Molecular surveillance of drug-resistant *Plasmodium vivax* using *pvdhfr*, *pvdhps* and *pvmdr1* markers in Nouakchott, Mauritania

Khadijetou Mint Lekweiry1–3, Ali Ould Mohamed Salem Boukhary1, Tiphaine Gaillard4, Nathalie Wurtz5,6, Hervé Bogreau5,6, Jamal Eddine Hafid2, Jean-François Trape7, Housem Bouchiba5,6, Mohamed Salem Ould Ahmedou Salem1, Bruno Pradines5,6, Christophe Rogier5,6†, Leonardo K. Basco5,7* and Sébastien Briolant5,6

1Laboratoire de Biotechnologies, Faculté des Sciences et Techniques, Université de Nouakchott, Mauritania; 2Laboratoire Aliments, Environnement et Santé (LAES), Faculté des Sciences et Techniques, Université Cadi Ayyad, Marrakech, Morocco; 3UFR Biologie et Santé, Département de Biologie, Faculté des Sciences Semlalia, Université Cadi Ayyad, Marrakech, Morocco; 4Laboratoire de Microbiologie, Hôpital d’Instruction des Armées Sainte Anne, Toulon, France; 5Unité de Parasitologie, Institut de Recherche Biomédicale des Armées, Le Pharo, Marseille, France; 6Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes (URMITE), Faculté de Médecine La Timone, Université de la Méditerranée, 13385 Marseille, France; 7Institut de Recherche pour le Développement (IRD), Unité Mixte de Recherche 198, Faculté de Médecine La Timone, Université de la Méditerranée, 13385 Marseille, France

†Present address: Institut Pasteur de Madagascar, B.P. 1274, 101 Antananarivo, Madagascar.

Corresponding author. Laboratoire de Parasitologie, Institut de Recherche Biomédicale des Armées, allée du médecin Colonel Eugène Jamot, Parc du Pharo, 13262 Marseille cedex 07, France. Tel: +33-4-91-15-01-49; Fax: +33-4-91-15-01-64; E-mail: lkbasco@yahoo.fr

Received 19 May 2011; returned 26 July 2011; revised 29 September 2011; accepted 8 October 2011

**Objectives:** *Plasmodium falciparum* and *Plasmodium vivax* occur in Mauritania. Drug-resistant *P. falciparum* has been reported, but the drug-resistance status of *P. vivax* is unknown. The aims of the present study were to determine the prevalence of mutant *pvdhfr*, *pvdhps* and *pvmdr1* genes and of *pvmdr1* gene amplification in *P. vivax* isolates in Nouakchott, the capital city of Mauritania, and to establish a baseline for molecular surveillance of drug-resistant *P. vivax* in the country.

**Patients and methods:** Between 2007 and 2009, 439 febrile patients were screened for malaria in Nouakchott. The sequences of *pvdhfr*, *pvdhps* and *pvmdr1* markers in 110 *P. vivax* isolates were determined by direct sequencing of PCR products. The *pvmdr1* gene copy number was determined by real-time PCR.

**Results:** The majority of the isolates with a successful PCR amplification (76/86, 88%) were characterized to be of the wild-type *pvdhfr* genotype, while the remaining 10 isolates carried the S58R and S117N double mutations. All isolates had the wild-type *pvdhps* genotype SAKAV. For *pvmdr1*, 75 of 103 (73%) had the wild-type Y976, and 28 (27%) carried the mutant F976. Most (98%) carried the mutant L1076 codon. Of 105 isolates, 102 (97%) had one copy and 3 (3%) had two copies of the *pvmdr1* gene.

**Conclusions:** The prevalence of mutations associated with antifolate resistance is low in Mauritania. Further studies are required to determine the roles of *pvmdr1* mutations and gene amplification in conferring drug resistance. These data will serve as a baseline for further monitoring of drug-resistant malaria.

