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Advancing research models and technologies to overcome biological barriers to *Plasmodium vivax* control

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Abstract

Malaria prevalence has declined in the past 10 years, especially outside of Sub-Saharan Africa. However, the proportion of cases due to *Plasmodium vivax* is increasing, accounting for up to 90–100% of the malaria burden in endemic regions. Nonetheless, investments in malaria research and control still prioritize *Plasmodium falciparum* while largely neglecting *P. vivax*. Specific biological features of *P. vivax*, particularly invasion of reticulocytes, occurrence of dormant liver forms of the parasite, and the potential for transmission of sexual stage parasites prior to onset of clinical illness, promote its persistence and hinder development of research tools and interventions. This review discusses recent advances in *P. vivax* research, current knowledge of its unique biology, and proposes priorities for *P. vivax* research and control efforts.

Keywords

Malaria; *Plasmodium vivax*; research

The Case for *P. vivax*-specific Research Innovations

Soon after the turn of this century, the fight against malaria entered a new era in which elimination, and ultimately eradication, is the goal [1]. A concerted international commitment to provide the scientific, political, and financial means to achieve this objective has since shown remarkable progress, achieving a 41% decrease in global malaria incidence and a 62% decline in mortality from 2000 to 2015 [2]. These gains reflect efforts mobilized predominantly against *Plasmodium falciparum*, the most prevalent and deadly of the five *Plasmodium* species that can infect and commonly cause malaria in humans, while largely neglecting malaria caused by *Plasmodium vivax* and other species. However, *P. vivax* contributes to greater morbidity and has a wider geographic distribution than *P. falciparum* (Figure 1), with approximately 35% of the world's population (2.5 billion people) at risk of infection [3]. Furthermore, having shown an apparently slower decline in prevalence than *P. falciparum*, it has become the predominant cause of malaria in nearly all endemic regions

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outside of sub-Saharan Africa, where it accounts for 41% of all reported clinical cases [4]. Although the overall incidence of malaria is declining in these areas, the prevalence of cases due to *P. vivax* is increasing, particularly in countries in the pre-elimination or elimination phase [4]. *P. vivax* is therefore likely to persist as a barrier to malaria elimination and eradication without continued investments in the development of species-specific research tools and interventions.

Advances concerning basic research tools have enabled breakthroughs in our understanding of *Plasmodium* biology and the subsequent discovery and development of diagnostics, anti-malarial drugs, and vaccines. Establishment of *in vivo* animal models, laboratory colonization of mosquito vectors, and perhaps most importantly, *in vitro* culture of erythrocytic asexual and sexual stages, opened new frontiers and enabled study of malaria outside of endemic areas. This progress has been facilitated largely by a significant increase in research and development (R&D) funding in recent decades, from \$121 million in 1993 to \$610 million in 2011 [5]. However, *P. vivax* remains largely neglected, accounting for no more than 5% of malaria R&D funding compared to nearly half of all research funds being dedicated to *P. falciparum* [5].

Unsurprisingly, this has resulted in the development of research tools and control strategies designed for the elimination of *P. falciparum*. While much of the research on *P. falciparum* has direct application to other *Plasmodium* species many control interventions have limited or no effectiveness against *P. vivax*. As a consequence, our understanding of the unique aspects of *P. vivax* biology and development of specific interventions to tackle them has lagged significantly behind.

Biological Barriers to *P. vivax* Research and Development

The life cycle of *P. vivax* is in general like that of *P. falciparum* in that it requires a competent female *Anopheles* mosquito for transmission and a human host for its survival and propagation. However, *P. vivax* exhibits a number of specific attributes to support its persistence in more diverse ecological habitats, and which hinder research and control efforts (Table 1). Following an infectious mosquito bite, sporozoites are delivered into the dermal tissues where they penetrate small blood vessels, circulate to the liver, and begin pre-erythrocytic or liver stage growth. Within hepatocytes, parasites replicate via asexual reproduction, termed **schizogony** (see Glossary), over a period of 7–9 days, after which thousands of merozoites are released into the blood circulation where they then invade red blood cells (RBCs) and begin the erythrocytic, or blood-stage cycle of infection. Alternatively, *P. vivax* sporozoites can pause in early liver stage development as small dormant forms, called **hypnozoites** (see Glossary), and then awaken weeks to years after the initial attack to cause relapsing infections. Hypnozoite activation is thought to cause the majority of blood stage infections, up to 90% in some areas [6].

