The Biology of *Plasmodium vivax*

John H. Adams¹ and Ivo Mueller²

¹Center for Global Health and Infectious Diseases, Department of Global Health, University of South Florida, Tampa, Florida 33612

²Population Health & Immunity Division, Walter & Eliza Hall Institute, Parkville, Victoria 3052, Australia

Correspondence: jadams3@health.usf.edu; ivomueller@fastmail.fm

Plasmodium vivax is the second most prevalent cause of malaria worldwide and the leading cause of malaria outside of Africa. Although infections are seldom fatal clinical disease can be debilitating and imposes significant health and economic impacts on affected populations. Estimates of transmission and prevalence intensity can be problematic because many episodes of vivax originate from hypnozoite stages in the liver that have remained dormant from previous infections by an unknown mechanism. Lack of treatment options to clear hypnozoites and the ability to infect mosquitoes before disease symptoms present represent major challenges for control and eradication of vivax malaria. Compounding these challenges is the unique biology of *P. vivax* and limited progress in development of experimental research tools, thereby hindering development of new drugs and vaccines. Renewed emphasis on vivax malaria research is beginning to make progress in overcoming some of these challenges.

Vivax malaria accounts for 14–80 million cases of clinical malaria each year with more than 70% of infections in Asia and the Americas (Mendis et al. 2001a; Price et al. 2007; WHO 2015). The last 10 years have seen a dramatic reduction in the burden of malaria, with many countries in the Asia-Pacific and the Americas with reductions of >90% in the number of clinical cases (WHO 2015). As a consequence, 34 countries are actively attempting to eliminate malaria and the leaders of Central American and East Asian countries have declared their intention to eliminate malaria from their regions by 2025 and 2030, respectively (PAHO 2013; APLMA 2014). In parallel to this reduction in overall incidence, a pronounced shift in species composition has been

observed with *Plasmodium vivax*, now the predominant *Plasmodium* spp. in the vast majority of countries outside Africa, and is generally seen as the major obstacle to achieving malaria elimination in Asia and the Americas. The challenges in controlling and eliminating vivax malaria are likely to be related to the following aspects of *P. vivax* biology: (1) its ability to relapse from long-lasting, dormant liver stages (the hypnozoites) (White and Imwong 2012), and (2) its high transmission potential caused by early and continuous production of gametocytes, high infectivity to mosquitoes, and shorter development cycle in the vector host compared to other *Plasmodium* spp. (Mueller et al. 2009). These characteristics facilitate *P. vivax* transmission by seasonal mosquito vectors and at lower am-

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bient environmental temperatures, thus extending the global distribution of *P. vivax* into temperate regions (Fig. 1) (Gething et al. 2011, 2012).

Although it has often been regarded as causing a benign self-limiting infection, there is increasing evidence that the overall burden, economic impact, and severity of disease from *P. vivax* have been underestimated (Price et al. 2007). Vivax malaria is known to incapacitate individuals of all ages resulting in repeated febrile episodes, severe anemia, respiratory distress, and, sometimes, poor outcomes in pregnancy. Increasing reports of clinical severity with complicated and lethal cases of vivax malaria, thus, challenge the perception of vivax malaria as benign (Anstey et al. 2012; Baird 2013). The increasingly widespread resistance to chloroquine, the primary frontline drug to treat vivax malaria (Baird et al. 1991; Ruebush et al. 2003; Teka et al. 2008; Ketema et al. 2009; Mohan and Maithani 2010; Rijken et al. 2011) represents an additional challenge to existing control and prevention programs for vivax malaria and highlights the critical need for more efforts toward development of new tools specifically targeting *P. vivax*.

Besides additional drugs targeting the dormant liver stages, the development of a vaccine against vivax malaria is especially important (Mueller et al. 2015). The rapid natural acquisition of immunity to *P. vivax* (Mueller et al. 2013) indicates that the development of *P. vivax* vaccines should be feasible. Nevertheless, understanding correlates of protective immunity is as challenging for vivax malaria as it is for *Plasmodium falciparum*, because in most endemic areas, transmission is intermittent, with lack of sustained acquired immunity. Immune responses to the *P. vivax* vaccine candidate antigen, Duffy-binding protein (DBP), are often weak, short-lived, and biased toward strain-specific alleles (VanBuskirk et al. 2004; Ceravolo et al. 2009; Cole-Tobian et al. 2009; Chootong et al. 2010), which may be due, in part, to a high level degree of allelic diversity. Therefore, an effective vaccine against vivax malaria may face a greater challenge to have a substantial level of immunity against diverse strains.

