Succinctus

Infection of laboratory colonies of *Anopheles* mosquitoes with *Plasmodium vivax* from cryopreserved clinical isolates

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ABSTRACT

Plasmodium vivax is the most geographically widespread malaria parasite. Unique features of transmission biology complicate *P. vivax* control. Interventions targeting transmission are required for malaria eradication. In the absence of an *in vitro* culture, transmission studies rely on live isolates from non-human primates or endemic regions. Here, we demonstrate *P. vivax* gametocytes from both India and Brazil are stable during cryopreservation. Importantly, cryopreserved gametocytes from Brazil were capable of infecting three anopheline mosquito species in feedings done in the United States. These findings create new opportunities for transmission studies in diverse locales.

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Despite decades of research, malaria remains a major public health problem. Malaria is caused by the asexual replication of the parasite, *Plasmodium*, in the blood of infected individuals and is transmitted by *Anopheles* mosquitoes. *Plasmodium vivax* is the most geographically widespread of the human malaria parasites—infecting an estimated 200 million people annually (Gething et al., 2012). Two unique features of *P. vivax* transmission present specific challenges for disease control. A dormant liver stage (hypnozoite) can reactivate and cause disease months to years after the initial infective mosquito bite was received (Krotoski et al., 1982). Also, the transmissible stage (gametocyte) often develops prior to the onset clinical symptoms, allowing asymptomatic transmission of the parasite (reviewed in Mueller et al., 2009). Owing to these facts, development of therapies that

target the transmission and formation of hypnozoites will greatly enhance disease control.

Recent advances in liver biology and the development of microscale liver platforms that support the development of *P. vivax* have greatly enhanced the ability to study *P. vivax* liver stages (March et al., 2013; Maher et al., 2014; Ng et al., 2015; Mikolajczak et al., 2015). These advancements have placed new demands on the production of *P. vivax* sporozoites. In the absence of an *in vitro* culture system, studies on the transmission stages of the parasite are restricted to standard membrane feeding assays (SMFAs) using blood isolated from non-human primates that have been experimentally infected with *P. vivax* (Collins et al., 2009) or from human patients infected with *P. vivax* in disease endemic areas (Bharti et al., 2006). The availability, ethical and financial issues surrounding non-human primates constrict these experiments in non-endemic regions (Coleman, 2011). Further, studies with human parasites are limited by transmission season, the need to travel to endemic regions and the variation in infectivity of gametocytes from different patients (Bharti et al., 2006).

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Given these issues, we set out to create a source for sporozoites from human infections, independent of both transmission season and endemic locales. Importantly, *P. vivax* has been cryopreserved for over 30 years (Rossan, 1985) and therefore provided an invaluable resource for our study. Here we report that *P. vivax* gametocytes obtained from two different geographic locations are stable during cryopreservation and that new gametocytes can develop in short-term *in vitro* cultures. Moreover, cryopreserved samples were capable of infecting three diverse species of *Anopheles* mosquitoes in laboratories at great distances from collection sites. This potentially transformative technique could allow transmission of human *P. vivax* and the study of liver stages in locations separate from collection sites.

