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Touchdown–touchup nested PCR for low-copy gene detection of benzimidazole-susceptible *Wuchereria bancrofti* with a *Wolbachia* endosymbiont imported by migrant carriers

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ABSTRACT

A novel, sensitive and specific touchdown–touchup nested PCR (TNPCR) technique based on two useful molecular markers, a *Wuchereria bancrofti β-tubulin* gene involved in benzimidazole susceptibility and a *Wolbachia ftsZ* gene involved in cell division, was developed to simultaneously detect the parasite *W. bancrofti* (W1) with its *Wolbachia* endosymbiont (W2) from both microfilaremic and post-treatment samples of at-risk migrant carriers infected with geographical *W. bancrofti* isolates. The detection and characterization of authentically low-copy gene-derived amplicons revealed no false positive identifications in amicrofilaremia with or without antigenemia. The W1-TNPCR was 100-fold more sensitive than the W2-TNPCR regardless of the microfilarial DNA isolation method and compared well with the thick blood film and membrane filtration techniques. These locus-specific TNPCRs could also detect *Wolbachia*-carrying *W. bancrofti* genotype in addition to a link to benzimidazole sensitivity among those with unknown infection origins that exhibited microfilaremia responsiveness against treatment with diethylcarbamazine plus albendazole. These TNPCR methods can augment the results of microscopic detection of the parasite because these methods enhance DNA isolation and PCR amplification capabilities.

1. Introduction

Lymphatic filariasis caused by *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* is one of the major public health problems in South-East Asia (SEA). This mosquito-borne disease accounts for the highest proportion of the population at risk, or half of the worldwide cases (WHO, 2001, 2008; Ottesen et al., 2008). Currently, this SEA poverty-attributed disease is controlled on a large-scale by an annual mass drug administration (MDA) using a single 6 mg/kg dose of diethylcarbamazine (DEC) plus 400 mg albendazole; this approach is part of the National Program to Eliminate Lymphatic Filariasis (PELF) guided by the World Health Organization (WHO). For monitoring and evaluating the effectiveness of the implementation of PELF (Kyelem et al., 2008), several key determinants focus not only on the microscopic detection of either microfilariae (Mf) present in the blood of infected persons or L1–L3 larvae in infected mosquitoes, but also on the detection of the parasite's antigen in humans (More and Copeman, 1990; Chanteau et al., 1994b; Weil et al., 1997) or the parasite's DNA in both mosquito and human (Lizotte et al., 1994; Chanteau et al., 1994a). Although the availability of these diagnostics (microfilariae and antigen) is not ideal for the surveillance and monitoring of the impacts of MDA on the parasite populations, field applications of microscopy-based methods are still part of the MDA 2-drug program.

The potential benefits of using advanced tools in the PELF should, therefore, not only be aimed at how to assess the responsible genotypes of the parasite populations in different settings of geographical MDA coverage, but also how to design strategic approaches to molecularly diagnose and monitor the infections under suppression by the MDA 2-drug regimen. Recent reports have shown promise for *W. bancrofti* Mf DNA detection by polymerase chain reaction (PCR)-based methods, and the choice of a PCR technique depends mostly on the purity and quantity of the microfilariae for different sample preparations (Cox-Singh et al., 2000; Pradeep Kumar et al., 2002; Fischer et al., 2003; Kanjanavas et al., 2005; Nuchprayoon et al., 2007). For instance, purified Mf DNA can be isolated either from a viable Mf population recovered by filtering microfilaremia through a Millipore membrane with a further Percoll density gradient centrifugation (Pradeep Kumar et al., 2002) or directly from microfilaremia-embedded Whatman

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FTA papers (Nuchprayoon et al., 2007), this DNA can then be used in PCR-based DNA fingerprinting studies. Furthermore, Giemsa-stained blood films of *W. bancrofti* microfilariae (WbMf) can be used to detect Mf DNA and *Wolbachia* DNA (Bisht et al., 2006). A *Wolbachia* endosymbiont is maternally transmitted vertically from a *W. bancrofti* female worm to its offspring, and intriguingly, it is clinically implicated in microfilaremia and drug responsiveness in infected patients (Taylor and Hoerauf, 1999; Fenn and Blaxter, 2004). Therefore, the molecular detection and monitoring of *Wolbachia*-carrying *W. bancrofti* infections that are genotypically associated with benzimidazole sensitivity are ideal for the reciprocal detection of the genetically-stable *W. bancrofti β-tubulin* isotype 1 (Wb*tubb*) gene responsible for benzimidazole susceptibility (Schwab et al., 2005; Hoti et al., 2009; Bhumiratana et al., 2010) and the *Wolbachia ftsZ* (WWb*ftsZ*) gene, which is involved in cell division (Bandi et al., 1998). Moreover, the possibility that the cross-border movement of migrant workers carrying *W. bancrofti* infections with unknown origins and exposed to multiple infective bites in multiple locations can spread the disease is very interesting. Our knowledge is quite rudimentary concerning the factors that shape *W. bancrofti* populations in these migrants when they are no longer receiving the MDA 2-drug regimen (Koyadun and Bhumiratana, 2005; Yongyuth et al., 2006; Bhumiratana et al., 2010). More interestingly, the question remains regarding the genetic stability of benzimidazole-susceptible *W. bancrofti* within a SEA region where migrant carriers naturally acquire the *Wolbachia*-carrying *W. bancrofti* infections from endemic settings with poor geographical MDA coverage.