Within RBCs, parasites undergo asexual replication via schizogony in 48-hour cycles in established infections. Unlike *P. falciparum*, which can invade and develop in RBCs of all ages, *P. vivax* has evolved to preferentially invade **reticulocytes** (see Glossary). These cells occur more frequently in the bone marrow than in circulation, resulting in low-density or

sub-microscopic parasitemia in the peripheral blood that are often undetectable. A small proportion of merozoites differentiate into male and female gametocytes, which following ingestion by a mosquito during bloodfeeding, undergo sexual reproduction and generation of infectious sporozoites via **sporogony** (see Glossary), thus completing the entire parasite life cycle. In contrast to *P. falciparum* gametocytes, which undergo prolonged development over 10–12 days, *P. vivax* gametocytes develop rapidly, possibly with the first generation of merozoites released from the liver, and appear in the blood nearly simultaneously with asexual blood stage parasites [7]. Therefore, transmission to mosquitoes can occur prior to the onset of clinical illness. This ability of *P. vivax* to form infectious gametocytes quickly and continuously, coupled with activation of hypnozoites, promotes highly effective and persistent transmission that renders ineffective the strategies designed to control falciparum malaria. Here, we review recent advances in models and technologies to enable *P. vivax* research, with a focus on these unique biological features. We furthermore identify gaps where investments are needed to better target research for development of interventions to intensify control of, and possibly eliminate, *P. vivax* malaria.

***In Vitro* Cultivation of Erythrocytic Stages**

A reliable, long-term, continuous *in vitro* culture system for the *P. vivax* asexual cycle remains elusive, despite attempts to do so over more than 100 years. Whereas the ability of *P. falciparum* and *P. knowlesi* to invade and develop within RBCs of all ages enabled establishment of robust and reproducible protocols for their long-term cultivation [8–11], similar methods have proven inadequate to achieve reproducible, stable *in vitro* growth of *P. vivax* [12–15]. This is most likely related to the marked tropism of *P. vivax* for reticulocytes. Progress to characterize and obtain viable host cells and optimize culture conditions for *P. vivax* has been slow. These efforts continue to support *ex vivo* invasion assays and short-term growth, but fall short of a continuous long-term *in vitro* culture system needed for experimental laboratory-based research.

Obtaining and Characterizing Reticulocytes

Given the low concentration (0.5–1.5%) and short duration (~24 hours) of reticulocytes in circulating blood, maintaining sufficient abundance is a major challenge for *P. vivax* culture. The most successful attempt at *P. vivax in vitro* culture resulted in a doubling of the population of a non-human primate (NHP) adapted strain at each of eight cycles when cultured in McCoy's 5A medium with regular supplementation of reticulocytes enriched from the blood of hemochromatosis patients [15]. Attempts to reproduce this protocol have been unsuccessful [16]. More recently, fresh and cryopreserved reticulocytes enriched from leukocyte-depleted human cord blood have been used, with short-term success [17–19]; however, variability among reticulocytes and the need to have continuous access to hemochromatosis patients and newborns limit these approaches. While blood from these sources have relatively higher concentrations of reticulocytes compared to normal human blood, enrichment is a crucial step in all of these protocols, and the method employed is likely to affect the *in vitro* invasion and development of *P. vivax*. Data suggests that while differential centrifugation is more laborious, reticulocytes enriched using this technique

better support *P. vivax* invasion than density gradients [18, 20] or immuno-magnetic purification [15, 16].