To determine whether gametocytes are capable of surviving cryopreservation, we collected six de-identified clinical isolates with mixed asexual and gametocyte stages at the Goa Medical College in Goa, India (Narayanasamy et al., 2012). We used standard protocols for cryopreservation and thawing. Prior to cryopreservation, clinical isolates were centrifuged at 800g for 5 min, plasma was aspirated and leukocytes were removed with CF11 (Sriprawat et al., 2009). Following washes with McCoy's 5A media (Sigma, USA), the packed volume (v) was estimated. Glycerolyte-57 (Fenwal-Baxter, USA), was added dropwise (until 1.667 volumes of v) to the sample with agitation. Samples were then distributed in 1 mL aliquots in cryovials (Falcon, USA). Cryovials were stored at −80 °C for 24 h before being transferred to liquid nitrogen. For thawing, cryovials were removed from the liquid nitrogen and rapidly thawed in a 37 °C water bath. The Glycerolyte-57 was removed using a two-step NaCl thawing procedure as in Noulin et al. (2012), omitting the final 0.9% NaCl step. The sample was washed with McCoy's 5A media supplemented with 20% AB+ heat-inactivated human serum (Interstate Blood Bank, Memphis, TN, USA). Gametocyte numbers were counted before cryopreservation and post-thaw (Fig. 1A). Although gametocyte numbers and stability vary between isolates, there were still appreciable levels of gametocytes remaining post-thaw (Fig. 1A)
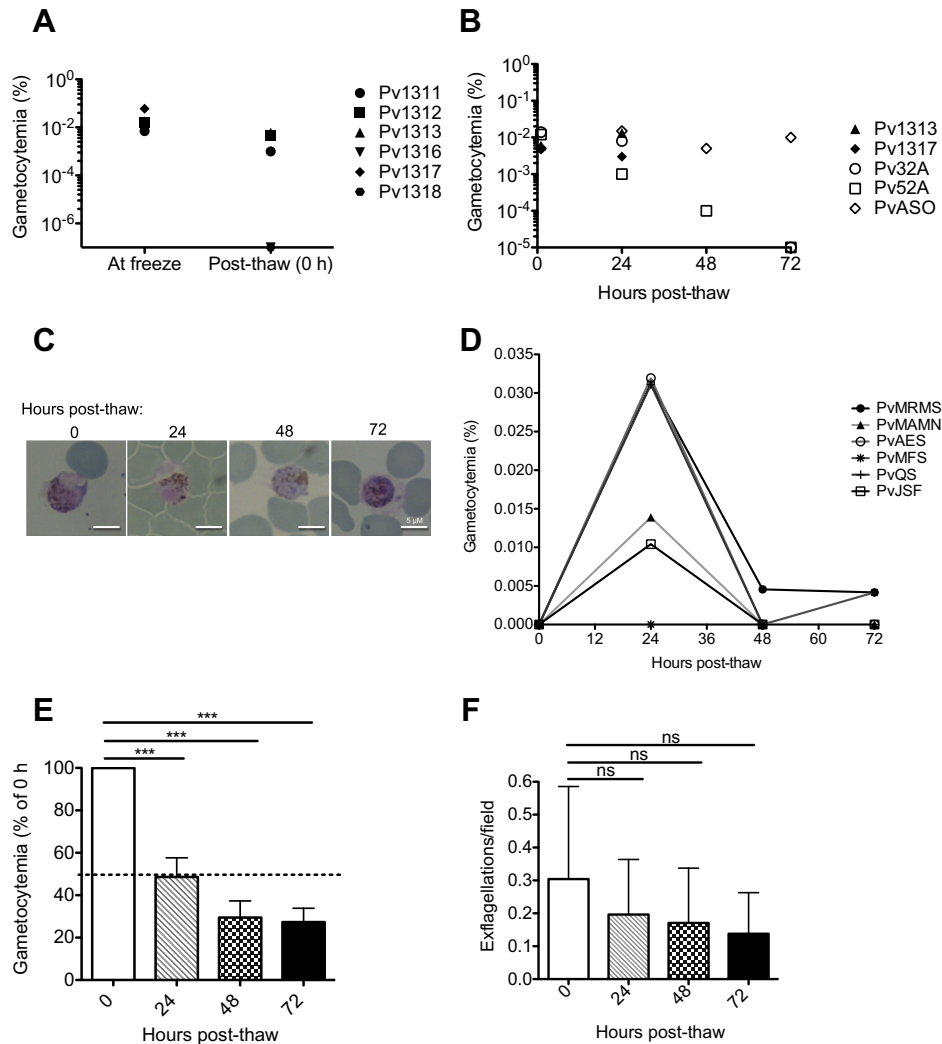

