Vivax series:

The genetic diversity of *Plasmodium vivax* populations

Liwang Cui[1], Ananias A. Escalante[2], Mallika Imwong[3] and Georges Snounou[4]

[1]Department of Entomology, Penn State University, 501 ASI, University Park, PA 16802, USA
[2]Centers for Disease Control and Prevention, National Center for Infectious Disease, Division of Parasitic Diseases, Mail Stop F12, 4770 Buford Highway, Chamblee, GA 30341, USA
[3]Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand
[4]Unité de Parasitologie Biomédicale, Institut Pasteur, 25 Rue du Dr Roux 75724, Paris, France

Little is known of the genetic diversity and population structure of *Plasmodium vivax*, a debilitating and highly prevalent malaria parasite of humans. This article reviews the known polymorphic genetic markers, summarizes current data on the population structure of this parasite and discusses future prospects for using knowledge of the genetic diversity to improve control measures.

Until the middle of the 1900s, *Plasmodium vivax* was the most globally widespread, and arguably the most prevalent, of the four malaria parasite species that infect humans (*Plasmodium falciparum*, *P. vivax*, *Plasmodium ovale* and *Plasmodium malariae*). The ability of this parasite to complete its sporogonic cycle at a minimum lower temperature of 16°C, compared with 21°C for *P. falciparum*, has substantially contributed to its success in establishing stable foci of transmission in the temperate zones. The global eradication campaign, carried out by the WHO in the mid-1950s, was most successful in the temperate endemic regions of Europe, Asia and the Americas and, as a result, the range of *P. vivax* was temporarily curtailed. However, the prevalence of *P. vivax* has been increasing lately, and the ~70–80 million cases currently recorded annually are of global public health importance [1]. Despite this, research aimed at understanding this parasite species has lagged by comparison with that carried out for *P. falciparum*. Unlike *P. falciparum*, *P. vivax* is rarely directly lethal and causes a form of malaria termed benign tertian malaria. However, the often-protracted course of a *P. vivax* infection is by no means clinically benign, and infections are a very important cause of morbidity and social economic loss [1]. Even today, there are sporadic reports of severe and fatal *P. vivax* infections [1,2]. As resources for malaria control are currently often directed against the more deadly *P. falciparum* malaria, the hidden burden of *P. vivax* malaria is likely to become increasingly apparent. Moreover, the recent emergence of chloroquine-resistant *P. vivax* strains is of great concern [3,4]. Clearly, a more comprehensive research effort directed at *P. vivax* malaria is urgently required.

The parasites that make up a *Plasmodium* spp. are heterogeneous for several important parameters, including: innate or acquired resistance to a particular drug; course and duration of the infection; symptoms; immunological crossreactivity; and transmissibility by anopheline vectors. A substantial proportion of our knowledge of the biological diversity of *P. vivax* results from observations of experimentally infected humans (Box 1). Relapses caused by a latent liver-stage parasite, the hypnozoite, are characteristic of *P. vivax* infections, and their pattern and frequency serves to distinguish tropical from temperate strains. The description of Russian and Chinese strains, by Nikolaev in 1949 and by Jiang and colleagues in 1982, where the primary infection occurs eight months or more following inoculation by an infected mosquito, led to establishing them as two subspecies, *Plasmodium vivax hibernans* and *Plasmodium vivax multinucleatum*, respectively. Whether these represent the same subspecies is unknown since the Russian strains had disappeared by the 1980s. A recent suggestion based on molecular polymorphisms and susceptibility of mosquitoes to infection [5] that New World *P. vivax* parasites might represent a separate subspecies, *Plasmodium vivax collins*, requires further biological studies before confirmation.

Parasite diversity is commonly observed within an endemic area, where different parasite strains circulate in the human host. The ability to distinguish between the different populations is a pre-requisite to understanding the local and global epidemiology of a parasite species. Studies on malaria parasite population diversity are not only of academic interest to biologists or population geneticists, but are of practical significance for the strategic development and deployment of control measures. On one hand, the parasite population structure, usually displaying considerable geographical variations, could affect the distribution of existing parasite strains, and this in turn determines the usefulness of a particular vaccine or drug formulation in a malaria-endemic region. On the other hand, parasite population structure will have significant influence on the gene flow and thus the rate at which new mutations leading to drug resistance or escape

Corresponding author: Liwang Cui (luc2@psu.edu).

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Box 1. Lessons from experimental human infections

Exploration of the genetic polymorphisms in *Plasmodium vivax* populations has only been initiated lately. Nonetheless, demonstration that diversity within this species is high has accrued from careful clinical observations. These were initiated by Italian malariologists late in the 19th century, who incidentally were the first to recognize that malaria parasites fell into separate species: *Plasmodium falciparum*, *P. vivax* and *Plasmodium malariae* (*Plasmodium ovale* was only described in 1922 by Stephens).

