Optimization of a flow cytometric assay to measure antimalarial drug effects

Denise Rodrigues Francisco
Under supervision of Prof. Dr. Thomas Hänscheid *and co-supervision of* Prof. Dr. Leonilde de Fátima Morais Moreira
Instituto Superior Técnico, Lisboa, Portugal
April 2017

Abstract

Malaria is still one of the most important parasitic diseases. The emergence of parasites, resistant to most available drugs, particularly to artemisinins which are the first line treatment, has been considered as a major public health problem. Thus, monitoring parasite drug sensitivity has a high priority. Although there are different methods available for the assessment of *P. falciparum* susceptibility *in vitro*, no single method is standard because all of them have associated limitations. Moreover, the common methods fail to detect artemisinin resistance. A novel sensitivity test has been developed which is based on a flow cytometer modification that allows the detection of hemozoin (Hz). Although it has the advantages of being rapid and reagent-free, it still has some limitations. The main goal of this project was to test various conditions to further optimize this method. Our results suggest that lysing the samples before the measurement decreases the limit of detection of the method to 0.05%. Even more interesting, it allowed the detection of inhibitory drug effects as early as 10 to 14 hours after incubation. A protocol of a tight synchronization of the parasite cultures was also tested to investigate the potential of this method to detect artemisinin resistance. Along with this, the drug concentration and the time of incubation were also altered. The survival rates obtained after only 30 hours allowed to differentiate between resistance and sensitive parasites. These preliminary results show that the Hz detection assay by flow cytometry can be a promising alternative method for drug sensitivity testing including artemisinins.

Keywords: Malaria; antimalarial drug resistance; antimalarial sensitivity test; flow cytometry; hemozoin; artemisinin resistance

Introduction

Malaria parasites have developed resistance to all the classes of antimalarial drugs. The recently described resistance to artemisinin is particularly concerning since it is the first-line treatment [1]. Consequently, sensitivity tests have become very important tools in controlling malaria. The WHO microtest and the isotopic assays have been used for more than two decades and have proven their reliability in the field and in well-equipped research laboratories, respectively [2]. However, both have limitations: the WHO microtest is laborious and results are subjective; and the isotopic assays has low sensitivity and involves radioactive material [2]. Flow cytometry and the ELISA based detection of HRPII are also reliable techniques however, both use expensive reagents and require sophisticated equipment [2]. Furthermore, all these methods have long turn around-times (48-72h) [3]; and they all fail to detect artemisinin resistance [4,5]. The novel Ring Stage Assay (RSA) has been developed and already proved to be capable of detecting artemisinin resistance [5]. This assay differs essentially in the conditions of the culture tested and in the way how we subjected them to the drug in cause. Despite the success of the RSA in detecting artemisinin

resistance, it requires a complex and meticulous synchronization protocol, and most importantly, it depends on tedious microscopy readouts [5]. This entails the subjectivity of the results and is very laborious and time-consuming.

Altogether, these limitations contribute to the continuous development and optimization of new sensitivity assays. In 2011, Frita *et al.* described a flow cytometer modification that allows to detect hemozoin [6]. Hz is a product of the parasite digestion of hemoglobin that increases along with the parasite maturation. Hz is capable of rotating the plane of polarized light. Depolarized light can be measured by the modified flow cytometer [6]. This allows to assess drug effects after only 24h of incubation and it does not require additional reagents [7].

However, some limitations still exist. The main goal of this project was to test sample conditions to further optimize the method. The objectives are divided in two parts: 1) Explore the effects of lysing samples before depolarized side-scatter measurement and 2) Investigate artemisinin resistance *in vitro* by Hz detection assay using a different protocol.

Materials and Methods

All reagents were obtained from Sigma Aldrich (St Louis, US.), unless stated otherwise. Uninfected human red blood cells were obtained from buffy coats that were collected weekly from the *Instituto Português do Sangue e da Transplatação*, Lisbon without any personal information. This study, including the origin and use of human blood, was approved by the Ethical Committee of the Faculty of Medicine, University of Lisbon.

Plasmodium falciparum continuous cultures

The *Plasmodium falciparum* susceptible strain (3D7), artemisinin-sensitive strain (1239) and artemisinin-resistant (1240) were grown in recently collected donor erythrocytes in complete malaria culture medium (CMCM). CMCM was prepared according to the recommendations of the Malaria Research and Reference Reagent Resource Centre (MR4) [8]. Parasites were cultured and maintained in a tissue culture flask with daily exchanges of CMCM containing 5% of recently collected human erythrocytes under an atmosphere of 5% O_2 and 5% CO_2, at 37ºC. As uninfected controls, erythrocytes from heathy donors were cultured as described above.

Synchronizing *Plasmodium falciparum* continuous culture

Sorbitol synchronization was performed when *P. falciparum* continuous cultures reached a parasitemia of more than 2% with a minimum of 50% rings. The culture medium was discarded and a volume of 5% (w/v) sorbitol corresponding to 10x the volume of the pellet was added to the pelleted cells and incubated at 37ºC for 10 minutes. Next, the culture was washed twice in RPMI-1640. CMCM was then added to the pellet and the synchronized culture was maintained at the conditions described above.

