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A qPCR-based Multiplex Assay for Detection of *Wuchereria bancrofti*, *Plasmodium falciparum*, and *Plasmodium vivax* DNA

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Summary

The purpose of this study was to develop multiplex qPCR assays for simultaneous detection of *Wuchereria bancrofti* (Wb), *Plasmodium falciparum* (Pf) and *P. vivax* (Pv) in mosquitoes. We optimized the assays with purified DNA samples and then used these assays to test DNA samples isolated from *Anopheles punctulatus* mosquitoes collected in villages in Papua New Guinea where these infections are co-endemic. Singleplex assays detected Wb, Pf, and Pv DNA in 32%, 19% and 15% of the mosquito pools, respectively, either alone or together with other parasites. Multiplex assay results agreed with singleplex results in most cases. Overall parasite DNA rates in mosquitoes (estimated by the Poolscreen2) for Wb, Pf, and Pv were 4.9%, 2.7%, and 2.1%, respectively. Parasite DNA rates were consistently higher in blood fed mosquitoes than in host seeking mosquitoes. Our results show that multiplex qPCR can be used to detect and estimate prevalence rates for multiple parasite species in arthropod vectors. We believe that multiplex molecular xenodiagnosis has great potential as a tool for non-invasively assessing the distribution and prevalence of vector-borne pathogens such as *W. bancrofti* and *Plasmodium* spp. in human populations and for assessing the impact of interventions aimed at controlling or eliminating these diseases.

Keywords

Wuchereria bancrofti; *Plasmodium falciparum*; *Plasmodium vivax*; Mosquitoes; qPCR; DNA; Multiplex

1. Introduction

Vector-borne parasites that cause malaria and lymphatic filariasis are co-endemic in some parts of Asia and Africa ^{1,2}. Papua New Guinea (PNG) has *Wuchereria bancrofti* and multiple species of *Plasmodium* that are transmitted by anopheline mosquitoes ^{3,4}. Female mosquitoes

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Authors' contributions: RUR and GW planned the study; RUR and YH performed real-time qPCR assays; RUR, YH, and GW analyzed the data; MS, SJL, and MJB organized mosquito sampling and DNA extractions in PNG; RUR and GW wrote the paper; all authors approved the final manuscript; RUR and GW are guarantors of the paper.

Conflicts of interest: None.

Ethical approval: The samples tested were field collected mosquitoes. Therefore ethical approval was not required.

feed on blood every few days for nutrition and egg production. Mosquitoes sometimes ingest blood parasites when they feed on humans in endemic areas. Since filarial infections may decrease mosquito survival, some authors have postulated that filariasis control programs could paradoxically increase malaria transmission in co-endemic areas⁵. On the other hand, malaria control interventions such as insecticide-treated bed nets may decrease filariasis transmission. Therefore, it is important to understand pathogen interactions in vectors and human hosts⁶. Improved diagnostic tools for detecting parasites in vectors could be used to non-invasively map the distribution of these infections and to assess the impact of interventions on filariasis and malaria infection rates in co-endemic areas.

Sensitive methods are available for detecting filarial and *Plasmodium* DNA⁷⁻¹¹. Several studies have shown that mosquito dissection and molecular xenomonitoring (MX, detection of a pathogen's DNA in vectors as a means of indirectly detecting human infections) are useful for assessing changes in prevalence rates of filarial parasites in human populations following mass drug administration (MDA)^{9,12-14}. MX could also be useful indirectly detecting and monitoring *Plasmodium* infections in humans. MX requires collection and pooling of vectors, isolation of genomic DNA from vectors, amplification of parasite DNA sequences, and detection of the amplified product.

Real-time, quantitative polymerase chain reaction (qPCR) assays are a major advance in the molecular detection of infectious agents. qPCR has been used to detect *Plasmodium* spp. in blood and in mosquito vectors^{7,15-17}. We recently reported qPCR methods for detecting *W. bancrofti* or *Brugia malayi* in blood and in mosquitoes^{10,18}. This report describes the development and technical evaluation of multiplex qPCR assays for simultaneous detection of DNA from *W. bancrofti* and *Plasmodium* spp. We also report results obtained with these assays with mosquito samples collected from an area in Papua New Guinea (PNG) that is co-endemic for filariasis and malaria. These results demonstrate the potential value of this approach for efficiently assessing the presence of these vector-borne pathogens in human populations.

