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A qPCR-based multiplex assay for the detection of *Wuchereria bancrofti*, *Plasmodium falciparum* and *Plasmodium vivax* DNA

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Summary The purpose of this study was to develop real-time multiplex quantitative PCR (qPCR) assays for the 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 PoolScreen 2 software, 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.

1. Introduction

Vector-borne parasites that cause malaria and lymphatic filariasis are co-endemic in some parts of Asia and Africa.[1,2] In Papua New Guinea (PNG), *Wuchereria bancrofti* and multiple species of *Plasmodium* are transmitted by anopheline

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mosquitoes.[3,4] Female mosquitoes feed on blood every few days for nutrition and egg production, and 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 areas.[5] 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 hosts.[6] 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* DNA.[7–11] Several studies have shown that mosquito dissection and molecular xenomonitoring (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.[9,12–14] Xenomonitoring could also be useful for indirectly detecting and monitoring *Plasmodium* infections in humans; a process 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 PCR (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 the simultaneous detection of DNA from *W. bancrofti* and *Plasmodium* spp. We also report results obtained from these assays on mosquito samples collected from an area in PNG that is co-endemic for filariasis and malaria.

2. Materials and methods

2.1. Parasite DNA samples

Genomic DNA was isolated from *W. bancrofti* (Wb) and *B. 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 CO_2 placed overnight inside houses in three villages (Buksak, Iguruwe and Naru) in Usino District, Madang Province, PNG during 2001–2003. This area is endemic for Wb, Pf and Pv.[4] Mosquitoes were pooled by household (1–23 mosquitoes/pool), and DNA was isolated as previously described.[10] Prevalence rates for Wb, Pf and Pv in the study area when mosquitoes collected were approximately 19%, 21% and 7.2%, respectively (authors' unpublished data).

2.3. Real-time PCR 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 labelled 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, USA) and Applied Biosystems (Foster City, CA, USA).

DNA (2 μl) was mixed with PCR master mix in 96-well microamp optical plates (Applied Biosystems) and all singleplex qPCR reactions were performed in a 25 μl 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 10–0.0001 ng. All multiplex qPCR reactions were performed with 2 μl of template, quantitect multiplex PCR master mix (Qiagen, Valencia, CA, USA), primers (400 nm) and probes (200 nm) in a final volume of 50 μl. 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, 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 *Aedes 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 described.[10] 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 of genomic DNA concentrations against C_t values.[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, USA) 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 2 software was used to estimate prevalence rates for parasite DNA in mosquitoes.[20]

3. Results

3.1. Sensitivity of singleplex and multiplex PCR assays in the laboratory

The sensitivities of singleplex qPCR assays were determined by using Wb, Pf and Pv genomic DNA as templates. 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 five 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 (Figure 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 the Wb DNA template. The analytical sensitivity of multiplex assays for detecting DNA from Wb and *Plasmodium* spp. was excellent when Qiagen Multiplex master mix was used (Figure 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 master mixes) 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—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 qPCR for detecting parasite 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 and 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 Figure 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

Table 1 Sensitivity of multiplex qPCR for detecting *Wuchereria bancrofti* (Wb) and *Plasmodium falciparum* (Pf) DNA[a]

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

[a] DNA was detected by multiplex PCR amplification of the Wb long DNA repeat and the Pf 18S rRNA sequences, respectively. +/+ indicates that multiplex qPCR was positive for both Wb and Pf with listed template concentrations.

