A sensitive, specific and reproducible real-time polymerase chain reaction method for detection of *Plasmodium vivax* and *Plasmodium falciparum* infection in field-collected anophelines

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We describe a simple method for detection of Plasmodium vivax *and* Plasmodium falciparum *infection in anophelines using a triplex TaqMan real-time polymerase chain reaction (PCR) assay (18S rRNA). We tested the assay on* Anopheles darlingi *and* Anopheles stephensi *colony mosquitoes fed with* Plasmodium*-infected blood meals and in duplicate on field collected* An. darlingi*. We compared the real-time PCR results of colony-infected and field collected* An. darlingi*, separately, to a conventional PCR method. We determined that a cytochrome* b*-PCR method was only 3.33% as sensitive and 93.38% as specific as our real-time PCR assay with field-collected samples. We demonstrate that this assay is sensitive, specific and reproducible.*

Key words: *Anopheles* - *Plasmodium* - TaqMan - real-time PCR

Here we describe a reliable, sensitive and specific real-time polymerase chain reaction (PCR) protocol to detect the two most common species of *Plasmodium* (*vivax* and *falciparum*) in *Anopheles* mosquito vector DNA, optimised with TaqMan reagents. It is essential to accurately identify mosquito vectors and their *Plasmodium* species infection status to calculate the entomological inoculation rate for monitoring and evaluating malaria transmission levels. A comparison was made between the commonly used cytochrome *b* PCR-based (*Cytb*-PCR) method for detecting *P. falciparum* and *P. vivax* (Hasan et al. 2009) infections and our real-time PCR method.

Colony *Anopheles darlingi* (Moreno et al. 2014) and *Anopheles stephensi* (provided by F Li and JM Vinetz, University of California, San Diego) were fed to repletion, using a membrane feeder, on *P. vivax* or *P. falciparum*-infected blood, respectively, then euthanised 14 days post-blood meal. Individual mosquito heads and thoraces were extracted manually or with a QIAcube using the DNeasy® Blood & Tissue Kit (Qiagen, Germany). DNA concentration of each extraction was determined using a Qubit® 2.0 fluorometer with the Qubit® dsDNA high sensitivity assay (Life Technologies, Thermo Fisher Scientific, USA). *Plasmodium* infection was detected with real-time PCR of the small subunit of the 18S rRNA gene, using a monoplex or triplex TaqMan assay (Life Technologies, Thermo Fisher Scientific) on the StepOnePlus Real-Time PCR System (Life Technologies, Thermo Fisher Scientific). These assays employed primers designed elsewhere (Rougemont et al. 2004, Shokoples et al. 2009, Diallo et al. 2012) with modified *Plasmodium* species-specific forward primers and probes. Modified primers were optimised for TaqMan assays using Primer Express® software v.3.0.1 (Life Technologies, Thermo Fisher Scientific). *Plasmodium* detection was achieved using genus specific primers and probe (Plasmo1-F: GTTAAGGGAGTGAAGACGATCAGA; Plasmo2-R: AACCCAAAGACTTTGATTTCTCATAA; Plasprobe: FAM-TCGTAATCTTAACCATAAAC-MGBNFQ) (Rougemont et al. 2004, Shokoples et al. 2009). *P. vivax* or *P. falciparum* species determination was achieved using forward primers and probes nested within the genus-specific product (Falc-F: GACTAGGTGTTGGATGAAAGTGTTAAA; Falciprobe: VIC-TGAAGGAAGCAATCTAAAAGTCACCTCGAAAGA-QSY; Vivax-F: GACTAGGCTTTGGATGAAAGATTTTAA; Vivaxprobe: NED-ATAAACTCCGAAGAGAAAA-MGBNFQ). Primers and probes were synthesised by Life Technologies. Each PCR reaction occurred in 20 µL containing 1x PerfeCTa qPCR ToughMix, Uracil N-glycosylase (UNG), ROX (Quanta Biosciences, USA), 0.3 µM of each primer, 0.1 µM of each probe and genomic DNA. Cycling conditions for both the monoplex and triplex assays included a 5 min UNG-activation hold at 45ºC and a denaturation step for 2 min at 95ºC, followed by 50 cycles of 95ºC denaturation for 15 s and 60ºC annealing/elongation for 1 min.