In this study, to address the problem of the low-copy of the Wb*tubb* and WWb*ftsZ* genes used as molecular markers, we have successfully developed a novel, sensitive and specific touchdown–touchup nested PCR (TNPCR) assay. The benzimidazole-susceptible *W. bancrofti* infections in imported bancroftian filariasis (IBF) cases, which were selected from the at-risk cross-border Myanmar migrants, were examined. Regarding the amplification efficiency (specificity and sensitivity) and characterization of the TNPCR products, two main determinants, the Mf DNA isolation method and exposure to the DEC plus albendazole treatment, were scrutinized to evaluate the empirical use of these locus-specific TNPCRs.

2. Materials and methods

2.1. Subject selection, recruitment and case definition

Night blood surveys of 1178 male and female Myanmar migrant workers aged $\geqslant$20 years were carried out in Phang-nga, southern Thailand, between 2007 and 2009, with the assistance of well-trained Myanmar translators. Of the nine microfilaremic IBF carriers, seven participants gave their informed consent. These included four that received treatment and had follow-up samples (namely, MKA2, MRA3, MME4 and MMO5) and three that had only pre-treatment samples (namely, MDA1, MMO6 and MMO7). As recommended by the provincial migrant worker health service program in Thailand, the four treated subjects were given a 300-mg oral-dose of DEC (for IBF treatment) and 400 mg of albendazole (for helminthiasis treatment) (Yongyuth et al., 2006). Intravenous blood samples of these microfilaremic (WbMf) individuals (approximately 4 ml each) were collected, near the time of microfilarial peak density (0100 h) into ethylenediamine tetra-acetic acid (EDTA), an anticoagulant, both before and at 1–2 months after treatment. Antigenemia was confirmed using the NOW® ICT Filariasis card test (Binax, USA) specific for the *W. bancrofti* circulating filarial antigen (WbAg) (Bhumiratana et al., 2005). The three untreated subjects whose microfilaremia samples were collected only pre-treatment were also examined for the presence of WbMf and WbAg, and eventually, they were given the same treatment that the treated subjects had received. At each time of blood collection, fresh specimens were stored in coolers during transfer to the laboratory, where they were then stored at 4 °C until use. Blood examination for species identification and microfilarial count was conducted using the thick blood film and membrane filtration techniques (Bhumiratana et al., 2005, 2010). Ethical clearance and approval for the study (MU 2007-059 and MU-IRB 2008/290.1301) was obtained from the Institutional Review Board at Mahidol University.

2.2. Genomic DNA extraction

2.2.1. Membrane filter-archived gDNA

One milliliter of each of the microfilaremia ($WbMf^+/Ag^+$) samples was separately filtered through a polycarbonate membrane (Bhumiratana et al., 2010). The isolation of DNA extracts archived from the Mf-containing filter (M-DNA) was performed using a QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Germany) with some modifications. Briefly, an M-DNA filter was initially placed onto a clean microscopic slide and aseptically cut into small pieces with scissors. These pieces were transferred into the bottom of a 2-ml sterilized homogenizer containing 200 μl of 0.9% normal saline solution in the presence of 20 μl of QIAGEN protease, followed by the addition of 200 μl of lysis buffer AL. The lysate was 30-stroke homogenized with a pestle. The lysis reaction was incubated in the homogenizer at 56 °C for 10 min. Then, 200 μl of absolute ethanol was added and the solution was mixed thoroughly by pulse-vortexing for 5 s. The clear lysate (approximately 400 μl) was subsequently filtered through a QIAamp spin column, followed by the downstream procedure described by the manufacturer. Finally, a pure eluted M-DNA solution with an A260/A280 ratio of 1.7–1.9 was obtained. The seven M-DNA samples from the four treated samples at the time-0 month and the three untreated samples, were further used to test the amplification efficiency of the locus-specific TNPCR methods.

2.2.2. Microfilaremia-archived gDNA

Two hundred microliters of each of the microfilaremia ($WbMf^+/Ag^+$) samples of the four subjects who were monitored both before and at post-treatment 1–2 months were used to prepare Mf DNA extracts (C-DNA), using the same QIAamp DNA Blood Mini Kit according to the procedure described by the manufacturer. There was no 2-month MRA3 sample due to the subject's migration. Finally, purified C-DNA was obtained from all eleven samples. Similar to the untreated M-DNA samples mentioned above, the seven C-DNA samples were also used to test the amplification efficiency of the locus-specific TNPCR methods.

2.2.3. Amicrofilaremia-archived gDNA

Importantly, due to human DNA contamination in both microfilaremic M-DNA and C-DNA samples, every microgram of purified gDNA usually contains more human DNA than *W. bancrofti* and *Wolbachia* DNA. That is, the negative control included amicrofilaremic migrant subjects, 10 $WbMf^-/Ag^+$ and 9 $WbMf^-/Ag^-$. Among them, there were four malaria cases co-infected with *Plasmodium falciparum* and *Plasmodium vivax* (two each with $WbMf^-/Ag^+$ and $WbMf^-/Ag^-$). Their resulting blood exams were all confirmed negative by the thick blood film ($WbMf^-$) and either concordantly negative ($WbAg^-$) or discordantly positive ($WbAg^+$) by the ICT card test. The purified DNA extracts (C-DNA) from these amicrofilaremia samples were obtained by using the same QIAamp DNA Blood Mini Kit as described above. In addition, unrelated gDNA purified by phenol/chloroform extraction was obtained from bacterial