RESEARCH ARTICLE

Genetic Analysis and Species Specific Amplification of the Artemisinin Resistance-Associated Kelch Propeller Domain in *P. falciparum* and *P. vivax*

Eldin Talundzic¹,²*, Stella M. Chenet¹, Ira F. Goldman¹, Dhruviben S. Patel¹, Julia A. Nelson², Mateusz M. Plucinski¹,³, John W. Barnwell¹, Venkatachalam Udhayakumar¹

¹ Centers for Disease Control and Prevention, Center for Global Health, Division of Parasitic Diseases and Malaria, 1600 Clifton Rd, Mail Stop D-67, Atlanta, Georgia, United States of America, ² Atlanta Research and Education Foundation/VA Medical Center, Decatur, Georgia, United States of America, ³ President’s Malaria Initiative, Atlanta, Georgia, United States of America

* etalundzic@cdc.gov

Abstract

*Plasmodium falciparum* resistance to artemisinin has emerged in the Greater Mekong Subregion and now poses a threat to malaria control and prevention. Recent work has identified mutations in the kelch propeller domain of the *P. falciparum* K13 gene to be associated artemisinin resistance as defined by delayed parasite clearance and *ex vivo* ring stage survival assays. Species specific primers for the two most prevalent human malaria species, *P. falciparum* and *P. vivax*, were designed and tested on multiple parasite isolates including human, rodent, and non-humans primate *Plasmodium* species. The new protocol described here using the species specific primers only amplified their respective species, *P. falciparum* and *P. vivax*, and did not cross react with any of the other human malaria *Plasmodium* species. We provide an improved species specific PCR and sequencing protocol that could be effectively used in areas where both *P. falciparum* and *P. vivax* are circulating.

To design this improved protocol, the kelch gene was analyzed and compared among different species of *Plasmodium*. The kelch propeller domain was found to be highly conserved across the mammalian *Plasmodium* species.

Introduction

Following the development and spread of resistance to antimalarials such as chloroquine and sulfadoxine-pyrimethamine, artemisinin-based combination therapy (ACT) was adopted as first-line treatment for uncomplicated *Plasmodium falciparum* malaria worldwide [1]. However, resistance to artemisinin, as measured by delayed parasite clearance, has now been confirmed in multiple countries in the Greater Mekong Subregion [2–10]. There is growing
that they have no competing interests.

**Competing Interests:** The authors have declared that they have no competing interests.

Concern that artemisinin resistance may spread from this region to Africa and other parts of Asia as was the case with chloroquine and sulfadoxine-pyrimethamine resistance [11–13].

While *in vivo* therapeutic efficacy studies (TES) are considered the gold standard for determining anti-malarial efficacy, the WHO recommends that data from these studies be complemented with molecular markers of drug resistance [1]. After a long search to identify a specific locus implicated in artemisinin resistance, the kelch propeller domain of the K13 gene (*PF3D7_1343700*) on chromosome 13 was recently identified as a molecular marker of artemisinin resistance [14]. Several mutations in the kelch propeller domain have now been associated with *in vitro* ring stage survival assays and delayed parasite clearance rates in patients treated with artemisinins [8, 14]. As a result, sequencing the kelch propeller domain of the K13 gene is becoming an important tool in the global surveillance of antimalarial drug resistance in *P. falciparum* [8–10, 14–19].

While *P. falciparum* is the dominant species that causes human malaria worldwide and contributes the most to mortality, *P. vivax* is more prevalent than *P. falciparum* in many regions of the Greater Mekong Subregion [20]. Interestingly, a recent study showed that non-synonymous mutations in the *P. vivax* ortholog K12 gene are already circulating at very low frequencies in Cambodia [21]. While chloroquine remains the primary treatment option for *P. vivax* infections, ACTs have also been found to be efficacious in clinical trials in Asia and may be used as an alternative treatment to chloroquine [22, 23]. In addition, widespread use of ACTs for *P. falciparum* infection may exert some indirect selective pressure on the *P. vivax* kelch gene in individuals with mixed *P. falciparum* and *P. vivax* infections. Moreover, in areas where mefloquine has been extensively used as the first-line treatment in falciparum-uncomplicated malaria, high frequencies of *P. falciparum* and *P. vivax* isolates with increased *mdr-1* copy numbers [24] have been observed. These findings suggest that antimalarial drugs used to treat *falciparum* malaria may have a significant impact on sympatric *Plasmodium* species [21].

