Comparison of WHO Mark III and HRP II ELISA for in vitro sensitivity of Plasmodium falciparum

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ABSTRACT

Background & objectives: Antimalarial drug resistance is a serious challenge to malaria control worldwide. In vitro sensitivity assays provide an early indication of emerging drug resistance. In vitro susceptibility of field and culture adapted Plasmodium falciparum isolates to different antimalarials was compared using two methods: World Health Organization (WHO) micro-test (MARK III) and histidine rich protein II (HRP II) based enzyme-linked immunosorbent assay (ELISA).

Methods: In total, 50 P. falciparum isolates were collected from five states, viz. Chhattisgarh, Meghalaya, Mizoram, Tripura and Odisha of India during December 2011-September 2014. The isolates were revived and evaluated for their susceptibility to chloroquine (CQ), monodesethylamodiaquine (AQ), mefloquine (MQ), quinine (QN) and artemisinin (ART) using the WHO micro-test (Mark III) and HRP II ELISA. The data were analyzed using non-linear regression analysis.

Results: The geometric mean (GM) IC₅₀ values of different antimalarials for WHO Mark III assay were comparatively lower than HRP II ELISA assay. The GM IC₅₀ value for CQ was 59.5 nM (95% confidence interval [CI]: 49.35–71.73 nM) and 78.34 nM (95% CI: 64.57–95.03 nM) for Mark III and HRP II ELISA, respectively. Similarly, the values of GM IC₅₀ for AQ, MQ, QN and ART by Mark III and HRP II ELISA were 13.31, 7.07, 146.4, 0.43 nM and 22.02, 11.46, 258.7, 1.00 nM, respectively. On analyzing statistically, the results of both assays were comparable (R² = 0.96, p < 0.001; mean log difference at IC₅₀ = 0.037).

Interpretation & conclusion: The HRP II ELISA assay showed a reliable sensitivity in comparison to WHO Mark III micro-test complemented with distinguishing features such as high specificity, ease of performance, and notable consistency.

Key words Drug sensitivity; HRP II ELISA assay; in vitro; malaria; Plasmodium falciparum; WHO Mark III assay
The antigen is expressed only by *P. falciparum* trophozoites. The stability of HRP II protein is major advantage for *in vitro* drug susceptibility assays. The amount of HRP II secreted varies between parasite strains. The amount of HRP II found in culture samples is directly related parasite growth and development. The HRP II assay practices a longer culture incubation time (72 h instead 48 h) than most other assays which give advantage of testing the slow metabolizing drugs with no change in the procedure. A simple, commercially available double-site sandwich, ELISA test kit can be used for the assessment of parasite growth and development simply by measuring the amount of HRP II produced. The simplicity of use and low resource settings has made ELISA-based *in vitro* tests a successful tool for antimalarial susceptibility testing.

The aim of this study was to assess the *in vitro* susceptibilities of field and culture adapted *P. falciparum* isolates to different antimalarials through two widely used *in vitro* assays, viz. modified WHO Mark III micro-test and HRP II ELISA test. These *in vitro* studies may be very helpful in evaluating the national guideline for malaria treatment.

**MATERIAL & METHODS**

**Study sites & parasites collection**

In total 50 *P. falciparum* isolates were collected from five states of India [Chhattisgarh (n = 10), Meghalaya (n = 6), Mizoram (n = 4), Odisha (n = 10), and Tripura (n = 10)] from December 2011 to September 2014. These samples also included culture adapted samples (n = 10) from the Malaria Parasite Bank of National Institute of Malaria Research (NIMR), New Delhi.

**Ethical consideration**

This work is a part of the project “Characterization of Indian *P. falciparum* isolates with special reference to *in vitro* susceptibilities and molecular markers for antimalarials resistance”, which has been approved by the ethical committee of NIMR, New Delhi.

**Processing of the samples**

The collected and cultured samples were revived *in vitro* using Trager and Jensen method in RPMI (Roswell Park Memorial Institute)-1640 medium. The parasite isolates were revived and maintained in culture for 7–8 cycles.