More recent advances in regard to hematopoietic stem cells (HSC) provided a reproducible *in vitro* source of fresh reticulocytes to support *P. vivax* culture [21–23]. Relatively large quantities of HSCs can be enriched from human cord blood by magnetic microbead selection on the basis of their expression of either CD34 or CD133, and then used immediately or cryopreserved as aliquots for initiating new cultures as needed. CD34/CD133 HSCs cultured in the presence of specific cytokines and growth factors (stem cell factor, IL-3, hydrocortisone, erythropoietin), with or without co-culture with mouse stromal cells, can produce erythroid cell populations with up to 20% reticulocytes. Unfortunately, the many variables inherent in optimizing production of HSC-derived reticulocytes, as well as the high cost, have limited its use to support *P. vivax* culture. In addition, the presence of fetal hemoglobin within these HSC-derived reticulocytes and the low expression level of some surface receptors (e.g., DARC) may negatively impact parasite growth [17, 24].

It should be noted that reticulocytes are a heterogeneous population of erythrocytes that, once released into the peripheral circulation, undergo significant morphological, biochemical, and metabolic changes during their rapid maturation to **normocytes** (see Glossary). A recent report has demonstrated that clinical isolates of *P. vivax* preferentially invade immature reticulocytes expressing high levels of the transferrin receptor, CD71, which are typically restricted to the bone marrow [25]. This is a significant step forward in characterizing the preferred host cell for *P. vivax*, but identification of additional reticulocyte surface markers, particularly the receptor(s) for *P. vivax* invasion, would further advance these efforts. Since small numbers of very immature stages of reticulocytes are present in peripheral blood, continued efforts to cultivate *P. vivax* may benefit by further improving enrichment methods for the young subpopulation of reticulocytes that better support invasion.

Culture Conditions

Given the limited success to date of efforts to cultivate *P. vivax in vitro*, it is evident that optimal culture conditions have yet to be determined and are unlikely to be due to limited availability of reticulocytes. Historically, McCoy's 5A medium has better supported *P. vivax* multiplication than the RPMI 1640 medium that is used for *P. falciparum* and *Plasmodium knowlesi* culture, although the formulation of the two media differs only slightly in the concentrations of a few vitamins and amino acids. Addition of a more stable form of L-glutamine to McCoy's 5A medium was recently found to promote longer persistence of parasites *in vitro* [16], suggesting that *P. vivax* is sensitive to the byproducts (ammonia and pyrrolidone carboxylic acid) resulting from the spontaneous degradation of this amino acid. Reports on the benefit of shaking *P. vivax* cultures are conflicting [14, 15, 17], but given the fragility of mature *P. vivax* schizonts, shifting from static to shaking conditions at the appropriate time may improve parasite maturation, egress, and/or re-invasion [16]. Finally, a higher starting hematocrit (12% vs. 6%) better supports parasite development, and may reflect the need for a particular parasite or total cell density to promote cell to cell communication [16].

***In vivo* Models**

Non-human Primates

NHP models have been critical to *P. vivax* research for over 50 years, and remain highly relevant in the absence of an *in vitro* culture system. A number of *P. vivax* isolates from diverse endemic regions have been adapted to chimpanzees and New World monkeys of the genera *Aotus* (owl monkeys) and *Saimiri* (squirrel monkeys) [26, 27]. These models facilitate study of all *P. vivax* life stages, including hypnozoites [28], and provide a critical renewable source of parasite material for in-depth omics studies [29, 30], adaptation to *in vitro* culture (as described above), and perhaps most importantly, a reliable laboratory method for transmission to mosquitoes. NHP models are also useful for evaluating pre-erythrocytic [31–33], blood-stage [34–37], and transmission-blocking [38–40] candidate vaccines, as well as antimalarial drugs [41–44]. In recent years, numerous countries, including the United States, Australia, Japan, and New Zealand, and the European Union have banned or severely limited chimpanzee-based research. Large investments to further primate research, including the use of chimpanzees, in countries such as China and Thailand, present new opportunities for conducting *P. vivax* research [45]. However, in light of continuing ethical, financial, and availability issues surrounding the use of other primates, alternative *in vivo* models for *P. vivax* research should be pursued.

