

A Direct from Blood Reverse Transcriptase Polymerase Chain Reaction Assay for Monitoring *Falciparum* Malaria Parasite Transmission in Elimination Settings

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Abstract. We describe a novel one-step reverse transcriptase real-time PCR (direct RT-PCR) for *Plasmodium falciparum* malaria parasites that amplifies RNA targets directly from blood. We developed the assay to identify gametocyte-specific transcripts in parasites from patient blood samples, as a means of monitoring malaria parasite transmission in field settings. To perform the test, blood is added directly to a master mix in PCR tubes and analyzed by real-time PCR. The limit of detection of the assay on both conventional and portable real-time PCR instruments was 100 parasites/mL for 18S rRNA, and 1,000 parasites/mL for asexual (PFE0065W) and gametocyte (PF14_0367, PFGEXP5) mRNA targets. The usefulness of this assay in field studies was explored in samples from individuals living in a high-transmission region in Cameroon. The sensitivity and specificity of the assay compared with a standard two-step RT-PCR was 100% for 18S rRNA on both conventional and portable instruments. For PF14_0367, the sensitivity and specificity were 85.7% and 70.0%, respectively, on the conventional instrument and 78.6% and 90%, respectively, on the portable instrument. The concordance for assays run on the two instruments was 100% for 18S rRNA, and 79.2% for PF14_0367, with most discrepancies resulting from samples with low transcript levels. The results show asexual and sexual stage RNA targets can be detected directly from blood samples in a simple one-step test on a field-friendly instrument. This assay may be useful for monitoring malaria parasite transmission potential in elimination settings, where sensitive diagnostics are needed to evaluate the progress of malaria eradication initiatives.

INTRODUCTION

Malaria is a disease of global concern that has been met with concerted efforts to move countries toward disease control, regional elimination, and ultimately global eradication.¹ In 2015, an estimated 212 million malaria cases occurred globally, resulting in 429,000 deaths. Since 2000, malaria case incidence and mortality were reduced by 41% and 62%, respectively, mostly from the broad implementation of malaria control interventions.² Significant progress toward elimination has also been achieved, with 17 countries reaching elimination status between 2000 and 2015. In 2015, an additional 19 countries reported 1,000 or fewer indigenous cases, at least 10 of these being on track for elimination status by 2020.² However, many challenges to achieve and sustain elimination status remain,³ not the least of which is preventing the highly efficient transmission of malaria parasites by mosquitoes.⁴ The potential for ongoing transmission partly stems from the high proportion of submicroscopic infections that remain undetected in settings where malaria parasite transmission intensity is reduced.^{5,6} Current diagnostic techniques, such as light microscopy and rapid diagnostic tests (RDTs), do not have the sensitivity to detect all infections.⁷ Molecular methods have been developed with increased sensitivity, but these methods remain to be implemented routinely within elimination programs.⁸

Although there is sufficient evidence that submicroscopic infections contribute to malaria parasite transmission in some settings of intense malaria parasite transmission,⁹ this evidence is inconclusive in settings of lower endemicity.^{10–12} Mature, infectious gametocytes are present in the majority of symptomatic and asymptomatic malaria infections, often at low concentrations.¹³ Transmissibility or infectiousness to mosquitoes is increasingly appreciated as a complex phenomenon, related in a nonlinear manner to parasite density, varying independently of transmission intensity, and influenced by human immune responses and vector susceptibility to infection.⁶ Despite these issues, monitoring submicroscopic infections, particularly gametocytes, remains of central importance to broaden our understanding of transmission dynamics in low-endemic elimination settings. Presently, there are few tools available to accomplish this goal, especially in field settings.

Molecular diagnostic tools such as nucleic acid tests provide the most accurate epidemiological data in all aspects of malaria control. In elimination settings, implementation of these tests for surveillance may support the prevention of reintroduction of malaria.⁸ For monitoring transmission potential, RNA-based testing shows particular promise, including reverse transcriptase polymerase chain reaction (RT-PCR),¹⁴ quantitative nucleic acid sequence-based amplification assay (QT-NASBA),¹⁵ and RT-loop-mediated isothermal amplification (RT-LAMP)¹⁶ methods. The targeting of sexual stage specific transcripts such as Pfs25,^{17,18} Pfs230,¹⁹ and Pfg377²⁰ allows the detection of gametocytes at unsurpassed sensitivity. The sensitivities of these methods are relatively high, reporting detection of Pfs25 at 20

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gametocytes/mL for both QT-NASBA and RT-PCR detection of Pfs25.^{21,22} One drawback is the need for nucleic acid purification prior to amplification, a step that is difficult to perform in the field. For molecular diagnostics in general, the cost and complexity of these tests has precluded their use in low-resource settings.

We addressed these challenges in previous work by developing a PCR test for malaria parasites that does not require nucleic acid purification²³ and incorporated this test into a desiccated hydrogel matrix for use in a customized, portable real-time PCR machine.²⁴ In the present study, we describe the development of an RT-PCR method to detect gametocytes directly from small blood volumes in a one-tube assay. The reaction involves the addition of blood directly to a master mix, reverse transcription of template RNA in the blood to cDNA, followed by PCR amplification of the cDNA template. This reaction uses a commercial enzyme mixture for reverse transcription and PCR that is resistant to inhibitors in blood. The enzymes also tolerate a high concentration of SYBR Green DNA intercalating dye, which is necessary to generate sufficient fluorescence signal in the presence of blood. This assay was optimized to detect 18S rRNA on a conventional real-time PCR instrument and adapted for detection of a gene expressed in mature gametocytes (PF14_0367).²⁵ As a first step in showing the potential for this assay to detect gametocytes in the field, we tested it on a blind panel of blood samples from gametocytemic individuals from Cameroon using a commercially available portable real-time PCR instrument.

