diagnostic test kit, and principally the same when use in qualitative and quantitative detection of active *W. bancrofti* infection, but more sensitive and specific than the ICT Filariasis; • Highly reproducible when lots of large-scale serum/plasma samples (100 μl each of freshly prepared or frozen samples) are analyzed in the public health reference laboratory or research institutes in order to assess the human infection rates, and to monitor and evaluate the infection or drug responses in individuals or in the target population under the circumstances described above;	• Costly, labor-intensive and intrusive • Cannot differentiate the status between infection and disease, occurrence and recurrence, or sensitivity and resistance • Primarily requires standardization and quality control of lots of venous blood samples (subsequently prepared for sera or plasma) that are collected, transported, stored and analyzed; • Also requires well-trained field workers and laboratory personnel	More & Copeman, 1990 Turner et al, 1993 Chanteau et al, 1994a; 1994b Lammie et al, 1994 McCarthy et al, 1995 Rocha et al, 1996 Nicolas, 1997 Ismail et al, 1998 Eberhard, 1997 Simonsen and Dunyo, 1999 Nuchprayoon et al, 2003 Bhumiratana et al, 2004; 2005
Polymerase chain reaction (PCR)	• Very highly sensitive and specific for the microfilaremic infections with *W. bancrofti* and *B. malayi* in humans and mosquitoes distinguishable from other filarial nematode parasites such as *O. volvulus*, *D. immitis*, *D. repens* and *B. pahangi*; • Highly reproducible and practical when lots of samples as low as 20 μl human blood, individual mosquito (dissected or whole body), or mosquito pool, are analyzed in the public health reference laboratory or research institutes in order to assess the human and	• Too costly, labor-intensive, intrusive, in-house developed tool • Primarily requires standardization and quality control of lots of samples (finger-prick or venous blood, or pooled wild-caught mosquitoes) that are collected, transported, stored and analyzed; • Also requires well-trained field workers or	Chanteau et al, 1994c Lizotte et al, 1994 McCarthy et al, 1996 Siridewa et al, 1996 Williams et al, 1996 Zhong et al, 1996 Ramzy et al, 1997 Nicolas et al, 1999 Cox-singh et al, 2000 Thanomsub et al, 2000 Pradeep Kumar et al, 2002

Tools	Advantages for the applications	Chief obstacles when applied or used in demonstrated areas	References
	mosquito infection rates, and to monitor and evaluate the infections or drug responses in individuals or in the target population under the circumstances described above; • Can differentiate the status between infection (microfilaremia) and disease, occurrence (new infection) and recurrence, or sensitivity and resistance; • Detection limit of as low as one Mf per blood volume tested (up to 1 ml), or one juvenile larva (L1, L2 or L3) per pooled mosquitoes (up to 100)	laboratory personnel and accessory equipments for DNA preparation and analysis of PCR products; • Cannot differentiate larval stages (L1, L2 or L3) in infected mosquitoes	Fischer et al, 2003 Kanjanavas et al, 2005 Nuchprayoon et al, 2005; 2007 Rao et al, 2006 Mishra et al, 2007 WHO, 2009 Bhumiratana et al, 2010 Pechgit et al, 2011 Takagi et al, 2011

Table 3. Direct determination tools for use as part of the Global Program to Eliminate Lymphatic Filariasis

To meet this objective, a large-scale transmission control requires a current magnitude and geographical distribution of the disease in the at-risk population. To understand the extent to which the target population needs to be designed for MDA and monitored whether the MDA implementation is effective, such surveillance and monitoring systems are required. To identify the communities with, or at risk of, the infection, for instance, the direct assessment techniques (Table 3) are required for practical use both in initial surveillance for filarial infection and in monitoring and evaluating the effectiveness of mass treatment, as part of the GPELF (WHO, 1999a; 1999b; Ottesen, 2000). In this regard, the mass treatment with more effective antifilarial drug regimens as well as the availability of other existing and alleviating control measures has been deliberately implemented to meet such these highly achievable objectives of the elimination. Nonetheless, in addition to what is recommended by WHO, the GPELF requires for ground-breaking development of systems, protocols and tools that will be able to be convincingly applied to or routinely used in the PELF to fix undesirable events of mass treatment impacts in different complex epidemiological settings (Kyelem et al, 2008; Ottesen et al, 2008; WHO, 2008; 2010).

