An assay for quantification of male and female gametocytes in human blood by qRT-PCR in the absence of pure gametocyte standards

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Abstract

Background Malaria transmission from humans to mosquitoes requires the presence of gametocytes in human peripheral circulation, and the dynamics of transmission are determined largely by the density and sex ratio of the gametocytes. Molecular methods are thus employed to measure gametocyte densities, particularly when assessing transmission epidemiology and the efficacy of transmission-blocking interventions. However accurate quantification of gametocytes with molecular methods requires pure male and female gametocytes as reference standards, which are not widely available.

Methods qRT-PCR assays were used to quantify levels of sex-specific mRNA transcripts in Plasmodium falciparum female and male gametocytes (pfs25 and pfMGET respectively) using synthetic cRNA standards and in-vitro cultured gametocytes. Assay were validated and assay performance was investigated using blood samples of clinical trial participants (ClinicalTrials.gov reference number NCT02431637 and NCT02431650) using these standards and compared to absolute quantification by droplet digital PCR (ddPCR).

Results The number of transcript copies per gametocyte were determined to be 279.3 (95% CI 253.5 - 307.6) for the female-specific transcript pfs25, and 12.5 (95% CI 10.6 - 14.9) for the male-specific transcript pfMGET . These numbers can be used to convert from transcript copies/mL to gametocyte/mL. The reportable range was determined to be 5.71x10 6 to 5.71 gametocytes/mL for pfs25, and 1.73x10 7 to 5.59 for pfMGET. The limit of detection was 3.9 (95% CI 2.5-8.2) gametocytes/mL for pfs25, and 26.9 (95% CI 19.3 - 51.7) gametocytes/mL for PfMGET . Both assays showed minimal intra-assay and inter-assay variability with coefficient of variation < 3%. No cross-reactivity was observed in both assays in uninfected human blood samples. Comparison of results from ddPCR to qRT-PCR assays on clinical blood samples indicated high level agreement (ICC=0.998 for pfs25 and 0.995 for pfMGET).

Conclusions We developed and validated qRT-PCR assays that are able to accurately quantify levels of female and male P. falciparum gametocytes at submicroscopic densities. The assays showed excellent reproducibility, sensitivity, precision, specificity and accuracy. The methodology will enable the estimation of gametocyte density in the absence of pure female and male gametocyte standards, and will facilitate clinical trials and epidemiological studies.

Background

Successful transmission of Plasmodium infection from humans to mosquitoes requires that a female Anopheles mosquito imbibes at least one mature male and one mature female gametocyte in a blood meal. The density and the ratio of female to male gametocytes in peripheral blood are therefore key determinants of the dynamics of transmission, transmission epidemiology in endemic settings, and for evaluating the efficacy of transmission blocking interventions, such as drugs and vaccines (1–6). Thus, techniques to accurately detect and quantify gametocytemia are required (7). Successful transmission has been reported to occur at submicroscopic gametocyte levels both in endemic areas during asymptomatic infections, and in volunteer infection studies (VIS), also referred to as controlled human malaria infection (CHMI)-transmission studies (6, 8, 9). Understanding the contribution of submicroscopic infections to the human infectious reservoir would greatly assist elimination efforts.

Gametocytemia has traditionally been measured by thick- or thin-film microscopy; however, both methods have limited sensitivity and cannot accurately measure gametocytemia below 10,000 gametocytes per mL (10). To overcome the limited sensitivity of microscopy, quantitative reverse transcription PCR (qRT-PCR) and quantitative nucleic acid sequence based amplification (NASBA) assays targeting gametocyte specific mRNA transcripts have been developed (6, 11–15). The highly conserved pfs25 gene transcript is present in abundance in female gametocytes, and is therefore commonly targeted for quantification by qRT-PCR (7, 14). More recently, male-specific gametocyte mRNA transcripts have been described (5, 16, 17), and among them pfMGET (Pf3D7_1469900) shows an abundant transcription profile (5) making it an appropriate candidate for quantitation of male gametocytes. The use of both female- and male-specific assays allows evaluation of the gametocyte sex ratio, a parameter of interest in quantifying infectivity to mosquitoes, particularly at lower gametocyte densities (18). The Pfs25-PfMGET combination for sex ratio determination was recently validated by immunofluorescence assays on field samples from gametocyte donors (19).This ratio may be particularly important for assessment of gametocytocidal drug activity, as the less abundant male gametocytes may be more readily sterilised or killed by some antimalarial drugs (14, 20, 21).