**Keywords:** *P. vivax*, tertian malaria, drug resistance, molecular epidemiology, chloroquine, antifolate

**Introduction**

*Plasmodium vivax*, the causative agent of relapsing benign tertian malaria, remains the second most common cause of malaria in the world with more than 80 million clinical cases annually. It is the most widely distributed human malaria parasite. It occurs in Central and South America, Asia, the Middle East and parts of Africa.1 Unlike *Plasmodium falciparum*, *P. vivax* rarely causes mortality, but it is responsible for considerable morbidity and economic loss in endemic countries.2–5

Mauritania (latitude 15–27°N, longitude 5–17°W) is situated in West Africa and is predominantly a Saharan country, with the northern two-thirds of its surface area occupied by the Sahara desert and the other third in the sub-Saharan south. The annual rainfall is low and increases from north to south. There is only one rainy season, from July to September. Malaria transmission, predominantly *P. falciparum* and, to a lesser extent, *P. vivax*, occurs in the southern one-third of the country bordering Senegal, Mali and the Atlantic coast.5,6 Chloroquine-resistant *P. falciparum* has been reported, both in
vivo and in vitro, from Mauritania.\textsuperscript{7–9} Due to the lack of reliable health information as well as insufficient resources to confirm presumptive clinical diagnosis of malaria with laboratory examinations, the epidemiology of malaria in general, and in particular for \textit{P. vivax}, is not well known in the country.

The presence of \textit{P. vivax} in Mauritania was first reported in 1948.\textsuperscript{10} In a later study conducted in 1996, infections in 28 of 77 patients (36\%) with malaria, confirmed by microscopic examination of blood smears, were due to \textit{P. vivax}, suggesting autochthonous \textit{P. vivax} transmission in some patients who had never travelled outside Nouakchott.\textsuperscript{11} More recently, Mint Lekweiry et al.\textsuperscript{12} have shown a high proportion of \textit{P. vivax} malaria (73.8\%, of which 3.3\% were mixed \textit{P. vivax}/\textit{Plasmodium ovale}), followed by \textit{P. ovale} (24.6\%) and \textit{P. falciparum} (1.6\%), by microscopic examination of blood smears and PCR, among 61 febrile patients in Nouakchott. At least two of the \textit{P. vivax}-infected patients had never travelled outside Nouakchott and were considered to be autochthonous cases. The presence of \textit{P. vivax} reported in these three studies, conducted in local populations, has further been confirmed by reports on imported \textit{P. vivax} malaria originating from within Mauritania.\textsuperscript{13–15}

The official national statistics suggest an increasing prevalence of malaria in Mauritania over the past two decades, of which 90\% were due to \textit{P. falciparum}.\textsuperscript{16} The environmental factors that probably explain this increasing prevalence of \textit{P. falciparum} malaria include the development of hydro-agricultural projects in the south as well as at oases in the north. The extent of malaria imported from neighbouring countries is unknown. Furthermore, the emergence and spread of antimalarial drug resistance is becoming an important factor.\textsuperscript{5,7,15} As for \textit{P. vivax}, the status of drug resistance is currently unknown in Mauritania.

Several mutations in drug resistance markers have been identified in \textit{P. vivax}. Most of these genes had initially been identified and analysed in \textit{P. falciparum}, and orthologous genes have been determined in \textit{P. vivax}. Resistance to antifolates (pyrimethamine and sulfadoxine) is associated with specific point mutations in genes encoding dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) in both \textit{P. falciparum} and \textit{P. vivax}, and an increasing number of specific mutations results in higher levels of resistance.\textsuperscript{17–21} Chloroquine resistance is associated with mutations in the gene encoding the \textit{P. falciparum} chloroquine-resistance transporter (\textit{pfcrt}), but mutations in the corresponding \textit{P. vivax} chloroquine-resistance transporter orthologue (\textit{pvcrto}) are not associated with chloroquine resistance.\textsuperscript{7,23} The \textit{P. falciparum} multidrug-resistance gene 1 (\textit{pfmdr1}) encodes a transmembrane transporter and has been associated with chloroquine, amino-alcohol and artesiminin resistance (\textit{pfmdr1} mutations) and mefloquine (also an amino-alcohol) resistance (\textit{pfmdr1} gene amplification).\textsuperscript{24} Mutations in the orthologous gene, \textit{pvmdr1}, have been reported to be associated with chloroquine resistance, while gene amplification may confer resistance to amodiaquine, mefloquine and artesunate.\textsuperscript{25,26} However, a role of \textit{pvmdr1} in conferring drug resistance is still not clear.\textsuperscript{22,27–30}

Based on the currently available data on drug resistance markers in \textit{P. vivax}, the objectives of the present study were to determine the prevalence of mutant \textit{pvmdr1} (\textit{pvdhps} and \textit{pvdhfr} genes and \textit{pvmdr1} gene amplification in isolates obtained from symptomatic patients residing in Nouakchott, and to establish a baseline for molecular surveillance of drug-resistant \textit{P. vivax} in the country. The chloroquine-resistance marker \textit{pvcrto} was not included in this study because its association with a resistant phenotype is not clearly established.

