

Emerging *Plasmodium vivax* resistance to chloroquine in South America: an overview

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The global emergence of Plasmodium vivax strains resistant to chloroquine (CQ) since the late 1980s is complicating the current international efforts for malaria control and elimination. Furthermore, CQ-resistant vivax malaria has already reached an alarming prevalence in Indonesia, East Timor and Papua New Guinea. More recently, in vivo studies have documented CQ-resistant P. vivax infections in Guyana, Peru and Brazil. Here, we summarise the available data on CQ resistance across P. vivax-endemic areas of Latin America by combining published in vivo and in vitro studies. We also review the current knowledge regarding the molecular mechanisms of CQ resistance in P. vivax and the prospects for developing and standardising reliable molecular markers of drug resistance. Finally, we discuss how the Worldwide Antimalarial Resistance Network, an international collaborative effort involving malaria experts from all continents, might contribute to the current regional efforts to map CQ-resistant vivax malaria in South America.

Key words: *Plasmodium vivax* - chloroquine - resistance - Latin America

Plasmodium vivax, the most widespread human malaria parasite, is estimated to cause up to 390 million clinical cases each year in Latin America, the Middle East, Central, South and Southeast Asia, Oceania and East Africa (Price et al. 2007, Battle et al. 2012), locations where 2.49 billion people are currently at risk of infection (Gething et al. 2012). Nearly half a million slide-confirmed malaria infections (80% due to *P. vivax*) are annually reported in 21 endemic countries across the Americas and the Caribbean (Arevalo-Herrera et al. 2012, da Silva-Nunes et al. 2012), with 137 million people at risk of malaria (Gething et al. 2012). Nonetheless, these figures are likely to be underestimates because of limitations in the coverage of malaria notification and diagnosis in many endemic areas. Nine Amazonian countries (Bolivia, Brazil, Colombia, Ecuador, Guyana, French Guyana, Peru, Suriname and Venezuela) together account for 90% of these infections.

The emergence of *P. vivax* strains resistant to chloroquine (CQ), which was first documented in Papua New Guinea (PNG) in 1989 (Rieckmann et al. 1989), complicates the current international efforts for malaria control and elimination. CQ resistance in vivo is formally defined as the persistence of asexual *P. vivax* blood stages despite adequate whole blood or plasma levels of CQ and its main active metabolite, desethylchloroquine (DCQ) (Baird et

al. 1997). By strictly applying this definition, *P. vivax* CQ resistance has been confirmed largely through the malaria endemic world, reaching an alarming prevalence in Indonesia and PNG (Baird 2009, Baird et al. 2012).

The first published reports of CQ-resistant vivax malaria in Latin America were from Colombia (Arias & Corredor 1989) and Brazil (Garavelli & Corti 1992); however, antimalarial treatment had not been supervised and drug levels were not measured in either of these early studies. Consequently, it was only in 1996 that CQ resistance was formally documented in a *P. vivax* strain from this region. Briefly, a Canadian patient returning from Guyana, who had been treated with CQ (25 mg/kg over 3 days) and primaquine (PQ) (15 mg base/day for 14 days), had a parasite recrudescence on day 11 of PQ treatment. His whole-blood CQ levels were in the therapeutic range (> 100 ng/mL) at the time of treatment failure. A second patient, also returning from Guyana, had a late recurrence (6 weeks after initial CQ-PQ treatment), again with therapeutic blood levels of CQ at presentation (Phillips et al. 1996). Subsequent reports of *P. vivax* CQ resistance originated from Colombia [3 parasite recurrences among 27 patients (11.1%)] (Soto et al. 2001) and Peru [4 recurrences among 212 patients (1.9%)] (Ruebush et al. 2003) (Figure). The patients enrolled in these studies were treated with 25 mg of CQ/kg over three days, with no PQ being co-administered, but CQ resistance was confirmed in only two of them (both from Peru) by measuring drug levels at the time of recurrence.

More recently, two additional reports of in vivo CQ resistance in *P. vivax* were from Manaus, the major Brazilian port city in the Amazon Basin. In the first study, 11 of 109 (10.1%) patients treated with CQ alone (25 mg/kg over 3 days) had *P. vivax* recrudescence despite adequate plasma levels of CQ (de Santana Filho et al. 2007). Subsequently, another single-arm clinical trial

doi: 10.1590/0074-0276130579

Financial support: Bill and Melinda Gates Foundation (to WWARN) MUF is supported by a senior research fellowship from CNPq.