Humanized Mice

In recent years, several humanized mouse models, which are reliant on immunodeficient mouse strains to support xenotransplantation, have been developed to support studies on different stages of the *Plasmodium* life cycle. To date, only the NOD [46] and NSG [47] mouse models have been implemented for *P. falciparum* asexual and sexual stage [48] development. In terms of blood-stage development, these mice can support limited engraftment and differentiation of HSCs following treatment with, or transgenic expression of, human cytokines such as interleukin-3, erythropoietin, and thrombopoietin, which offers the potential that they may be suitable for the study of *P. vivax* [49]. In addition, clodronate-treated NSG mice are receptive to direct engraftment by intravenous injection of human RBCs to levels sufficient for support of *P. falciparum* infections [47, 50].

For pre-erythrocytic stage studies, the livers of multiple types of immunodeficient mice have been repopulated sufficiently with human hepatocytes to support *Plasmodium* liver stage development. The Alb-uPA liver-humanized mouse has been shown to be a suitable model for *P. falciparum* liver stage infection [51–53], while FRG KO huHep mice have also been successful in supporting *P. vivax* sporozoite infection, liver stage development, and hypnozoite formation [54, 55]. When backcrossed on the NOD background, FRG-NOD huHep mice further support the transition of *P. falciparum* pre-erythrocytic merozoites to blood stage infection [55]. The DRAG humanized mouse model also supports the complete *P. falciparum* life cycle, including transmission to mosquitoes [56], while TK-NOG humanized mice allow liver and blood stage development of both *P. falciparum* and *Plasmodium ovale* [57]. The presence of late-developing *P. ovale* schizonts in the livers of these mice suggests the eventual activation of quiescent parasites. Therefore, TK-NOG mice may also serve as a suitable model for *P. vivax* liver stage infection. Mouse models that

support humanized liver tissue engraftment and/or human reticulocytes could be crucial to furthering our understanding of hypnozoite biology and relapse, and erythrocytic host cell requirements for parasite invasion and growth.

Controlled Human Malaria Infections

Deliberate infection of patients with *P. vivax* began more than a century ago, with the development of malariotherapy to treat neurosyphilis [58]. Infections of prisoners from the 1940s to the 1970s provided a wealth of information on *P. vivax* biology, antimalarial efficacy, and immunization with irradiated sporozoites [59–62]. In recent years, **controlled human malaria infection** (CHMI; See Glossary) models for *P. vivax* have been developed in Colombia, the United States, and Australia. CHMI initiated via mosquito bite closely resembles natural infection [63–69], but relies on access to and production of infected mosquitoes or sporozoites, and risks hypnozoite formation and potential relapse. Thus, these studies will require pre-screening of participants for Duffy blood group, as well as glucose-6-phosphate dehydrogenase (G6PD) deficiency and intermediate metabolizer CYP2D6 phenotypes in order to avoid hemolysis induced by treatment with primaquine. While CHMI initiated with blood-stage parasites does not mimic natural *P. vivax* infection [70, 71], it circumvents these issues, and allows for control over parasite strain, inoculum size, and study location. Assuming the risks of *P. vivax* CHMI are managed effectively, it has the potential to provide a platform for early proof-of-concept drug and vaccine testing, as well as small, inexpensive Phase II efficacy trials.

Sexual Stages and Experimental Transmission

Studies on *P. vivax* gametocytes, mosquito stages, and transmission rely on either experimentally infected NHPs or human subjects, although moving forward, humanized mouse models may also be suitable for this purpose. Protocols have been established to infect mosquitoes by direct skin feeding [72], and isolated blood can be used in **membrane feeding assays** (see Glossary) to infect mosquitoes [73–78] and generate mosquito life stages, including gametes and ookinetes, *in vitro* [79, 80]. Given the historical reliance on chimpanzees for robust production of NHP-adapted *P. vivax* sporozoites [81–84], and the recent phase-out of chimpanzee-based research in most countries, it will be crucial to improve existing New World monkey models to maximize *P. vivax* transmission and sporozoite production. Development of protocols to infect mosquitoes with *P. vivax* following short-term culture of cryopreserved clinical isolates [85], and to cryopreserve infectious sporozoites [86–88], will help to overcome limitations surrounding NHP and human research and expand capabilities to investigate *P. vivax* transmission and liver stage biology in both *in vivo* and *ex vivo* models. When coupled with G6PD and CYP2D6 screening for CHMI as described above, such advances may also further expand our capabilities to explore novel approaches to *P. vivax* vaccination, including chemoprophylaxis with sporozoites, as has recently been demonstrated for *P. falciparum* [89].

