RUNNING TITLE: Plasmodium sporozoite cryopreservation

A simple and efficient method for cryopreservation and recovery of viable *Plasmodium vivax* and *P. falciparum* sporozoites

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Abbreviations:

CSP (circumsporozoite surface protein)
DMSO (dimethyl sulfoxide)
RPMI (RPMI 1640 medium)
PBS (phosphate-buffered saline)
FBS (fetal bovine serum)
BSA (bovine serum albumin)

Abstract

Plasmodium falciparum and *P. vivax* sporozoites are the crucial stages of malaria parasites that initiate infection in humans. However, studies to develop new vaccines and drugs targeting these infective stages remain insufficient due to limited availability of sporozoites for research. This is a consequence of relatively few facilities that are established to produce sporozoites of human malaria parasites, sporozoites remaining viable for only a few days, and infected mosquitoes being a biohazard, making them difficult to transport. Cryopreservation of sporozoites offers the potential to alleviate these limitations and enhance sporozoite availability. These experiments were performed to evaluate methods for cryopreservation of *P. vivax* and *P. falciparum* sporozoites. Sporozoites, isolated in sterile buffer from infected mosquitoes by manual dissection of salivary glands, were cryopreserved using several types of commercially available serum-free cryoprotective solutions. The efficiency of cryopreservation was validated by a standard *in vitro* gliding motility assay as a measure of sporozoite activity. Viability of infective sporozoites was defined as percent gliding of sporozoites attached to the coverslip. Significant differences were observed among the cryopreservation media and protocols evaluated, with CryoStor CS2 giving the best results for both *P. falciparum* and *P. vivax*, whereas Hestar 200 worked efficiently only for *P. vivax* sporozoites. Further improvement in recovery of viable sporozoites would be anticipated using automated controlled-rate freezing equipment. Our results demonstrate cryopreservation provides an alternative for experimental studies that currently rely on fresh *P. falciparum* and *P. vivax* sporozoites.

1. Introduction

1.1. Background

Malaria is one of the major deadly infectious diseases worldwide, especially in the tropics where environmental factors are favorable for the mosquito vectors and access to health care limited [1]. The infection cycle of malaria parasites starts when a female *Anopheles* takes a blood meal and injects sporozoites into the host's skin during the bite. Infectious sporozoites are typically inside mosquito salivary glands for a limited period, and lose their infectious capability even more quickly if removed by dissection [2-4]. Sporozoites enter a blood vessel in the skin of the human host and travel through circulation to reach the liver and infect hepatocytes. Within hepatocytes all *P. falciparum* sporozoites initiate exoerythrocytic development by asexual multiplication; transforming to produce thousands of merozoites to start the blood-stage cycle that can lead to clinical illness and death. In addition to this direct asexual development process, some exoerythrocytic forms of *P. vivax* can become dormant, appearing to suspend their development a few days into the infection. These dormant exoerythrocytic forms of *P. vivax*, also known as hypnozoites, can renew their development process after different intervals leading to a relapse infection of the blood stage [5]. The relapsing nature of *P. vivax* combined with suboptimal antimalarial drug treatments to cure patients of hypnozoites represents a major challenge to eliminate malaria [6]. Apicomplexa species are obligate intracellular parasites that exhibit a form of locomotion known as gliding motility, using a membrane-associated actin-myosin motor complex [7]. Gliding motility is a substrate dependent form of locomotion powered by a sub-pellicular actin myosin motor complex that does not involve significant changes in cell shape [8]. *Plasmodium* sporozoites need gliding motility to migrate from the injection site in the skin to the liver and then infection of hepatocytes. Since it is difficult to image *in vivo* tissue migration for sporozoites as a experimental method, especially for the human malaria parasites, an *in vitro* gliding motility assay is typically performed as a quantitative measure of sporozoite functionality [9, 10]. During the gliding movement sporozoites leave a trail of circumsporozoite surface protein (CSP) that can be visualized by antibody staining as definite path of locomotion. Typically sporozoites migrate in a circular pattern on the glass coverslip with a majority of the sporozoites gliding in counter clockwise direction [11]. The extent of migration of sporozoites attached to the coverslip can be used as a surrogate measure of viability [12].