However, it was the instigation by Julius Wagner-Jauregg in 1917 of malaria-induced fever therapy for neurosyphilis that provided the basis for a most unique set of observations. The therapeutic success earned Wagner-Jauregg a Nobel prize, and led to the establishment of numerous centres of malariotherapy. A wealth of information on the biology, immunology and clinical features of malaria parasites was gathered from direct observations of induced malaria infections, principally *P. vivax*, in humans. Observations, such as the one provided in Fig. Ia (reproduced, with permission, from Ref. [59]), led to the recognition of clinical, immunological and biological diversity among the different *P. vivax* isolates. It remains to be established whether multiple infections of red blood cells by some *P. vivax* strains (Fig. Ib, reproduced from Ref. [60]), or morphological differences that were observed for a given strain infecting different hosts (Fig. Ic, reproduced from Ref. [61]), have a genetic basis.

The fact that late relapses were only observed when the infections were initiated by sporozoites, but not by infected blood, led Raffaele, James and others in the 1930s to question Fritz Schaudin's 1903 observation of the direct invasion of red blood cells by the sporozoite. The hepatocyte was ultimately demonstrated, by Shortt and Garnham in 1948 [62], to be the site of the first schizogony in the mammalian host, but the demonstration of the hypnozoite was only made in 1980 [63,64]. Molecular studies on the pre-erythrocytic stages, sporozoites and the liver stages have been severely restricted by the lack of sufficient material. Methods for the *in vitro* culture of hepatic stages in primary human hepatocytes have been devised [65], but the logistic burdens required to dispose concurrently of healthy human hepatocytes in culture and mosquitoes bearing infectious *P. vivax* sporozoites are a formidable deterrent.

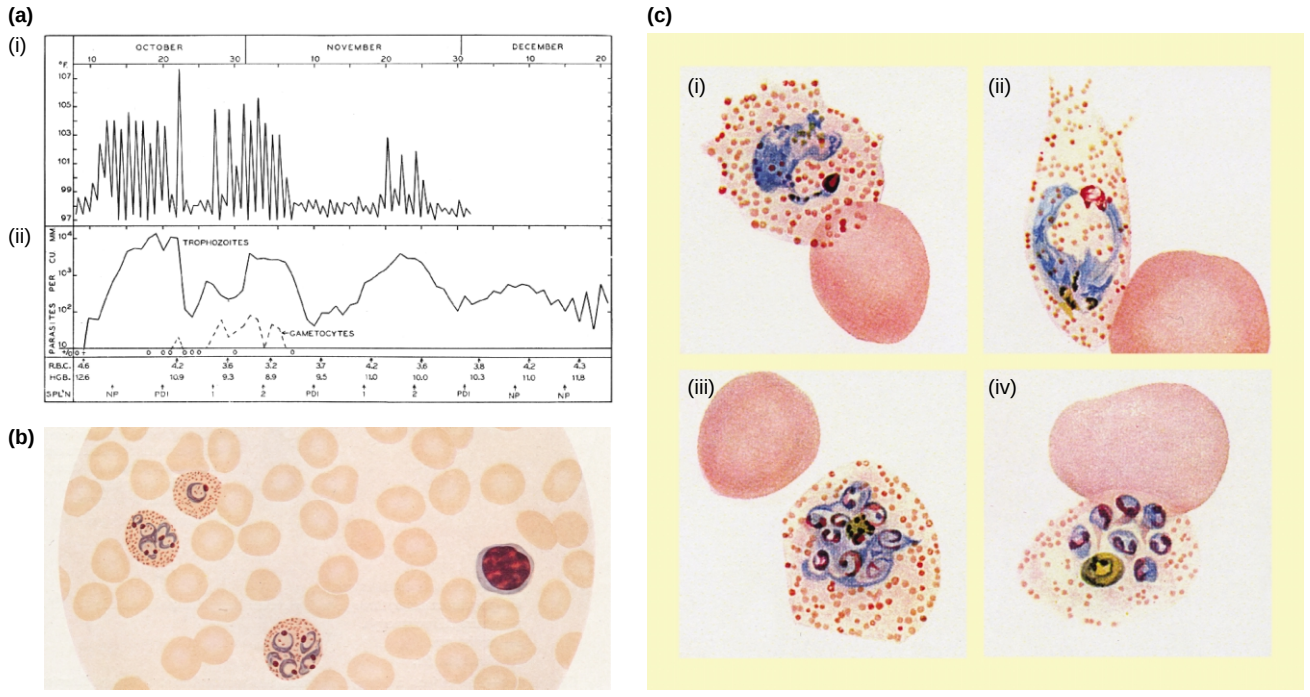

Fig. I.

from vaccine-induced responses will spread. Extensive efforts in this field over the past two decades have contributed to our understanding of the genetic structure of *P. falciparum* populations (reviewed in Refs [6,7]). The genetic diversity of *P. vivax* parasites has been less investigated.

Population genetic studies of malaria parasites

Two basic approaches have been followed in population genetic studies of malaria parasites: (1) those that aim to understand the parasite population genetic structure and intrahost dynamics, including the stability of strains and complexity of infections; and (2) studies assessing the diversity of specific genes encoding vaccine antigens and those associated with resistance to antimalarial drugs.