Fig. 1. Gametocytes from cryopreserved clinical isolates of *Plasmodium vivax* are stable and can exflagellate. (A) *Plasmodium vivax* clinical isolates from Goa, India were cryopreserved and thawed using traditional methods. The gametocytemia was assessed prior to cryopreservation and after thawing. While the gametocyte numbers decreased, we found that the gametocytes were able to survive the cryopreservation and thawing procedure. The difference in gametocyte numbers pre- and post-thaw is not statistically significant (two-way ANOVA, Bonferroni post-test, GraphPad v5). Moreover (B), we found that gametocytes were stable post-cryopreservation and new gametocytes could develop from the cryopreserved rings in both Goan isolates (Pv1313 and Pv1317) and from isolates from Acrelândia, Brazil (Pv32A, Pv52A, PvASO). (C) No overt differences were observed in the morphology of the thin smears of gametocytes at 0–72 h post-thaw. (D). The ability of cryopreserved rings to develop gametocytes *in vitro* was confirmed by removing the gametocytes, when thawed, on a magnetic column and checking the gametocytemia every 24 h post-thaw. Cryopreserved gametocytes were isolated and checked for their stability *in vitro* (E) and ability to exflagellate (F). Statistical significance was determined by a Tukey multiple comparison test. ns, not significant. ***$P < 0.005$.

and the difference between pre- and post-thaw was not statistically significant. This indicates that gametocytes could remain intact during the cryopreservation and thawing procedures, which has not been previously reported. Two isolates, Pv1313 and Pv1317, were also cultured for 24 h in complete McCoy's media (for 1 L: McCoy's 5A powder (Sigma), 50 mg of hypoxanthine (Sigma), 17.1 g of sucrose (Sigma), 109.6 mg of D-glucose (Sigma), 5.96 g of HEPES (Sigma), $1\times$ GlutaMAX (Gibco, USA), 2 g of sodium bicarbonate (Sigma), 20% AB+ heat-inactivated human serum) in flat-bottomed dishes (Falcon) at a 4–5% final hematocrit in a hypoxia chamber (Billups Rothenberg, USA) with $10\%O_2$, $5\%CO_2$, nitrogen balance.

Importantly, gametocytes remained stable in culture (Fig. 1B). In Pv1313, it appeared that new gametocytes developed from the cryopreserved rings as gametocyte numbers increased while in culture for 24 h (Fig. 1B). To confirm whether the observations made in India are broadly reproducible, we repeated these same experimental procedures using cryopreserved clinical isolates that were collected in Acrelândia, northwestern Brazil (da Silva-Nunes et al., 2006) and shipped from the University of Sao Paulo, Brazil to the infection facilities at the Harvard T.H. Chan School of Public Health, Boston, MA, USA. The samples were washed, leukocytes were removed and samples were cryopreserved as above. The cryovials were then transferred from liquid nitrogen to dry ice in Brazil and shipped frozen to Boston where those were stored in liquid nitrogen until required to be thawed. Encouragingly, we found that the *P. vivax* gametocytes from Brazilian clinical isolates were also able to survive cryopreservation and remained stable in short-term *in vitro* cultures for up to 72 h (Fig. 1B). No obvious differences were observed in morphology of the gametocytes when thawed and cultured *in vitro* (Fig. 1C). We observed not only that the gametocytes remained stable *in vitro* but also the potential development of new gametocytes from rings in sample PvASO (Fig. 1B). To determine whether gametocytes could develop from the cryopreserved rings *in vitro*, we thawed six Brazilian isolates and removed the gametocytes by running those through an LS-MACS magnetic column (Miltenyi, USA) immediately post-thaw according to Ribaut et al. (2008) and Vera et al. (2015) but with the addition of a 23 G needle to the end of the column to modulate the flow rate and maximise parasite binding. Gametocytes, which contain hemozoin, will bind to the magnetic column, while rings and uninfected cells will flow through (Ribaut et al., 2008). The flow-through (rings and uninfected cells) was collected and cultured as before. Smears were checked every 24 h for asexual parasitemia and gametocytemia. We found that gametocytes were able to develop *in vitro* with a peak at 24 h (Fig. 1D).

