Quantitative Determination of Plasmodium vivax Gametocytes by Real-Time Quantitative Nucleic Acid Sequence-Based Amplification in Clinical Samples

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Abstract. Microscopic detection of Plasmodium vivax gametocytes, the sexual life stage of this malaria parasite, is insensitive because P. vivax parasitaemia is low. To detect and quantify gametocytes a more sensitive, quantitative real-time Pvs25-QT-NASBA based on Pvs25 mRNA was developed and tested in two clinical sample sets from three different continents. Pvs25-QT-NASBA is highly reproducible with low inter-assay variation and reaches sensitivity approximately 800 times higher than conventional microscopic gametocyte detection. Specificity was tested in 104 samples from P. vivax-, P. falciparum-, P. malariae-, and P. ovale-infected patients. All non-vivax samples were negative in the Pvs25-QT-NASBA; out of 74 Pvs18-QT-NASBA positive samples 69% were positive in the Pvs25-QT-NASBA. In a second set of 136 P. vivax microscopically confirmed samples, gametocyte prevalence was 8%, whereas in contrast 66% were positive by Pvs25-QT-NASBA. The data suggest that the human P. vivax gametocyte reservoir is much larger when assessed by Pvs25-QT-NASBA than by microscopy.

INTRODUCTION

Plasmodium vivax accounts for over half of all malaria cases outside Africa, with an estimated 130 to 435 million new infections annually and 75 million acute clinical episodes.1–3 It is the predominant Plasmodium species in South and Central Asia, North Africa, the Pacific, and the Americas and, contrary to what is generally assumed, P. vivax infections may not always follow a benign course.4

Routine laboratory diagnosis of malaria is usually based on microscopic detection of Plasmodium parasites in Giemsa-stained blood slides, which enables differentiation between the different species of malaria and detection of gametocytes. However, this technique is relatively laborious and the detection limit, ~20 parasites/μL, may not always be sufficiently sensitive. Studying gametocytaemia in P. vivax is difficult because infections often result in relatively low parasitaemia of usually <2% with only a fraction of asexual parasites developing into a sexual stage; in addition, mixed infections with other Plasmodium species are common.5–7 Furthermore, P. vivax gametocytes cannot be cultured in vitro.8

Relatively little is known about the in vivo kinetics of P. vivax gametocytes and the epidemiology of transmission. It is believed that P. vivax gametocytes evade trapping and clearance in the sinusoids of the spleen by increasing their deformability.9 This may lead to local differences in gametocyte densities (for example, in capillary versus venous blood). Furthermore, P. vivax gametocytaemia was found to be more common after treatment with drugs that have weak antimalarial activity.10

Molecular amplification techniques achieve a low detection limit with high specificity. Some of these tests have been adapted to enable quantification of malaria parasites in a blood sample.11–16 Real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) is relatively easy to perform compared with real-time polymerase chain reaction (PCR), as only a small blood volume is needed, whereas up to 48 samples can be extracted and quantified with a sensitivity of 20 parasites/μL in as little as 4 hours.17

The QT-NASBA assays have been developed to detect the four human Plasmodium species.16,17 For quantification of mature gametocytes of P. falciparum a Pf625 NASBA has been developed detecting RNA expressed by the sexual-stage-specific Pf625 gene.15 With this assay the prevalence of sub-microscopic gametocytaemia was shown to be high, indicating that the human reservoir of P. falciparum transmission is higher than expected.18,20

Recently, a report was published describing a reverse transcription (RT)-PCR assay based on RNA of Pvs25 to quantify P. vivax gametocytaemia, however, data on specificity, sensitivity, reproducibility, and inter-assay variation were lacking.21

This study describes the development of a P. vivax gametocyte-specific NASBA assay based on RNA of Pvs25. Specificity, sensitivity, reproducibility, and inter-assay variation were tested by using in vitro Pvs25 RNA copies and patient material.