METHODS

Parasite cultures and parasite dilution series. *Plasmodium falciparum* 3D7 strain MRA-102 was obtained from the Malaria Research and Reference Reagent Resource Center (BEI Resources, Manassas, VA) and grown in human erythrocytes in vitro at 3% hematocrit as described.²⁶ NF54 parasites were maintained in vitro in a semiautomated culture system.^{27–29} Briefly, parasites were grown in RPMI-1640 medium supplemented with human serum (complete medium) and 5% hematocrit (Sanquin) and medium was refreshed twice daily. *Plasmodium* asexual parasites at 10% density were synchronized by the selection of late trophozoites and schizonts on a 63% Percoll density gradient. This was followed by a 5% sorbitol treatment after 5 hours, killing the remaining schizonts. Five hours later, samples containing early ring-stage parasites were harvested. Gametocyte cultures were started

at 0.5% asexual parasitemia and treated with N-acetylglucosamine on day 7 to eliminate asexual parasites.^{30,31} Stage V parasites were obtained on day 14. The presence of asexual and sexual stage parasites was confirmed on Giemsa-stained slides, and parasite concentration was determined using a Burkert-Turk cell counting chamber. Ten-fold serial dilutions of parasites were made in uninfected EDTA blood to be used directly as templates in amplification reactions. Nucleic acid was purified from the dilution series by MagNAPure LC Total Nucleic Acid Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany). Extracted nucleic acid was treated with RQ1 DNase I (Promega, Madison, WI) for 30 minutes at 37°C to remove genomic DNA while keeping RNA intact. This purified RNA was used as template in amplification reactions.

Patient samples. Deidentified samples were used from a separate study on the transmissibility of *P. falciparum* infections to *Anopheles gambiae* s.s. mosquitoes. All procedures used in that study were approved by the Cameroonian national ethical committee (2015/04/583/CE/CNERSH/SP) that monitored progress in annual reports. Caretakers of study participants provided written informed consent prior to inclusion in the study. Microscopically detectable gametocyte carriers aged 5–15 years were recruited from schools and communities surrounding Yaoundé, Cameroon, as described previously.³² Whole blood samples collected by venipuncture into heparinized tubes were stored directly at –80°C prior to use in the assay. Asexual and sexual stage densities were estimated in Giemsa-stained thick smears by counting parasite numbers per thousand white blood cells (WBCs), and converting to a density value assuming the standard of 8,000 WBC/μL of blood. For this cohort, values ranged from 8–1,496 gametocytes/μL of blood, with a median value of 80 gametocytes/μL and a standard deviation of 332 gametocytes/μL. From cohort members for whom ≥ 1 mL of whole blood was remaining after transmissibility assessments, 1 mL blood samples were anonymized prior to shipment to Alberta, Canada. Participants gave explicit consent for their material to be analyzed outside Cameroon.

Direct RT-PCR assay. The direct RT-PCR was performed in a 25 μL reaction containing 1× blood RT-PCR buffer, 0.5 μL RT polymerase mix (Direct Blood RT-PCR kit; VitaNavi Technology, Manchester, MO), 40× SYBR Green (Thermo Fisher Scientific, Waltham, MA), 1 μL Ribolock RNase Inhibitor (Thermo Fisher Scientific), and primers at a final concentration of 200 nM, with the exception of the PFGEXP5 primers, which were used at a final concentration of 400 nM (Table 1). A new 5'

TABLE 1
Primers used in this study

Target	DNA sequence (5'–3')	Reference
18S rRNA forward	GCCGTTTTAGTTCGTGAAT	This study
18S rRNA reverse	GCAGGTTAAGATCTCGTTCG	This study
Pf_A18S_fw	TCCGATAACGAACGAGATCTTAAC	22
Pf_A18S_rev	ATGTATAGTTACCTATGTTCAATTTCA	22
PF14_0367 forward	GTTACATTTTCGACCCAGCATAAAT	25
PF14_0367 reverse	TCCCTGTGTTTTGCTCATCTTC	25
PFE0065w forward	GCAAAACAAGCCGTACATGTTG	25
PFE0065w reverse	TTGCTAGGTAATATCCTTTTCTTTTCC	25
PFGEXP5 forward	GGGCTGTTATGTATATTTTTATTGAATTTT	This study
PFGEXP5 reverse	CATTCGTTTCATTTTCACCACTTC	This study

consensus primer was designed for 18S rRNA amplification; the 18S rRNA 3' consensus primer is a modified sequence from a previous study.³³ This primer set reduces nonspecific amplification that may originate from off-target primer interactions in the reverse transcription step. The blood component of the reaction mixture was added in a 2.5 μ L volume, either as uninfected blood controls, parasite serial dilutions in blood, or patient blood samples. In experiments where purified parasite RNA was tested, 2.5 μ L purified RNA and 2.5 μ L uninfected blood were added to the reaction. Thermocycling was performed in both conventional and portable real-time PCR instruments. For the conventional instrument, reactions were run in 96-well PCR plates on a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) and in 200 μ L low profile PCR 8-strip tubes (BioRad) in the portable real-time PCR instrument (Open qPCR, Chai Biotechnologies Inc, Santa Clara, CA). The RT-PCR thermocycling program was as follows: 60°C reverse transcription for 30 minutes, 3 minute 95°C denaturation, 45 cycles of 95°C for 20 seconds, 60°C for 30 seconds, 70°C for 30 seconds, 2 minute 70°C final extension, and melt curve analysis from 65°C to 95°C.

The 18S rRNA and PF14_0367 RT-PCR assays were performed in duplicate on patient samples in the clinical panel, in both the conventional and portable real-time PCR instruments. For 18S rRNA and PF14_0367 in the conventional instrument and 18S rRNA in the portable instrument, 1 \times enzyme concentration was used. Both 1 \times and 5 \times enzyme concentrations were compared for the initial optimization of PF14_0367 in the portable instrument and 5 \times enzyme concentration was used to run the clinical samples on this instrument.

A two-step RT-PCR was performed as a reference standard for assessing the performance of the direct RT-PCR with samples from the clinical panel. In the first step, RNA was purified from patient blood samples as described earlier and reverse transcribed (High capacity cDNA Reverse Transcription Kit; Thermo Fisher Scientific). The resulting cDNA was used in the second step as template for quantitative PCR using the GoTaq qPCR master mix and Bryt Green dye (Promega) on the BioRad CFX instrument. Duplicate reactions were performed using primers targeting 18S rRNA (Pf_A18S fw and rev in Table 1) and PF14_0367 (Table 1). Thermocycling conditions included an initial denaturation of 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Data analysis. BioRad CFX Manager 3.1 software was used for conventional RT-PCR post-amplification data analysis. Quantification cycle (Cq) determination mode was set to single threshold with baseline-subtracted curve fit and a user-defined threshold of 50 relative fluorescence units (RFUs) for analysis of parasite serial dilutions in blood. The limit of detection (LOD) of the assay was defined as the lowest parasite dilution that met the following criteria: 1) at least two of three curves crossing the threshold at or before the cycle cutoff value of 40 cycles for 18S rRNA and 45 cycles for all other targets; and 2) melting temperatures (Tm) within acceptable ranges as defined by positive controls for 18S rRNA (76–79°C) and PF14_0367 (77–80°C). The two-step RT-PCR reaction used as a reference standard in the clinical panel was analyzed in the same way with a user-defined threshold of 200 RFU. Melt curve analysis was not performed for this reaction.