3. Parasite infection and drug responsiveness

3.1 Microfilaremia and drug-responsive microfilaremia

In an endemic population that represents the infection and disease dynamics in nature, the asymptomatic microfilaremia is a stage of the active infection with filarial adult worms (Srividya et al, 1991; Meyrowitsch et al, 1995), which regulate host immune responses (hypoimmune responsiveness) in infected individuals (Bundy et al, 1991; Grenfell et al, 1991; Maizels and Lawrence, 1991; Mitchell, 1991; Grenfell and Micheal, 1992; Ottesen, 1992;

Simonsen et al, 1996; 2008; Ravindran et al, 2003). This phenomenon results in immunotolerance, i.e., a prolonged induction of the balance of immune defense to the parasites stimulated by the adult worms, in most asymptomatic microfilaremic persons. The female adult worm involves in regulation of the host microfilaremia. The fecundicity (a period of pregnancy) allows its fertilization to produce a diverse number of the offspring microfilariae. Although the proportion of microfilariae can be removed from the blood circulation in patients, there are the plenty of microfilariae, which circulate in the peripheral blood and show the appearance both nocturnally and diurnally. This microfilarial periodicity or circadian cycle of the parasite in humans is clinically unimportant for treatment but very important for its epidemiologic implication, which plays a significant role in diagnosis, surveillance and epidemiology. The parasite infection is the foundation for the processes that not only determine the infection prevalence but also monitor and evaluate the effectiveness of the treatment with the antifilarial drugs in infected individuals. In this regard, the amount of microfilaremia seems to be a function of naturally-acquired infection loads, which refers to as the most viable microfilariae, and drug-responsive microfilaremia refers to as the affected parasite population that harbors a diverse range of viable and non-viable microfilariae (Pechgit et al, 2011). These outcome indicators are useful for monitoring and evaluating the benzimidazole susceptibility of the filarial nematode parasites in the population in areas of the PELF implementing the MDA 2-drug regimen either albendazole plus DEC or albendazole plus ivermectin.

However, the MDA 2-drug regimen is not the only factor that shapes the parasite population under complex epidemiological settings. Of note, the *W. bancrofti* populations have ability to provoke the genetic variability that shows the important implications in the endemic populations targeted by the MDA (Pradeep Kumar, 2000). The existence of genetic diversity of *W. bancrofti* populations that has greater heterogeneity under DEC therapy and vector control gives rise to questioning about the development of drug resistance in LF, which possibly occurs in the target populations. The selection pressure is an intensity of selection affecting the frequency of genes in a parasite population. The selection that increases or decreases the susceptibility of the parasite population depends on the frequency of the alleles involved. The genetic polymorphism occurring in the *W. bancrofti* population under the selection pressure(s) may evoke gradually under specified conditions to yield the fitness, which can be determined by a genotype in the parasite population. The increase in the parasite fitness can be estimated by the equilibrium frequencies of the alleles (genotypes) at heterozygote advantage in a hypothethical population. That is, rapid establishment of advantageous alleles in the *W. bancrofti* population, called "selection sweep", may evoke with advantageous drug-resistant genotypes epidemiologically linked to other factors shaped by the host and environment (Schwab et al, 2006: 2007; Churcher et al, 2008). Eventually, it may reduce the genetic variation in the population.

3.2 Benzimidazole-susceptibility of the parasite

This chapter emphasizes microfilaremia responsiveness in the population under the suppression of the PELF implementing MDA 2-drug regimen, 6 mg/kg DEC plus 400 mg albendazole. The microfilaremia responses against the DEC are the foundations of understanding how the albendazole exerts the effects on the parasite population in addition to what is observed by DEC alone. The DEC is known as the oldest of the antifilarial drugs used in the LF control. The single-dose drug acts as microfilaricide as does the effective ivermectin (de Silva et al, 1997; Ottesen, 2000; Molynuex et al, 2003; Ottesen et al, 2008)

