A COLORIMETRIC IN VITRO DRUG SENSITIVITY ASSAY FOR PLASMODIUM FALCIPARUM BASED ON A HIGHLY SENSITIVE DOUBLE-SITE LACTATE DEHYDROGENASE ANTIGEN-CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract. We report a double-site enzyme-linked lactate dehydrogenase immunodetection assay (DELI), a highly sensitive antigen-capture enzyme-linked immunosorbent assay, which proved to be more sensitive for the detection of Plasmodium falciparum than thick blood smears, as sensitive as the polymerase chain reaction, and probably more reliable. This technique can help to detect infra-microscopic parasitemias (one parasite in $10^9$–$10^{10}$ red blood cells) from biological samples, and being quantitative, provide a fast substitute to thick smears for epidemiologic purposes. The technique can also be used to measure the in vitro drug sensitivity of P. falciparum with greater ease, much greater speed, and simpler equipment than that required for the isotopic microtest. Results obtained with four antimalarial drugs upon 16 strains closely paralleled those obtained by the isotopic assay ($R = 0.95$). In contrast with the latter, much lower parasite densities could be tested in the DELI assay (as low as 0.005%), thereby extending the number of isolates that can be investigated. The ease of implementation and low cost of the DELI-microtest may contribute to a revived interest in using in vitro methods to survey resistance to antimalarial drugs, so as to better predict future in vivo drug failures and provide public health recommendations.

INTRODUCTION

In view of the fast spread of antimalarial drug resistance, there is now an increased need for diagnosis-based treatment of malaria so as to use adequately more expensive compounds. Therefore, there have been several recent studies aimed at developing rapid individual diagnosis by immunochromatographic systems, i.e., dipsticks methods. Despite the simplicity of the antigen-capture procedure used in the dipstick methodology, monoclonal antibodies have been identified that are able to detect minute amounts of parasite antigens, corresponding to parasitemias as low as one infected red blood cell (IRBC) in $10^5$ normal RBCs (NRBCs). We reasoned that if a simple monoclonal antibody capture assay could reach these levels, a two-site enzyme-linked immunosorbent assay (ELISA) using a labeled second antibody directed to a distinct epitope of the same antigen should achieve greater sensitivity.

Two monoclonal antibodies (MAbs) specific for Plasmodium falciparum lactate dehydrogenase (pLDH) were selected and used to develop a double-site enzyme-linked LDH immunodetection assay (DELI). Results showed a remarkable level of sensitivity. In addition, the linear correlation between optical density and parasite density, together with the ease of the method, was thought to be a useful and practical tool to measure the differential parasite growth in the presence of increasing drug concentrations, i.e., to develop a simple colorimetric drug sensitivity assay. Results from parallel experiments performed using the isotopic drug sensitivity assay suggested that this was the case.

MATERIALS AND METHODS

Strains. This study was carried out using eight culture-adapted strains of P. falciparum: the chloroquine-resistant Palo Alto (FUP/C) strain, the chloroquine-sensitive strain NF 54 strain, the 3D7 clone derived from the latter, the highly chloroquine/pyrimethamine-resistant strain T23, and the FCIP-S47, FCIP-150, FCIP-55, and FCIP-C isolates adapted to culture, which showed different profiles to antimalarial compounds. Strains were maintained at a 5% hematocrit in complete culture medium at 37°C in a candle jar. This medium contained RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD), 35 mM HEPES (Sigma, St. Louis, MO), 24 mM NaHCO$_3$, 0.5% Albumax (Gibco-BRL), 1 mg/liter of hypoxanthine (Sigma), and 5 μg/ml of gentamicin (Gibco-BRL). The cultures were synchronized twice a week by plasmagel (Bellen, Neuilly-Sur-Seine, France) flotation. Fresh isolates. Eight fresh isolates were obtained from symptomatic African patients infected with P. falciparum. After informed consent was obtained, venous blood was collected if the parasitemia was at least 0.2%, the minimal value established as necessary for the isotopic-microtest, and studied within 5 hr.

Other samples. Parasite detection was performed with other types of samples. The first group of samples was a series of 100 fingertip blood samples collected on Whatman (Hillsboro, OR) paper, stored at 4°C for six months, and compared with results from Giemsa-stained thick blood smears examined by microscopy at 1,000× (20 min per smear). Pieces (0.5-cm diameter) of blood-blotted paper were punched, dipped into 500 μl of phosphate-buffered saline (PBS), and used in the DELI method. The second group was two series of thin blood smears prepared using 20 μl of IRBC pellets with various parasitemia levels (using the range of standard parasitemias described below), which were dried overnight at room temperature and then scraped into 200 μl of PBS. The third group was three series of either whole frozen blood, serum, or RBC pellets obtained from 31 individuals.