Tight synchronization

Tight synchronization was performed as a sequence of sorbitol treatments with a purification by Percoll gradient. After three sorbitol synchronizations, synchronous schizonts were incubated for 15 minutes at 37ºC in RPMI-1640 supplemented with 15 U/mL of sodium heparin (Rotexmedica, Luitre, France). Then, the schizonts culture was purified on a 35%/75% (v/v) Percoll gradient, washed in RPMI-1640 and cultured with fresh uninfected erythrocytes. After 3 hours, cultures were treated again with sorbitol. The hematocrit was adjusted to 2% and parasitemia to 1%. These steps were performed immediately before the beginning of the Ring Stage Assay.

Microscopy

Parasitemia, parasite maturation and synchronicity of the culture was assessed by the observation of blood smears stained with Giemsa (Merck, Darsmstadt, Germany). Smears were fixed in absolute methanol and stained with 10% Giemsa-solution prepared in 0.4% PBS for 20 minutes. Microscopy pictures were taken using a Leica DM2500 (Leica, Solms, Germany).

SYBR green staining

A volume of 5 µL (~ 800 000 cells) of each culture sample was stained with 1x of a DNA-specific dye SYBR green I (Invitrogen, Carlsbad, USA). Samples were stained for 20 minutes, in the dark, after which a volume of 1 ml of PBS was added and samples were analyzed using a 535/45 nm bandpass filter.

Flow cytometer and the modification (depolarized side-scatter detection)

The Cyflow® Blue (Partec, Münster, Germany) is a portable five parameter flow cytometer with a blue laser excitation (488 nm) and detectors for: forward scatter (FSC), side scatter (SSC), green fluorescence (FL1), orange fluorescence (FL2) and red fluorescence (FL3). For this study, the flow cytometer was modified as described elsewhere [6]. Basically, a second SSC detector was created with a 50%/50% beam splitter between the new and the pre-existent SSC detector. A polarizer was placed 90 degrees to the polarization plane of the laser light, in front of one of the SSC detectors, allowing detection of depolarized light.

Flow cytometric analysis

Flow cytometry data was analyzed using FlowJo software (version 9.0.2, Tree Star Inc., Oregon, US.). Flow cytometry modification allowed to assess parasite maturation by measuring the percentage of depolarizing events. These events were defined in plots of SSC versus depolarized-SSC. The uninfected control was always used as a mean of comparison to exclude any background signal. Parasitemia was assessed by analysing the percentage of SYBR green positive cells. Plots green fluorescence (FL1) versus red fluorescence (FL3) were used to allow the differentiation between weakly stained cells and auto-fluorescence cells. Selection of positive events was done at each time-point using the uninfected samples stained with SYBR green, from corresponding time-point, as negative control. Parasitemia was determined by quantifying the percentage of SYBR green positive cells.

Drug preparation

Doubling concentrations ranging from 12 to 200 nM for chloroquine (CQ), 12 to 972 nM for piperaquine (PQ) and 0.24 to 64 nM for dihydroartemisinin (DHA) were tested for lysing experiments. A concentration of 1400 nM of DHA was prepared for RSA experiments.

Hemozoin detection sensitivity assay - Standard protocol

A volume of 100 µL of a blood suspension from a ring-stage synchronized culture at 2.5% hematocrit and approximately 1% parasitemia were incubated with the same volume of increasing concentrations of antimalarial drugs or with CMCM (for the drug free control and uninfected controls) in 96 well-plates, for 48 or 72 hours, at 37ºC in a 5% CO_2 atmosphere. At each time-point, 5 µL of the blood suspension present in each well was used and stained with SYBR green as described above. Samples were analyzed in triplicates and in each measurement approximately 100,000 events were analyzed. At specific time-points, 50% inhibitory concentrations (IC50) were calculated based on a nonlinear regression model (sigmoidal dose-response/variable slope) using the SigmaPlot – Systat Software (Chicago, US.).

Hemozoin detection sensitivity assay - Lysis protocol

For lysis experiments, the same standard protocol was followed as described above but with an additional step of lysing the samples before the flow cytometric measurement was performed. Samples were lysed by adding to the content of each well 320 µl of lysis solution. The lysis solution was prepared with 20 mM of NaOH and TritonX-100 at 0.063%. The limit of detection of the method using lysed samples was determined using a schizont culture at 5% parasitemia and 5% hematocrit. The parasitemia was then diluted to 1%, 0.5%, 0.1%, 0.05% and 0.01% by adding uninfected RBCs.

Ring stage assay protocol

Tightly synchronized ring-form cultures were incubated with 700 nM of DHA for 6 hours, as described elsewhere [5]. After that, the drug was washed away. Washed cells were put back in culture with CMCM. Parasite maturation and growth was assessed by flow cytometry every 6 hours during a whole life-cycle (48h) and then at 72h of incubation. Microscopy was used to assess to parasitemia and parasite survival at 72h of incubation. Parasite survival was determined by counting the proportion of viable parasites (in approximately 2000 erythrocytes) that developed into second-generation rings or trophozoites with normal morphology at 72h after drug removal. The parasite survival rate was expressed as a ratio of viable parasites exposed to DHA in comparison to viable parasites in the drug free control.