For the portable real-time PCR instrument, Open qPCR software version 1.0.2 (Chai Biotechnologies Inc) was used for post-amplification analysis. Cq and Tm values were calculated directly by the instrument software and reported. The software calculates Cq values for each individual curve by the second derivative maximum method, thus a single threshold is not used for all curves in the experiment (as in the commercial instrument). Raw data output from the software was used to generate amplification curves (baseline subtracted RFU versus PCR cycle) and melting curves ($-d[RFU]/dT$ versus temperature) in Microsoft Excel (Microsoft Corporation, Redmond, WA). The analytical sensitivity of the assay was defined as earlier, with a Cq cutoff of 45 and a Tm within acceptable ranges for 18S rRNA (76–80°C) and PF14_0367 (78–80°C). The clinical sensitivity and specificity of the direct RT-PCR assay for 18S rRNA and PF14_0367 transcripts on conventional and portable instruments were calculated in comparison to the reference standard RT-PCR. The concordance and kappa statistic were also determined for both assays between the conventional and portable instruments. For the comparison of sensitivities and specificities of the PF14_0367 assay on the two instruments, *P* values were calculated using McNemar's exact test, and the proportion of agreement and 95% confidence intervals were reported (Supplemental Table 3).

RESULTS

Detection of parasite rRNA in a direct blood RT-PCR reaction. To optimize the direct RT-PCR assay for detection of RNA targets in blood, we chose *P. falciparum* 18S small subunit rRNA, a commonly used target that we had studied previously with a direct-from-blood PCR diagnostic.²³ For this target, there are 5–8 18S rRNA gene copies³⁴ and thousands of transcripts per cell.³⁵ In the first enzymatic step of this reaction, the ribosomal RNA is reverse transcribed into cDNA; in the second step, the 18S cDNA and 18S genomic DNA serve as templates for the PCR reaction. The LOD of this assay was first evaluated for purified parasite RNA added to blood. RNA was purified from 10-fold dilutions of ring-stage parasites in uninfected blood, added to a new aliquot of uninfected blood and tested by direct RT-PCR (Figure 1A and B). The LOD was 10 parasites/mL blood, a value that exceeds the sensitivity of microscopy (10,000–100,000 parasites/mL).³⁶ Next, we tested the LOD with intact ring-stage parasites serially diluted in uninfected blood and added directly to the reaction (Figure 1C and D). In this case, the LOD was 100 parasites/mL. The difference in sensitivity may reflect the increased availability of purified RNA compared with RNA released from infected red blood cells. Raw data and statistical analysis of Cq values for the direct RT-PCR assay on the conventional real-time PCR instrument are presented in Supplemental Table 1.

Detection of mRNA by direct blood RT-PCR in asexual parasites. In the 18S rRNA RT-PCR assay, amplification occurred from both rRNA and genomic DNA targets. To determine whether the assay can strictly detect messenger RNA apart from the genomic DNA sequence, we selected PFE0065w skeleton-binding protein 1 mRNA, a target transcript expressed in ring-stage parasites.³⁷ The LOD for this target was 1,000 parasites/mL (Figure 2A and B). The use of intron-spanning primers confirmed that the

