A HISTIDINE-RICH PROTEIN 2–BASED MALARIA DRUG SENSITIVITY ASSAY FOR FIELD USE

HARALD NOEDL, BERNHARD ATTLMAYR, WALther H. WERNSDORFER, HERWIG KOLLARITSCh, and ROBERT S. MILLER

Department of Immunology and Medicine, United States Army Medical Component, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; Department of Specific Prophylaxis and Tropical Medicine, Institute of Pathophysiology, University of Vienna, Vienna, Austria

Abstract. With the spread of antimalarial drug resistance, simple and reliable tools for the assessment of antimalarial drug resistance, particularly in endemic regions and under field conditions, have become more important than ever before. We therefore developed a histidine-rich protein 2 (HRP2)–based drug sensitivity assay for testing of fresh isolates of Plasmodium falciparum in the field. In contrast to the HRP2 laboratory assay, the field assay uses a procedure that further simplifies the handling and culturing of malaria parasites by omitting centrifugation, washing, the use of serum, and dilution with uninfected red blood cells. A total of 40 fresh Plasmodium falciparum isolates were successfully tested for their susceptibility to dihydroartemisinin, mefloquine, quinine, and chloroquine (50% inhibitory concentration [IC50] = 3.43, 61.89, 326.75, and 185.31 nM, respectively). Results very closely matched those obtained with a modified World Health Organization schizont maturation assay (R2 = 0.96, P < 0.001; mean log difference at IC50 = 0.054).

INTRODUCTION

Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today. It has been implicated in the spread of malaria to new areas and in re-emergence of malaria in areas where malaria had previously been eradicated, but above all it has been identified as a factor of economic constraints for malaria control.1 Particularly due to the financial restrictions in the fight against the resurgence of malaria, the considerable use of the limited available resources is essential.2 Simple and reliable methods for the assessment of antimalarial drug resistance, particularly under field conditions, have therefore become more important than ever before.

There are essentially two different approaches to the assessment of drug resistance in malaria parasites: in vivo and in vitro assays.3 Although in vitro tests offer a number of advantages when it comes to measuring the intrinsic drug resistance of malaria parasites, in vivo tests are essential when it comes to assessing the clinical efficacy of antimalarial drugs in certain populations. Ideally, in vivo and in vitro tests should therefore be used in parallel. Most of the traditional in vitro assays, such as the isotopic assay and the World Health Organization (WHO) schizont maturation assay, have been in use for more than 20 years.4,5 However, these tests have a number of weaknesses that limit their usefulness particularly in the field. The isotopic assay has the disadvantage of requiring high parasite densities, expensive and highly specialized equipment, and the handling of radioactive material. The WHO microtest is particularly labor-intensive and requires highly trained personnel to limit individual variability. We recently developed a novel enzyme-linked immunosorbent assay (ELISA)–based in vitro drug sensitivity assay for Plasmodium falciparum that overcomes many of the problems traditionally associated with in vitro tests.6 It measures the production of histidine-rich protein 2 (HRP2) and its inhibition by antimalarial drugs during 72 hours of culture. The aim of the present study was to establish and validate a simplified HRP2-based drug sensitivity assay for testing of fresh P. falciparum isolates in the field.

MATERIALS AND METHODS

All culture and ELISA procedures were performed at a small temporary field laboratory set up at the Malaria Clinic of Mae Sot, along the Thailand-Myanmar border. The test procedure is a simplified HRP2 drug-sensitivity assay modified for field use.6

Parasite isolates. A total of 46 fresh P. falciparum isolates were collected from adult, symptomatic outpatients (9 woman and 37 men) at the malaria clinics in Mae Sot, So Oh, and Chedi Koh in Tak Province, in close proximity to the Thailand-Myanmar border. Written informed consent was obtained from all study participants and the study protocol was reviewed and approved by the Ethical Review Boards of the Thai Ministry of Public Health (Nonthaburi, Thailand) and the Human Use Review Committee of the U.S. Army (Washington, DC). The parasitized blood samples were tested in the HRP2 field drug sensitivity assay as well as in a modified WHO schizont maturation assay. Plasmodium falciparum parasite samples were only selected for a minimum parasite density (≥ 0.01% infected red blood cells [IRBC], equivalent to approximately ≥ 500 parasites/μL). No other parasitologic criteria (such as healthy looking rings or minimum size of the rings) were used. Only samples with parasite densities > 1% IRBC (50,000 parasites/μL) were diluted with uninfected red blood cells (blood group O) to < 1% (preferably to 0.17%, the geometric mean of the optimal culture parasite density range of 0.03–1%).