In this article, we review the unique challenges associated with *P. vivax* experimental research, recent advances overcoming these obstacles, and breakthroughs needed to effectively deal with vivax malaria.

THE UNIQUE BIOLOGY OF *P. vivax*

Significant differences in the biology of *P. vivax* compared to *P. falciparum* are obvious by simple examination of a Giemsa-stained blood smear (Fig. 2) (Coatney et al. 1971). A typical parasitemia in vivax malaria is relatively low, which is attributed to restricted infection of only reticulocytes, often necessitating the use of thick smears to concentrate the blood for reliable diagnosis (Galinski and Barnwell 2008). Although not fixing the blood significantly increases sensitivity, it comes at the cost of retaining good morphology, which can present a challenge for parasite identification for nonexpert microscopists. Important for distinguishing different *Plasmodium* species is the observation of unique morphological features, such as the small dark granules in the reticulocyte cytoplasm, known as Schüffner's dots, representing caveola-vesicle complexes of parasite origin exported into the cytoplasm of the infected reticulocyte (Aikawa et al. 1975; Udagama et al. 1988), which increase in abundance as *P. vivax* develops. A second distinctive morphological feature for *P. vivax* is sexual-stage parasites have a round shape similar to the asexual stages. Finally, all *P. vivax* stages of blood-stage development can be observed in the peripheral blood.

The ability of *P. vivax* to remain in circulation is thought to be possible because of the increased plasticity of infected reticulocytes that is observed as the parasites develop, which is in contrast to the increased rigidity that occurs for *P. falciparum*-infected erythrocytes (Suwanarusk et al. 2004). Increased deformability is needed to periodically squeeze through the splenic chords and thereby evade removal from circulation (Handayani et al. 2009). Infected red blood cell (RBC) rigidity for *P. falciparum* is linked to cytoadherence and sequestration, but PfEMP1 knob proteins that are the key mediator of antigenic variant are absent in *P. vivax*.

Figure 1. The spatial global distribution of *Plasmodium vivax* malaria endemicity in 2010. Mean point estimates of the age-standardized annual *P. vivax* parasite prevalence in 2- to 10-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 greater than or equal to 90% are hatched to provide additional context for the impact of *P. vivax* on the local population within these areas. Areas in light gray: *P. vivax* free; dark gray: unstable transmission; hatched marks: unstable transmission and high Duffy negative. Areas from red to light blue indicate $PvPR_{1-99} > 7\%$ to 0%, respectively. (From Gething et al. 2010; reprinted under the Creative Commons Attribution 3.0 Unported License.)

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Figure 2. Light microscopic images of Giemsa-stained developmental stages of *Plasmodium vivax* during the blood-stage asexual cycle from early to late stages (left to right, respectively).

Antigenic variation originally described in the closely related vivax-like primate malaria *Plasmodium knowlesi* (Brown and Brown 1965) and is likely common to most, if not all, malaria parasites that are mediated by a large family of small surface variant antigens (Jemmely et al. 2010). In *P. vivax*, the VIR protein products of the *vir* multigene family are proposed to be the key molecule for antigenic variation in *P. vivax* (del Portillo et al. 2001). More recently, VIR proteins expressed on the reticulocyte surface are suggested to have a role mediating cytoadherence to endothelial cells and placenta, albeit much weaker than for *P. falciparum* (del Portillo et al. 2001; Fernandez-Becerra et al. 2005). Therefore, the lack of knobs and a strong sequestration mechanism does not mean that there is a lack of antigenic variation and sequestration, although cytoadherence to endothelium is less prevalent and possibly occurring only in more virulent infections (del Portillo et al. 2001; Fernandez-Becerra et al. 2005; Carvalho et al. 2010; Chotivanich et al. 2012). The lack of robust continuous culture methods have greatly hampered laboratory-based experimental studies of this and other unique biological properties of *P. vivax*.

In addition to their round morphology, *P. vivax* gametocytes are noteworthy for their very early appearance during the course of infection. In part, this may be caused by a rapid development time with gametocytes often detected within 3 days after the first asexual parasites are observed (Bousema and Drakeley 2011). In controlled experimental infections gametocytes appeared in the peripheral blood within 7 days of direct *P. vivax* sporozoite infection (Vallejo et al. 2016), indicating that in most natural infections mosquito transmission can

occur before onset of clinical symptoms. Although early transmissibility can lead to rapid transmission with outbreaks spreading quickly, a side benefit attributed to this phenomenon is limiting the rapid spread of drug resistance for *P. vivax*. It is important to note that because transmission usually occurs before infections are treated, an effective transmission-blocking vaccine would help contain relapse infections that result from radical cure treatment failures (Douglas et al. 2013).