In some cases, (Fig. 1A–D), we observed exflagellation of cryopreserved gametocytes post-thaw. For a more robust analysis of gametocyte kinetics and exflagellation, we thawed 14 Brazilian isolates. As before, the gametocytes were specifically isolated on a magnetic column. The magnetic column was used in a room where the temperature was maintained at 37 °C to minimise exflagellation during the isolation procedure. Gametocytes were placed in an *in vitro* culture as before and gametocytemia and exflagellation were measured every 24 h using standard methods (Vera et al., 2015). Despite the gametocytemia dropping significantly over 72 h (Fig. 1E), we found that gametocytes were able to exflagellate immediately post-thaw and up to 72 h *in vitro* (Fig. 1F) without a significant decrease in the number of exflagellations observed. This may indicate gametocytes of varying ages can survive cryopreservation.

Given the stability of gametocytes during cryopreservation and their ability to exflagellate post-thaw, we next sought to determine whether the cryopreserved gametocytes could infect laboratory colonies of *Anopheles* mosquitoes held at the Boston facility. Colonies of an Indian (*Anopheles stephensi*), central American (*Anopheles*

albimanus, Santa Tecla strain) and African (*Anopheles gambiae* G3 strain) anopheline species were reared according to standard protocols (Baldini et al., 2013). For the infections, we used SMFAs (Bharti et al., 2006). Isolates were thawed and cultured as above. All reagents and pipettes were pre-warmed to 37 °C and samples were kept at 37 °C at all times to decrease the chances of premature exflagellation. Either post-thaw or following the short-term *in vitro* culture, the excess media was removed, ~20 μL (one drop from a 200 μL pipette tip) of fresh O+ blood was added and the isolates were resuspended to a final 40–50% hematocrit with heat-inactivated AB+ human serum. Female mosquitoes were starved overnight and placed in small cages in an isolated chamber. Clinical isolates were then placed in water-jacketed glass membrane feeders covered with Parafilm and female mosquitoes were allowed to feed for 20–30 min until the majority of the mosquitoes were engorged. Unfed females were removed. Mosquitoes were incubated at 26–28 °C. On day 8 post-feed, mosquitoes were dissected and evaluated for the presence of oocysts by staining the midguts with 2 mg/mL of Mercurochrome for 15 min. Images were obtained on a Zeiss microscope and analysed using ImageJ (Abràmoff et al., 2004).

Initial feeds with *A. stephensi* on three Brazilian clinical *P. vivax* isolates showed that oocysts could develop (Fig. 2A) and that the optimal time to feed post-thaw for both prevalence of infection and oocyst numbers was after a short-term *in vitro* culture for 24 h (Fig. 2B). This is in line with the peak of gametocyte development *in vitro* (Fig. 1D), gametocyte stability post-thaw (Fig. 1E) and exflagellation (Fig. 1F). We also tested two other species of laboratory colonies of *Anopheles* mosquitoes, *A. gambiae* and *A. albimanus*, on additional Brazilian clinical isolates. As with *A. stephensi*, both *A. gambiae* and *A. albimanus* supported oocyst development (Fig. 2C). Of the three *Anopheles* spp., *A. stephensi* exhibited the best success both in terms of oocyst numbers and overall prevalence (Fig. 2C) (statistically significant compared with *A. albimanus*). Although we observed variability in infectivity, the numbers of observed oocysts were in line with those found with live SMFAs in disease endemic countries (Bharti et al., 2006; Zollner et al., 2006; Basseri et al., 2008; Joshi et al., 2009; Bahia et al., 2011; Solarte et al., 2011; Rios-Velásquez et al., 2013; Mathias et al., 2014; Moreno et al., 2014; Vera et al., 2015).

Interestingly, we observed that slight modifications to the media for the short-term *in vitro* cultures could increase oocyst numbers. Our original feeds (Fig. 2B) were performed with media containing both antibiotics and glutamine. In early studies, we found that removal of the antibiotics and addition of a stabilised form of glutamine (GlutaMAX, Gibco), increased the mean oocysts from 0.423 to 0.81 and the range increased from 0–2 to 0–20 (Fig. 2B compared with Fig. 2C). We also observed that increasing gametocytemia at the time of feeding correlated with a higher number of oocysts (Fig. 2D) (Spearman correlation done using GraphPad Software v5). Taken together, both the health and overall numbers of gametocytes are essential for oocyst development.