Antibodies. Two MAbs, 17E4 and 19G7, developed by Flow Laboratories, Inc. (Portland, OR) used in the DELI parasite detection method were kindly provided by Dr. M.
Makler. Monoclonal antibody 17E4 specifically reacts with *P. falciparum* LDH, whereas MAbs 19G7 reacts with both *P. falciparum* and *P. vivax* LDH. The former was used as the capture antibody and the latter was used as the detection antibody. When these two MAbs are used in the DELI method, it is specific for the detection of *P. falciparum* LDH only. Monoclonal antibody 19G7 was biotinylated as follows: 1 ml of MAb 19G7 at a concentration of 1 mg/ml was dialyzed against 0.1 M NaHCO₃, overnight at 4°C, and 10 µl of 0.1 M biotin (Boehringer Mannheim, Meylan, France) was then added and incubated for 1 hr at room temperature. The solution was then dialyzed overnight against PBS at 4°C. The biotinylated MAb 19G7 was diluted (v/v) in glycerol (Pro-labo, Fontenay-sous-bois, France). Both antibodies were aliquoted and stored at 4°C.

**Standard parasitemias.** A range of standard parasitemias was included in each plate as positive controls. Cultures were synchronized by plasmagel flotation, returned to culture, and after re-invasion had occurred, re-synchronized by treatment with sorbitol. From sorbitol-synchronized cultures at ring stage, 10 ml of culture adjusted to a 5% hemocytocrit and a 1% parasitemia was prepared for each manipulation, an initial dilution (1:1,000 for these stock solutions, which were extemporaneously prepared for each manipulation, an initial dilution (1:1,000 for these stock solutions, which were extemporaneously prepared for each manipulation, an initial dilution (1:1,000 for these stock solutions, which were extemporaneously prepared for each manipulation, an initial dilution (1:1,000 for these stock solutions, which were extemporaneously prepared for each manipulation, an initial dilution (1:1,000 for these stock solutions, which were extemporaneously prepared for each manipulation, an initial dilution (1:1,000 for these stock solutions, which were extemporaneously prepared for each manipulation, an initial dilution (1:1,000 for these stock solutions, which were extemporaneously prepared for each manipulation, an initial dilution (1:1,000 for 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to remove the plasma and most of the white blood cells present in the buffy coat.

**Isotopic microtest.** The IRBCs from cultures or isolates were resuspended at a 0.5% parasitemia, 2% hematocrit in a hypoxanthine-free culture medium supplemented with 2 \( \mu \text{Ci/ml of } ^3\text{H}-\text{hypoxanthine} \) (New England Nuclear, Boston, MA). Two hundred microliters of this suspension were distributed to each well in the antimalarial pre-dosed plates, using a Multipette-plus (Eppendorf) and sterile Combitips (Eppendorf). The final drug concentrations ranged from 5.4 to 2,870 nM for chloroquine, 6.5 to 3,333 nM for quinine, and 1.4 to 722.8 nM for both mefloquine and sodium artesunate. The plates were incubated at 37°C for 48 hr in a plastic candle jar and freeze-thawed thereafter. Hemolyzed microcultures were collected on glass fiber filters (GF/C; Whatman) using a cell harvester (Skatron Inc, Sterling, VA), and incorporated radioactivity was evaluated using a liquid scintillation counter (Oy Wallac, Turku, Finland).

**DELI microtest.** The IRBC suspension was prepared in complete culture medium under the same conditions of parasitemia and hematocrit as for the isotopic microtest and distributed in the same manner in the appropriate antimalarial pre-dosed plates. The plates were incubated at 37°C for 48 hr in a candle jar and were thereafter freeze-thawed. At this stage, it was necessary to first dilute the hemolyzed microcultures because a starting parasitemia of 0.5% yielded absorbance values that were too high to be interpretable. Thus, the hemolyzed microcultures were diluted 1:100 in PBS. One hundred microliters of the diluted microcultures were added to each well of the MAb-coated plates to perform the DELI method as described above.