qRT-PCR methods have superior sensitivity over microscopy, allowing the quantification of gametocyte densities across the epidemiologically relevant range (1). These assays require the use of reference standards to generate standard curves for quantification. These ideally consist of biological reference standards, namely pure populations of female and male gametocytes. However, preparation of such material requires laborious approaches to culture and FACS-sort gametocyte reporter lines that are not readily available to all laboratories (22). An alternative method for generation of standard curve material is the use of synthetic standards such as cRNA, which can be prepared by any laboratory. Although this approach permits quantification of the absolute number of mRNA transcripts, the assay does not allow the quantification of gametocyte numbers unless we know how many copies of the target mRNA transcript are expressed per gametocyte. As the number of mRNA transcripts expressed per gametocyte differs for each target (5, 22), the expression level of each must be known in order to convert transcripts per mL to gametocytes per mL. By determining these conversation factors it would significantly improve the assessment of gametocyte density and sex ratios by qRT-PCR using synthetic cRNA standards.

Here, we describe the validation of two qRT-PCR assays that enable the accurate and sensitive detection and quantification of female (pfs25) and male (pfMGET) gametocytes in whole blood samples without the need for purified female and male gametocytes reference standards.

Methods

RNA extraction

250∝ l of packed red blood cells (pRBCs) from each clinical trial participant (see below) were stored (1:5) in RNA Protect Cell Reagent (Qiagen, Australia) at -80 °C until RNA extraction. Prior to RNA extraction, a known concentration of equine arteritis virus (EAV) culture was spiked into each sample as an internal control to monitor extraction efficiency and qRT-PCR inhibition (23). RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen, Australia) following manufacturer's instructions with treatment of DNase on-column

digestion using RNase-Free DNase set (Qiagen, Australia) to eliminate genomic DNA. 100 ∝l of RNA extract was eluted.

Female and male gametocyte qRT-PCR assays

Sex-specific qRT-PCR assays were used to measure RNA transcripts specific to female and male gametocytes (Table 1). The previously described female marker assay was designed to target the P. falciparum gametocyte surface protein (pfs25) mRNA (Genbank accession number AF154117) (11). The male marker assay targeted the exon-exon junction of the P. falciparum PF3D7_1469900 mRNA transcript (Genbank accession number XM_001348805) recently characterised as male gametocyte-enriched transcript (pfMGET) (5, 6).

Table 1
List of oligonucleotides used in this study.

Oligo names	Sequences	Target	References
pfs25 (female gametocytes)	Forward: 5'-AAATCCCGTTTCATACGCTTGTAA-3'	P. falciparum pfs25 mRNA	(11)
	Reverse: 5'-CAGTTTTAACAGGATTGCTTGTATCTAATATAC-3'		
	Probe: 5'-FAM-ACCAAATGAATGTAAGAATGTAACTTGTGGTAACGGT-BHQ1-3'		
pfMGET (male gametocytes)	Forward: 5'- AAAATTCGGTCCAAATATAAAATCCTG-3'	P. falciparum PF14_0063 (PF3D7_1469900) mRNA	(5, 6)
	Reverse: 5'- CTTCATCAATTAAAAATCCCTTTTTTGT-3'		
	Probe: 5'-FAM – CCTGGTAAAAAACAGCTCCAGCA – BHQ1-3'		

qRT-PCR was conducted with One-Step RT-PCR mix (Qiagen, Australia) using methods previously described (6, 11) with 0.45 µM of each primer and 0.18 µM of Taqman probe in each PCR reaction. Amplification was performed in a Rotorgene 6000 or Q instrument (Qiagen, Australia) under the following cycling conditions: 50 °C reverse transcription for 30 min, 95 °C incubation for 15 min, followed by 45 cycles of 95 °C for 15 sec and 60 °C for 60 sec. Additional PCR reactions with heat inactivated reverse transcriptase were included to ensure genomic DNA was undetected in each qRT-PCR reaction. RNA extraction and PCR efficiency were monitored through the use of EAV RT-PCR assay (6).