while its macrofilaricidal activity is not definitely effective against adult worms (Eberhard et al, 1991; Norões et al, 1997; Dreyer et al, 1998; Rajendran et al, 2002; 2004; Oliveira-Menezes et al, 2007). The adult worm loads that are age-dependent (Lammie et al, 1994; Rajendran et al, 2002) are susceptible to treatment with the DEC alone or even combination with albendazole (Norões et al, 1997; Rajendran et al, 2002). It was seen that DEC alone disturbs the microfilarial sheath of some filarial species while it has effects on the oogenesis and fertilization of female adult worms. Nonetheless, little is known about the filarial nematode parasites whether they evoke resistance mechanism against DEC due to the lack of deepening its mechanism of action, particularly the availability in parasite tissues and the selectivity on parasite targets. Oliveira-Menezes et al (2007) demonstrated that DEC has minor effects on alterations of the cuticle or surface of both male and female adult worms; these responsible parasites were collected from the *W. bancrofti*-infected patients treated with DEC, as compared to those isolated from the untreated patients. Additionally, such alterations are seen in adult worms recovered from the patients treated with DEC plus albendazole. The possible explanation is that the potential adulticidal effect of albendazole relative to what is observed by the DEC alone. The subtle alterations imply the distinct morphologic characteristics of the parasite itself or the complex host-parasite interactions, and if implemented and continuously prolonged, the annual mass treatment with DEC plus albendazole has not yet become an apparent issue, particularly the impacts on the parasite population adaptation. Of note, the *O. volvulus* parasite develops the mechanism involved in resistance to ivermectin (Awadzi et al, 2004). Do the filarial nematode parasites have mimicry in the resistance mechanisms to ivermectin and albendazole? However, detailed study of the parasite resistance to albendazole has not been established.

Focus is on the MDA 2-drug that acts as effective microfilaricide while its aberrant activity that influences microfilaremia response to its efficacy in microfilaricidal activity. Provided this phenomenon occurs, the expected outcomes of such drug failure will impact on solving the solutions and paving the implications of how they will adapt under the certain circumstances and how we will also mitigate their adaptation. Most studies enlightened the understanding of this effective deworming MDA 2-drug, which plays the significance of reduction of the infection prevalence. A single-dose combined treatment with DEC plus albendazole has short- and long-term effects on *W. bancrofti* microfilaremics in the endemic populations (Ismail et al, 1998; Ottesen et al, 1999; El Setouhy et al, 2004; Rajendran et al, 2004). Compared to those receiving DEC alone, an additional benefit of the combined drugs results in decline in annual cyclic infection prevalence due to progressive reduction in density of *W. bancrofti* microfilaremia. Although its macrofilaricidal effect on clearance of *W. bancrofti* antigenemia has been reported (McCarthy et al, 1995; Eberhard, 1997; Rajendran et al, 2002; 2004; Koyadun et al, 2003; Bhumiratana et al, 2004; Siriaut et al, 2005; Bhumiratana et al, 2005; Yongyuth et al, 2006), the DEC alone or co-administered with the albendazole does not clear rapidly the antigenemia. The MDA with the DEC alone will recover an increase in the antigenemia prevalence of *W. bancrofti* unless there is yearly-round MDA in the population (Rajendran et al, 2002). A 400 mg single oral-dose albendazole regimen is broad-spectrum effective against helminthiases (Albonico, 1994; de Silva et al, 1997; Beach et al, 1999; Ottesen et al, 1999; Horton, 2000) and, as co-administered orally with DEC, a synergistic and long-term effect on geohelminths has been proven useful for 'beyond-lymphatic filariasis' elimination program (Ottesen et al, 1997; 1999; Ismail et al, 1998; Horton et al, 2000; Ottesen, 2000; Mani et al, 2002; 2004; Molynuex et al, 2003; Yongyuth et al, 2006).