Results

Depolarized side scatter detects hemozoin in non-lysed and lysed samples

The flow cytometric analysis of non-lysed and lysed samples is shown in Figures 1a) and 1b), respectively. For both non-lysed and lysed samples, depolarizing events are easily detectable in the infected samples in comparison with the uninfected control.

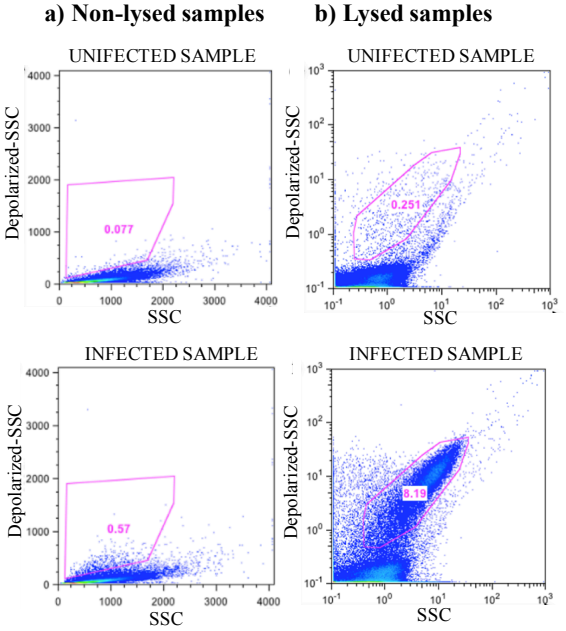

Figure 1. Gating strategy to detect depolarizing events in *P. falciparum* culture. Flow cytometric analysis of an uninfected and an infected synchronized culture of *P. falciparum* (3D7) with 1% parasitemia, after 24h of incubation. In a) intact samples were analyzed and in b) samples were subjected to the lysis procedure. Gain values had to be adjusted and scale was altered for logarithmic to analyze lysed samples.

Detection of drug effects using lysed samples

Lysing the samples allowed to detect free hemozoin and to distinguish between infected and uninfected samples. In order to verify if drug effects could be detected earlier with the lysis protocol, *P. falciparum* 3D7 parasites were incubated with several drugs and parasite growth was assessed at different times over a whole 48-hour life cycle. Time curves of *P. falciparum* 3D7 in the presence of CQ, PQ and DHA are shown in Figure 2. When exposed to increasing concentrations of chloroquine, the difference between inhibiting and non-inhibiting concentrations in *P. falciparum* 3D7 strain started to be visible after 8 hours of incubation and could be clearly detected at 10h of incubation (Figure 2A). The inhibitory effects of piperaquine in lysed samples started to be detectable at 10 hours of incubation being more evident at 12h (Figure 2B).

