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.

Colonies of Anopheles darlingi (Moreno et al. 2014) and Anopheles stephensi (provided by F Li and JM Vinetz, University of California, San Diego) were fed topletion, using a membrane feeder, on Plasmodium (provided by F Li and JM Vinetz, University of California, San Diego) were fed topletion, using a membrane feeder, on

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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: Plasmodium 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 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-
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):

\[
\text{Sensitivity} = \frac{TP}{TP+FN} \times 100\%
\]

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):

\[
\text{Specificity} = \frac{TN}{TN+FP} \times 100\%
\]

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:

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

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:

\[
\Pr(e) = \left(\frac{\# \text{ pos. by Cytb-PCR}}{\text{total samples tested}} \times \frac{\# \text{ pos. by real-time PCR}}{\text{total samples tested}}\right) + \left(\frac{\# \text{ neg. by Cytb-PCR}}{\text{total samples tested}} \times \frac{\# \text{ neg. by real-time PCR}}{\text{total samples tested}}\right)
\]

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 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 \( \chi^2 = 1.653, p > 0.1 \).

Anopheles 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.

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