Few reports established the evidence that the human filarial nematode parasites provoke molecular mechanism involved in benzimidazole sensitivity/resistance until recently findings of the genetically-induced resistance against benzimidazole compounds have been well documented in veterinary nematode parasites (Beech et al, 1994; Kwa et al, 1995; Humbert et al, 2001; Bennett et al, 2002; Drogemuller et al, 2004; Robinson et al, 2004; Cole et al, 2006; Ghisi et al, 2007). Resistance to albendazole in veterinary nematodes is known to be caused by either one of two single amino acid substitutions from phenylalanine to tyrosine in parasite β-tubulin at position 167 or 200. The genetically stable *W. bancrofti* β-tubulin gene responsible for a molecular mechanism of drug resistance has been proposed as that of the veterinary helminth parasites is performed under selection of albendazole and ivermectin. The *W. bancrofti* population isolated from the patients treated with a combination of albendazole and ivermectin had significantly higher genotypic frequencies associated with resistance at position 200 (Schwab et al, 2005). A resistance mutation was not detected at position 167. Hoti et al (2003, 2009) reported that the polymorphism in the codon of this residue in *W. bancrofti* populations representing geographically distant areas of India, through sequencing exon 5 region of *β-tubulin* isotype 1 gene. The nucleotide sequence data showed that *W. bancrofti* isolates from wide geographic areas of India had codon for Phe (TTC) at position 200, suggesting that the parasite might be genetically sensitive to benzimidazole. Similarly, Bhumiratana et al (2010) and Petchgit et al (2011) demonstrated that the *W. bancrofti* population recovered from the dynamic cross-border migrant population from areas that have been targeted by the MDA 2-drug regimen (300 mg DEC plus 400 mg albendazole) elicits the genetic background of benzimidazole susceptibility; a resistance mutation has not been observed at position 167 or 200. However, the albendazole, anthelmintic benzimidazole, is being co-administered with an antifilarial drug such as DEC, part of the PELF implementing in many endemic countries. But this drug is known to result in the faster development of drug resistance in the veterinary nematode parasites and hence it is necessary to monitor drug sensitivity among the responsible *W. bancrofti* populations.

4. Molecular diagnosis and monitoring of benzimidazole susceptibility

4.1 Parasite beta-tubulin encoding gene as molecular marker

Molecular mechanisms of benzimidazole resistance in nematode parasites are hypothesized. However, detailed study of benzimidazole resistance in trichostrongylids found that the β-tubulin encoding gene involved in benzimidazole susceptibility is responsible for the genetic inheritance of resistance in the veterinary nematode parasites under selection with benzimidazole that involves one of two single amino acid substitutions from phenylalanine (Phe) to tyrosine (Tyr) in parasite β-tubulin at position 167 or 200 (Beech et al, 1994; Kwa et al, 1993; 1994; 1995; Roos et al, 1990; Elard et al, 1996; Elard and Humbert, 1999; Humbert et al, 2001; von Samson-Himmelstjerna et al, 2002; Winterrowd et al, 2003; Drogemuller et al, 2004; Cole et al, 2006; Ghisi et al, 2007). The potential point mutation occurs at the DNA level by nucleotide substitution for the codon for amino acid position 200 of the *β-tubulin* gene, a substitution of TTC (Phe) with TAC (Tyr). This irreversible change brings about distinguishment of the responsible parasite population between benzimidazole-sensitive and -resistant nematodes. This principal mechanism for benzimidazole resistance is postulate to involve changes in the selectivity of the benzimidazoles on the primary structure of β-tubulin molecules, a building block of the microtubule in the parasites (Lacey, 1988; Lacey and Gill, 1994; Robinson et al, 2004).

