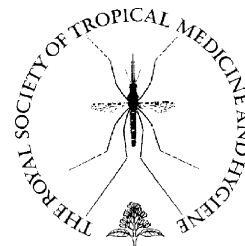




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Performance and reliability of the SYBR Green I based assay for the routine monitoring of susceptibility of *Plasmodium falciparum* clinical isolates

Marie Ange Rason^a, Tantely Randriantsoa^a, Herilalaina Andrianantenaina^a,
Arsène Ratsimbaoa^b, Didier Menard^{a,*}

^a Malaria Unit Research, Institut Pasteur de Madagascar, BP 1274, Antananarivo 101, Madagascar

^b Epidemiology Unit, Institut Pasteur de Madagascar, BP 1274, Antananarivo 101, Madagascar

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Summary The performance and the reliability of a SYBR Green I fluorescence-based assay to assess drug susceptibility in routine monitoring were evaluated in 138 *Plasmodium falciparum* clinical samples. Blood samples were studied for susceptibility to four antimalarial drugs by the SYBR Green I based assay, with the traditional [³H]-hypoxanthine isotopic assay as a reference. The two methods were observed to have similar geometric means of IC50s and IC90s, and high correlation ($r=0.93$ for IC50s and $r=0.94$ for IC90s) for the drugs tested. The strength of agreement estimated by using concordance coefficient correlation was from almost perfect to substantial for IC50s. Our data demonstrate (i) the reliability of a simple, rapid and easy to use fluorescence-based assay for the routine monitoring of susceptibility of *P. falciparum* clinical isolates, and (ii) the possible switch from the traditional in vitro drug sensitivity assay to the SYBR Green I method, because previous data acquired by the isotopic assay were comparable with those obtained by the SYBR Green I method. We conclude that this assay will provide an easier method for testing drug susceptibility of malaria parasites, especially in malaria-endemic countries, where there is massive implementation of new artemisinin-based combination therapies.

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1. Introduction

Malaria remains one of the leading causes of childhood morbidity and mortality worldwide, especially in Africa (Bremar et al., 2004; Hay et al., 2004; Snow et al.,

* Corresponding author. Tel.: +261 20 22 412 72;
fax: +261 20 22 415 34.
E-mail address: dmenard@pasteur.mg (D. Menard)

2005; Trape, 2001). For several decades efforts to control malaria have been severely compromised by the emergence of *Plasmodium falciparum* resistance to inexpensive and widely used drugs, such as chloroquine and sulfadoxine-pyrimethamine (Bjorkman and Bhattarai, 2005). As long as chemotherapy remains a key factor in the fight against malaria, constant monitoring of parasite susceptibility to antimalarial drugs is of the utmost importance for the development of therapeutic guidelines and policies. For this purpose, various approaches have been developed. The most traditional approach is the assessment of therapeutic responses (in vivo test). This 'gold standard' method has enabled determination of the thresholds of treatment failure that are crucial for adjusting antimalarial drug policies (WHO, 2003). However, the risk of loss of patients during long follow-up periods and the logistical demands of clinical response studies in endemic areas have led to the development of laboratory strategies for identifying antimalarial drug resistance. Laboratory strategies include in vitro drug sensitivity tests and evaluation of molecular markers associated with drug resistance (Bickii et al., 1998; Warhurst, 2001). Several in vitro drug sensitivity assays, based on culturing *P. falciparum* isolates in the presence of a range of antimalarial drug concentrations are available (Noedl et al., 2003), and include the WHO microtest (WHO, 1990), the isotopic assay (Desjardins et al., 1979; Elabbadi et al., 1992) and colorimetric methods, based on the measurement of the levels of parasite lactate dehydrogenase (pLDH) (Druilhe et al., 2001; Makler et al., 1993; Piper et al., 1999) or histidine-rich protein II (Noedl et al., 2002). Each of these commonly used assays has unique advantages as well as a number of known drawbacks. Recently, a microfluorimetric method using SYBR Green I for assessing susceptibility of parasites to antiplasmodial compounds was reported (Bennett et al., 2004; Smilkstein et al., 2004). This fluorescence-based method has, in particular, the advantages of being a simple, one-step procedure.