In areas where both *P. falciparum* and *P. vivax* coexist, kelch gene amplification must be species specific; otherwise, non-specific amplification using *P. falciparum* and *P. vivax* clinical isolates, might lead to incorrect classification of polymorphisms. Although microscopy remains standard practice for malaria diagnosis, molecular methods such as PCR based protocols for the confirmation of *Plasmodium species* can also be used [25]. Here, we provided a *P. falciparum* and a *P. vivax* species-specific protocol for accurately amplifying and sequencing the kelch propeller domain of these two species.

**Materials and Methods**

**Parasite isolates**

The following species and strains of parasites archived at the Malaria Laboratory, Research and Development Unit, Center for Disease Control and Prevention (CDC) were analyzed: *P. falciparum* (strains: W2, 7G8, FCR3, Dd2, 3D7, HB3, Honduras I/DCD, Panama II, Brazil, Malayan IV, and Santa Lucia), *P. vivax* (strains: India VII, Nicaragua I, Belem, Mauritania II, Ecuador I, Eritrea I, Indonesia XIX, and Vietnam II), *P. malariae* (Uganda I), *P. ovale* (Nigeria I), *P. reicheni*, *P. cynomolgi* (strains: PTI, Berok, Cambodia, Smithsonian, Gombok), *P. fieldi* (AB intortulus), *P. simiovale*, *P. simium*, *P. knowlesi* (strains: Philippines, Malayian), *P. gonderi*, *P. hylobati*, *P. inui* (Taiwan, Philippines), *P. berghei*, and *P. yoelii*. DNA was isolated using the commercially available QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). Genomic DNA was eluted with 100 μl of elution buffer and stored at −20°C for use in PCR assays. All isolates were screened using PET-PCR [26, 27] and confirmed to be positive for *Plasmodium*. 
Species specific primer design and genetic analysis

Primers specific for the kelch K13 gene (PF3D7_1343700) for *Plasmodium falciparum* and the ortholog kelch K12 gene in *Plasmodium vivax* were designed using the Geneious Pro R8 software (www.geneious.com). Briefly, Geneious Pro R8 was used to perform a BLAST search using the reference *P. falciparum* K13 gene sequence (PF3D7_134700) and download matching and published kelch gene *Plasmodium* sequences (Accession number: NC_009917, *P. vivax*; NC_020405, *P. cynomolgi* strain B; NW_000841840P. *inui*; NC_020405, NW_875089; *P. chabaudi*; and NW_965350, *P. yoelii*; CDO666221, protein, *P. reichenowi*). Sequences were aligned and analyzed for regions of sequence identity. Using the principle of allelic exclusion, different sets of species specific primers were designed and mapped onto the aligned sequences, aiming for primers that differentiate species by destabilizing the 3' end via mismatched nucleotides. We tested *P. falciparum* and *P. vivax* species specific primers on various *P. falciparum* and *P. vivax* strains derived from various geographical origins as well as on other human, non-human primate, and rodent malaria species. Lastly, our protocol was compared to an original published protocol for the amplification of the K13 gene in *P. falciparum* [14].

PCR amplification and sequencing of the *Plasmodium* kelch propeller domain

The kelch gene from different *Plasmodium* species was amplified using a nested PCR approach. The species specific primers used for amplification of the kelch propeller domain are shown in Table 1. Two μL of genomic DNA was amplified using 0.5 μM of each primer, 0.2mM dNTP, 2 mM MgCl₂ for the primary and secondary reactions, and 1 U Expand High Fidelity Taq (Roche CA, USA). For the primary reaction the following cycling parameters were used: 5 min at 94°C, 35 cycles at 94°C for 30 sec, 46°C for 60 sec (for *P. falciparum* primers) or 61°C for 60 sec (for *P. vivax* primers), 72°C for 90 sec, and final extension for 5 min at 72°C. For the nested PCR, 1μL of 1/5 diluted primary PCR product was used as template. For the nested PCR reaction the following cycling parameters were used: 2 min at 94°C, 35 cycles at 94°C for 30 sec, 55°C for 30 sec (for *P. falciparum* primers) or 59°C for 30 sec (for *P. vivax* primers), 72°C for 90 sec, and final extension for 5 min at 72°C. PCR products were confirmed using a 2% agarose gel electrophoresis and Gel red (Biotium, Hayward, CA USA). For cycle sequencing, 2.0uL Big Dye and Dye Buffer, 0.32 uL Primer (stock primer concentration of 10uM), 1.0uL of 1/5 diluted secondary PCR product, and 5.32uL of sterile water were used per reaction with the following cycling parameters: 96.0°C for 60 sec, 30 cycles 96.0°C for 10s, 50.0°C for 5 sec, and 70.0°C for 4 min. The original K13 amplification protocol [14] was used for comparison with the protocol