Synthetic complementary RNA (cRNA) standard preparation and standard curves construction

Standard curves were generated from serial-diluted synthetic cRNA controls. Synthetic linear dsDNA string containing qRT-PCR target sequences with T7 promoter (Thermo Fisher Scientific, Australia) was in-vitro transcribed to cRNA using HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs) following the manufacturer's instructions, and then subjected to two cycles of DNase digestion (RNase-free DNase set, Qiagen, Australia) to eliminate synthetic DNA contamination. cRNA was then purified using the Qiagen RNeasy mini kit (Qiagen, Australia). Quantity (copy numbers) of neat cRNA control was calculated based on molecular

weight measured by the High Sensitivity RNA Qubit assay (Thermo Fisher Scientific). Serial dilutions (cRNA copy number equivalent to pfs25: 5.71×10^6 to 5.71 gametocytes/mL; pfMGET: 1.73×10^7 to 1.73×10^2 gametocytes/mL) were prepared in uninfected human whole blood extracts to reflect similar matrix specimen type from clinical trials. Dilution series were analysed in replicates to generate standard curves for transcript quantification. The linear regression using an established external standard curve (pfs25 slope: -3.395, pfMGET slope: -3.236) was imported into all qRT-PCR runs within Rotorgene software with fixing to the highest standard to achieve final quantification of RNA copies.

Assay sensitivity

To determine assay sensitivity or limit of detection (LOD), neat cRNA was serial-diluted in uninfected human blood extracts to produce 14 concentrations of pfs25 cRNA (equivalent to the range of 5.71×10^6 to 0.18 gametocytes/mL) and 11 concentrations of pfMGET cRNAs (equivalent to the range of 1.73×10^7 to 5.59 gametocytes/mL), with focus on more dense dilutions in the lower range for more accurate determination of assay limits. Replicates of each dilution and a negative control (uninfected human blood extracts) were analysed on separate qRT-PCR runs (6 for pfs25 and 3 for pfMGET) on a Rotorgene Q instrument (Qiagen, Australia) with same batch of mastermix used.

Assay specificity

Analytical specificity was determined by comparing the sequence of the nucleic acid target (primer and probe sequences) to sequences available on publicly accessible databases using BLAST search tool (NCBI) to check its specificity to P. falciparum pfs25 and PF3D7_1469900 (pfMGET) transcripts. No cross reactions were observed for other malarial mRNA transcripts and human genomic DNA or mRNA transcripts. Blood from malaria-naïve volunteers enrolled in clinical trials (pfs25 n = 66, pfMGET n = 11) was assessed for assay specificity.

Female and male gametocyte ddPCR

Since no “gold standards” were available for RNA quantification, ddPCR was utilised to generate absolute quantification of RNA copy numbers to confirm the accuracy of RNA estimates in both cRNA standards and infected blood. The female and male gametocyte qRT-PCR assays were adapted to the droplet digital PCR (RT-ddPCR) format. cRNA controls and RNA extracts from participants (see below) were analysed on QX200 ddPCR system (BioRad, Australia). The RT-ddPCR reactions were prepared using One-Step RT-ddPCR Advanced Kit for Probes (BioRad, Australia). Each 20 µl of ddPCR reaction contained 5 µl of 1 × One-Step RT-ddPCR Supermix, 0.45 µM of each primer, 0.18 µM of Taqman probe, and 5 µl of RNA template. The RT-ddPCR mix was loaded onto the QX-200 AutoDG automated droplet generator (BioRad, Australia) to partition the reaction into nanolitre-sized droplets before being transferred to a 96 well plate for thermal cycling. Amplification was performed on a C1000 Touch thermal cycler (BioRad, Australia) with the following conditions: reverse transcription at 50 °C for 1 hour, enzyme activation at 95 °C for 10 minutes, 40 cycles of 95 °C for 30 seconds and 60 °C for 1 minute with ramp rate settings to 2 °C/sec, with final step of enzyme deactivation at 98 °C for 10 minutes and 12 °C forever. Amplification products were read on the QX-200 Droplet Reader (BioRad, Australia) and quantification was determined using the associated QuantaSoft analysis software (BioRad, Australia). Reactions containing uninfected human blood extracts were used to determine the negative

amplitude threshold for quantification analysis of samples. Quantification results by ddPCR were compared to the results by qRT-PCR.

