

## *Plasmodium chabaudi*: Reverse transcription PCR for the detection and quantification of transmission stage malaria parasites

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### Abstract

We have developed two reverse transcription polymerase chain reaction (RT-PCR) techniques to detect and quantify the transmission stages (gametocytes) of *Plasmodium chabaudi* malaria parasites. Both the qualitative and quantitative techniques are based on the amplification of mRNA coding for the *P. chabaudi* protein Pcs230, which is expressed exclusively in gametocytes. The quantitative RT-PCR (qRT-PCR) technique was developed and validated by examining serial dilutions of known gametocyte densities. The method generated a high correlation between calibration curves of blind samples ( $R^2 = 0.86$ ). The technique was found to be specific, reproducible, and time efficient for quantification of both patent and sub-patent gametocytemia with a sensitivity level 100–1000 times greater than microscopy. The qualitative RT-PCR (RT-PCR) technique was used to monitor the persistence and dynamics of *P. chabaudi* gametocytes following acute infection. Mice in two independent experiments were sampled for up to 87 days post-infection. RT-PCR showed that gametocytes can persist for up to 8 weeks, post-infection, whereas microscopy could only detect gametocytes up to 6 weeks. Potential applications of the above techniques for studying the ecology, evolution, and epidemiology of malaria transmission are discussed.

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*Index Descriptors and Abbreviations:* *Plasmodium chabaudi*; Gametocyte; Malaria transmission; pcs230; Chronic infection; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; dNTPs, deoxyribonucleotide triphosphate mix; RT-PCR, reverse transcription of the RNA followed by PCR amplification of the cDNA; cDNA (RNA to complementary DNA); qPCR, quantitative real time PCR; qRT-PCR, quantitative real time reverse transcription PCR

### 1. Introduction

Molecular methods are now becoming widely utilized to study malaria parasites both in field and laboratory settings. The power of these techniques is increasingly clear, particularly for studying the ecology and evolution of parasites. In nature, malaria infections often occur at sub-patent levels where parasites cannot be detected by microscopy. This is very common in areas such as eastern Sudan, where the transmission of *Plasmodium falciparum* is highly sea-

sonal and chronic sub-patent infections are prevalent in the dry season (Abdel-Wahab et al., 2002; Babiker and Walliker, 1997; Hamad et al., 2002).

Numerous studies have been conducted to examine the diversity and dynamics of asexual forms of malaria parasites, but the ecology of gametocytes is still poorly understood. Recently, reverse transcription PCR (RT-PCR) techniques have been used to detect RNA of proteins that are expressed exclusively in gametocyte stages, distinguishing gametocytes from asexual stages of *P. falciparum*, even at sub-patent levels (Babiker et al., 1999; Menegon et al., 2000; Niederwieser et al., 2000; Schneider et al., 2004). Some gametocyte-specific protein genes are polymorphic, allowing the technique to be extended to distinguish game-

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tocytes of different genotypes within a single infection (Abdel-Wahab et al., 2002). RT-PCR has shown that *P. falciparum* is capable of producing gametocytes, even when it exists as asymptomatic, sub-patent infection, demonstrating the potential of such infections to sustain the cyclical malaria epidemics in areas of seasonal transmission (Babiker et al., 1999). A shortcoming of the existing RT-PCR protocol is that it cannot quantify gametocytes (Abdel-Wahab et al., 2002; Babiker et al., 1999, 2000; Menegon et al., 2000). Such quantification is important to gain a better understanding of the transmissibility of individual parasite clones that exist in mixed clone infections and the ecological factors that influence them.

Recently, real-time quantitative PCR (qPCR) has been developed and successfully utilized to study the dynamics of different malaria species and genetically distinct clones in mixed infections (Cheesman et al., 2003; De Monbrison et al., 2003; De Roode et al., 2004; Perandin et al., 2004). This method enables accurate quantification of genomic DNA of specific malaria parasite clones within multiple infections, but cannot selectively quantify different life-stages. However, quantitative reverse transcription PCR techniques (qRT-PCR) have also been developed and utilized for the quantification of RNA coding for various genes, including those of malaria parasites (Bruna-Romero et al., 2001; Nirmalan et al., 2002). The technique has been utilized for the quantification of malaria parasite liver stages and a related but different method (QT-NASBA) has been used to examine dynamics of *P. falciparum* gametocytes (Schneider et al., 2004; Witney et al., 2001).