1.2. Objectives

The study presented here describes a simple effective method for cryopreservation for *P. vivax* and *P. falciparum* sporozoites. *Plasmodium* sporozoite cryopreservation was performed using commercially available serum-free cryogenic solutions following protocols easily replicated by other laboratories. The method provides a procedure that can be completed within a reasonable time to minimize loss of viability and maximize recovery of infective sporozoites. The efficiency of cryopreservation and viability of sporozoites was evaluated by performing *in vitro* gliding motility assay of frozen thawed samples by comparing with fresh sporozoites. This initial study presents unique

approach to further evaluate cryogenic solutions and conditions for more efficient cryopreservation of *P. falciparum* and *P. vivax* sporozoites.

2. Materials and Methods

2.1. Ethics Statement

The Human Subjects Protocol number TMEC 11-033 was approved by the Ethical Review Committee of Faculty of Tropical Medicine, Mahidol University, Thailand. All subjects or their legal guardians provided written informed consent to participate in the study.

2.2 *Plasmodium vivax* sporozoites

The Ethical Review Committee of Faculty of Tropical Medicine, Mahidol University, approved a Human Subjects protocol for this study. Blood was drawn by venipuncture from individuals diagnosed with a *P. vivax* infection into a tube containing heparin. *Anopheles dirus* mosquitoes were maintained at the Mahidol Vivax Research Unit insectary, Faculty of Tropical Medicine, Mahidol University, Thailand. Mosquitoes were fed on infected blood by a direct membrane feeding system [13] and after being examined for parasite infection mosquitoes were transported in sealed containers to University of South Florida, Tampa, USA. Infected mosquitoes were fed on 10% sugar solution and maintained at 26°C, 75% humidity in closed environment chamber until dissection. A preliminary determination of mosquito infection level was performed 7 days of post-infective blood meal by mercurochrome staining (1% v/v) and midgut oocyst count.

2.3 *Production of Plasmodium falciparum* sporozoites

"The following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Plasmodium falciparum* NF54, MRA-1000, deposited by Megan Dowler, Walter Reed Army Institute of Research." *Plasmodium falciparum* NF54 (MRA1000, MR4 [14]) line maintained as asexual and sexual blood stage cultures in complete RPMI1640 (Corning) with 10% Human AB serum (Interstate Blood Bank) supplemented with 6µg/ml hypoxanthine (Sigma) in 6-well plate according to candle jar method [15, 16]. Gametocyte cultures were initiated with about 1% parasitemia at 4% hematocrit in 5ml/well culture and grown for 16 days to produce infectious gametocytes. Four – six day old *An. stephensi*, maintained at USF insectary by standard methods [16], were infected using a Hemotek electric membrane feeding system (Discovery Workshop) with mature stage V gametocyte cultures with good exflagellation [17, 18]. Infected mosquitoes were maintained at 26°C, 75% humidity in closed environment chamber fed on 10% sugar solution. Mosquito infectivity was determined by mercurochrome (1% v/v) midgut oocyst count after 10 days of post-infective blood meal.

2.4 *Mosquito dissection and sporozoites preparation for cryopreservation*

Infected mosquitoes were chilled at -20C for 5 min and washed with 70% ethanol followed by rinse in 1xPBS containing mixture of penicillin (50 IU)-streptomycin (50µg/ml) and neomycin (0.025 µg/ml) (GIBCO) before dissection. Clean sporozoites were obtained by manual dissection of salivary glands in sterile incomplete RPMI1640 (Corning) on day 14-20 post-infective blood meal. Free sporozoites were obtained by trituration of pooled salivary glands in each batch with plastic pestle in a microcentrifuge tube in 100µl incomplete RPMI1640. Sporozoite counts were made by hemocytometer to determine yield. Sporozoites were maintained on ice all the time. A sporozoites suspension was prepared in incomplete RPMI1640 at 10,000 sporozoites/µl and mixed with chilled cryogenic solutions in the ratio of 1:3. The mixture was dispensed at 100µl volume with 250,000 sporozoites per pre-chilled cryogenic vial (Matrix). Using a manual cooling method, the cryogenic vials with sporozoites suspension were incubated on ice for 30 min, transferred to -80C for one hour, and immediately transferred to liquid nitrogen tank in vapor phase for long-term storage (Cryoplus 3).