2. Materials and methods

2.1 Parasite DNA samples

Genomic DNA was isolated from from *W. bancrofti* (Wb) and *Brugia malayi* as previously described^{10,18}. DNA from *P. falciparum* (Pf) and *P. vivax* (Pv) was kindly provided by Drs. D. Goldberg, M. Klemba and J. Barnwell.

2.2 Mosquito collection

Host-seeking ("empty") and blood-fed *Anopheles punctulatus* mosquitoes were collected with CDC light traps without CO₂ placed overnight inside houses in 3 villages (Buksak, Iguruwe and Naru) in Usino District, Madang province, PNG. This area is endemic for Wb, Pf and Pv⁴. Mosquitoes were pooled by household (1-23 mosquitoes/pool), and DNA was isolated as previously described¹⁰. Prevalence rates for Wb, Pf, and Pv in the study area when mosquitoes were collected were approximately 19%, 21%, and 7.2%, respectively (authors' unpublished data).

2.3 Real-time polymerase chain reaction (PCR) assays for detection of *Wuchereria bancrofti*, *Plasmodium* DNA in singleplex and multiplex assays

The primer and probe sequences used to amplify target sequences (18S rRNA gene for Pf and Pv; long DNA repeat, LDR for Wb) have been previously described^{10,16}. Probes were labeled with the reporter dye 6-carboxyfluorescein (FAM) and VIC or NED at the 5' end. The quencher dyes used were 6-carboxytetramethyl-rhodamine (TAMRA) or Black Hole Quencher (BHQ-1) at the 3' end. Probes for *Plasmodium* spp. were tagged with a minor groove binder (MGB) at

the 3' end. Primers and probes were synthesized commercially by Integrated DNA Technologies (IDT, Coralville, IA) and Applied Biosystems, Foster City, CA.

Two μ l of DNA was mixed with PCR master mix in 96-well MicroAmp optical plates (Applied Biosystems, Foster City, CA) and all singleplex qPCR reactions were performed in a 25 μ l volume as previously described^{10,16}. Singleplex assays were performed to assess assay sensitivity and efficiency using serially diluted Wb, Pf and Pv genomic DNA.

Multiplex assays were designed to detect DNA from two parasite species at a time (Wb and Pf, Wb and Pv, or Pf and Pv). The sensitivity and efficiency of multiplex assays were measured using mixtures of genomic DNA in a checkerboard fashion with DNA templates in the range of 10 ng to 0.0001 ng. All multiplex qPCR reactions were performed with 2 μ l of template, QuantiTect multiplex PCR master mix (Qiagen, Valencia, CA), primers (400 nM) and probes (200 nM) in a final volume of 50 μ l. Thermal cycling and data analysis for singleplex and multiplex assays were performed with an ABI Prism 7300 Real-time PCR System (Applied Biosystems) using ABI sequence detection software. Water was used as a "no template" control (NTC), and DNA from Wb, Pf and Pv served as positive control samples for qPCR reactions. Other negative control samples included DNA isolated from laboratory reared *Ae. aegypti* mosquitoes, DNA isolated from persons with no history of exposure to filariasis or malaria, and *B. malayi* DNA. All qPCR assays were carried out in duplicate, and cycle threshold (C_t) values for each sample were determined as previously described¹⁰. Samples that did not produce fluorescence signals above the threshold by 40 cycles were considered to be negative. Discrepant samples with a single positive well were retested. qPCR efficiencies for each target were determined from the slopes of standard curves generated by plotting graphs with genomic DNA concentrations against C_t values (ABI Prism qPCR User Bulletin 2).¹⁹

2.4 Cloning of PCR products for sequencing

Selected PCR products amplified from genomic DNA in mosquito pools that were positive for LDR, Pf, and Pv were cloned into the pCR4-TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced. These sequences were compared to reference sequences in GenBank (AY297458; M19173; U03079).