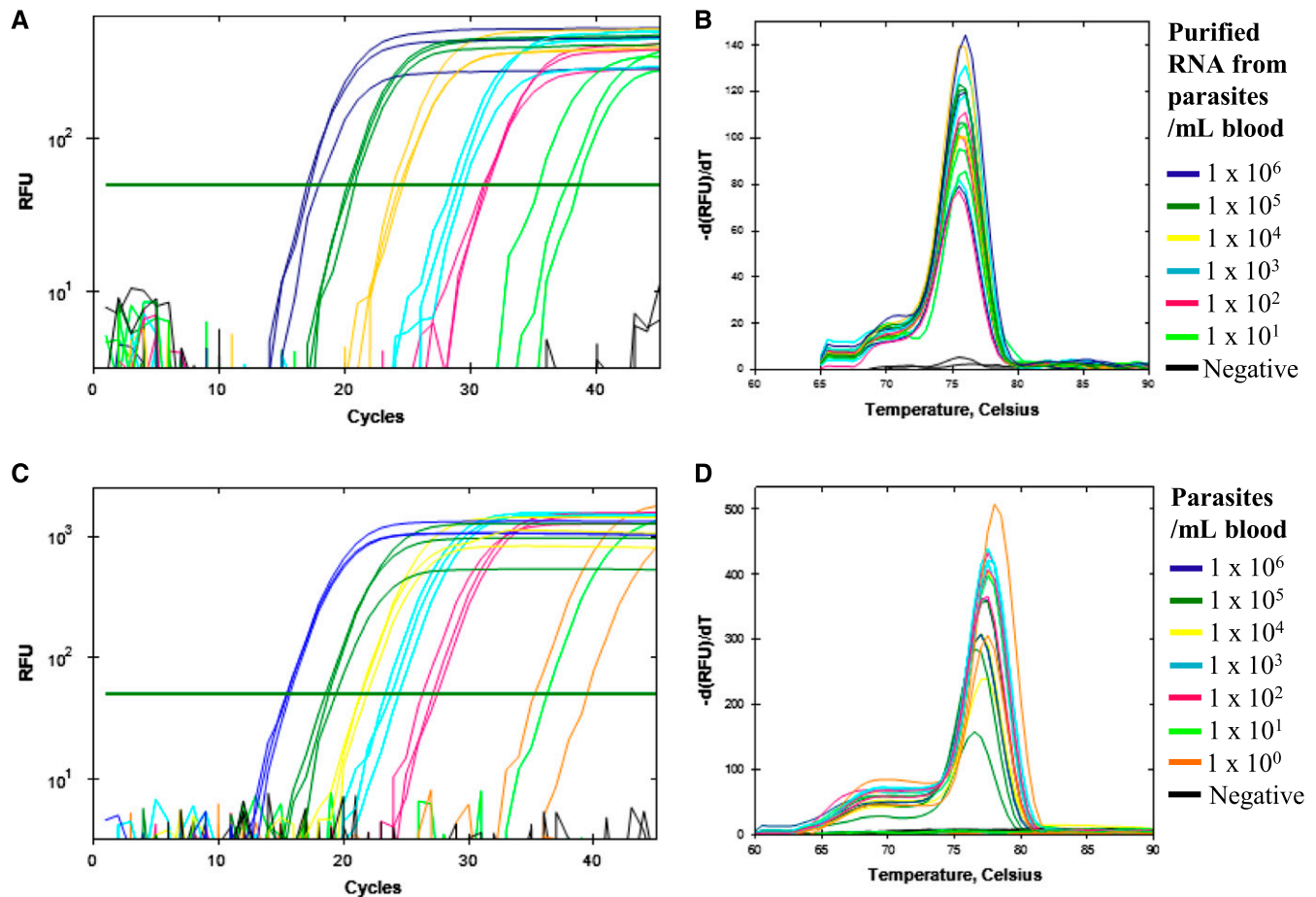


FIGURE 1. *Plasmodium falciparum* 18S rRNA detection in blood by direct reverse transcriptase polymerase chain reaction (RT-PCR). (A) Real-time amplification and (B) melting curves for 18S rRNA direct RT-PCR. Total nucleic acid was first purified from serial dilutions of *P. falciparum* 3D7 ring-stage parasites in uninfected blood. This purified nucleic acid was added back to blood in the direct RT-PCR reaction. (C) Amplification and (D) melting curves for 18S rRNA using the intact unpurified parasite serial dilutions in blood added directly to the reaction. The limit of detection for 18S rRNA from purified parasite nucleic acid is 10 parasites/mL and from unpurified parasite serial dilutions is 100 parasites/mL. These reactions were performed on a conventional real-time PCR instrument (Bio-Rad CFX Connect) with a threshold setting of 50 relative fluorescence units (horizontal line). This figure appears in color at www.ajtmh.org.

reverse transcription step of the reaction was working, as these primers specifically amplify only RNA.²⁵ In addition, we found that inclusion of RNase inhibitor was essential for high sensitivity detection of RNA. Omitting RNase inhibitor resulted in a decrease in signal for both purified RNA and parasites added directly to the reaction (Supplemental Figure 1).

Monitoring *P. falciparum* gene expression in gametocytes.

Next, we tested the direct RT-PCR reaction on parasite sexual stage mRNA targets. These included sexual stage transcripts from *P. falciparum* gametocyte exported protein-5 (PFGEXP5)³⁸ and PF14_0367.²⁵ PFGEXP5 is one of the earliest sexual stage markers identified to date, expressed in sexually committed ring-stage parasites, whereas PF14_0367 is a mature gametocyte marker of unknown function.²⁵ The LOD of the RT-PCR reaction for these targets in gametocytes serially diluted in blood was 1,000 parasites/mL (Figure 3). Although the assay has a similar sensitivity for both asexual- and sexual-stage mRNA targets, the amplification curves cross the threshold at different cycles, reflecting the differences in the efficiencies of each primer set and the abundance of these mRNA species. The melt

curves for both PFGEXP5 and PF14_0367 also displayed some peaks that did not align with the major product peak, indicating the presence of nonspecific amplification with these primers, mostly at the lower parasite concentrations. Based on the relatively robust amplification of PF14_0367, we chose this as a candidate gametocyte marker for further study. To confirm that PF14_0367 was expressed only in gametocytes, we compared expression in purified asexual ring-stage parasites and mature stage V gametocytes, showing increased levels of expression in gametocytes (Figure 4). 18S rRNA expression, which is not expected to differ between sexual and asexual stages, was analyzed in parallel.

Performance of the direct blood RT-PCR assay on a portable real-time PCR instrument. To evaluate the potential of the direct RT-PCR assay to be used in field settings, we ran the assay on a portable 16-well real-time PCR machine (Open qPCR, Chai Biotechnologies, Inc). This instrument was chosen for its ability to generate real-time PCR data, ease of use, portability, and low cost. On this instrument, the RT-PCR assay had a LOD of 100 parasites/mL for 18S rRNA, similar to the conventional instrument, and 10,000 parasites/mL for

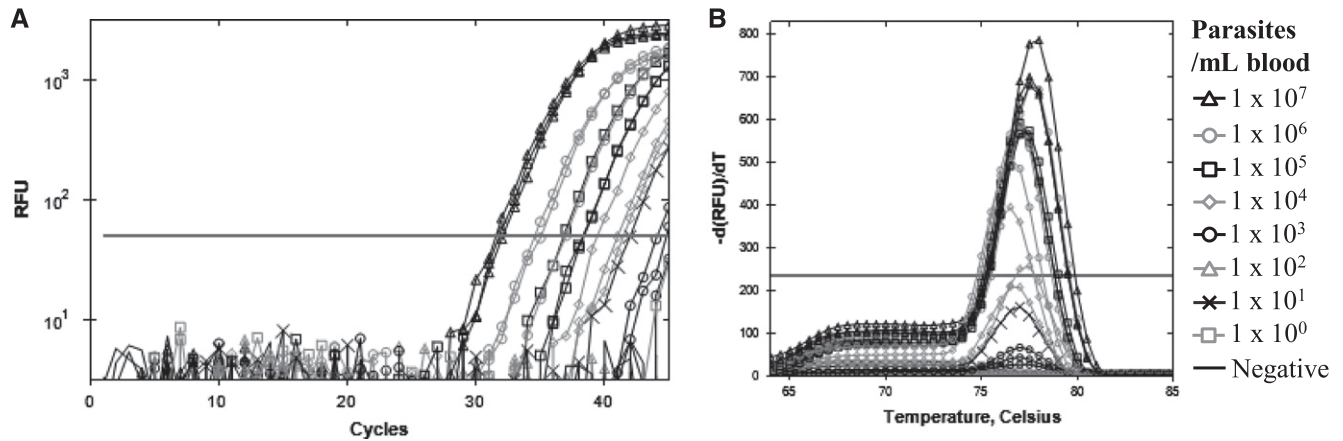


FIGURE 2. Detection of *Plasmodium falciparum* PFE0065w (SBP1) mRNA from asexual stages in blood. (A) Real-time amplification and (B) melting curves for PFE0065w mRNA direct reverse transcriptase polymerase chain reaction (RT-PCR) using *P. falciparum* 3D7 ring-stage serial dilutions in blood. The limit of detection for PFE0065w mRNA is 1,000 parasites/mL. This reaction was performed in the conventional real-time PCR instrument.

