VIABILITY AND INFECTIVITY OF CRYOPRESERVED *PLASMODIUM VIVAX* SPOROZOITES

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Abstract. *Plasmodium vivax* presents a great challenge to malaria control because of the ability of its dormant form in the liver, the hypnozoite, to cause relapse in otherwise fully recovered patient. Research efforts to better understand *P. vivax* hypnozoite biology have been hampered by the limited availability of its sporozoite form responsible for liver infection. Thus, the ability to cryopreserve and recover *P. vivax* sporozoites is an essential procedure. In this study, protective effects of hydroxyethyl starch (HES) alone and in combination with other cryoprotectants on *P. vivax* sporozoite recovery, viability and *in vitro* infectivity of a human liver HC-04 cell line were investigated. Sporozoites were harvested from *P. vivax*-infected female *Anopheles* mosquitoes and cryopreserved at a freezing rate of -1°C/minute to a final temperature of -80°C before being stored in a vapor phase liquid nitrogen tank. Cryopreserved sporozoites were thawed at 37°C and recovery of intact sporozoites assessed using a hemocytometer. Sporozoite viability and *in vitro* infectivity was measured using a gliding and an indirect immunofluorescence assay, respectively. A combination of 10% HES + 50% fetal bovine serum was the best cryopreservant compared to HES solution alone or mixed with cryopreservants such as dimethyl sulfoxide (DMSO) and sucrose. A mixture of bovine serum albumin, DMSO and sucrose in RPMI 1640 medium constituted an alternative cryopreservant. Sporozoites recovered from all cryopreservation media exhibited motility and infectivity of < 0.1% and < 0.001%, respectively. Thus, there is an urgent need for a vast improvement in cryopreservation procedures of viable and infective *P. vivax* sporozoites necessary for advancing research on hypnozoite biology.

Keywords: *Plasmodium vivax,* cryopreservation**,** malaria, sporozoite

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INTRODUCTION

Malaria is caused by infection with *Plasmodium* parasites, which are transmitted to humans through the bites of infected female *Anopheles* mosquitoes. Malaria parasites in the form of sporozoites injected from mosquito salivary

glands into the host blood stream and, upon reaching the liver, sporozoites traverse liver sinusoidal barrier including liver-resident macrophages (Kupffer cells) and migrate through a number of hepatocytes before finally invading hepatocytes, in which they undergo intra-cellular differentiation and multiplication (Lindner *et al*, 2012). After 6-8 days of development in the liver, tens of thousands of merozoite forms are released into the blood stream ready to red blood cells and initiate the blood stage cycle of infection, which is associated with the clinical symptoms of malaria disease.

Of the five *Plasmodium* species that infect humans, *P. vivax* is the most prevalent with almost half of the world's population at risk (WHO, 2014). Although *P. vivax* infection results in mild clinical symptoms, severe outcomes have been reported and are becoming more increasingly detected (Price *et al*, 2009). In addition, *P. vivax* has an ability to remain dormant in the liver, known as hypnozoites, which can cause infections (relapses) weeks, months or even years after the primary infection (Wells *et al*, 2010). To date, primaquine is the only approved drug that effectively eliminates hypnozoites, but there is a risk of severe hemolytic toxicity in individuals with glucose-6-phosphate dehydrogenase (G6PD)-deficiency and reports of primaquine tolerance in patients have compromised its clinical use (Chiang *et al*, 2012; Townell *et al*, 2012; Bright *et al*, 2013). An alternative drug, targeting *P. vivax* hypnozoites, tafenoquine, is currently in Phase III clinical trials, but the possibility of hemolytic crisis in G6PD-deficient patients, although low, has to be taken into consideration (Campo *et al*, 2015). Thus, there still is a need to develop safer drugs to eradicate liver-stage *P. vivax*.