In order to assess the performance and the reliability of this method for the routine monitoring of susceptibility of *P. falciparum* isolates to antimalarial drugs, we designed a study to report the results of in vitro drug sensitivity assays by using 50% and 90% inhibitory concentration (IC₅₀ and IC₉₀) values of clinical isolates collected from malaria symptomatic patients in Madagascar. Each clinical isolate was tested for its susceptibility to chloroquine, monodesethylamodiaquine, quinine and dihydroartemisinin by using the traditional [³H]-hypoxanthine accumulation method and the SYBR Green I based assay.

2. Materials and methods

2.1. Parasites

2.1.1. Reference culture-adapted strains

Two cloned strains of *P. falciparum* obtained from the Malaria Research Reference Reagent Resource Centre (MR4/ATCC, Manassas, VA, USA) were used as controls: one chloroquine-resistant strain (FCM29 Cameroon) and one chloroquine-susceptible strain (3D7 Africa).

2.1.2. Clinical samples

As part of the surveillance of antimalarial drug resistance in Madagascar, clinical isolates were collected during 2006 from symptomatic patients prior to treatment in six health centres throughout Madagascar. The health centres were located in areas exhibiting three of the four epidemiological patterns of malaria transmission: Ihosy in South Madagascar (sub-desert stratum, epidemic prone), Maevatanana and Miandrivazo in West Madagascar (tropical stratum, seasonal and endemic area) and Tsiroanomandidy, Saharevo and Moramanga in the foothills of the Central Highlands of Madagascar (highlands stratum, low-endemic area). Venous blood samples (10 ml) were collected in tubes coated with EDTA (Vacutainer tubes, Becton Dickinson, Rutherford, NJ, USA), from malaria-positive patients (>2 years old). Parents or guardians gave their consent for participation in the study. Malaria positivity was evaluated by using a rapid diagnostic test based on the detection of *Plasmodium*-specific lactate dehydrogenase (pLDH) (OptiMAL-IT, DiaMed AG, Cressier sur Morat, Switzerland). Positive patients were treated with artesunate-amodiaquine combination, according to the National Malaria Control Programme (NMCP), and samples were sent to the Malaria Research Unit of the Institut Pasteur of Madagascar at Antananarivo in a controlled cool box at 4 °C. Blood smears were stained with 10% Giemsa for 10 min and were examined to check for mono-infection with *P. falciparum*. Parasite densities were determined from thick blood smears by counting the number of asexual parasites per 200 white blood cells (WBCs) (or per 500, if the count was less than 10 parasites/200 WBCs), assuming a WBC count of 8000/μl. In vitro assays were performed on blood samples with the following criteria: parasite density ≥0.1%, transportation within 48 h after blood extraction, and no declared antimalarial drug intake during the last 7 days. Each isolate was tested for its susceptibility to one to four drugs, depending on the whole-blood volume available; priority was given to the isotopic assay. For each sample, the isotopic assay and the SYBR Green I based assay were run on the same parasite suspension.

2.2. In vitro assay

The following four antimalarial drugs in the appropriate solvent were distributed on 96-well tissue culture plates (4 × 24 wells) and dried: chloroquine diphosphate (Sigma Aldrich, Saint-Quentin Fallavier, France), 12.5–1600 nM; monodesethylamodiaquine (RCC Ltd, Itingen, Switzerland), 2.5–320 nM; quinine (Sigma Aldrich), 25–3200 nM and dihydroartemisinin (Sigma Aldrich), 0.25–40 nM. Each batch of plates was controlled by measuring the susceptibilities of strains FCM29 Cameroon and 3D7 Africa to the corresponding drugs, and was used within 6 weeks. For each drug tested, three control wells were drug free, and each concentration was studied in duplicate or triplicate. The blood samples were washed three times with a solution of RPMI 1640 (Invitrogen, Cergy Pontoise, France) plus 25 mM HEPES (Sigma Aldrich) and 25 mM NaHCO₃ (Sigma Aldrich). The blood samples were then resuspended in the same culture medium supplemented with 10% (v/v) AB⁺ human serum (Abcys Biowest, Paris, France), but without hypoxanthine supplementation. If necessary, a dilution was performed by adding uninfected erythrocytes to obtain a 0.1–0.5%

parasite density and a 1.5% haematocrit. The plates were incubated for 42 h (isotopic assay) or 72 h (SYBR Green I assay) at 37 °C in a humidified incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) flushed daily for 1–2 min with a gas mixture containing 5% CO₂, 5% O₂, and 90% N₂, then frozen and kept at –20 °C.