MATERIALS AND METHODS

Selection of primers and molecular beacon and synthesis of in vitro Pvs25 RNA. Plasmodium vivax forward (5′-CTC CTA CTA CAG CCT CTT CG-3′) and reverse (5′-CGT AAA GCC TTC CAT ACA CTG-3′) primers were used to amplify a 553-bp region of the Pvs25 gene by PCR. The amplified fragment was cloned into a pCR II vector in Escherichia coli TOP10F cells (both from Invitrogen, Carlsbad, CA). The cloned fragment was subsequently sequenced at the department of Anthropogenetics (Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands) to confirm a perfect homology with all Pvs25 entries available. In vitro Pvs25 RNA was synthesized using the mMessage mMachine T7 kit (Ambion, Austin, TX) and after transcription RNA was purified by LiCl-precipitation, as described in the mMessage mMachine protocol.

Primers and molecular beacon for the P. vivax sexual stage real-time QT-NASBA were based on Pvs25 mRNA (Genbank...
acquisition number [AY639972]. Forward primer was 5′-AAT TCT AAT ACG ACT CAC TAT AGG GAA AAA ATT CTC ATC TTC TGG ATT GGG G-3′ (position 426 to 446, including a T7 promoter sequence), reverse primer was 5′-CTT GTG TGC TTC ATG ATG TAT GTC GTC-3′ (position 314 to 334), and Pvs25 molecular beacon was 5′-6FAM-CGC GAT GCA TTG TTG AGT ACC TCT CGG AAT CGC G-Dabcyl-3′ (position 365 to 386, a hairpin structure with a stem of 6 paired nucleotides at both ends). All three sequences showed a 100% homology with all P. vivax entries known at that time in a BLAST database search and none with human or other invertebrate sequences.

**Clinical samples.** Blood samples (set 1, N = 104, 38 full blood samples and 66 blood spots on filter paper) of individuals suspected of P. vivax infection were screened for *Plasmodium* infection by their respective species-specific 18S real-time QT-NASBA, as described previously.23 Blood samples were collected from returned travelers (N = 22) with clinical malaria symptoms from the (out) patient clinic of the Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands) and from field studies in Indonesia (N = 37), Kenya (14), Mali (2), Senegal (4), Turkey (21), Vietnam (2), and Zambia (2). Blood samples (50 μL) or blood spots on filter paper (3 MM; Whatman, Hillsboro, OR; containing 50 μL blood equivalent) were mixed with guanidium isothiocyanate lysis buffer and nucleic acids were extracted as described previously.23

Set 2 was obtained from 136 individuals attending outpatient clinics in West Sumba and West Papua, Indonesia; thin and thick blood smears were prepared for microscopy. Blood samples were aseptically collected by finger prick from *P. vivax*-infected individuals by using capillary tubes, and 100-μL blood was spotted onto Whatman filter paper, which stabilizes RNA.24 The filter papers were transported at ambient temperature to the laboratory and kept at 4°C until use. Blood spots on filter paper were mixed with guanidium isothiocyanate lysis buffer and nucleic acids were extracted, as described previously.23 Blood collection was carried out with the approval of the Eijkman Institute for Molecular Biology Research Ethics Committees (Jakarta, Indonesia).

**Pvs25 real-time QT-NASBA.** The NASBA was performed using NucliSens Basic Kits for amplification (BioMérieux, Boxtel, The Netherlands) at a KCl concentration of 60 mM. The reaction mixture, including both primers (20 pmol/assay), molecular beacon (10 pmol/assay), and 2.5 μL of sample, was incubated for 2 minutes at 65°C and subsequently for 2 minutes at 41°C. After addition of 2.5 μL of enzyme mix (AMV-RT, RNase H and T7 RNA polymerase) amplification was followed for 90 minutes at 41°C in a NucliSens EasyQ Analyzer (BioMérieux, Boxtel, The Netherlands). Afterwards, time to positivity (TTP [in minutes]; the time point during amplification at which fluorescence, detecting target amplicons, exceeded the threshold, calculated as mean fluorescence of the three negative controls plus 20 standard deviations), as described previously,17 quantification (in copy numbers) was performed by using a 10-fold dilution series of *in vitro* Pvs25 RNA, which was aliquoted to use in every assay, ranging from 10^6 to 10^9 copies/μL.

**Statistical analysis.** Regression of the *in vitro* Pvs25 mRNA 10-fold diluted standard curves was analyzed using Microsoft Excel (Microsoft Corp., Redman, WA). For analysis of the inter-assay variations; mean and SD were calculated for every dilution step of all 18 dilution series. For intra-assay variation analysis, mean and SD were calculated for 47 replicates. Subsequently, inter- and intra-assay variations were calculated as (SD/mean) × 100%.

