Characterization of treatment failure in *Plasmodium vivax* efficacy trials by genotyping neutral and drug resistance associated markers

Celine Barnadas¹²³, Cristian Koepfli⁴, Harin A Karunajeewa⁵*, Peter M Siba¹, Timothy ME Davis⁵, Ivo Mueller¹³⁶

¹ Vector Borne Diseases Unit, PNG Institute of Medical Research, Papua New Guinea.
² Center for Global Health & Diseases, Case Western Reserve University, Cleveland, OH, USA.
³ Infection & Immunity Division, Walter & Eliza Hall Institute, Melbourne, Australia.
⁴ Swiss Tropical and Public Health Institute, Switzerland.
⁵ School of Medicine and Pharmacology, University of Western Australia, Australia.
⁶ Barcelona Centre for International Health Research, Barcelona, Spain.

*Current address: Division of Medicine, Western Hospital, Footscray, Victoria, Australia

Corresponding author: Dr Ivo Mueller, Infection and Immunity Division, Walter & Eliza Hall Institute, 1G Royal Parade, Parkville, Victoria 3052, Australia. Telephone +613 9345 2936.

Email ivomueller@fastmail.fm
Abstract

Plasmodium vivax intervention trials customarily report uncorrected treatment failure rates. Application of recrudescence-reinfection genotyping and drug resistance single-nucleotide polymorphism typing to a 4-arm comparative efficacy trial illustrates that molecular approaches can assist in understanding the relative contributions of true drug resistance (recurrent with same genotype) and new infections to treatment failure. The PCR-corrected adequate clinical and parasitologic response may constitute an informative secondary endpoint in future P. vivax drug trials.
Approximately 40% of the world’s population is at risk of vivax malaria (7). Recent interest in this infection has been heightened by the emergence of chloroquine (CQ) resistance in 1989 (21) and subsequent reports of severe disease (6, 25). There has been a resultant increase in monitoring of *Plasmodium vivax* drug sensitivity through efficacy trials (2, 12, 19, 20, 22) and identification of molecular markers of resistance (1, 8, 16, 24).

Recurrent *P. vivax* parasitemia in intervention trials may indicate not only treatment failure but also activation of liver-stage hypnozoites (relapses) or a new infection (17). Two studies of patients not at risk of re-infection found that most relapses were genetically distinct from the primary infections (4, 10). Standardized gentotyping protocols characterizing treatment failure have not yet been developed for antimalarial trials in vivax malaria. However, candidate markers on genes coding for surface proteins (2, 9, 14) or neutral markers such as microsatellites (10, 13, 14) could, if sufficiently polymorphic, allow discrimination between strains in assessing post-treatment recurrence in a way analogous to that established for falciparum malaria (26). In a recent study of small numbers of children in Papua New Guinea (PNG) treated with amodiaquine or CQ plus sulfadoxine-pyrimethamine (SP), the authors recommended use of two highly polymorphic markers associated with a very low probability of independent infections carrying the same alleles (14). We have utilized this approach in a retrospective analysis of samples taken from a larger number of PNG children participating in an efficacy trial comparing CQ-SP and three artemisinin combination therapies (ACTs) (12), and performed a complementary analysis of 4-aminoquinoline and SP drug resistance markers (3, 5, 15, 24).

The study was conducted in Madang and East Sepik provinces between 2005 and 2007 (12), and involved 195 children aged 0.5 to 5 years with >250 *P. vivax* asexual forms/µL and no
features of severe malaria who were randomly assigned to CQ-SP, artesunate-SP (ARTS-SP),
dihydroartemisinin-piperaquine (DHA-PIP) or artemether-lumefantrine (AL). The non-PCR-
corrected clinical and parasitologic failure rates were 49.0%, 48.7%, 15.8% and 51.5%,
respectively, after 28 days follow-up, and 87.0%, 66.7%, 30.6% and 69.7% after 42 days.
There was no difference between the rate of recurrent *P. vivax* parasitemia between the CQ-
SP, ARTS-SP and AL arms (Figure 1A; log-rank test, *P*=0.28).