PF14_0367, 10-fold less sensitive than the conventional instrument (Figure 5). To boost the sensitivity of the reaction for PF14_0367, we increased the enzyme levels in the reaction 5-fold, achieving a LOD of 1,000 parasites/mL (Figure 5E). Raw data and statistical analysis of Cq values for the direct RT-PCR

assay on the portable real-time PCR instrument are presented in Supplemental Table 2.

The blood RT-PCR assay can successfully identify gametocytes in individuals from a high transmission setting. The ability to detect gametocytes in patient blood samples is a

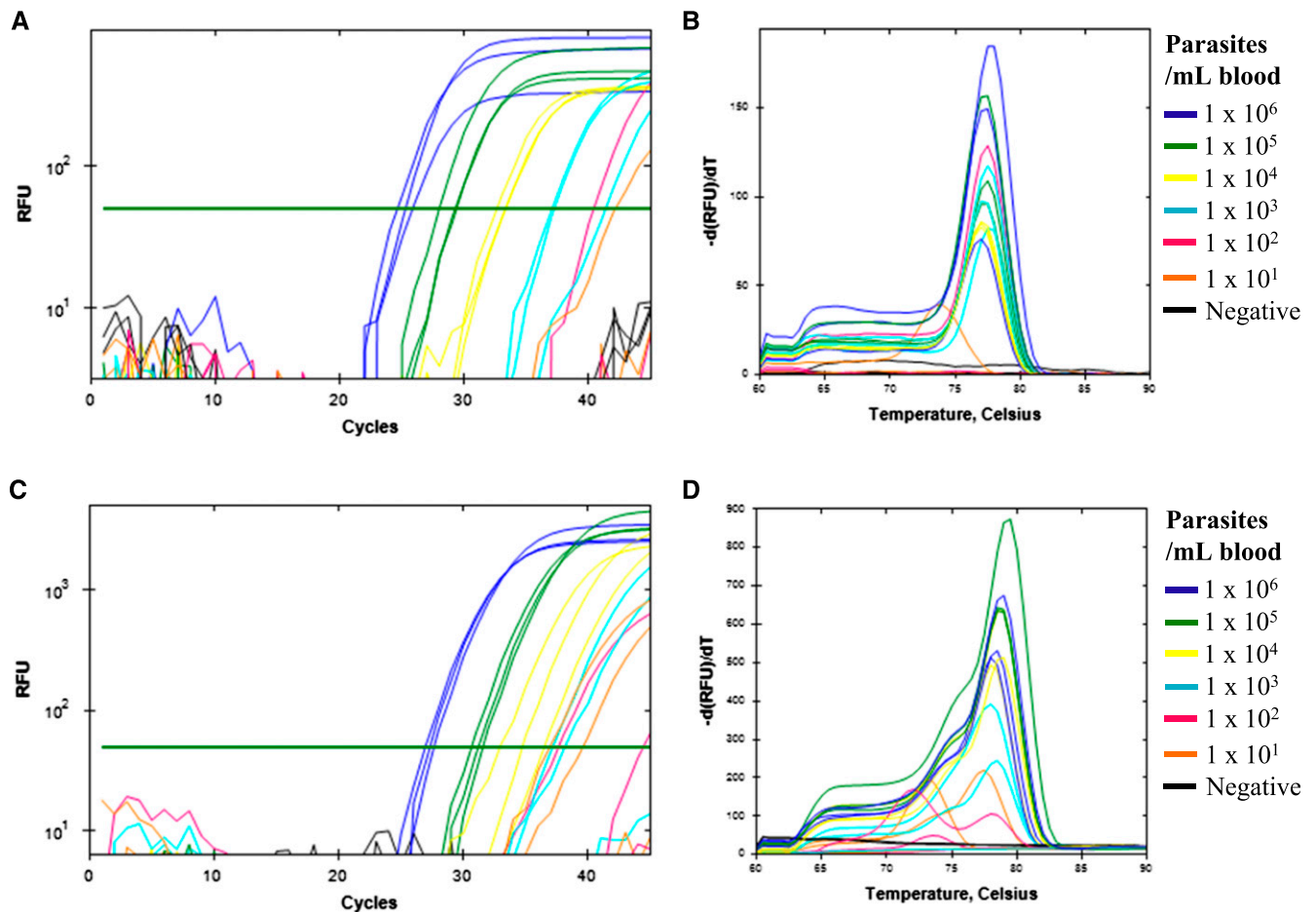


FIGURE 3. Detection of *Plasmodium falciparum* gametocyte mRNA targets in blood. (A) Real-time amplification and (B) melting curves for PF14_0367 mRNA direct reverse transcriptase polymerase chain reaction (RT-PCR). (C) Real-time amplification and (D) melting curves for PFGEXP5 mRNA direct RT-PCR. Both mRNA targets were detected in *P. falciparum* NF54 gametocyte serial dilutions on the conventional instrument at a limit of detection of 1,000 parasites/mL. This figure appears in color at www.ajtmh.org.

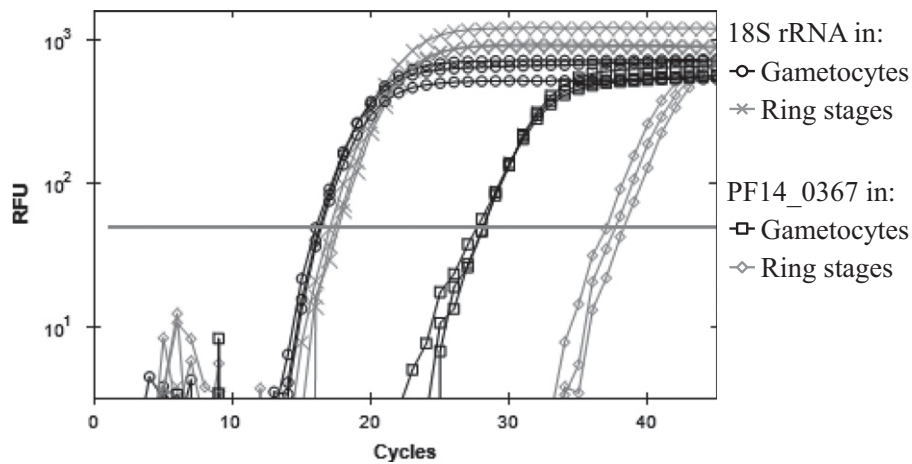


FIGURE 4. Detection of *Plasmodium falciparum* PF14_0367 mRNA and 18S rRNA in sexual- and asexual-stage parasites by direct RT-PCR. *Plasmodium falciparum* strain NF54 stage V gametocytes and asexual ring stages purified 10 hours after merozoite invasion at 1×10^6 parasites/mL blood. Arrows indicate the amplification curves for 18S rRNA in gametocytes and ring stages (0.8 cycle shift), and Pf14_0367 (8.3 cycle shift).