The studies on population structure quantify how alleles are spatially dispersed and how the overall genetic diversity is organized. These studies provide important indications as to the origin, dispersal and stability of multilocus genotypes. This information is essential for predicting and monitoring the effectiveness of intervention strategies such as: (1) the usefulness of combining specific drugs or therapeutic regimens; (2) the dispersion of drug resistance and the emergence of multidrug-resistant parasites; (3) the long-term impact of physical interventions such as bednets; and (4) the origin of genotypes that

might elude the immune response elicited by multivalent vaccines. Knowledge of the genetic diversity can also be used to assess geographical differentiation and explain the spatial distribution of alleles at a given loci. For example, it can provide baseline information for surveillance systems directed to identifying the origin of isolates in areas with occasional imported malaria cases.

Analysis of defined genes provides information about how different alleles are generated and maintained in the population. Specifically, it addresses the relevance of factors such as intragenic recombination and natural selection on the observed polymorphisms. This approach answers fundamental questions related to the association of specific alleles with drug resistance, and how the genetic variation at a given locus affects vaccine development and deployment.

Polymorphic molecular markers and *P. vivax* population diversity

High levels of polymorphism have been described for many parasite molecules, particularly antigens that might be under selection by host immunity. Initially, techniques employing monoclonal antibodies and isozymes detected high levels of polymorphism in *P. vivax* isolates collected in the field [8,9]. A major obstacle for this type of approach, and indeed for research on *P. vivax* in general, is the difficulty in maintaining this pathogen *in vitro*. The recent demonstration of continuous culture of *P. vivax* [10] is a major step forward, although the need for a constant supply of human reticulocytes is highly restrictive. With respect to genetic analyses, this limitation can be largely overcome by the detection of diversity at the nucleotide level using the powerful technique of polymerase chain reaction (PCR), alone or in combination with sequencing and restriction analysis. The lack of suitable genetic markers, such as microsatellites, single nucleotide polymorphisms (SNPs) or repetitive regions, has limited the number of field studies addressing basic molecular epidemiological questions. However, it is expected that the complete sequence of the parasite will increase the number of genetic markers when it is released. Nevertheless, polymorphic markers encoding parasite surface antigens have been used as genetic markers to study parasite diversity. Commonly used methods for large-scale population analysis, often developed for *P. falciparum*, include the design of allele family-specific primers for PCR detection of different alleles and their size polymorphism [e.g. genes of the merozoite surface proteins (MSP), *PfMSP1* and *PfMSP2*], repeat region size polymorphism [genes for the glutamate-rich protein *PfGLURP* and the circumsporozoite protein (CSP) *PfCSP*], or PCR/restriction fragment size polymorphism (e.g. *PfMSP2* and *PvMSP3α*). Occasionally, allele families are determined by hybridizing the PCR product to allele-specific probes (e.g. *PfMSP2* and *PvCSP*). Currently, DNA markers for genotyping *P. vivax* include the genes encoding MSP1 and MSP3α, apical membrane antigen 1 (AMA1), CSP, GAM1 and dihydrofolate reductase (DHFR).

MSP

To date, genes encoding seven MSPs have been cloned from *P. vivax*, all named in accordance with the *P. falciparum* orthologues. *PvMSP1* and *PvMSP3α* are highly polymorphic and are currently used for genotyping *P. vivax* field isolates [11,12]. The primary structure of *PvMSP1*, originally characterized from two monkey-adapted *P. vivax* strains (Belem and Salvador-1), exhibits conserved, semi-conserved and polymorphic regions [11,13]. This gene has a similar genetic organization to that of *PfMSP1* and *P. yoelii MSP1*, with several regions highly conserved among species [termed 'interspecies conserved blocks' (ICBs)]. Sequence analysis of the region between ICB5 and ICB6 from parasite isolates from Brazil [14], Sri Lanka [15], Colombia [16], Papua New Guinea (PNG) [17] and Thailand [18] demonstrates the dimorphic nature of this region and suggests intragenic recombination as a mechanism of generating antigenic diversity. Other regions (between ICB2 and ICB4, ICB4 and ICB5, ICB8 and ICB10) also exhibit varying degrees of polymorphism [19,20]. Although most studies with *PvMSP1* show evidence of genetic recombination of different alleles, and some even detect geographical clusters of specific variations [19], sample sizes are too small to allow the accurate determination of allele frequencies.

In addition to the length polymorphism of the PCR product, extensive polymorphism at the nucleotide level has been observed by PCR-RFLP in the *PvMSP3α* gene in *P. vivax* isolates from diverse geographical regions [21,22]. The polymorphism and deletions are largely restricted to the central domain containing coiled-coil heptad repeats [23,24]. In malaria-hyperendemic Papua New Guinea, 24 alleles with varying frequencies are observed from 74 samples from asymptomatic patients [22].

