Maintaining Plasmodium Falciparum Gametocyte Infectivity During Blood Collection and Transport for Mosquito Feeding Assays in the Field

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Research

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Abstract

Background

Mosquito feeding assays using venous blood are commonly used for evaluating the transmission potential of malaria infected individuals. To improve the accuracy of these assays, care must be taken to prevent premature activation or inactivation of gametocytes before they are fed to mosquitoes. This can be challenging in the field where infected individuals and insectary facilities are sometimes very far apart. In this study, a simple, reliable, field applicable method is presented for storage and transport of gametocyte infected blood using a thermos flask.

Methods

The optimal storage conditions for maintaining the transmissibility of gametocytes were determined initially using cultured *Plasmodium falciparum* gametocytes in standard membrane feeding assays (SMFAs). The impact of both the internal thermos water temperature (35.5 – 37.8°C), and the external environmental temperature (room temp – 42°C) during long-term (4hr) storage, and the impact of short-term temperature changes (room temp – 40°C) during membrane feeding assays was assessed. The optimal conditions were then evaluated in direct membrane feeding assays (DMFAs) in Burkina Faso and The Gambia where blood from naturally infected gametocyte carriers was offered to mosquitoes immediately and after storage in thermos flasks.

Results

Using cultured gametocytes in SMFAs it was determined that an internal thermos water temperature of 35.5°C and storage of the thermos flask between RT (~21.3°C) and 32°C was optimal for maintaining transmissibility of gametocytes for 4 hours. Short-term storage of the gametocyte infected blood at temperatures up to 38°C (range: RT, 30°C and 38°C) did not have a negative effect on gametocyte infectivity. Using samples from natural gametocyte carriers (47 from Burkina Faso and 16 from The Gambia), the prevalence of infected mosquitoes and the intensity of oocyst infection was maintained when gametocyte infected blood was stored in a thermos flask in water at 35.5°C for up to 4 hours.

Conclusions

This study determines the optimal long-term (4 hours) storage temperature for gametocyte infected blood and the external environment temperature range within which gametocyte infectivity is unaffected. This will improve the accuracy, reproducibility, and utility of DMFAs in the field, and permit reliable comparative assessments of malaria transmission epidemiology in different settings.

Background

In the past decade remarkable reductions in malaria burden have been achieved largely through widespread access to and use of artemisinin-based combination therapies and insecticide treated

bednets (1). However, since 2015 progress has stalled (2) and elimination is unlikely to be achieved with these conventional methods alone in most settings in Africa (3). One of the biggest challenges to malaria control and elimination is effectively interrupting the efficient process of malaria transmission. New tools or implementation strategies will likely be required that specifically aim to reduce malaria transmission (4). In order to effectively target transmission reduction and develop and implement transmission-blocking interventions a thorough understanding of the dynamics and epidemiology of malaria transmission in different settings is needed.

The transmission of malaria depends on the presence of mature sexual stage parasites (gametocytes) in the peripheral blood. During a blood meal, *Anopheles* mosquitoes must imbibe at least one male and one female gametocyte to become infected, but other host or parasite factors may also contribute to this, such a transmission reducing immunity. To understand how likely it is that individuals contribute to onward transmission, assays are needed for accurate assessment of gametocyte infectivity to mosquitoes. This is commonly measured by direct membrane feeding assay (DMFA) where venous blood is fed to mosquitoes, or direct skin feeding assays, where mosquitoes feed directly on the skin of the volunteer (5–11). In DMFA venous blood collected from malaria infected individuals is offered to female *Anopheles* mosquitoes using water-jacketed glass feeders. These feeders are connected to a circulating water bath to maintain gametocyte temperature during feeding. Maintaining gametocyte temperature is important since a drop in temperature may result in gametocyte activation (12) while high temperatures (~ 40 °C or higher) may inactivate gametocytes and gametes (13–16).