2.2.1. Isotopic measurement

For the isotopic assay, 1 ml RPMI 1640 with [³H]-hypoxanthine (40 mCi/litre; Amersham Biosciences, Orsay, France) was added to 20 ml of the homogeneous parasite suspension (0.4 µCi [³H]-hypoxanthine/well) at the beginning of the in vitro assay. After the plates had been thawed, the content of each well was harvested onto fibreglass paper (Printed Filtermat, Wallac, Turku, Finland). The fibreglass paper was dried and mixed with 2 ml of scintillation fluid (NBCS 204, Amersham Biosciences, Orsay, France). The level of parasite incorporation of radioactivity (in counts per minute) was measured with a liquid scintillation counter 1450 Microbeta TRILUX (Wallac). Tests were considered to be interpretable if [³H]-hypoxanthine incorporation gave > 1000 counts per minute in the drug-free wells. Growth curves were obtained, and IC₅₀ and IC₉₀ values were calculated by log-probit approximation.

2.2.2. Fluorescence intensity measurement

Plates were thawed for 2 h at room temperature and each sample was mixed by pipetting. One hundred microliters of the contents of each well were transferred to a new 96-well plate followed by 100 µl of SYBR Green I (SYBR Green I nucleic acid Gel Stain 10 000×, Sigma Aldrich) in lysis buffer (0.2 µl of SYBR Green I/ml of 2× lysis buffer). The lysis buffer contained Tris (20 mM, pH 7.5), EDTA (5 mM), saponin (0.008% wt/vol) and Triton X-100 (0.08% vol/vol) and was prepared in advance and stored at room temperature (Bacon et al., 2007). Plates were covered and incubated at room temperature for 1 h. Fluorescence intensity was measured with a FLX800 Microplate Fluorescence Reader (BioTek Instruments, Winooski, VT, USA) with excitation and emission wavelengths of 485 nm and 535 nm respectively. Values were expressed in relative fluorescence units. Growth curves were obtained using the same procedure as for the isotopic assay.

2.2.3. Statistical analysis

For all the clinical isolates for which interpretable results were obtained from both the isotopic and SYBR Green I assays, the geometric means of the IC₅₀s and IC₉₀s of each drug tested were compared by Mann-Whitney *U* tests. The strength of agreement between IC₅₀s and IC₉₀s values for the isotopic assay and the SYBR Green I assay were calculated by using the concordance correlation coefficient ρ_c . We chose this technique to avoid all the shortcomings associated with the panoply of usual procedures (Pearson's correlation coefficient *r*, paired *t*-tests, least squares analysis for slope and intercept, coefficient of variation, intraclass correlation coefficient) (Lin and Torbeck, 1998), and the following scale was used for interpretation: ρ_c value >0.9 was considered as 'almost perfect', from 0.8 to 0.9 as 'substantial', from 0.65 to 0.8 as 'moderate' and <0.65 as 'poor'. Clinical isolates were also identified as resistant or

susceptible for chloroquine, monodesethylamodiaquine and quinine, on the basis of an IC₅₀ cut-off (100 nM for chloroquine, 60 nM for monodesethylamodiaquine and 800 nM for quinine). The IC₅₀s between these two groups for each of the three drugs and for each assay method were compared by using Fisher's exact test. The relationship between the two measurement methods was studied by use of the individual log IC₅₀s and log IC₉₀s of the four drugs tested. The Bland and Altman plot was used to display, by horizontal lines, the mean difference of the log IC₅₀s or the log IC₉₀s and the mean difference plus and minus two times the standard deviation of the differences, with the latter corresponding to the limits of agreement of the two techniques. This method was more appropriate in the analysis of measurement method comparison data than the correlation coefficient or techniques such as regression analysis (Altman and Bland, 1983). For all tests, *P* values <0.05 were taken as indicative of statistically significant differences.