\textbf{Methods}

\textbf{Study area}

The study was conducted in Nouakchott (18°05′2″N; 15°58′42″W), the capital city of Mauritania (Figure 1). The city is situated in the Saharan zone, and the centre of the town is ≈4 miles from the Atlantic coast. The vegetation consists of orchards with diverse crops (date palm, vegetables, mint and alfalfa). The climate is Saharan with dry and wet seasons. The rainy season is short, extending from July to September with an average annual rainfall of 50–80 mm. The dry season is relatively cool (19–24°C) between November and March, and relatively hot (26–30°C) from April to June. The monthly average temperature ranges from 20.7°C (in January) to 33.3°C (in September), and the mean relative humidity varies from 33.4% to 79.1%. The current population in Nouakchott is estimated to be 661,000. The city has four hospitals and nine health centres.

\textbf{Patients and sample collection}

All febrile patients attending the National Hospital and Cheick Zayed Hospital between 2007 and 2009 were screened for malaria. After written informed consent had been obtained from at least one parent or legal guardian for febrile children and adolescents, and/or from febrile adolescents and adults, venous blood was collected in a EDTA-coated tube by venipuncture for microscopic examination of blood smear and a rapid diagnostic test (OptiMAL; DiMed GmbH, Cressier, Switzerland). A blood sample was spotted onto Whatman 3MM filter paper, dried and stored at room temperature until use. The remainder of the sample was centrifuged, and the plasma and red cell pellets were stored separately at −20°C. Artemether/lumefantrine fixed-combination was prescribed to patients with a positive rapid diagnostic test, in accordance with the national antimalarial drug policy of the country. The patients were not followed up. This study was reviewed and approved by the Mauritanian National Ethics Committee.
Table 1. Primers used for PCR, sequencing reactions and gene copy number determination

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Forward, reverse primers and probes</th>
<th>Amplicon size (bp)</th>
<th>Annealing temperatures (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pv dhfr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st PCR</td>
<td>forward, 5′-ACCATTTCCATAGGATTCACCTT-3′ \ reverse, 5′-CGCATTTGAGTTCGTCGAA-3′</td>
<td>961</td>
<td>53</td>
</tr>
<tr>
<td>2nd PCR, SR</td>
<td>forward, 5′-CCCAACACTAACAAAGGAATGATG-3′ \ reverse, 5′-GCCGTTGATCCTTGTGAAG-3′</td>
<td>632</td>
<td>65°, 50°</td>
</tr>
<tr>
<td>pv dhps</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st PCR</td>
<td>forward, 5′-GGAAAGGACTCGATCACTTATAA-3′ \ reverse, 5′-CGTCAAGTACGCCGTTAATAAA-3′</td>
<td>970</td>
<td>53</td>
</tr>
<tr>
<td>2nd PCR, SR</td>
<td>forward, 5′-GATGGCGGTTATTTGTCGAT-3′ \ reverse, 5′-GCCCTCAGCCTACGTCT-3′</td>
<td>767</td>
<td>65°, 50°</td>
</tr>
<tr>
<td>pv md r1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st PCR</td>
<td>forward, 5′-CGGAAACCTGAAAAGTACTCCCTA-3′ \ reverse, 5′-GCGGTAGTGGTCGTCGGAT-3′</td>
<td>762</td>
<td>53</td>
</tr>
<tr>
<td>2nd PCR, SR</td>
<td>forward, 5′-GAGGACGTCTAAAGGATCCT-3′ \ reverse, 5′-GGAAAGCCATTCGCTCAACTTATAA-3′ \ probe, reverse, 5′-VIC-AAGGATTCTACCAATCTCAATTGCG-TAMRA-3′</td>
<td>547</td>
<td>65°, 50°</td>
</tr>
<tr>
<td>pv md r1 copy number</td>
<td>reverse, 5′-AGTTTGTTGCGATCGTCTAATAC-3′ \ forward, 5′-GAGGACGTCTAAAGGATCCT-3′ \ probe, reverse, 5′-FAM-AGGCAATCGTGTGTCG-TAMRA-3′</td>
<td>82</td>
<td>60</td>
</tr>
<tr>
<td>p vald olase copy number</td>
<td>forward, 5′-AGTTTGTTGCGATCGTCTAATAC-3′ \ reverse, 5′-GAGGACGTCTAAAGGATCCT-3′ \ probe, reverse, 5′-FAM-AGGCAATCGTGTGTCG-TAMRA-3′</td>
<td>71</td>
<td>60</td>
</tr>
</tbody>
</table>

