Plasmodium falciparum isolates with increased pfmdr1 copy number circulate in West Africa

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Running Title: Duplicated pfmdr1 genotypes circulate in West Africa

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ABSTRACT

Amplification of pfmdr1 in *Plasmodium falciparum* is linked to resistance to aryl-amino-alcohols and in reduced susceptibility to artemisinins. We demonstrate here that duplicated *pfmdr1* genotypes circulate in West Africa. The monitoring of this prevalence in Africa appears essential for determining the antimalarial policy and to maintain the efficiency of ACT for as long as possible.
Plasmodium falciparum malaria remains a major cause of morbidity and mortality in tropical and sub-tropical areas. One of the main characteristics of *P. falciparum* is its ability to become resistant to all the treatments used. To limit the evolution and spread of drug resistance, the World Health Organization (WHO) recommendations are now based on artemisinin (ART)-based combination therapies (ACTs) to treat uncomplicated falciparum malaria. By May of 2008, 39 of the 42 countries in Africa where *P. falciparum* is endemic had adopted ACT (artemether-lumefantrine [AL] or artesunate-amodiaquine [AS + AQ]) as the first-line treatments (17). Even if accessibility to these compounds is still insufficient in Africa, the drug pressure by ART derivatives has dramatically increased in recent years. Furthermore, although mefloquine (MQ) is still not used in Africa, it is likely that it will be introduced within a few years. The current level of resistance to sulfadoxine-pyrimethamine (SP), the only malaria prophylaxis for pregnant women, is high and therefore SP is likely to be replaced by MQ (3) as has happened in Southeast Asia. Based on the expectation of new drug policies, the monitoring of the efficacy of ACTs, especially MQ, against *P. falciparum* is necessary.

Amplification of *pfmdr1* is a common molecular marker of ACT and MQ susceptibility. An increase in the *pfmdr1* copy number is associated with clinical failures and to *in vitro* resistance to aryl-amino-alcohols, particularly MQ, but also to lumefantrine (11, 13). *Pfmdr1* amplification has also been demonstrated to decrease the susceptibility to ART derivatives in the field as well as *in vitro* (2, 4, 8, 13) although the role of *pfatp6* polymorphism in the phenomenon is unclear.

Few data exist on *pfmdr1* amplification in Africa. One study carried out in 1995 in Lambarené (Gabon) found 5% of isolates with more than 1 copy number of *pfmdr1*, but this was not confirmed in 2002 (15). In Kenya, Holmgren *et al* (7) identified only one isolate with 2
pfmdr1 copies from 72 isolates tested. Except for one isolate from Ivory Coast in 1993 (1) no
pfmdr1 amplification has been identified in West Africa (6, 16).

Using falciparum malaria patients returning from Africa as a sentinel, we have investigated
whether pfmdr1 amplification has occurred in isolates from these patients.

DNA was obtained from patients who returned from West and Central Africa between
2005 and 2009 with falciparum malaria diagnosed in the Parasitology-Mycology Department
of the Toulouse University Hospital. The pfmdr1 copy number determination was carried out
by real time-PCR on a LightCycler480® (Roche Diagnostics). Primers were obtained from
Price et al (2004) (11). β-tubulin, was used as one copy reference gene. The reaction was
carried out in 10µl final volume in a 96-well plate (5µl of LightCycler 480 SYB Green I
Master mix, (Roche Diagnostics), 0.25mM of each primer, 2µl DNA sample). The
amplification program was 95°C / 10 min, then 95°C / 15 sec 63°C / 15 sec and 70°C / 10 sec
for 45 cycles. In each experiment, DNA from the laboratory strains FcM29-Cameroon (1 copy
pfmdr1) and Dd2 (2-3 copies pfmdr1) (16) were used as controls. The efficiency of each PCR
(pfmdr1 and β-tubulin) was determined using a scale dilution of the FcM29 DNA. The
determination of the copy number was done by comparison of the ratio of pfmdr1/β-tubulin
on the LightCycler480 V1.5.0 software (Roche Diagnostic) taking into account the efficiency
of each PCR. The PCR results of all the samples considered in this study could be localized to
the linear portion of the efficiency curve in terms of Cp (Crossing point) (Cp value between
20 to 35). The cut-off value of a multicopy was considered to be > 1.5. Each sample was
analyzed in triplicate and each one found with a copy number more than 1.5 pfmdr1 copies
was checked again.