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer Name</th>
<th>Primer</th>
<th>Annealing Temperature</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td>K13_Pf_F1</td>
<td>5’ -GCAAATAGTATCTCGAAT-3’</td>
<td>46°C</td>
<td>1° reaction</td>
</tr>
<tr>
<td></td>
<td>K13_Pf_R1</td>
<td>5’ -CTGGGAACATAA TAAGAT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K13_Pf_F2</td>
<td>5’ -GATAAACAAGGAAGAATATTCT-3’</td>
<td>54°C</td>
<td>2° reaction</td>
</tr>
<tr>
<td></td>
<td>K13_Pf_R2</td>
<td>5’ -CGGAATCTAATATGTTATGTTCA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>K13_Pvx_F1</td>
<td>5’ -CATTTCCAACTTCTCCGTC-3’</td>
<td>61°C</td>
<td>1° reaction</td>
</tr>
<tr>
<td></td>
<td>K13_Pvx_R1</td>
<td>5’ -TATCTGCCACTCATTCGTG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K13_Pvx_F2</td>
<td>5’ -CGAAAGTGAGGCTTTACTA-3’</td>
<td>59°C</td>
<td>2° reaction</td>
</tr>
<tr>
<td></td>
<td>K13_Pvx_R2</td>
<td>5’ -CCACCAGTGTGATGTAAC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0136099.t001
described in this study. Sanger sequencing of PCR products was performed using ABI 3130 (Applied Biosystems, CA, USA). The sequence data was analyzed using Geneious Pro R8 software (www.geneious.com).

**Plasmodium** kelch propeller sequences submitted to Genbank

All of the geographically distinct *P. falciparum* and *P. vivax* isolates sequenced for the propeller domain were identical on both the nucleotide and amino acid level to the *P. falciparum* 3D7 (PF3D7_134700) isolate and *P. vivax* Sal-1 (NC_009917) isolate already available in Genbank, respectively. The following non-human primate isolates were also successfully amplified and sequenced: *P. cynomolgi* (Gombok), *P. simium*, *P. simiovale* and *P. inui*. Any new sequences reported in this study were deposited in Genbank under the accession numbers: KT198970-KT198972.

**3D structural modeling of the loop region**

The protein structure of the Keap1 protein (Protein Data Bank: 2Z32) was used to generate the *P. falciparum* K13 propeller domain using the USCSF Chimera plugin MODELLER [28].

**Results**

Comparison of the kelch propeller domain in human, non-human primates, and rodent *Plasmodium* species

The *P. falciparum* (PF3D7_134700) K13 gene is 2,181 nucleotide base pairs, encoding a protein of 727 amino acids (Fig 1), whereas the *P. vivax* (Sal-1 NC_009917) ortholog K12 gene is

---

Fig 1. Schematic representation of the *P. falciparum* K13 gene. (A) Schematic depicting the *P. falciparum* K13 gene size and propeller domain region. A 3D model is shown for the predicted propeller domain. (B) Species specific nested PCR workflow for amplifying the K13 gene and propeller domain. The protein structure of the Keap1 protein (Protein Data Bank: 2Z32) was used to generate the 3D model of the *P. falciparum* K13 propeller domain.

doi:10.1371/journal.pone.0136099.g001
2,139 base pairs, corresponding to 713 amino acids. The difference in length is due to nucleotide deletions found at the 5’ end of the *P. vivax* sequence at positions: 12, 407, 446, 472, and 534 with respect to the *P. falciparum* sequence (Fig 2). Comparison of the entire coding region of the kelch gene between *P. falciparum* and *P. vivax* revealed the gene to be 80% identical at the nucleotide level and 88% identical at the amino acid level. However, when the comparison was restricted to the kelch propeller domain only, the two genes were 80% identical at the nucleotide level and 97% at the amino acid level (Table 2).