Genotyping based on length polymorphism of a region of *msp1* (*mspIF3*) and a microsatellite
MS16 was performed, a combination with a probability of <0.25% that two isolates carry the
same alleles (14). Recurrent infections occurring during 42 days follow-up that contained at
least one genotype present at baseline were classified as ‘recurrent infections with the same
genotype’ and recurrences with a different genotype as ‘new infections’. Since *P. vivax*
genotyping was not pre-specified and in view of limited volumes, usable blood samples on
the day of recurrent parasitemia were available for 70.1% and 70.3% of the samples to day 28
and 42, respectively. The present sub-study was approved by the PNG IMR Institutional
Review Board (approval 1029).

During 28 days follow-up, there were no significant differences between CQ-SP, ARTS-SP
or AL in the rates of either recurrent parasitemia with the same genotype (Figure 1B; *P*=0.74)
or new infections (Figure 1C; *P*=0.59). Up to day 42, there were significantly more cases of
recurrent parasitemia with the same genotype in the CQ-SP arm but not in the number of new
infections (Table 1). At days 28 and 42, fewer infections with both the same and different
genotypes were observed after DHA-PIP (Table 1). Day 42 treatment failure rates were
87.0%, 66.7%, 69.7% and 30.6% for CQ-SP, ARTS-SP, AL and DHA-PIP, respectively, and
51.4%, 28.1%, 22.2% and 9.7% after PCR correction.
To better understand these differences, we screened mutations in two *P. vivax* genes related to SP or 4-aminoquinoline resistance, namely *dhfr* (5) and *mdr1* (3, 23). Of patients allocated CQ-SP or ARTS-SP, 32 (69.6%) and 38 (71.8%), respectively, were infected with at least one triple or quadruple *dhfr* mutant parasite at enrolment (57L, 58R, 61M +/- 117T). While the small sample size does not allow firm conclusions regarding selection of mutant parasites, an increase in the frequency of triple/quadruple *dhfr* mutants was observed in the recurrent parasitaemias with the same genotype (based on *msp1/MS16* genotyping) in the CQ-SP (15/18; 83.3%) and ARTS-SP (9/9; 100%) arms. No such increase was observed in the AL arm (20/32; 62.5% vs 4/6; 66.7%). In the CQ-SP arm, the presence of parasites with triple/quadruple *dhfr* (57L-58R-61M/117T) plus *mdr1* 976F was associated with treatment failure (recurrent parasitaemia with same genotype) with an odds ratio of 3.9 (95% confidence intervals 0.6-28.2, *P*=0.08). Given the overall high levels of triple/quadruple *dhfr* mutants, this adds to emerging evidence that *mdr1* (976F) mutations may be involved in reduced *P. vivax* CQ sensitivity (16, 24).

Despite comparable non-PCR corrected adequate clinical and parasitologic responses (ACPRs), the genotyping/molecular marker data reveal between-treatment differences in recurrent parasitemia that reflects the pharmacodynamic and pharmacokinetic profiles of the antimalarial drugs (11, 18, 27). Given the high prevalence of quadruple *dhfr*-mutated parasites and the relatively short elimination half-lives of the components, SP is likely to have contributed little to either initial parasite clearance or to prevention of new (or relapsing) infections during follow-up in the CQ-SP and ARTS-SP arms. Thus, ARTS would have been primarily responsible for successful initial clearance in the latter arm. The predominantly late occurrence of recurrent parasitemia irrespective of origin in the CQ-SP arm indicates that CQ
remained partially effective despite the positive selection of mdr1 mutant (976F) parasites. The difference in efficacy between DHA-PIP and AL may largely reflect the terminal elimination half-lives of PIP (3-4 weeks) and lumefantrine (4-6 days), with long-lasting PIP suppression of re-infections and relapses regardless of genotype and plasma lumefantrine concentrations beyond two weeks post-treatment that were insufficient to prevent recurrence (20).

The present preliminary data highlight the important potential contribution that genotyping/molecular marker typing can make to improved characterization of recurrent parasitemia in P. vivax intervention trials. Since genotyping cannot differentiate between true failures and relapses with the same genotype, the primary endpoint should remain ACPR without genotyping. However, ACPR after genotyping based on epidemiologically appropriate markers could be added as a secondary endpoint. Further research may promote harmonization of P. vivax genotyping protocols and the adoption of consensus recommendations.

