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PROTOZOOLOGY - SHORT COMMUNICATION

A high number of *pfmdr1* gene copies in *P. falciparum* from Venezuela

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Abstract

Multidrug resistance in *Plasmodium falciparum* has been associated with gene amplification of *pfmdr1*. We studied the corresponding gene amplification in *P. falciparum* from blood samples of malaria patients in the Sifontes Municipality, Bolívar State, Venezuela, known as the highest region of incidence of malaria. Fifty-five *P. falciparum* DNA samples were extracted from different hosts and used for qPCR assessment of the copy number of *pfmdr1*. The assay detected four copies of the multidrug-resistant line *P. falciparum* Dd2 in comparison with the *P. falciparum* 3D7 that had only one copy. In the patients' samples, the copy number of *pfmdr1* was a single copy in 80% and 20% left distributed in different copy numbers up to seven.

Keywords Malaria · *P. falciparum* · *pfmdr1* gene · Venezuela

Introduction

The appearance or dissemination of drug-resistant *Plasmodium falciparum* parasites limits global malaria control (Blasco et al. 2017). Therefore, efforts to raise and sustain surveillance of drug-resistant malaria are encouraged (World Health Organization 2017). Several methods can be used to follow drug-resistant malaria, such as the efficacy of antimalarial agents (in vivo or in vitro) and molecular markers to study the genetics of *P. falciparum*. This last method is one of the most convenient because it allows the analysis of many blood samples collected from patients. The genetic analysis can be performed on samples collected on filter papers as dried blood spots that facilitate their transport and storage conditions. This technique is convenient especially for studies in remote areas.

The increased copy number of the *pfmdr1* gene is associated with parasites resistant to different antimalarial drugs, especially the current antimalarial combination therapies (ACTs) used for uncomplicated *P. falciparum* malaria (Kremsner and Krishna 2004; Gil and Krishna 2017). The majority of countries in Africa (except Ethiopia) has only one copy of the *pfmdr1* gene in *P. falciparum* populations (Costa et al. 2017; Gil and Krishna 2017), while in the Americas, the amplification rate has oscillated between 6 and 38% (Griffing et al. 2010; Inoue et al. 2014; Labadie-Bracho and Adhin 2013; Legrand et al. 2012). Contrary to these continents, countries from Southeast Asia showed the highest *pfmdr1* amplification in *P. falciparum* isolates (Price et al. 2004, 2006), although parasites from Southwest Asia exhibited no gene amplification (Awab et al. 2013; Khattak et al. 2013; Pirahmadi et al. 2013).

The gene is highly polymorphic, with more than 100 polymorphisms (SNPs), with N86Y, Y184F, D1246Y, S1034C, and N1042D being the most studied (Veiga et al. 2016). SNP variation exists among *P. falciparum* populations from different geographical regions. Notably, the 86N allele is present in duplicated *pfmdr1* genes in Southeast Asia and South America, while in Africa, these genes contain the 86Y allele (Gil and Krishna 2017). Although several SNPs of the *pfmdr1* gene could significantly affect parasite susceptibility to antimalarials such as quinine, mefloquine, artemisinin,

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halofantrine, and lumefantrine (Duraisingh et al. 2000; Reed et al. 2000; Sidhu et al. 2005), the *pfmdr1* increased copy numbers are claimed to be the principal reason for resistance to current antimalarials (Price et al. 2004). This assessment was confirmed when one of the two copies of the *pfmdr1* gene in the drug-resistant FCB line was knocked down, and in consequence, a decrease in the IC50s took place in mefloquine, lumefantrine, quinine, and artemisinin, commonly used antimalarials (Sidhu et al. 2006).

Venezuela has been using ACT since 2004. Griffing et al. (2010) reported the presence of amplification up to four times of the *pfdmr1* gene copies of *P. falciparum* DNA samples from the Sifontes Municipality, Bolivar State, Venezuela, collected in 2003–2004. In this region, malaria infections by *P. falciparum* have increased on an alarming scale (Pacheco et al. 2019; WHO 2017). Therefore, the regular checking of this molecular marker can be used to promptly detect the parasite's resistance to antimalarial drugs and guide therapy. Then, after 12 years, we reexamined the *pfmdr1* gene copy number in parasite DNA from blood samples of malaria patients in the Sifontes Municipality, an area with more malaria cases in the country.