We report here the first known observation that *P. vivax* gametocytes remain stable during cryopreservation and these gametocytes can infect geographically diverse *Anopheles* spp. in laboratories not located in from endemic regions. Although our initial study was not designed to assess sporozoite production, we observed the occasional formation of sporozoites in both oocysts and salivary glands at day 14 post-feed. We were not able to confirm whether the sporozoites were able to infect liver stages but these observations are promising. There are many points at which the system could be improved. We observed that there is a positive correlation between gametocyte density and oocyst numbers, which has been previously reported using SMFAs from live clinical isolates and laboratory colonies of *Anopheles* (Rios-Velásquez et al., 2013; Moreno et al., 2014). This suggests that if the overall number
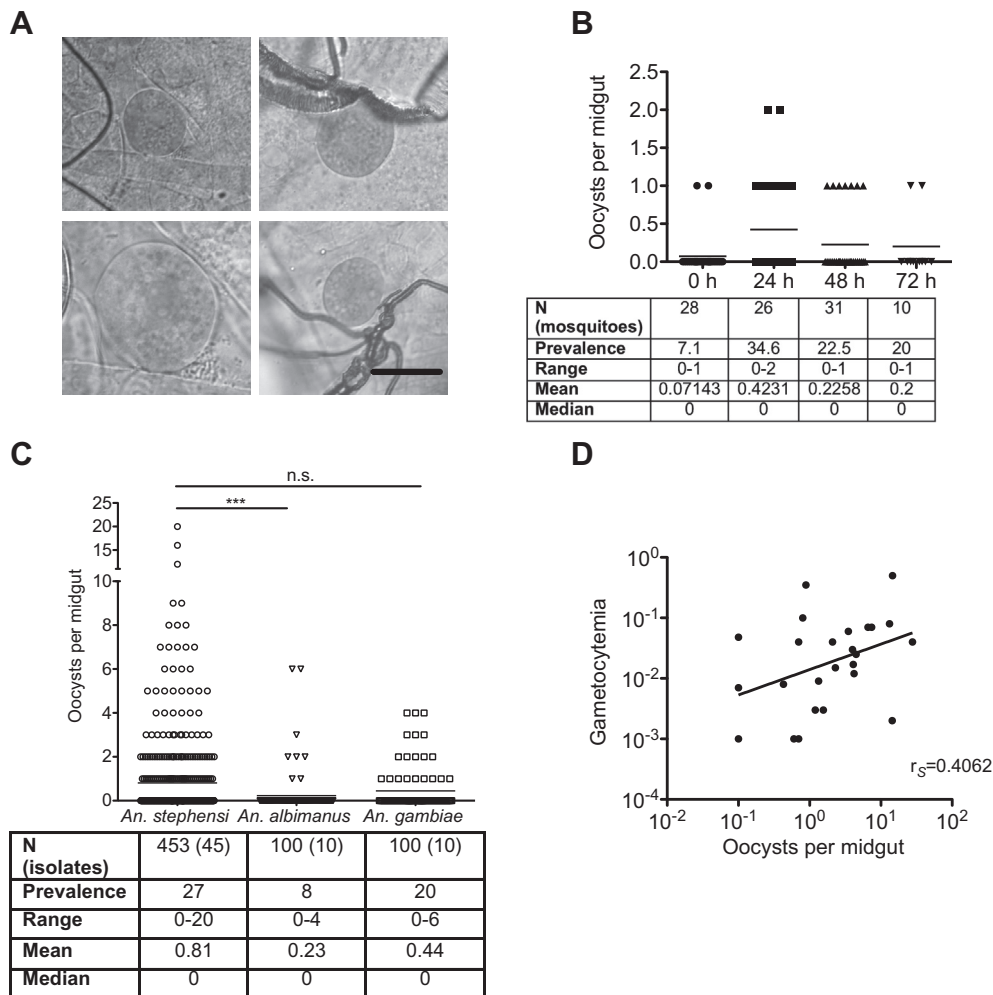
Fig. 2. Gametocytes can be transmitted to laboratory colonies of *Anopheles* mosquitoes. Given the stability of the gametocytes and the potential to develop post-thaw, we tested feeding laboratory colonies of *Anopheles* mosquitoes on cryopreserved *Plasmodium vivax* clinical isolates in our laboratory in Boston, MA, USA. Encouragingly, we found that oocysts could develop (A). Scale bar = 20 μM. The peak of oocyst development occurred when mosquitoes were fed on clinical isolates which had been thawed and short-term *in vitro* cultured for 24 h (B). Results are shown for three independent feeds of *Anopheles stephensi* with three unique Brazilian clinical isolates. Each dot represents a single mosquito. The summary table includes the total number (*N*) of mosquitoes dissected for all three isolates. Three different species of laboratory colonies of *Anopheles* mosquitoes were fed on independent *P. vivax* clinical isolates (C). Each dot represents the number of oocysts for a single mosquito. The solid line represents the mean. The summary table shows the number of mosquitoes (*N*) dissected with the number of clinical isolates shown in the parentheses. All isolates that were fed to either *Anopheles albimanus* or *Anopheles gambiae* were also fed to *A. stephensi*. In all cases, *A. stephensi* harboured higher numbers of oocysts in their midguts compared with the other two species (statically significant by a Mann–Whitney test, GraphPad v5). Oocyst numbers were correlated with gametocytemia (Spearman correlation, *P* = 0.0439), but not with the asexual parasitemia at the time of the feed (D). n.s., not significant.