Blood drawn for DMFAs must therefore be fed to mosquitoes as quickly as possible after collection to eliminate any detrimental effect of temperature change (17). This can be logistically challenging in the field where participants in DMFA experiments may be recruited far from the insectary facilities, resulting in either lengthy travel for volunteers, or restricting the available population for infectivity assessment to those near the facilities. To overcome these limitations, a method to store and transport gametocyte infected blood from the field to the laboratory and prevent premature activation or inactivation of gametocytes before mosquito feeding is needed. In this study, the temperature range for storage of gametocyte infected blood to maintain gametocyte infectivity is determined and a simple, cheap, field applicable method for collecting and transporting blood is presented.

Methods

Mosquito rearing

Anopheles stephensi mosquitoes Sind-Kasur Nijmegen strain (18) (Radboudumc) were reared at 30°C, and *Anopheles coluzzii* mosquitoes (Burkina Faso and The Gambia) were reared at 27°C. All were kept at ~ 70–80% relative humidity (RH) on a 12 hour day/night cycle and were fed on 5–10% glucose. Female mosquitoes between 2–6 days post emergence were used for all mosquito feeding assays. After feeding on gametocyte infected blood, mosquitoes were stored either at 26°C (Radboudumc) or 27°C (Burkina Faso and The Gambia)

Standard membrane feeding assays (SMFAs)

SMFAs were performed at Radboudumc, Nijmegen, The Netherlands, using cultured *Plasmodium falciparum* gametocytes as previously described (19). In brief, *P. falciparum* parasites were cultured in an automated incubator under a continuous gas flow of 4% CO_2, 3% O_2 and 93% N_2. Gametocytes were produced from asynchronous cultures (day 0, 1% parasitemia and 5% red blood cells) which were harvested at day 15, and gametocyte infected blood-meals were prepared as previously described (19). For the long-term storage experiments, gametocyte infected blood-meals were either fed immediately to mosquitoes or were stored for 4 hours in a thermos flask filled with water at 35.5 °C, 37 °C or 37.8 °C, measured using a calibrated probe thermometer accurate to +/- 0.2 °C (Traceable® Ultra digital thermometer, VWR 620–2079) before feeding to mosquitoes. Thermos flasks (Stainless king food flask 470 ml, Thermos) were stored either at room temperature (RT; mean 21.3 °C, range: 20.4 °C-22.1 °C) or in an incubator set at 32 °C or 42 °C. For the short-term storage experiments, gametocyte infected blood-meals were stored for 15 minutes and RT or in a heat block at a range of temperatures (30 °C, 35.5 °C, 38 °C, 40 °C or 42 °C) before feeding to mosquitoes. For all SMFAs, female mosquitoes were offered the infected blood-meal in glass mini-feeders (300uL) attached to a circulating water bath set at 39 °C and allowed to feed in the dark for 15 minutes (19). A total of 30–60 mosquitoes in 2 or 3 cups were used for each experimental condition. Mosquitoes were maintained on 5% glucose and dissected 6–8 days after feeding and the number of developed oocysts per mosquito was determined by microscopy after 1% mercurochrome midgut staining.

Gametocyte positive blood samples

Gametocyte positive human blood samples were obtained from individuals as part of ongoing studies in Burkina Faso and The Gambia. The Burkina Faso study was approved by the London School of Hygiene and Tropical Medicine ethics committee (Review number: 14724), The Centre National de Recherche et de Formation sur le Paludisme institutional review board (Deliberation N° 2018/000002/MS/SG/CNRFP/CIB) and the Ethics Committee for Health Research in Burkina Faso (Deliberation N° 2018-01-010). The Gambia study was approved by the London School of Hygiene and Tropical Medicine ethics committee (Review number:15993) and by The Gambia Government/MRC Joint Ethics Committee (SCC1621). All participants gave informed consent before inclusion in the studies.

In Burkina Faso, participants were microscopy-positive gametocyte carriers aged 10–15 years old recruited from screening campaigns in the Saponé health and demographic surveillance system area, ~ 45 kilometres Southwest of Ouagadougou. In the Gambia, participants were microscopy-positive gametocyte carriers aged > 2 years, passively recruited from four health facilities (Basse Hospital, Sabi, Sotuma Sere, Gambissara) in the South Bank of the Upper River Region in The Gambia.