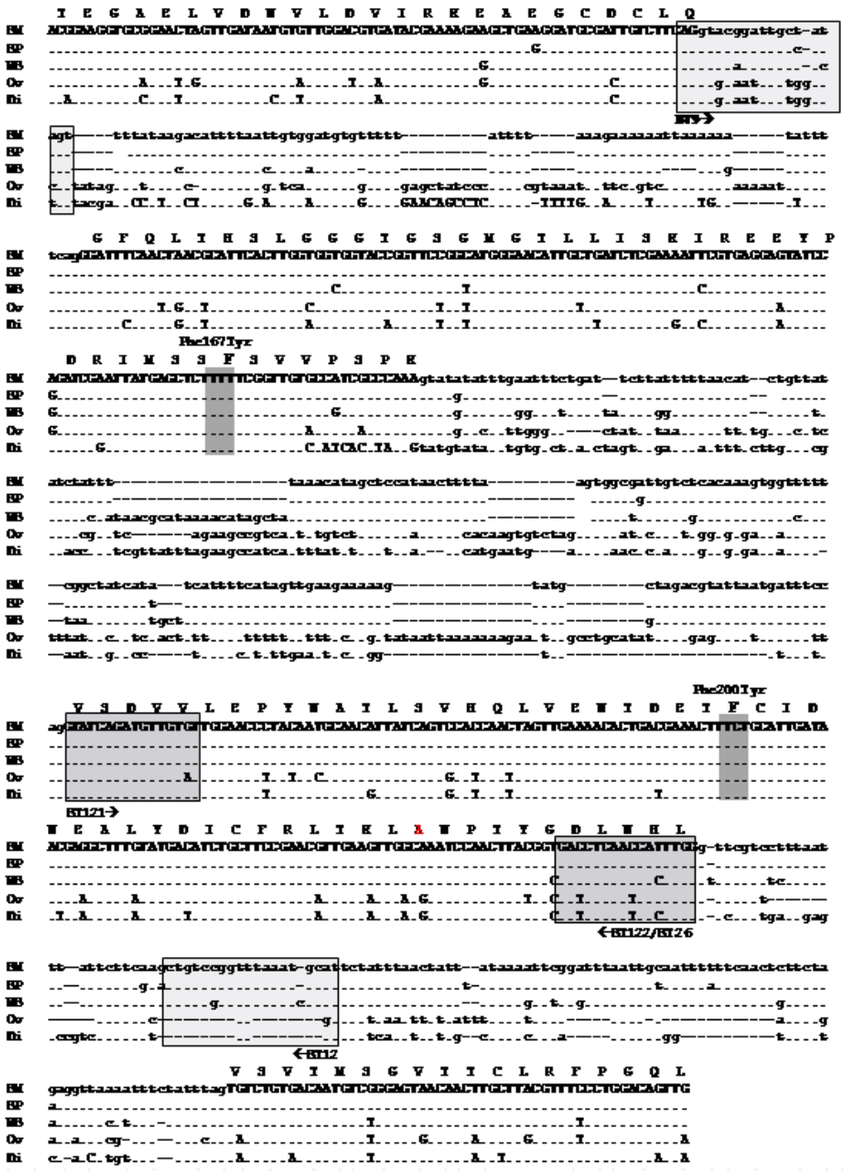

Fig. 3. ClustalW alignment of the filarial *β-tubulin* gene. The partial nucleotide sequence representatives (accession no. and positions): *Brugia malayi* (BRQD553TR, 3-789), *Brugia pahangi* (M36380, 2267-3054), *Wuchereria bancrofti* (AY705383, 109-916), *Onchocerca volvulus* (AF019886, 1582-2400) and *Dirofilaria immitis* (HM596854, 1462-2244) are shown as coding (upper case) and non-coding (lower case) sequences. The deduced amino acid sequences for the conserved domains are shown for all taxa aligned; *D. immitis* and *O. volvulus* have one amino acid substituted at position Ala218Thr. The gap is performed on the maximum homology (insertion/deletion), which represents conserved (•) and degenerate nucleotide residues and the regions designed to amplify specifically the target sequences based on the Wb*tubb* primer sets (light-gray boxes), both forward (→) and reverse (←). Hypothetically, two amino acid substitutions at positions Phe167Tyr and Phe200Tyr (dark-gray boxes) retained in DNA fragments (141 and 174 bp) could be identified using the PCR detection system described by Bhumiratana et al (2010) and Pechgit et al (2011).

Intriguingly, such mimicry in molecular mechanism for benzimidazole resistance in the filarial nematode parasites has been increasingly investigated, based basically on the molecular characterization of the homologous β-*tubulin* gene retained in their genome and the advantageous fitness of benzimidazole-resistant genotypes in the population (Roos et al, 1995; Elard et al, 1998; Elard et al, 1999; Silvestre et al, 2001; Silvestre and Humbert, 2002).