To assess its ability to measure the drug response of low parasitemias, two strains (NF54 and FCIP-55) were tested against four antimalarial drugs using an initial parasitemia of either 0.5%, 0.05%, or 0.005%. These microtests had to be diluted in PBS 1:100, 1:10, or used undiluted, respectively, before performing the DELI-method.

Drug sensitivity was expressed as the concentration of drug that resulted in a 50% inhibition of parasite growth (IC\(_{50}\)), i.e., an inhibition by 50% compared to control wells without drug of either \( ^3\text{H}-\text{hypoxanthine} \) counts per minute or of maximal optical density (OD) values. The cut-off IC\(_{50}\) values between sensitivity and resistance for each drug had been determined previously for the isotopic assay and were > 100 nM for chloroquine, > 300 nM for quinine, and > 30 nM for mefloquine.26,21 For the comparative assessment of the DELI assay and the isotopic sensitivity assay, only those results derived from cultures having a satisfactory growth rate were used (i.e., where the difference between starting parasitemia and final parasitemia was at least 5%).

**Adaptation of the DELI microtest to field conditions.** In view of the high sensitivity of the method, most parasitemias currently observed under field conditions are too high and would result in maximal OD values in the DELI assay. Thus, most of them require an intermediate dilution step that can be either of the initial parasitemia (in NRBCs) or alternatively and preferentially of the hemolyzed culture used to perform the DELI method. To speed up this dilution step, we designed a more simple “rack dilution” method, using Eppendorf tips distributed in a 96-tip rack. All 96 tips were dipped simultaneously into the culture plate up to the bottom of the well, taken out, and then dipped into a MAb 17E4 coated 96-well plate (as described in above DELI method) filled with 100 \( \mu \text{l per well of PBS buffer; this resulted in a dilution of approximately 1:200} \). The amount of liquid remaining in the end of each tip was found to be reproducible, as shown by the systematic comparison of two series of standard parasitemias of IRBCs, which were diluted either by multipipette or by this technique. The resulting ODs were measured in a spectrophotometer. Duplicate plates of drug-sensitivity assays diluted by each method were compared.

To determine which dilution of hemolyzed culture had to be used in the DELI method if the starting parasitemia was not initially evaluated, we tested serial dilutions of control wells of the microtest at the end of the culture period. The presence of the pLDH in each dilution was then detected by the Optimal® dipstick (Flow Laboratories, Inc.). It was found that a dilution 10 times greater than the last dilution yielding a positive signal by the dipstick was appropriate for running the DELI method.

However, for fast field use, a second methodology can be proposed. Both undiluted as well as rack-diluted cultures were tested directly by the DELI assay and the one that yielded a drug concentration–dependent response was used to calculate the IC\(_{50}\). This allows one to accommodate in a single culture plate isolates from various patients who have a wide range of initial parasite densities without any prior estimation of them. This is straightforward for field applications because it avoids both microscopy and prior use of dipsticks and is therefore more practical even if it indicates that two ELISA plates have to be run instead of one.

**RESULTS**

**Detection of low parasitemias.** Initial studies were performed using established strains of *P. falciparum* adjusted to various parasite densities. A typical example of results obtained using either parasites at the ring stage or mature schizont stage obtained from synchronized cultures is shown in Figure 1. A linear relationship between parasite density and absorbance values was found and a clear difference between schizont and ring stages was observed, indicating as expected, a higher content of pLDH at the schizont stage.

Having obtained evidence that the DELI method could specifically detect pLDH, we initiated a series of systematic studies aimed at finding optimal conditions to reach the best possible sensitivity. Large differences were observed depending on each parameter, i.e., the lowest positive density detectable varied from 1 IRBC in 10\(^5\) to 10\(^7\) NRBCs. Some of these combinations provided a stronger signal; however, the discriminating ability, i.e., the difference in absorbance between NRBCs and IRBCs was not directly proportional to the maximal absorbance obtained (i.e., for some results, the background increased as much as the signal). This study led to select the conditions that are described in the Materials and Methods for further studies.

Using the above selected conditions, we then examined the level of detection achieved by the method using patient isolates. These were chosen because they often contain parasites that are at an earlier stage of development of blood schizogony than even the best synchronized rings from culture, i.e., tiny rings, and thus may produce less pLDH and
be more difficult to detect. A typical example is shown in Figure 2. The method was able to detect a parasitemia as low as 1 IRBC in \(10^8\) NRBCs. However, to obtain this extremely high sensitivity the assay conditions had to be carefully monitored: minor modifications in the handling of the plates, in washing, and in the electrostatic forces (depending on the surface on which the plate was handled) resulted in a loss of sensitivity. Of the first 30 plates handled in this manner by two different investigators, sensitivity varied by two orders of magnitude, from one IRBC in \(10^6\) to \(10^8\) NRBCs.