of gametocytes that are successfully cryopreserved could be increased, we might be able to boost infections. In addition, we found pronounced donor-to-donor variability with respect to infectivity, which has been previously reported (Zollner et al., 2006). Ideally, blood from a single donor who presents with a high gametocytemia and gives mosquitoes infections which result in a large number of oocysts could be cryopreserved and used for subsequent experiments.

We found that three *Anopheles* spp. from a wide geographical range could be infected with the clinical isolates from Brazil, with the best competence in *A. stephensi*. This was surprising as it implies that parasites from endemic areas can be used with different laboratory mosquito colonies. However, since the oocyst numbers and overall prevalence were low, we plan to test geographically matched mosquitoes and parasites. A previous study using *P. vivax* infections in Manaus, Brazil found that three South American species, *Anopheles darlingi*, *Anopheles aquasalis* and *Anopheles albitarsis*, were highly susceptible to infection (Rios-Velásquez et al., 2013), suggesting matching the parasite

with its natural vector could increase prevalence and oocysts and sporozoite numbers. Additionally, inhibition of the mosquito immune response by knockdown of the JAK/STAT pathway could greatly enhance the number of *P. vivax* oocysts (Bahia et al., 2011). Taken together, our results open new avenues for the study of *P. vivax* infections.

Acknowledgements

References

Abràmoff, M.D., Magalhães, P.J., Ram, S.J., 2004. Image processing with imageJ. Biophoton. Int. 11, 36–41.

Bahia, A.C., Kubota, M.S., Tempone, A.J., Araújo, H.R.C., Guedes, B.a M., Orfanó, A.S., Tadei, W.P., Ríos-Velásquez, C.M., Han, Y.S., Secundino, N.F.C., Barillas-Mury, C., Pimenta, P.F.P., Traub-Csekö, Y.M., 2011. The JAK-STAT pathway controls *Plasmodium vivax* load in early stages of *Anopheles aquasalis* infection. PLoS Negl. Trop. Dis. 5, e1317.

Baldini, F., Gabrieli, P., South, A., Valim, C., Mancini, F., Catteruccia, F., 2013. The interaction between a sexually transferred steroid hormone and a female protein regulates oogenesis in the malaria mosquito *Anopheles gambiae*. PLoS Biol. 11, e1001695.

Basseri, H.R., Doosti, S., Akbarzadeh, K., Nateghpour, M., Whitten, M.M., Ladoni, H., 2008. Competency of *Anopheles stephensi* mysorensis strain for *Plasmodium vivax* and the role of inhibitory carbohydrates to block its sporogonic cycle. Malar. J. 7, 131.