Sample collection and processing. After disinfection of the skin, 1 mL of whole blood was collected by venipuncture using a sterile disposable phlebotomy tool and a heparinized container. Thick and thin blood films were prepared, stained in 2% Giemsa solution, and the parasite density was assessed. To obtain a total of 25 mL of cell medium mixture at approximately 1.5% hematocrit (assuming a 40% hematocrit in the original blood sample), 0.94 mL of whole blood were mixed with 24.06 mL of complete RPMI 1640 medium (10.43 grams of RPMI 1640 powder, 6 grams of HEPES, 25 mg of gentamicin, 0.5% [(w/v) albumin [Albumax I; Gibco, Bangkok, Thailand], and distilled water to obtain one liter of medium; before use 28 mL of 7.5% NaHCO3 solution were added).
**Culture plates.** Stock solutions (1 mg/mL) in ethanol were prepared from mefloquine hydrochloride (Mr = 414.778), chloroquine diphosphate (Mr = 515.867), quinine sulfate dihydrate (Mr = 782.954), and dihydroartemisinin (DHA) (Mr = 284.35). These were diluted with distilled water to obtain the desired test concentrations (mefloquine hydrochloride = 3.2–206.3 ng/mL, chloroquine diphosphate = 16.1–1,033.1 ng/mL, quinine sulfate dihydrate = 24.1–1,543.2 ng/mL, and DHA = 0.2–9.4 ng/mL). Serial two-fold dilutions (seven concentrations and one drug-free control well) of the drugs (25 μL/well) were dispensed in duplicate into standard 96-well microculture plates (Costar 3599; Costar, Cambridge, MA) by a semiautomated microdilution technique. The plates were dried overnight in an incubator at 37°C and stored at 4°C.

**Culture.** Using a multichannel pipette, 200 μL of cell medium mixture (CMM) were dispensed into each well of the predosed culture plates (including around 10 drug-free wells that serve as additional controls). The plates were then incubated in a candle jar at 37°C for 72 hours. After the first 24 hours of incubation, the contents of four control wells was harvested, transferred into a microcentrifuge tube, and stored at −20°C. This sample may be used in the ELISA to subtract the background (i.e., parasite growth within the first 24 hours). After the end of the 72-hour incubation time, thick and thin blood films were prepared from one of the control wells to check for adequate parasite growth and reinvasion. The culture plates were then transferred into a simple household freezer (approximately −15°C) and frozen-thawed twice.

**Enzyme-linked immunosorbent assay.** Commercial ELISA kits were used to quantify the amount of HRP2 (Malaria Ag CELISA; Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). However, basically any ELISA specific to HRP2 may be used (by using a simple generic HRP2 double-site sandwich ELISA, the overall cost for the ELISA may be drastically reduced). The culture samples were diluted directly on the ELISA plates with distilled water according to their starting parasite densities to obtain the equivalent of approximately 0.01–0.1% (ideally 0.05%; e.g., if the initial parasite density in the culture was 0.25%, one part (20 μL) of the hemolyzed CMM was added to four parts (80 μL) of water to obtain 100 μL of sample with the equivalent of a 0.05% parasitemia). This was done by first adding the water to the ELISA plates and then transferring and carefully mixing the hemolyzed CMM in each well using a multichannel pipette. In addition to the samples from the predosed wells, 100 μL of the control sample frozen after 24 hours and diluted in the same way were added to two wells to determine background HRP2 concentrations.

The ELISA plates were then incubated at room temperature for one hour. Subsequently, the plates were washed three times with washing solution, and 100 μL of the diluted antibody conjugate were added to each well. After further incubation for one hour, the plates were washed three times and 100 μL of the diluted tetramethylbenzidine chromogen were added to each well. The plates were then incubated for an additional 15 minutes in the dark, and 50 μL of the stopping solution were added. Spectrophotometric analysis was performed using a small, field-suitable ELISA plate reader (Tecan Sunrise Absorbance Reader; Tecan Austria GmbH, Groedig, Austria) at an absorbance maximum of 450 nm.