DNA pools of five mosquitoes were made using equal amounts of gDNA (ng) per mosquito. Mosquito DNA pools were tested initially with a monoplex assay for *Plasmodium* spp detection using only *Plasmodium*

doi: 10.1590/0074-02760150031
Financial support: NIH [R01 AI110112 (to JEC), U19 AI089681 (to JMV)]
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Received 20 February 2015
Accepted 30 April 2015

genus-specific primers and probe. For this assay, up to 15 ng of gDNA was used per reaction with a maximum volume of 8.6 µL. Controls consisted of water as a negative control, a no-template control using gDNA from uninfected, colony *An. darlingi* and a positive control of 1,000X diluted MR4 MRA-102G (reagent obtained through the MR4 as part of the BEI Resources Repository, National Institute of Allergy and Infectious Diseases, National Institutes of Health: *P. falciparum* genomic DNA from *P. falciparum* 3D7, MRA-102G) (Rosario 1981, Walliker et al. 1987). Amplification began at approximately cycle 35 (*Plasmodium* spp range 32-34, *P. vivax* range 32-36 and *P. falciparum* range 34-38), with a cut-off of 50 cycles to define *Plasmodium* positive samples. *Plasmodium* spp positive *An. darlingi* and *An. stephensi* were then tested using the triplex assay to confirm infection status and verify the specificity of the assay by determining the *Plasmodium* species. In these individual triplex reactions, up to 15 ng of gDNA was used per reaction with a maximum volume of 7 µL. Cycling conditions were the same as for the monoplex assay.

Previous studies (Rao et al. 2009, Sandeu et al. 2012, Marie et al. 2013, Ngo et al. 2014) have developed real-time PCR assays to detect *Plasmodium* infections in mosquito vectors, but none in the major Neotropical vector *An. darlingi*. Our motivation for developing this assay was to reliably detect *Plasmodium*-infected field-caught mosquitoes in malaria endemic regions of Latin America to incriminate anopheline vectors. Therefore, we tested the monoplex assay on pools of five field-caught *An. darlingi* mosquitoes from localities near Iquitos, Peru, in duplicate. In all cases, pools identified as positive in monoplex assay were positive in both replicates. Individual mosquitoes from each of the positive pools were tested with the triplex assay to determine infection status and corresponding *Plasmodium* species. At least one *Plasmodium*-positive *An. darlingi* was identified in each positive pool. Throughout the course of development and analyses, this assay proved very reliable under a number of different circumstances: (i) individual *Plasmodium* positive mosquitoes were identified in pools of DNA from five mosquitoes *via* monoplex assay (Fig. 1) and verified in individual mosquito triplex assay, (ii) positive controls were accurately and reliably identified in triplex assay (Fig. 2), (iii) mixed infections were identified in some mosquito samples (Fig. 3) and, finally, (iv) *Plasmodium*-positive mosquitoes that measured as low as < 0.5 ng/µL in Qubit® assay amplified and showed infection in the triplex assay.

The results from real-time PCR *Plasmodium* detection were compared with the results of the *Cytb*-PCR method for detection of *Plasmodium*, which amplifies the *Plasmodium Cytb* gene. This PCR method was carried out according to Hasan et al. (2009) and infection was determined through visualisation of PCR product on agarose gel. To compare the results from the two assays, sensitivity and specificity calculations, Cohen's kappa (κ) (for concordance) and McNemar's test (for discordance) were implemented. In these comparisons, the *Cytb*-PCR results were compared to our assay's results for two important reasons. First, while testing samples

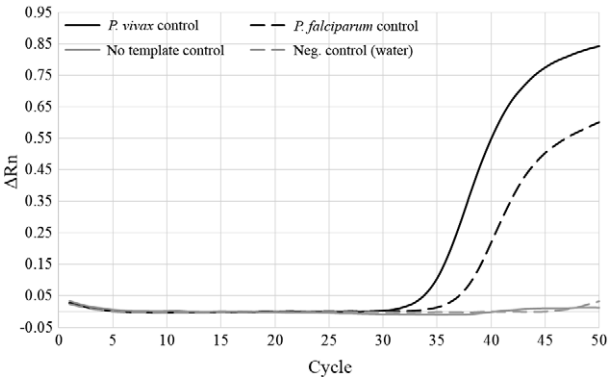

Fig. 1: real-time polymerase chain reaction (PCR) amplification plot of a monoplex *Plasmodium* spp assay. The four quantitative PCR controls are shown: *Plasmodium vivax* infected *Anopheles darlingi* (black, solid line), *Plasmodium falciparum* infected *Anopheles stephensi* (black, dashed line), uninfected *An. darlingi* (no template control) (grey, solid line) and water (negative control) (grey, dashed line). ΔRn: baseline corrected normalised fluorescence.