**RESULTS AND DISCUSSION**

**In vitro RNA samples.** *In vitro* Pvs25 RNA was synthesized for quantification since cultivation of *P. vivax* gametocytes is, till now, not possible.5 *In vitro* Pvs25 RNA synthesis and purification resulted in one distinct band (no degradation) after agarose gel electrophoresis enabling exact quantification of the copy number of *in vitro* Pvs25 RNA (Figure 1B). The concentration of the synthesized product was 0.9 μg/μL, as determined by spectrophotometry corresponding to 2.4 × 10^12 copies *in vitro* RNA/μL of the 682 nucleotide fragments of Pvs25.

After optimization of the assay, the analytical sensitivity of the QT-NASBA assay was determined by 10-fold dilution series of *in vitro* Pvs25 RNA. The lower detection limit for reliable quantification was 100 copies/μL (Figure 1A); 50 copies/μL could also be detected albeit in a RNA background only. Log input and mean TTP measured in 18 dilution series (10^2 to 10^8 copies/μL) correlated significantly (R^2 = 0.996 and P < 0.01). Furthermore, an initial inter-assay variation of 10% was found in dilution series data. This variation steadily remained below 10% on expansion of the dataset (Table 1) and, consequently, an inter-assay variation of below 10% was considered highly reproducible. Intra-assay variation was below 5%.

**Clinical samples.** Specificity of the Pvs25 real-time QT-NASBA assay was assessed using 104 microscopically screened samples from individuals clinically suspected of a *Plasmodium* infection (set 1). Seventy-four samples were positive in the *P. vivax* 18S real-time QT-NASBA (Pv18S). The 30 Pv18S NASBA negative samples were positive in their species-specific 18S NASBA (4, 5, and 8 samples were positive in the 18S NASBA specific for *P. ovale, P. malariae*).

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Gametocyte prevalence in samples of clinical individuals suspected to have \textit{Plasmodium vivax} infection (set 1, \(N = 104\)) as determined by \textit{Pv}18S and \textit{Pvs}25 Real-time QT-NASBA.*

<table>
<thead>
<tr>
<th>(P.) vivax parasitaemia ((Pv18S) NASBA)</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>51 (69%)</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>23 (31%)</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>30</td>
</tr>
</tbody>
</table>

*QT-NASBA = quantitative nucleic acid sequence-based amplification.

**Table 2**

Gametocyte prevalence in clinical samples of \textit{Plasmodium vivax}-infected individuals from West Sumba and West Papua (set 2, \(N = 136\)) as determined by microscopy and \textit{Pvs}25 real-time quantitative nucleic acid sequence-based amplification (QT-NASBA). All samples were positive by \textit{Pv}18S NASBA.

**Figure 2.** Gametocyte prevalence in clinical samples of \textit{Plasmodium vivax}-infected individuals from West Sumba and West Papua (set 2, \(N = 136\)) as determined by microscopy and \textit{Pvs}25 real-time quantitative nucleic acid sequence-based amplification (QT-NASBA). All samples were positive by \textit{Pv}18S NASBA.

\*TTP = time to positivity.