We report here on the utilization of PCR and RT-PCR protocols to detect parasites and monitor the longevity of *Plasmodium chabaudi* infections and their gametocyte production over time. Additionally, we developed RT-PCR and qPCR methods into a protocol for qRT-PCR that can specifically quantify gametocytes of the rodent malaria parasite *P. chabaudi*.

## 2. Materials and methods

### 2.1. *Plasmodium chabaudi* infections

Two independent experiments were carried out to monitor the longevity of gametocyte production of the *P. chabaudi* rodent malaria parasite clone CR that is known to have moderate virulence in mice (Mackinnon and Read, 1999). Infections were established by inoculating 5 (experiment I) and 10 (experiment II) C57Bl/6J 5 to 8-week-old inbred female mice (Harlan Scientific, UK) with  $10^6$  *P. chabaudi* parasites, into the peritoneal cavity, as described elsewhere (Mackinnon and Read, 1999). Mice were maintained at 21 °C ( $\pm 1$  °C) and fed ad libitum SDS 41B (Harlan Scientific, UK) rat and maintenance diet and 0.05% PABA supplemented drinking water to enhance parasite growth. Blood samples (10  $\mu$ l for DNA extraction and 20  $\mu$ l for RNA extraction), thin blood smears, and red blood cell counts (on 2  $\mu$ l of blood) from flow-cytometry (Coulter

Electronics) were taken 5 days a week, for 3 weeks, starting on day 3 post-infection (PI). After day 22 PI, samples were taken 2–3 times a week until day 87 (experiment I) or day 70 (experiment II). Larger blood volumes were required for RNA extraction to increase sensitivity for gametocytes which are in low abundance compared to asexual stages. Thin blood smears were stained with a 20% Giemsa solution and examined under a microscope at 1000 $\times$  magnification to determine parasitemia and gametocytemia. Parasite and gametocyte densities were calculated by multiplying parasitemia and gametocytemia, respectively, by red blood cell density, measured with flow-cytometry.

For qRT-PCR, separate infections of the *P. chabaudi* clone CR were established in three NIH mice using methods described above. The density of gametocytes (gametocytemia) was determined using Giemsa stained thin blood smears, and blood samples were taken for RNA extraction when gametocytes were visible microscopically. These mice were not used for the monitoring of chronic infections via RT-PCR.

### 2.2. Molecular methods for detecting parasites

#### 2.2.1. DNA extraction

For general PCR, DNA was extracted from 10  $\mu$ l of blood taken from the tail of *P. chabaudi* infected mice and added to 200  $\mu$ l of citrate saline (0.85% NaCl, 1.5% trisodium citrate), on ice. The samples were centrifuged at 11,000 rcf for 2–3 min to pellet red blood cells and the supernatant was removed. The blood pellet was stored at  $-80$  °C until DNA extraction. DNA was extracted using InstaGene Matrix (Bio-Rad) according to the manufacturer's protocol.

#### 2.2.2. RNA extraction

Parasite RNA was extracted using two methods:

(i) For RT-PCR, RNA was extracted from 20  $\mu$ l of whole blood, using TRIzol Reagent for total RNA isolation, according to the manufacturer's protocol (Invitrogen Life Technologies). On each day of sampling, 20  $\mu$ l of blood was taken from the tail of each mouse and added to 200  $\mu$ l of citrate saline on ice. The samples were centrifuged at 11,000 rcf for 3 min at room temperature to pellet the blood and the supernatant was removed. A 10 $\times$  volume (200  $\mu$ l) of TRIzol was added to each pellet and the samples were vortexed immediately and stored at  $-80$  °C until RNA extraction. RNA samples were subjected to DNase treatment to remove co-extracted DNA in a 10  $\mu$ l reaction containing 5  $\mu$ l RNA extract, 5 mM Tris, pH 7.6, 33 mM MgCl<sub>2</sub>, 0.27 U RNasin Ribonuclease Inhibitor and 4.2 U DNase I (Roche, UK), with incubation at 37 °C for 15 min and 75 °C for 10 min.