The nematode parasites possess the single-copy homologous *β-tubulin* (*tubb*) gene that encodes a β-tubulin polypeptide, 448 amino acids (Met1 to Glu448). Hypothetically similar to that of trichostrongylids, the binding of benzimidazoles to conserved domains (of the exons 4 to 6) leads to blocking an assembly of tubulin (TUBB), and thus disrupting structural formation of microtubule (cytoskeleton protein) in the nematode parasites. The nucleotide sequences of the homologous *β-tubulin* gene as molecular marker and other related TUBB gene family of the nematode parasites can be retrieved from the genome databases: the GenBank at the National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/genbank/, the European Molecular Biology Laboratory (EMBL) http://www.ebi.ac.uk/, and the DNA DataBank of Japan (DDBJ) http://www.ddbj.nig.ac.jp/. The website of nematode and neglected genomics (http://www.nematodes.org/fgn/index.html) establishes genome database, especially for the filarial genome project (FGP), which includes published complete *B. malayi* genome. Meanwhile, the homologous sequences of *B. malayi β-tubulin* gene can be obtained from the TIGR genome database (http://www.tigr.org/tdb/e2k1/bma1).

The structural organization of homologous *tubb* genes of two filarial nematode parasites, *B. pahangi* (Guenette et al, 1991) and *D. immitis* (Bourguinat et al, 2011), has been shown for the establishment of complete coding sequences that span 9 discrete exons: exon 1 (Met1 to Lys19), exon 2 (Phe20 to Asp55), exon 3 (Gly56 to Gln131), exon 4 (Gly132 to Lys174), exon 5 (Val175 to Leu228), exon 6 (Val229 to Gln292), exon 7 (Met293 to Arg324), exon 8 (Glu325 to Thr386) and exon 9 (Ala387 to Glu448). The homology is 78% at DNA level due to bias of codon usage and insertion/deletion of intron sequences (Fig. 3). Among these, the exons 4 and 5 confer hypothetical point mutation at amino acid positions Phe167Tyr (or TTT/TAT) and Phe200Tyr (or TTC/TAC), based only on the second nucleotide base changed in the codons. In the homologous segment of its closely related taxa, *W. bancrofti β-tubulin* (Wb*tubb*) gene that possesses two distinct exons, 4 (Gly132 to Lys174) and 5 (Val175 to Leu228), with flanking intron sequences (Fig. 3) shares the homology at DNA level with *B. malayi* and *B. pahangi* (93% similarity), compared to *O. volvulus* and *D. immitis* (76% similarity) (Bhumiratana et al, 2010; Pechgit et al, 2011). This target DNA has been proved useful for designing Wb*tubb* locus-specific primers to discriminate between Wb*tubb* and other homologs of human and animal filariids. Based on its usefulness in molecular diagnosis and monitoring of the infection carrying the benzimidazole-sensitive or resistant phenotypes, the PCR applications of this molecular marker for *W. bancrofti* have been well documented (Hoti et al, 2003; 2009; Schwab et al, 2005; Bhumiratana et al, 2010; Pechgit et al, 2011).

4.2 Polymerase chain reaction-based approaches

In contrary to the antigen detection methods such as ICT Filariasis and Og4C3 ELISA that provide the proof of *W. bancrofti* antigenemic infection in human blood, the microfilarial DNA detection by PCR provides the evidence of *W. bancrofti* microfilaremic infection in human blood and mosquito (Table 3). As a result of the existence of genetically stable