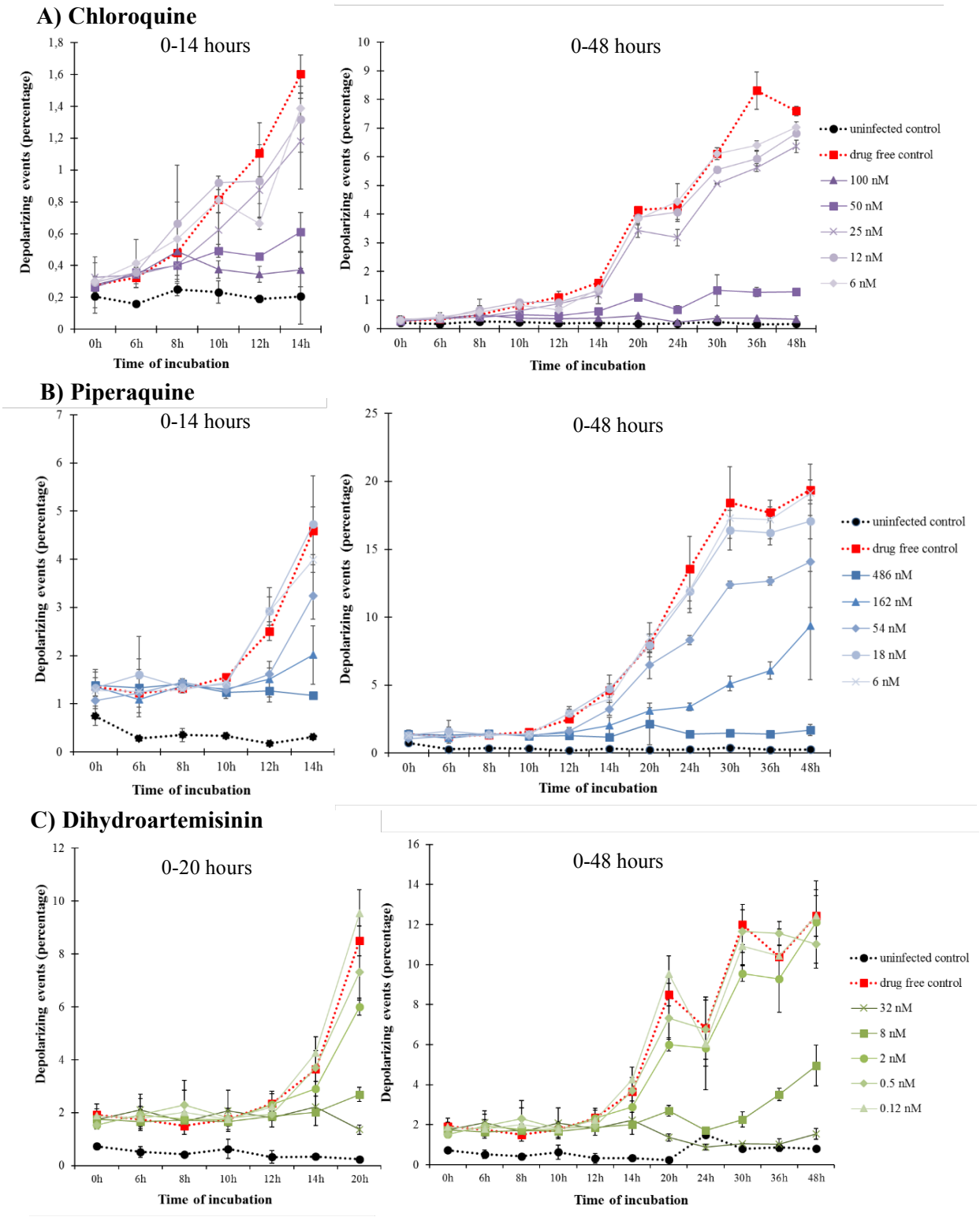

Figure 2. Time curves of *P. falciparum* in the presence of A) Chloroquine, B) Piperaquine and C) Dihydroartemisinin.

Uninfected control and a ring-stage synchronized culture (0.5%-1% parasitemia) of *P. falciparum* 3D7 strain were incubated for 48h with increasing concentrations of the drugs tested. Two plots from 0h to 14h of incubation (0h to 20h for dihydroartemisinin) and from 0h to 48h of incubation are represented. The samples were lysed before the measurement. Each time-point represents the mean value of triplicate measurements (± one SD).

Regarding dihydroartemisinin, the difference between inhibiting concentrations and drug free control could be detected starting at 12 hours of incubation but are more perceptible at 14 hours, considering the standard deviations (Figure 2C).

Comparison of IC50 values

The analysis of *P. falciparum* sensitive-strain (3D7) in the presence different drugs, predicted that it was possible to observe the first signs of drug effects earlier than the standard protocol. The IC50 values were calculated at different incubation times and compared in Table 1 to determine the earliest time-point that would allow to reliably calculate IC50 values. Some values could not be determined because at these time-points, the maximum concentration of the drug tested did not inhibited 50% of the parasite population.

Table 1. IC50 values of antimalarial drugs tested against *P. falciparum* (3D7) at different time-points measuring lysed samples.

IC50 - Lysed samples			
	CQ	**PQ**	**DHA**
10h	94,7 nM	*	*
12h	42,9 nM	330,2 nM	*
14h	41,4 nM	119,6 nM	*
20h	42,2 nM	116,8 nM	4,6 nM
24h	34 nM	79,3 nM	3,5 nM

*values were not determined because the maximum concentration of the drug tested did not inhibit 50% of the parasite population.

Determination of the Hz assay limit of detection using lysis protocol

Infected lysed samples showed a depolarizing population that could be identified and gated. Additionally, measuring lysed samples provided an earlier detection of antimalarial drug effects. Therefore, the limit of detection of the Hz detection assay was investigated using the lysis protocol. Five different parasitemias of 0.01%, 0.05%, 0.1%, 0.5% and 1% were tested. The higher parasitemia tested (1%) corresponded to the higher percentage of depolarizing events (28.3%). The lower parasitemia tested (0.01%) showed no evident difference from the uninfected control. However, the depolarizing events present in the sample with a 0.05% parasitemia were clearly above (3.8%) the uninfected control (0.3%).

Detection of *in vitro* artemisinin-resistant parasites – Ring Stage Assay

The potential of the hemozoin assay to detect artemisinin resistance *in vitro* was explored. A RSA was performed where two strains were tested: an artemisinin-sensitive strain (1239) and an artemisinin-resistant strain (1240). The percentage of depolarizing events decreased at 24h in the sensitive parasites while in the resistant parasites the percentage of depolarizing events was similar to the drug free control (Figure 3).

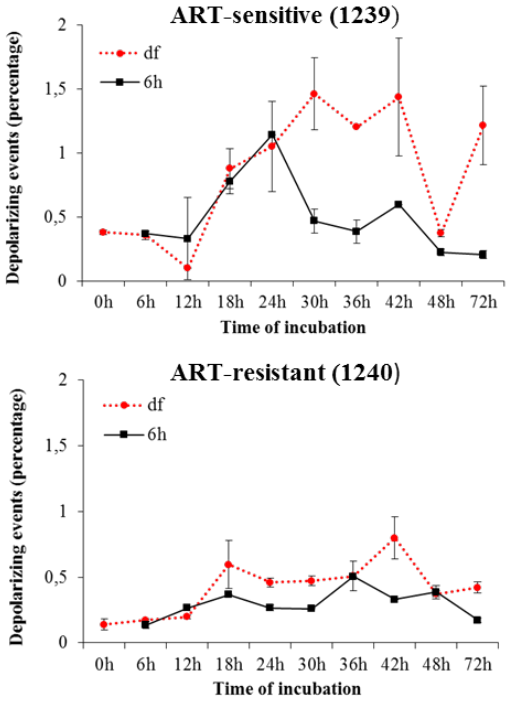

Figure 3. Comparison of inhibitory effects of DHA on sensitive and resistant parasites by flow cytometric Hz detection assay. A drug free control (red spotted line) and a tightly synchronized cultures of *P. falciparum* 1239 and 1240 strains (black lines) were incubated for 72h in the absence of drug treatment and after a 6h pulse of DHA 700 nM, respectively. The measurements were performed every 6h until the 48h and then at 72h of incubation. Each time-point represents the mean value of triplicate measurements (± one SD).

