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SHORT COMMUNICATION

Lack of multiple copies of *pfmdr1* gene in Papua New Guinea

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Summary We describe here the results of an analysis of *Plasmodium falciparum* multidrug resistance protein 1 (*pfmdr1*) gene copy number from 440 field isolates from Papua New Guinea. No multiple copies of the gene were found, which corresponds to the lack of usage of mefloquine. These data extend regional knowledge about the distribution of multidrug-resistant *P. falciparum*.
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1. Introduction

Recent in vitro and in vivo studies showed an association between artesunate–mefloquine therapy failure and genetic changes in *Plasmodium falciparum* multidrug resistance protein 1 (*pfmdr1*) gene (Price et al., 2004; Sidhu et al., 2006). These findings underline the contribution of *pfmdr1* copy number to the susceptibility of *P. falciparum* to antimalarial drugs. Most malaria-endemic countries have recently adopted artemisinin combination therapies (ACTs) as first-line therapy for *P. falciparum* infections because of increasing resistance to all currently used antimalarial drugs.

As the world's most multidrug-resistant *P. falciparum* parasites are found along the borders of Thailand and are also emerging elsewhere in Southeast Asia, many studies on *pfmdr1* gene copy number have been performed on samples from Southeast Asia. Studies in Africa indicate that *pfmdr1* gene copy number is rare in the field outside Southeast Asia (Ursing et al., 2006). However, no research has been conducted on *pfmdr1* copy number in Papua New Guinea (PNG), where drug pressure of mefloquine on *P. falciparum* has been minimal so far. Until recently, standard first-line

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treatment in PNG for malaria consisted of chloroquine or amodiaquine plus sulfadoxine–pyrimethamine. In order to describe the baseline situation, we determined *pfmdr1* copy numbers in 440 field isolates from PNG. With this analysis we extend the knowledge about the global distribution of multidrug-resistant *P. falciparum*.

2. Materials and methods

Pre-treatment fingerprick samples were collected from 440 patients within several in vivo drug efficacy studies conducted between 2003 and 2005 in three health centres in Simbu, East Sepik and Madang Province in PNG (Marfurt et al., 2007).

The *pfmdr1* gene copy number was assessed as described previously (Price et al., 2004). The assays were run in triplicate on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Rotkreuz, Switzerland) and every run contained two calibrator DNA samples from clones 3D7 and W2-mef, having *pfmdr1* copy numbers of 1 and 3, respectively. The Ct threshold was set manually at 0.05 for *pfmdr1* and at 0.03 for β-*tubulin*, respectively, and the baseline was calculated automatically. Results were analysed by the comparative ΔΔCt method described previously (Price et al., 2004). Assays were repeated if one of the following results was obtained: no Ct value for more than one sample in the triplicate; calibrator copy number $\neq$ 3; ΔΔCt spread >1.5; Ct values >35.

The multiplicity of infection was determined as described previously by fragment-sizing of *P. falciparum* merozoite surface protein 2 (*pfmsp2*) using GeneMapper Software, v3.7 (Applied Biosystems) (Falk et al., 2006). The primary and nested PCR protocol was changed to 2 min at 94 °C followed by 25 cycles (30 s at 94 °C, 45 s at 50 °C and 1 min 30 s at 70 °C) with a final elongation for 10 min at 70 °C each. Of the diluted nested PCR product (1:10 in H_2O), 2.5 μl were combined with 10 μl diluted ROX-500 size standard (1:40 in H_2O; Applied Biosystems). Assays were repeated if one of the following results was obtained: no sizing data; *pfmsp2* PCR artefacts; no *pfmsp2* PCR product.

Samples were excluded from the final analysis if one or more of the following results were obtained after repetition of the assay: no Ct value for more than one sample in triplicate; ΔΔCt spread >1.5; Ct values >35; *pfmsp2* PCR artefacts; no sizing data; no *pfmsp2* PCR product.

3. Results

Of the 440 samples analysed, 35 were excluded from the final analysis (15 no Ct value for more than one sample in triplicate; one Ct value >35; three no Ct value for more than one sample in triplicate and no *pfmsp2* PCR product; six no sizing data; three *pfmsp2* PCR artefacts; seven no *pfmsp2* PCR product). The remaining 405 samples included in the final analysis showed only one copy of the *pfmdr1* gene. The mean multiplicity of infection found in the patients was 1.56 ± 0.76 (mean ± SD), with 240 patients showing single infections.

4. Discussion

The number of samples excluded from the final analysis might be explained by the fact that field samples sometimes contain very low levels of parasite DNA, thus leading to indeterminable Ct values in the real-time PCR or missing *pfmsp2* PCR products.

Mefloquine use has not been significant yet in PNG, and the occurrence of only one gene copy of *pfmdr1* strengthens the assumption that mefloquine selects for copy number increase. Studies that have tested different *P. falciparum* isolates from PNG for mefloquine resistance showed full in vitro susceptibility (Hombhanje, 1998). By contrast, other authors previously have shown a high frequency of *pfmdr1* sequence polymorphisms, confirming that chloroquine selects for polymorphisms (Nagesha et al., 2001).

The multiplicity of infections found is similar to results in other settings in PNG (Cortes et al., 2004). However, it is possible that a small percentage of the parasites in multiple infections carry more than one copy of *pfmdr1* and will not appear as multiple copies in the analysis due to lack of statistical power. In addition, the fact that within the large number of single infections there was no gene amplification supports the idea that *pfmdr1* is still not amplified in PNG.

In conclusion, we show that drug-resistance-associated *pfmdr1* gene amplifications have not yet been selected in PNG. Therefore mefloquine and artesunate might still be highly effective first-line treatment options against *P. falciparum* infections.

Authors' contributions: HPB conceived the study; JM, SB, IM, JCR, PS and BG designed the study; JM collected samples; EMH, DM and AR carried out the laboratory work; EMH and HPB analysed the data; SB, IM, JCR, PS and BG interpreted the data; EMH and HPB wrote the manuscript. All authors read and approved the final manuscript. HPB is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: The study was approved by the Papua New Guinea Medical Research Advisory Committee.

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