**Modified WHO schizont maturation assay.** A morphologic assay based on the WHO schizont maturation test was used to obtain data for the comparison with a traditional drug sensitivity assay.7,8 The culture samples were treated in the same way as those for the HRP2 assay and cultured on the same culture plates. Since the parasite samples were not preselected, the samples had to be monitored continuously for parasite maturation and harvested whenever the parasites had reached the schizont stage (mean incubation time for successfully cultured samples = 29 hours, range = 19–45 hours). Thick blood films were prepared from the cell sediment of each well and stained in 2% Giemsa stain. These were microscopically evaluated by counting the number of schizonts with three or more chromatins against 200 asexual parasites.

**RESULTS**

A total of 46 samples were collected from symptomatic *P. falciparum* malaria patients with a mean age of 29 years (range = 20–55 years). One sample was contaminated, five samples did not show adequate growth in either the HRP2 or the modified WHO assay, and 40 isolates (87%) were successfully tested in both assays. The geometric mean parasite density of the cultured samples was 0.27% (range = 0.01–0.95%). Eight blood samples with parasite densities > 1% (range = 1.6–5.8%) were diluted with uninfected whole blood (blood group O) to < 1% before culture. All isolates were tested in duplicate for their susceptibility to DHA, mefloquine, quinine, and chloroquine. The geometric mean IC50 for the 40 isolates in the HRP2 field assay were 3.43 nM (95% confidence interval [Cl] = 2.73–4.31 nM), 61.89 nM (95% Cl = 49.47–77.42 nM), 326.75 nM (95% Cl = 268.18–398.12 nM), and 185.31 nM (95% Cl = 163.11–210.53 nM) for DHA, mefloquine, quinine, and chloroquine, respectively (Table 1). The corresponding results obtained with the modified WHO schizont maturation assay were 2.42 nM (95% Cl = 1.92–3.04 nM), 75.20 nM (95% Cl = 58.25–97.08 nM), 543.76 nM (95% Cl = 447.11–661.31 nM), and 214.21 nM (95% Cl = 188.52–243.39 nM), respectively.

In a correlation analysis, the results obtained from the HRP2 drug sensitivity tests with all four antimalarials showed a highly significant linear association with those obtained from the WHO assay at the IC50 level (R2 = 0.98, P < 0.001), as well as at IC90 level (R2 = 0.96, P < 0.001) (Figure 1). When correlating the results obtained from both assays for individual drugs, the correlation coefficients at the IC90 level were R = 0.82 (R2 = 0.67, P < 0.001) for DHA, R = 0.83 (R2 = 0.69, P < 0.001) for mefloquine, R = 0.74 (R2 = 0.55, P < 0.001) for quinine, and R = 0.50 (R2 = 0.25, P = 0.001) for chloroquine, respectively.

The mean difference in log IC50 for DHA, mefloquine, quinine, and chloroquine determined by the HRP2 drug susceptibility assay and the WHO schizont maturation assay was
Geometric mean 50% and 90% inhibitory concentrations (nM) with 95% confidence intervals for 40 field isolates of *Plasmodium falciparum* tested with dihydroartemisinin, mefloquine, quinine, and chloroquine determined by the HRP2 field drug sensitivity assay and a modified WHO schizont maturation assay.

<table>
<thead>
<tr>
<th></th>
<th>HRP2 assay</th>
<th>WHO assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHA</td>
<td>3.43 (2.73–4.31)</td>
<td>2.42 (1.92–3.04)</td>
</tr>
<tr>
<td>MEF</td>
<td>61.89 (49.47–77.42)</td>
<td>75.20 (58.25–97.08)</td>
</tr>
<tr>
<td>QNN</td>
<td>326.75 (268.18–398.12)</td>
<td>543.76 (447.11–661.31)</td>
</tr>
<tr>
<td>CHL</td>
<td>185.31 (163.11–210.53)</td>
<td>214.21 (188.52–243.39)</td>
</tr>
</tbody>
</table>

*HRP2* = histidine-rich protein 2; WHO = World Health Organization; IC = inhibitory concentration (at 50% and 90%); DHA = dihydroartemisinin; MEF = mefloquine; QNN = quinine; CHL = chloroquine.

Together with simplicity, both in implementation and in execution, high sensitivity is therefore one of the key factors for the success of field drug resistance assays. In this study, samples with parasite densities as low as 0.01% (approximately 500 parasites/μL) were successfully tested. However, from our experience the optimal range for testing starts at 0.03% (1,500 parasites/μL), which includes the vast majority of symptomatic *P. falciparum* malaria cases. Its sensitivity is therefore comparable to that of the WHO schizont maturation assay and approximately 10 times higher than that of the isotopic assay.