Bharti, A.R., Chuquiyauri, R., Brouwer, K.C., Stancil, J., Lin, J., Llanos-Cuentas, A., Vinetz, J.M., 2006. Experimental infection of the neotropical malaria vector *Anopheles darlingi* by human patient-derived *Plasmodium vivax* in the Peruvian Amazon. Am. J. Trop. Med. Hyg. 75, 610–616.

Coleman, K., 2011. Caring for non-human primates in biomedical research facilities: scientific, moral and emotional considerations. Am. J. Primatol. 73, 220–225.

Collins, W.E., Sullivan, J.S., Strobert, E., Galland, G.G., Williams, A., Nace, D., Williams, T., Barnwell, J.W., 2009. Studies on the Salvador I strain of *Plasmodium vivax* in non-human primates and anopheline mosquitoes. Am. J. Trop. Med. Hyg. 80, 228–235.

Gething, P.W., Elyazar, I.R.F., Moyes, C.L., Smith, D.L., Battle, K.E., Guerra, C.A., Patil, A.P., Tatem, A.J., Howes, R.E., Myers, M.F., George, D.B., Horby, P., Wertheim, H.F., Price, R.N., Müeller, I., Baird, J.K., Hay, S.I., 2012. A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. PLoS Negl. Trop. Dis. 6, e1814.

Joshi, D., Choochote, W., Park, M.-H., Kim, J.-Y., Kim, T.-S., Suwonkerd, W., Min, G.-S., 2009. The susceptibility of *Anopheles lesteri* to infection with Korean strain of *Plasmodium vivax*. Malar. J. 8, 42.

Krotoski, W.A., Collins, W.E., Cogswell, F.B., Gwadz, R.W., Sinden, R., Koontz, L.C., 1982. Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection. Am. J. Trop. Med. Hyg. 31, 1291–1293.

March, S., Ng, S., Velmurugan, S., Galstian, A., Shan, J., Logan, D.J., Carpenter, A.E., Thomas, D., Sim, B.K., Mota, M.M., Hoffman, S.L., Bhatia, S.N., 2013. A microscale human liver platform that supports the hepatic stages of *Plasmodium falciparum* and *vivax*. Cell Host Microbe 14, 104–115.

Maher, S.P., Crouse, R.B., Conway, A.J., Bannister, E.C., Adams, J.H., Kyle, D.E., Saadi, W.M., 2014. Microphysical space of a liver sinusoid device enables simplified long-term maintenance of chimeric mouse-expanded human hepatocytes. Biomed. Microdev. 16, 727–736.

Mathias, D.K., Jardim, J.G., Parish, L.A., Armistead, J.S., Trinh, H.V., Kumpitak, C., Sattabongkot, J., Dinglasan, R.R., 2014. Differential roles of an anopheline midgut GPI-anchored protein in mediating *Plasmodium falciparum* and *Plasmodium vivax* ookinete invasion. Infect. Genet. Evol. 28, 635–647.

Mikolajczak, S.A., Vaughan, A.M., Kangwanrangsan, N., Roobsoong, W., Fishbaugher, M., Yimamnuaychok, N., Rezakhani, N., Lakshmanan, V., Singh, N., Kaushansky, A., Camargo, N., Baldwin, M., Lindner, S.E., Adams, J.H., Sattabongkot, J., Kappe, S. H.I., 2015. *Plasmodium vivax* liver stage development and hypnozoite persistence in human liver-chimeric mice. Cell Host Microbe 17, 526–535.

Moreno, M., Tong, C., Guzmán, M., Chuquiyauri, R., Llanos-Cuentas, A., Rodriguez, H., Gamboa, D., Meister, S., Winzeler, E.A., Maguina, P., Conn, J.M., Vinetz, J.E., 2014. Infection of laboratory-colonized *Anopheles darlingi* mosquitoes by *Plasmodium vivax*. Am. J. Trop. Med. Hyg. 90, 612–616.