A major obstacle to achieving the objective of *P. vivax* control and ultimately complete elimination is the limited knowledge of hypnozoite biology. This is due in part to the lack of a reliable method for *in vitro* culture of liver stage parasites, although new culture techniques have recently been reported (March *et al*, 2013; Maher *et al*, 2014; Patrapuvich *et al*, 2015). However, access to *P. vivax* sporozoites remains a major bottleneck to advances in research on hypnozoites, both in the area of drug discovery and vaccine development. In order to obtain *P. vivax* sporozoites, female *Anopheles* mosquitoes have to be fed on blood obtained from *P. vivax*-infected patients through an artificial membrane-feeding device (Sattabongkot *et al*, 2003). In addition, creating of a reliable source of sporozoites requires a well-established insectary at field sites (Campo *et al*, 2015). The main disadvantages of these methods are a reduction in parasite viability during transportation of infected mosquitoes to the laboratory and the logistical and regulatory challenges. Continuous *in vitro* culture of blood stage *P. vivax* has yet to be established as a feasible alternative to *in vivo* infected blood (Roobsoong *et al*, 2015).

Cryopreservation offers another strategy to overcome limitations in obtaining access to *P. vivax* sporozoites. Retention of sporozoite viability after a year of cryopreservation in plasma or serum was reported (Jeffery and Rendtorff, 1955). Other effective cryopreservants include a combination of serum and hydroxylethyl starch (HES) (Leef *et al*, 1979; Hollingdale *et al*, 1985) and bovine serum albumin (Lamke and Liljedahl, 1976).

The present study demonstrates the utilization of HES in cryopreservation of *P. vivax* sporozoites. In addition, recovery of sporozoite following freezing, viability and *in vitro* infectivity of human hepatocyte HC-04 cells were examined.

MATERIALS AND METHODS

Collection of *P. vivax*-infected *Anopheles* mosquitoes

Blood samples were collected from *P. vivax*-infected individuals in Kanchanaburi and Tak Provinces, Thailand. Laboratory-reared female *Anopheles dirus* were membrane-fed with patients' blood samples and subsequently kept at 26°C and 75% humidity with access to cotton wool soaked in 10% sucrose solution (Sattabongkot *et al*, 2003). Mosquito infection was monitored at day 7 post-feeding for midgut oocysts using a mercurochrome staining method (Usui *et al*, 2011). Salivary gland sporozoites were examined on day 14 post-feeding.

Experimental protocols were approved by the Ethical Review Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (human use protocol TMEC 11-033). Written approval consents were obtained prior to blood collection. Infected subjects were treated with radical curative regimen of chloroquine and primaquine according to WHO guideline (WHO, 2015).

Salivary gland sporozoites

Salivary gland sporozoites were dissected from anesthetized infected mosquitoes using a standard protocol (Kennedy *et al*, 2012). In brief, salivary glands of 50 mosquitoes infected for 4-22 days were dissected and placed in 50 µl of either ice-cold RPMI 1640 medium (Gibco, Gaitherberg, MD), Schneider's Drosophila medium (Gibco) or Grace's insect medium (Gibco), and ground with a sterile pestle. Released sporozoites were counted in a hemocytometer and kept on ice until used.

Cryopreservation of *P. vivax* sporozoites

A suspension of $1x10^6$ *P. vivax* sporozoites was sedimented in a cryo-vial (CryoKing®) at 13,500*g* for 10 minutes at 4°C, re-suspended in 100 µl of freshly prepared freezing media (Table 1) at a concentration of 10^4 sporozoites/µl and frozen at a rate of -1°C/minute to -80°C in a controlled-rate freezer (Thermo Scientific Nalgene, Rockford, IL). After storage at -80°C for 24 hours, samples were transferred and stored in the vapor phase of liquid nitrogen.

Thawing of *P. vivax* sporozoites

Frozen *P. vivax* sporozoites were thawed by shaking in a water bath at 37°C for 30 seconds and the sporozoite suspensions were mixed with 500 µl of complete medium [1:1 MEM:Ham's F12 media supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin], then sedimented at 13,500*g* for 10 minutes at room temperature. Sporozoites were re-suspended in 200 µl of complete medium. Recovery of intact sporozoites was determined by counting in a hemocytometer and recorded as percent cryopreserved parasites.