Acknowledgments

The intervention trial was sponsored by WHO Western Pacific Region, Rotary Against Malaria (PNG), and the National Health and Medical Research Council (NHMRC) of Australia (grant 353663). The present sub-study was funded by NHMRC project grant 1010203. TMED was supported by an NHMRC Practitioner Fellowship.
References


Table 1: Genotyping results of recurrent parasitemia during 6 weeks of follow-up.

<table>
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<tr>
<th></th>
<th>CQ-SP</th>
<th>ART-SP</th>
<th>AL</th>
<th>DHA-PIP</th>
<th>p*</th>
<th>p**</th>
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<tr>
<td>28 day assessment</td>
<td>51</td>
<td>39</td>
<td>33</td>
<td>38</td>
<td>0.97</td>
<td>&lt;0.001</td>
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<td>Non-corrected ACPR</td>
<td>26 (51.0%)</td>
<td>20 (51.3%)</td>
<td>16 (48.5%)</td>
<td>32 (84.2%)</td>
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<td>Evaluable by PCR</td>
<td>44</td>
<td>34</td>
<td>29</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>no recurrent parasitaemia</td>
<td>26 (59.1%)</td>
<td>20 (55.8%)</td>
<td>16 (55.2%)</td>
<td>32 (94.1%)</td>
<td>0.44</td>
<td>0.03</td>
</tr>
<tr>
<td>same genotype</td>
<td>10 (22.7%)</td>
<td>4 (11.8%)</td>
<td>6 (20.7%)</td>
<td>1 (2.9%)</td>
<td>0.51</td>
<td>0.005</td>
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<td>new infection</td>
<td>8 (18.2%)</td>
<td>10 (29.4%)</td>
<td>7 (22.6%)</td>
<td>1 (2.9%)</td>
<td>0.51</td>
<td>0.005</td>
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<tr>
<td>PCR-corrected ACPR</td>
<td>34 (77.3%)</td>
<td>30 (88.2%)</td>
<td>23 (78.3%)</td>
<td>33 (97.1%)</td>
<td>0.44</td>
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<td>42 day assessment</td>
<td>46</td>
<td>39</td>
<td>33</td>
<td>36</td>
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<tr>
<td>Non-corrected ACPR</td>
<td>6 (13.0%)</td>
<td>13 (33.3%)</td>
<td>10 (30.3%)</td>
<td>25 (69.4%)</td>
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<tr>
<td>Evaluable by PCR</td>
<td>35</td>
<td>32</td>
<td>27</td>
<td>31</td>
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<tr>
<td>no recurrent parasitaemia</td>
<td>6 (17.1%)</td>
<td>13 (40.6%)</td>
<td>10 (37.0%)</td>
<td>25 (80.6%)</td>
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<tr>
<td>same genotype</td>
<td>18 (51.4%)</td>
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<td>6 (22.2%)</td>
<td>3 (9.7%)</td>
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<td>0.01</td>
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<tr>
<td>new infection</td>
<td>11 (31.4%)</td>
<td>10 (31.3%)</td>
<td>11 (40.7%)</td>
<td>3 (9.7%)</td>
<td>0.94</td>
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<tr>
<td>PCR-corrected ACPR</td>
<td>17 (48.6%)</td>
<td>23 (71.9%)</td>
<td>23 (71.9%)</td>
<td>28 (90.3%)</td>
<td>0.03</td>
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</tbody>
</table>

* Chi-squared test for CQ-SP, ART-SP and AL treatments arms
** Chi-squared test for DHA-PIP and the other three arms combined
PCR-corrected ACPR: sum of no recurrent parasitemia and new infections.
**Figure 1:** Kaplan-Meier Curves for time first *P. vivax* infection during 6 weeks of follow-up. A) Time to any recurrent *P. vivax* parasitaemia; B) Time to recurrent infection with same genotype; C) Time to recurrent infection with new genotype