SR, sequencing reaction.
*Annealing temperature for the 2nd PCR.
*bAnnealing temperature for the SR.

Parasite (and human) DNA was extracted from filter papers according to the modified protocol described by Kain and Lanar. Briefly, a 2 mm diameter disc was punched out from the filter paper and placed in a 0.2 mL microtube, and 65 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) was added. The samples were incubated at 50°C for 15 min, followed by 90°C for 15 min to elute DNA.

Specific synthetic oligonucleotides were designed from the nucleotide sequences of the *P. vivax* ARI/Pakistan isolate for *pv dhfr* (GenBank accession no AY981231), Brazilian clinical isolate for *pv dhps* (accession no AY186730) and the Sal-1 strain for *pv md r1* (accession no AY618622) (Table 1). The reaction mixture for the primary PCR amplification consisted of 3 μL of DNA template, 0.5 μmol of the forward and reverse primers, 0.2 mM deoxynucleoside triphosphate mixture (dGTP, dATP, dTTP and dCTP), 2.5 mM MgCl2, 1.0 μL of PCR reaction buffer (Eurogentec, Angers, France) and 0.03 U/μL Taq DNA polymerase (Eurogentec) in a final volume of 20 μL. PCR was performed using a thermal cycler (Biometra, Göttingen, Germany) with the following programme for the three genes: denaturation at 94°C for 5 min, annealing at 53°C for 1 min and extension at 72°C for 1 min, for a total of 45 cycles, followed by a final extension at 72°C for 10 min. The secondary nested PCR was performed with a similar PCR mixture as for the primary amplification but using 2 μL of the primary PCR product. The same thermal cycling programme was used for secondary amplification (except that the annealing temperature was 65°C). The amplified nested PCR products were visualized by agarose gel electrophoresis and ultraviolet transillumination. The expected band sizes for *pv dhfr*, *pv dhps* and *pv md r1* were 632, 767 and 547 bp, respectively.

For the sequencing reactions, the secondary amplified product (2 μL) was marked with fluorescent nucleotides in the following mixture: 3.2 pmol of primers (Table 1), 2 μL of BigDye buffer and 2 μL of BigDye v1.1 (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μL. The thermal cycler was programmed as follows: 96°C for 10 s, 50°C for 5 s and 60°C for 4 min for 25 cycles. Residual dye terminators (BigDye terminator V3.1) were removed by the BigDye Xterminator purification kit according to the manufacturer’s instructions. An ABI Prism 3100 automated DNA sequencer (Applied Biosystems) was used to sequence the extension product. Sequences were analysed by using the BioEdit sequence alignment editor software version 7.0.9.0.

For TaqMan real-time PCR (7900HT FastReal-Time PCR system; Applied Biosystems) was used to estimate *pv md r1* (PVX_080100) copy numbers relative to the single-copy gene *p vald olase* (PVX_118255). The oligonucleotide primers and probes (Eurogentec, Seraing, Belgium) were designed by using Primer Express software (version 2.0) (Table 1). Individual PCRs were performed using TaqManUniversal PCR Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 200 nM TaqMan and 5 μL of template DNA in a final volume of 25 μL. The reaction mixtures were prepared at 4°C in a 96-well optical reaction plate (Applied Biosystems) covered with optical adhesive covers. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicate and analysed with
SDS software (version 2.2.1; Applied Biosystems). The PCR efficiencies of all primer pairs were evaluated using a series of dilutions of \(P.\) \(\text{vivax}\) genomic DNA and were found to be sufficiently close to obviate the need for any correction factor. Therefore, the \(2^{-\Delta \Delta Ct}\) method (where \(Ct\) indicates the threshold cycle) for relative quantification was used, adapted to estimate the number of copies of \(pvmdr1\) with the formula \(\Delta \Delta Ct = (Ct_{\text{wild-type}} - Ct_{\text{pvaldolase}})\) sample \(\text{sample} - (Ct_{\text{wild-type}} - Ct_{\text{pvaldolase}})\) calibrator.\(^{36,37}\) Genomic DNA extracted from \(P.\) \(\text{vivax}\) was used for calibration, and \(pvaldolase\) served as the control gene in all experiments.

