

## Short Report: Molecular-Based Assay for Simultaneous Detection of Four *Plasmodium* spp. and *Wuchereria bancrofti* Infections

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**Abstract.** Four major malaria-causing *Plasmodium* spp. and lymphatic filariasis-causing *Wuchereria bancrofti* are co-endemic in many tropical and sub-tropical regions. Among molecular diagnostic assays, multiplex polymerase chain reaction (PCR)-based assays for the simultaneous detection of DNAs from these parasite species are currently available only for *P. falciparum* and *W. bancrofti* or *P. vivax* and *W. bancrofti*. Using a post-PCR oligonucleotide ligation detection reaction-fluorescent microsphere assay (LDR-FMA), we developed a multiplex assay that has the capability to simultaneously detect all four *Plasmodium* spp. and *W. bancrofti* infections in blood samples. Compared with microfilarial positivity in the blood, the LDR-FMA assay is highly concordant (91%), sensitive (86%), and specific (94%), and has high reproducibility for *Plasmodium* spp. (85–93%) and *W. bancrofti* (90%) diagnoses. The development of this assay for the simultaneous diagnosis of multiple parasitic infections enables efficient screening of large numbers of human blood and mosquito samples from co-endemic areas.

Globally, malaria and lymphatic filariasis are the most threatening of the mosquito-transmitted parasitic diseases.<sup>1</sup> Among the three parasites that cause lymphatic filariasis, *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*, *W. bancrofti* is the most widely distributed and is responsible for 90% of lymphatic filariasis infections (bancroftian filariasis) worldwide.<sup>2</sup> Malaria and bancroftian filariasis are co-endemic in many tropical and sub-tropical regions, such as Southeast Asia, including the western Pacific, Africa, and Central and South America, and are transmitted by a number of common vector species.<sup>3,4</sup> Thus, co-infections with malaria and bancroftian parasites in humans<sup>5–7</sup> and mosquitoes<sup>7,8</sup> are found in these regions.

Because of their significant impact on public health, global campaigns with a variety of approaches have been launched for the control/elimination of these diseases.<sup>9,10</sup> These approaches range from the treatment of clinical patients to the control of disease transmission by preventative chemotherapy and vector control.<sup>9,10</sup> However, challenges lie ahead for the success of these control/elimination programs without thoughtful and appropriate use of highly sensitive and specific diagnostic methods.

Parasitologic diagnosis of malaria and bancroftian filariasis is typically made by microscopic examination of stained blood smears or membrane filtrates.<sup>5–7</sup> In addition to microscopic detection of microfilariae, detection of circulating filarial antigen(s) by enzyme-linked immunosorbent assay and immunochromatographic test are other commonly used methods to diagnose bancroftian filariasis.<sup>11–13</sup> A number of polymerase chain reaction (PCR)-based assays are available to separately detect malaria<sup>14,15</sup> and bancroftian filariasis<sup>16–19</sup> parasites. However, only two assays are available to detect these parasite species simultaneously: a multiplex PCR assay for detection of *Plasmodium falciparum* and *W. bancrofti* in humans,<sup>20</sup> and a real-time multiplex quantitative PCR assay

for detection of *P. falciparum* and *W. bancrofti*, or *P. vivax* and *W. bancrofti* in mosquitoes.<sup>21</sup>

Malaria is endemic at altitudes below 1,300–1,600 meters in Papua New Guinea and is the leading cause of illness and death in this country.<sup>22</sup> Four major parasite species, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, are transmitted in Papua New Guinea and mixed-species infections are common.<sup>23,24</sup> Recently, we developed a 96-well format, post-PCR ligation detection reaction-fluorescent microsphere assay (LDR-FMA) for multiplex detection of the four major *Plasmodium* spp.,<sup>25</sup> and validated its utility in diverse epidemiologic settings.<sup>26,27</sup> Bancroftian filariasis is also endemic in several areas in Papua New Guinea and is a major cause of chronic and acute morbidity.<sup>28</sup>