All of the geographically distinct *P. falciparum* and the *P. vivax* isolates sequenced for the kelch propeller domain were identical on both the nucleotide and amino acid level to the *P. falciparum* 3D7 (PF3D7_134700) isolate and *P. vivax* Sal-1 (NC_009917), respectively. A total of eight amino acid differences within the kelch propeller domain (spanning 301 amino acids in length) between all of the *P. falciparum* and *P. vivax* strains were found at the following positions: 448, 517, 519, 568, 578, 605, 691, and 708 (Table 3). The kelch propeller domain is relatively conserved among the rodent and primate species of *Plasmodium* (Table 2, S1 and S2 Figs). Nucleotide and amino acid comparison for the various *Plasmodium* species is presented in Table 2.

**Kelch propeller domain species specific amplification**

The final *P. falciparum* nested PCR product size using species specific primers was 784 bp for *P. falciparum* and 792 bp for *P. vivax*. The *P. falciparum* primers designed in this study
amplified all _P. falciparum_ strains tested, but not any other _Plasmodium_ species (Fig 3). In contrast, the previously published protocol showed non-specific amplification with other _Plasmodium_ species, S3 Fig.

The _P. vivax_ primers amplified eight different _P. vivax_ strains derived from various geographical regions as well as five closely related non-human primate malaria parasites: _P. cynomolgi-Gombok_ strain, _P. simium_, _P. simiovale_, _P. inui_, and _P. hylobati_ (Fig 4). However, only _P. cynomolgi-Gombok_ strain, _P. simium_, _P. simiovale_, and _P. inui_ could be sequenced. Interestingly, of the five _P. cynomolgi_ strains tested, only the Gombok strain showed cross reactivity with the _P. vivax_ primers (Fig 4). Neither the _P. falciparum_ nor _P. vivax_ primers amplified the other human malaria parasites _P. malariae_, _P. ovale_ and _P. knowlesi_, or the rodent malaria parasites _P. bergehi_ and _P. yoelii_ (Figs 3 and 4).

Table 2. Genetic distance matrix of kelch propeller domain of various _Plasmodium_ species. Amino acid and nucleotide sequence percent (%) identity (e.g. percent of residues that are identical) are shown in the table. Amino acid similarity is shown in bold numbers and nucleotide similarity in italicized numbers.

<table>
<thead>
<tr>
<th></th>
<th>P.fal</th>
<th>P.rei</th>
<th>P.viv</th>
<th>P.cyn</th>
<th>P. cyn G</th>
<th>P.kno</th>
<th>P.inu</th>
<th>P.sim</th>
<th>P.sim</th>
<th>P. yoe</th>
<th>P. cha</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.fal</td>
<td>-</td>
<td>100</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>P.rei</td>
<td>N/A</td>
<td>-</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>98</td>
<td>96</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>P.viv</td>
<td>80</td>
<td>N/A</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>99</td>
<td>100</td>
<td>100</td>
<td>99</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>P.cyn</td>
<td>82</td>
<td>N/A</td>
<td>95</td>
<td>-</td>
<td>100</td>
<td>99</td>
<td>100</td>
<td>100</td>
<td>99</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>P. cyn G</td>
<td>83</td>
<td>N/A</td>
<td>94</td>
<td>96</td>
<td>-</td>
<td>99</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>94</td>
<td>95</td>
</tr>
<tr>
<td>P.kno</td>
<td>82</td>
<td>N/A</td>
<td>92</td>
<td>94</td>
<td>93</td>
<td>-</td>
<td>99</td>
<td>99</td>
<td>98</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>P.inu</td>
<td>81</td>
<td>N/A</td>
<td>94</td>
<td>96</td>
<td>96</td>
<td>93</td>
<td>-</td>
<td>100</td>
<td>99</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>P.sim</td>
<td>83</td>
<td>N/A</td>
<td>95</td>
<td>97</td>
<td>96</td>
<td>94</td>
<td>97</td>
<td>-</td>
<td>100</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>P.sim</td>
<td>80</td>
<td>N/A</td>
<td>100</td>
<td>95</td>
<td>94</td>
<td>92</td>
<td>94</td>
<td>95</td>
<td>-</td>
<td>93</td>
<td>94</td>
</tr>
<tr>
<td>P. yoe</td>
<td>87</td>
<td>N/A</td>
<td>79</td>
<td>81</td>
<td>82</td>
<td>82</td>
<td>80</td>
<td>81</td>
<td>79</td>
<td>-</td>
<td>99</td>
</tr>
<tr>
<td>P. cha</td>
<td>86</td>
<td>N/A</td>
<td>80</td>
<td>81</td>
<td>82</td>
<td>82</td>
<td>80</td>
<td>81</td>
<td>80</td>
<td>97</td>
<td>-</td>
</tr>
</tbody>
</table>