Direct membrane feeding assays (DMFAs)

Thermos flask water temperature setup

Thermos flasks (Stainless king food flask 470 ml, Thermos) were filled (to 2 cm below the top of the flask) with water between 35.5-36**°**C, measured with a calibrated probe thermometer (accurate to +/- 0.2 °C, Traceable® Ultra digital thermometer, VWR 620–2079) and immediately sealed. Gametocyte infected blood samples were collected in Lithium heparin vacutainers and either fed to mosquitoes immediately (control) or transferred to the thermos flask immediately after phlebotomy. Themos flasks were used within 1 hour of it being filled with water. Thermos flasks were then stored at ambient temperature for a maximum of 4 hours from the time of filling and the stored blood sample was then used for mosquito feeding.

Feeding procedures

For all DMFAs, female mosquitoes were offered the infected blood-meal in glass mini-feeders (300uL) attached to a circulating water bath at 37 °C-39 °C and allowed to feed in the dark for 15–20 minutes. A total of 3 cups of 30 mosquitoes were used for all experimental conditions in The Gambia, and 2 cups of 60 mosquitoes were used for all experimental conditions in Burkina Faso. Mosquitoes were maintained on 5–10% glucose until 7–8 days after feeding when they were dissected and the number of developed oocysts per mosquito was determined by microscopy after 0.5% mercurochrome midgut staining (20).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (ver. 8). Mosquito infectivity in SMFAs was analysed by comparing groups with the Kruskal-Wallis test comparing each experimental condition to the control with Dunns multiple comparison test. Mosquito infectivity in DMFAs was analysed by comparing oocyst intensity (average oocysts per mosquito) or prevalence of infection (% of infected mosquitoes) by Wilcoxon matched-pairs signed rank test. Agreement between the immediate DMFAs or DMFAs with stored gametocytes was analysed using Spearmans correlation of % mosquitoes infected before and after gametocyte storage and Bland-Altman analysis to visualize and test whether systematic bias in infection rates occurred between the two methods.

Results

Optimal storage conditions for cultured gametocytes for SMFAs

To determine the optimal storage conditions for cultured gametocytes that maintains their infectivity, SMFAs were performed where two conditions were evaluated; (i) the starting water temperature in the thermos flask and (ii) the temperature the sealed thermos flask was stored at. In a series of experiments, mosquito blood-meals were prepared with mature stage 5 gametocyte cultures and either fed immediately to mosquitoes (control) or stored in a thermos flask for 4 hours. The thermos flasks were filled with water at either 37.8 °C, 37 °C or 35.5 °C and after addition of the mosquito blood-meal the thermos flasks were sealed and stored at either room temperature (RT), 32 °C or 42 °C. The impact of storing the cultured gametocytes in the thermos flask was assessed by comparing the level of

transmission, as measured by the intensity of mosquito oocyst infection in the immediate control SMFA to the SMFA performed with the stored gametocytes.

There was no reduction in transmission when the gametocytes infected blood-meal was stored for up to 4 hours in a thermos containing water at 35.5 °C and the thermos flask was stored between RT (~ 21.3 °C) and 32 °C. However, when the thermos was stored at higher temperatures (42 °C) there was a statistically significant reduction in intensity of oocyst infection in 50% of the experiments (Fig. 1 and Table 1). When the water in the thermos was higher, at 37 °C or 37.8 °C, a statistically significant reduction in transmission was occasionally observed after gametocyte infected blood-meal storage, even when the thermos was kept between RT (~ 21.3 °C) and 32 °C. This was more frequent when the water was 37.8 °C (Table 1 and Figure S1). This indicates that in order to maintain infectivity of gametocytes they should be stored in a thermos filled with water that is 35.5 °C and the thermos should be stored between 21.3 °C and 32 °C.