Materials and methods

P. falciparum DNA extraction from samples

Fifty-five DNA samples of *P. falciparum* from Venezuela were used in this study. Previously, blood samples from patients with malaria-related symptoms were taken from patients by ear prick. Most patients came from San Isidro parish to be treated at the Field Research Center Dr. Francesco Vitanza, a public health center in the city of Tumeremo, during epidemiologic week 50 (December 12–17) in 2016.

First, the *P. falciparum* infection was detected immediately by microscopical observation. Then, PCR diagnosis was performed later in BIOMED-UC, Maracay, Aragua State, since we found that PCR typing has higher sensitivity (97.2%) and specificity (100%) than microscopy (Abou Orm et al. 2014). The DNA was extracted from individual samples using a classical phenol/chloroform method resuspended in 50 μL of sterilized water and stored at − 80 °C. Detection and typing of *Plasmodium* spp. in blood samples were performed using an assay already described (Snounou et al. 1993).

Real-time PCR assays

qPCR was conducted in a reaction volume of 25 μL in a 96-well plate containing 0.05 μg of template and 200 nM forward and reverse primers, using *GoTaq* qPCR Master Mix (Promega Corporation, USA), on the 7500 qPCR System (Applied Biosystems, Massachusetts, USA) and the default run program: 10 min of preincubation at 95 °C followed by 40 cycles for 15 s at 95 °C and 1 min at 60 °C. Specific primers for the *pfmdr1* gene were previously reported (Ferreira et al. 2006) as well as primers for the seryl-tRNA synthetase gene used as the endogenous reference to normalize the data (Salanti et al. 2003).

Seryl-tRNA synthetase and *pfmdr1* primer pairs were evaluated, and for each one, the following was found: (a) the observed efficiency was close to 100% (Supplementary file 1) and (b) the amplification specificity was exhibited through the production of a singular peak in the melt curve analysis (Supplementary file 2). Therefore, the $2^{-\Delta\Delta CT}$ method of relative quantification was used to estimate the copy number in the *pfmdr1* gene. The calibrator used here was the genomic DNA of *P. falciparum* 3D7 (CDC Atlanta, USA) that possesses only one copy of the gene (Ferreira et al. 2006; Price et al. 2004). We also used the genomic DNA of *P. falciparum* Dd2 as a positive control (Wellems et al. 1990). The Dd2 parasite is polymorphic, and according to the polymorphism, the *pfmdr1* copy number varies (Friedrich et al. 2014). Here we used DNA from a Dd2 strain having four copies of the gene, calculated by CDC.

Each qPCR experiment was repeated three times with three replicates of each one. The average and standard deviation (SD) of the C_Ts from the three replicates were determined, and the average was only approved if the SD was < 0.38 (Pfaffl 2001). Repeatability and reproducibility for the assay were calculated by a percent coefficient of variance (%CV) within and between assays in 10% of the sample (Supplementary file 3).

The *N*-fold copy number of the *pfmrd1* gene relative to *P. falciparum* 3D7 in each sample was calculated using geometric means among the three experiments. The frequency of the copy number was clustered in discrete values according to the following criteria: 1 copy number $\geq 0.7 \leq 1.5$, 2 copy numbers $\geq 1.6 \leq 2.5$, and 3 copy numbers $\geq 2.6 \leq 3.5$, and the sequence continued using the same pattern (Supplementary file 4).