The key distinguishing feature of *P. vivax* biology is, however, its ability to form long-lasting liver stages, the hypnozoites (Krotoski et al. 1982). On entry into the liver, *P. vivax* sporozoites can take two distinct pathways. Some develop directly into liver-schizonts, which after 8 days, release merozoites to initiate the asexual cycle in the blood, although others, the hypnozoites, “arrest” their development beginning about the third day after infection (Coatney et al. 1971; Mikolajczak et al. 2015). Although hypnozoites are probably metabolically active, they do not divide and remain dormant for weeks if not months before reactivating. The frequency and timing of these relapses varies globally (White 2011), with the longest relapse periods present in temperate strains (Battle et al. 2014), indicating that relapse periodicity is under natural selection (White et al. 2016). The mechanisms underlying both commitment to dormancy and reactivation are poorly understood. However, it is increasingly clear that relapses from emerging such hypnozoites may contribute up to 80% of all *P. vivax* blood-stage infections (Betuela et al. 2012; Robinson et al. 2015). As there are no diagnostic tests that can diagnose dormant liver-stage infections, undetectable hypnozoite carriers are an important

potential source of reintroduction of *P. vivax* and, if not treated appropriately, can cause new blood-stage infections months after the primary infection. Unfortunately, the only currently available drug to clear hypnozoites is primaquine, which can cause severe hemolysis in people with glucose 6-phosphate dehydrogenase (G6PD) deficiency (Baird et al. 2012). Given the lack of a cheap, reliable point-of-care G6PD test, many *P. vivax* patients do not currently receive adequate antihypnozoite treatment (Baird 2015).

GENETIC DIVERSITY/POPULATION OF *P. vivax*

Although its closest non-ape *Plasmodium* spp. are parasites of Southeast Asia monkeys, *P. vivax* is now thought to have originated in Africa with its closest relatives still circulating in African chimpanzee and gorilla populations (Liu et al. 2014). *P. vivax* shows a higher degree of genetic diversity and more global structure than *P. falciparum*. Comparative analyses revealed that *P. vivax* genomes show twice as much single-nucleotide polymorphism (SNP) diversity as *P. falciparum* and also have high microsatellite and gene family variability (Neafsey et al. 2012). Although *P. falciparum* microsatellite diversity is closely associated with regional levels of endemicity, *P. vivax* shows a high genetic diversity across all endemicities (Barry et al. 2015).

The global *P. vivax* population is nevertheless more strongly structured than that of *P. falciparum*. A comparative analyses of microsatellite diversity in a global panel of 841 *P. vivax* isolates showed very strong geographical structure with clearly distinct populations in South America, Africa and India, Southeast Asia, and the Southwest Pacific (Koepfli et al. 2015b). Similar global structure is also evident in sequences encoding for vaccine candidate antigens (Arnott et al. 2013) and has recently been confirmed using genome-wide SNPs (Hupalo et al. 2016; Pearson et al. 2016).

Contrary to the pronounced global structure, most studies found limited or no population structure at local or regional scale (Imwong et al. 2007; Getachew et al. 2015), even in areas

where *P. falciparum* did show substantial population structure (Jennison et al. 2015; Noviyanti et al. 2015). This is likely to be caused by the generally higher genetic complexity of *P. vivax* infections (Barry et al. 2015), which increase the likelihood of genetic recombination and result in large effective population sizes. The exception to this pattern is found in the Americas, where pronounced local population structure is observed (Ferreira et al. 2007; Van den Eede et al. 2010; Delgado-Ratto et al. 2016), despite high continent-wide genetic diversity. This pattern is thought to be a reflection of both multiple introduction and more recent bottlenecks caused by intensified control.

Together, these patterns suggest a capacity for greater functional variation, a more distinct history of global colonization and a higher ability to maintain genetic diversity in the face of intensifying control efforts. More detailed studies of global and local *P. vivax* diversity are required to develop tools that will allow examination of the impact of control and elimination programs on the parasite gene pool. Of particular importance for local elimination would be to develop tools that allow for differentiation of locally acquired infections from imported *P. vivax* infections.