Optimal membrane feeder temperature for cultured gametocytes in SMFAs

Since the optimal temperature for 4 hours of gametocyte storage was determined to be 35.5 °C, lower than expected. The standard temperature used in the membrane feeders for SMFAs (37 °C) was examined to determine whether this might also have a negative effect on gametocyte transmission to mosquitoes. To evaluate this in a series of experiments, cultured gametocyte blood-meals were stored for 15 minutes (the standard duration of mosquito feeding in a SMFA) at either 35.5 °C (safe temperature for 4 hour storage) or a range of higher and lower temperatures (RT, 30 °C, 38 °C, 40 °C and 42 °C).

Short-term storage of the gametocyte blood-meal at ≥ 40 °C resulted in significant reduction in transmission, but short-term storage at temperatures lower than this (38 °C, 30 °C and RT) did not have a negative effect (Fig. 2 and Table 2). This indicates that the temperature of the membrane feeders should not exceed 38 °C for a 15 minute mosquito feed, but lower temperatures (as low as 21.3 °C) are not detrimental for this short duration.

Validation of optimal gametocyte thermos storage conditions using natural gametocyte carriers

To assess if the optimal thermos storage conditions determined using cultured gametocytes were also suitable for storage of natural gametocytes, paired experiments (n = 47 in the Burkina Faso and n = 16 in the Gambia) were performed where blood was collected from gametocyte infected individuals and either fed immediately to mosquitoes in a DMFA or stored in the thermos flask for up to 4 hours before DMFA. The starting temperature of the water in the thermos flask was 35.5 °C – 36 °C, and the thermos flasks were stored at ambient lab temperature. The prevalence of infected mosquitoes and the intensity of mosquito infection was similar for the DMFA performed immediately compared to the DMFA performed after 4 hours storage in a thermos flask (Fig. 3A + B). When comparing results from the two assays, there

was a strong correlation (Spearman's r = 0.9431, p < 0.0001) and good agreement using the Bland-Altman analysis (mean difference in mosquito infection rate = -0.07%).

Discussion

In this study, a method to maintain *P. falciparum* gametocyte infectivity during blood collection in the field and transportation to the insectary for mosquito feeding assays is presented. The optimal storage conditions preventing gametocyte activation or inactivation were first assessed with cultured gametocytes and then confirmed using natural gametocyte carriers from two malaria endemic settings. The findings corroborate that temperature can play a key role in maintaining the infectivity of *P. falciparum* gametocytes. Infectivity was not affected when gametocytes were stored in thermos flasks in water at 35.5ºC for up to 4 hours and the thermos flask was stored at ambient temperatures ranging from 21.3ºC to 32ºC.

DMFAs are more commonly used than direct skin feeding assays to assess the infectiousness of gametocyte carriers to mosquitoes, largely because they minimise the discomfort experienced by volunteers, which is particularly important when sampling young children. DMFAs may therefore be more readily acceptable by both local communities and ethics committees (21), especially when repeated assessments of infectivity are made. The relationship between gametocyte density and infection success in mosquitoes has been well studied, but differs slightly by settings (11, 21, 22). These differences could plausibly be due to the different populations, different levels of malaria exposure and resulting levels of transmission-blocking immunity, different mosquito species, or parasite genotypes (8, 9, 11). However, for reliable assessments of infectivity by DMFA it is crucial to minimize technical differences in how the assays are performed between settings (21, 22). Here, evidence is presented that variation in blood storage temperature and duration of storage, as well as feeder temperature, could have a significant impact on transmission.

The results show that the optimal temperature for longer-term storage (4 hours) is 35.5 ºC. The maintenance of gametocyte infectivity at this temperature is consistent with previous studies which showed a drop in temperature of at least 5ºC from the standard 37 °C in the human body, is required to activate *P. falciparum* gametocytes (12). Also consistent with previous data, our results show that as little as 15 minutes (mimicking the time the gametocytes are present in the feeder) at 40ºC or 42ºC is sufficient to inactivate gametocytes, with transmission being almost completely prevented after storage at 42ºC (13, 14, 16, 23). Taken together, these data suggest that the temperature in the feeders should not reach 40 °C during DMFAs.