The potential of the DELI method was investigated using other types of biological samples. In addition to frozen RBC pellets, we investigated the sensitivity reached using finger-tip blood samples collected on filter paper. Using first a range of standard parasitemia as described in the Materials and Methods, the sensitivity of detection by DELI method from blotted paper samples was found to be of the order of 1 IRBC in \(10^6\) to \(10^8\) NRBCs, a satisfactory sensitivity considering the small amount of blood collected. The results obtained using patients’ blood indicated that all of the individuals with a positive thick smear were also positive in the DELI detection method. Studies using scraped thin smears as the starting material also showed detection of parasite densities as low or lower than those that can be detected by thick blood smears (e.g., 1 IRBC in \(10^6\) NRBCs). Finally, the assessment of pLDH was also done with either whole blood, serum samples, or RBC pellets from 31 individuals. Results obtained using these samples were positive in all of the thick smear-positive individuals and negative in controls. However, the use of serum yielded less sensitive detection of parasites than whole blood or RBC pellets (by one order of magnitude). Moreover, it was soon realized that the serum detection level was highly dependent on the degree of hemolysis, a factor difficult to control when separating serum; thus, this material was not used further.

**Evaluation of drug responses using the DELI microtest.** In preliminary experiments, the method was used for the determination of the IC\(_{50}\) values of the FCIP-C strain and the 3D7 clone for chloroquine and quinine. Parasites were cultured in the presence of two-fold dilutions of drugs in conditions similar to those of the isotopic microtest. The plates were then frozen, thawed, and the supernatants were used in the DELI assay to measure the resulting parasite densities. A typical result of the DELI microtest is shown in Figure 3. Optical densities were measured using an ELISA plate reader for a quantitative assessment of the IC\(_{50}\). However, the first estimate of the drug response pattern of the strain is readily apparent by eye. No changes in the measured OD were found when plates were either immediately processed, kept frozen for 1–3 months, or subjected to several rounds of freezing and thawing. This makes it possible to keep the plates frozen until the DELI method can be performed.

**Correlation between the DELI and isotopic assays.** The correlation between results of the DELI microtest and the isotopic microtest was examined using various strains and isolates tested against several antimalarials. An example is shown in Figure 4. Strain 3D7, which is susceptible to chloroquine, yielded IC\(_{50}\) values of 15.1 nM and 15.7 nM as determined by isotopic and DELI methods, respectively, whereas strain PA (FUP/C), which is resistant to chloroquine, showed IC\(_{50}\) values of 387.5 nM and 396.1 nM, respectively.

Further studies were performed testing six other strains and eight isolates with chloroquine, quinine, mefloquine, and sodium artesunate. These parasites had, as shown by the isotopic microtest, a wide range of responses to these drugs. Although some degree of variation in the IC\(_{50}\) was seen, a close correlation between the isotopic and colorimetric methods was found. The correlation coefficient was \(r = 0.96\) for chloroquine, \(r = 0.95\) for quinine, \(r = 0.96\) for mefloquine, and \(r = 0.96\) for sodium artesunate. The correlation coeffi-
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FIGURE 3. Results from a typical double-site enzyme-linked lactate dehydrogenase immunodetection (DELI) assay. Nine decreasing concentrations of quinine (rows A, B, C, and D) and of chloroquine (rows E, F, G, and H) were distributed in wells 1–9 of a 96-well plate to assess the drug sensitivity of strain FCIP-C and clone 3D7. Wells 10 and 11 are positive controls (without drug). In rows A, B, E, and F one can observe the parasite growth of the drug-resistant FCIP-C strain in wells with high concentrations of quinine and chloroquine, whereas for clone 3D7 growth is prevented by low concentrations of both antimalarials.

FIGURE 4. Dose-response curves of the chloroquine-resistant PA strain (squares) and the chloroquine-sensitive 3D7 strain (circles) using either the isotopic method (open symbols) or the double-site enzyme-linked lactate dehydrogenase immunodetection (DELI) assay (closed symbols). Results are expressed as % optical density (OD) values or % incorporated radioactivity in control wells without drug subtracted from background values observed at the highest drug concentration.