THE ROLE OF ASYMPTOMATIC INFECTIONS

Although *P. vivax* has a lower pyrogenic threshold than that of *P. falciparum* (Hemmer et al. 2006), asymptomatic *P. vivax* infections are very common at all levels of endemicity and often account for 90%–100% of all infections detected in cross-sectional surveys (Anstey et al. 2012). The vast majority of these *P. vivax* infections are of very low density, with submicroscopic infections accounting for 67% of all infections (Cheng et al. 2015). This is significantly higher than for *P. falciparum* in the same studies.

Several factors are thought to contribute to this very high rate of asymptomatic infections. Most importantly, clinical immunity to *P. vivax* infections is acquired rapidly. When malaria therapy patients were experimentally infected with *P. vivax*, effective clinical immunity, even

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against heterologous challenge, was often attained after as few as one to five *P. vivax* infections (Mueller et al. 2013; Snounou and Perignon 2013). Under natural exposure, clinical immunity to *P. vivax* is also acquired significantly more rapidly than to *P. falciparum*, both in high transmission settings such as Papua New Guinea, where the incidence of *P. vivax* malaria starts declining in the second year of life (Lin et al. 2010) and clinical disease is virtually absent in children >5 years, although *P. falciparum* episodes are common in primary school children (Michon et al. 2007), as well as in lower transmission settings such as Thailand (Phimphaphi et al. 2008), Sri Lanka (Mendis et al. 2001b), and Vanuatu (Maitland et al. 1996). With little evidence for naturally acquired immunity against (preerythrocytic) infections (Mueller et al. 2013), these patterns are indicative that, even in low transmission settings, semi-immune individuals develop effective blood-stage immunity characterized by a very good control of blood-stage parasitemia, which results in significant numbers of submicroscopic infections. The main driver for this rapid acquisition of immunity is the higher force of infections (Koepfli et al. 2013), caused largely by genetically distinct but related relapses (Bright et al. 2014) that account for up to 80% of all *P. vivax* blood-stage infections (Robinson et al. 2015).

The role of these asymptomatic infections in maintaining *P. vivax* transmission is not yet well understood. Most, if not all, of these asymptomatic infections produce gametocytes (Wampfler et al. 2013; Barbosa et al. 2014) with gametocyte densities closely correlated with overall parasitemia levels and thus at low levels (Koepfli et al. 2015a). Nevertheless, the asymptomatic cases are infectious to anopheline vectors, albeit at a substantially lower level than clinical cases (Pethleart et al. 2004; Alves et al. 2005), and may contribute substantially to maintaining *P. vivax* transmission given the high prevalence of the “silent” infections.

These asymptomatic infections may thus be a major impediment to the elimination of *P. vivax* malaria as they may not be efficiently cleared unless specifically targeted with active

case detection and/or mass drug administration. Such a strategy is logistically challenging and would again require treating large numbers of individuals with asymptomatic infections, even those without detected blood-stage infections, with primaquine to clear the hypnozoite reservoir.

ADVANCES IN ENABLING TECHNOLOGIES TO SUPPORT *P. vivax* RESEARCH

The unique biology of *P. vivax* has presented major challenges for applying modern approaches in fundamental and translational research. In particular, the blood-stage requirement to infect reticulocytes and the hypnozoite liver stage have been difficult obstacles to overcome for laboratory-based research studies, thereby limiting most research efforts to using *P. vivax* from clinical isolates or nonhuman primate infections. In recent years, greater efforts from multiple laboratories have begun to provide new approaches to create more useful enabling technologies for research studies on the liver stages (Dembale et al. 2011; March et al. 2013; Mikolajczak et al. 2015). Unfortunately, similar success for developing a robust continuous culture system for blood-stage parasites has not been as successful with capabilities being limited primarily for short-term assays (Russell et al. 2011; Noulin et al. 2013; Roobsoong et al. 2015).

Because reticulocytes rapidly develop to mature erythrocytes at 37°C, a reticulocyte’s half-life is shorter than the blood-stage asexual developmental cycle time. Therefore, reticulocytes must be replenished as each new generation matures or there is nothing for the parasites to infect. An important strategy to overcome the reticulocyte problem was exemplified in the Golenda study (Golenda et al. 1997). This 19-year-old study from the Walter Reed Army Institute of Research remains the gold standard for sustained growth of in vitro blood-stage culture of *P. vivax*. In the Golenda study, blood-stage parasites were grown for six to eight asexual cycles using an inventive, but complex labor-intensive culturing protocol not easily replicated. The success of the Golenda study was likely because

of a combination of variables that included use of the primate-adapted Chesson strain, obtaining reticulocytes from hemochromatosis patients undergoing traditional bloodletting treatments, which induces a reticulocytopenia, and differential centrifugation methods for reticulocyte enrichment. Other important handling features of the protocol included exposure of the static cultures to intermittent agitation along with periodic addition of fresh reticulocytes. Of these variables, many subsequent studies identified the critical limiting factor for *P. vivax* blood-stage culture to be sustaining reticulocyte levels. Consequently, studies now include regular addition of relatively pure reticulocyte preparations so as to not dilute the culture with mature erythrocytes that cannot be invaded by *P. vivax*.