2.5 *Sporozoites gliding motility assay*

The quality and viability of sporozoites before and after cryopreservation was determined by gliding motility assay (Nardin et al 1982). Briefly about 40,000 sporozoites were suspended in 300µl complete hepatocyte media (Invitrogen) containing 10% FBS (Hyclone) and incubated on *Plasmodium* species-specific anti-CSP antibody pre-coated circular coverglass in a 24 well plate at 37C for 30 min in 5% CO₂. Gliding trails were fixed with 4% paraformaldehyde (Alfa Aesar) for 15 min and blocked with 1% BSA (Fisher) for 15 min. The gliding trails were stained with *Plasmodium* species-specific primary anti-CSP antibody (10µg/ml) and developed with secondary antibody (dilution 1:1000) Alexafluor 488 green (Invitrogen). Finally, a coverglass was mounted on slide in ProLong Antifade (Invitrogen). Gliding assays were performed with fresh sporozoites immediately after dissection prior to cryopreservation and post-thaw.

2.6 *Analysis of cryopreserved sporozoites*

Cryopreserved sporozoite samples were revived by two methods. Samples were removed from cryostorage and either held at room temperature until ice crystals disappeared (1-2 min) or by dipping the vial in 37C water bath for 1 min. Samples were diluted with equal volume of complete hepatocyte media. Sporozoite counts were determined once again with hemocytometer to determine number of recovered sporozoites according to formula mentioned below. Finally, about 40,000 sporozoites were diluted in 300µl complete hepatocyte media to perform gliding motility assay as mentioned above.

$$\text{Sporozoite recovery (\%)} = \frac{\text{Number of sporozoites recovered post – thaw}}{\text{Number of sporozoites before cryopreservation}} \times 100$$

2.7 *Determination of sporozoite viability*

Visualization of antibody stained sporozoites gliding trails was performed under 40x Zeiss scope A1 viewing several random microscopic fields counting 100 sporozoites to determine percent gliding. All trails, including with or without intact sporozoites, were accounted. A gliding trail equal to the length of sporozoite or longer counted as valid trail for the current study. The viability of sporozoites calculated as percent gliding from fresh and cryopreserved sporozoites according to formula mentioned below.

$$\text{Sporozoite viability (\%)} = \frac{\text{Percent sporozoites gliding post - thaw}}{\text{Percent sporozoites gliding for fresh}} \times 100$$

2.8 Statistical analysis

Non-parametric methods were used for statistical analysis since the data did not follow a normal distribution. Differences between cryogenic solutions for both percent recovery and percent viability were evaluated using a Bonferroni multiple comparison test. A Kruskal-Wallis test was used to evaluate the statistical difference between the cryogenic solutions and the thawing method for both percent recovery and percent viability. The thawing method had no effect on either the percent recovery or the percent viability. There was a significant difference between cryogenic solutions for both percent recovery and percent viability, which was further evaluated using a Bonferroni multiple comparison test.

3. Results

3.1 Standardization of sporozoite extraction and cryopreservation

A simple easily reproducible protocol was developed to evaluate methods for effective cryopreservation of *Plasmodium* sporozoites (Figure 1). Standard procedures were used to produce *Plasmodium vivax* and *P. falciparum* sporozoites from clinical isolates and a laboratory-cultured line (NF54), respectively. The salivary glands of infected mosquitoes were removed by dissection under aseptic conditions and sporozoites were released into sterile RPMI1640 medium. In order to standardize the procedures in each experiment, the concentration of sporozoite was adjusted to 10,000 per μl , aliquots of these sporozoites were mixed with one of several cryopreservation media, or RPMI1640 as a control, and then 250,000 sporozoites were dispensed into commercial cryogenic vials (Table 1). Under all conditions tested there was a high percentage of sporozoites recovered although not all were viable (Tables 2 & 3).

3.2 Evaluation of different cryoprotectant solutions to preserve viable sporozoites

As a quantifiable surrogate of sporozoite viability, in this study we used gliding motility of *P. vivax* and *P. falciparum* sporozoites to evaluate experimental differences and determine the best cryopreservation media and protocol (Figures 2 & 3). There were considerable differences in the level of gliding among the conditions tested, but some sporozoites retained gliding motility under all conditions tested (Tables 2 & 3). For each dissection batch of the *P. vivax* or *P. falciparum* sporozoites, the baseline gliding motility was determined soon after dissection for samples kept only in RPMI1640 on ice and

without cryopreservation (Figures 2A & 3A, respectively). The effect of each experimental difference in the cryopreservation protocol was calculated as a percentage relative to the respective baseline of its experimental batch (Tables 2 & 3).