Reproducibility of the DELI microtest. Reproducibility was assessed by repeating both the colorimetric and the isotopic microtests nine times, by two different investigators, using a single strain against three antimalarial drugs. Determination of the IC_{50} values of this strain against each antimalarial drug showed that the reproducibility was similar using both methods mean ± SD = 322.8 ± 61 nM and 376 ± 74 nM for chloroquine, 335.3 ± 88 nM and 296.3 ± 73 nM for quinine, and 24.6 ± 3 nM and 25.7 ± 4 nM for mefloquine. Thus, both techniques exhibited the same degree of variation from assay to assay and from one investigator to another.

Sensitivity of the DELI microtest. As shown in Table 1, the DELI microtest was sensitive enough to precisely determine the IC_{50} values of strains even at starting parasitemias of 0.005%. The results found at various initial parasitemias correlated with those obtained using the isotopic method (which could not be carried out at parasitemia lower than 0.2%). Moreover, the IC_{50} was not greatly influenced by the initial parasite density. This was unexpected since it is efficient for paired values derived from the four antimalarials in the 16 parasite strains and isolates was r = 0.95 and is shown in Figure 5.
known that quinolines concentrate heavily in IRBCs; thus, the drug concentrations could vary depending on parasite concentrations.

DISCUSSION

The results show that the DELI method can reliably detect *P. falciparum* parasitemias from various biological samples, preferentially frozen whole blood or frozen RBC pellets. The degree of sensitivity reached after optimizing the method was surprisingly high because it was as sensitive as the polymerase chain reaction (PCR), currently considered the most sensitive method available. Both techniques can show variations in the degree of sensitivity reached, i.e., in the PCR from one amplification to another, or with the DELI depending on the way the ELISA is being handled. This possibility of variation from experiment to experiment makes it imperative to include controls having defined parasite densities in each plate so as to assess the real sensitivity obtained in each experiment. In addition, the DELI method has major advantages over the PCR. It is quantitative whereas PCR is mostly qualitative. It may be more reliable as we have shown, as have others, that DNA contamination can easily occur in laboratories where parasite DNA is regularly handled, indicating that specifically designed rooms are needed to totally exclude DNA contamination. In addition, the DELI method is cheaper and faster than PCR.

Since the DELI detection method is suited for epidemiologic purposes, it stands in contrast to the dipstick method, which is better suited for the diagnosis of individual cases suspected of having a fever related to malaria. However, it has suboptimal sensitivity. Nevertheless, the high level of sensitivity reached in ELISA plates using the same reagents as those used in dipsticks also suggests that the sensitivity of the latter can probably be increased so as to improve rapid individual diagnosis.

For drug sensitivity studies, comparative results between DELI and isotopic microtests clearly demonstrate the practical feasibility of the method and its reliability. The DELI

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**TABLE 1**

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<thead>
<tr>
<th>Strain: Parasitemia</th>
<th>Chloroquine</th>
<th>Quinine</th>
<th>Mefloquine</th>
<th>Sodium artesunate</th>
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<td>FCIP-55:</td>
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<tr>
<td>0.5%</td>
<td>268.4</td>
<td>370.0</td>
<td>317.3</td>
<td>273.5</td>
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<tr>
<td>0.05%</td>
<td>290.1</td>
<td>310.8</td>
<td>317.3</td>
<td>273.5</td>
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<tr>
<td>0.005%</td>
<td>348.4</td>
<td>350.2</td>
<td>317.3</td>
<td>273.5</td>
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<td>NF54:</td>
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<tr>
<td>0.5%</td>
<td>20.0</td>
<td>21.1</td>
<td>330.0</td>
<td>375.0</td>
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<tr>
<td>0.05%</td>
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<td>408.0</td>
<td>42.3</td>
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<td>0.005%</td>
<td>17.3</td>
<td>420.0</td>
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*IC₅₀ concentrations of strains FCIP-55 and NF54 were determined using starting parasitemias of 0.5% for the isotopic microtest and 0.5%, 0.05%, and 0.005% for the double-site enzyme-linked lactate dehydrogenase immunodetection (DELI) assay. Values are in nanomoles (nM).*