AMA1

AMA1, a protein essential for erythrocyte invasion, is highly conserved among *Plasmodium* spp. Similar to *PfAMA1*, *PvAMA1* shows limited sequence polymorphism [25,26]. Moreover, *PvAMA1* might have only a few predominant haplotypes and display very limited genetic diversity within a geographic region, although a more comprehensive study of the whole gene is necessary to confirm this observation.

CSP

CSP genes have been cloned from numerous *Plasmodium* spp, including *P. vivax* [27]. Being one of the prime targets for anti-infection vaccines, CSP has been studied extensively in terms of antigenicity and polymorphism. In all *Plasmodium* spp., it is a single-copy gene encoding structurally similar CSP, where a central domain of tandemly repeated sequences is flanked by predominantly nonrepeated conserved sequences. The central repetitive domain varies in sequence and length among *Plasmodium* spp. Analysis of PvCSP sequences revealed that parasites have repeats belonging to one of two types of nonapeptide repeat units (GDRAA/DGPQA or ANGAGNQPG), named respectively VK210 or VK247, for the sample number from which they were originally described [28]. Both variants have a worldwide distribution [29]. Analysis of the *PvCSP* sequences from southeast Asian isolates indicates very limited polymorphisms outside of the repeat region [30]. Currently, *PvCSP* has been employed successfully in

epidemiological studies of *P. vivax* malaria using two well-established methods: molecular hybridization analysis of parasite DNA isolated from blood samples [31] and ELISA to detect sporozoite proteins in mosquitoes artificially infected or collected from the field [32,33].

Other genes

The *PvGAM1* gene, named because of its expression in gametocytes, displays polymorphic deletions near the 3′ end in a limited number of Sri Lankan isolates [34]. Its potential as a molecular marker for genotyping is compromised due to artefacts associated with amplification of this region [35]. The reason for the artefacts is not clear; the presence of GC-rich regions, especially with consecutive runs of G, close to the site of the deletion might be implicated.

In addition to point mutations that are associated with resistance to pyrimethamine, the *P. vivax DHFR* gene also has a size polymorphism resulting from the deletion of five or six amino acids. Four allelic variants have been observed to date [36,37]. However, the fact that the gene is under drug selection must be taken into account if this polymorphism is to be used as a genetic marker.

Population structure of *P. vivax*

Given that meiotic recombination takes place in the vector as part of the *Plasmodium* life cycle, the rate of genetic recombination and the effective population size are both expected to be linked to the transmission intensity. In agreement, studies carried out on *P. falciparum* have shown high genetic diversity and failed to detect multilocus associations in Africa with a high transmission rate, and have found low genetic diversity and strong linkage disequilibria in areas with low transmission [38,39].

There are no studies on *P. vivax* using this type of approach, but the available evidence suggests that the situation could be similar. In a hyperendemic area such as Papua New Guinea, intragenic recombination and high genetic diversity have been reported in genes encoding malarial vaccine antigens such as MSP1 and MSP3α [17,22]. Surprisingly, even in hypoendemic areas such as Thailand and Brazil, *PvMSP1*, *PvMSP3α* and *P. vivax* thrombospondin-related adhesive protein (*PvTRAP*) also display high levels of diversity and evidence of genetic recombination [24,40]. By contrast, relatively low genetic diversity has been detected in the re-emerging *P. vivax* malaria focus in Korea [41], probably owing to the epidemic nature where few strains might be involved. For *PvCSP*, parasite isolates appear to cluster geographically; for example, a 36-base insertion 3′ to the central repeats is found only in parasite samples from China and the Korean Peninsula [42,43]. Unfortunately, since the information available is derived from antigens, geographical differentiation caused by positive natural immune selection rather than reduced gene flow cannot be ruled out. Recently, studies on the susceptibility of mosquitoes to infection have provided grounds to speculate about the geographically varying frequencies of the two *PvCSP* repeat variants. Laboratory-bred *Anopheles albimanus* and *Anopheles pseudopunctipennis* from southern Mexico were found to be differentially susceptible to infections by VK210- and VK247-bearing *P. vivax* [44], and the prevalence of the two parasite types correlated with the distribution of the two mosquito vectors [45]. It remains to be determined whether ookinete destruction and oocyst development arrest in the unsusceptible vector [46] or a polymorphic gene genetically closely linked to *PvCSP* accounts for these observations.

Multiple clonal infections (i.e. the simultaneous infection of a host by more than one strain of the same parasite species) are common in malaria. For *P. falciparum*, the levels of multiplicity of infection are partially correlated with the levels of transmission intensities. For *P. vivax* malaria, the proportion of mixed-strain infections estimated in Papua New Guinea, India and Thailand ranges from ~30% to 65% [17,22,24,47]. In Papua New Guinea, as many as six parasite clones have been found in a single host [22]. Although not directly comparable, since different sampling strategies and genotyping methods were used, these values are strikingly high in regard to the low endemicity of *P. vivax* malaria in certain regions such as Thailand. This could be because of biological features of the *P. vivax* parasite, such as earlier gametocytogenesis and relapse. The production of gametocytes during the presymptomatic period might allow for a more efficient transmission to the mosquito vector before drug treatment is initiated, while regular relapses would also enhance transmission and increase the probability of detecting mixed infections as further inoculations occur. Consequently, the multiplicity of infections is likely to facilitate genetic recombination of parasites and the generation of novel strains. Similar to *P. falciparum* [48], the natural populations of *P. vivax* also appear to have random mating as demonstrated using the two *PvCSP* repeat types [33]. This might also contribute to the high levels of genetic diversity of *P. vivax* populations in low-endemic areas [24].