**Data interpretation**

The key codons associated with pyrimethamine resistance in \(P.\) \(\text{vivax}\) were determined by Eldin de Pecoulas et al.\(^{32,36}\) The complete panel of combinations of \(pvdhfr\) mutations reported so far from different endemic regions has been reviewed by Hawkins et al.\(^{37}\) In the present study, the wild-type \(pvdhfr\) genotype was defined as residues I13-P33-F57-S58-T61-S117-I173, while the mutant \(pvdhfr\) genotype was defined as at least one amino acid change in the corresponding residues, 13L-33L-57L-58R-61M-117N-T173L/F.

The key \(pfdhps\) codon mutations that are associated with sulfadoxine resistance (5436A/F, A437G, K540E, A581G and A613S) correspond to S382A/C-A383G-K512M/T/E-A553G/C and V585, respectively, in \(pvmdr1\).\(^{38}\) So far, only V585 has been observed. The wild-type codon at position 585 is still undetermined. In the present paper, the wild-type \(pvdhfr\) genotype is defined as S382-A383-K512-A553-V585, and mutant genotype is defined as at least one of the following residues: 382A/C-383G-512M/T/E-553G/C and any amino acid residue other than Val in position 585.

In \(P.\) \(\text{falciparum}\), mutations in the following \(pfmdr1\) codons have been associated with drug resistance: N86, Y184, S1034, N1042 and D1246, which correspond to Y976-F1076L have been suggested to be the key changes that contributed to a chloroquine-resistant phenotype.\(^{25,38}\) The amino acid substitutions Y976, S1071, N1079 and D1291 are not associated with a chloroquine-resistant phenotype.\(^{25}\) Field studies conducted in Brazil, Papua New Guinea, Thailand and Madagascar have confirmed the presence of these two mutations.\(^{22,27,28,38,39}\) The \(pfmdr1\) genotypes were therefore defined as wild type (Y976-F1076L) and mutant (976F-1076L). The presence of mixed alleles was deduced when a double peak was observed in the electrophogram.

Since \(pfmdr1\) (also probably \(pvmdr1\)) gene amplification is correlated with both mefloquine and lumefantrine resistance, and artenmether/ lumefantrine is one of the current artemisinin-based combination therapies (ACTs) employed in Mauritania, \(pvmdr1\) copy number was determined. One copy was considered as an indicator of susceptibility to amino-alcohols, while two or more \(pvmdr1\) gene copies suggested drug resistance.

**Results**

Of a total of 439 febrile patients screened for malaria, 110 were \(P.\) \(\text{vivax}\) positive by microscopy and/or rapid diagnostic test, 1 patient had mixed \(P.\) \(\text{falciparum}\) and \(P.\) \(\text{vivax}\), and none had \(P.\) \(\text{falciparum}\) or \(P.\) \(\text{ovale}\) or pure \(P.\) \(\text{falciparum}\). All 110 pure \(P.\) \(\text{vivax}\) samples, as well as the one \(P.\) \(\text{falciparum}/P.\) \(\text{vivax}\) co-infection, yielded the expected band sizes by PCR. Sequences showed evidence for mixed \(pvdhfr\) alleles in three \(P.\) \(\text{vivax}\) isolates. \(pvdhfr\), \(pvmdr1\) and \(pfdhps\) sequence data were available from 78% (86/110), 85% (94/110) and 94% (103/110) of the samples, respectively. At least one of the molecular markers was successfully amplified and sequenced for each \(P.\) \(\text{vivax}\) isolate.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pvdhfr)</td>
<td></td>
</tr>
<tr>
<td>wild-type genotype</td>
<td>76 (88)</td>
</tr>
<tr>
<td>mutant genotypes (double mutations)</td>
<td>7 (8)</td>
</tr>
<tr>
<td>(IPFR^{RTS/NI})</td>
<td>3 (4)</td>
</tr>
<tr>
<td>(pvmdr1)</td>
<td></td>
</tr>
<tr>
<td>wild-type Y976 codon</td>
<td>75 (73)</td>
</tr>
<tr>
<td>mutant Y976 codon</td>
<td>28 (27)</td>
</tr>
<tr>
<td>wild-type F1076 codon</td>
<td>2 (2)</td>
</tr>
<tr>
<td>mutant L1076 codon</td>
<td>101 (98)</td>
</tr>
<tr>
<td>wild-type codon</td>
<td>2 (2)</td>
</tr>
<tr>
<td>single mutant genotype</td>
<td>73 (71)</td>
</tr>
<tr>
<td>double mutant genotype</td>
<td>0</td>
</tr>
<tr>
<td>(pvmdr1) copy numbers</td>
<td></td>
</tr>
<tr>
<td>one</td>
<td>102 (97)</td>
</tr>
<tr>
<td>two</td>
<td>3 (3)</td>
</tr>
</tbody>
</table>