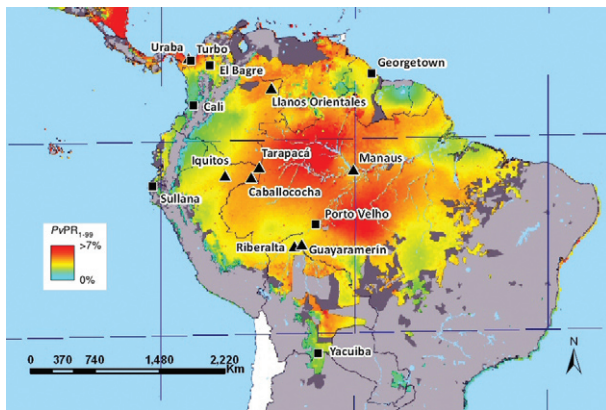
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Received 13 December 2013

Accepted 14 March 2014

in Manaus documented *P. vivax* recurrences in seven of 135 (5.2%) patients treated with CQ (25 mg/kg over 3 days) and PQ (30 mg base/day for 7 days); all of them had CQ levels above 100 ng/mL of whole blood at the time of recurrence (Marques et al. 2013). These studies indicate that *P. vivax* CQ resistance has occurred in at least three Latin American countries (Guyana, Peru and Brazil), affecting patients treated with either CQ alone or with a CQ-PQ combination.

However, other recent clinical trials have failed to confirm *P. vivax* CQ resistance in Guyana (Baird et al. 2002), Bolivia (Añez et al. 2012) and Colombia (Castillo et al. 2002, Alvarez et al. 2006, Carmona-Fonseca & Maestre 2009). The patients treated with CQ alone who had treatment failures in Guayaramerín (8 of 79 subjects followed over 28 days) and Riberalta (5 of 81 subjects followed over 28 days), both in Bolivia, had blood levels of CQ and DCQ < 70 ng/mL at the time of parasite recurrence; as a consequence, CQ resistance could not be confirmed (Añez et al. 2012). Furthermore, one late treatment failure (on day 28 of CQ - only treatment) was recently observed in a small study (20 patients) in Tarapacá, Colombia, but the CQ concentration had not been measured at the time of parasite recrudescence (Osorio et al. 2007). Although CQ treatment failures have been reported in these study sites in Bolivia (Añez et al. 2012) and Colombia (Osorio et al. 2007) (Figure), the available data do not provide evidence for in vivo CQ resistance.



Map showing sites of recent in vivo studies of chloroquine treatment for *Plasmodium vivax* infections in South America (published between 2000-2013). Triangles indicate study sites where treatment failures have been reported, while squares indicate sites with no treatment failures observed over 28 days of follow-up. Note the close proximity between two sites in Colombia (Uraba and Turbo) and two sites in Bolivia (Riberalta and Guayaramerín). Clinical data are from Villalobos-Salcedo et al. (2000) (Porto Velho, Brazil), Soto et al. (2001) (Uraba and Llanos Orientales, Colombia), Baird et al. (2002) (Georgetown, Guyana), Ruebush et al. (2003) (Iquitos, Caballococha and Sullana, Peru), Alvarez et al. (2006) (Turbo and El Bagre, Colombia), de Santana Filho et al. (2007) (Manaus, Brazil), Osorio et al. (2007) (Tarapacá, Colombia), Carmona-Fonseca and Maestre (2009) (Turbo and El Bagre, Colombia), Añez et al. (2012) (Riberalta, Guayaramerín and Yacuiba, Bolivia) and Marques et al. (2013) (Manaus, Brazil). Shading patterns vary according to *P. vivax* malaria prevalence (P_vPR) data estimated by Gething et al. (2012).

A study comparing two CQ-PQ regimens in 73 Brazilian subjects showed no *P. vivax* recurrences up to 30 days after treatment (Villalobos-Salcedo et al. 2000). Nevertheless, most of these studies were underpowered to detect rare events due to small sample sizes. Furthermore, no CQ resistance data from in vivo studies are publicly available for *P. vivax* populations from other Latin American countries. In conclusion, the extent to which CQ-resistant *P. vivax* currently represents a significant threat to malaria control efforts in Latin America remains to be determined.

CQ-PQ as a combination therapy - The reasons why CQ resistance tends to spread much slower in *P. vivax* than in *Plasmodium falciparum* remain open to speculation. The finding that PQ reverses CQ resistance in *P. falciparum* (Bray et al. 2005) suggests that a similar effect might occur in *P. vivax* isolates simultaneously exposed to both drugs [reviewed by Egan (2006)]. Over the past 60 years, CQ and PQ have been combined for the radical cure of *P. vivax* infections (i.e., eradication of blood stages and hepatic hypnozoites) in Latin America, but not in other regions where glucose-6-phosphate dehydrogenase (G6PD) deficiency is common due to the risk of severe PQ-induced haemolysis. CQ and PQ administration may be either simultaneous or sequential (PQ is usually given on day 28 of CQ treatment).