Mueller, I., Galinski, M.R., Baird, J.K., Carlton, J.M., Kochar, D.K., Alonso, P.L., del Portillo, H.A., 2009. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. Lancet Infect. Dis. 9, 555–566.

Narayanasamy, K., Chery, L., Basu, A., Duraisingh, M.T., Escalante, A., Fowble, J., Guler, J.L., Herricks, T., Kumar, A., Majumder, P., Maki, J., Mascarenhas, A., Rodrigues, J., Roy, B., Sen, S., Shastri, J., Smith, J., Valecha, N., White, J., Rathod, P. K., 2012. Malaria evolution in South Asia: knowledge for control and elimination. Acta Trop. 121, 256–266.

Ng, S., Schwartz, R.E., March, S., Galstian, A., Gural, N., Shan, J., Prabhu, M., Mota, M. M., Bhatia, S.N., 2015. Human iPSC-derived hepatocyte-like cells support *Plasmodium vivax* liver-stage infection *in vitro*. Stem Cell Rep. 4, 348–359.

Noulin, F., Borlon, C., van den Eede, P., Boel, L., Verfaillie, C.M., D'Alessandro, U., Erhart, A., 2012. Cryopreserved reticulocytes derived from hematopoietic stem cells can be invaded by cryopreserved *Plasmodium vivax* isolates. PLoS One 7, e40798.

Ribaut, C., Berry, A., Chevalley, S., Reybier, K., Morlais, I., Parzy, D., Nepveu, F., Benoit-Vical, F., Valentin, A., 2008. Concentration and purification by magnetic separation of the erythrocytic stages of all human *Plasmodium* species. Malar. J. 7, 45.

Rios-Velásquez, C.M., Martins-Campos, K.M., Simões, R.C., Izzo, T., dos Santos, E.V., Pessoa, A.C., Lima, J.B., Monteiro, W.M., Secundino, N.F., Lacerda, M.V., Tadei, W. P., Pimenta, P.F., 2013. Experimental *Plasmodium vivax* infection of key *Anopheles* species from the Brazilian Amazon. Malar. J. 12, 460.

Rossan, R.N., 1985. Cryopreservation of the blood stages of *Plasmodium falciparum* and *Plasmodium vivax* for *in vivo* studies. Am. J. Trop. Med. Hyg. 34, 207–208.

da Silva-Nunes, M., Malafronte, R.D.S., Luz, B.D.A., De Souza, E.A., Martins, L.C., Rodrigues, S.G., Chiang, J.O., Vasconcelos, P.F., Muniz, P.T., Ferreira, M.U., 2006. The Acre Project: the epidemiology of malaria and arthropod-borne virus infections in a rural Amazonian population. Cad Saude Publica 22, 1325–1334.

Solarte, Y., Manzano, M.R., Rocha, L., Hurtado, H., James, M.A., Arévalo-Herrera, M., Herrera, S., 2011. *Plasmodium vivax* sporozoite production in *Anopheles albimanus* mosquitoes for vaccine clinical trials. Am. J. Trop. Med. Hyg. 84, 28–34.

Sriprawat, K., Kaewpongsri, S., Suwanarusk, R., Leimanis, M.L., Lek-Uthai, U., Phyo, A. P., Snounou, G., Russell, B., Renia, L., Nosten, F., 2009. Effective and cheap removal of leukocytes and platelets from *Plasmodium vivax* infected blood. Malar. J. 8, 115.

Vera, O., Brelaz, P., Albrecht, L., Monick, K., Pimenta, P.F.P., Monteiro, W.M., Lacerda, M.V., Lopes, S.C., Costa, F.T., 2015. A purification methodology for viable and infective *Plasmodium vivax* gametocytes compatible with transmission blocking assays. Antimicrob. Agents Chemother. 59, 6638–6641.

Zollner, G.E., Ponsa, N., Garman, G.W., Poudel, S., Bell, J.a, Sattabongkot, J., Coleman, R.E., Vaughan, J.A., 2006. Population dynamics of sporogony for *Plasmodium vivax* parasites from western Thailand developing within three species of colonized *Anopheles* mosquitoes. Malar. J. 5, 68.