Female and male gametocyte reporter lines

Gametocytes were cultured and maintained using the PfDynGFP/PfP47mCherry reporter line as previously described (5, 22), and were sorted using the Coulter Epics Elite flow cytometer (Beckman Coulter) or the BD FACS Aria SORP flow cytometer keeping cells at 4 °C in SA buffer at Radboud University (Nijmegen, Netherlands) (22). The sorted female and male gametocytes (10^6 gametocytes/mL) were stored frozen in RNA Protect Cell Reagent (Qiagen, Australia).

Gametocytes were thawed and RNA extracted using methods described above. A 10-fold dilution series of the sample was made ranging from 10^6 to 10 gametocytes/mL. Each sample dilution series was run in duplicate over three days (a total of 36 PCR reactions). The pfs25 and pfMGET mRNA transcript numbers were determined in these samples using the qRT-PCR assays with cRNA standards. The study design was a randomised block design with the dilution series as treatments, daily runs as blocks and technical replicates used to estimate intra-assay variability. An analysis of variance on the residual difference in $\log_{10}$ copies per gametocyte between $\log_{10}$(copies/mL) and $\log_{10}$(gametocytes/mL) was used to obtain mean and 95% confidence intervals for each conversion factor to translate from copies/mL to gametocytes/mL.

Gametocyte positive human blood samples

Gametocyte positive human blood samples (pfs25 n = 33, pfMGET n = 36) were obtained from previously reported CHMI-transmission studies. These studies were single-centre, open-label clinical trials run concurrently between 2015 and 2016 at Q-Pharm Pty Ltd (Brisbane, Australia) (6). Participants were healthy, malaria-naïve adults aged between 18 and 55 years. Participants were inoculated with ~ 2,800 parasite infected RBCs on day 0 and were treated with 480 mg piperaquine 7 or 8 days after inoculation to clear asexual parasitemia. Whole blood samples were collected before and after piperaquine treatment and were analysed by female- and male- specific qRT-PCR and subsequently ddPCR for verification of quantification as described above. These clinical trials were approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee, and all participants gave written informed consent before inclusion in the study. The clinical trials are registered with ClinicalTrials.gov (NCT02431637 and NCT02431650).

Statistical analysis

Statistical analysis of the conversion factors for both assays, reportable range, precision and sensitivity was conducted in SPSS version 22 (IBM Corp, Armonk, NY). The reportable range dataset with large linear range (14 dilutions pfs25, 11 dilutions pfMGET) was used for these analyses. LOD at which 95% of samples tested positive was calculated using Probit regression. Intra-assay variability, as the standard deviation for intra-assay variability, was estimated as the variability between technical replicates pooled across standards. The inter-assay variability was determined using standard curve data from historical records of 17 runs of 7 study cohorts for pfs25 and 29 runs from 15 study cohorts for pfMGET, with each standard analysed separately using ANOVA with the standard deviation estimated as the variability between assay runs pooled within cohorts. Relative variability was measured as the percent coefficient of variation (%CV) for each standard concentration. Linear regression was used to assess the relationship between $\log_{10}$ concentration of standard

relative to the highest standard and C_q value. Accuracy was assessed using intraclass correlation coefficient (ICC), paired t-test and Passing-Bablok regression to examine differences in gametocyte per mL of whole blood between qRT-PCR and ddPCR using R Studio (ver. 1.1.442, R version 3.4.4).

Results

Estimating transcript numbers per gametocyte using purified in-vitro cultured female and male gametocytes

A dilution series of purified female and male gametocytes of known concentration obtained from the PfDynGFP/PfP47mCherry reporter line were analysed by qRT-PCR, and used to calculate the number of copies of each mRNA target expressed per gametocyte for converting copies/mL to gametocytes/mL. The conversion factors were determined to be 279.3 (95% CI 253.5–307.6) for the female-specific marker pfs25 and 12.5 (95% CI 10.6–14.9) for pfMGET. The pooled inter-assay standard deviation was calculated to be 0.105 (pfs25) and 0.180 (pfMGET). The pooled intra-assay standard deviation was calculated to be 0.044 (pfs25) and 0.080 (pfMGET).