2.5 Data analysis

The Kruskal-Wallis test was used to assess the statistical significance of differences in C_t values obtained by singleplex and multiplex assays. Poolscreen software (Version. 2) was used to estimate prevalence rates for parasite DNA in mosquitoes²⁰.

3. Results

3.1 Sensitivity of singleplex and multiplex PCR assays

The sensitivities of singleplex qPCR assays were determined by using Wb, Pf, and Pv genomic DNA as templates. Cycle threshold (C_t) values obtained with LDR and 18S primers and probes were inversely proportional to the amounts of DNA template tested. C_t values showed a reproducible linearity over 5 orders of magnitude, and amplification efficiencies were close to 100 % for all three target sequences. The singleplex assays detected 0.1 pg of template DNA (Fig. 1A-C). These results are consistent with published reports^{10,16}.

Figure 2A shows typical amplification plots for Wb-LDR and Pf-18S templates in a multiplex reaction. Multiplex assays were specific; no FAM fluorescence was detected with Pf or Pv DNA templates, and no VIC or NED fluorescence was detected with Wb DNA template. The analytical sensitivity of multiplex assays for detecting DNA from Wb and *Plasmodium* spp. was excellent when Qiagen Multiplex Mastermix was used (Fig. 2B). TaqMan buffer, routinely

used for the singleplex assays, did not yield optimal target amplifications in multiplex PCR. However, optimized multiplex and singleplex assays (with their respective mastermixes) were equally sensitive for all three targets. Results of a representative checkerboard experiment obtained with a multiplex assay for Wb and Pf DNA are shown in Table 1. The multiplex assay detected both Wb and Pf genomic DNA in the range of 10 ng - 0.0001 ng. However, the multiplex assay failed to detect very low concentrations of one target when the concentration of the second target was very high (Table 1).

All of the singleplex and multiplex assays were highly specific; positive signals were not observed with DNA from humans, *B. malayi*, or uninfected mosquitoes.

3.2 Sensitivity of singleplex and multiplex qPCR for detecting *Wuchereria bancrofti* and *Plasmodium* DNA in field-collected *Anopheles punctulatus* mosquitoes

Four hundred and twelve mosquito pools were tested in this study; the mean number of mosquitoes per pool was 8.1 (median 5). One hundred ninety five pools (47%) were blood-fed or gravid mosquitoes and 217 pools (53%) were host-seeking ("empty").

Singleplex qPCR detected many mosquito pools positive for one or more parasite species (Table 2 and Fig. 3). Of 412 mosquito pools tested, 32%, 19% and 15% were positive for Wb, Pf, and Pv DNA, respectively, either alone or together with DNA from another parasite species. All parasite sequences amplified from mosquitoes (two for each parasite species) matched known target gene sequences for 18S (Pf or Pv) and LDR (Wb).

Mosquito DNA samples were also tested by multiplex PCR. Sensitivities of multiplex and singleplex assays were very similar (Table 2), and the concordance between these assays was excellent (Table 2). However, multiplex assays detected Pf or Pv DNA in a few samples that were negative by singleplex (Table 2). Repeat singleplex assays on these samples were negative. All pools that were negative in multiplex assays were negative for the same targets in singleplex assays. Mosquito pools sometimes contained DNA from multiple pathogens: Wb and Pf DNA were detected in 8.25% of pools, Wb and Pv in 5.8%, and Pf and Pv in 6.5%. C_t values obtained in multiplex assays for Wb and Pf DNA were significantly lower than those obtained in singleplex assays performed on the same specimens ($P < 0.05$).

Table 3 shows parasite DNA rates in mosquitoes estimated by Poolscreen2 software. *W. bancrofti* DNA rates were higher than Pf or Pv rates. Parasite DNA rates were uniformly higher in fed/gravid mosquitoes than those in "empty" mosquitoes. Pf DNA rates were slightly higher than Pv rates, but confidence limits for the two *Plasmodium* DNA rates overlapped. Parasite DNA rates estimated with multiplex and singleplex assays were similar.