Comparison of survival rates between artemisinin-resistant and artemisinin-sensitive parasites using different methods.

Survival rates are calculated in the RSA instead of IC50 since only a single concentration of the drug is tested. The survival rates of two different *P. falciparum* strains (1239 and 1240) were calculated using different methods: 1) Hz detection assay: measured maturation at 30h of incubation; 2) SYBR Green analysis: measured parasitemia at 48h of incubation; and 3) Microscopy: assessed parasitemia at 72h of incubation. All the methods showed a higher percentage of survival in the artemisinin-resistant parasites in comparison to lower survival rates detected in the artemisinin-sensitive parasites. However, the difference between resistant and sensitive strains was higher when survival rates were determined by microscopy.

Table 2. Survival rates obtained and respective difference between sensitive and resistant strains.

Method	Survival rates (%)		Difference (%)
	1239	1240	
Hz detection	30	54	24
SYBR Green	18	46	28
Microscopy	5	44	39

Detection of delayed growth in artemisinin-resistant parasites

The development pattern of artemisinin-sensitive and artemisinin-resistance parasites was monitored *in vitro* over 48h. The parasite development of three different *P. falciparum* strains was analyzed by hemozoin detection, by SYBR Green and by microscopy (Figure 4). Parasites were tightly synchronized and their development in the absence of drug treatment was assessed every 6 hours during a whole life cycle of 48 hours and at 72h of incubation. All methods showed that, artemisinin-resistant parasites had a longer cycle in comparison to the artemisinin-sensitive strains. With SYBR Green and microscopy it was observed that artemisinin-resistant parasites have an approximately 6h delay in comparison to artemisinin-sensitive ones. Microscopy showed that artemisinin-resistant parasites had a ring-stage phase of 18 hours instead of 12 hours observed in the

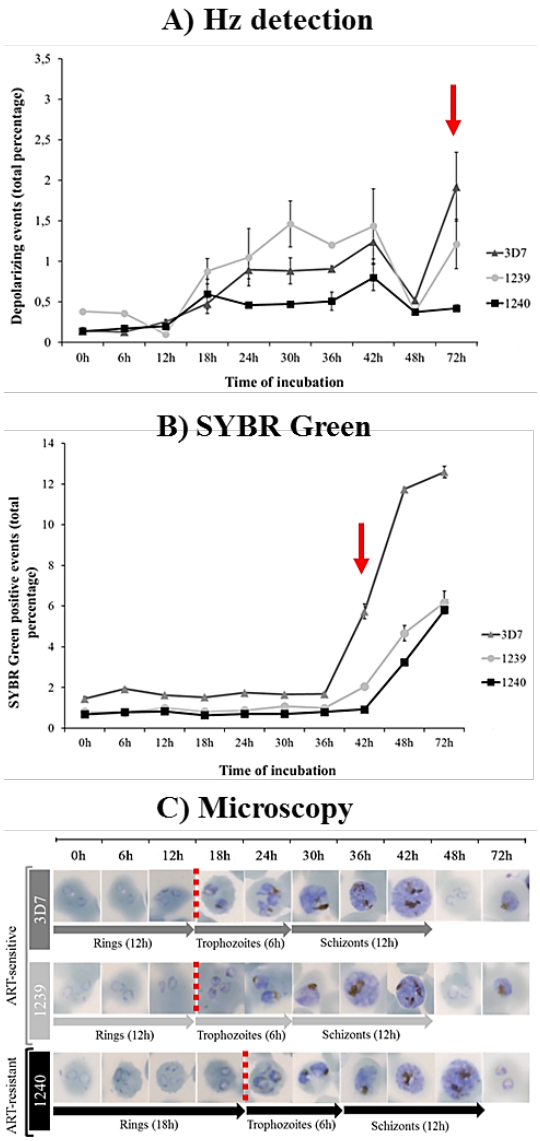

Figure 4. Assessment of parasite development by different methods.

Strains represented in grey corresponds to artemisinin-sensitive parasites (3D7 and 1239) while artemisinin resistance strain (1240) corresponds to the black colour. Parasites development was assessed by A) flow cytometric Hz detection assay analysis B) SYBR Green analysis and C) microscopy observation of blood smears. In A) the percentage of depolarizing events (hemozoin-containing cells) is shown which is related to parasite maturation over time. In B) the SYBR Green positive events are shown which correspond to parasitemia. Each time-point represents the mean value of triplicate measurements (± one SD). In C) pictures of blood smears representatives of each time-point show the maturation forms of parasites.

artemisinin-sensitive strains. The other stages of development seem to have the same duration in all strains. The difference between resistant and sensitive strain growth could only be detected after 72 hours of incubation by the Hz detection assay.

