Limited Degradation of the *Plasmodium falciparum* Gametocyte Marker *pfs25* mRNA Exposed to Tropical Temperatures: Considerations for Malaria Transmission Field Studies

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Abstract. An important aspect of many malaria molecular epidemiology and transmission studies is RNA-based detection of gametocytes. Ensuring RNA stability represents a challenge in tropical, resource-limited environments, as RNA may quickly degrade when samples are not preserved under adequate conditions. This study investigated the degradation of *pfs25* messenger RNA (mRNA), the most widely used *Plasmodium falciparum* gametocyte marker, in whole blood spiked with cultured *P. falciparum* gametocytes, exposed to different temperatures for up to 48 hours, and collected with different anticoagulants. The levels of *pfs25* mRNA were similar between samples stored at 4°C and 30°C for up to 48 hours before stabilization with RNeasy Protect (Qiagen, Hilden, Germany). We observed that *pfs25* mRNA in heparin-collected blood degraded less than that in ethylenediaminetetraacetic acid (EDTA)-collected blood over the 48-hour period. For field studies aiming for *P. falciparum* gametocyte detection, immediate stabilization of blood samples is not necessary, as the *pfs25* transcript is relatively stable, more so in heparin than EDTA collection tubes.

As malaria transmission decreases, surveillance of the human infectious reservoir, including reliable estimation of gametocyte carriers in endemic areas, is essential.^{1–3} Molecular epidemiology studies of malaria rely on adequate stabilization and storage of blood samples to ensure the integrity of nucleic acids, particularly RNA, which is prone to rapid degradation.⁴ Molecular quantification of *Plasmodium falciparum* gametocytes can be achieved by reverse transcription quantitative polymerase chain reaction (RT-qPCR) targeting the messenger RNA (mRNA) of the *pfs25* gene, the most widely used marker of mature, stage V *P. falciparum* gametocytes.⁵

When collecting blood samples for gametocyte detection, sample collection and stabilization are important aspects to ensure mRNA integrity.^{6,7} If the whole blood sample collected in the field is stored at tropical temperatures for extended periods (e.g., the period it takes to reach a field laboratory for processing and freezing), mRNA degradation is thought to occur.⁸ Therefore, the original gametocyte density may not be deducible from samples that were exposed to temperatures below 37°C for extended periods. Although using appropriate storage media, such as RNeasy Protect (Qiagen, Hilden, Germany), may stabilize mRNA, the handling of blood samples at the collection site might not be logistically feasible or introduce risks of contamination.

The stability of *pfs25* transcripts under field conditions has been investigated previously, but only with respect to dried blood spots.^{6,7} Our study investigated the stability of *pfs25* mRNA in whole blood samples collected with either ethylenediaminetetraacetic acid (EDTA) or heparin as anticoagulants and stored over 48 hours at either 4°C or 30°C to emulate ice pack field conditions or ambient/tropical temperature. We aimed to assess the extent of expected mRNA degradation and inform future studies that collect blood samples in remote field settings for subsequent *P. falciparum* gametocyte detection. In malaria studies, heparin is sometimes avoided as it interferes with erythrocyte invasion of the parasite and has also been reported to be a potential inhibitor of PCR.^{9,10} On the other hand, EDTA sample col-

lection and subsequent storage at 4°C and 20°C has been linked to RNA degradation.⁸

Plasmodium falciparum gametocytes (NF54 strain) were cultured as described previously.¹¹ When outside the incubator, cultures were kept at 37°C on a slide warmer. Of the stage V gametocyte culture, 10 mL was split into two fractions and centrifuged at 500 × g for 5 minutes at 37°C. Gametocytemia was determined as the percentage of gametocyte-infected red blood cells in 5,000 erythrocytes in the resulting pellets. The pellets were then diluted with whole blood collected in tubes containing either 2.6 mg/mL of potassium EDTA (Sarstedt, Germany) or 15–17 USP/mL of lithium heparin (Becton Dickinson, Franklin, Lakes, NH) to 500 gametocytes/μL. Triplicate aliquots of 50 μL were removed from each of the heparin and EDTA preparations and added to 250 μL of commercially available RNA stabilization reagent (RNeasy Protect). The samples were then placed at –80°C. These samples represent the heparin and EDTA baseline gametocyte samples (time point 0).