4. Discussion

The purpose of this study was to develop and evaluate improved qPCR methods for detecting parasite DNA in field samples. We developed multiplex assays that can simultaneously detect two parasite DNA templates. These assays could be used by clinical laboratories to detect blood parasites in individuals or to screen samples in blood banks in co-endemic areas. However, we believe the main value of such assays will be in public health applications such as MX. The multiplex and singleplex assays we tested detect low levels of parasite DNA. Technical tests showed that multiplex assays failed to detect low DNA concentrations of one target when the concentration of other target was very high. This interference was not unexpected, because the PCR amplifying an abundant template can deplete reagents needed for amplification of a less abundant template. However, this is unlikely to pose a problem in practice, because the high concentrations of DNA template that caused interference are not likely to be encountered in field samples.

Prior reports have described simultaneous detection of filarial and malaria DNA by conventional PCR methods^{21,22}. However, we have recently reported that qPCR assays are more sensitive than conventional PCR for detecting filarial DNA^{10,18}. Mishra et al.²² have recently reported detection of *B. malayi* and *W. bancrofti* DNA by multiplex qPCR analysis of laboratory samples spiked with these templates. The present study used singleplex and multiplex assays to detect Wb and *Plasmodium* spp DNA in pooled, wild-caught mosquitoes. We tested mosquitoes that were blood-fed or gravid (which had recently fed on blood) separately from empty mosquitoes that were seeking blood meals. As expected, parasite DNA rates were higher in blood-fed or gravid mosquitoes than in host seeking mosquitoes, some of which had never fed before. This emphasizes the importance of mosquito sorting for quantitative studies of mosquito populations. In addition, our results demonstrate the utility of multiplex MX for detecting the presence of these parasites in communities.

The large number of DNA samples from mosquitoes collected in PNG permitted us to compare the sensitivity of singleplex and multiplex qPCR assays in terms of the number of positive samples detected. The two methods had high rates of agreement, although multiplex assays detected a few Pf and Pv-positive samples that were not detected by singleplex assays. This result could be related to different mastermixes used in the singleplex and multiplex assays. However, singleplex assays did not work well with multiplex mastermix (data not shown).

Our results show that multiplex PCR assays can be used to detect multiple parasites in arthropod vectors. Similar multiplex assays could also be used to test human blood samples for multiple pathogens. The protocols we used worked well for detecting two DNA templates, but they did not work well for detecting three templates (data not shown). This may be related to the fluorescent dyes used in our probes and technical limitations of the sequence detection instrument we employed. It should be feasible to substitute pan-*Plasmodium* PCR reagents for the Pf and Pv reagents we used to detect Wb plus *Plasmodium* species DNA in a duplex assay⁷. *Plasmodium*-positive samples could then be retested with species-specific reagents to identify the species present in the sample. Technical advances should make triplex or higher level parasite DNA assays feasible in the future.

Multiplex MX provides a convenient and practical method for noninvasively detecting the presence of multiple vector-borne pathogens in human populations. In addition, the high-throughput capacity of qPCR makes multiplex MX a practical tool for measuring parasite DNA rates in mosquitoes and changes in DNA rates following intervention activities.

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Figure 1.

Sensitivity of singleplex qPCR in detecting *Wuchereria bancrofti* (Wb), *P. falciparum* (Pf) and *P. vivax* (Pv). Genomic DNA templates from Wb (A), Pf (B) and Pv (C) were tested in duplicate. The X axis shows DNA template values on a Log scale with “0” representing 1 ng. Cycle threshold values (C_t) (y-axis) were plotted against Log template DNA (x-axis) concentrations to generate a standard curve. PCR efficiencies were close to 100%.

Figure 2.