Gliding motility assay

Gliding motility assay was performed as previously described (Lupton *et al*, 2015). In short, glass cover slips coated with 10 µg/ml of anti-*P. vivax* circumsporozoite surface protein antibodies (anti-PvCSP) (kindly provided by RA Wirtz, CDC, USA) in phosphate-buffered saline (PBS) were placed in wells of a 24-well plate (Jet Bio-Filtration, Guangzhou, China) and sporozoite suspension ($3x10^4$ in complete medium) was introduced into the well. The plate was centrifuged at 1,500*g* for 5 minutes at room temperature and incubated at 37°C for 30 minutes. The glass cover slips were treated with 4% paraformaldehyde for 20 minutes at room temperature followed by 1% BSA

Table 1
Recipe for preparation of cryopreservant media.

Reagent	HES	10% HES + 50% FBS	HES + 10% DMSO	HES + 10% DMSO + 250 mM sucrose	RPMI + 0.5% BSA + 10% DMSO + 250 mM sucrose
HESTAR-200™ 6% (Claris Lifesciences, India)	1 ml	0.1 ml	0.9 ml	0.8 ml	-
FBS	-	0.5 ml	-	-	-
MEM/Ham's F12	-	0.4 ml	-	-	-
DMSO	-	-	0.1 ml	0.1 ml	0.1 ml
2.5 M sucrose	-	-	-	0.1 ml	0.1 ml
RPMI 1640	-	-	-	-	0.7 ml
5% BSA	-	-	-	-	0.1 ml

BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HES, hydroxylethyl starch.

solution for 15 minutes at room temperature. Then cover slips were treated with 10 μg/ml mouse primary anti-PvCSP in 1% BSA solution, followed by goat secondary Alexa Fluor® 488-conjugated anti-mouse IgG antibodies (1:500 dilution; Invitrogen, Carlsbad, CA). Sporozoite CSP gliding trails on the cover slips were visualized under a fluorescent microscope (ZEISS AXIO Scope.A1, Jena, Germany). As control, pre-frozen sporozoite gliding motility was determined by counting the numbers of sporozoites that completed at least one circle per 300 sporozoites. Gliding motility of thawed cryopreserved sporozoites was determined by counting sporozoites that have gliding trails per total sporozoites on entire coverslip.

Sporozoite infectivity assay

Human hepatocyte HC-04 cells ($3x10^5$) grown in complete medium in an 8-well Lab-Tek II plate (Nalgene Nunc International, Naperville, IL) at 37°C for 18-20 hours were used for sporozoite infectivity assay as previously described (Sattabongkot *et al*, 2006). Medium was removed by aspiration and $3x10^5$ pre-cryopreserved or 3-7.4 $x10^5$ thawed sporozoites in 150 μl of infection medium (1:1 MEM:Ham's F12 media supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B) were added to HC-04 cells. After 4 hours of incubation at 37°C, uninvaded sporozoites were removed by aspiration, then 400 μl aliquot of fresh infection medium was added and the cell culture was maintained at 37°C for 4 days with daily change of medium prior to visualization of the liver stage parasites (EEs) by indirect immunofluorescence assay (IFA). Cells were washed three times with PBS, incubated with 4% paraformaldehyde for 20 minutes and permeabilized by treating with 0.1% Triton X-100. Cells then were added with 3% BSA solution, followed by mouse anti-PbHsp70 antibodies (clone 4C9) (Tsuji *et al*, 1994), then goat secondary IgG Alexa Fluor® 488-conjugated anti-mouse antibodies (1:500 dilu-