3.3 Statistical comparisons of results with cryoprotectant solutions tested

We were able to compare the relative effect of each experimental difference in the cryopreservation protocol, using the quantifiable gliding activity of sporozoites, to determine the most effective cryopreservation method. Cryostor CS2 and Hestar 200 were significantly better for cryopreservation of *P. vivax* sporozoites than the other cryopreservation media evaluated (Figure 4A). In contrast, only Cryostor CS2 worked well for cryopreservation of *P. falciparum* sporozoites (Figure 4B). As part of developing a simple protocol, two simple thawing methods for the cryopreserved sporozoites were evaluated, placing the sporozoite cryogenic tubes on the laboratory bench top to reach ambient temperature or placing the cryogenic vial in a 37C water bath. There was no significant difference between the two thawing methods tested for either *P. vivax* or *P. falciparum* sporozoites (data not shown).

4. Discussion

It is expected that most malaria research laboratories currently engaged in infecting mosquitoes can adopt the protocols developed in this study. The cryopreservation media used in this study are relatively inexpensive commercially available reagents. No special equipment, other than -80C freezer and LN storage, was required to cryopreserve *P. vivax* and *P. falciparum* sporozoites. With further improvements we are expecting to optimize these procedures by adjusting the rate of freezing and thawing, which are likely to improve viability. However, regulating these steps is likely to require specialized equipment that may not be available to all laboratories.

An important aspect of the method we have developed is the cryopreservation medium does not require the addition of serum. While serum is generally appreciated as a good cryoprotective additive and is a renewable resource, its complex components vary and when present can complicate potential downstream uses for basic experimental and clinical research. Previous studies attempting to cryopreserve sporozoites of mammalian *Plasmodium* have reported variable results but generally required the presence of serum. The first successful cryopreservation of human malaria sporozoites, freezing to -70C, required the presence of human serum or plasma [19]. Initial studies of *P. berghei* sporozoites found also a much better recovery of viable sporozoites when whole mouse serum was included in the cryopreservation medium [20]. Furthermore, in this study of *P. berghei* sporozoites hydroxyethyl starch retained highest viability during cryopreservation at low temperatures in comparison to dimethyl sulfoxide (DMSO), glycerol, polyvinylpyrrolidone. Cryopreserving irradiated *P. berghei* sporozoites in the presence of serum has been successful, also [21]. In a surprising contrast, cryopreservation of *P. gallinaceum* sporozoites did not use any cryogenic solution but preserved sporozoites *in situ* in infected *Aedes aegypti* [22].

Commercially available cryogenic solutions usually contain an active ingredient, which is responsible for maintaining integrity of cellular membranes and hence viability during cryopreservation and post-thaw recovery. In addition cryogenic solutions like biological media contain other additives to maintain buffering capacity to minimize cellular damage. The cryogenic solutions used generally for cell preservation, like the ones we evaluated for *P. vivax* or *P. falciparum* sporozoites, can be broadly divided into two categories intracellular and extracellular cryoprotectants. Also, cryogenic solutions can be classified based on the molecular weight of active component and its ability to penetrate cells during freezing [23, 24]. Intracellular solutions have low molecular weights and amphipathic properties, permeate cells and are effective in minimizing cell damage in biological systems. Such solutions include DMSO and glycerol, which are in Cryostor CS2 and Glycerolyte 57, respectively. Glycerol protects RBCs by limiting the rise in electrolyte concentration that occurs when water is converted to ice and by inhibiting the rate of ice crystal growth [25]. Extracellular solutions operate mainly by forming a shield around the cell to minimize the effects of dehydration during the freezing process such as hydroxyethyl starch [26], which is in Hestar 200, Voluven, and Hetastarch. Hydroxyethyl starch is non-toxic, poorly immunogenic, and often used as a plasma volume expander [27]. Although our study found only the cryoprotectant solution with DMSO worked successfully for both *P. vivax* and *P. falciparum*, future studies may be able to improve the protocol for cryopreserving *P. falciparum* sporozoites with hydroxyethylstarch, also.