significant step toward developing a better diagnostic tool for malaria parasite transmission. A blind panel of 24 blood samples from individuals living in an area of high malaria parasite transmission in Cameroon was tested for 18S rRNA and PF14_0367 expression on conventional and portable real-time PCR instruments (Table 2). Each sample was tested in duplicate; Cq values were returned for all 96 runs in the conventional instrument and 93/96 runs in the portable instrument (Table 2). Two runs generated no Cq values despite the presence of a curve and one run generated a low Cq value that was considered an instrument Cq calculation error based on examination of the amplification curve. Cq values for 18S rRNA expression suggest a significant parasite burden in the majority of patients. Negative controls showed no amplification for all targets and platforms. The sensitivity and specificity for 18S rRNA and PF14_0367 assays on both instruments were calculated in comparison to a standard two-step RT-PCR method (Table 3). This method uses RNA purified from the blood as a template and is considered a highly sensitive and specific assay for parasite transcripts. In both the conventional and portable instruments, the sensitivity and specificity for 18S rRNA was 100%, with 100% concordance between the two instruments. The sensitivity and specificity of the PF14_0367 assay on the conventional instrument were 85.7% and 70%, respectively. Many of the discrepant results were from samples with low-target transcript levels. Three of 24 results were likely false positives, with two of these having Cq values greater than 39. Two of the 24 results were false negatives, with one of these (patient 21) having relatively low Cq values in the standard RT-PCR and low parasite densities by microscopy. The sensitivity and specificity of the PF14_0367 assay in the portable instrument were 78.6% and 90%, respectively. One of 24 results was likely a false positive, matching one of the false positives in the conventional instrument (patient 18). Three of 24 results were false negatives, with one matching a false negative in the conventional instrument (patient 21), and the others corresponding to samples that had relatively low Cq values in the standard RT-PCR. For the PF14_0367 assay, the

concordance between the two instruments was 79.2% ($\kappa = 0.583$, 95% confidence interval [CI] = 0.269–0.898), with the discordant results observed in samples with low-target transcript levels. Comparing the sensitivities and specificities of the two assays revealed no significant difference in the performance of the direct RT-PCR on the conventional or portable real-time PCR instruments (Supplemental Table 3).

DISCUSSION

The World Health Organization Global Technical Strategy for Malaria provides a 15-year framework for malaria elimination that includes transforming malaria surveillance into a core intervention as one of its three strategic “pillars”.³⁹ For the goals of this strategy to be realized, highly sensitive field-friendly surveillance tools must be developed.⁴⁰ In this study, we present a one-step RT-PCR assay that detected asexual malaria parasites with a LOD of 10 parasites/mL. This assay, if its sensitivity is confirmed in field settings, is markedly more sensitive than currently available microscopy and HRP2-based RDTs and has similar or higher sensitivity compared with a LAMP assay targeting *P. falciparum* mitochondrial targets.⁴¹ The high sensitivity for gametocyte-specific mRNA directly from blood on a portable real-time PCR instrument indicates the tool may be highly relevant in monitoring malaria transmission potential. The assay is sensitive (1,000 parasites/mL) and specific for detection of mature gametocyte targets. Although lower sensitivities for gametocytes have been reported with QT-NASBA (20 parasites/mL)²¹ and RT-LAMP (2 parasites/mL),¹⁶ the advantage of the RT-PCR method reported here is that it does not require nucleic acid purification and uses small volumes of blood (2.5 μ L). The RT-PCR method may lose some sensitivity because it is performed directly in the presence of blood, which can degrade the RNA target and inhibit polymerase activity.^{42,43} This assay also has some variability in amplification and melt curve profiles for asexual- and sexual-stage transcripts emphasizing the need for optimization of primer sets, reaction conditions, and enzyme