Heparin and EDTA gametocyte preparations were further split for incubation at 4°C or 30°C resulting in four experimental treatments (i.e., heparin or EDTA at 4°C or 30°C). All samples were held at these storage conditions over 48 hours. At 2, 4, 6, 8, 12, 24, and 48 hours after time point 0, 50 μL blood from each treatment was added to tubes containing 250 μL of RNeasy Protect, in triplicate, and the tubes were immediately placed at –80°C.

After 1 week of –80°C storage, the RNeasy Protect-preserved samples were thawed and RNA extraction was performed using a Qiagen RNeasy 96 Plus Kit as described elsewhere⁵ with the slight modification to elute the extracted RNA in 60 μL of RNase-Free Water (Qiagen, Hilden, Germany).

TaqMan one-step RT-qPCR targeting the *pfs25* transcript was used to detect and quantify the presence of gametocytes in the experimental samples.^{5,12} The assay and primers have been described previously.⁵ Each detection experiment included duplicate plasmid controls containing the *pfs25* target product (at 10⁴, 10³, 10², 10, 5, and 1 copies/μL) to estimate gametocyte densities as *pfs25* transcript copies/μL.⁵

To determine whether patterns of RNA decay in the *pfs25* assays were due to total RNA degradation, gametocyte-specific RNA degradation, or technical artefact of the gametocyte-specific assay, TaqMan one-step RT-qPCR assays targeting the human beta-globin transcript, which is the most abundant

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transcript in erythrocytes, were conducted in parallel. The primers and probe used in this assay have been described previously.¹³ The assay was performed using the TaqMan RNA-to-Ct™ 1-Step Kit (Applied Biosystems, Carlsbad, CA) in a total volume of 12 μ L containing 6 μ L of 2 $\times$ RNA-to-CT™ Mastermix (Applied Biosystems), 670 nM of each of the primers, and 335 nM of the probe. Cycling parameters included reverse transcription for 30 minutes at 48°C, Taq DNA polymerase activation for 10 minutes at 95°C, and 45 cycles of 15 seconds at 95°C denaturation and 1 minute at 58°C annealing, extension, and detection. Positive RNA controls of known concentration, which were serially diluted, were used as standards for quantification, and thus a standard curve of beta-globin (ng/ μ L) was generated. Both *pfs25* and beta-globin assays were run in 384-well plates on a Roche LightCycler480® (Roche, Basel, Switzerland) in triplicate. Spearman rank correlation analysis between replicates indicated minimal intra-assay variation (*pfs25* replicate 1 versus 2, $r = 0.98$ and replicate 1 versus 3, $r = 0.95$; beta-globin replicate 1 versus 2, $r = 0.96$ and replicate 1 versus 3, $r = 0.95$).

Temperature did not affect the *pfs25* mRNA content of samples stabilized over 48 hours significantly (4°C = 3,680 versus 30°C = 3,420 copies/ μ L, Mann-Whitney $P = 0.6007$; Figure 1A). For human beta-globin data, there was moderate

evidence that the 4°C storage stabilized human mRNA better than the storage at 30°C (4°C = 2.57 versus 30°C = 1.36 ng/ μ L Mann-Whitney $P = 0.0105$; Figure 2A). The greatest reduction in human mRNA content occurred between 12 and 48 hours (pre-24 hours median ng/ μ L = 1.97 versus post-24 hours median ng/ μ L = 0.59, Mann-Whitney $P < 0.0001$; Figure 2B).

These data suggest that storing blood in RNAprotect at ambient (tropical) temperature for up to 48 hours does not lead to complete degradation of the *pfs25* and beta-globin transcripts, as both are detectable at 48 hours. However, given there was a significant reduction in detectable beta-globin transcript after the 12-hour time point, it may be prudent to stabilize and freeze the sample within 24 hours of collection.

To investigate the effect of anticoagulant, data were stratified by EDTA and heparin collection methods. A significant decrease of *pfs25* content over 48 hours was observed in the EDTA samples (EDTA pre-8 hours median *pfs25* copies/ μ L = 7,048 versus EDTA post-8 hours median *pfs25* copies/ μ L = 1,963, Mann-Whitney $P = 0.0014$) compared with the heparin samples (heparin pre-8 hours median *pfs25* copies/ μ L = 4,280 versus heparin post-8 hours median *pfs25* copies/ μ L = 3,715, Mann-Whitney $P = 0.5345$). Time degradation profiles of *pfs25* EDTA and heparin at each time point are given in Figure 1B and C, respectively.