3. Results

A total of 249 clinical isolates from *P. falciparum* infected blood were collected from February to September 2006 in Ihosy (*n*=15), Maevatanana (*n*=28), Miandrivazo (*n*=83), Tsiroanomandidy (*n*=53), Saharevo (*n*=29) and Moramanga (*n*=41). Among these isolates, 193/249 (77.5%) had interpretable in vitro assay results by the isotopic method and 139/170 (81.7%) by the SYBR Green I based assay. A total of 138 blood samples collected from patients with malaria were successfully analysed using the isotopic and SYBR Green I methods. The failure rates of the isotopic assay and the SYBR Green I based assay were similar (*P*=0.36). Comparisons of the IC₅₀ and IC₉₀ for these isolates obtained by the two methods are illustrated in Table 1 and Figures 1 and 2. The mean IC₅₀s and IC₉₀s were not significantly different

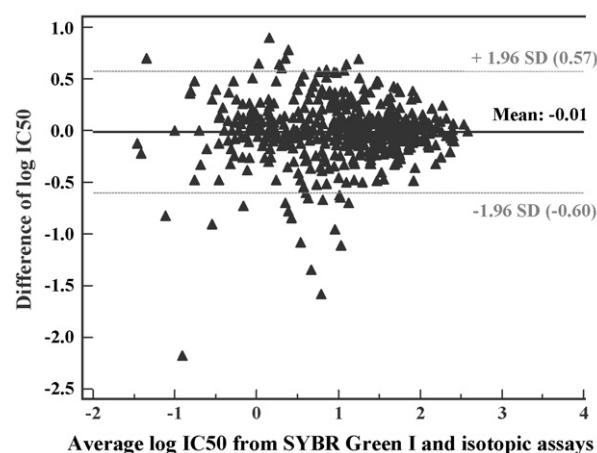


Figure 1 Bland and Altman graph of the difference in log IC₅₀s between the two methods (SYBR Green I and isotopic assays) for chloroquine, monodesethylamodiaquine, quinine and dihydroartemisinin plotted against the mean values. The estimated mean difference (– 0.01) and its confidence interval (2 SD) were calculated and are drawn as horizontal lines. As the IC₅₀s increase, no tendency for greater or smaller differences between the methods is observed on the plot.

Table 1 Comparison of the isotopic and SYBR Green I measurements for clinical isolates of *Plasmodium falciparum* tested against four drugs

Drug tested	No. of isolates tested	Geometric mean (nM) [95% CI]		P-value ^a	ρ _c ^b
		Isotopic assay [³ H]-hypoxanthine	SYBR Green I		
50% inhibitory concentration					
Chloroquine	138	20.2 [16.5–27.7]	22.3 [18.7–26.7]	0.46	0.93
Monodesethylamodiaquine	138	7.6 [6.6–8.8]	7.2 [6.3–8.3]	0.61	0.92
Quinine	135	48.7 [40.6–58.4]	52.3 [44.7–61.3]	0.56	0.90
Dihydroartemisinin	130	0.80 [0.65–0.97]	0.80 [0.65–0.96]	0.99	0.81
90% inhibitory concentration					
Chloroquine	138	116.6 [98.0–138.8]	119.4 [102.3–139.5]	0.84	0.68
Monodesethylamodiaquine	138	32.9 [28.8–37.7]	35.5 [30.8–40.8]	0.45	0.73
Quinine	135	342.1 [296.7–394.4]	335.8 [287.7–391.9]	0.86	0.72
Dihydroartemisinin	130	4.4 [3.8–5.1]	4.6 [3.95–5.46]	0.66	0.58

^a Significances by Mann-Whitney *U* tests are given for the difference in inhibitory concentrations measured with the two assays.