Molecular evolution of antigens

For malarial vaccine antigens, the observed high levels of genetic diversity have been attributed to positive natural selection. This interpretation is supported, among other lines of evidence, by the observation that nonsynonymous nucleotide substitutions are more common than synonymous substitutions [49,50]. Synonymous substitutions are likely to be neutral, or nearly so, whereas nonsynonymous substitutions could be functionally constrained and, thus, subject to negative natural selection and are eliminated from the population. It is expected that the genetic polymorphism resulting from random accumulation of mutations will exhibit more synonymous than nonsynonymous substitutions. An excess of nonsynonymous substitutions can be taken as evidence that these mutations might confer an adaptive advantage so they are maintained by natural selection. Under this scenario, the action of the host immune system on the parasite population allows the accumulation and frequent switch of suitable mutations.

Several molecular evolutionary studies have been performed on the *PfAMA1* gene [50–52], where the rate of nonsynonymous substitution is found to be much higher than that of synonymous substitution, which is suggestive of positive diversifying selection probably by

host immunity. This is particularly evident in the regions that serve as T helper (Th)-cell epitopes [51]. There is also indication of high recombination rates of the molecule, demonstrated by the high recombination parameter value and the rapid decline in linkage disequilibrium with increasing nucleotide distance [52]. In the specific case of *P. vivax*, the available information suggests that there is no such overwhelming pattern of more nonsynonymous than synonymous substitutions as in *P. falciparum*. A plausible explanation is that its genome does not have the confounding factor of a strong codon bias. Specifically, the average effective number of codons, a statistic that measures codon bias, is 54.44 for *P. vivax*, based on 15 genes. This value contrasts with the reported 36.82 for *P. falciparum*. The available evidence shows that there are more synonymous than nonsynonymous substitutions in partial sequences of genes such as *PvAMA1* and *PvMSP1* [26], and evidence for positive natural selection has been found in *PvTRAP* [40]. This might also be true for *PvCSP*, where amino acid substitutions are mostly localized in the regions that are probably under strong immune selection (e.g. regions that harbor T-cell determinants) [30].

It has been suggested that synonymous substitutions might not be neutral in the case of *PvAMA1*, in that its rate is lower than the one observed on *PvMSP1* [26]. The interpretation of this result demands a further clarification of what neutrality means. The neutral theory is compatible with the widely observed phenomenon that genes evolve at different rates. The rate of substitutions is affected by the overall forces of selection (negative or positive) acting at that locus and closely linked loci, the population history and its effective population size, among other factors. These arguments support the comparison among alleles at a given locus of the synonymous and nonsynonymous substitution rates. The observation that the rate of synonymous substitutions in one part of a gene is lower when compared with partial sequences from other genes does not imply that the silent substitutions are more or less neutral; indeed, the available theory explains such patterns. However, we agree that the effect of factors such as local codon bias needs to be explored. As we stated earlier, although the *P. vivax* genome is not particularly biased, the effective number of codons for *PvAMA1* is 44.3, which is lower than observed for other merozoite genes such as *PvMSP1* (average 55.04 with 61 for MSP1). Studies using complete sequences are needed to address these issues. It is worth noticing that the comparison of synonymous and nonsynonymous substitutions is not the only approach for studying the effect of selection. There are several methods available that have been used mostly in *P. falciparum* [50,53,54]. For *P. vivax*, there is an advantage over *P. falciparum* given that comparative studies are possible due to the availability of isolates from closely related species (e.g. *P. simium*) [55,56].

Genetic diversity and malaria control

The efficacy of control measures is generally assessed from their impact on the number of infected individuals/-mosquitoes, clinical cases and treatment failures, and so on. However, it is possible to envisage situations where intervention only marginally affects such measurable outcomes over the period of observation. Parasite genetic diversity could then be exploited to detect subtle changes in the population of the pathogen.

Changes in the overall genetic composition of the population or the multiplicity of infection observed in individuals can provide an indication of an effect resulting from an intervention such as the deployment of bednets or vaccination. An increasing number of *P. vivax* antigens are now under development as vaccine candidates: CSP, MSP1, AMA1, Pvs25, Pvs28, TRAP and the Duffy binding protein (DBP). Many of these antigens are polymorphic and the induced immune responses might be more effective against parasites carrying particular allelic variants. Determination of allelic frequencies should be included in future field-based trials involving vaccination with polymorphic antigens.