**WHO Mark III micro-test**

The sensitivity of Indian *P. falciparum* isolates to different antimalarials was assessed by using a modified version of the standard WHO Mark III micro-test. *In vitro* assays were carried out on samples with parasitaemia >0.3% by microscopy. The initial parasitaemia was adjusted to 0.3–1% when required, by the addition of fresh uninfected erythrocytes. Microtitre plates were coated with five different antimalarials in concentrations recommended by WHO, viz. chloroquine (CQ) = 20–1280 ng/ml, amodiaquine (AQ) = 5–320 ng/ml, mefloquine (MQ) = 2.5–160 ng/ml, quinine (QN) = 50–3200 ng/ml, and artemisinin (ART) = 0.3–16 ng/ml. Each well of a 96-well tissue culture plate was filled with 90 µl drug solution of appropriate concentration plus 10 µl parasite suspension of about 5% haematocrit. Control wells with parasitized blood contained no drug. The plates were incubated at 37°C in CO₂ incubator having a gas mixture of 90% N₂, 5% CO₂ and 5% O₂ for 25–30 h. At the end of the incubation period, suspended medium was removed while the blood within each well was used to make thick smears on a microscope glass slide. These were air-dried, stained with JSB (Jaswant Singh-Bhattacharji) stain and examined under microscope at 100 × magnification. The number of schizonts with three or more nuclei against 200 asexual parasites was counted for each sample. All the tests were carried out in duplicate.

**HRP II ELISA**

ELISA plates were dosed with appropriate concentrations of antimalarials same as done for WHO Mark III test. The cell medium mixture (CMM) was prepared by adding 0.94 ml of the parasitized blood sample to 24.06 ml of RPMI-1640 medium in a sterile disposable tube. The pre-dosed plates were loaded with 200 µl of the resulting CMM to each well (starting with well A and proceeding to higher drug concentrations). The plates were incubated for 72 h at 37°C in CO₂ incubator. After incubation the plates were further processed immediately or stored below –20°C. After 72 h, another slide was prepared to determine parasitaemia. Four to 10 fold increase in parasite density within 72 h was considered as adequate growth. The plates were removed from the incubator and separately transferred into a freezer, and kept there until all wells were completely frozen (preferably overnight). The plates were thawed. Primary IgM antibody (MPFM-55A, Immunology Consultants Laboratories, Inc, Newberg, OR, USA) was diluted to 1 µl/ml in phosphate buffer saline (PBS); 100 µl of the diluted antibody was transferred to each well of the ELISA plate using a multichannel pipette. The plates were sealed and incubated at 4°C overnight. Contents of the ELISA plate were
discarded and the plate was dried by gentle tapping. About 200 µl of blocking solution (2% bovine serum albumin in PBS) was added to each well followed by incubation of the plates at room temperature for 2 h. The content was discarded and the plate was dried. The plates were washed three times with PBS-Tween 20 (0.05%) washing solution, sealed air-tight in plastic bag and freezeed at −20°C.

In total, 100 µl of sample from the culture plate was transferred to the ELISA plate. Plate was incubated for 1 h at room temperature in humid chamber, washed three times in PBS/Tween (200 µl/well) and banged dry. Secondary antibody (IgG) (MPFG-55P, Immunology Consultants Laboratories, Inc. Newberg, OR, USA) was diluted to 0.2 µg/ml in 2% BSA and 1% Tween 20 and 100 µl of the same was transferred to the ELISA plate. It was incubated for 1 h at room temperature in humid chamber, washed three times in PBS/Tween and banged to dry. Then 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogen (Ameresco, US) (100 µl) was added and plates were incubated for 5–10 min at room temperature in dark. The reaction was stopped by adding 50 µl of 1 M sulphuric acid and absorbance was read using an ELISA plate reader (Spectrostar Nano, BMG LABTECH, Germany) at 450 nM.

**Data management and statistical analysis**

The drug concentration that inhibits schizogony by 50% (IC$_{50}$) relative to the drug-free control samples of each *P. falciparum* isolate for both in vitro assays was estimated from dose-response curves by non-linear regression analysis using HN-NonLin software v1.11. The IC$_{50}$ cut-off values for determining sensitivity to antimalarials based on the WHO micro-test protocol were: 160 nM/l for CQ, 80 nM/l for AQ, MQ, and 800 nM/l for QN. The cut-off IC$_{50}$ value of 10.5 nM/l for artesunate was based on a previous study, as WHO protocol does not recommend cut-off for artemisinin. The confidence interval (CI) at 0.05 significance level was calculated by GraphPad Prism software v 5.0 package (GraphPad Software, San Diego, CA, USA).

Individual inhibitory concentrations (IC$_{50}$, IC$_{90}$ and IC$_{99}$) for both the assays were determined by non-linear regression analysis. Bland-Altman plots were performed to assess agreement between two methods. Standard correlation analysis was used to establish linear association between inhibitory concentrations obtained by two assays with selected drugs. Non-parametric procedures or log transformations were used for data that were not normally distributed.