(ii) For qRT-PCR, RNA was extracted using the Applied Biosystems 6100 equipment and protocols. This method allows simultaneous processing of 96 samples and is more time efficient for use with the 96-well qRT-PCR format. To extract gametocyte RNA, 20  $\mu$ l of blood was taken from the

mouse tail and added to 200  $\mu$ l of citrate saline pre-warmed to 37 °C to avoid alterations in expression of RNA believed to occur at temperature changes (Fang and McCutchan, 2002). Samples were centrifuged at 11,000 *ref* for 3 min at room temperature, the supernatant was removed, and 20  $\mu$ l  $\text{Ca}^{2+}/\text{Mg}^{2+}$  free RNase/DNase free PBS (Gibco) and 40  $\mu$ l of 2 $\times$  Nucleic Acid Purification Lysis Solution (Applied Biosystems) were added to the blood pellet. The samples were then stored at –80 °C. For completion of the extraction protocol, the samples were thawed and incubated at room temperature for 1 h to digest blood. RNA was extracted using the Applied Biosystems Isolation of total RNA from Whole Blood Chemistry protocol on the ABI Prism 6100 Nucleic Acid PrepStation with the RNA Blood-DNA method (Applied Biosystems). RNA was eluted in 100  $\mu$ l of Nucleic Acid Purification Elution Solution and stored at –80 °C until required for quantification.

### 2.2.3. PCR and RT-PCR

Primers for PCR and RT-PCR used for longevity experiments were developed to amplify the gametocyte-specific *P. chabaudi* gene *pcs230* (GenBank Accession No. EAA15629.1 and EAA22479.1). The *pcs230* sequence was determined by the *P. chabaudi* sequencing project and can be obtained from <ftp://ftp.sanger.ac.uk/projects/P.chabaudi>. This gene belongs to the *p\_s48/45* super-family that encode gametocyte-specific surface proteins in malaria parasites and is only expressed in mature late stage gametocytes (Bozdech et al., 2003; Hall et al., 2005; Le Roch et al., 2003; Lobo and Kumar, 1998; Niederwieser et al., 2000; Thompson et al., 2001; Williamson et al., 1993). To increase the sensitivity of detection, two rounds of PCR were performed, using outer primers, Pc230(O1), 5'-AAAGATTCAGGGCATGGCG-3' and Pc230(O2), 5'-TTGCCCCA CTTTTGAGCTAC-3' and inner primers, Pc230(N1), 5'-ATCTGTTATGCCTTATGGGAG-3' and Pc230(N2), 5'-CTTGATTAATACTTAGATACACCATATGAG-3'. The optimal annealing temperature for each set of primers was determined on a ThermoHyaid PCR machine using a temperature gradient PCR on samples known to be positive for *P. chabaudi* DNA.

To determine longevity of *P. chabaudi* infection, PCR was performed using Roche 2 $\times$  PCR Master mix in 20  $\mu$ l reactions containing 2  $\mu$ l of genomic DNA, 0.5 U *Taq* DNA polymerase, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTPs, and 0.5  $\mu$ M of primers Pc230(O1) and Pc230(O2). Samples underwent PCR on an MJ Research DNA Engine, PTC-200; using the following conditions: denaturation at 94 °C/180 s, 35 cycles of amplification [94 °C/30 s, 51 °C/30 s, and 70 °C/60 s], and a terminal extension at 70 °C/600 s. Positive DNA controls and negative controls (all reagents + 2  $\mu$ l water) were run for each reaction. The outer PCR products were then diluted 10 $\times$  and 2  $\mu$ l of the diluted product underwent nested PCR using primers Pc230(N1) and Pc230(N2) in a 20  $\mu$ l PCR using PCR Master mix (Roche) and the PCR conditions described above.

For the detection of longevity of gametocyte production in *P. chabaudi* infection, a nested RT-PCR was carried out to detect *pcs230* mRNA. RNA samples underwent a one-step RT-PCR using the AccessQuick RT-PCR system (Promega). This protocol generates cDNA from RNA and then amplifies the cDNA through PCR, in a single reaction. The RT-PCR was carried out in a 20  $\mu$ l volume on the MJ Research DNA Engine PTC-200, with a reaction mixture using the AccessQuick Master Mix reagents consisting of 2  $\mu$ l of RNA, 2 U of AccessQuick reverse transcriptase (Promega), 2 U *tfl* DNA polymerase, 0.2 mM dNTPs, 1.5 mM  $\text{MgCl}_2$ , 10  $\mu$ l AccessQuick Master Mix (supplied by Promega as part of kit), 0.5  $\mu$ M of primers Pc230(O1) and Pc230(O2), and 6  $\mu$ l AccessQuick H<sub>2</sub>O (Promega). The RT-PCR and subsequent amplification of cDNA took place under the following conditions, reverse transcription at 50 °C/60 min, denaturation at 94 °C/3 min, 39 cycles of amplification [94 °C/30 s, 61 °C/30 s, and 70 °C/6 s], and a terminal extension at 70 °C/600 s. Simultaneously, an aliquot of 2  $\mu$ l RNA from each sample was subjected to the same PCR protocol using the primers and the above conditions minus reverse transcriptase, to detect any carry over genomic DNA that might have been co-extracted with the RNA samples. Positive RNA controls, positive DNA controls, and negative controls (all reagents + 2  $\mu$ l water) were also included in each run. All outer RT-PCR products were diluted 1/10 then underwent a second PCR under the same conditions as outlined for the PCR of DNA.