Discussion

Rebelo *et al.* described that drug effects can be consistently detected at 18 hours of incubation in standard protocol [7]. This means that the lysis procedure decreased the time of incubation needed to observe inhibitory effects by 4 to 8 hours depending on the drug tested. Interestingly, the first sign of drug effects for chloroquine could be detected starting at 8 hours of incubation which has never been reported by other methods. Although 10-14 hours is still a long period, it comes closer to the ideal of a "same-day" assay read-out. One possible reason for this is based on the number of cells analyzed per sample. In the standard protocol, for each sample measurement only 5µL of the culture is used. The lysis protocol implies lysing the whole well of the culture, meaning that 200µL is used. Consequently, with the lysis protocol we analyze free Hz in bulk coming from the lysis of a number of cells forty times higher than the number of intact cells analyzed in the standard protocol. Therefore, when the standard protocol is performed, the amount of Hz within each cell may not be sufficient to be detectable in early time-points. However, if we lysed the samples even if the amount of Hz per cell is lower, the quantity of Hz coming from the total number of cells can be enough to be detected. Thus, the inhibitory effects of the drugs can be detected earlier. This also explains the fact that in lysed samples, an accentuated decrease is not detected at 48h, as it has been previously observed in non-lysed samples [7]. When lysed samples are analyzed the total amount of hemozoin present in the sample will be detected, therefore a cumulative effect of the Hz produced from different cycles is quantified.

The IC50 values obtained tend to decrease from the earlier to the later time-point. The analysis of IC50 values reported for the same strain and drugs led us to consider more similar the results obtained with the time-point of 24h of incubation (Table I in Supplemental file) but overall, our method obtains increased IC50 values. Comparing IC50 can be misleading for several reasons. Owing to the peculiarities of each assay, it is impossible to perform them all under the same conditions. For instance, in our method parasitemias of around 1% were used and compared with methods that use lower parasitemias ranging from 0.25 to 0.5%, as the standard [H3]hypoxanthine method [9]. When a higher number of parasites is inoculated the inhibitory drug concentration for artemisinin, artesunate, chloroquine and mefloquine tend to increase, which is called inoculum effect [10,11].

The implementation of a lysis step in the Hz detection assay improved the limit of detection from 0.3% to 0.05% parasitemia. Other commonly used assays as HRP II or WHO schizont maturation test can detect parasitemias as low as 0,01% [12]. The novel MOT method can even detect lower parasitemias [13]. However, 0.05% is below the limit threshold of the standard [H^3]hypoxanthine method (0.25-0.5%) [9] and SYBR Green (0.5-1%) assays [14, 15, 16]. In further studies, it would also be important to verify if inhibitory drug effects could be detected with an initial parasitemia equal to the minimal limit of detection determined with lysed samples instead of the 1% used. Overall, the implementation of a lysis step in the Hz detection assay makes the method more sensitive without compromising the ease of execution and application in the field.

This study also allowed to verify that flow cytometry could be a useful tool for artemisinin resistance testing. The artemisinin-resistant (1240) and sensitive (1239) strains were reported to have survival rates of 88% and 0.1%, respectively [17]. Results obtained in this study using the same assay (RSA 0-3h) were not consistent to the ones described since the median survival rates determined by microscopy were 44% for resistant and 5% for sensitive strain (Table 2). Even though there is evidence for heritability in artemisinin resistance, the phenotype appears unstable, so it could be partially lost over time [18]. However, in most studies survival rates of artemisinin-resistance strains tend to range between 4%-30% [5,19,20].

By performing a 0-3h RSA, it was possible to distinguish at 30h of incubation a resistant parasite from a sensitive parasite in the presence of the drug with the Hz detection method (Figure 3). Survival rates obtained with the Hz assay showed higher values for resistant parasites than for the sensitive strain (1239). However, the difference between the strains was lower than the values obtained by microscopy. The increased survival rates of both strains determined by the Hz assay might be due to the fact only 30% of all parasites were typically detected by depolarization measurements as compared to microscopy or SYBR Green. There is no clear explanation for that but a possible reason is the fact that only parasites that contain a high amount of Hz were detected. Thus, a mature parasite with less Hz might not be considered within the depolarization population. Consequently, the drug free control used to calculate these survival rates may not represent the whole population of parasites. Though, the depolarizing peak in drug free control occurs at 42h so in future experiments it would be interesting to measure parasite maturation at 42h instead of the 30h used. This might approximate the survival values to the ones obtained by microscopy.

The increased survival rates obtained by SYBR Green might be explained by the fact that this method, contrarily to microscopy, does not distinguish a viable from a dead parasite because the stain binds to both.