Therefore, CQ-resistant *P. vivax* first emerged and became widespread in Melanesia and parts of Southeast Asia, regions where PQ is not widely used because of the relatively high prevalence of G6PD deficiency in human populations (Howes et al. 2012). Moreover, a recent meta-analysis of four clinical trials carried out in Asia (Thailand and Pakistan) and Africa (Ethiopia), with a total of 1,423 patients, showed that CQ alone (25 mg/kg over 3 days) is less effective against *P. vivax* asexual blood stages than CQ (25 mg/kg over 3 days) co-administered with PQ (15 mg of PQ base/day for 14 days), with a relative risk of parasite recrudescence of 1.92 (95% confidence interval, 1.59-2.34) over 28 days of follow-up (Naing et al. 2010). Although these data suggest that *P. vivax* CQ resistance might not be a significant reason for concern in countries where CQ and PQ are routinely co-administered (Ferreira & da Silva-Nunes 2010), there are clearly documented examples of *P. vivax* infections acquired in Guyana (Phillips et al. 1996) and Brazil (Marques et al. 2013) that were resistant to CQ-PQ combination therapy.

P. vivax CQ resistance monitoring: a role for ex-vivo assays? - In addition to in vivo studies, *P. vivax* drug susceptibility may also be assessed ex vivo (testing parasites directly from infected patients) using short-term schizont maturation tests in the presence of increasing drug concentrations (Russell et al. 2003, Tasanori et al. 2006). Theoretically, in vitro analysis allows for a more objective assessment of drug resistance patterns than in vivo tests, with no influence of confounding factors such as patients' acquired immunity and antimalarial drug absorption and metabolism (Price et al. 2012). However, the comparison of data generated in different laboratories is affected by factors such as previous use of antimalarials, synchronicity of infection in the original clinical samples,

time delays in processing these samples, variations in methods used for sample cryopreservation and thawing (if any) and differences in assay protocols, including methods for quantifying parasite growth (Russell et al. 2008). Moreover, no standard *P. vivax* strains with different drug resistance patterns are available for testing the reproducibility of assays in different laboratories. Interestingly, *P. vivax* displays striking stage-specific differences in CQ susceptibility: only the early ring stages are affected by CQ exposure, whereas late trophozoites and schizonts are almost completely CQ resistant (Russell et al. 2008).

Given these reasonable limitations, it is far from surprising that ex vivo drug susceptibility assays have rarely been used to evaluate CQ resistance in Latin American isolates of *P. vivax*. Nevertheless, two recent reports from Manaus revealed similar prevalence of CQ resistance in local *P. vivax* isolates. Both studies used similar assay protocols, with parasite growth being measured by antigen-capture dehydrogenase immunodetection assay and the same definition of CQ resistance [50% inhibitory concentration (IC_{50}) > 100 nM] was used. These studies showed a rate of 9.8% of CQ-resistant isolates among 132 clinical samples collected and processed between 2004-2007 (Pratt-Riccio et al. 2013) and 10.7% among 112 samples collected between 2007-2008 (Chehuan et al. 2013). Such figures are consistent with the known recrudescence rate of 10.1% in the same area among *P. vivax* patients treated with CQ alone (de Santana Filho et al. 2007). Unfortunately, no further ex vivo CQ-resistance data are available for *P. vivax* samples from other regions of Brazil and elsewhere in Latin America.

Molecular mechanisms and markers of *P. vivax* CQ resistance - Our knowledge about the mechanisms and molecular markers of CQ resistance in *P. vivax* remains limited and several factors may account for this. First, the absence of an effective and reproducible protocol for the continuous in vitro culture of this parasite, such as the one available for *P. falciparum*, has precluded the determination of clear-cut IC_{50} values for CQ, which would allow establishing the associations between phenotypes and genotypes [for example, single-nucleotide variants (SNVs)]. Second, current in vivo studies for *P. vivax* are unable to distinguish a recrudescence of CQ-resistant parasites from a relapse or a new infection (which may be due to either a major or a minor parasite subpopulation in the original infection) (Price et al. 2012). As such, resulting associations between putative molecular markers of resistance and the pre-treatment of parasites may be spurious. Third, as efforts on genetic research on malaria have historically been spear-headed by studies on *P. falciparum*, we have largely used the resulting CQ resistance markers to infer those that would be appropriate for *P. vivax*. However, due to intrinsic biological dissimilarities, extrapolation from *P. falciparum* may not reflect the true drug resistance mechanisms of *P. vivax*, thus limiting the number of markers studied.