In Vitro Models for Liver Stage Development and Hypnozoites

HepG2 [90] and HC04 [91] immortalized human hepatoma cell lines have successfully been used to study *P. vivax* liver stage infection and evaluate antimalarial drugs *in vitro*. However,

these hepatocytoma cells exhibit aberrant gene expression, signaling, metabolism, and abnormal cellular architecture that fail to adequately recapitulate that of normal primary human hepatocytes *in vivo*. Furthermore, to attain even low infection efficiencies (0.05–2%) requires substantial numbers of sporozoites, and excessive proliferation of both HepG2 and HC04 cells limits their use and prohibits long-term culture that is necessary to study hypnozoites. Use of primary human hepatocytes may solve these problems, but reports to date are that even in co-cultures optimized with supportive stromal cells, *P. vivax* infection efficiencies of hepatoma cell lines are poor [92, 93], and are highly variable among donors. Other promising modifications to *in vitro* cultures of primary human hepatocytes include the use of microfluidic devices with flow [94], or the use of hepatocyte-like cells derived from human induced pluripotent stem cells (iPSC) originating from human fibroblasts, which can be permissive to *P. vivax* [95] and may also support genetic modification of hepatocytes. Long-term *in vitro* cultivation of *Macaca fascicularis* primary hepatocytes with the closely related simian malaria parasite *Plasmodium cynomologi* has recently been reported to support hypnozoite persistence and reactivation for up to 40 days [96], and offers a viable alternative. These innovations further enable our ability to study *P. vivax* liver stage biology and reflect remarkable progress towards development of a high-throughput *in vitro* assay for discovery and evaluation of novel drugs that target pre-erythrocytic stages and hypnozoites.

Development of Genetic Tools

Completion of the first *P. vivax* reference genome sequence generated from the NHP-adapted Salvador I strain in 2008 [29] together with rapidly advancing genomics technologies, have led to a growing number of *P. vivax* whole genomes being sequenced. In-depth genomics approaches have provided new insights concerning *P. vivax* genetic diversity and population genetics, mechanisms of human RBC invasion [97–99], characterization of multi-gene families, parasite evolution [100], drug resistance [101], and relapse [102]. Recent advances in single-cell genomics [103, 104] and techniques for utilizing cryopreserved *Plasmodium* samples [105] provide a promising approach for detailed analyses of noncultivable parasites from complex multi-species or multi-clonal infections, which are common among *P. vivax* infections in humans and NHPs [105–107]. The known *P. vivax* genome has further facilitated development of platforms for immunoscreening [108–110] and protein-protein interactions [111] to identify novel vaccine candidate antigens, and provides a basis for transcriptomic [66, 112–115] and proteomic [30, 116, 117] studies.

Genetic Modification

Whereas *P. falciparum* research has greatly benefited from the use of genetic modification tools, development of *P. vivax* transgenic parasites has suffered from the lack of an *in vitro* culture system, low parasite densities observed in NHP and patient infections, and the existence of only a limited number of genetic markers and drugs for selection. Despite these challenges, significant steps have been taken towards rendering *P. vivax* more amenable to molecular investigation. Transient episomal expression of a luciferase reporter was achieved *ex vivo* using blood-stage parasites isolated from a *Saimiri* monkey [118]. More recently, zinc finger nucleases (ZFNs) have been used to stably introduce drug-resistance mutations

into the dihydrofolate reductase (DHFR) gene of *P. vivax* parasites *in vivo* in *Saimiri* monkeys [119]. Development of a stable transgenic fluorescent or luciferase-expressing *P. vivax* line that could be used in the aforementioned *in vivo* and *in vitro* models would greatly advance efforts to understand the unique features of *P. vivax* biology and will be fundamental for high-throughput drug and vaccine research.