FIGURE 1. Messenger RNA levels of *Plasmodium falciparum pfs25* transcript ($\log_{10}$ transcript copies/ μ L) detected by reverse transcription quantitative polymerase chain reaction over 48 hours. (A) Samples stored at 4°C or 30°C (ethylenediaminetetraacetic acid [EDTA] and heparin samples combined). (B) Samples in EDTA (4°C or 30°C samples combined). (C) Samples in heparin (4°C or 30°C samples combined). The error bars represent standard errors of the mean. *Pfs25* concentration followed an exponential decay pattern in the samples prepared with blood collected in EDTA (best fit lines in B represent the best fit and 95% confidence interval of an exponential decay function). No degradation was observed in the samples prepared from blood collected in heparin.

FIGURE 2. Messenger RNA (mRNA) levels of the human beta-globin transcript ($\log_{10}$ ng/ μ L) detected by reverse transcription quantitative polymerase chain reaction. (A) Over 48 hours in samples stored at 4°C vs. 30°C, (B) at each time point in samples stored at 30°C, (C) in ethylenediaminetetraacetic acid (EDTA), and (D) in heparin. The error bars represent standard errors of the mean. The greatest decrease in mRNA levels occurred after 24 hours of storage at 30°C. Beta-globin also followed an exponential decay pattern in the samples prepared with blood collected in EDTA (best fit lines in C represent the best fit and 95% confidence interval of an exponential decay function). No degradation was observed in the samples prepared from blood collected in heparin (D).

Human beta-globin mRNA decay analysis resulted in similar profiles as those determined with the *P. falciparum* gametocyte assays for both EDTA (pre-8 hours median ng/ μ L = 4.6 versus EDTA post-8 hours median ng/ μ L = 1.6, Mann-Whitney P = 0.0008; Figure 2C) and heparin samples (pre-8 hours median ng/ μ L = 1.6 versus post-8 hours median ng/ μ L = 1.7, Mann-Whitney P = 0.9751; Figure 2D).

As heparin has been shown to inhibit PCR,^{9,10} heparin concentrations in a representative subset of heparinated RNA samples (N = 16; specifically one replicate from each time point, 0, 2, 4, 6, 8, 12, 24, and 48 hours and from both incubation temperatures 4°C and 30°C) was measured as previously described.¹⁴ Heparin was not detected in the mRNA samples (Supplemental Figure 1). It is therefore unlikely that residual heparin influenced the RT-qPCR assays. These findings suggest that both gametocyte and human mRNA degraded to a higher degree in the blood samples collected with EDTA, and thus heparin may be preferable to EDTA when collecting blood samples for RNA-based assays.⁸

RNA stabilization is a relevant concern for many field studies in remote tropical environments. Transferring samples directly into RNA stabilization reagent at the time of blood collection adds an additional logistic challenge for collection teams and may place samples at risk of contamination.

Although short-term ice pack storage conditions are possible for some collection sites, it may be an added logistical challenge for others. This study shows that storage at either 4°C or 30°C did not significantly affect the mRNA stability of *P. falciparum* gametocyte marker *pfs25* over a 48-hour period and of human mRNA up to 24 hours, suggesting that it may be feasible to keep blood samples at tropical ambient conditions for the time required to reach a centralized field laboratory and conduct downstream processing (usually within 24–48 hours). The choice of anticoagulant, however, does seem to affect mRNA stability significantly. In blood samples collected in EDTA, degradation of both *pfs25* and human beta-globin transcripts was observed at both temperatures whereas mRNA levels did not decrease significantly in samples collected into heparin.

We observed variability between the starting concentrations of *pfs25* and beta-globin mRNA levels in both EDTA and heparin samples. We are confident to rule out inhibition of PCR by heparin, but inherent sources of variability may exist, originating from complex sample preparation (which should also be taken into account when processing field samples for gametocyte quantification) and inhibition during RNA extraction (e.g., through DNase treatment). This may lead to different overall extraction efficiencies between samples.

However, the higher instability of mRNA in EDTA samples is clearly significant, as the decay was observed within the first 8 hours in the EDTA samples but not in the heparin samples. The EDTA-related decrease is consistent with a previous study that demonstrated ex vivo instability of mRNA in whole blood collected in EDTA tubes.⁸

In conclusion, this study shows that sample storage at ambient (tropical) temperature is feasible up to 24 hours and that cooling and/or immediate placement into RNA preservation reagents may not be crucial if samples are collected into heparin. For samples collected into EDTA, transfer into an RNA stabilizing agent should occur within 6–8 hours after collection to avoid excessive RNA degradation.

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