### 2.2.4. Detection limits of PCR and RT-PCR

To determine the sensitivity of nested PCR and RT-PCR, samples of known parasite and gametocyte density determined by microscopy (parasites or gametocytes/ $\mu$ l of blood) of *P. chabaudi* were serially diluted. Eight independent samples of extracted DNA were subjected to 10-fold serial dilutions, creating a dilution series ranging over seven orders of magnitude from undiluted (neat) to diluted 1/10<sup>7</sup> (actual parasite density range tested:  $1.72 \times 10^5$ – $4.3 \times 10^{-3}$  parasites/ $\mu$ l). Since nested PCR of DNA amplifies genomic DNA from all stages of the parasite, counts of both ring stage parasites and gametocytes were used to determine parasite densities from thin blood smears. To establish the sensitivity of RT-PCR, 11 samples of RNA were subjected to 10-fold serial dilutions, creating a dilution series ranging from neat to diluted 1/10<sup>7</sup> for seven samples. None of the samples 1–7 were positive below a dilution of 10<sup>–5</sup>, so four more samples were diluted only to 1/10<sup>6</sup>. The overall dilution range tested for RT-PCR was  $1.3 \times 10^4$ – $1.0 \times 10^{-4}$  parasites/ $\mu$ l.

### 2.2.5. qRT-PCR

A qRT-PCR technique was performed to amplify and quantify RNA coding for the gametocyte-specific protein Pcs230, using the Applied Biosystems TaqMan One-Step RT-PCR Master Mix Reagents Kit, on the ABI Prism 7000 sequence detection system. Two primers (Pc230RT-1, 5'-TCTAGTACATGCTTTGAAGAAGTAATAGAATATAATCCTAATA-3', and Pc230RT-2, 5'-TGCAACGACTT

TCTATAGCTAGTAGGT-3') and a fluorescent labeled probe (Pc230RT-probe, 5'-AAAAATGGGATCGAAAT AAAA-3') were designed by Applied Biosystems Assays-by-Design Service from the *pcs230* gene sequence. As part of this service, Applied Biosystems optimized all primers and probe amplification and annealing conditions and combined them in a master mix.

A one-step qRT-PCR assay was used to generate cDNA from RNA and the cDNA quantified, in a single reaction (Applied Biosystems). This method assumes that reverse transcription takes place at a constant rate between all samples (Nirmalan et al., 2002). The reaction mix consisted of 8.5  $\mu$ l of extracted RNA, 1  $\mu$ l of 20 $\times$  Assay-by-Design Mix containing primers and probes at a reaction concentration of 900 and 250 nM, respectively (supplied by Applied Biosystems), and the following components from the Applied Biosystems TaqMan One-Step RT-PCR Master Mix Reagents Kit: 10  $\mu$ l 2 $\times$  AmpliTaq Gold DNA Polymerase mix (contains AmpliTaq Gold DNA polymerase, dNTP's with dUTP and buffer components), 5  $\mu$ l of 40 $\times$  RT enzyme mix (contains MultiScribe Reverse Transcriptase and RNase Inhibitor). Samples were loaded onto 96-well optical reaction plates and placed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems), then underwent the following reaction conditions: reverse transcription at 48  $^{\circ}$ C for 30 min, denaturation at 95  $^{\circ}$ C for 10 min, and 50 cycles of amplification [95  $^{\circ}$ C for 15 s and 60  $^{\circ}$ C for 60 s]. Data were visualized using the ABI Prism 7000 SDS Software (Applied Biosystems). Simultaneously, eight RNA samples underwent conventional nested PCR using primers Pc230(O1), Pc230(O2), Pc230(N1), and Pc230(N2) to test for DNA contamination.