**RESULTS**

**WHO Mark III result**

Among the field and culture isolates, only 10% were found resistant to CQ with geometric mean (GM) IC$_{50}$ value of 59.5 against the cut-off value of 160 nM. All the isolates were found sensitive for— AQ with the GMIC$_{50}$ value of 13.3 nM against 80 nM, MQ with GMIC$_{50}$ of 7.07 nM against 649 nM, QN with 146.4 nM against 800 nM and ART with 0.43 nM against 10.5 nM.

**HRP II ELISA result**

The HRP II assay revealed that 18% of field and culture isolates were resistant to CQ with GMIC$_{50}$ value of 78.34 against the cut-off value of 160 nM. For AQ, 8% of the isolates were resistant with the GMIC$_{50}$ value of 22.02 nM against 80 nM cut-off. Similarly 4% of the isolates were resistant to QN with GMIC$_{50}$ value 258.7 with respect to the cut-off value of 800 nM. None of the isolates were found to be resistant to MQ and ART as all the IC$_{50}$ values were below the cut-off value of 640 nM and 10.5 nM, respectively.

**Comparative results of two in vitro assays**

The GMIC$_{50}$ for 50 isolates in the modified WHO Mark III assay was 59.5 nM (95% confidence interval [CI] : 49.35–71.73 nM) for CQ whereas the corresponding result in HRP II ELISA assay was 78.34 nM (95% CI = 64.57–95.03 nM). Similarly, for AQ, the GMIC$_{50}$ by WHO Mark III assay was 13.31 nM (95% CI: 11.06–16.02 nM) which is slightly higher than the 22.02 nM (95% CI: 18.03–26.9 nM) by HRP II ELISA assay. The WHO Mark III assay result of GMIC$_{50}$ for MQ was 7.07 nM (95% CI = 5.93–8.43 nM) in comparison to 11.46 nM (95% CI: 9.44–13.9 nM) obtained by HRP II ELISA result. Also, the result of GMIC$_{50}$ was higher for QN, i.e. 258.7 nM (95% CI: 207.6–322.4 nM) by HRP II ELISA assay than the WHO Mark III assay result, i.e. 146.4 nM (95% CI: 124.3–172.6 nM). A nominal difference was observed in the GM for ART in both the assays, i.e. 0.43 nM (95% CI: 0.37–0.50 nM) versus 1.00 nM (95% CI: 0.81–1.23 nM) for WHO Mark III assay and HRP II ELISA assay, respectively (Table 1 and Fig. 1).

The correlation analysis results obtained by the HRP II ELISA drug sensitivity tests with all five antimalarials showed a highly significant linear association with those obtained by the WHO Mark III assay at the IC$_{50}$ level (RIC$_{50}$ = 0.94, R$^2$ = 0.92, p < 0.001), as well as at IC$_{90}$ level (RIC$_{90}$ = 0.96, R$^2$ = 0.94, p < 0.001) (Fig. 2). When correlating the results obtained with both the assays for
individual drugs, the correlation coefficients at the IC\textsubscript{50} level were $R = 0.52$ ($R^2 = 0.34$, $p < 0.001$) for CQ, $R = 0.73$ ($R^2 = 0.53$, $p < 0.001$) for AQ, $R = 0.82$ ($R^2 = 0.76$, $p < 0.001$) for QN, $R = 0.72$ ($R^2 = 0.65$, $p < 0.001$) for MQ, and $R = 0.98$ ($R^2 = 0.89$, $p < 0.001$) for ART, respectively. The mean difference for CQ, AQ, MQ, QN and ART, determined by the modified WHO Mark III assay and HRP II ELISA assay was in limits of agreement, i.e. 148.3 and 100.6 for CQ, 58.3 and 34.4 for AQ, 29.4 and 17.2 for MQ, 781.4 and 425.3 for QN, and 3.4 and 1.7 for ART at the IC\textsubscript{50} level. The agreement of the results (mean difference and limits of agreement) obtained by both the assays are plotted as Bland-Altman plots shown in Fig. 3.

**DISCUSSION**

The monitoring and prevention of drug resistance have become highly significant than ever before, as malaria parasites have advanced their approaches to escape almost any existing antimalarial treatment. *In vitro* sensitivity assays are simple and effective methods for surveillance of antimalarial drug resistance. The key factors for the success of drug resistance assays are high sensitivity, together with ease of implementation and execution. These can detect even a minute change in the drug.
Fig. 3: Bland-Altman plot of the difference in log IC₅₀ values for: (a) chloroquine (CQ); (b) amodiaquine (AQ); (c) mefloquine (MQ); (d) quinine (QN); and (e) dihydroartemisinin (ART), determined by the modified WHO Mark III micro-test and HRP II assay plotted against their mean values.
sensitivity pattern of the isolates of particular areas and can produce an early sign of change in the parasite’s susceptibility pattern even before it is detected clinically. In this study, the drug sensitivity pattern of five antimalarials in field and culture isolates of *P. falciparum* from diverse regions of India was evaluated by two *in vitro* methods namely, modified WHO Mark III assay and HRP II ELISA assay.