Mutant genotype amino acid substitutions associated with resistance are shown in bold. The prevalences of isolates with novel mutations (synonymous and non-synonymous) in \(pvdhfr\), \(pvmdr1\) and \(pvmdr1\) were 94.2%, 5.3% and 94.2%, respectively. The prevalences of isolates with novel non-synonymous mutations in \(pvdhfr\), \(pvmdr1\) and \(pvmdr1\) were 15.1%, 9.3% and 91%, respectively.

\(pvdhfr\) was successfully amplified and sequenced from 86 isolates. The majority of the isolates (76 of 86 (88%)) were characterized by the wild-type \(pvdhfr\) genotype IPFSTSI (codons 13, 33, 57, 58, 61, 117, 173) (Table 2). The remaining 10 isolates (12%) carried the double mutation S58R and S117N, either as pure alleles (\(n = 7\)) or mixed alleles (\(n = 3\)). The mutant genotypes of these 10 isolates were \(IPFR^{RTS/NI}\) for the pure alleles and \(IPFS/RTS/NI\) for mixed alleles (mutated codons shown in bold face). Nine novel mutations were found. Five mutations were synonymous (A45G, T114C, T207C, C360T and C493T in the nucleotide sequence) and four mutations were non-synonymous: G190A, G229C, A361T and C510A in the nucleotide sequence, corresponding to V64M, E77Q, I121F and C170stop in the amino acid sequence, respectively. As reported in earlier studies,\(^{12,36}\) a 4 amino acid repeat unit, GGDN, was observed between residues 88 and 106. Three amino acid repeats were found in all isolates. \(pvmdr1\) sequence data were available from 94 isolates. All were characterized by the wild-type genotype SAKAV (codons 382, 383, 512, 553, 585). Five novel mutations were found in five different isolates at positions 388, 400, 455, 505 and 550 (C1163T, C1199T, A1363G, G1513A and T1649C in the nucleotide sequence) and four mutations were non-synonymous: G190A, G229C, A361T and C510A in the nucleotide sequence, corresponding to V64M, E77Q, I121F and C170stop in the amino acid sequence, respectively.
the mutation Y976F. Two isolates (2%) had the wild-type F1076, and 101 (98%) carried the mutation F1076L, while 28 (27%) harboured the double mutation. Two novel mutations were observed, a synonymous change (C3064T; 1022 in the amino acid sequence) and a non-synonymous change at the position 958 (C2873T, corresponding to T958M).

The copy numbers of the *pvmdr1* gene were successfully evaluated in 105 isolates. Of these isolates, 102 (97%) had one copy of *pvmdr1* and three had two copies (Table 2). Of these three isolates, two had the double mutant genotype F976-L1076, and the other had the single mutant genotype Y976-L1076. The genotypes of individual isolates or groups of isolates are presented in Table 3.