With the 4 hour storage experiments, not only temperatures above 40C but also lower temperatures appeared to reduce gametocyte infectivity. It was surprising to find that storing the gametocytes in the thermos flask in water at 37ºC for 4 hours was associated with reduced transmission efficiency in some experiments. This was seen most often when the ambient temperature was high (i.e. 32ºC or 42ºC), and not when it was the typical room temperature in an air-conditioned European laboratory (~ 21.3 °C). The

temperature of 37ºC would be hypothesized to be ideal for gametocytes, as it mimics their natural environment in the body, and previous studies have indeed shown that *P. falciparum* gametocyte activation was prevented when they were maintained at 37ºC for 1 hour (24). In agreement with this, short-term storage for 15 minutes at temperatures up to 38 ºC in our study also did not reduce transmission. This suggests that either the duration of exposure to high temperatures, or the fact that the gametocytes are stored in venous blood collected in lithium heparin anticoagulant, or both, may also be important factors for gametocyte inactivation, although this is not evaluated in this study.

Altogether, the data demonstrates that temperature fluctuations influence gametocyte infectivity, seen most acutely with higher temperatures, including high ambient temperatures, and temperature should thus be controlled when collecting and transporting gametocyte infected blood in regions where temperatures could reach over 40 ºC. An effect of temperature on gametocyte infectivity has been seen before, however, the current study adds value by a large number of replicates in the SMFA with varying thermos and ambient temperatures. This allowed an informed decision on the optimal temperature conditions for use in DMFA experiments using natural gametocytes carriers in the field. With the proposed approach, samples can be transported from more remote settings to the insectary within 4 hours without affecting gametocyte infectivity and thus maintaining assay quality.

This method will facilitate widespread, accurate assessment of malaria transmission dynamics in the field and this understanding will contribute to malaria control strategies as we move towards elimination.

Abbreviations

DMFA – direct membrane feeding assay, SMFA – standard membrane feeding assays, RT – room temperature, RH - relative humidity

Declarations

Ethics approval and consent to participate: Data within this manuscript was generated using blood samples from clinical trial participants. These participants were enrolled in clinical trials that were reviewed and approved by the LSHTM ethics committee (Review number: 14724), The CNRFP institutional review board (Deliberation N° 2018/000002/MS/SG/CNRFP/CIB) and the Ethics Committee for Health Research in Burkina Faso (Deliberation N° 2018-01-010). The Gambia protocol was approved by the LSHTM ethics committee (Review number:15993) and SCC/Ethics Committee The Gambia Government/MRC (Review number: 1621) Ethics Committee. All participants gave informed consent before inclusion in the studies. The clinical trials are registered with ClinicalTrials.gov; Burkina Faso study (NCT03705624) and The Gambia study (NCT04053907).

Consent for publication: Not applicable

Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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

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Author contributions: KAC, TB and MM were involved in study conception and experimental design; WMG, ZS, KAC, HMS, MvB, and GG performed the experiments; AO, MS and AT coordinated participant recruitment in Burkina Faso, HMS, AA, LJ, MMC, PMG, MM and UDA coordinated participant recruitment in The Gambia. HMS and KAC analysed the data and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Tables

Table 1. Effect of gametocyte blood-meal storage on infectivity to mosquitoes

Mosquito infection rate (intensity of oocyst infection) was compared when a gametocyte infected blood-meal was fed to mosquitoes immediately or stored in a thermos flask for 4 hours at a range of temperatures. A total of 30-60 mosquitoes in 2 or 3 cups were used for each experimental condition and each experiment was repeated 3 to 4 times. Groups were compared by Kruskal-Wallis comparing each experimental condition to its own control with Dunns multiple comparison test. Graphs showing the full results of the experiments are presented in Figure 1 and Figure S1.