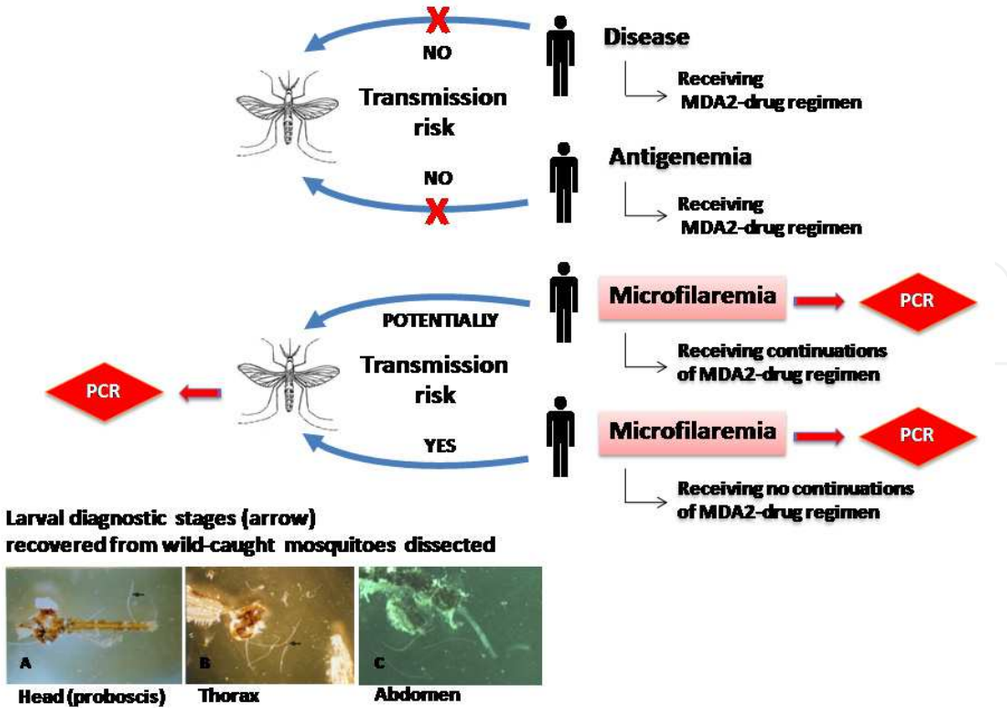

Fig. 4. A purposed scheme for PCR detection of *W. bancrofti* benzimidazole-susceptible isolates in human blood and mosquito.

Primer Name	Sequence (5′ to 3′)	Direction	Length (bases)	Expected amplicon size (bp)	Hypothetical nucleotide positions	Susceptible(S)/ resistant(R) genotypes investigated	Reference
BT9	CAGGTACAGATT GCTACAGT	Forward	20	607[c]	(TTT)167(TAT)	S/Phe167	Bhumiratana
BT12	GCGATTTAAACC CGACAGC	Reverse	19		(TTC)200(TAC)	S/Phe200	et al (2010)
BT121[a]	GGATCCGTATCA GATGTTGTG	Forward	21	174[d]	(TTC)200(TAC)	S/Phe200	Bhumiratana
BT122[b]	GAATTCCAAGTG GTTGAGGTCG	Reverse	22				et al (2010)
Wt2F	GTATCAGATGTT GTGTTG	Forward	18	475[e]	(TTC)200(TAC)	S/Phe200	Hoti et al
Wt2R	ACGACTTGAATG AGTTGTC	Reverse	19				(2003)
Wbbt2 F	TATCAGATGTTG TGTTGG	Forward	18	475[f]	(TTC)200(TAC)	S/Phe200	Hoti et al
Wbbt2 R	CTGTTGAG AAGTTCAGCA	Reverse	19				(2009)

5′modifications with additional recognition sequences: [a]*Bam*H I (GGATCC) and [b]*Eco*R I (GAATTC).
[c d]Retrieved *Wuchereria bancrofti* genome accession nos.: AY705383 and GU190718–24.
[e]Retrieved *Brugia pahangi* genome accession nos.: M36380.
[f]Retrieved *Wuchereria bancrofti* genome accession nos.: EF190199-190209, EF492870-492878.

Table 4. The *β-tubulin* isotype 1 gene-specific primers used in the PCR amplification of *W. bancrofti* benzimidazole-susceptible isolates