#### 2.2.6. Validation of qRT-PCR

The qRT-PCR technique was validated by comparing threshold cycle values (number of cycles after which a sample crosses the threshold of positivity) generated from serial dilutions of samples of known gametocytemia. We expected the threshold cycle to be negatively correlated with the number of gametocytes present in a sample, showing the same value when the numbers of gametocytes in two different samples are equal. Therefore, a strong correlation in best fit lines for threshold cycle versus gametocyte number generated from serial dilutions with known numbers of gametocytes will demonstrate that gametocytes are accurately quantified. To further test the robustness of the method, we compared the accuracy of threshold cycle values calculated for two different types of serial dilutions, performing replicates of each set. The first set of dilution series was prepared blindly with extracted RNA from samples of known gametocyte counts (determined after qRT-PCR, via microscopy). Eleven, 10-fold serial dilutions (neat to 1/10<sup>7</sup>) of extracted RNA (from three different mice) diluted in water were prepared. A second type of dilution curves were set up blindly to dilute actual parasites by using uninfected blood from NIH mice to dilute samples of *P. chabaudi* infected whole blood with known gametocyte counts (num-

bers calculated after qRT-PCR, via microscopy). Two, independent, 10-fold serial dilutions ranging from neat to 1/10<sup>7</sup> were generated in this manner and the dilution series samples were extracted on the ABI Prism 6100 Nucleic Acid PrepStation with the RNA Blood-DNA method (Applied Biosystems). The dilution of samples with the uninfected whole blood method more accurately simulates quantification of gametocytes from mice harbouring different levels of gametocyte density, and therefore accurately demonstrates the sensitivity of extraction as well as qRT-PCR.

All 13 sets of RNA dilutions were quantified for *pcs230* mRNA using the TaqMan One-Step RT-PCR Master Mix Reagents Kit on the ABI Prism 7000 sequence detection system (Applied Biosystems). The data from all curves were pooled and analysed using Minitab statistical software (version 13.1). Threshold cycle values were regressed against the log<sub>10</sub> of the number of gametocytes within samples using ANCOVA. All sets of serial dilutions were compared using a regression to estimate the validity of the qRT-PCR method.

### 3. Results

#### 3.1. Detection of *P. chabaudi* infection and gametocytes

Conventional PCR of genomic DNA showed a mean minimum detection limit of  $3.68 \pm 2.11$  ( $\pm 1$  SE) parasites/ $\mu$ l blood. With regard to gametocytes, RT-PCR was found to detect a mean minimum of  $27.1 \pm 11.7$  ( $\pm 1$  SE) gametocytes/ $\mu$ l blood. However, in 5 out of 11 sets of serial dilutions of gametocyte-infected blood, qualitative RT-PCR was detected down to 1 gametocyte/ $\mu$ l of blood. These detection limits are similar to those found for the detection of *P. falciparum* asexual forms and gametocytes (Babiker et al., 1999; Roper et al., 1996). In general, the sets of dilution series that started with initial higher gametocytemia resulted in RT-PCR product at lower dilutions (Fig. 1). In one case, a neat sample did not give an RT-PCR product, likely due to pipetting error.

Overall, significantly more blood samples were found to harbour *P. chabaudi* infection revealed by PCR ( $\chi^2 = 16.9$ ,  $P < 0.0001$ ,  $df = 1$ ), and gametocytes detectable by RT-PCR ( $\chi^2 = 24.4$ ,  $P < 0.0001$ ,  $df = 1$ ), than by microscopy. Across the 424 blood samples examined, 161 (38%) were gametocyte-positive by nested RT-PCR, whereas only 97 (23%) were gametocyte-positive by microscopy. There were 14 instances (3.30%) where gametocytes were detected by microscopy but not with nested RT-PCR, likely to be due to failed RNA extraction. PCR product from DNA was seen in 110 (27%) out of 405 blood samples when RT-PCR for gametocyte RNA was negative. Because both gametocytes and asexual parasites contain DNA but only gametocytes contain Pc230 RNA, we believe these samples represent cases where asexual parasites were present but gametocytes were absent. Additionally, it has been shown that PCR of parasite DNA is not possible after the parasites have been cleared by the host (Jarra and Snounou, 1998).