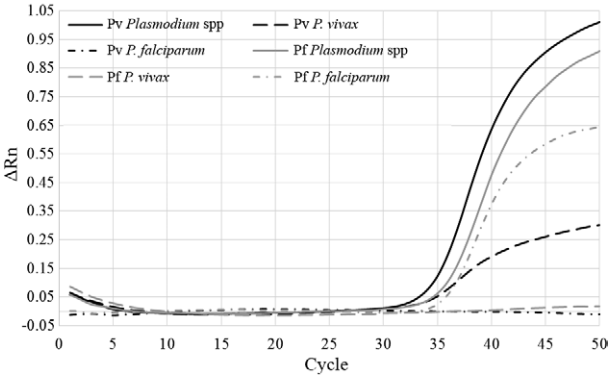

Fig. 2: real-time polymerase chain reaction (PCR) amplification plot of a triplex *Plasmodium* spp assay. The two quantitative PCR positive controls are shown: *Plasmodium vivax* infected *Anopheles darlingi* (black lines) (solid line: *Plasmodium* spp positive; dashed line: *P. vivax* species positive; dashed/dotted line: *Plasmodium falciparum* negative) and *P. falciparum* infected *Anopheles stephensi* (grey lines) (solid line: *Plasmodium* spp positive; dashed line: *P. vivax* negative; dashed/dotted line: P. *falciparum* positive). ΔRn: baseline corrected normalised fluorescence.

with the *Cytb*-PCR protocol, we ran into problems that suggested the results could not be replicated, such as nonspecific binding (laddering of the PCR product on agarose gel) and inconsistency of results between our laboratory and our collaborating International Centers of Excellence for Malaria Research laboratory in Iquitos. Samples with laddering included one band that corresponds to the correct PCR product size, but there were also samples that appeared positive without the laddering effect. Second, multiple reports have shown real-time PCR detection of *Plasmodium* is much more sensi-

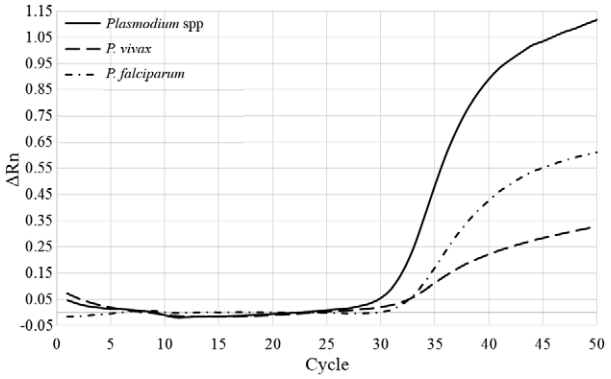

Fig. 3: real-time polymerase chain reaction (PCR) amplification plot of a triplex *Plasmodium* spp assay showing a sample with a mixed *Plasmodium vivax*/*Plasmodium falciparum* infection (solid line: *Plasmodium* spp positive; dashed line: *P. vivax* positive; dashed/dotted line: *P. falciparum* positive). ΔRn: baseline corrected normalised fluorescence.

tive than conventional PCR-based methods and provide faster, less time-consuming results with a reduced risk of contamination (Rougemont et al. 2004, Shokoples et al. 2009, Marie et al. 2013, Lau et al. 2015).