^b Concordance coefficient correlation.

for clinical isolates of *P. falciparum* tested against four drugs (Table 1). The prevalence of resistance determined for clinical isolates by the isotopic assay and the SYBR Green I based assay was similar for chloroquine ($P=0.69$), monodesethylamodiaquine ($P=0.47$) and quinine ($P=0.56$). The strength of agreement between the two methods was almost perfect for IC₅₀s of chloroquine, monodesethylamodiaquine and quinine, and substantial for IC₅₀s of dihydroartemisinin, but moderate for IC₉₀s of chloroquine, monodesethylamodiaquine and quinine, and poor for IC₉₀s of dihydroartemisinin (Table 1). The correlation between the log IC₅₀s and log IC₉₀s obtained by the two assays revealed a significant correlation for chloroquine ($n=138$; $r=0.83$, 95% CI 0.77–0.88; $P<0.001$ and $n=138$; $r=0.79$, 95% CI 0.72–0.84; $P<0.001$), for monodesethylamodiaquine ($n=138$; $r=0.68$, 95% CI 0.58–0.76; $P<0.001$ and $n=138$;

$r=0.71$, 95% CI 0.62–0.78; $P<0.001$), for quinine ($n=135$; $r=0.78$, 95% CI 0.70–0.84; $P<0.001$ and $n=135$; $r=0.72$, 95% CI 0.63–0.79; $P<0.001$) and for dihydroartemisinin ($n=130$; $r=0.77$, 95% CI 0.69–0.83; $P<0.001$ and $n=130$; $r=0.78$, 95% CI 0.70–0.84; $P<0.001$).

For all tests with all drugs ($n=541$), r was 0.93 for IC₅₀s and 0.94 for IC₉₀s. In the Bland and Altman plot, the mean difference in the log IC₅₀s (Figure 1) and the log IC₉₀s (Figure 2) of the four drugs determined by the isotopic assay and the SYBR Green I was -0.01 (limits of agreement, $+0.57$ to -0.60) and -1.2 (limits of agreement, $+79.3$ to -81.7), respectively. This suggests that globally higher IC₅₀s and IC₉₀s were obtained with the SYBR Green I based assay than by the isotopic assay. No tendency for greater or smaller difference between the two methods was found as the IC₅₀s or IC₉₀s increased.

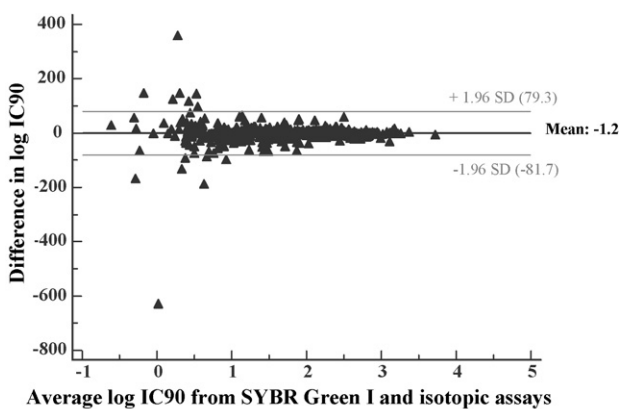


Figure 2 Bland and Altman graph of the difference in log IC₉₀s between the two methods (SYBR Green I and isotopic assays) for chloroquine, monodesethylamodiaquine, quinine and dihydroartemisinin plotted against the mean values. The estimated mean difference ($+1.2$) and its confidence interval (2 SD) were calculated and are drawn as horizontal lines. As the IC₉₀s increase, no tendency for greater or smaller differences between the methods is observed on the plot.

4. Discussion

Currently, and especially since the massive implementation of artemisinin-based combination therapies (ACTs) as first-line treatment, reliable methods such as in vitro drug susceptibility testing for the assessment of the sensitivity of *P. falciparum* parasites are useful tools to provide guidelines for public health policies in monitoring temporal changes in resistance. After more than two decades of use, both the WHO microtest and the isotopic assays have proven their reliability. However, both assays are associated with serious drawbacks: the WHO assay with the subjectivity of the results and the amount of labour involved, and the isotopic assay with the fact that it involves the handling of radioactive material. The new SYBR Green I assay overcomes many of these disadvantages and might be a method of choice, especially in malaria-endemic countries. This method is relatively simple and quick to perform, does not require highly specialized equipment and has the potential to replace traditional assays in the routine monitoring of susceptibility of *P. falciparum* isolates to antimalarial drugs. Since it was originally reported by two independent groups (Bennett et