The main gene investigated within the context of CQ resistance in *P. vivax* is *pvcrt*, the homologue of the *P. falciparum* CQ resistance transporter (*pfCRT*), in which a K76T substitution is known to be a crucial determinant of CQ resistance. A heterologous expression study

in which *pvcrt* was transfected into *P. falciparum* and *Dictyostelium discoideum* showed that *pvcrt* overexpression was able to reduce CQ susceptibility in both organisms (Sá et al. 2006), suggesting that *pvcrt* may be able to modulate CQ transport and accumulation in *P. vivax*. In a different study, parasites from a traveller with severe vivax malaria acquired in the Brazilian Amazon showed a 22-fold increase in the levels of *pvcrt* transcription compared to the parasites from three patients with uncomplicated malaria (Fernández-Becerra et al. 2009). Although some reports of drug resistance have originated from regions where severe vivax disease is common (Tjitra et al. 2008), a direct association between *pvcrt* overexpression, CQ resistance and malaria morbidity could not be established (Fernández-Becerra et al. 2009). Importantly, in vivo drug trials testing the clinical response of *P. vivax* to CQ failed to show a clear association between SNVs in *pvcrt* and treatment outcome (Nomura et al. 2001, Suwanarusk et al. 2007, Orjuela-Sánchez et al. 2009, Ganguly et al. 2013), suggesting that SNVs in this gene are not appropriate molecular markers for the surveillance of CQ resistance in this parasite.

The *P. vivax* homologue of the *P. falciparum* multi-drug resistance gene 1 (*pvmdr1*) has also been examined from the perspective of CQ resistance. In a study of natural parasite populations of *P. vivax* from Papua, Indonesia, where CQ resistance is highly prevalent, a sequence polymorphism in *pvmdr1*, translating into an Y976F substitution, was found in all patients presenting at a local health facility. The same substitution, however, was rare in Thailand, where *P. vivax* CQ resistance remains infrequent (Suwanarusk et al. 2007). In a different study, another sequence polymorphism in *pvmdr1* (F1076L) was identified in *P. vivax* samples from Thailand and Indonesia (Brega et al. 2005), countries where CQ resistance is known to occur, especially in the latter area. These polymorphisms are relatively infrequent in Latin America, where *P. vivax* CQ resistance remains relatively uncommon (Vargas-Rodríguez et al. 2012), though the Y976F substitution may reach a high prevalence in Eastern Amazonia (Gama et al. 2009). However, the absence of clinical correlates of CQ efficacy in these studies precludes the establishment of direct associations between the genetic polymorphisms analysed and treatment outcomes. The hypothesis that *pvmdr1* polymorphisms play a major role in modulating CQ responses is further challenged by the finding of CQ-resistant isolates with the wild-type version of the gene (Sá et al. 2005) or with other SNVs in the same gene (Orjuela-Sánchez et al. 2009).

Genome-wide association studies can circumvent the limitations faced by single-gene approaches, as described previously, because they do not assume that the molecules are involved in resistance to any given drug. As such, these unconstrained methodologies may help deepen our knowledge about the mechanisms of CQ resistance and identify useful molecular markers for resistance surveillance. Such strategies are now beginning to be incorporated into *P. vivax* drug resistance research and they have already yielded interesting results. Recently, Chan et al. (2012) used whole-genome sequenc-

ing (WGS) of five *P. vivax* isolates from Madagascar and Cambodia, obtained directly from patients' blood samples and from the monkey-adapted Belem strain. In doing so, these authors demonstrated that it is feasible to perform WGS of *P. vivax* field isolates and generated a catalogue of approximately 80,000 SNPs distributed throughout the genome that will enable large-scale genotyping studies and contribute to a better understanding of *P. vivax* traits, such as drug resistance (Chan et al. 2012). In a different work, a single uncultured isolate of *P. vivax* obtained from a patient from Peru with clinical malaria was subjected to WGS (30× coverage) (Dharia et al. 2010). The authors reported that a number of genes contained high ratios of nonsynonymous-to-synonymous polymorphisms, including the *P. vivax* multidrug resistance-associated protein (*PvMRPI*), an ABC transporter previously shown to be linked to quinoline and antifolate responses in *P. falciparum* (Dharia et al. 2010). More recently, Bright et al. (2013) sequenced the entire genome of a *P. vivax* isolate derived from a traveller returning to Canada from Sudan and reported the identification of several SNVs as candidate molecular markers of drug resistance, which also included mutations in the *pvmrpl* gene (Bright et al. 2013).