Female and male gametocyte qRT-PCR assay performance

Sex-specific qRT-PCR assays were validated to quantify mRNA transcript levels specific to female (pfs25) and male (pfMGET) gametocytes. Both assays showed reliable amplification across a large linear range with good precision, sensitivity and specificity. The reportable range was determined to be 5.71×10^6 to 5.71 gametocytes/mL for pfs25, and 1.73×10^7 to 5.59 for pfMGET. The relationship between $\log_{10}$ concentration of standard relative to the highest standard and C_q value for pfs25 had slope − 3.35 (95% CI -3.38 to -3.32), intercept 17.18 (95% CI 17.05 to 17.31), coefficient of determination (R^2) of 0.99 and mean squared error of 0.37, and that for pfMGET had slope − 3.30 (95% CI -3.35 to -3.26), intercept 16.65 (95% CI 16.44 to 16.86), coefficient of determination (R^2) of 0.99 and mean squared error of 0.40. The amplification efficiency was 98.8% for pfs25 and 100.8% for pfMGET. The linearity of calibration across the lower dilutions in both series extended the reportable range and lower limit of quantification to 0.18 gametocytes/mL for pfs25 and to 5.59 gametocytes/mL for pfMGET. No amplification was observed in the negative control extracts (pfs25 n = 33, pfMGET n = 6) from uninfected human blood extracts.

The $LOD_{95\%}$ for these assays was determined to be 3.9 gametocytes/mL of whole blood (95% CI 2.5–8.2) for pfs25, and 26.9 gametocytes/mL of whole blood (95% CI 19.3–51.7) for pfMGET when a starting volume of 250∝ l of packed RBCs (equivalent to 500∝ l whole blood) is analysed.

There was a tendency for both intra- and inter-assay variability to increase at lower dilutions but the %CV was less than 3% for each standard in both assays. The standard deviation measure for intra-assay variability pooled across all dilutions was 0.52 C_q units for pfs25 and 0.51 C_q units for pfMGET, indicating minimal variability between replicates pooled across standards (Tables 2 and 3). The overall standard deviation measures for inter-assay variability were 0.50 C_q units for pfs25 and 0.46 C_q units for pfMGET (Tables 4 and 5), which were higher than the pooled intra-assay variability at similar dilutions (0.30 for pfs25 and 0.18 for pfMGET.

Table 2

pfs25 (female gametocyte) qRT-PCR assay for assessing intra-assay variability between technical replicates

Standard (gametocytes/mL)	Detected (% positive)	Mean C_q (SD)	Range	%CV
5.71×10^6	36/36 (100.0%)	17.14 (0.19)	16.67–17.62	1.1
5.71×10^5	34/34 (100.0%)	20.53 (0.23)	19.87–20.96	1.1
5.71×10^4	35/35 (100.0%)	23.94 (0.18)	23.60–24.33	0.7
5.71×10^3	36/36 (100.0%)	27.13 (0.19)	26.57–27.49	0.7
5.71×10^2	36/36 (100.0%)	30.57 (0.22)	30.09–31.29	0.7
5.71×10^1	36/36 (100.0%)	33.87 (0.25)	33.10–34.50	0.8
1.15×10^1	21/21 (100.0%)	36.15 (0.45)	35.36–36.94	1.2
7.16	21/21 (100.0%)	37.26 (0.55)	36.40–38.87	1.5
5.71	14/15 (93.3%)	38.09 (0.92)	36.81–39.65	2.4
2.86	18/21 (85.7%)	38.58 (1.09)	37.23–40.96	2.8
1.43	16/21 (76.2%)	39.77 (1.01)	38.43–42.32	2.5
0.72	32/42 (76.2%)	40.07 (0.80)	38.92–41.8	2.0
0.36	8/21 (38.1%)	40.42 (0.80)	38.99–42.17	2.0
0.18	3/20 (15.0%)	40.51 (n/a)	40.43–40.56	n/a
Negative control	0/33	Not detect		
Total	346/395 (87.6%)	33.14 (0.52^)	16.67–42.32	

C_q: quantification cycle, SD: standard deviation, %CV: percent coefficient of variation, n/a: not applicable, Negative control: uninfected human blood control, ^ Overall SD.