We have been using the density of microfilariae in blood and an enzyme-linked immunosorbent assay (detection of Og4C3 antigen and anti-Bm14 IgG4) as measures of *W. bancrofti* infection in our ongoing lymphatic filariasis-related epidemiologic studies.<sup>29–31</sup> However, with decreasing prevalence of *W. bancrofti* infections, lower microfilaremia, and increasing importance of xenodiagnosis of infection in mosquitoes because of the anticipated success of filariasis elimination programs, DNA-based methods may be more efficient for performing the population-level diagnostic surveillance. Expanding our existing post-PCR LDR-FMA assay, we report the development of a multiplex assay that has the capability to simultaneously detect *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, and *W. bancrofti* infections with high sensitivity and specificity in blood samples.

The study was performed according to protocols approved by Institutional Review Boards of University Hospitals Case Medical Center (Protocol 08-05-13) and the Papua New Guinea Institute of Medical Research (Protocol 07-16). Further approval was obtained from the Papua New Guinea Medical Research Advisory Committee (Protocol 6.09). Informed consent was obtained from all study participants at the time of enrollment.

This new assay involves a multiplex PCR to amplify genomic regions from *Plasmodium* spp. (small subunit ribosomal RNA gene fragment)<sup>25</sup> and *W. bancrofti* (long DNA repeat region),<sup>18</sup> followed by a multiplex LDR-FMA to detect *P. falciparum*,

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*P. vivax*, *P. malariae*, and *P. ovale*,<sup>25</sup> and *W. bancrofti* in a sequence-specific manner. The PCR reagents and conditions for *Plasmodium* spp. amplification have been described.<sup>24,25</sup>

For the multiplex PCR, we evaluated the dNTP concentrations (dATP, dTTP, dGTP, and dCTP) from 200 μM to 800 μM to ensure nucleotide availability for the amplification of both *Plasmodium* spp. and *W. bancrofti* genomic regions, and added 0.12 μM of each of *W. bancrofti* UP (5'-GATGGTGTATAATAGCAGCA-3') and *W. bancrofti* DN (5'-GTCATTATTCTCCGTCGACTGTC-3') amplification primers to the PCR master mixture. The dNTP concentration that performed with consistently high efficiency was 400 μM. The PCR products were subjected to electrophoresis on agarose gels to visualize distinct *Plasmodium* spp. (491–500 basepairs)<sup>25</sup> and *W. bancrofti* (174 basepairs) amplicons. The PCR products were then subjected to LDR-FMA as described,<sup>25</sup> with minor modifications that included use of LDR primers: a *W. bancrofti*-specific primer (tacacttatacaaatcttacaatcTATATCTGCCATAGAAATAACTA [sequence in lower case letters represents a 24-basepair oligonucleotide tag]) and a *W. bancrofti* common primer (Phos 5'-CGGTGGATCTCTGGTTACTACTCTG-3'/Biotin). In the LDR-fluorescent microsphere hybridization solution containing *Plasmodium* species-specific fluorescent microspheres,<sup>25</sup> we added *W. bancrofti* -specific fluorescent microsphere #3. Our *W. bancrofti* PCR and LDR primer sequences are based on the *W. bancrofti* sequence in GenBank (accession no. AY297458).<sup>18</sup>

To confirm the specificity of our *W. bancrofti* PCR primers, we amplified an approximately 170-basepair genomic DNA region from one microfilaria isolated from a person in Papua New Guinea and sequenced it. We also sequenced the PCR products from two microfilaria-positive persons from Papua New Guinea whose blood samples were collected as a part of ongoing studies.<sup>32</sup> Furthermore, we sequenced the PCR product from five pooled third-stage larvae dissected from mosquitoes that were collected in the Dreikikir District (East Sepik Province, Papua New Guinea), where malaria and lymphatic filariasis are endemic.<sup>24,29–31</sup> All sequences were 100% identical with the *W. bancrofti* sequence in GenBank (accession no. AY297458).