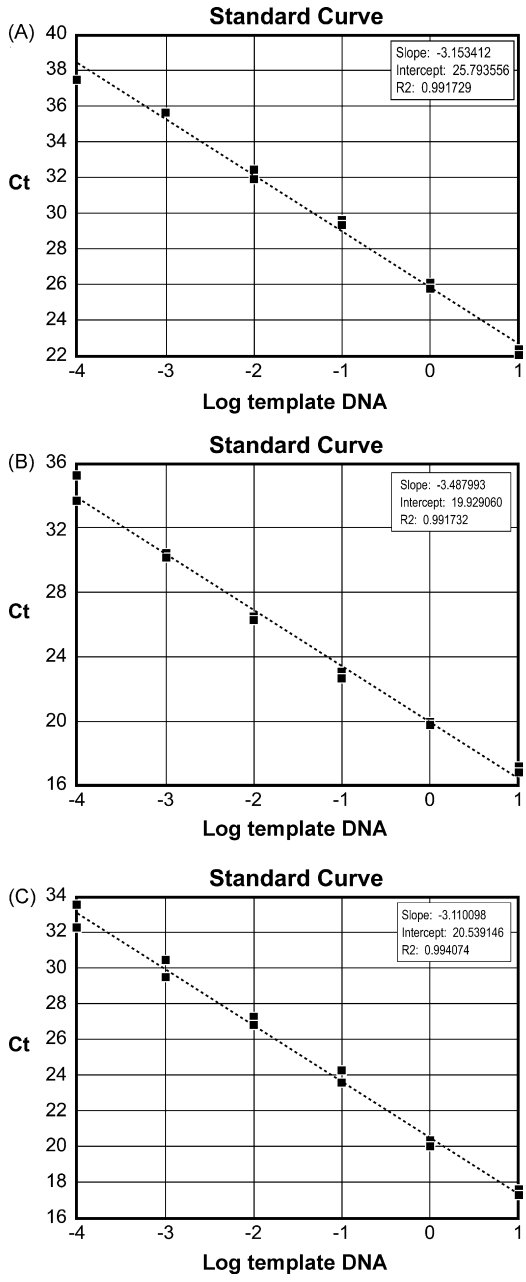

Figure 1 Sensitivity of singleplex qPCR in detecting *Wuchereria bancrofti* (Wb), *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv). Genomic DNA templates from Wb (A), Pf (B) and Pv (C) were tested in duplicate. The x-axis shows DNA template values on a log scale with '0' representing 1 ng. Cycle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

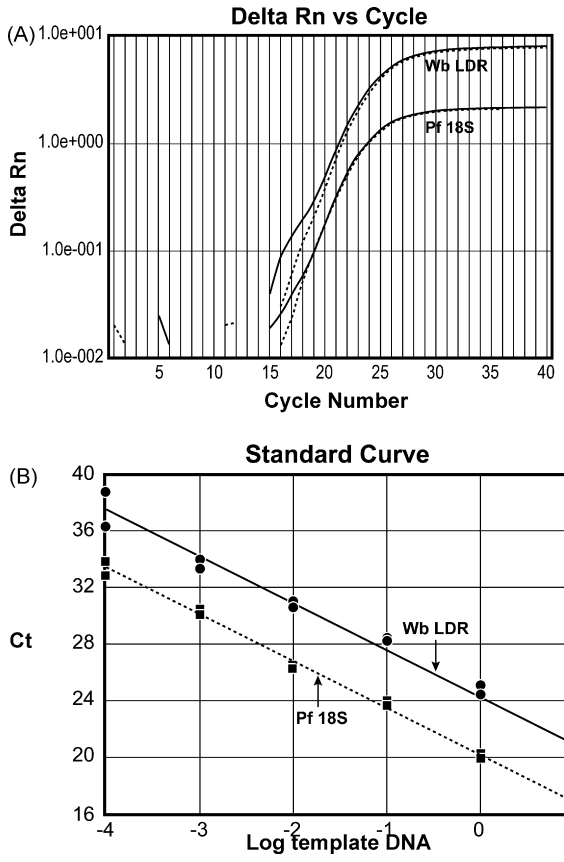

Figure 2 Panel A: amplification plot of fluorescence units (y-axis) vs. cycle number (x-axis) shows the sensitivity of multiplex PCR for detecting *Wuchereria bancrofti* (Wb) and *Plasmodium falciparum* (Pf) DNA. Genomic DNA templates (1 ng) from Wb and Pf were tested in a single well. Panel B: sensitivity 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 concentrations to generate standard curves with reproducible linearity. The reaction efficiencies were close to 100%.