When the growth pattern of the strains tested was investigated in the absence of antimalarial drugs it was confirmed that resistant parasites showed a delayed growth, as has been reported [21]. However, accordingly to our results, the whole life cycle duration was increased by 6h since the trophozoite stage was not shorten contrarily to what was previously described [21]. The detection of a prolonged life cycle in resistant parasites was common to all the methods and was caused by a longer ring stage observed microscopically. It was already described that resistant parasite forms which are less susceptible to artemisinin were precisely the young ring forms [19]. Taking into account the short half-life of this drug of approximately 1 hour [22], the prolonged ring stage has been suggested as a parasite mechanism of survival [21]. The idea is that resistant parasites in the presence of artemisinins can extend the ring stage until the drug effect is lost so they can proceed their growth later. Nonetheless, the delayed growth is not common to all resistant clones since a case was already reported where the resistant clone had a shorter life cycle of 36 hours [21]. Furthermore, previous results obtained in field studies demonstrated that strains with comparable susceptibility profiles to a determined drug can have different growth patterns *ex vivo* [23]. Thus, if the detection of delayed growth to determine artemisinin resistance works remains unclear.

Overall, although the results suggest that the Hz assay could be used for testing artemisinin resistance, more studies are required since we based our observations only on one resistant strain (MRA-1240). Further studies should include other culture-

adapted resistant strain. Ideally, also a field-study should be conducted in Southeast Asia, where artemisinin resistance has been reported, to evaluate the usefulness of Hz assay with strains obtained directly from patients. Nonetheless, the synchronization protocol used for the RSA is very time-consuming and laborious. The synchronization protocol should be simplified and optimized in future researches.

Despite the remaining open questions, the results strongly suggest that the Hz detection assay may play an important role in antimalarial drug testing, including artemisinin, in the future.

References

[1] Bloland PB. (2001) Drug resistance in malaria. Geneva, Switzerland: World Health Organization 41:45–53. Available from: doi:10.1007/978-1-4613-1267-3.

[2] Noedl H, Wongsrichanalai C and Wernsdorfer WH. (2003) Malaria drug-sensitivity testing: New assays, new perspectives. Trends Parasitol 19:175–181.

[3] Basco LK. (2007) Field application of *in vitro* assays for the sensitivity of human malaria parasites to antimalarial drugs. Geneva, Switzerland: World Health Organization. 191 p.

[4] Dondorp AM, Nosten F, Yi P, Das D, Phio AP, Tarning J, et al. (2009) Artemisinin Resistance in *Plasmodium falciparum* Malaria. N Engl J Med 361:455–467.

[5] Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, et al. (2013) Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: In-vitro and ex-vivo drug-response studies. Lancet Infect Dis 13:1043–1049.

[6] Frita R, Rebelo M, Pamplona A, Vigario AM, Mota MM, Grobusch MP, et al. (2011) Simple flow cytometric detection of haemozoin containing leukocytes and erythrocytes for research on diagnosis, immunology and drug sensitivity testing. Malar J 10:74.

[7] Rebelo M, Sousa C, Shapiro HM, Mota MM, Grobusch MP and Hänscheid T. (2013) A novel flow cytometric hemozoin detection assay for real-time sensitivity testing of *Plasmodium falciparum.* PLoS ONE 8(4):e61606.

[8] Mphande F, Nilsson Sa and Bolad A. (2008) Culturing of erythrocytic asexual stages of *Plasmodium falciparum* and *P. vivax*. In: Moll K, Ljungström I, Perlmann H, Scherf A, Wahlgren M, editors. Methods in Malaria Research. 5th edition, Manassas, Virginia; 1–10.

[9] Desjardins RE, Canfield CJ, Haynes JD and Chulay JD. (1979) Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother 16:710–178.

[10] Duraisingh MT, Jones P, Sambou I, Seidlein LV, Pinder M, Warhurst DC. (1999) Inoculum effect leads to overestimation of in vitro resistance for artemisinin derivatives and standard antimalarials: a Gambian field study. Parasitology 119: 435–440

[11] Gluzman IY, Schlesinger PH, Krogstad DJ. (1987) Inoculum effect with chloroquine and *Plasmodium falciparum*. Antimicrob Agents Chemother 31:32–36.

[12] Noedl H, Wernsdorfer WH, Miller RS and Wongsrichanalai C. (2002) Histidine-Rich Protein II: a novel approach to malaria drug sensitivity testing. Antimicrob Agents Chemother 46:1658–1664.

[13] Mens PF, Matelon RJ, Nour BY, Newman DM and Schallig HD (2010) Laboratory evaluation on the sensitivity and specificity of a novel and rapid detection method for malaria diagnosis based on magneto-optical technology (MOT). Malar J 9:207.

[14] Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P and Riscoe M. (2004) Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother 48:1803–1806.

[15] Bacon DJ, Latour C, Lucas C, Colina O, Ringwald P and Picot S. (2007) Comparison of a SYBR green I-based assay with a histidine-rich protein II enzyme-linked immunosorbent assay for *in vitro* antimalarial drug efficacy testing and application to clinical isolates. Antimicrob Agents Chemother 51:1172–1178.

[16] Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC. (2007) Assessment and continued validation of the malaria SYBR Green I-based fluorescence assay for use in malaria drug screening. Antimicrob Agents Chemother 51:1926–1933.

[17] Bei resources [Internet]. Massanas: American Type Culture Collection. Catalog; Product Information Sheet of IPC5188/MRA-1239; 2016. Product Information Sheet of IPC5202/MRA-1240; 2015. [cited 2017 Fev 15]; Available from: https://www.beiresources.org/Catalog

[18] Chotivanich K, Tripura R, Das D, Yi P, Day NP, Pukrittayakamee S, et al. (2014) Laboratory detection of artemisinin-resistant *Plasmodium falciparum*. Antimicrob Agents Chemother 58:3157–3161.