The emergence and spread of drug resistance are major problems in the control of malaria. Early detection and monitoring of resistant *P. vivax* parasites is needed to avoid the disastrous humanitarian problems that have resulted from the spread of drug-resistant *P. falciparum* parasites. Direct monitoring requires knowledge of the genetic basis of resistance. The enzyme targets of anti-folate drugs pyrimethamine and sulfadoxine are DHFR and dihydropteroate synthase (DHPS). Rapid molecular monitoring of anti-folate resistance in *P. falciparum* has been made possible with the determination of the point mutations associated with such resistance. Recent analysis of *pvdhfr* has brought to light five residues putatively associated with resistance [37]. Protocols for the detection of these mutations in field samples have been elaborated. However, a similar molecular approach for monitoring resistance to chloroquine or primaquine in *P. vivax* awaits the discovery of the target proteins of these drugs. Since *in vitro* culture of *P. vivax* is not easily available, monitoring of drug resistance relies exclusively on *in vivo* drug efficacy trials. When conducted in an endemic area, as is frequently the case, these studies suffer from a major limitation: the inability to distinguish true recrudescences (which denotes a treatment failure) from a re-infection during the follow-up period. This has been largely solved by the development of PCR-based practical protocols for parasite genotyping [57]. Standard protocols, similar to those developed for *P. falciparum* and based on the *PvCSP* and *PvMSP1* genes, have been developed (M. Imwong *et al.* unpublished) and should be included in future drug efficacy trials.

Perspective

The molecular analysis of parasite populations is a relatively recent research tool. Although these studies have so far been limited to a few polymorphic genes, several important insights in the biology and immunology of the parasite have accrued. With the advent of relatively cheap and rapid sequencing of whole genomes, as well as the development of high-throughput post-genomic analyses, the forthcoming completion of the *P. vivax* genome should substantially increase the potential for discovery. Genome-wide comparisons among the *Plasmodium* spp. might then provide a clearer indication of the phylogenetic position of *P. vivax*. Wholesale comparison of the genomes

of different *P. vivax* lines should provide a broader array of genetic markers, including microsatellite and noncoding DNA stretches. In this context, a challenge for the future is the development of strategies to analyze on a population level the parasite multigene families that are associated with pathology and virulence, for example the *var* family of *P. falciparum*, the *py235* family of *P. yoelii* and the recently described *vir* genes of *P. vivax* [58]. Ultimately, in the absence of acceptable experimental models of the infection, parasite genotyping strategies are crucial for deriving knowledge of parasite survival strategies from observations of natural infections.

References

1 Mendis, K.N. *et al.* (2001) The neglected burden of *Plasmodium vivax* malaria. *Am. J. Trop. Med. Hyg.* 64, 97–106

2 Beg, M.A. *et al.* (2002) Cerebral involvement in benign tertian malaria. *Am. J. Trop. Med. Hyg.* 67, 230–232

3 Rieckmann, K.H. *et al.* (1989) *Plasmodium vivax* resistance to chloroquine? *Lancet ii*, 1183–1184

4 Baird, J.K. *et al.* (1991) Resistance to chloroquine by *Plasmodium vivax* in Irian Jaya, Indonesia. *Am. J. Trop. Med. Hyg.* 44, 547–552

5 Li, J. *et al.* (2001) Geographic subdivision of the range of the malaria parasite *Plasmodium vivax*. *Emerg. Infect. Dis.* 7, 35–42

6 Babiker, H.A. and Walliker, D. (1997) Current views on the population structure of *Plasmodium falciparum*: implications for control. *Parasitol. Today* 13, 262–267

7 Awadalla, P. *et al.* (2001) The question of *Plasmodium falciparum* population structure. *Trends Parasitol.* 17, 351–353

8 Myint-Oo (1986) Isoenzyme variation in schizonts of *Plasmodium vivax* from Burma. *Trans. R. Soc. Trop. Med. Hyg.* 80, 1–4

9 Udagama, P.V. *et al.* (1987) Demonstration of antigenic polymorphism in *Plasmodium vivax* malaria with a panel of 30 monoclonal antibodies. *Infect. Immun.* 55, 2604–2611

10 Golenda, C. *et al.* (1997) Continuous *in vitro* propagation of the malaria parasite *Plasmodium vivax*. *Proc. Natl. Acad. Sci. U. S. A.* 94, 6786–6791

11 Del Portillo, H.A. *et al.* (1991) Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different *Plasmodium* species. *Proc. Natl. Acad. Sci. U. S. A.* 88, 4030–4034

12 Galinski, M.R. *et al.* (1999) *Plasmodium vivax* merozoite surface protein-3 contains coiled-coil motifs in an alanine-rich central domain. *Mol. Biochem. Parasitol.* 101, 131–147

13 Gibson, H.L. *et al.* (1992) Structure and expression of the gene for Pv200, a major blood-stage surface antigen of *Plasmodium vivax*. *Mol. Biochem. Parasitol.* 50, 325–333