Sensitivity, or the ability of an assay to correctly determine whether a sample is truly positive, is calculated by dividing the number of true positives (TP) (both assays agree that a sample is positive) by the sum of the TPs and the false negatives (FN):

$$Sensitivity = \frac{TP}{TP+FN} * 100\%$$

In this case, FNs are those samples positive by real-time PCR, but negative by *Cytb*-PCR. Specificity is the ability of an assay to correctly determine whether a sample is truly negative. This is calculated by dividing the number of true negatives (TN) (both assays agree that a sample is negative) by the sum of the TNs and the false positives (FP):

$$Specificity = \frac{TN}{TN+FP} * 100\%$$

FPs are samples negative by real-time PCR assay but positive by *Cytb*-PCR assay (Gerstman 2008). Cohen's κ, used to measure agreement, determines at what percentage of samples the results from the two assays agree, correcting for random chance. κ ranges between -1, complete disagreement and 1, complete agreement and is calculated by first determining the percentage of the samples with results that agree between the two assays:

$$[\Pr(a) = \frac{TP+TN}{total\ samples\ tested}]$$

Next, the probability of random chance agreement is calculated by the product of the percentages of samples that were considered positive by each assay, added to the product of the percentages of samples that were considered negative by each assay:

$$\Pr(e) = (\frac{\#\ pos.\ by\ \text{Cytb-}PCR}{total\ samples\ tested} * \frac{\#\ pos.\ by\ real\text{-}time\ PCR}{total\ samples\ tested}) + (\frac{\#\ neg.\ by\ \text{Cytb-}PCR}{total\ samples\ tested} * \frac{\#\ neg.\ by\ real\text{-}time\ PCR}{total\ samples\ tested})$$

Finally, κ is calculated (Cohen 1960) by

$$\kappa = \frac{\Pr(a)-\Pr(e)}{1-\Pr(e)}$$

The test statistic for McNemar's test for discordance is calculated as

$$\chi^2 = \frac{(FP-FN)^2}{FP+FN}$$

and follows a chi-squared distribution with one degree of freedom (McNemar 1947).

When we compared our real-time assay to the *Cytb*-PCR assay using laboratory-infected mosquitoes, we found few differences. The *Cytb*-PCR assay was 85% as sensitive and 82.50% as specific as our real-time PCR assay and showed substantial agreement (κ = 0.68). In addition, McNemar's test showed no significant discordance between assays (χ^2 = 0.077; p > 0.5) (Table I). However, in comparisons using field-collected *An. darlingi*, *Cytb*-PCR is 3.33% as sensitive and 93.38% as specific as our real-time PCR assay (Table II). Additionally, the *Cytb*-PCR protocol neither agrees nor disagrees with our assay, after accounting for random chance (κ = -0.04) and is not significantly discordant from our real-time PCR assay (χ^2 = 1.653; p > 0.1), due to the large number of truly negative samples tested (Table II). In Latin America, the percentage of *Plasmodium*-infected

TABLE I

Comparison of results from the cytochrome *b*-polymerase chain reaction (*Cytb*-PCR) and real-time PCR *Plasmodium* detection assays with laboratory infected *Anopheles darlingi* and *Anopheles stephensi*

	Real-time PCR positive (n)	Real-time PCR negative (n)	Sensitivity (%)	Specificity (%)
Cytb-PCR positive	34	7	85	82.50
Cytb-PCR negative	6	33	-	-

Cohen's kappa = 0.68; McNemar's test χ^2 = 0.077, p > 0.5.

TABLE II

Comparison of results from the cytochrome *b*-polymerase chain reaction (*Cytb*-PCR) and real-time PCR *Plasmodium* detection assays using field-caught *Anopheles darlingi*

	Real-time PCR positive (n)	Real-time PCR negative (n)	Sensitivity (%)	Specificity (%)
Cytb-PCR positive	1	20	3.33	93.38
Cytb-PCR negative	29	282	-	-

Cohen's kappa = -0.04; McNemar's test χ^2 =1.653, p > 0.1.

anopheline vectors varies greatly and is highly dependent upon vector, season, host availability and location (da Silva-Nunes et al. 2012). The comparisons between these assays represent a real-world scenario, as would be encountered by vector biologists testing field-collected samples, in addition to comparing results of known infected mosquitoes from laboratory colonies (as above).

In conclusion, we have demonstrated that the assays we describe are sensitive, specific and reproducible alternative to the *Cytb*-PCR-based detection of *P. vivax* and *P. falciparum* in field collected *Anopheles* samples.

ACKNOWLEDGEMENTS

To Carlos Tong (Universidad Peruana Cayetano Heredia, Peru), for providing *P. vivax* infected *An. darlingi*, and to Dr Fengwu Li (Division of Infectious Diseases, UCSD), for providing *P. falciparum* infected *An. stephensi*.

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