When performed under the same conditions, the results of the HRP2 field assay were close to those obtained with the WHO assay. The previous validation of the HRP2 assay with culture-adapted parasite strains suggests that the results would most likely also be very similar to those obtained with the traditional isotopic drug sensitivity assay, which could not be used in this study because many of the samples had parasite densities too low for the isotopic assay and because the nature of the field site would not allow for the use of radioactive material.

Compared with the HRP2 drug sensitivity assay applied to laboratory strains of *P. falciparum*, the field test uses a further simplified design to adapt for the working conditions in the field. Centrifugation, washing of the samples, the use of serum
in culture, and dilution with uninfected red blood cells are omitted (except if the parasite density reaches more than 1%). Thus, the handling of the parasites could be reduced to less than 10 minutes from blood draw to the initiation of the culture.

Instead of using human serum as a protein source for the parasites, commercial albumin was used. The principal advantage of using albumin instead of human serum is the standardized quality. Furthermore, the use of albumin eliminates the need for storing and handling of serum in the field. Although results obtained with commercial albumin may not necessarily be identical with those obtained with human serum, results in the long run will be easier to compare due to consistent protein contents and quality in the culture medium.12

The IC50s obtained from the HRP2 assay did not show any significant association with the parasite densities, suggesting that within the test range, the parasitemia does not have a major impact on the outcome of the tests. In well-equipped laboratories, however, the parasitemia may be adjusted by diluting the original blood sample with uninfected red blood cells (blood group O) as described previously to fully eliminate any influence from the inoculum effect.6,13 The natural background associated with the use of HRP2 as an indicator of parasite growth reflects the high stability of this protein and therefore of the background.14 This background may be excluded by subtracting the result obtained from a control sample after 24 hours of culture (or, if not available, by subtracting the lowest test value in each test).

Apart from the minimum parasite density, no other parasitologic criteria (such as healthy looking rings or minimum size of the rings) were used. Compared with an unmodified WHO drug sensitivity assay (incubation time not exceeding 30 hours), the success rate of the HRP2 assay (87%) was considerably higher than that of the WHO test (61%). Some of the tests in this study had to be incubated for up to 45 hours to produce valid WHO results. The long incubation time of the HRP2 assay largely overcomes these issues and also allows for the testing of slow-acting drugs (such as antifolate antimalarials and antibiotics). From our experience, the main criterion for the success of the HRP2 test is the reinvasion of the parasites into previously uninfected red blood cells after the first cycle is completed. This may easily be assessed in a thin blood film prepared after 72 hours of culture. Even most samples that showed no schizont maturation after 24 hours were successfully tested in the HRP2 assay.

The application of in vitro drug sensitivity techniques in the field is particularly challenging. Field testing requires a highly sensitive and simple assay that gives a high yield with field isolates and that works within a wide range of parasite densities. The simplified technique for the HRP2 field assay fulfills all these requirements. The test is highly sensitive, non-isotopic, semi-automated, and particularly simple to establish and perform. The culture procedure in the field is similar to the WHO microtest. However, the reading of the results is automated and may either be performed with a portable ELISA plate reader in the field or after transporting the frozen culture plates to a central laboratory.

Received January 20, 2004. Accepted for publication June 21, 2004.

Acknowledgments: We thank the staff of the Malaria Clinic in Mae Sot for their active support in collecting the samples and Ruth Ellis for her review of the manuscript.

Financial support: This work was supported by the Austrian Science Fund Project No. 15754-B02 and the U.S. Department of Defense Global Emerging Infections System (GEIS) program.

Disclaimer: The opinions reflected herein reflect those of the authors and do not necessarily reflect the official views of the U.S. Army or the U.S. Department of Defense.

Authors’ addresses: Harald Noedl, Bernhard Attlmayr, Walther H. Wernsdorfer, and Herwig Kollaritsch, Department of Specific Prophylaxis and Tropical Medicine, Institute of Pathophysiology, University of Vienna, Kinderspitalgasse 15, A-1095, Vienna, Austria. Robert S. Miller, Armed Forces Research Institute of Medical Sciences, 316/6 Rajivji Road, Bangkok 10400, Thailand.

Reprint requests: Harald Noedl, Department of Specific Prophylaxis and Tropical Medicine, Institute of Pathophysiology, Vienna Medical School, Kinderspitalgasse 15, A-1095, Vienna, Austria; Telephone: 43-1-4277-64882, Fax: 43-1-403-854390, E-mail: harald.noedl@univie.ac.at.

REFERENCES