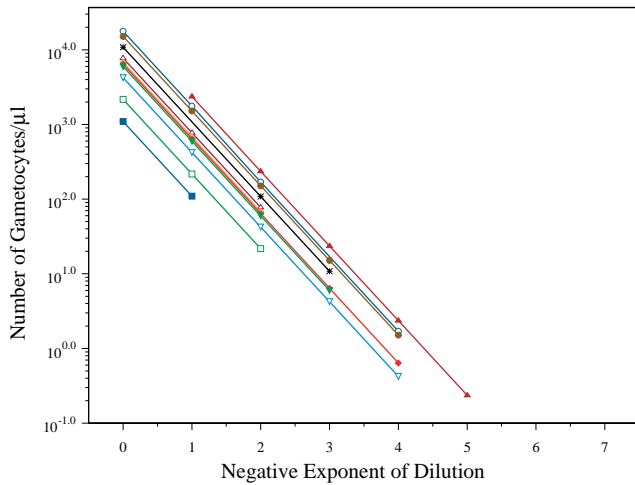


Fig. 1. Detection level of gametocytes with RT-PCR of RNA coding for the *Pcs230* gene. Each line represents a separate dilution series of independent samples. Y-axis shows number of gametocytes in the sample estimated by microscopy. X-axis shows the position of the sample in the dilution series with 0 being the neat (undiluted) sample and 7 being the seventh dilution in the series (i.e., the neat sample diluted  $10^{-7}$ ). A dilution series up to  $10^{-7}$  was performed on seven samples and up to  $10^{-6}$  on four samples. Where symbols are present, positive RT-PCR was obtained. Where symbols are absent, RT-PCR was negative. In one case, RT-PCR on the undiluted sample was negative likely due to pipetting error.

### 3.2. Persistence of *P. chabaudi* asexuals and gametocytes in infected mice from longevity experiments

Figs. 2A and B show *P. chabaudi* parasite density in chronic infections revealed by microscopy, nested PCR, and RT-PCR for one of the five mice in experiment I and one of the 10 mice in experiment II, respectively. All of the remaining mice in both experiments show the same pattern as those in Figs. 2A and B (see Supplementary data). In both experiments, gametocytes were often detected using RT-PCR several days after they were no longer detectable by microscopy. Mice controlled initial parasitemia after about 3 weeks. Subsequently, the infections showed one or a few transient peaks of patent parasitemia that recurred between days 30 and 60, during which time gametocytes were frequently only detectable by RT-PCR. In most mice, parasites were not detected after day 50 PI and none were detectable either by microscopy or molecular methods after day 60. The median time ( $\pm 1$  SE) to clearance of gametocytes (i.e., time for 50% of mice to clear gametocytes) was  $36 \pm 3$  days derived by RT-PCR compared to  $18 \pm 2$  days by microscopy (Kaplan-Meier test).

### 3.3. qRT-PCR

The qRT-PCR technique for gametocyte-specific *pcs230* RNA in 13 separate serial dilutions of gametocytes detected approximately one gametocyte per reaction, corresponding to  $\leq 1$  gametocyte/ $\mu$ l blood (Fig. 3). As expected, the number of gametocytes was highly related to the threshold cycle