Traditional sources of reticulocytes to support *P. vivax* culture for most researchers have been placenta cord blood and adult peripheral blood, which are both readily available (Noulin et al. 2013). In both cases, reticulocytes constitute a subset of the total erythroid cell population and must be enriched (Udomsangpetch et al. 2007; Kumar et al. 2015). Over time, protocols have evolved to enable enrichment of nearly pure populations of reticulocytes from both of these traditional sources to overcome a historical limitation of attempts to establish *P. vivax* blood-stage cultures. Parallel to improvements in traditional approaches for enriching reticulocytes were the improvements and lowered costs in the hematopoietic stem cell (HSC) ex vivo production methods for creating reticulocytes (Douay and Giarratana 2005; Giarratana et al. 2005). These HSC reticulocytes have provided a third source, which also revealed the capacity of *P. vivax* to infect nucleated erythroid progenitor cells (Panichakul et al. 2007; Noulin et al. 2014). In addition, ex vivo reticulocytes produced from HSC can be experimentally manipulated to tease apart specific molecular interactions required for parasites infection (Crosnier et al. 2011; Egan et al. 2015). Despite being able to sustain abundant reticulocytes in vitro, a concomitant success in maintaining continuous long-term *P. vivax* culture has not been achieved yet, indicating factors

beyond just reticulocytes are required to sustain parasites in vitro (Martin-Jaular et al. 2013; Roobsoong et al. 2015). Nonetheless, the value of studies reliant on traditional sources of *P. vivax* have improved considerably for support of fundamental and translational research (Price et al. 2010; Rijken et al. 2011; Marfurt et al. 2012; Lee et al. 2014; Malleret et al. 2015; Cho et al. 2016).

The basic capability to study complete in vitro development of *P. vivax*, *P. falciparum*, and other malaria parasites with primary hepatocytes and hepatocytoma cells has been established for many years (Mazier et al. 1984; Sattabongkot et al. 2006). With the increased awareness of the force of infection in *vivax* malaria caused by relapse (Betuela et al. 2012; Robinson et al. 2015), along with emergence of chloroquine resistance in many endemic regions (Baird et al. 1991; Ruebush et al. 2003; Teka et al. 2008; Ketema et al. 2009; Mohan and Maithani 2010; Rijken et al. 2011), there has been a renewed effort to develop higher throughput, more efficient, and longer duration systems for liver-stage studies, especially of hypnozoites (Wells et al. 2010; Campo et al. 2015). These efforts to improve capabilities for laboratory-based studies of the liver stages are yielding exciting results. Multiple new experimental platforms have emerged permitting researchers access to this least studied phase of *P. vivax* and human malaria parasites. Improvements in modified culture devices for in vitro enhancement of primary cell properties have progressed greatly to provide stability of viable primary human hepatocytes enhancing their potential use for malaria research (Dembele et al. 2011; March et al. 2013; Dembele et al. 2014; Maher et al. 2014; Ng et al. 2015). However, so far, the most significant innovations established are in vivo humanized mouse models (Shultz et al. 2007; Peltz 2013). These human liver-chimeric mice support complete liver-stage development, including persisting hypnozoites, of *P. vivax* and *Plasmodium ovale* (Mikolajczak et al. 2015; Soulard et al. 2015). These mouse models offer the potential to explore liver-stage biology and evaluate antimalarial candidates, in ways that were previously only possible

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through the use of primate models (Coatney et al. 1971).

CONCLUDING REMARKS

If the ambitious goals for the elimination of malaria are to be achieved, new tools specifically targeting *P. vivax* will be required. Accelerating the development of new drugs and vaccines will require both a better understanding of the unique *P. vivax* biology and the development of key research tools such as in vitro culture and in vivo models. Although significant progress has been made, many challenges remain.

Addressing these will require novel interdisciplinary research approaches that bridge from the field to the bedside and from the laboratory to the vivax research community backed by substantial investments in both basic and applied research.

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