**FIGURE 5.** Correlation between the 50% inhibitory concentration (IC₅₀) values for chloroquine (open squares), quinine (closed triangles), mefloquine (closed squares), and sodium artesunate (open circles) determined both by the double-site enzyme-linked lactate dehydrogenase immunodetection (DELI) assay and the isotopic method using 8 strains and 8 isolates.
microtest was not only able to distinguish sensitive from resistant parasites, but in addition, supplied IC₅₀ values similar to those obtained using the isotopic microtest. This shows that despite the difference in the measurement made, nucleic acid synthesis in one and protein synthesis in the other, the results for different concentrations of drugs with different biological targets, such as chloroquine and pyrimethamine, would be comparable by both methods. Thus, the DELI method can be applied either to study new drugs with standardized parasite isolates, or conversely, to study the response of field isolates to existing antimalarial drugs. The correlation obtained between both microtests has practical implications: results obtained with the isotopic microtest could be compared in the future with those eventually obtained with the colorimetric DELI microtest.

Furthermore, the remarkable sensitivity of the DELI microtest has practical advantages in drug resistance field studies. When the isotopic microtest is used under field conditions, the need to identify first those donors having parasite densities > 0.2% has been in practical terms a time-consuming step and therefore is an additional cost to the costly isotopic microtest. In contrast, the high sensitivity of the DELI microtest will now allow one to measure the drug response at low parasites densities. Given the fact that 40–60% of the individuals from hyperendemic or holoendemic areas show a detectable parasitemia by thick smear and 90% are positive by the PCR or by DELI detection method, it also implies that samples from nearly all individuals from those areas could be incorporated into the assays without prior parasite screening. Thus, this technique is not only faster to perform but would also save time at the level of screening individuals susceptible of being included in the assay, providing by including low-grade parasitemia a more complete picture of parasites resistance in a given area. However, it must be emphasized that the DELI assay, a two-site ELISA, differs from the previously reported pLDH enzymatic assay revealed by the Malstaf9 (Flow Laboratories, Inc.) substrate, 40-42 and has a much higher sensitivity than the latter (which could only detect parasitemias ≥ 1%).

The progressive resistance of *P. falciparum* to all antimalarial drugs has emphasized the need to determine the drug sensitivity patterns of this parasite in the endemic areas so that rational prophylaxis and treatment schemes can be implemented. Techniques aimed at assessing drug resistance available to date can be classified into two main categories, *in vivo* and *in vitro* assays. Generally speaking, *in vivo* and *in vitro* assays are complementary. *In vitro* assays include mainly two types: 1) the so-called World Health Organization (WHO) microtest, 44 in which results are assessed microscopically, a time-consuming process, and 2) the isotopic microtest as originally described by Desjardins and others, 45 of which there are several slight modifications aimed at better adaptation to field conditions. 46-50 However, the isotopic assay has the main disadvantage of being isotopic, i.e., it requires the use of radioactive materials that are dangerous to handle and short-lived. It also requires well-trained personnel, and heavy and expensive equipment, which is difficult to maintain in tropical areas.

This state of affairs has resulted, somewhat paradoxically, in a progressive move from *in vitro* assays towards *in vivo* assays in tropical regions. However, the original *in vivo* assay requires 28–35 days of follow-up outside the transmission area to detect early resistance cases at the RI level. For this reason, it has been also progressively abandoned in favor of a seven-day assay, 51 or a hybrid assay that takes 14 days, 52-53 which detect mostly RII–RII levels of resistance (except when combined with an expensive genotyping by PCR that is seldom used). The history of drug resistance surveyed properly by either *in vitro* or *in vivo* teaches us that before treatment failure occurs, it is preceded by a progressive decrease in sensitivity easily detected by a shift in IC₅₀ values *in vitro*. 54-55 The increase in resistance is then detectable by *in vivo* means at the RI level and it is only several years later that the increase in the degree of resistance results in RII or RIII levels (or the early treatment failure [ETF] and late treatment failure [LTF], following the recently proposed WHO classification). Finally, the limitations found with the existing *in vivo* and *in vitro* assays indicate that current data regarding the spread of resistance are scarce and rarely up-dated.

Since the DELI assay is easier to perform, faster to implement, and cheaper than *in vitro* isotopic assays, we hope that it may contribute to a revived interest in performing *in vitro* assays, which are clearly complementary to *in vivo* studies. Within the present context of fast-evolving drug resistance patterns, we believe that *in vitro* assays have one major advantage, i.e., they provide predictive data showing a change in sensitivity long before true resistance occurs at RI level. 21,56 The present DELI assay may satisfy this need as it stands today; however, further improvements can be made to simplify its use and make it easily available to remote, poorly equipped, field areas.

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