Results and discussion

The amplification of the *pfmdr1* gene contributes to an increased risk of treatment failure to different antimalarials like mefloquine, lumefantrine, artemisinin, and quinine (Sidhu et al. 2006). Previous work in the Sifontes Municipality, Bolívar State, Venezuela, assessed the presence of a copy number in the *pfmdr1* gene from *P. falciparum*–infected blood samples collected between 2003 and 2004 (Griffing et al. 2010). The use of ACT has been continued in the last years in the country; therefore, it is important to know if there is a change in the number of copies of the *pfmdr1* gene in newly collected samples. In this study, we used a qPCR assay to

Table 1 Estimated *pfmdr1* gene copy number of Dd2 DNA relative to 3D7 DNA

DNA	RQ[1]	RQ Min[1]	RQ Max[1]	RQ mean[2]	RQ SD[2]
3D7	1	0.997	1.033		
	0.995	0.973	1.135	0.996	0.004
	0.993	0.963	1.402		
Dd2	3.940	3.892	4.095		
	4.031	3.910	4.140	3.994	0.048
	4.012	3.903	4.173		

RQ relative quantification, *Min* minimum, *Max* maximum, *SD* standard deviation

[1] Each value of RQ (including RQ Min and RQ Max) represents the mean of triplicates of one independent experiment. Three experiments were run per sample, and RQ mean showed the mean of all

[2] Mean and standard deviation of the gene copy number estimates (RQ) considering each value of RQ[1]

determine the *pfmdr1* copy number in 55 *P. falciparum*–infected patient samples from the Sifontes Municipality.

The efficiency of the assay for *pfmdr1* and seryl-tRNA synthetase genes was close to 100% which means that the amplification efficiencies of both genes were approximately equal (Supplementary file 1). The assay showed specificity (Supplementary file 2), repeatability, and reproducibility (Supplementary file 3). To validate the assay furthermore, we compare the copy number of the *pfmdr1* gene between the DNAs *P. falciparum* 3D7 and *P. falciparum* Dd2. The DNA 3D7 carries one copy of the gene (Ferreira et al. 2006; Price et al. 2004) whereas the Dd2 used in the assay has four copies (Wellems et al. 1990). The number of experimental runs, range, mean, and standard deviation of the copy number of the *pfmdr1* gene for the *P. falciparum* DNAs 3D7 and Dd2 in comparison with 3D7 are presented in Table 1. After validating the assay, we used it on DNA extracted from 55 *P. falciparum*–infected blood samples from the Sifontes Municipality. 80% (44/55) of the *P. falciparum* from malaria patients carried only one copy of the *pfmdr1* gene and 20% was left distributed in different copy numbers (Fig. 1). The estimated copy number of the *pfmdr1* gene for all samples is presented in Supplementary file 4.

According to the earlier study in Venezuela (Griffing et al. 2010), of the 12% of the samples with increased copy number of *pfmdr1*, 63.6% ($n = 7/11$) exhibited only two copies of *pfmdr1*. After more than a decade (12–13 years), we found