\(P.\) falciparum, respectively, and 13 samples in both the \textit{P. falciparum} and \textit{P. malariae} NASBA), but negative in the \textit{Pvs}25 real-time QT-NASBA assay showing 100% specificity for \textit{P. vivax}. Fifty-one out of the 74 \textit{Pv}18S NASBA positive samples (69%) were positive in the \textit{Pvs}25 real-time QT-NASBA (Table 2) (TTP ranging from 37.69 to 76.52 minutes). No blood smears were available to confirm microscopically the presence of asexual parasites and absence of \textit{P. vivax} gametocytes. To overcome this limitation, the \textit{Pvs}25 NASBA was tested on a second set of field samples collected in a \textit{P. vivax} endemic area (Sumba and Papua). Microscopically positive samples for \textit{P. vivax} parasites and/or gametocytes (Figure 2) were selected and confirmed by the \textit{Pv}18S NASBA. However, \textit{P. vivax} gametocytes were detected microscopically in only 8.1% (11/136), whereas 66.2% (90/136) of the samples were positive in the \textit{Pvs}25 NASBA. These results are consistent with those obtained with the first set in which 69% (51/74) were positive. These data suggest that the \textit{P. vivax} gametocyte reservoir may be much larger than previously indicated by microscopic analysis. Little is known about the incidence and prevalence of gametocytes in \textit{P. vivax}. Some studies indicate that gametocytes appear simultaneously with the first asexual parasites, findings supported by a field study from Afghanistan and Pakistan in which all microscopically confirmed patients were positive for \textit{P. vivax} gametocytes.\(^{25}\) However, others report that the presence of gametocytes could not be demonstrated during the first days (7–12 days) and at the end of a microscopically positive \textit{P. vivax} infection; more recent studies showed that only 47 of 212 (22%) and 77 of 349 (22%) \textit{P. vivax}-infected patients were carriers of gametocytes.\(^{10,26,27}\) In both clinical sample sets used in our study, 141 out of a total of 210 \textit{Pv}S18 positive samples (67%) contained gametocytes as determined by the \textit{Pvs}25 NASBA.

The detection limit of the \textit{Pfs}25 real-time QT-NASBA assay, \(4 \times 10^3 \) to \(4 \times 10^7 \) copies \textit{in vitro} \textit{Pfs}25 RNA/\(\mu\)L, corresponds to \(10^7\) \textit{P. falciparum} gametocytes/mL.\(^{15}\) On the basis of these data, we concluded that the sensitivity of the \textit{Pvs}25 real-time QT-NASBA assay is high because it is able to detect 100 copies/\(\mu\)L, which corresponds to a minimum of 25 gametocytes/mL. Confirming the presence of gametocytes in \textit{Pvs}25 negative/\textit{Pv}18S positive samples is not easy, as the gold standard “microscopy” and the molecular amplification techniques available, even the \textit{Pvs}25 NASBA, are not sensitive enough. Recently, a real-time RT-PCR based on the \textit{Pvs}25 gene was described.\(^{21}\) The PCR determined gametocytaemia was weakly but significantly associated with the number of microscopically determined gametocytes \((R^2 = 0.26)\). A limitation of the study was that gametocyte prevalence was assessed by membrane feeding experiments in which transmission blocking is a confounder, and not by PCR. Furthermore, validation of this PCR with respect to specificity, sensitivity, reproducibility, and inter-assay variation is not described in the article or in papers cited therein.

In conclusion, our study shows that the \textit{Pvs}25 real-time QT-NASBA assay may be a valuable tool to detect \textit{P. vivax} gametocytes and to quantify gametocytaemia. The results indicate that gametocyte prevalence in endemic areas may be much larger than previously indicated by microscopic analysis. This NASBA offers a relatively easy, specific, and more sensitive alternative to microscopy in studies on \textit{P. vivax} epidemiology, transmission and impact of drugs on gametocytaemia and can contribute to detection of gametocytes for control, elimination, and eventually eradication of \textit{P. vivax}.

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**Table 1**

Inter-assay variation in \textit{Pvs}25 standard curve series was calculated as SD/mean × 100%. The mean and SD of the TTP values (minutes) were calculated over 18 measurements for every dilution step.*

<table>
<thead>
<tr>
<th>Log copies in \textit{Pvs}25 RNA/(\mu)L</th>
<th>Mean TTP (minutes)</th>
<th>Variation</th>
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<tbody>
<tr>
<td>8</td>
<td>30.52</td>
<td>7.69</td>
</tr>
<tr>
<td>7</td>
<td>36.39</td>
<td>8.66</td>
</tr>
<tr>
<td>6</td>
<td>42.95</td>
<td>9.04</td>
</tr>
<tr>
<td>5</td>
<td>48.58</td>
<td>8.58</td>
</tr>
<tr>
<td>4</td>
<td>57.12</td>
<td>9.22</td>
</tr>
<tr>
<td>3</td>
<td>65.83</td>
<td>6.67</td>
</tr>
<tr>
<td>2</td>
<td>71.67</td>
<td>8.10</td>
</tr>
</tbody>
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*\(\mu\)L, which corresponds to a minimum of 25 gametocytes/mL.
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