**Discussion**

Until 2005, uncomplicated malaria (*P. falciparum* and *P. vivax*) had been treated with chloroquine as the first-line and sulfadoxine/pyrimethamine as the second-line drug in Mauritania. A large majority of patients had been treated on the basis of presumptive diagnosis. For malaria prevention in pregnant women, chloroquine had been prescribed for weekly intake. Since 2006, artesunate/amodiaquine and artemether/lumefantrine have been introduced in the country for the first-line treatment of *P. falciparum* malaria.40 Sulfadoxine/pyrimethamine is employed for intermittent preventive treatment in pregnant women. Due to the scarcity of *P. vivax* infections, there is currently no defined national policy on the treatment of *P. vivax* infections in Mauritania. Quinine is still reserved for the treatment of severe malaria. These antimarial treatment policies have not been based on clinical studies on therapeutic efficacy. In fact, between 1996 and 2010 only two studies have been performed in Mauritania, both in 1998 on chloroquine efficacy.41,42

The results of the present study confirm the occurrence of *P. vivax* transmission in Nouakchott. In an earlier study conducted with 416 febrile patients in 1996 in Nouakchott,
61.0% (47/77) of malaria-positive cases were due to \textit{P. falciparum}, 36.4% (28/77) were due to \textit{P. vivax}, 1.3% (1/77) were due to \textit{P. ovale} and 1.3% (1/77) were due to \textit{P. malariae}. In the present study, the relative proportion of \textit{P. falciparum} (0.9%) and \textit{P. vivax} (100%, including one \textit{P. falciparum}/\textit{P. vivax} mixed infection) showed a large predominance of \textit{P. vivax}. The underlying causes of this inversion of infection showed a large predominance of \textit{P. falciparum}, mostly in Asia and parts of South America \textit{P. vivax} surveys are currently being undertaken to characterize the urban site, no specific entomological data are available. Further surveys are currently being undertaken to characterize the epidemiology of \textit{P. vivax} malaria in Nouakchott.

The results of the present study also suggest that, compared with many other countries where \textit{P. vivax} is endemic, the prevalence of mutant \textit{dhfr} alleles associated with pyrimethamine resistance is low in Mauritania. \textit{P. vivax} malaria that is clinically resistant to standard drugs, such as chloroquine, sulfadoxine/pyrimethamine, mefloquine and primaquine, is known to be present in certain foci, mostly in Asia and parts of South America where \textit{P. falciparum} and \textit{P. vivax} co-exist and the latter has been exposed to antifolate drugs. Molecular assays conducted in areas where \textit{P. falciparum} and \textit{P. vivax} transmission is moderate (French Guiana, Brazil, Vanuatu, Papua New Guinea, Indonesia, East Timor, Philippines, China, Vietnam, Thailand, India, Sri Lanka and Madagascar) have shown a high prevalence of mutant \textit{pvdhfr}, mostly \textit{F57L/I}, \textit{S58R} and \textit{S117N/T}, and a few cases of \textit{I173L/F}. Similarly, \textit{pvdhfr} mutations at positions 382, 383, 512 and 553 (but not at position 585) have been reported from these countries. By contrast, in Iran and Afghanistan, where malaria transmission occurs seasonally at a relatively low level and where \textit{P. vivax} largely predominates over \textit{P. falciparum}, the prevalence of mutant \textit{pvdhfr} was low: mutant \textit{R58} in 17.5% and 4%, and mutant \textit{N117} in 26% and 12% of isolates in Iran and Afghanistan, respectively. Furthermore, in these two countries, only a single isolate (1 of 171 Afghan isolates (0.6%) and none of 189 Iranian isolates) carried the mutation \textit{A383G} in \textit{pvdhps}, and all isolates carried the wild-type \textit{A553}. Likewise, in Korea, where \textit{P. vivax} is a re-emerging parasite, single \textit{N117} or \textit{T117 dhfr} mutants (14%) and double mutants involving codons 57, 58, 117 or 173 occur at a low prevalence. Further phenotypic studies (in vitro and clinical responses) are required to determine whether these genotype variations reflect drug susceptibility or geographic variation independent of drug susceptibility.

The \textit{pvdhfr} and \textit{pvdhps} genotypes of Mauritanian isolates collected in Nouakchott were 88% and 100% wild-type, respectively. Moreover, \textit{pvdhfr} mutations involved only two (amino acids 58 and 117) of the seven alleles that have been reported to be associated with antifolate resistance. Therefore, the current \textit{P. vivax} drug-resistant status in Nouakchott is characterized by a high proportion of drug-susceptible molecular profiles.