Heterologous Expression of *P. vivax* Genes

In the absence of robust genetic modification systems, heterologous expression of *P. vivax* genes in other *Plasmodium* species, and even other organisms, can provide important information about parasite biology and permit evaluation of antimalarial drugs and candidate vaccine antigens. Episomal overexpression of the *P. vivax* chloroquine resistance transporter (PvCRT-O) in *P. falciparum* and *Dictyostelium discoideum* allowed for analyses of drug sensitivity and PvCRT function in chloroquine transport and accumulation [120]. Similarly, **double crossover-homologous recombination** (see Glossary) and **piggyBac transposition systems** (see Glossary) have been used to stably integrate wild type and mutant *P. vivax* DHFR alleles in *P. falciparum* [121] and *P. berghei* [122] to confirm the involvement of these mutations in pyrimethamine resistance. The rise of CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 nuclease)-based genome editing tools, which have recently been successfully established for *P. falciparum* [123–126], should be used to improve targeting efficiency and reduce the time required to stably modify target genes, potentially without the need for drug-selectable marker genes [127, 128]. Development of chimeric *P. berghei* expressing *P. vivax* circumsporozoite protein (CSP) [129, 130] and cell traversal protein for ookinetes and sporozoites (CelTOS) [131] genes has provided a mechanism for early *in vivo* evaluation of the safety and efficacy of candidate vaccine antigens that target these proteins. *P. falciparum* and *P. berghei* are quite genetically and evolutionarily distinct from *P. vivax*, and differences in gene expression, regulation [132], or function may limit the utility of these models.

Simian Models for *P. vivax*

To improve upon genetic modification strategies for *P. vivax*, there is opportunity to harness recent innovations for two closely related simian malaria parasites, *P. cynomolgi* and *P. knowlesi*. Development of a transgenic fluorescent *P. cynomolgi* reporter line using an episomal centromere has enabled imaging of live parasites in infected rhesus monkey blood, infected mosquitoes, *in vitro* liver stage assays, and purification of hypnozoites for further study [133]. *P. knowlesi* grown in either rhesus monkey [11] or human erythrocytes [10] is also genetically tractable, and homologous recombination techniques have recently been utilized to investigate erythrocyte invasion and remodeling by this parasite [134, 135]. It has been demonstrated that *P. knowlesi* recognizes regulatory sequences for other *Plasmodium* species and can be used to express heterologous constructs [136], including the *P. vivax* PHISTb gene, which was shown to be associated with the host cell cytoskeleton [137]. Recent efforts to optimize direct electroporation of *P. knowlesi* schizonts may also have direct application to the genetic transformation of *P. vivax* [138]. The adaptation of *P. knowlesi* to long term *in vitro* culture and the proven methods for its genetic modification provide new insights and should be applied to efforts for *P. vivax*.

Concluding Remarks and Future Perspectives

Overcoming decades of neglect, an international cadre of dedicated scientists has persisted, advancing research technologies and models for *P. vivax* research to uncover new facets of this parasite's unique biology with the goal of developing *P. vivax*-specific interventions. New opportunities abound, with recent advances having been made in stem cell research and characterizing reticulocyte invasion, introducing human and animal models for the complete life cycle, and investigating hepatocyte biology. There are clear priorities on which we should focus to expand upon existing tools and maximize our capabilities (**see Outstanding Questions Box**). Namely, we must 1) identify and source the preferred host cell for invasion and blood stage development, 2) recapitulate *in vitro* the hepatocyte phenotypes required to support pre-erythrocytic development and hypnozoite formation, 3) improve access to a renewable source(s) of single clonal *P. vivax* populations, and 4) establish genetic modification methods for stable parasite transfection.