Table 3

pfMGET (male gametocyte) qRT-PCR assay for assessing intra-assay variability between technical replicates

Standard (gametocytes/mL)	Detected (% positive)	Mean C_q (SD)	Range	%CV
1.73×10^7	15/15 (100.0%)	16.56 (0.06)	16.44–16.70	0.4
1.73×10^6	15/15 (100.0%)	19.86 (0.06)	19.70–20.08	0.3
1.73×10^5	15/15 (100.0%)	23.19 (0.06)	23.03–23.32	0.3
1.73×10^4	15/15 (100.0%)	26.57 (0.10)	26.41–26.76	0.4
1.73×10^3	15/15 (100.0%)	29.94 (0.20)	29.68–30.52	0.7
1.73×10^2	14/14 (100.0%)	33.28 (0.39)	32.63–33.93	1.2
86.19	24/24 (100.0%)	34.35 (0.40)	33.32–35.08	1.2
43.10	24/24 (100.0%)	35.47 (0.92)	34.09–37.13	2.6
21.55	22/24 (91.7%)	36.47 (0.75)	34.94–37.25	2.1
11.17	14/24 (58.3%)	36.59 (0.49)	35.47–37.21	1.3
5.59	8/24 (33.3%)	36.73 (0.64)	35.89–37.47	1.7
Negative control	0/6	Not detect		
Total	181/209 (86.6%)	30.34 (0.51^)	16.44–37.47	

C_q: quantification cycle, SD: standard deviation, %CV: percent coefficient of variation, Negative control: uninfected human blood control, ^ Overall SD.

Table 4

Pfs25 (female gametocyte) qRT-PCR assay for assessing inter-assay variability between runs for 7 clinical study cohorts

Standard (gametocytes/mL)	n	Mean C_q (SD)	Range	%CV
5.71×10^6	17	16.73 (0.27)	15.66–17.43	1.6
5.71×10^5	17	19.93 (0.36)	18.71–20.82	1.8
5.71×10^4	17	23.32 (0.31)	22.47–24.63	1.3
5.71×10^3	17	26.60 (0.45)	25.12–27.87	1.7
5.71×10^2	24	30.04 (0.45)	29.07–31.21	1.5
5.71×10^1	11	34.02 (0.38)	32.94–34.97	1.1
5.71×10^0	13*	37.14 (0.87)	35.79–38.42	2.3
Total	116	26.29 (0.50^)	15.66–38.42	

C_q: quantification cycle, SD: standard deviation, %CV: percent coefficient of variation ^ Overall SD, * out of 16 runs.

Table 5

pfMGET (male gametocyte) qRT-PCR assay for assessing inter-assay variability between runs for 15 clinical study cohorts

Standard (gametocytes/mL)	n	Mean C_q (SD)	Range	%CV
1.73×10^7	28	16.42 (0.26)	15.76–17.57	1.6
1.73×10^6	29	19.68 (0.28)	19.11–20.90	1.4
1.73×10^5	29	22.97 (0.28)	22.32–24.13	1.2
1.73×10^4	29	26.32 (0.33)	25.65–28.07	1.3
1.73×10^3	29	29.71 (0.34)	28.85–31.30	1.2
1.73×10^2	29	33.08 (0.64)	32.11–35.42	1.9
1.73×10^1	21*	36.30 (0.90)	34.57–37.67	2.5
Total	194	26.00 (0.46^)	15.76–37.67	

C_q: quantification cycle, SD: standard deviation, %CV: percent coefficient of variation, * out of 29 runs, ^ Overall SD.

No off target primer or probe interactions were identified by search of GenBank (BLAST, NCBI) indicating good analytical specificity of the two assays. Diagnostic specificity for both pfs25 and pfMGET was demonstrated using blood samples from malaria-naïve individuals (pfs25 n = 66, pfMGET n = 11), all being negative, to give a specificity of 100% with lower bounds of 95% confidence of 95.6% and 76.2%, respectively.

Absolute quantification by ddPCR verifies qRT-PCR quantification

To confirm the accuracy of the gametocyte-specific qRT-PCR assays, a collection of gametocyte-positive blood samples from two clinical trials were used to compare the quantitative results of the qRT-PCR to those from droplet digital PCR technology. Only samples with gametocyte densities greater than one gametocyte/mL as measured by qRT-PCR were included in the analysis for pfs25 (n = 21) and pfMGET (n = 36). ICC values were very high, 0.998 (95% CI 0.996–0.999) for pfs25 and 0.995 (95% CI 0.990–0.998) for pfMGET, indicating excellent consistency between methods. Overall qRT-PCR had higher mean gametocyte pfs25 values than droplet digital PCR (mean difference 0.21, 95% CI 0.17–0.26) but values for pfMGET were similar (mean difference 0.1, 95% CI -0.04-0.05). There was no evidence of non-linearity by Passing-Bablok regression nor of a systematic difference for each qRT-PCR assay (pfs25 intercept 0.15 [95% CI -0.08–0.26], pfMGET intercept 0.12 [95% CI 0.00–0.30]) (Fig. 1). There was no evidence of a proportional difference for pfs25 (slope 1.03 [95% CI 0.99–1.09]); however there was evidence of a slight proportional difference for pfMGET between methods at lower concentrations (slope 0.95 [95% CI 0.89–0.98]).