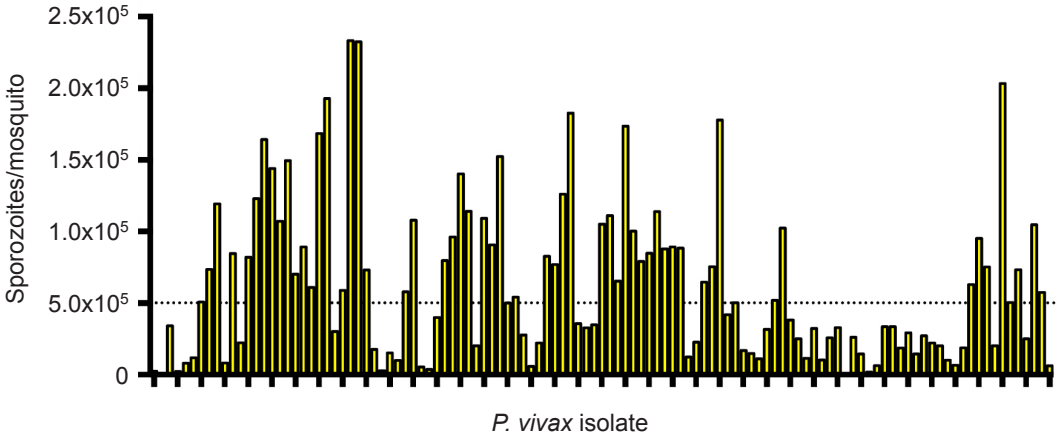

Fig 1–*P. vivax* sporozoite production. *P. vivax* sporozoites were dissected from *An. dirus* mosquitoes membrane-fed on 115 patients' blood samples collected at Tak and Kanchanaburi Provinces, Thailand during 2012-2014.

tion; Invitrogen) followed by Alexa Fluor 568 phalloidin (1:1,000, Invitrogen) and 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Samples were covered with Prolong Gold Antifade reagent (Invitrogen), sealed under cover slip and viewed under a fluorescent microscope (400x magnification; ZEISS AXIO Scope. A1 equipped with AxioVision Rel 4.8 Software). Percent sporozoite infectivity was determined by comparing the number of infected hepatocytes to total number of sporozoite inoculum.

Statistical analysis

At least three independent experiments were conducted in duplicate and results expressed as mean ± SEM. Difference of the data was determined using standard Student's *t*-test and statistical significance is accepted when a *p*-value is < 0.05.

RESULTS

Supply of *P. vivax* sporozoites

Owing to the lack of *P. vivax in vitro* blood stage culture, sporozoite supply has to rely on mosquito feeding of *P. vivax*-infected patients' blood. The numbers of salivary gland sporozoites from infected mosquitoes varied with different batches of patients' blood samples. Among 115 *P. vivax* cases collected during 2012 to 2014, 50% had infection rates resulting in production of > 50,000 salivary gland sporozoites per mosquito (Fig 1). In order to reduce mosquito debris and microbial contaminants during salivary gland dissection, only mosquitoes with ≥ 50,000 sporozoites were chosen for further study.

***P. vivax* sporozoite collecting media**

Ideally, a medium used for collecting sporozoites harvested from infected mosquito salivary glands should contain components that retain both viability and infectivity of the sporozoites. A comparison of human hepatocyte HC-04 cell infectivity of fleshly isolated sporozoites collected in RPMI 1640 and two insect media, Schneider's and Grace's, in the absence of added serum albumin so as not to cause sporozoite activation before reaching the hepatocyte cells (Vanderberg, 1974),

Table 2
Gliding motility and *in vitro* infectivity of *P. vivax* sporozoites following cryopreservation and thawing compared to freshly prepared sporozoites from mosquito salivary glands.