W. bancrofti β–tubulin gene, the nested PCR amplification can work well with the microfilaremic infection that responds to treatment with MDA 2-drug regimen (DEC plus albendaozle) (Pechgit et al, 2011). This newly developed PCR assay in addition to promising advanced tool (Hoti et al, 2003; 2009; Bhumiratana et al, 2010) has the potential benefits in the molecular diagnosis and monitoring of the infection, as compared to the other PCR amplification methods previously described elsewhere (Table 3). The concepts for PCR assays based on the Wb*tubb* locus-specific primers (Table 4) have been proposed in two applicable formats: the locus-specific nested PCR and allele-specific nested PCR. These applications have established the advantages on how to circumvent some common counterintuitive problems of conventional PCR with regards to both parasite genome analysis and low-copy gene detection; such detailed study has been well established by Pechgit et al (2011). The *W. bancrofti* microfilarial DNA detection methods depends much on the purity and quantity of the microfilariae recovered from different blood sample preparations. The purified aggregate parasite number in the absence of human host white blood cells, for example, are ideal for the quality of DNA extract, which serves as target sequences in the PCR reactions. In general, most PCR methods for the detection of *W. bancrofti* distinguishable from other filarial nematode parasites in human and mosquito is based on the repetitive *Ssp* I sequences, which are highly copy number per haploid genome. However, PCR amplification based on this *Ssp* I locus provides the positive identifications of the parasite infection existed in specimens of choice. The assay does not determine the infection that responds to benzimidazole sensitivity/resistance; such responsible *W. bancrofti* parasite population is amplified based on the *β–tubulin* gene which is single copy in haploid genome. Therefore, the amplification is performed using the Wb*tubb* locus-specific nested PCR and allele-specific nested PCR that provides the proof of the *W. bancrofti* infection carrying benzimidazole-sensitive/resistant phenotypes; methodologically, the technical requirements for their applications have been described by Pechgit et al (2011) and Hoti et al (2009). More specific, based on our experience, the Wb*tubb* locus-specific nested PCR with thermocycling modifications using touchdown and touchup cycles has been applied or used in detection and characterization of *W. bancrofti* infection both in human blood from patients untreated or treated with DEC plus albendazole and in wild-caught mosquito, provided such infections carrying benzimidazole-sensitive/resistant strains are the same source of the parasite population (Fig. 4). Hypothesis is that whether the parasite infection is genetically predisposed to the MDA 2–drug regimen (DEC plus albendazole) in areas under suppression of PELF, it will have frequencies of benzimidazole-susceptible homozygous allele (*SS*) greater than benzimidazole-susceptible heterozygous allele (*Sr*) and homozygous resistant allele (*rr*), which are associated with albendazole resistance, unless the parasite fitness is increased. This also permit the monitoring and evaluation of the parasite fitness to better understand theoretically and hypothetically evolutionary biology and ecology of the parasite, by which the human hosts play a key as a major source of selective pressures on the adaptation of parasite population constrained by environmental conditions.

5. Future perspectives

The GPELF has been deployed into the endemic countries implementing MDA 2-drug regimes (i.e., single annual doses of albendazole in combination with DEC or ivermectin) to reduce microfilaremia prevalence to levels low enough (principally lower than transmission threshold) to interrupt transmission of the disease in the absence of vector control. Based on scientific information on drug resistance to anthelmintics, the issue of albendazole resistance

in the *W. bancrofti* parasite has assumed increasing importance since the GPELF is implemented on a large-scale in at-risk populations in different complex epidemiological settings, and predictably, the implementation phrase of the program will increase. Many studies have shown some vulnerability in how the parasite has the ability to evoke molecular mechanisms for resistance to anthelminthics as the nematodes of veterinary importance have developed resistance against both albendazole and ivermectin. Furthermore, there have been lines of evidence that the vulnerability of helminthiasis control programs that employ the MDA with these drugs is associated with several factors that facilitate the promotion of drug-resistant strains. At the same time, the factors are considered concerning the new options of drug combinations with different parasite targets or modes of actions to keep active shelf-life of DEC because the filarial nematode parasites have long life-span and complex life-cycle development. Likewise, to better understand what is relative to achieve MDA's goal to confront growing trend of drug resistance, it is essential for the development of molecularly diagnosing and monitoring the benzimidazole sensitivity/resistance in areas of long-running PELF program implementation using albendazole plus DEC or albendazole plus ivermectin. More applicable tools which will be validated in effective manner are also required to explore the genetic basis for resistance to anthelminthics.

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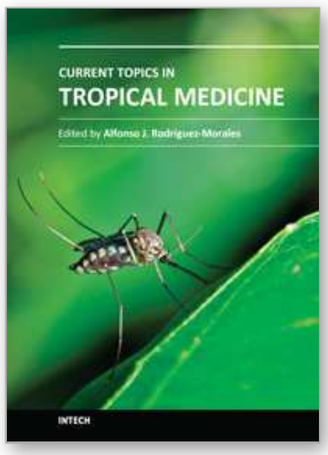

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