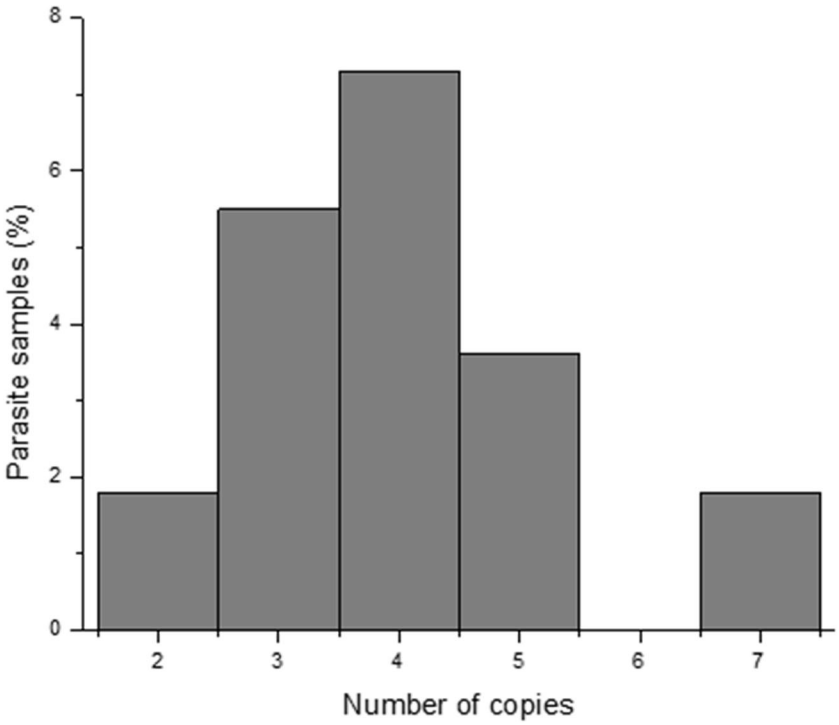

Fig. 1 Percentage of parasite infected samples with increased *pfmdr1* gene copynumber. The 2−ΔΔCT method of relative quantification was used to estimate the copynumber in the *pfmdr1* gene as the observed efficiency of the qPCR was close to 100%for the *pfmdr1* gene and for the seryl-tRNA synthetase gene used as the endogenousreference. The calibrator used here was the genomic DNA of P. *falciparum* 3D7 thatpossesses only one copy of the gen. Each qPCR experiment was repeated three timeswith three replicates of each one. Repeatability and reproducibility for the assaywere calculated in 10% of the sample (Supplementary file 3). The N-fold copynumber of the pfmrd1 gene relative to P. *falciparum* 3D7 in each sample wascalculated using geometric means among the three experiments. Fifty five (55)samples were analyzed for the detection of *pfmdr1* gene copy number. Of these,eleven (11) samples had more than one copy. The percentages of the multicopysamples were plotted against their number of copies that were grouped into bins of1-copy units. The number of experimental repeats, range, and mean ± standarddeviation of the estimated copy number for the P. *falciparum* samples are shown in Supplementary file 4.

that the *pfmdr1* gene presented more copy number since 20% ($n = 11/55$) of the samples had multiple copies to a maximum of 7. The distribution of the copies was also different. Of the 11 samples with increased copy number of *pfmdr1*, two copies were identified in 9.1% ($n = 1$) of the samples and more than two copies in 90.9% ($n = 10$), and 63.6% ($n = 7$) had equal or greater than four copies.

The number of copies of the gene tends to increase with time that could mean that the use of ACT treatment this last decade potentiates the selection pressure on parasites with raised *pfmdr1* gene copy number and possibly with a minor sensitivity to antimalarial drugs. Reports indicate that *pfmdr1* is subject to a strong positive selection (Cheeseman et al. 2016).

Studies in Southeast Asia determined that increased *pfmdr1* copy number is associated directly with a lack of success in healing treatments using MFQ or ACT therapy (Price et al. 2004, 2006; Gil and Krishna 2017). The importance of *pfmdr1* gene amplification was shown in dihydroartemisinin (DHA)-resistant lines of *P. falciparum* selected in vitro (Cuia et al. 2012). These clones presented a susceptibility to DHA diminished by more than 25-fold, and this was in parallel to an increase of 8 to 9 copies of the *pfmdr1* gene. The clones regained their DHA susceptibilities with the removal of the drug from the culture, and the *pfmdr1* copy number returned to four which was the number of the original strain.

The *pfmdr1* gene presents the highest rates of amplification in Asia, and on the contrary, low amplification rates are found in most cases in Africa and America (Costa et al. 2017; Gil and Krishna 2017). Brazil and French Guiana, neighbors of Venezuela, show around a 30% *pfdmr1* amplification with the copy number ranging from 2 to 4 copies (Inoue et al. 2014; Legrand et al. 2012). We found up to seven copies of the *pfdmr1* gene in the *P. falciparum* DNA from parasite-infected blood samples in Venezuela. If the number of copies in vivo reflected a drug-resistance degree like those in vitro experiments, then a failure of ACT treatment may be expected over time in Venezuela. Therefore, the constant monitoring of the raised *pfmdr1* gene copy number in *P. falciparum* is an urgent need to reduce the strength of this serious health threat in the country.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval Ethical approval for the study was obtained from the Bioethics Committee of the Biomedical Research Institute of the University of Carabobo (protocol number CBIIB-UC/2016-3). The patients enrolled in the study were all adult and signed written informed consent.

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