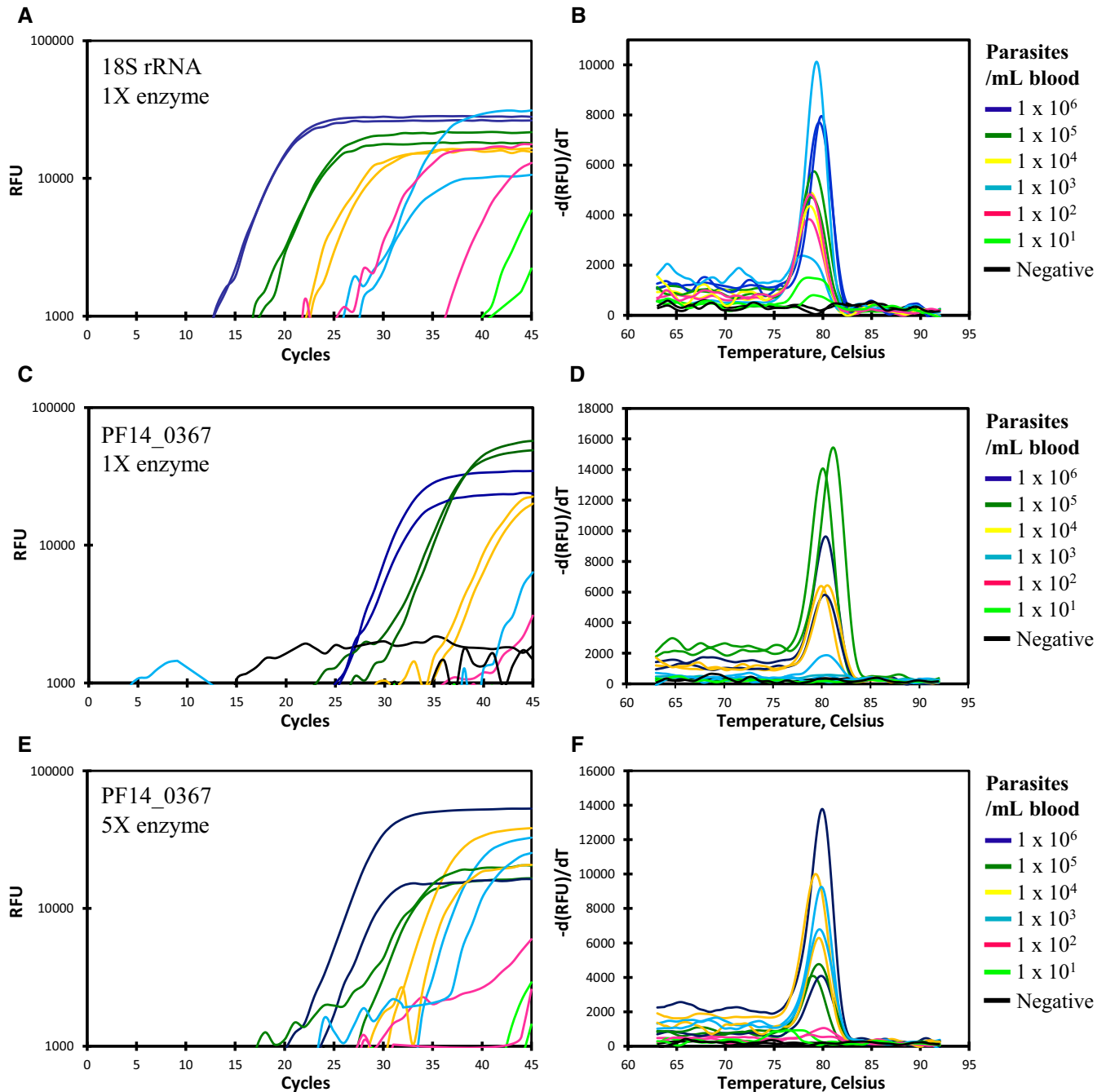


FIGURE 5. Detection of *Plasmodium falciparum* 18S rRNA and PF14_0367 mRNA in a portable real-time PCR instrument by direct RT-PCR. *Plasmodium falciparum* RNA targets were tested in a 16-well portable real-time PCR instrument. Amplification (A, C, and E) and melt curve (B, D, and F) data from the instrument are shown. For the 18S rRNA assay, standard (1 \times) concentration of enzyme was used, and produced a LOD of 100 parasites/mL (A and B). For the PF14_0367 assay, mRNA was detected in 3D7 gametocytes at a LOD of 10,000 parasites/mL with 1 \times concentration of enzyme (C and D) or 1,000 parasites/mL with 5 \times concentration of enzyme (E and F). Individual C_q values obtained directly from the instrument are listed in Supplemental Table 2. This figure appears in color at www.ajtmh.org.

concentration for each target. For simplicity of experimental design and interpretation of SYBR-based amplification and melting curves, internal controls were not used in this study. Nonetheless, the method presented here is sufficiently sensitive and robust in detecting gametocytes in blood samples on both a conventional real-time PCR instrument used in research laboratory settings and a commercially available portable real-time PCR machine intended for use in field settings.

The choice of PF14_0367 as a gametocyte marker is supported in this study in Figure 4, showing greatly increased expression in purified sexual compared with asexual stages. Detection of the PF14_0367 transcript at low levels in asexual stages most likely arises from contaminating sexual stages in the preparation, and is not expected to interfere with interpretation of the results.

TABLE 2

Direct RT-PCR detection of 18S rRNA and PF14_0367 gametocyte mRNA in individuals from a high malaria transmission setting in Cameroon

Patient*	18S rRNA RT-PCR					PF14_0367 RT-PCR				
	Conventional		Portable		Ref§	Conventional		Portable		Ref
	Cq†	Tm (°C)‡	Cq	Tm (°C)	Cq	Cq	Tm (°C)	Cq	Tm (°C)	Cq
1	17.9	77	18	78.5	16.7	33.4	78.5	31.9	79.2	39.4
	18	76.5	20	79.1	16.7	32.7	78	32.4	79.6	38.2
2	14.4	77.5	16.8	79.3	17.8	32.1	78.5	33	79.8	35.7
	14.3	77.5	16.5	79.1	17.8	32.1	78.5	32.9	80	34.7
3	20.6	77.5	27	78.9	22.8	36.2	79	36.2	79.7	31.7
	21.2	77.5	26.8	79.8	22.8	35.5	79	36	79.6	32
4	26.3	76.5	31.1	79.1	29.5	-	-	-	-	-
	26.4	77	NC	-	29.5	-	-	-	-	-
5	17.4	77	18.5	78	14.4	33.7	78.5	31.3	78.8	27.3
	17.3	77	19.4	78.4	14.4	33.1	78	30.8	79	26.7
6	25.4	77	31.9	79.3	25.6	-	-	-	-	-
	26.1	77.5	33.5	79.8	25.6	-	-	-	-	-
7	20.1	77	24.9	78.3	14.1	38.9	78.5	-	-	29.4
	19.8	76.5	24.6	79.5	14.3	39	78.5	-	-	29.1
8	16.2	76.5	19.6	77.8	15.6	34.7	77.5	33	78.8	33.2
	16.2	76.5	18.6	78.1	15.6	34.1	77.5	33.9	79.4	34.1
9	21.3	76	NC	76.4	24.9	39.3	77.5	39.6	78.3	35.8
	21.3	76	22.3	76.8	24.9	40.1	78.5	39.9	78.8	-
10	18.3	77	25.2	77	17.7	38.7	78	-	-	-
	17.7	76.5	22.9	78.1	17.7	37.9	79	-	-	-
11	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
12	21.4	77.5	26.2	79	20.7	36.1	79	37.3	79.2	35.3
	21.4	77.5	26.5	78.7	20.8	36.2	79	38.4	79.1	34.5
13	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
14	26.5	76.5	29.6	79	27.1	-	-	-	-	-
	26.2	77	29.6	78.7	27.2	-	-	-	-	-
15	18.3	77	19.1	78	16.4	32.3	78	31	79	24.7
	18	77	19.8	78.6	16.4	32.5	78	31.1	79.3	25.6
16	21	77.5	28.7	78.5	19.5	-	-	-	-	-
	21.5	77.5	29	78.9	19.5	41.8	78.5	-	-	-
17	20	77	23.7	78.1	18.5	34.9	79	33.9	79.3	32.1
	19.7	77	26.1	78.5	18.6	34.3	78.5	30.6	78.6	30.8
18	17.4	77	22.8	78.3	16.8	40.2	78	38.6	79.7	-
	17.3	76.5	25.8	78.3	16.9	39.5	78.5	40.4	79.7	-
19	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
20	21.7	76.5	27.1	79.5	22.4	41.5	79	-	-	-
	21.6	77	25.4	79.5	22.5	39.9	79	-	-	-
21	28.6	77	35.9	79	27.8	-	-	-	-	28.4
	28.4	77.5	NC	-	27.5	-	-	-	-	29.5
22	18.2	76	24.2	78.7	20.2	38.5	77	39.4	79	36.1
	18.5	76.5	23.5	79.2	20.2	37.8	78	39	79.1	35.6
23	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
24	23.4	76.5	29.9	79	22.6	-	-	40	79.1	40.1
	23.3	77	29	80.2	22.6	-	-	42.1	78.9	-