The recent discovery that *P. vivax* merozoites have a predilection for a subset of immature, reticulocytes expressing high levels of CD71 that are largely restricted to the bone marrow has important implications for establishing an *in vitro* continuous culture system for this parasite. Further characterization of these cells, development of improved methods to obtain them, and clear definition of the culture conditions required to support *P. vivax in vitro* invasion, growth, and egress are clearly needed. However, while establishing a continuous culture system is correctly often described as the major challenge facing the *P. vivax* research community, development of CHMI, improved NHP and humanized mouse models, and especially *in vitro* hepatocyte culturing, provide a greatly expanded framework for conducting a plethora of *ex vivo* studies, including the evaluation of antimalarial drugs and vaccines. Renewable sources of well-characterized, single clonal populations of *P. vivax*, perhaps grown in NHPs, would allow us to better leverage these models. By further harnessing recent advances in genetics to develop transgenic fluorescent or luciferase-expressing *P. vivax* reporter lines, these models could support *in vivo* live imaging, particularly for the study of hypnozoites, and high-throughput assays for drug discovery and evaluation. The closely related *P. cynomolgi* and *P. knowlesi* provide suitable models to pursue investigations in the absence of *P. vivax*, including reticulocyte invasion and hypnozoite formation and reactivation. These endeavors will improve upon existing resources and provide essential new tools to advance *P. vivax* research and development efforts, and accelerate progress towards the elimination and eradication of this parasite.

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Glossary

Controlled human malaria infection (CHMI)

the intentional infection of human volunteers with *Plasmodium* by mosquito-bite or injection of sporozoites or blood-stage parasites

Double-crossover homologous recombination

A form of genetic modification in which foreign DNA with sequences identical to those flanking the target gene is introduced into the cell, causing the target gene DNA to be replaced with the foreign DNA sequence in between those homologous regions during replication

Hypnozoite

a persistent, non-growing and non-dividing uninucleate hepatic stage parasite observed in infections caused by relapsing species, such as *P. vivax*, *P. ovale*, and *P. cynomolgi*. Hypnozoites may remain dormant for weeks to years, and are apparently only sensitive to treatment with primaquine

Membrane feeding assay

a functional assay to measure transmission of *Plasmodium* parasites to mosquitoes. *Plasmodium*-infected blood is delivered to mosquitoes via an artificial membrane attached to a water-jacketed glass feeder that is maintained at 37°C via circulating water bath

Normocyte

amature erythrocyte that is normal in size, shape, and color

***piggyBac* transposition system**

A form of genetic modification in which the *piggybac* transposon can be mobilized between vectors and chromosomes via a cut and paste mechanism to target genes of interest located between specific inverted terminal repeat sequences (ITRs). The *piggybac* transposase recognizes the ITRs located on both ends of the transposon vector, removes the DNA sequence between them, and integrates it into TTAA chromosomal sites

Relapse

blood-stage infection resulting from activation of a hypnozoite(s) in the liver

Reticulocyte

an immature erythrocyte lacking a nucleus, and containing granular or reticular matter that can be stained with new methylene blue or Giemsa for identification

Schizogony

haploid asexual replication by multiple fission of the nucleus of the parasite within a host cell, followed by segmentation of the cytoplasm to give rise to multiple identical daughter cells. When occurring within the liver, a *Plasmodium* sporozoite gives rise to merozoites that are released into the bloodstream. During blood-stage infection, a merozoite gives rise to additional merozoites that maintain the erythrocytic cycle

Sporogony

formation of sporozoites within the mosquito vector during the sexual stage of the *Plasmodium* life cycle. Fusion of male and female gametocytes gives rise to a motile zygote,

or ookinete, that invades and traverses the mosquito midgut where it undergoes division by multiple fission within an oocyst, resulting in production of sporozoites

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Trends Box

- The predilection of *P. vivax* for immature red blood cells (RBCs), its ability to form hypnozoites, and early transmission of gametocytes are significant barriers to research and control
- Improved understanding of the preferred host cell for *P. vivax* invasion (reticulocytes expressing high levels of CD71) has enabled development of *ex vivo* assays and short-term growth, but long-term continuous culture remains elusive.
- Non-human primates (NHPs) remain the best model for *P. vivax* research, but controlled human malaria infections (CHMI), and pending further studies, humanized mice, are promising alternatives.
- In-depth genomics and systems biology approaches have provided new insight into *P. vivax* genetics, RBC invasion, multi-gene families, evolution, drug resistance, and relapse.
- Genetic modification of *P. vivax* remains challenging, but successes with *P. cynomolgi* and *P. knowlesi* may provide insight necessary to achieve this goal.