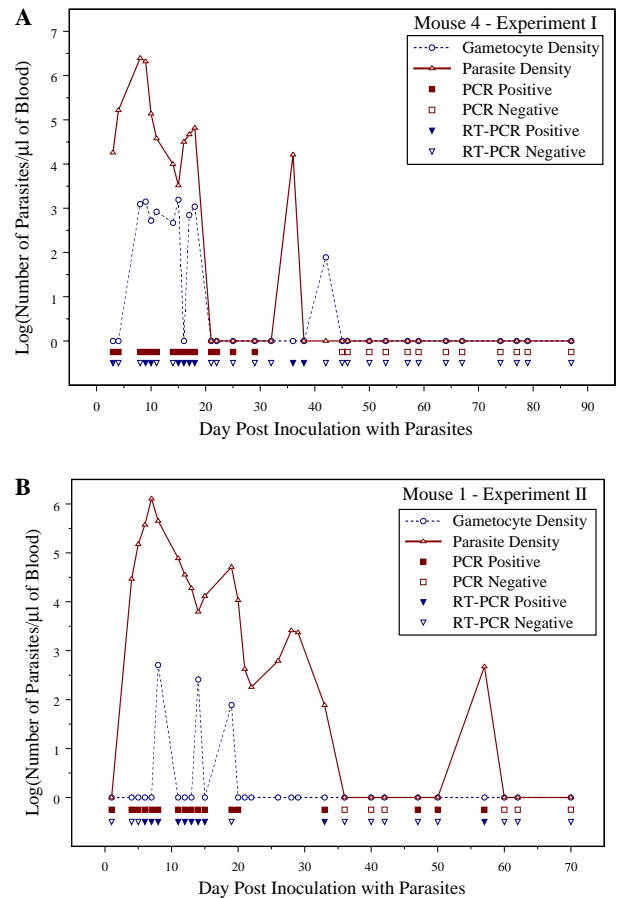


Fig. 2. *Plasmodium chabaudi* asexual forms and gametocytes detected by microscopy, PCR, and RT-PCR in a mouse from experiment I (A) and one from II (B). Red solid lines and blue dotted lines represent asexual stages and gametocytes density, respectively, as determined by microscopy. Squares show qualitative PCR for respective days, filled squares are positive samples, empty squares are negative samples, absence of a square means no sample taken. Triangles represent qualitative RT-PCR samples for *Pcs230* gene on respective days, illustrated in the same manner. PCR and qualitative RT-PCR were more sensitive for detecting asexuals and gametocytes than microscopy as indicated by filled squares and triangles, respectively. No RNA samples showed an RT-PCR product when reverse transcriptase was omitted, thus excluding DNA contamination during RNA extraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

( $R^2 = 0.86$ ,  $F_{1,32} = 174$ ,  $P < 0.0001$ ), with no significant differences in the slope of the relationship between the 13 dilution series. Specifically, there was no difference in slope between the two dilution series made from samples diluted in whole blood and 11 dilution series made extracted RNA diluted in water ( $P > 0.05$  in all cases). The common slope ( $\pm 1$  SE) of the combined 13 dilution series was  $CT = -3.20(\pm 0.24)$ . Our results are very close to the optimum obtainable slope of  $-3.3$  for PCR amplification during qRT-PCR.

## 4. Discussion

We have previously developed an RT-PCR technique to detect RNA encoding proteins that are expressed

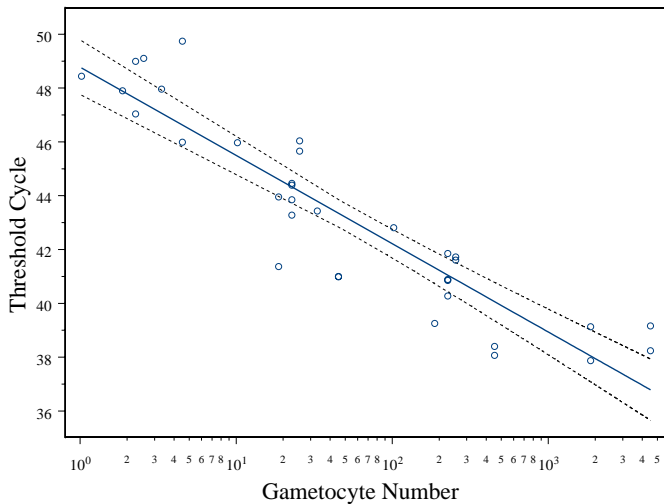


Fig. 3. Threshold cycle (determined by qRT-PCR) and gametocyte number (determined by microscopy) for 13 dilution series (11 from diluted extracted RNA, 2 from dilutions of actual parasites with whole blood). The lines are the least squares regression line (solid) with 95% confidence intervals (dotted). There is a strong correlation between threshold cycle and gametocyte number ( $R^2 = 0.86$ ,  $F_{1,32} = 174$ ,  $P < 0.0001$ ; slope  $\pm 1$  SE =  $-3.20 \pm 0.24$ ). See text for further details.

exclusively in *P. falciparum* gametocytes (Babiker et al., 1999; Menegon et al., 2000). Here, we have extended this protocol and established robust and sensitive RT-PCR and qRT-PCR methods to monitor the persistence of *P. chabaudi* gametocytes throughout infection. The techniques are based on the amplification of *pcs230* mRNA, which is expressed exclusively in gametocytes (Hall et al., 2005; Niederwieser et al., 2000; Thompson et al., 2001).