Medium	Number added[a]	Before cryopreservation			Number added[a]	After cryopreservation and thawing		
		Gliding (%)	Number invaded	Infectivity (%)		Gliding (%)	Number invaded	Infectivity (%)
HES	300,000	29	153	0.05	320,000	0.02	5	0.0016
	300,000	37	182	0.06	299,000	0.2	0	0
	300,000	69	34	0.01	350,000	0	2	0.0006
10% HES + 50% FBS	300,000	29	153	0.05	630,000	0.3	8	0.0013
	300,000	37	182	0.06	545,000	0	0	0
	300,000	69	34	0.01	589,300	0	2	0.0003
	300,000	90	124	0.04	550,000	0	3	0.0005
RPMI + 10% DMSO	300,000	29	153	0.05	740,000	0.03	2	0.0003
+250 mM sucrose +	300,000	37	182	0.06	421,400	0	0	0
0.5% BSA	300,000	23	66	0.02	534,000	0	5	0.0009
	300,000	90	124	0.04	580,000	0	9	0.0016

[a]Total number of frozen sporozoites recovered from cryopreservation and added to HC-04 cell culture for infectivity assay. Data are mean values from each experiment conducted in duplicate. BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HES, hydroxylethyl starch.

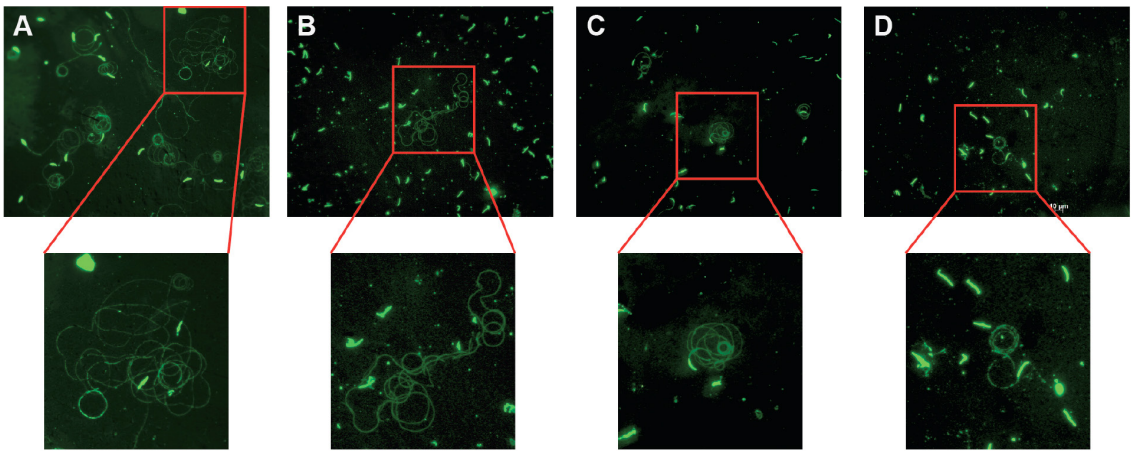

Fig 2 –Gliding motility of freshly prepared and cryopreserved *P. vivax* sporozoites. Tracks of motile sporozoites on glass coverslips coated with 10 μg/ml anti-PvCSP were visualized under a fluorescent microscope (ZEISS AXIO Scope.A1) following treating with 10 μg/ml mouse primary anti-PvCSP antibodies and goat secondary IgG Alexa Fluor® 488-conjugated anti-mouse antibodies (1:500 dilution; Invitrogen). Representative images are shown for (A) freshly prepared sporozoites and sporozoites cryopreserved in (B) hydroxylethyl starch (HES) solution, (C) 10% HES + 50% fetal bovine serum and (D) RPMI 1640 medium + 0.5% bovine serum albumin + 10% dimethyl sulfoxide + 250 mM sucrose. CSP, circumsporozite surface protein.