al., 2004; Smilkstein et al., 2004), several studies have been published on this technique, but most of them have only evaluated its performance in drug sensitivity screening against *P. falciparum* reference strains. Our study provides, for the first time, an extensive assessment and validation of the malaria SYBR Green I based fluorescence assay under routine condition, on a large number of *P. falciparum* field isolates. The method described is based on the detection of *Plasmodium* DNA in short-term cultures using a 96-well format, allowing efficient, quantitative measurements of antiplasmodial activity for a large number of samples. Clinical samples, without removal of WBCs, were first incubated with the test drug for 72 h, followed by addition of SYBR Green I, followed by a 5–30 min incubation period prior to the measurement of fluorescence. For each sample, a paired comparison of both assays was performed. Geometric means of IC50s and IC90s and paired *t* test for comparison of paired IC50s and IC90s determined by the two methods were not significantly different for the four drugs tested. Moreover, the correlation between the log IC50s and log IC90s obtained by the two assays revealed significant correlation for the four antimalarial drugs and the strength of agreement estimated by using concordance coefficient correlation between the two methods were from almost perfect to substantial for IC50s. These observations are consistent with those from previous studies (Bacon et al., 2007; Bennett et al., 2004; Johnson et al., 2007; Smilkstein et al., 2004). As the monitoring of the antimalarial drug resistance is primarily based on the temporal evolution of the geometric mean of the IC50s, our results clearly show that the switch from the traditional in vitro drug sensitivity assay to the SYBR Green I method is possible. Previous data acquired by the isotopic assay is comparable to those obtained by the SYBR Green I method. In addition to the microfluorimetric method, other non-radioactive assays exist and are beginning to be widely used to determine susceptibility levels. In these ELISA-based methods, parasite growth and development is assessed by measuring either the enzymatic activity of the parasite lactate dehydrogenase (pLDH) (Druilhe et al., 2001; Makler et al., 1993) or the production of a histidine- and alanin-rich protein produced by *P. falciparum* (HRP-2) (Noedl et al., 2002). Recently published data suggest that the results obtained with these assays are comparable with those obtained by the WHO microtest and the isotopic assays (Kaddouri et al., 2006; Noedl et al., 2005). Compared to the SYBR Green I based assay, the main advantages of these methods are (i) their sensitivity enabling their use for any fresh isolate irrespective of parasite density (0.002% for HRP-2 and 0.005% for pLDH), and (ii) the possibility to be performed in isolated laboratories. On the other hand, their main drawbacks are the availability of monoclonal antibodies and their cost. According to several authors (Bacon et al., 2007; Kaddouri et al., 2006; Smilkstein et al., 2004), the development of SYBR Green I assay will make in vitro drug sensitivity testing more affordable for researchers in countries most affected by malaria and considerably cheaper than traditional radioisotope-based drug sensitivity assays, pLDH assays or HRP-2 assays using an in-house ELISA method.

In conclusion, this report demonstrates the reliability of a simple, rapid, inexpensive and easy to use fluorescence-based assay for the routine monitoring of susceptibility of *P. falciparum* clinical isolates. The availability of this assay will

provide an easier method for drug susceptibility testing of malaria parasites, especially in malaria-endemic countries, where there is massive implementation of new combined therapeutic such as ACTs.

Authors' contributions: MAR supervised the in vitro assays and performed the fluorescence intensity measurements; TR and HA carried out the in vitro assays and the isotopic measurements; AR supervised the collection of samples throughout the national network for the surveillance of malaria resistance (RER – Réseau d'Etude de la Résistance); DM designed the study protocol and carried out analysis and interpretation; MAR and DM drafted the manuscript. All authors read and approved the final manuscript. MAR and DM are guarantors of the paper.

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Conflicts of interest: None declared.

Ethical approval: The study protocol was reviewed and approved by the Ethics Committee of the Ministry of Health of Madagascar, N°007/SANPF/2007.

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