Table 2 Results of singleplex and multiplex assays for detecting *Wuchereria bancrofti* (Wb), *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv) DNA in *Anopheles punctulatus* mosquito pools[a]

Parasite	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

[a] 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 Wb, 99% for Pf and 97% for Pv.

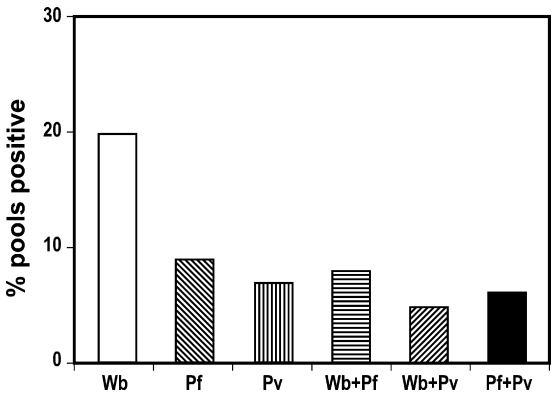

Figure 3 Results of singleplex qPCR assays for *Wuchereria bancrofti* (Wb), *Plasmodium falciparum* (Pf), and *Plasmodium vivax* (Pv) DNA in 412 pools of *Anopheles punctulatus* from Papua New Guinea. Data shown are the percentage of pools positive for DNA from one or more parasite species. DNA from 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.

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, 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 assays (Table 2). Repeat singleplex assays of 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 PoolScreen 2 software. Wb 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 xenomonitoring. 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

Table 3 Parasite DNA rates in *Anopheles punctulatus* mosquitoes estimated from singleplex and multiplex qPCR assays of mosquito pools

Assay	PoolScreen estimates[a]		
	Total	Blood fed/gravid	Empty[b]
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)

Wb: *Wuchereria bancrofti*; Pf: *Plasmodium falciparum*; Pv: *Plasmodium vivax*.
[a] Data shown are maximum likelihood values (% with 95% CI in parentheses) estimated with PoolScreen 2 software.
[b] Empty mosquitoes were host-seeking mosquitoes (not gravid with no blood visible in the midgut).

one target when the concentration of the 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 the 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 the detection of *B. malayi* and *W. bancrofti* DNA by multiplex PCR analysis of laboratory samples spiked with these templates. The present study used singleplex and multiplex qPCR 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 xenomonitoring 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 samples positive for Pf and Pv that were not detected by singleplex assays. This result could be related to the different master mixes used in the singleplex and multiplex assays. However, singleplex assays did not work well with multiplex master mixes (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 primer sequences selected, 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 in order to detect Wb plus *Plasmodium* species DNA in a duplex assay.[7] *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 xenomonitoring 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 xenomonitoring a practical tool for measuring parasite DNA rates in mosquitoes and changes in DNA rates following intervention activities.

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

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Conflicts of interest: None.

Ethical approval: The samples tested were field-collected mosquitoes; therefore ethical approval was not required.