We identified 4 out of 131 isolates (3%) with a pfmdr1 copy number greater than 1.
The 4 patients were cured by quinine treatment. These 4 isolates were all from West Africa
and were identified in 2008 and 2009. No isolates with \textit{pfmdr1} amplification were detected in Central Africa. However the low number of samples from this area was not sufficient to enable a conclusion on the prevalence to be reached (Table 1, Figure 1). It would therefore be interesting to determine the exact prevalence of clones with \textit{pfmdr1} amplification and to follow its evolution in a more extended study in Africa and at multiple test sites. This monitoring could be achieved through the WorldWide Antimalarial Resistance Network (WWARN), now operational (12).

The presence of these \textit{pfmdr1} multicopy clones in West Africa could be linked to rare but actual clinical MQ failures (5) and to the reduced susceptibility of few isolates to MQ (10, 14) \textit{in vitro} observed in this area.

In South-East Asia, specifically in some parts of Thailand and Cambodia, the high level of isolates with a \textit{pfmdr1} copy number greater than 1 (30 - 40\%) was due to MQ monotherapy for many years as the first-line treatment for uncomplicated falciparum malaria (11). As this treatment is not used in Africa, the prevalence of clones with \textit{pfmdr1} amplification remains low at the moment. Nevertheless, because of the widespread prescription of lumefantrine (aryl-amino-alcohol), the partner drug of artemether in ACT (AL) in Africa, monitoring \textit{pfmdr1} now appears necessary to determine the possible role of AL in the selection of \textit{pfmdr1} copy number amplification. Moreover, in addition to fake formulations, many drugs are used without any the authorization of the Ministries of Public Health. In the case of Africa, contrary to WHO recommendations, artesunate and MQ in monotherapy and MQ + SP already circulate (9). A misuse of these antimalarial drugs could lead to a rapid increase in the prevalence of strains with amplified \textit{pfmdr1}, since the transmission level of malaria in Africa is the highest of all endemic areas. This situation could accelerate the emergence of resistance to ACTs. Identification of these 4 isolates with \textit{Pfmdr1} amplification in 2008 and 2009 thus raises the question of the emergence of this genotype in West Africa.
Based on the role of pfmdr1 amplification in the susceptibility of P. falciparum to ART derivatives and aryl-amino-alcohol, avoid the selection of strains with duplicated pfmdr1 in Africa is essential. This will be a difficult challenge but vital to ensure a longer period of efficacy for the ACTs.

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From 131 isolates (2005: n = 5, 2006: n = 35, 2007: n = 33, 2008: n = 34, 2009: n = 24) only four had \textit{pfmdr1} copy number amplification. The 127 other isolates showed a mean value of \textit{pfmdr1} copies of 1.08 ± 0.14. Ninety nine of these 131 isolates were from West Africa (38 Ivory Coast, 18 Burkina Faso, 10 Senegal, 9 Guinea Conakry, 6 Mali, 6 Benin, 4 Togo, 3 Mauritania, 2 Nigeria, 2 Ghana, 1 Liberia) and 32 from Central Africa (17 Cameroon, 8 Central Africa Republic, 4 Gabon, 2 Congo, 1 Chad).
Table 1: Value and country of origin of the 4 isolates with a *pfmdr1* copy number greater than 1

<table>
<thead>
<tr>
<th>Value of <em>pfmdr1</em> copy number (mean of the triplicate assays ± SD)</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.87 ± 0.1</td>
<td>Ivory Coast</td>
</tr>
<tr>
<td>2.03 ± 0.09</td>
<td>Ivory Coast</td>
</tr>
<tr>
<td>1.94 ± 0.2</td>
<td>Burkina Faso</td>
</tr>
<tr>
<td>1.76 ± 0.08</td>
<td>Togo</td>
</tr>
</tbody>
</table>