Outstanding Questions

- What are the primary receptors for *P. vivax* merozoite invasion of reticulocytes? Can subpopulations of cells expressing these receptors be identified and enriched from existing sources, or can stem cells be modified to express them in order to facilitate *in vitro* culture?
- Considering its similar pathobiology, can *P. cynomolgi* be adapted for blood-stage *in vitro* growth in human erythrocytes to provide insights into *P. vivax* biology and evaluate new therapies?
- Can *P. vivax* be further adapted to New World monkeys to produce robust, highly transmissible infections without the need for sporozoites derived from chimpanzee infections?
- Can humanized mice be engrafted with both primary human hepatocytes and CD71⁺ reticulocytes to levels that support liver-to-blood-stage transition of *P. vivax*?
- What hepatocyte phenotype(s) and growth conditions support improved *ex vivo* invasion efficiencies and long-term growth of *P. vivax*? Are these comparable to what is achieved in natural human infections?
- Can one or more of the laboratory models validate early transmissibility to mosquitoes, especially whether first-generation merozoites emerge out of the liver develop into infectious gametocytes.
- Can a repository of different *P. vivax* strains consisting of a single, genetically clonal population of parasites be established to allow for routine use in experimental models?
- Can advances in genomics, particularly single-cell approaches, be utilized to decipher the basis of unique *P. vivax* biology, namely determinants of hypnozoite formation, early transmission, and reticulocyte invasion, for development of more *vivax* effective targeted therapies?
- Can transfection methods developed using the closely related *P. knowlesi* and *P. cynomolgi* be adapted to genetically modify *P. vivax*? Can we develop alternative drug-selectable markers that will facilitate selection of genetically modified *P. vivax in vivo*?

Figure 1. The spatial distribution of *Plasmodium falciparum* and *Plasmodium vivax* malaria in 2010.

A) Estimates of the age-standardized annual mean *P. falciparum* parasite rate in two to ten year olds (*PfPR*_{2–10}), within the spatial limits of stable transmission, stratified into four levels of risk. Areas of no risk and unstable risk (*PfAPI* < 0.1%) are also shown [139]. B) Mean point estimates of the age-standardized annual mean *P. vivax* parasite prevalence in two to ten year olds, within the spatial limits of stable transmission. Areas of no risk and unstable risk are also shown. Areas where Duffy negative prevalence was estimated as 90% are hatched to provide additional context for the impact of *P. vivax* on the local population within these areas [3]. Maps downloaded from the Malaria Atlas Project <http://www.map.ox.ac.uk/> and reproduced under the Creative Commons Attribution 3.0 Unported (CC BY 3.0) license (<https://creativecommons.org/licenses/by/3.0/>).

Table 1.

Biological features and available research tools for *P. vivax* as compared to other human and simian *Plasmodium* species.

	Human		Simian	
	<i>P. vivax</i>	<i>P. falciparum</i>	<i>P. cynomolgi</i>	<i>P. knowlesi</i>
Pre-erythrocytic cycle				
Hypnozoites	Yes	No	Yes	No
Erythrocytic cycle				
Duration (h)	48	48	48	24
Host cell	Reticulocytes	Normocytes, Reticulocytes	Reticulocytes	Normocytes, Reticulocytes
Gametocytes				
Time to maturation	2–3d	12–14d	58h	31h
Lifespan	3d	2.5d	12h	5h
<i>In vitro</i> cultivation				
Asexual blood stages	Short-term <i>ex vivo</i>	Continuous <i>in vitro</i>	Short-term <i>ex vivo</i>	Continuous <i>in vitro</i>
Gametocytes	No	Yes	No	No
Liver stages	Yes	Yes	Yes	No
<i>In vivo</i> models				
Old World monkeys	No development	Complete	Complete	Complete
New World monkeys	Partial (no hypnozoites)	Complete	Partial (no gametocytes)	Partial (no gametocytes)
Humanized mice	Liver stage	Complete	No	No
CHMI	Yes	Yes	No	No
Research tools				
Genetic modification	Episomes, Point mutations	Episomes, mutagenesis, homologous recombination	Episomal centromeres	Episomes, homologous recombination