References

1. Ravindran B, Sahoo PK, Dash AP. Lymphatic filariasis and malaria: concomitant parasitism in Orissa, India. *Trans R Soc Trop Med Hyg* 1998;**92**:21–3.
2. Chadee DD, Rawlins SC, Tiwari TS. Short communication: concomitant malaria and filariasis infections in Georgetown, Guyana. *Trop Med Int Health* 2003;**8**:140–3.
3. Burkot TR, Molineaux L, Graves PM, Paru R, Battistutta D, Dagoro H, et al. The prevalence of naturally acquired multiple infections of *Wuchereria bancrofti* and human malarias in anophelines. *Parasitology* 1990;**100**:369–75.
4. Muller I, Bockarie M, Alpers M, Smith T. The epidemiology of malaria in Papua New Guinea. *Trends Parasitol* 2003;**19**:253–9.
5. Pichon G. Limitation and facilitation in the vectors and other aspects of the dynamics of filarial transmission: the need for vector control against *Anopheles*-transmitted filariasis. *Ann Trop Med Parasitol* 2002;**96**(Suppl 2):S143–52.
6. Muturi EJ, Jacob BG, Kim CH, Mbogo CM, Novak RJ. Are coinfections of malaria and filariasis of any epidemiological significance? *Parasitol Res* 2008;**102**:175–81.
7. Rougemont M, Van Saanen M, Sahli R, Hinrikson HP, Bille J, Jaton K. Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. *J Clin Microbiol* 2004;**42**:5636–43.
8. Williams S, Laney S, Bierwert L, Saunders LJ, Boakye DA, Fischer P, et al. Development and standardization of a rapid PCR-based method for the detection of *Wuchereria bancrofti* in mosquitoes for xenomonitoring the human prevalence of bancroftian filariasis. *Ann Trop Med Parasitol* 2002;**96**(Suppl 2):S41–46.
9. Bockarie MJ, Fischer P, Williams SA, Zimmerman PA, Griffin L, Alpers MP, et al. Application of a polymerase chain reaction-ELISA to detect *Wuchereria bancrofti* in pools of wild-caught *Anopheles punctulatus* in a filariasis control area in Papua New Guinea. *Am J Trop Med Hyg* 2000;**62**:363–7.
10. Rao RU, Atkinson LJ, Ramzy RM, Helmy H, Farid HA, Bockarie MJ, et al. A real-time PCR-based assay for detection of *Wuchereria bancrofti* DNA in blood and mosquitoes. *Am J Trop Med Hyg* 2006;**74**:826–32.
11. Goodman DS, Orelus JN, Roberts JM, Lammie PJ, Streit TG. PCR and mosquito dissection as tools to monitor filarial infection levels following mass treatment. *Filaria J* 2003;**2**:11.
12. Ramzy R, Farid H, Kamal H, Ibrahim GH, Morsy ZS, Faris R, et al. A polymerase chain reaction-based assay for detection of *Wuchereria bancrofti* in human blood and *Culex pipiens*. *Trans R Soc Trop Med Hyg* 1997;**91**:156–60.
13. Weil GJ, Ramzy RM. Diagnostic tools for filariasis elimination programs. *Trends Parasitol* 2007;**23**:78–82.
14. Farid HA, Morsy ZS, Helmy H, Ramzy RM, El Setouhy M, Weil GJ. A critical appraisal of molecular xenomonitoring as a tool for assessing progress toward elimination of lymphatic filariasis. *Am J Trop Med Hyg* 2007;**77**:593–600.
15. Polanco JC, Rodriguez JA, Corredor V, Patarroyo MA. *Plasmodium vivax*: parasitemia determination by real-time quantitative PCR in Aotus monkeys. *Exp Parasitol* 2002;**100**:131–4.
16. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, et al. Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. *J Clin Microbiol* 2004;**42**:1214–9.
17. Bell AS, Ranford-Cartwright LC. A real-time PCR assay for quantifying *Plasmodium falciparum* infections in the mosquito vector. *Int J Parasitol* 2004;**34**:795–802.
18. Rao RU, Weil GJ, Fischer K, Supali T, Fischer P. Detection of *Brugia* parasite DNA in human blood by real-time PCR. *J Clin Microbiol* 2006;**44**:3887–93.
19. Applied Biosystems. User Bulletin 2: ABI PRISM7700 Sequence Detection System. Foster City: Applied Biosystems; 2001. http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf [accessed 3 July 2008].
20. Katholi CR, Toe L, Merriweather A, Unnasch TR. Determining the prevalence of *Onchocerca volvulus* infection in vector populations by polymerase chain reaction screening of pools of black flies. *J Infect Dis* 1995;**172**:1414–7.
21. Chansiri K, Kwoasak P, Tananyutthawongese C, Sukhumsirichart W, Sarataphan N, Phantana S. Detection of *Plasmodium falciparum* and *Wuchereria bancrofti* infected blood samples using multiplex PCR. *Mol Cell Probes* 2001;**15**:201–7.
22. Mishra K, Raj DK, Dash AP, Hazra RK. Combined detection of *Brugia malayi* and *Wuchereria bancrofti* using single PCR. *Acta Trop* 2005;**93**:233–7.