Cq = quantification cycle; RT-PCR = reverse transcriptase polymerase chain reaction; Tm = melting temperature.

* Duplicate reactions performed for each patient sample are shown.

† Quantification cycle value. Negative is indicated as -, judged by cutoff values for each target and instrument. NC indicates (1) no amplification curve but Cq value was assigned, or (2) presence of an amplification curve with no Cq value assigned; both considered negative.

‡ Melting temperature. Negative (-) indicates no value assigned by the instrument.

§ Reference standard RT-PCR.

The performance of the RT-PCR assay on field samples from a region of high transmission in Cameroon indicates it will be valuable as a transmission surveillance tool. To assess the performance of this test, results were compared with an RT-PCR reference standard using purified RNA from the panel as template. The clinical sensitivity and specificity of this test for malaria detection were excellent in both conventional and portable instruments. With regard to monitoring gametocyte transcripts in clinical samples, the assay performance was also promising, considering

the relatively low levels of transcripts compared with 18S rRNA. To reliably detect the lower levels of PF14_0367 transcripts, the assay performed in the portable instrument required increased enzyme compared with the 18S rRNA assay. The sensitivities of the conventional and portable PF14_0367 assays based on comparison to the reference standard (85.7% versus 78.6%) were not significantly different, which was not unexpected considering the small sample size. False negatives in both instruments tended to correspond to samples with relatively high Cq values,

TABLE 3

Clinical sensitivity and specificity of 18S rRNA and PF14_0367 RT-PCR assays performed in conventional and portable real-time PCR instruments

Target	Instrument	Sensitivity (%)	95% CI	Specificity (%)	95% CI
18S rRNA	Conventional	100.0	83.2–100.0	100.0	39.8–100.0
18S rRNA	Portable	100.0	83.2–100.0	100.0	39.8–100.0
PF14_0367	Conventional	85.7	57.2–98.2	70.0	34.8–93.3
PF14_0367	Portable	78.6	49.2–95.3	90.0	55.6–99.8

95% CI = 95% confidence interval; RT-PCR = reverse transcriptase polymerase chain reaction.

suggesting these may have been low transcript samples on the cusp of detection, or samples containing reaction inhibitors. Much the same can be said for the specificities of the PF14_0367 assay in the portable and conventional instruments (90% versus 70%), which were also not significantly different and tended to occur in samples with high Cq values. It is possible that the test performance has been affected by the potential for single-stranded RNA target to be degraded, especially in low transcript samples. Taken together, these results show much promise for the further development of a direct RT-PCR assay. Future studies with an expanded cohort of patients of varying levels of parasitemia, including asymptomatic and submicroscopic patients, will clarify these issues.

The portable instrument is a rugged, easily transportable, AC-powered unit that performs 16 reactions per run including controls. It is capable of onboard Cq and melting curve processing and results display, avoiding the need for a computer to run the instrument. Issues with venipuncture may be overcome by fingerprick collection of blood, as the RT-PCR assay runs on small sample volumes. However, to enable implementation in the field, several challenges remain to be addressed. Samples currently require cold storage to preserve the RNA target, as do the liquid components of the RT-PCR assay. In a previous study, we adapted PCR diagnostics for the field using desiccated hydrogels²⁴ that preserve enzyme activity at room temperature. For that assay, samples could be added directly after collection to desiccated hydrogels and run on the portable real-time PCR instrument. We envision a similar approach for the RT-PCR assay, which would be particularly effective in avoiding degradation of the RNA template. The relatively low throughput of the instrument may still require some cold storage of samples in larger studies; however, the low cost may make it possible to have multiple instruments running at once. The detection of RNA targets present in low abundance, as was the case with PF14_0367, may also require increased enzyme and modified master mix formulations, increasing the cost of the assay in some cases. Despite these challenges, the success of the direct RT-PCR on the portable real-time PCR instrument coupled with the previous success of the hydrogel assay provides a clear path for further testing in the field.

The detection of gametocytes by sensitive molecular methods may be particularly relevant for epidemiological studies examining the human infectious reservoir and assessing the efficacy of transmission-interrupting regimens.⁶ Detection of gametocytes at sensitivities below the threshold of microscopy, coupled with mosquito feeding assays, may shed new light on infectiousness in many areas of study such as age-stratified populations, in longitudinal studies measuring duration of infectiousness, and in

regions of low parasite density targeted by malaria elimination programs. Similarly, it is expected that sensitive tools for gametocyte detection will be useful to measure the efficacy of transmission-blocking vaccines currently in preclinical development and expected to enter clinical testing in the next 5 years.⁴⁴

CONCLUSION

The shift in focus from malaria control to elimination programs requires a change in strategy that is underscored by the need for more sensitive surveillance tools. The development of molecular tests, particularly those of high sensitivity and versatility to detect not only the presence of malaria parasites, but other important characteristics such as transmission potential, will be of great value in sustaining elimination efforts and preventing reintroduction of disease. The use of simple, RNA-based diagnostics such as the direct RT-PCR assay described here, addresses many of the specific needs of countries on the path to malaria eradication.

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