Discussion

A good understanding of the infectious reservoir of malaria and malaria transmission dynamics is required to inform the development and implementation of transmission-blocking interventions. To determine the relative contribution of submicroscopic gametocytemia to transmission (8, 9), molecular assays able to accurately detect very low gametocyte densities have been developed (5, 6, 15). Here, we report the validation of qRT-PCR assays for female and male gametocytes using cRNA standards. Use of these standards overcomes previous limitations of qRT-PCR assays that required purified and well-characterised female and male gametocyte reference materials to generate standard curves. To enable interpretation of such qRT-PCR data, we have determined the copy number of each transcript per gametocyte (279.3 for pfs25 and 12.5 for pfMGET). These figures are very similar to those described by Meerstein-Kessel et al who used the same transgenic NF54 parasite line, pfs25 231.7 (95% CI 199.1–269.8) and pfMGET 9.8 (95% CI 8.9–10.2) (24) and to those estimated from in-vivo samples.

These two assays were validated and shown to be highly sensitive with their LOD for female gametocytes determined to be 3.9 gametocytes/mL, and 26.9 gametocytes/mL for male gametocytes. In addition, the specificity, and reproducibility of these two assays was confirmed. The accuracy of our calculations is supported by the fact that absolute quantification of male and female gametocytes in clinical samples using ddPCR assay were very close to those derived from qRT-PCR assays in terms of agreement, paired, systematic or proportional differences. This supports our estimates of transcript abundance, as well as demonstrating that qRT-PCR is sufficiently accurate where ddPCR is not available. A significant advantage of these assays is

that they are sufficiently sensitive to quantify submicroscopic female and male gametocyte levels and determine gametocyte sex ratios in natural infections or during transmission studies.

Determining the numbers of transcripts per gametocyte is challenging due to the need for pure populations of both female and male gametocytes. These samples are difficult to obtain from natural infections due to the presence of both gametocyte sexes and ring-stage parasites in peripheral circulation during most infections. Pure gametocyte samples can be obtained from in-vitro cultured parasites; however, it is not currently possible to efficiently separate the female and male populations from wild type parasite cultures. Therefore, in this study we utilized a previously described transgenic NF54 parasite line, PfDynGFP/PfP47mCherry, to generate gametocytes in-vitro (5, 22). Gametocytes produced using this line have fluorescent markers in the female and male gametocytes enabling purification of the two populations by FACS. Although this is currently the best method available for generating pure populations of female and male gametocytes, there are a number of limitations. Firstly, the variability in levels of transcription of the two chosen targets may vary with gametocyte age. Although we used a stage-V gametocyte culture, low-level contamination of different stage gametocytes cannot be ruled out, and copy numbers at different stages of gametocyte maturation was not evaluated. In addition, it is plausible that transcript levels may differ between in-vivo and in-vitro generated gametocytes. Likewise, it is unknown if there are differences in levels of these two transcripts in different strains of P. falciparum. However, in support of our calculations, the copy numbers per gametocyte were similar to those estimated from in-vivo samples during a CHMI-transmission study (6).

Conclusions

We validated P. falciparum male and female gametocyte-specific qRT-PCR assays that can quantify gametocyte densities with excellent reproducibility, sensitivity, specificity and accuracy. Moreover, sex-specific mRNA transcript levels per gametocyte were determined, enabling accurate quantification of gametocyte densities in the absence of pure female and male gametocyte standards. The methodology described here can enable the wider use of qRT-PCR assays for detecting and quantifying gametocytemia over a broad range of gametocyte densities, including submicroscopic gametocytemia. This will facilitate studies that either evaluate transmission-blocking interventions, or studies aiming to improve understanding of the infectious reservoir, which will be increasingly more valuable as we move towards elimination.