The specificity of the assay was further demonstrated by using *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, and *W. bancrofti* genomic DNA samples (Figure 1). Using these genomic DNA samples individually in LDR-FMA reactions containing primers and microspheres for all five species, we found that the assay detected only the parasite species DNA present, and background signals for all other species DNAs were below a median fluorescence intensity of 500. We then performed multiplex PCR LDR-FMA diagnosis to detect *Plasmodium* spp. and *W. bancrofti* infections in the blood samples from 517 persons living in the Dreikikir District (East Sepik Province, Papua New Guinea).

Using this assay, we found that 443 persons (86%) were infected with at least one of the parasites. Overall infection counts were *P. falciparum* 346 (67%), *P. vivax* 176 (34%), *P. malariae* 116 (22%), *P. ovale* 35 (7%), and *W. bancrofti* 175 (34%). Reproducibility of these results was tested for 174 samples (34%). In this analysis, we observed 85–93% concordance for *Plasmodium* spp. diagnosis and 90% concordance for *W. bancrofti* diagnosis. Thus, development of this 96-well format assay for simultaneous diagnosis of multiple para-

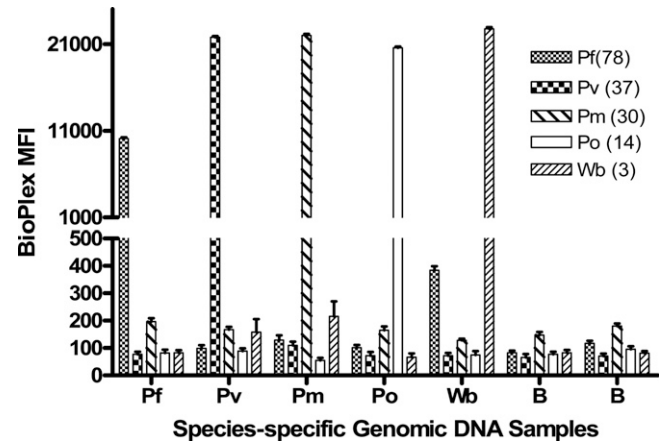


FIGURE 1. Detection of species-specific DNAs by the five-species multiplex polymerase chain reaction oligonucleotide ligation detection reaction–fluorescent microsphere assay (LDR-FMA). Data represent a summary of five positive-control experiments detecting *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *Wuchereria bancrofti* genomic DNAs. Whereas genomic DNAs were added individually, LDR-FMA reactions included oligonucleotide primers and microspheres representing all five species. Numbers in parentheses next to parasite-species designations in the legend ((78), (37), (30), (14), (3)) identify FlexMap™ microspheres (Luminex Corp., Austin, TX). B identifies two blank samples to which no genomic DNAs were added.

sitic infections enables efficient screening of large numbers of samples.

Finally, we categorized *Plasmodium* spp. and *W. bancrofti* infections into parasite assemblages, which are shown in Table 1.

TABLE 1  
Prevalence (counts) of not infected and all infections detected by ligation detection reaction–fluorescent microsphere assay\*

Infection status	No. of persons
Not infected	74
Pf	100
Pv	30
Pm	7
Po	2
Wb	25
Pf, Pv	51
Pf, Pm	27
Pf, Po	4
Pv, Pm	8
Pv, Po	1
Pm, Po	1
Pf, Pv, Pm	22
Pf, Pv, Po	5
Pf, Pm, Po	5
Pv, Pm, Po	2
Pf, Pv, Pm, Po	3
Pf, Wb	68
Pv, Wb	11
Pm, Wb	6
Po, Wb	2
Pf, Pv, Wb	22
Pf, Pm, Wb	16
Pf, Po, Wb	4
Pv, Pm, Wb	1
Pv, Po, Wb	1
Pf, Pv, Pm, Wb	14
Pf, Pv, Po, Wb	1
Pf, Pv, Pm, Po, Wb	4

\*Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*; Wb = *Wuchereria bancrofti*.