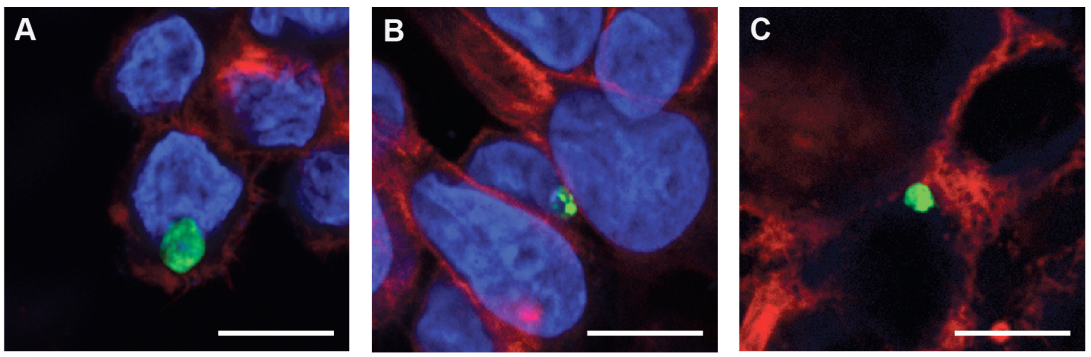

Fig 3–*In vitro* growth of intra-hepatocytic *P. vivax* in human hepatocyte HC-04 cells. Freshly prepared (A), cryopreserved in hydroxylethyl starch (HES) solution (B) and cryopreserved in 10% HES + 50% fetal bovine serum (C) sporozoites were co-cultured for 4 days with HC-04 cells and intra-cellular parasites visualized by indirect immunofluorescence assay using antibodies against parasite HSP-70 (green). Red = Alexa Fluor 568 phalloidin-labelled hepatocyte F-actin, blue = DAPI-labelled hepatocyte nucleus. Bar = 10 μm.

following 4 days of co-culture showed using IFA that sporozoites maintained in RPMI 1640 medium are significantly more infective (0.044 ± 0.004%) than those in Schneider's (0.012 ± 0.004%) or Grace's medium (0.014 ± 0.006%) (p = 0.005 and 0.016, respectively).

Recovery of intact *P. vivax* sporozoites after cryopreservation

When HES alone was used for cryopreservation of *P. vivax* sporozoites,

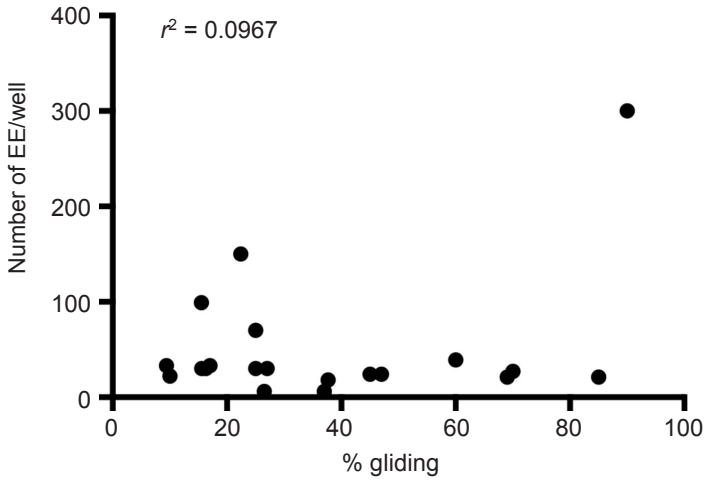

Fig 4–Association of gliding motility and infectivity of 20 freshly isolated *P. vivax* sporozoites. Gliding motility was determined from tracks of motile sporozoites on glass coverslips as described in legend to Fig 2. Percent gliding motility was determined by counting numbers of sporozoites that completed at least one circle per 300 sporozoites. Infectivity assay was performed as described in legend to Fig 3. Sporozoite infectivity was determined by comparing the number of infected hepatocytes per well containing an inoculum of $3x10^5$ sporozoites. Data are mean values from each experiment performed in duplicate.

recovery based on numbers of intact thawed sporozoites was 37 ± 1.2% and is raised significantly to 59 ± 2.5% when a mixture of 10% HES + 50% FBS was used (p = 0.0001), but this value is not significantly from those achieved with other cryopreservant mixtures employed: HES + 10% DMSO (55 ± 14%), HES + 10% DMSO + 250 mM sucrose (51 ± 13 %) and RPMI 1640 + 0.5% BSA + 10% DMSO + 250 mM sucrose (56 ± 16%). Trehalose, widely used as a cryoprotective agent (Richards *et al*, 2002; Stoll *et al*, 2012), did not provide any improvement over sucrose (data not shown).