Abbreviations

CHMI
controlled human malaria infection; CI:confidence interval; Cq:quantification cycle; cRNA:complementary RNA; CV:coefficient of variation; ddPCR:droplet digital PCR; EAV:equine arteritis virus; ICC:intraclass correlation coefficient; FACS:fluorescent activated cell sorting; LOD:limit of detection; mRNA:messenger RNA; pRBCs:packed red blood cells; qRT-PCR:quantitative reverse-transcriptase PCR; SD:standard deviation; TBIs:transmission-blocking interventions; ULoQ:upper limit of quantification; VIS:volunteer infection study.

Declarations

Ethics approval and consent to participate: This study used blood samples collected from human subjects enrolled in two clinical trials (ClinicalTrials.gov reference number NCT02431637 and NCT02431650). Both of these clinical trials were approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee (Brisbane, Australia) and all participants gave written informed consent before enrollment.

Consent for publication: Not applicable.

Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Author contributions: KAC, TB and JSM were involved in study conception and experimental design; CW and KAC performed the experiments; KAC, CW, EB, LM and SL analysed the data; KAC, CW, EB and SL wrote the manuscript. All authors reviewed the manuscript.

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Figures

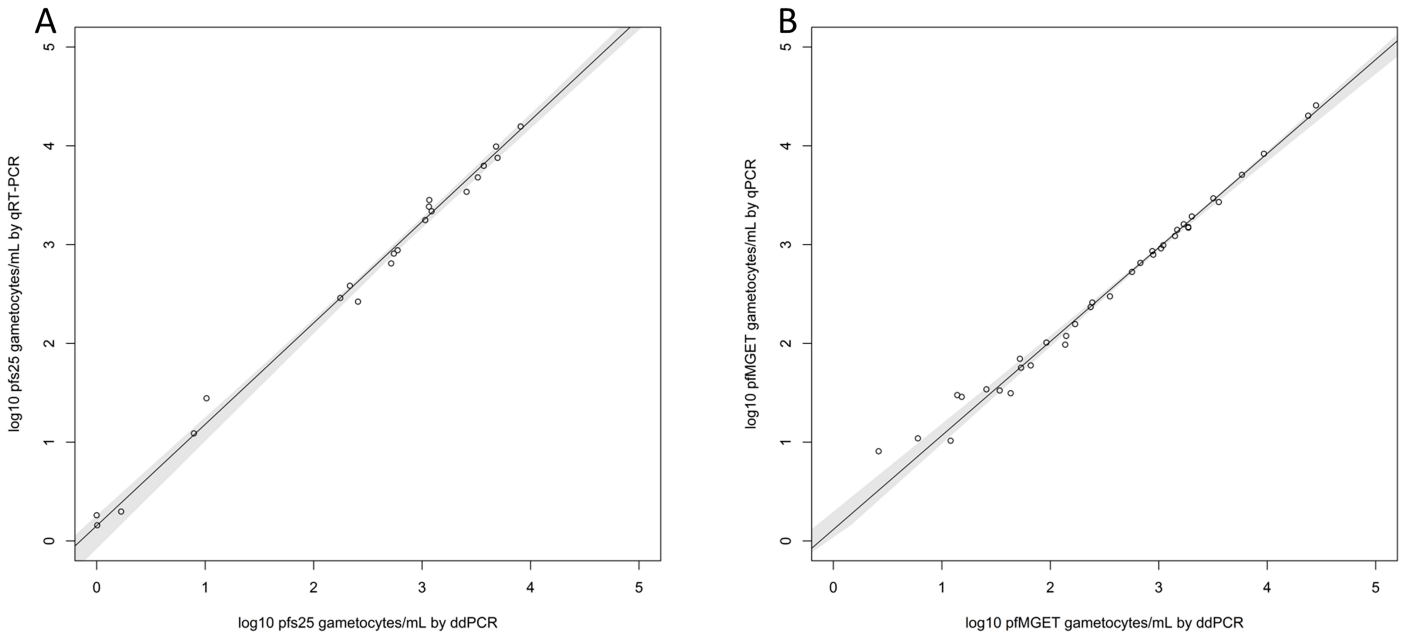

Figure 1

The plots show log10 gametocytes/mL by ddPCR and qRT-PCR. The solid black line represents the fitted Passing–Bablok regression line. The 95% confidence bounds, in grey, were calculated using the bootstrap quantile method. The female pfs25 assay is shown to the left and the male pfMGET assay is shown to the right.