14 Porto, M. *et al.* (1992) Second form in a segment of the merozoite surface protein 1 gene of *Plasmodium vivax* among isolates from Rondônia (Brazil). *Mol. Biochem. Parasitol.* 54, 121–124

15 Premawansa, S. *et al.* (1993) *Plasmodium vivax*: recombination between potential allelic types of the merozoite surface protein MSP1 in parasites isolated from patients. *Exp. Parasitol.* 76, 192–199

16 Mancilla, L.I. *et al.* (1994) *Plasmodium vivax*: dimorphic DNA sequences from the MSP-1 gene code for regions that are immunogenic in natural infections. *Exp. Parasitol.* 79, 148–158

17 Kolakovich, K.A. *et al.* (1996) *Plasmodium vivax*: favored gene frequencies of the merozoite surface protein-1 and the multiplicity of infection in a malaria endemic region. *Exp. Parasitol.* 83, 11–18

18 Putaporntip, C. *et al.* (1997) Interallelic recombination in the merozoite surface protein 1 (MSP-1) gene of *Plasmodium vivax* from Thai isolates. *Mol. Biochem. Parasitol.* 84, 49–56

19 Cheng, Q. *et al.* (1993) Polymorphism in *Plasmodium vivax* MSA1 gene – the result of intragenic recombinations? *Parasitology* 106, 335–345

20 Gutierrez, A. *et al.* (2000) *Plasmodium vivax*: polymorphism in the merozoite surface protein 1 gene from wild Colombian isolates. *Exp. Parasitol.* 95, 215–219

21 Bruce, M.C. *et al.* (1999) Polymorphism at the merozoite surface protein-3α locus of *Plasmodium vivax*: global and local diversity. *Am. J. Trop. Med. Hyg.* 61, 518–525

22 Bruce, M.C. *et al.* (2000) Genetic diversity and dynamics of *Plasmodium falciparum* and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology* 121, 257–272

23 Rayner, J.C. *et al.* (2002) Extensive polymorphism in the *Plasmodium vivax* merozoite surface coat protein MSP-3α is limited to specific domains. *Parasitology* 125, 393–405

24 Cui, L. *et al.* Genetic diversity and multiple infections of *Plasmodium vivax* malaria in western Thailand. *Am. J. Trop. Med. Hyg.* (in press)

25 Cheng, Q. and Saul, A.J. (1994) Sequence analysis of the apical membrane antigen I (AMA-1) of *Plasmodium vivax*. *Mol. Biochem. Parasitol.* 65, 183–187

26 Figtree, M. *et al.* (2000) *Plasmodium vivax* synonymous substitution frequencies, evolution and population structure deduced from diversity in AMA 1 and MSP 1 genes. *Mol. Biochem. Parasitol.* 108, 53–66

27 Arnot, D.E. *et al.* (1985) Circumsporozoite protein of *Plasmodium vivax*: gene cloning and characterization of the immunodominant epitope. *Science* 230, 815–818

28 Rosenberg, R. *et al.* (1989) Circumsporozoite protein heterogeneity in the human malaria parasite *Plasmodium vivax*. *Science* 245, 973–976

29 Kain, K.C. *et al.* (1992) Circumsporozoite genotyping of global isolates of *Plasmodium vivax* from dried blood specimens. *J. Clin. Microbiol.* 30, 1863–1866

30 Qari, S.H. *et al.* (1992) Polymorphism in the circumsporozoite protein of the human malaria parasite *Plasmodium vivax*. *Mol. Biochem. Parasitol.* 55, 105–113

31 Kain, K.C. and Lanar, D.E. (1991) Determination of genetic variation within *Plasmodium falciparum* by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. *J. Clin. Microbiol.* 29, 1171–1174

32 Burkot, T.R. *et al.* (1992) The population dynamics in mosquitoes and humans of two *Plasmodium vivax* polymorphs distinguished by different circumsporozoite protein repeat regions. *Am. J. Trop. Med. Hyg.* 47, 778–786

33 Rosenberg, R. *et al.* (1992) Random mating of natural *Plasmodium* populations demonstrated from individual oocysts. *Mol. Biochem. Parasitol.* 53, 129–133

34 Snewin, V.A. *et al.* (1995) A new polymorphic marker for PCR typing of *Plasmodium vivax* parasites. *Mol. Biochem. Parasitol.* 71, 135–138

35 Imwong, M. *et al.* (2001) *Plasmodium vivax*: polymerase chain reaction amplification artifacts limit the suitability of pvgam1 as a genetic marker. *Exp. Parasitol.* 99, 175–179

36 Eldin de Pécoulas, P. *et al.* (1998) Sequence variation in the *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase gene and their relationship with pyrimethamine resistance. *Mol. Biochem. Parasitol* 92, 265–273

37 Imwong, M. *et al.* (2001) Association of genetic mutations in *Plasmodium vivax dhfr* with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. *Antimicrob. Agents Chemother.* 45, 3122–3127