Panel A: Amplification plot of fluorescence (y-axis) vs cycle number (x-axis) shows the sensitivity of multiplex PCR for detecting *Wuchereria bancrofti* (Wb) and *Plasmodium falciparum* (Pf) DNA. Genomic DNA templates (1 ng) from Wb and Pf were tested in a single well. Panel B: Sensitivity of multiplex qPCR in detecting Wb and Pf. Genomic DNA templates from Wb and Pf were tested in duplicate. The x-axis shows DNA template values on a Log scale with “0” representing 1 ng. Cycle threshold values (y-axis) were plotted against Log template DNA (x-axis) concentrations to generate standard curves with reproducible linearity. The reaction efficiencies were close to 100%.

Figure 3.

This figure shows results of singleplex qPCR assays for *Wuchereria bancrofti* (Wb), *P. falciparum* (Pf), and *P. vivax* (Pv) DNA in 412 pools of *Anopheles punctulatus* from Papua New Guinea. Data shown are the percent of pools positive for DNA from one or more parasite species. DNA from all three parasites was detected in these mosquitoes. This indicates that all three parasites were present in mosquitoes (and in humans) in the study area.

Table 1
Sensitivity of multiplex qPCR for detecting *Wuchereria bancrofti* and *Plasmodium falciparum* DNA

Pf DNA (ng)	10	1	Wb DNA(ng) 0.1	0.01	0.001	0.0001
10	+/+	+/+	+/+	+/+	+/-	+/-
1	+/+	+/+	+/+	+/+	+/-	+/-
0.1	+/+	+/+	+/+	+/+	+/+	+/+
0.01	+/+	+/+	+/+	+/+	+/+	+/+
0.001	-/-	+/+	+/+	+/+	+/+	+/+
0.0001	-/-	-/-	+/+	+/+	+/+	+/+

Wb, *Wuchereria bancrofti*, Pf, *P. falciparum*. Wb and Pf DNA were detected by multiplex PCR amplification of LDR and 18S sequences, respectively. +/- indicates indicate that multiplex qPCR was positive for both Wb and Pf with listed template concentrations.

Results of singleplex and multiplex assays for detecting *Wuchereria bancrofti* and *Plasmodium* DNA in *Anopheles punctulatus* mosquito pools*

Parasites	Singleplex assay			Multiplex assay		
	Wb	Pf	Pv	Wb/Pf	Wb/Pv	Pf/Pv
Wb	131	-	-	131	131	-
Pf	-	79	-	79	-	80
Pv	-	-	60	-	61	62

* 412 mosquito pools were tested by singleplex and multiplex qPCR assays. Results shown are the number of pools positive for each parasite DNA target sequence. Overall concordance rates for multiplex and singleplex assays were 100% for *Wuchereria bancrofti* (Wb), 99% for *Plasmodium falciparum* (Pf), and 97% for *P. vivax* (Pv).

Table 3

Parasite DNA rates in *Anopheles punctulatus* mosquitoes estimated from data from singleplex and multiplex qPCR assays of mosquito pools

Parasites		Poolscreen estimates		
	Assay	Total	Blood fed/gravid	“Empty”
Wb	Singleplex	4.9 (4.0-6.0)	7.2 (5.4-9.2)	3.7 (2.7-4.8)
	Multiplex	4.9 (4.0-6.0)	7.2 (5.4-9.2)	3.7 (2.7-4.8)
Pf	Singleplex	2.7 (2.1-3.4)	5.2 (3.7-7.0)	1.4 (0.9-2.1)
	Multiplex	2.7 (2.1-3.5)	5.4 (3.9-7.2)	1.4 (0.9-2.1)
Pv	Singleplex	2.0 (1.4-2.6)	2.3 (1.4-3.5)	1.8 (1.2-2.6)
	Multiplex	2.1 (1.6-2.8)	2.6 (1.7-3.9)	1.8 (1.2-2.6)

Data shown are maximum likelihood values (% with 95% confidence intervals in parentheses) estimated with Poolscreen2 software. Wb, *Wuchereria bancrofti*; Pf, *Plasmodium falciparum*; Pv, *P. vivax*. “Empty” mosquitoes were host-seeking mosquitoes (not gravid with no blood visible in the midgut).