_P.fal_ = _P. falciparum_; _P.rei_ = _P. reichenowi_; _P.viv_ = _P. vivax_; _P.cyn_ = _P. cynomolgi_; _P.cyn G_ = _P. cynomolgi-Gombok_; _P.kno_ = _P. knowlesi_; _P.inu_ = _P. inui_; _P.sim_ = _P. simiovale_; _P.sim_ = _P. simium_; _P.yoe_ = _P. yoelii_; _P.cha_ = _P. chabaudi_; N/A = not available.

doi:10.1371/journal.pone.0136099.t002

Table 3. _P. falciparum_ non-synonymous amino acid changes in the kelch propeller domain as compared to _P. vivax_. Relative to alignment between _P. falciparum_ 3D7 and _P. vivax_ Sali.

<table>
<thead>
<tr>
<th>Codon Position</th>
<th>Amino Acid</th>
<th>Nucleotide</th>
<th>Side-Chain Polarity</th>
<th>Side-Chain Charge</th>
<th>Amino Acid</th>
<th>Nucleotide</th>
<th>Side-Chain Polarity</th>
<th>Side-Chain Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>448</td>
<td>I</td>
<td>ATA</td>
<td>Nonpolar</td>
<td>Neutral</td>
<td>M</td>
<td>ATG</td>
<td>Nonpolar</td>
<td>Neutral</td>
</tr>
<tr>
<td>517</td>
<td>V</td>
<td>GTA</td>
<td>Nonpolar</td>
<td>Neutral</td>
<td>T</td>
<td>ACT</td>
<td>Polar</td>
<td>Neutral</td>
</tr>
<tr>
<td>519</td>
<td>Y</td>
<td>TAT</td>
<td>Polar</td>
<td>Neutral</td>
<td>F</td>
<td>TTT</td>
<td>Nonpolar</td>
<td>Neutral</td>
</tr>
<tr>
<td>568</td>
<td>V</td>
<td>GTG</td>
<td>Nonpolar</td>
<td>Neutral</td>
<td>I</td>
<td>ATC</td>
<td>Nonpolar</td>
<td>Neutral</td>
</tr>
<tr>
<td>578</td>
<td>A</td>
<td>GCT</td>
<td>Nonpolar</td>
<td>Neutral</td>
<td>S</td>
<td>TCC</td>
<td>Polar</td>
<td>Neutral</td>
</tr>
<tr>
<td>605</td>
<td>E</td>
<td>GAA</td>
<td>Polar</td>
<td>Negative</td>
<td>D</td>
<td>GAT</td>
<td>Polar</td>
<td>Negative</td>
</tr>
<tr>
<td>691</td>
<td>E</td>
<td>GAA</td>
<td>Polar</td>
<td>Negative</td>
<td>D</td>
<td>GAT</td>
<td>Polar</td>
<td>Negative</td>
</tr>
<tr>
<td>708</td>
<td>L</td>
<td>CTT</td>
<td>Nonpolar</td>
<td>Neutral</td>
<td>I</td>
<td>ACT</td>
<td>Nonpolar</td>
<td>Neutral</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0136099.t003
Discussion

In some endemic regions often *P. vivax* is more prevalent than *P. falciparum* [29, 30], and co-infections can be common. Artemisinin combination therapy has been in use since the early 2000s in numerous malaria endemic regions where *P. falciparum* and *P. vivax* co-exist [1]. While drug resistance in *P. falciparum* has primarily been the recent focus of research, the study of resistance in *P. vivax* has received limited attention. For example, to date only one other paper investigating kelch propeller domain mutations in *P. vivax* has been published.

**Fig 3.** *P. falciparum* K13 gene species-specific amplification. The *Plasmodium falciparum* species specific primers were tested on multiple *Plasmodium* species, including human, rodent, and non-human primate malaria parasites from various geographical regions. The expected K13 propeller domain PCR product for *P. falciparum* is 784 base pairs. Panels (A) and (B) show human malaria parasites tested; Panel (C) shows non-human primate and rodent malaria parasites tested. Blue text denotes samples that were amplified by the PCR protocol.

doi:10.1371/journal.pone.0136099.g003
In this study, we successfully designed a species specific protocol for the detection of K13 propeller domain mutations associated with artemisinin drug resistance in *P. falciparum*. The previously published protocol [14] was found to show non-specific amplification with multiple *Plasmodium* species (S3 Fig), including the two major human malaria species *P. falciparum* and *P. vivax*. While we were unable to successfully sequence the full length *P. vivax* kelch propeller domain using the previous protocol, we did obtain truncated sequence data for *P. vivax*.