Thermos water temperature	Thermos storage temperature	Total number of mosquitoes evaluated per condition (controls)	Number of experiments	Percentage of experiments with a significant reduction in infectivity after 4 hours storage
37.8°C	RT	110 (110)	3	66% (2/3)
	32°C	120 (120)	3	33.3% (1/3)
	42°C	150 (150)	4	75% (3/4)
37°C	RT	180 (178)	3	0% (0/3)
	32°C	140 (140)	3	33.3% (1/3)
	42°C	180 (180)	3	66.7% (2/3)
35.5°C	RT	160 (160)	3	0% (0/3)
	32°C	140 (140)	3	0% (0/3)
	42°C	200 (200)	4	50% (2/4)

Figures

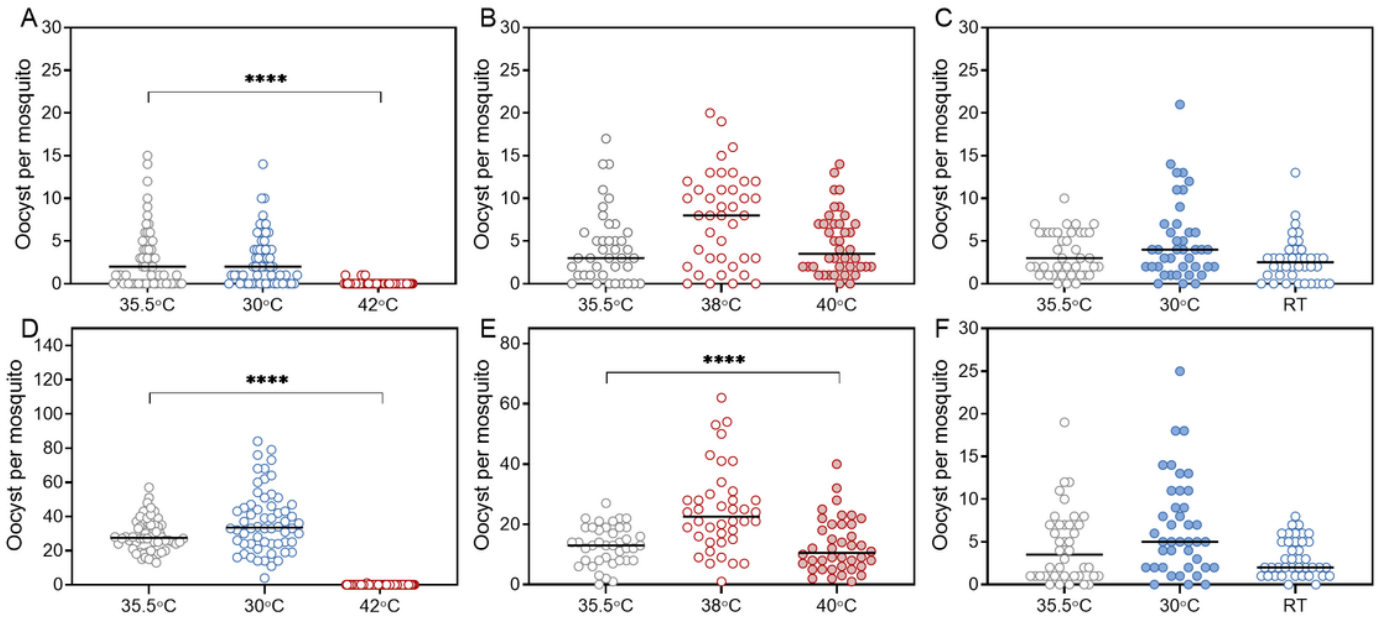

Figure 2

Impact of short-term gametocyte blood-meal storage in gametocyte infectivity Mosquito infection rate was evaluated from gametocyte blood-meals that were stored for 15 minutes at a range of temperatures (RT, 30°C, 38°C, 40°C and 42°C) and compared the control storage at 35.5°C (safe storage temperature for 4 hour storage). A total of 30-60 mosquitoes in 2 or 3 cups were used for each experimental condition. Each experiment was performed twice, with graphs A-F representing the results from independent experiments. Graphs show the intensity of oocyst infection (number of oocysts per mosquito) and the lines indicate group medians. Groups were compared by Kruskal-Wallis comparing each experimental condition to the control (35.5°C) with Dunns multiple comparison test.

Supplementary Files

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