5. Conclusion

This study demonstrates an excellent method for cryopreservation of *P. vivax* and *P. falciparum* sporozoites using commercially available reagents and standard laboratory equipment. Importantly, this easily reproducible protocol is achieved without the need for the addition of serum or plasma in the cryopreservation media. This simple protocol should help facilitate a greater availability of sporozoites of the human malaria parasites and more widespread studies of their critical infective pre-erythrocytic stages for drug and vaccine efficacy evaluations.

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Table 1

Product information about cryogenic solutions used in this study.

Cryoprotectant solutions	Vendor	Catalog number	Concentration of active ingredient in stock solution	Concentration of active ingredient in cryopreservation storage
CryoStor CS2	Sigma	C3124	2% DMSO	1.50%
Hestar 200	Claris	G/LVP-5	6% Hydroxyethyl starch	4.5%
Voluven	Hospira	PB-1909	6% Hydroxyethyl starch	4.5%
Hetastarch	B Braun	L6511	6% Hydroxyethyl starch	4.5%
Glycerolyte 57	Fenwal	4A7833	50-60% Glycerin	37.5-45%

Table 2Recovery and viability of *P. vivax* sporozoites after cryopreservation.

Cryogenic solutions	Thaw method	% recovered (AVG±SD)	% viable (AVG±SD)
CryoStor CS2	RT	95.6±5.2	82.7±5.8
	37C	96.7±1.3	83.6±1.0
Hestar 200	RT	88.8±2.8	80.4±13.0
	37C	86.0±5.7	81.0±0.2
Voluven	RT	80.2±11.6	5.2±3.4
	37C	82.8±16.2	5.9±4.2
Hetastarch	RT	81.2±10.2	18.9±3.5
	37C	83.8±18.2	16.2±1.4
Glycerolyte 57	RT	72.5±10.6	1.3±0.1
	37C	76.5±1.0	1.3±0.1
RPMI1640	RT	72.6±4.5	3.4±0.9
	37C	75.0±1.9	3.4±1.0

Table 3Recovery and viability of *P. falciparum* sporozoites after cryopreservation.

Cryogenic solutions	Thaw method	% recovered (AVG±SE)	% viable (AVG±SE)
CryoStor CS2	RT	92.0±11.3	62.5±0.0
	37C	90.5±10.6	73.9±12.1
Hestar 200	RT	82.0±1.4	9.1±9.2
	37C	81.5±6.4	11.0±12.0
Voluven	RT	90.0±12.7	19.4±11.6
	37C	81.5±3.5	17.7±11.5
Hetastarch	RT	72.9±2.0	9.6±4.1
	37C	77.0±2.2	10.8±5.9
Glycerolyte 57	RT	69.0±3.4	5.0±2.4
	37C	72.1±11.1	6.7±0.0
RPMI1640	RT	66.3±2.5	5.5±3.5
	37C	70.5±0.7	5.0±1.5

Fig. 1. Flow chart for sporozoite preparation, cryopreservation and post-thaw analysis

Figure 2.

Fig. 2. Gliding motility assays of *P. vivax* sporozoites. (A) Freshly dissected sporozoites kept in RPMI only without cryopreservation. (B-F) Sporozoite gliding post-thaw when using cryogenic solutions (B) CryoStor CS2, (C) Hestar 200, (D) Voluven, (E) Hetastarch, and (F) Glycerolyte 57.

Figure 3.

Fig. 3. Gliding motility assay of *P. falciparum* sporozoites. (A) Freshly dissected sporozoites in RPMI only without cryopreservation. (B-D) Sporozoite gliding post-thaw when using cryogenic solutions (B) CryoStor CS2, (C) Hestar 200, (D) Voluven (data not shown: Hetastarch, Glycerolyte 57).

Fig. 4. Statistical analysis of the distribution of percent viability of (A) *P. vivax* sporozoites (Kruskal Wallis $p=0.0034$) and (B) *P. falciparum* sporozoites (Kruskal Wallis $p=0.0111$) analyzed by cryogenic solution. The y-axes on the plots are percent (%) viability for each Cryogenic Solution on the x-axes.

CS2 Cryopreserved *P. vivax* sporozoite
gliding post thaw