Viability and infectivity of cryopreserved *P. vivax* sporozoites

Viability of *P. vivax* sporozoites was measured in terms of their gliding motility, which in freshly prepared *P. vivax* sporozoites ranged from 29% to 90%, but dropped drastically following cryopreservation (0.05 ± 0.03%), with optimum viability being achieved with a mixture of 10% HES + 50% FBS (Table 2). Fig 2 shows typical gliding trails for freshly prepared and thawed sporozoites.

In vitro infectivity of HC-04 cells by cryopreserved sporozoites was only ≤5% of freshly prepared samples (Table 2). Smaller EE forms were obtained from cryopreserved compared with freshly prepared sporozoites, indicative of slower/retarded growth (Fig 3). No correlation between freshly isolated *P. vivax* sporozoite viability and infectivity of HC-04 cells was observed (Fig 4).

DISCUSSION

The availability of *P. vivax* sporozoites only from mosquitoes fed on blood from infected subjects has severely hampered drug discovery and vaccine development of pre-erythrocytic stages of this highly prevalent parasite. Considerable amounts of *P. vivax* sporozoites (≥ 50,000 per mosquito) have been acquired through artificial membrane feeding technique (Sattabongkot *et al*, 2003). As transportation of infected mosquitoes among laboratories in not a feasible undertaking and *P. vivax* sporozoite viability rapidly decreases once removed from mosquito salivary

glands (Beier, 1998). One resource is shipment of cryopreserved *P. vivax* sporozoites freshly dissected from mosquitoes.

Although a suitable solution was found to maintain viable *P. vivax* sporozoites following their removal from mosquitoes, disappointing recoveries of both viability and infectivity of a human hepatocyte cell line were obtained despite the use of a variety of cryopreservant solutions. Those few hardy *P. vivax* sporozoites that managed to invade and develop in hepatocytes into merozoites, albeit at a slower rate than freshly prepared samples. Taking into account this *caveat*, the procedure may suffice for *in situ* studies of liver stage *P. vivax* obtained from cryopreserved sporozoites.

Similar results were reported for rodent sporozoites using HES as a cryoprotective agent (Hollingdale *et al*, 1985). A comparison of infectivity of freshly prepared and commercially available cryopreserved *P. vivax* sporozoites in a micro patterned co-culture liver stage model showed approximately 90% loss in infectivity of the latter sporozoites (March *et al*, 2013). Until a better cryopreservation technique is developed, the only other alternative is to increase the numbers of sporozoites to be frozen in each batch.

A relationship between gliding motility and infectivity of sporozoites has been reported (Vanderberg, 1974, 1975). However, this was not borne out in this study. It is recommended to carry out both assays on each batch of *P. vivax* sporozoites rather than depend on viability (gliding motility) assay alone.

In conclusion, cryopreserved *P. vivax* sporozoites are the only means for supporting liver-stage malaria research community when access to sporozoite-infected mosquitoes is not readily available. However, the extremely low viability and liver infectivity of cryopreserved *P. vivax* sporozoites constitute a very serious impediment and the situation provides an opportunity for a more concerted effort on the part of *P. vivax* research community to help hurdle this stumbling block, particularly as regards development of better cryopreservation technologies and improvement in efficiency of *P. vivax* sporozoite liver infectivity and intrahepatocyte development.

Addendum: While this manuscript was in preparation, Singh *et al* (2015) reported similar results on the viability of *P. vivax* sporozoites cryopreserved in RPMI 1640 medium.

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