38 Anderson, T.J. *et al.* (2000) Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol. Biol. Evol.* 17, 1467–1482

39 Urdaneta, L. *et al.* (2001) Evidence for clonal propagation in natural isolates of *Plasmodium falciparum* from Venezuela. *Proc. Natl. Acad. Sci. U. S. A.* 98, 6725–6729

40 Putaporntip, C. *et al.* (2001) Diversity in the thrombospondin-related adhesive protein gene (TRAP) of *Plasmodium vivax*. *Gene* 268, 97–104

41 Lim, C.S. *et al.* (2000) Analysis of *Plasmodium vivax* merozoite surface protein-1 gene sequences from resurgent Korean isolates. *Am. J. Trop. Med. Hyg.* 62, 261–265

42 Mann, V.H. *et al.* (1994) Sequence variation in the circumsporozoite protein gene of *Plasmodium vivax* appears to be regionally biased. *Mol. Biochem. Parasitol.* 68, 45–52

43 Lim, C.S. *et al.* (2001) The analysis of circumsporozoite-protein gene sequences from South Korean isolates of *Plasmodium vivax*. *Ann. Trop. Med. Parasitol.* 95, 229–235

44 Gonzalez-Ceron, L. *et al.* (1999) Differential susceptibilities of *Anopheles albimanus* and *Anopheles pseudopunctipennis* to infections with coindigenous *Plasmodium vivax* variants VK210 and VK247 in southern Mexico. *Infect. Immun.* 67, 410–412

45 Rodriguez, M.H. *et al.* (2000) Different prevalences of *Plasmodium vivax* phenotypes VK210 and VK247 associated with the distribution of *Anopheles albimanus* and *Anopheles pseudopunctipennis* in Mexico. *Am. J. Trop. Med. Hyg.* 62, 122–127
46 Gonzalez-Ceron, L. *et al.* (2001) *Plasmodium vivax*: ookinete destruction and oocyst development arrest are responsible for *Anopheles albimanus* resistance to circumsporozoite phenotype VK247 parasites. *Exp. Parasitol.* 98, 152–161
47 Joshi, H. *et al.* (1997) Genetic structure of *Plasmodium vivax* isolates in India. *Trans. R. Soc. Trop. Med. Hyg.* 91, 231–235
48 Anderson, T.J. *et al.* (2000) Do malaria parasites mate non-randomly in the mosquito midgut? *Genet. Res.* 75, 285–296
49 Hughes, M.K. and Hughes, A.L. (1995) Natural selection on *Plasmodium* surface proteins. *Mol. Biochem. Parasitol.* 71, 99–113
50 Escalante, A.A. *et al.* (1998) Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum*. *Genetics* 149, 189–202
51 Verra, F. and Hughes, A.L. (2000) Evidence for ancient balanced polymorphism at the Apical Membrane Antigen-1 (AMA-1) locus of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 105, 149–153
52 Polley, S.D. and Conway, D.J. (2001) Strong diversifying selection on domains of the *Plasmodium falciparum* apical membrane antigen 1 gene. *Genetics* 158, 1505–1512
53 Conway, D.J. (1997) Natural selection on polymorphic malaria antigens and the search for a vaccine. *Parasitol. Today* 13, 26–29
54 Escalante, A.A. *et al.* (2002) Polymorphism in the gene encoding the Pfs48/45 antigen of *Plasmodium falciparum*. XI. Asembo Bay Cohort Project. *Mol. Biochem. Parasitol.* 119, 17–22
55 Escalante, A.A. *et al.* (1995) Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. *Mol. Biol. Evol.* 12, 616–626
56 Escalante, A.A. *et al.* (1998) The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome. *Proc. Natl. Acad. Sci. U. S. A.* 95, 8124–8129
57 Snounou, G. and Beck, H.-P. (1998) The use of PCR-genotyping in the assessment of recrudescence or reinfection after antimalarial treatment. *Parasitol. Today* 14, 462–467
58 Del Portillo, H.A. *et al.* (2001) A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. *Nature* 410, 839–842
59 Boyd, M.F. ed. (1949) *Malariology* W.B. Saunders Co
60 Young, M.D. and Eyles, D.E. (1949) Parasites resembling *Plasmodium ovale* in strains of *Plasmodium vivax*. *J. Natl. Malaria Soc.* 8, 219–223
61 Field, J.W. *et al.* (1956) *The Microscopic Diagnosis of Human Malaria*, Government Press
62 Shortt, H.E. and Garnham, P.C.C. (1948) The pre-erythrocytic development of *Plasmodium cynomolgi* and *Plasmodium vivax*. *Trans. Roy. S. Trop. Med. Hyg.* 41, 785–795
63 Krotoski, W.A. *et al.* (1980) Relapses in primate malaria: discovery of two populations of exoerythrocytic stages. Preliminary note. *Br. Med. J.* 280, 153–154
64 Krotoski, W.A. *et al.* (1982) Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection. *Am. J. Trop. Med. Hyg.* 31, 1291–1293
65 Mazier, D. (1984) Cultivation of the liver forms of *Plasmodium vivax* in human hepatocytes. *Nature* 307, 367–369