Overall infection prevalences of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* and the results regarding single-species and mixed-species infections (Table 1) were consistent with our previous observations from the same area.<sup>24</sup> Most *W. bancrofti* infections (150 of 175) were observed in various *Plasmodium* spp. assemblages containing the most prevalent *P. falciparum* infections (Table 1), which is similar to other findings.<sup>5-7</sup>

For diagnosis of *Plasmodium* spp. infections, we have observed that LDR-FMA is significantly more sensitive than light microscopy.<sup>25-27</sup> In the present study, we compared microfilarial positivity in the blood, determined by microscopic examination of membrane filtrates,<sup>31</sup> with the *W. bancrofti* LDR-FMA results (Table 2). We observed high concordance (91%) between the two methods, and high sensitivity (86%) and specificity (94%) of the LDR-FMA. When we further analyzed 26 false-negative results (microfilariae positive and LDR-FMA negative), we found that 69% (18 of 26) of them had low microfilaria counts (1–5 microfilariae/mL). Thus, false-negative results could be attributed to low parasite density, technical issues related to genomic DNA extraction and PCR/LDR-FMA, and/or *W. bancrofti* sequence polymorphism. Conversely, 19 false-positive results (microfilariae negative and LDR-FMA positive) could be attributed to misreadings during microscopy. Furthermore, false-negative and false-positive results may be partly attributed to non-uniform distribution of microfilariae in the blood.<sup>33</sup>

*Wuchereria bancrofti* is most commonly diagnosed when microfilariae or circulating parasite antigen(s) are detected in the blood.<sup>10</sup> Antibody testing<sup>10</sup> and parasite DNA detection in either human blood samples<sup>20</sup> or mosquitoes<sup>21</sup> could also be used for this purpose. All of these diagnostic tests have their own advantages and limitations, depending upon the lymphatic filariasis-related epidemiologic and clinical characteristics of the region.<sup>10</sup> In the regions where *W. bancrofti* and multispecies *Plasmodium* infections are co-endemic, sensitive and specific molecular diagnosis of the full spectrum of these infections is highly desirable. Furthermore, the availability of accurate tools to monitor presence or absence of parasites in humans and mosquitoes is essential to better assess the effectiveness of intervention programs aimed at controlling or eliminating these diseases. In this direction, the multiplex post-PCR LDR-FMA assay described here represents a significant advance because it detects the four major *Plasmodium* spp. and *W. bancrofti* infections in humans with high sensitivity and specificity in various assemblages.

Using the same assay, studies to detect these infections in mosquitoes collected from the same area in Papua New Guinea are underway. It is envisaged that because of its high-throughput format and high sensitivity and specificity, this multiplex assay has the potential to serve as a useful monitoring tool

during and after implementation of large-scale control/elimination programs. In this regard, the assay can be successfully implemented whether DNA extraction was performed using whole blood, mosquitoes, or filter paper-dried blood spots. In addition to the four major *Plasmodium* spp. and *W. bancrofti*, future investigations can also include detection of the recently emerged human malaria parasite *P. knowlesi*<sup>34</sup> and another important, but less prevalent, filariasis parasite, *B. malayi*, for worldwide application of this assay to the control/elimination programs targeting malaria and lymphatic filariasis.

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TABLE 2

Comparison between microfilaria counts and *Wuchereria bancrofti* LDR-FMA results\*

LDR-FMA	Microfilariae	
	Positive	Negative
Positive	156	19
Negative	26	316

\*Concordance between microfilariae detection by microscopy and ligation detection reaction-fluorescent microsphere assay (LDR-FMA) was calculated as  $(156 + 316)/517 = 91\%$ . Sensitivity of the LDR-FMA assay was calculated as  $156/(156 + 26) = 86\%$ , and specificity of the assay was calculated as  $316/(316 + 19) = 94\%$ .



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