![Fig 4. *P. vivax* K12 gene species-specific amplification.](image_url)

The *Plasmodium vivax* species specific primers were tested on multiple *Plasmodium* species, including human, rodent, and non-human primate malaria parasites from various geographical regions. The expected K12 propeller domain PCR product for *P. vivax* is 792 base pairs. Panels (A) and (B) show human malaria parasites tested; Panel (C) shows non-human primate and rodent malaria parasites tested. Blue text denotes samples that were amplified by the PCR protocol.

[21]. In this study, we successfully designed a species specific protocol for the detection of K13 propeller domain mutations associated with artemisinin drug resistance in *P. falciparum*.
vivax using the published P. falciparum primers [14]. This can cause some uncertainty in the interpretation of sequence data.

The P. falciparum primers in our study were able to amplify different strains of P. falciparum indicating that this protocol will be useful for amplify and sequence the kelch propeller domain from several P. falciparum isolates from various regions around the world (Brazil, Honduras, Panama, and Malaysia). Importantly, the P. falciparum primers did not show amplification of any other human Plasmodium parasites (Fig 3).

Similarly, the P. vivax primers did not cross react with P. falciparum or any other human malaria parasites. Further, the P. vivax primers amplified successfully P. vivax strains collected from different geographical origins (Fig 3). The P. vivax primers showed evidence of some cross-reaction with P. cynomolgi-Gombok strain, P. simium, P. simiovale, and P. inui (Fig 4). This was expected, since most of these species are evolutionarily and genetically related to P. vivax [31]. Out of the five P. cynomolgi lab isolates tested only the Gombok strain cross-reacted with the P. vivax primers, which could be attributed to the known diversity within the two major subgroups of P. cynomolgi [31]. Although this primer set was cross reactive with some of the non-human primate malaria parasites, this may not be a limitation for the amplification of field samples since most of these non-human primate parasites are not commonly transmitted naturally to humans. The only currently known zoonotic malaria is caused by P. knowlesi, which showed no cross reaction with either the P. falciparum or P. vivax species specific protocol.

Given that currently the K13 gene can serve as a effective molecular marker of artemisinin resistance, we emphasize the importance of using our species specific protocol for routine screening of K13 artemisinin associated resistant alleles.

Supporting Information

S1 Fig. Nucleotide sequence alignment of the kelch propeller domain of various Plasmodium species. Sequences of the kelch propeller domain are shown for P. falciparum, P. vivax, P. cynomolgi strain B, P. cynomolgi Gombok, P. knowlesi, P. inui, P. simiovale, P. simium, P. yoelii, and P. chabaudi. Nucleotides are highlighted based on disagreement with the reference P. falciparum K13 sequence. The yellow annotation below the P. falciparum sequence indicates the gene region that encodes the kelch propeller domain. (PDF)

S2 Fig. Amino acid sequence alignment of the kelch propeller domain of various Plasmodium species. Translation of kelch propeller domain nucleotide sequences are shown for P. falciparum, P. reichenowi, P. vivax, P. cynomolgi strain B, P. cynomolgi Gombok, P. knowlesi, P. inui, P. simiovale, P. simium, P. yoelii, and P. chabaudi. Highlighted are amino acid bases that are in disagreement with the reference P. falciparum kelch propeller sequence. The yellow annotation below the P. falciparum sequence indicates the gene region that encodes the kelch propeller domain. (PDF)

S3 Fig. K13 gene amplification using a previously described protocol. The original K13 gene amplification protocol was tested on multiple Plasmodium species, including human, rodent, and non-human primate malaria parasites from various geographical regions. Rows (A) and (B) show human malaria parasites tested; Row (C) shows non-human primate and rodent malaria parasites tested. Results are shown separately for the primary and secondary reactions. Blue text denotes samples that were amplified by the PCR protocol. (TIFF)
Acknowledgments

We acknowledge support from the Advanced Molecular Detection Initiative at the CDC. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Author Contributions

Conceived and designed the experiments: ET. Performed the experiments: ET IFG JAN DSP. Analyzed the data: ET. Contributed reagents/materials/analysis tools: JWB. Wrote the paper: ET. Helped edit the manuscript: VU SMC MMP JWB.

References