We found that the qRT-PCR detection limit ranged from 1 to 10 gametocytes/ $\mu$ l of blood making it 100–1000 times more sensitive than microscopy, with similar sensitivity levels to those found using QT-NASBA for *P. falciparum* gametocytes (Schneider et al., 2004). This very low limit of detection was reproducible in 11 independent sets of serial dilutions of gametocytes. Such very low gametocytemia would require an experienced microscopist to examine more than 1000 fields of a well-stained thick blood smear, which could take at least 50 min (Babiker et al., 1999). Additionally, quantification by microscopy is often inaccurate when gametocytes persist at the low levels ( $<0.01$  gametocytes/red blood cell) common in natural malaria infections (L. Crooks, *Transmission Investment in Malaria*, Ph.D. Thesis, University of Edinburgh, 2004; Taylor and Read, 1997). Thus, comparatively, the qRT-PCR technique for gametocytes presented here provides a specific and highly sensitive estimate to gametocyte density, especially at low levels of gametocytemia. It is theoretically possible that different stages of gametocytes may express varying levels of *Pcs230* RNA. However, even when using blood samples from mice on different days post-inoculation, our results showed a strong correlation between expected and observed gametocyte values, showing that expression level is not a source of a large degree of error.

Overall, our protocol is time efficient and capable of processing 96 samples in 8 h. It, therefore, has great epidemiological potential, and can easily be adapted to study human malaria parasites. This method differs from current quantification protocols in that it is, to our knowledge, the first to quantify *P. chabaudi* gametocytes. Since this technology only requires the design of probes and primers and does not require the use of gene constructs as internal standards, assays can rapidly and cheaply be developed. In addition, by utilizing polymorphic gametocyte-specific protein genes, the presented method can easily be adapted for tracking an individual gametocyte genotype when the infection is composed of multiple genotypes. Through the application of newly described sex specific gametocyte protein genes (e.g., Khan et al., 2005), the method can also be developed for the individual quantification of male or female gametocytes.

We used the developed RT-PCR technique to monitor longevity of gametocyte production of two *P. chabaudi* clones. Our results demonstrated that *P. chabaudi* persisted at sub-patent levels, and continued to produce gametocytes for several weeks as chronic infections. This result agrees with that seen in natural *P. chabaudi* infections in the natural host *Thomomys rutilus*, where chronic infections lasting up to 2 years are often observed (Landau et al., 1999). In the present study, *P. chabaudi* infection persisted for up to 60 days post-infection, however, the duration of our study was limited to 87 days, and therefore it is possible that additional parasite recrudescence phases may have been missed. In addition, all infections were composed of a single *P. chabaudi* clone, whereas, in a recent study of *P. chabaudi*, infections consisting of three clones were found to persist longer than did single-clone infections (De Roode et al., 2003).

The techniques presented here allow a range of hypotheses to be tested. For instance, chronic persistence of malaria infections in the face of sustained immune attack is probably due to antigenic variation by parasites (Phillips et al., 1997). Antigenic variation is often viewed as an adaptation to enhance transmission (e.g., Antia et al., 1996). Techniques such as that presented here make it possible to determine when transmission stages are present in chronic infections, and hence the extent to which antigenic variation can actually contribute to parasite fitness. Of greater practical significance to malaria epidemiology are the findings that chemotherapy led to increased rates of gametocytogenesis in acute infections of *P. chabaudi* and increased gametocyte density in infections of *P. falciparum* (Ali et al., 2005; Buckling et al., 1997, 1999a,b); if the same occurs in chronic asymptomatic infections, inappropriate drug use could enhance malarial transmission. Moreover, multi-clone infections are the rule rather than the exception in malaria (Awadalla et al., 2001; Babiker and Walliker, 1997). The dynamics of individual clones in mixed infections is complex (Bruce et al., 2000; De Roode et al., 2005; Read and Taylor, 2001) and the transmission consequences of these dynamics are very poorly understood. We are currently developing allele-specific gametocyte qRT-PCR,

which will make it possible to track gametocyte densities of individual clones in multiple-clone infections. Such studies will shed more light on the dynamics of malaria parasites and assess biological and environmental factors that influence their gametocytogenesis in nature (Bousema et al., 2004; Price et al., 1999). Gametocyte-specific quantification thus makes it possible to conduct detailed studies of malaria transmission biology and should help to improve predictions of malaria parasite responses to control measures.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.expara.2005.08.013](https://doi.org/10.1016/j.expara.2005.08.013).

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