A role for the Worldwide Antimalarial Resistance Network (WWARN) in Latin America - The WWARN (wwarn.org) is a global network of academic experts aiming to improve the extent and quality of data, fill regional research and data gaps and strengthen resources to collate available data, with the ultimate goal of producing reliable information to help identify and track the global spread of malaria drug resistance (Sibley et al. 2008). Established in 2009 with funds from the Bill and Melinda Gates Foundation, WWARN has its secretariat in Oxford (United Kingdom), with regional centres in Bangkok (Thailand), Dakar (Senegal), Nairobi (Kenya), Cape Town (South Africa) and, since 2012, a Latin American representation based in São Paulo, Brazil.

As a multidisciplinary network, WWARN focuses on five aspects of drug resistance: clinical, molecular, in vitro, pharmacology and antimalarial quality. Over the past five years, WWARN has developed customised tools and services designed to facilitate quality-assured data collection, analysis and reporting. Over 350 clinical, molecular, in vitro and pharmacokinetic data sets (comprising 90,000 patient records, mainly from falciparum malaria studies) have been uploaded into the WWARN repository. This repository allows for automated quality assurance and the integration of data from many different sources, building a comprehensive picture of resistance while protecting the rights of the data owners.

In addition to the data repository, WWARN has developed several freely available tools, services and training materials to support the malaria research community (wwarn.org/toolkit). One of these online tools is the Parasite Clearance Estimator (PCE) software (Flegg et al. 2011) ([available from wwarn.org/pce/](http://wwarn.org/pce/)), which provides standardised estimates of parasite clearance rates in terms of slope half-life (Flegg et al. 2013). PCE has been used to compare data derived from several clinical

trials of artemisinin combination therapies (ACTs) for falciparum malaria and can also be used to analyse *P. vivax* clearance rates following CQ treatment.

Another popular WWARN software is the In Vitro Analysis and Reporting Tool (IVART) (Woodrow et al. 2013) ([available from wwarn.org/toolkit/data-management/ivart](http://wwarn.org/toolkit/data-management/ivart)). IVART automatically generates IC₅₀ estimates and plots data obtained with different in vitro assay formats and readouts. Although IVART has been extensively used in *P. falciparum* drug resistance studies, it can also be applied to ex vivo schizont maturation inhibition assays of *P. vivax*.

Mapping and containing *P. vivax* CQ resistance in Latin America poses a major challenge for local researchers and regional networks. Presently, drug resistance surveillance activities have mainly focused on ACT efficacy against *P. falciparum*, with scarce data on vivax malaria therapies (de Santana Filho et al. 2007). Accordingly, the newly established WWARN representation in Latin America has been collaborating with researchers to collate and analyse available in vivo and in vitro data (both published and unpublished) and develop an accurate picture of *P. vivax* CQ resistance in the region. Moreover, WWARN is working in close collaboration with local clinical and laboratory-oriented researchers to facilitate the integration and harmonisation of study protocols, aiming to generate high-quality in vivo data and clinical samples for the ex vivo and molecular characterisation of CQ-resistant *P. vivax* isolates using state-of-the-art techniques. This multidisciplinary effort was launched during a WWARN-sponsored regional workshop held in São Paulo in August 2013 and now includes malaria experts from five Latin American countries.

WWARN invites Latin American researchers to become involved in this initiative, both by sharing data and study protocols and by offering suggestions and comments to improve the design of new studies of malaria drug resistance in the region. Working together, we can make a difference.

Concluding remarks - Recent advances in our capacity to generate genome data *de novo* and to rapidly analyse these data have opened up unprecedented opportunities for deepening our knowledge about drug resistance in *P. vivax*. It has already been possible to identify genetic signatures of positive selection in areas of CQ resistance using genome-wide association studies. In the future, it should also be possible to compare the genomes of confirmed CQ-resistant parasites with those from sensitive ones or to identify CQ resistance *loci* using Linkage Group Selection on genetic crosses. Ultimately, such strategies will allow the identification of new putative molecular markers of CQ resistance in *P. vivax*, the value of which will rest on the demonstration that such markers effectively predict the outcome of treatment in vivo.

ACKNOWLEDGEMENTS

To Dr Márcia C de Castro (Harvard School of Public Health), for help in preparing the Figure, and to Prof Carol H Sibley and Dr Philippe Guérin, for their comments on earlier drafts of this article.

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