[19] Witkowski B, Khim N, Chim P, Kim S, Ke S, Kloeung N, et al. (2013) Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in western Cambodia. Antimicrob Agents Chemother 57:914–923

[20] Kite WA, Melendez-Muniz VA, Barros RR, Wellems TE and Sa JM. (2016) Alternative methods for the *Plasmodium falciparum* artemisinin ring-stage survival assay with increased simplicity and parasite stage-specificity. Malar J 15:94.

[21] Hott A, Casandra D, Sparks KN, Morton LC, Castanares GG, Rutter A, et al. (2015) Artemisinin-resistant *Plasmodium falciparum* parasites exhibit altered patterns of development in infected erythrocytes. Antimicrob Agents Chemother 59:3156–3167.

[22] White NJ. (2014) Malaria. In: Farrar J, Hotez PJ,

Junghanss T, Kang G, Lalloo D and White NJ, editors. Manson's Tropical Infectious Diseases, 23rd edition. London: W.B. Saunders;532–600.e1.

[23] Rebelo M, Tempera C, Fernandes JF, Grobusch MP and Hänscheid T. (2015) Assessing anti-malarial drug effects ex vivo using the haemozoin detection assay. Malar J 14:140.

Supplemental file

Table I. IC50 values of several antimalarial drugs against P. falciparum culture-adapted strain (3D7) determined by different in vitro sensitivity tests.

Drug	[H^3]hypoxanthine	WHO schizont maturation assay	SYBR Green	HRPII- assay	pLDH-assay
CQ	15.7 nM [1] 33.5 nM [2] 9.56 nM [3] 29.6 nM [4] 22.76 nM [5] 6.5 nM [6]	14.79 nM [3]	8.1 nM [7] 11.54 nM [3] 22.2 nM [8] 31.10 nM [9]	7.5 nM [7] 10.10 nM [3]	40.5 nM [2] 14.97 nM [3]
PQ	16.9 nM [4] 36.90 nM [5] 27 nM [6]	–	26.33 nM [9]	–	–
DHA	5.27 nM [1] 4.2 nM [4] 2.0 nM [6]	–	3.78 nM [8] 2.30 nM [9] 22.1 nM [10]	2.30 nM [11]	–

References

[1] Duraisingh MT, Roper C, Walliker D and Warhurst DC. (2000) Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of *Plasmodium falciparum*. Molecular Microbiology 36:955-961.

[2] Kaddouri H, Nakache S, Houzé S, Mentré F and Le Bras J. (2006) Assessment of the drug susceptibility of *Plasmodium falciparum* clinical isolates from Africa by using a *Plasmodium* lactate dehydrogenase immunodetection assay and an inhibitory maximum effect model for precise measurement of the 50-percent inhibitory concentration. Antimicrob Agents Chemother 50:3343–3349.

[3] Wein S, Maynadier M, Van Ba CT, Cerdan R, Peyrottes S, Fraisse L, et al. (2010) Reliability of antimalarial sensitivity tests depends on drug mechanisms of action. J Clin Microbiol 48:1651–1660.

[4] Wong RP, Salman S, Ilett KF, Siba PM, Mueller I, Davis TM. (2011) Desbutyl-lumefantrine is a metabolite of lumefantrine with potent in vitro antimalarial activity that may influence artemether-lumefantrine treatment outcome. Antimicrob Agents Chemother 55:1194–1198.

[5] Fivelman QL, Adagu IS and Warhurst DC. (2007) Effects of piperaquine, chloroquine, and amodiaquine on drug uptake and of these in combination with dihydroartemisinin against drug-sensitive and -resistant *Plasmodium falciparum* strains. Antimicrob Agents Chemother 51:2265–2267.

[6] Mwai L, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, et al. (2009) In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in pfcrt and pfmdr1. Antimicrob Agents Chemother 53:5069–5073

[7] Bacon DJ, Latour C, Lucas C, Colina O, Ringwald P and Picot S. (2007) Comparison of a SYBR green I-based assay with a histidine-rich protein II enzyme-linked immunosorbent assay for in vitro antimalarial drug efficacy testing and application to clinical isolates. Antimicrob Agents Chemother 51:1172–1178.

[8] He Z, Chen L, You J, Qin L, Chen X. (2010) In vitro interactions between antiretroviral protease inhibitors and artemisinin endoperoxides against *Plasmodium falciparum.* Int J Antimicrob Agents 35:191–193.

[9] Traore K, Lavoignat A, Bonnot G, Sow F, Bess GC, Chavant M, et al. (2015) Drying anti-malarial drugs in vitro tests to outsource SYBR green assays. Malar J 14:90.

[10] Wang Z, Parker D, Meng H, Wu L, Li J, Zhao Z, et al. (2012) In vitro sensitivity of *Plasmodium falciparum* from China-Myanmar border area to major ACT drugs and polymorphisms in potential target genes. PLoS ONE 7(5):e30927

[11] Held J, Soomro SA, Kremsner PG, Jansen FH, Mordmüller B. (2011) In vitro activity of new artemisinin derivatives against *Plasmodium falciparum* clinical isolates from Gabon. Int J Antimicrob Agents 37:485–488.