\textit{pvmrd1} mutations have been suggested to be associated with chloroquine resistance. The double mutations \textit{Y976F} and \textit{F1076L} have been observed in \textit{P. vivax} isolates from Thailand and Indonesia. Parasites from Thailand, Indonesia and Papua New Guinea carrying \textit{Y976F} are characterized by reduced chloroquine susceptibility in \textit{vitro}. Several studies suggest that \textit{F1076L} may not be an indicator of drug resistance, but rather a geographic variant since this mutation was observed in close to 100% of isolates from Madagascar, the Republic of Korea and Mauritania, about 30% in isolates from Thailand and Indonesia, and fewer (14%) in Brazil. Chloroquine- or amodiaquine-treatment failure may be associated with \textit{P. vivax} isolates carrying the \textit{Y976F} substitution. However, other studies did not confirm an association between \textit{pvmrd1} mutations and drug resistance. Unfortunately, in the present study in \textit{vitro} and clinical responses of \textit{P. vivax} isolates were not assessed, and the practical significance of mutant Mauritanian \textit{P. vivax} isolates for treatment cannot be determined.

\textit{pvmrd1} gene amplification has been suggested to be associated with amodiaquine, artesunate and mefloquine resistance in Thailand, in particular in areas where mefloquine has been employed intensively for over two decades. When \textit{pvmrd1} amplification occurs, it is usually a duplication (i.e. 2 gene copies). Amplification with 3 copies (none are reported >3) has been observed in only a few isolates. Our finding is in agreement with previous studies. A large majority of Mauritanian isolates had a single \textit{pvmrd1} gene copy, and only 3 of 105 isolates had two copies. Interestingly, these isolates with \textit{pvmrd1} amplification also carried mutations in residue 976 or 1076, or both. Further studies including in \textit{vitro} assays and/or clinical response are required to determine the phenotype of such isolates in Mauritania.

The origin of \textit{P. vivax} in Mauritania is not known. It may have been introduced from known \textit{P. vivax} foci of \textit{P. falciparum} in Algeria and Morocco through nomadic migration of some tribes, imported through human migration from neighbouring countries (Mali), or the Cape Verde islands, or by European travellers or explorers during the colonial period. The ethnic origin of the Moor people (mostly Duffy-positive blood group) favours the origin of \textit{P. vivax} in Mauritania. To explain the apparent homogeneity of \textit{P. vivax} isolates observed in the present study, further studies on the population genetics of the parasites, human hosts and mosquitoes are required. Moreover, the diversity of \textit{pvdhfr}, \textit{pvdhps} and \textit{pvmrd1} sequences, including novel mutations, may offer a complementary means to determine the origin of Mauritanian \textit{P. vivax} populations.

The present study is the first report based on molecular epidemiology on the status of drug-resistant \textit{P. vivax} in West Africa, and the second report on drug-resistant \textit{P. vivax} in the African continent, following one from Ethiopia in East Africa. Drug-resistant \textit{P. vivax} has not been reported elsewhere from the African continent, probably due to under-reporting, incomplete data or the absence of drug resistance. Furthermore, \textit{P. vivax} is relatively uncommon in Africa, except in east Africa, and is limited to foci in northern Africa, and some islands (Cape Verde, São Tomé y Príncipe and islands belonging to Equatorial Guinea). In Madagascar all four human malaria species co-exist, and drug-resistant \textit{P. falciparum} and \textit{P. vivax} have been reported. Data on the molecular markers of drug resistance in Mauritanian \textit{P. vivax} isolates in the present study will serve as a baseline for further monitoring of drug-resistant \textit{P. vivax} in Nouakchott.
Drug-resistant *Plasmodium vivax* in Mauritania

**Acknowledgements**

We are grateful to the staff of the National hospital and Chiekh Zayed Hospital for their aid in recruiting patients and the patients for kindly agreeing to participate in the study.

**Funding**

This study was supported by the Délégation Générale pour l’Armement (grant 10 CO 404), the Direction Centrale du Service de Santé des Armées and the French Agence Nationale de la Recherche (project RES-ATO, ANR-08-MIE-024). K. M. L. is a recipient of the 2009 International Fellowship of ‘For Women in Science Program’ granted by L’Oréal Foundation.

**Transparency declarations**

None to declare.

**References**