When both assays were performed under the same conditions, results of HRP II field assay were close to those obtained with the WHO assay, similar to the results of another study\(^\text{14}\). Additionally, HRP II drug sensitivity assay combines the advantages of the both *in vitro* assays, although it overlooks most of their disadvantages (Table 2). Further, the HRP II assay was easier, less labour intensive and faster to perform than the WHO Mark III assay. Commercial ELISA kits availability for the quantification of HRP II protein of *P. falciparum*, eliminates the necessity for standardization of the ELISA process, thereby making its execution faster than that of any other assay.

The results of the HRP II assay are analogous to those obtained by the modified WHO schizont maturation assays, although both methods used different end points. The shared component of both the methods is that they deal with parasite growth and development. Due to the unique features of every assay, it is not possible to perform all assays in the same condition. The modified WHO method is based on schizont maturation; it gives the estimate of a number of parasites which develops from ring stage (early trophozoite) to schizont. The assay is considered as more sensitive as it is based on the microscopic evaluation\(^\text{5}\). Also, it is more economical than the HRP II assay; however, it is more labour demanding and requires highly trained personnel to overcome the experimental inaccuracy caused by human errors.

On the other hand, HRP II assay measures the rise in HRP II levels with respect to gradually increasing incubation time, marked by the early schizont and trophozoite stages of the parasite\(^\text{8}\). ELISA assays are sensitive but they need minimum incubation time as in the case of HRP II, and an increase in the protein level directly gives measure of parasitaemia.

The comparable results obtained by both the assays reveal the consistency and reliabilities of *in vitro* assays for testing antimalarial susceptibilities. The benefits of using HRP II protein is its stability which results in the ability of this protein to be detected even when there is a small difference in parasite growth\(^\text{6}\). Although, the cost of performing ELISA test is little higher than WHO *in vitro* assay, it provides options for its execution with any of the HRP II specific ELISA for *P. falciparum*. Now-a-days, HRP II monoclonal antibodies are available in the market at very competitive price which can be used widely for the assay. The commercial kits have plus points like the ease of performance with standardized parameters, high sensitivity and reproducibility of assay.

Furthermore, to its prospective value, both as an implement for drug resistance surveillance and as a screening tool for new antimalarials, numerous added practices for the assay are under concern, such as, to evaluate the potential pharmacodynamics of antimalarial drugs, or to measure the quantity of antimalarials in the blood post-intake of drug. Alternative potential use of these *in vitro* assays is the analysis of the inhibitory activities of specimens which are potential vaccine candidates\(^\text{6}\).

### Table 2. Comparison of WHO Mark III (micro-test) assay and HRP II ELISA assay

<table>
<thead>
<tr>
<th>Factors/Characteristics</th>
<th>WHO Mark III assay</th>
<th>HRP II ELISA assay</th>
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<tbody>
<tr>
<td>Sample handling time</td>
<td>~ 24–36 h</td>
<td>~ 72 h</td>
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<tr>
<td>Ease of use</td>
<td>Labor intensive(^\text{5})</td>
<td>Comparatively less labour intensive(^\text{5})</td>
</tr>
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<td>Cost effectiveness</td>
<td>Low cost</td>
<td>High cost</td>
</tr>
<tr>
<td>Interpretation of result</td>
<td>It is subjective (depends on efficiency of microscopist)(^\text{6})</td>
<td>Colorimeter gives result</td>
</tr>
<tr>
<td>Availability of commercial kit</td>
<td>Not available</td>
<td>Available in variant of HRP II protein(^\text{6})</td>
</tr>
<tr>
<td>Accuracy of result</td>
<td>Not so accurate</td>
<td>HRP II protein is stable so result is more accurate</td>
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<tr>
<td>Equipment required</td>
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<td>Microscope and ELISA reader (Colorimeter)</td>
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<tr>
<td>Utility of assay for slow metabolizing drugs</td>
<td>Not helpful</td>
<td>Useful as incubation time is longer</td>
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<tr>
<td>Sensitivity</td>
<td>Comparatively lower than HRP II ELISA assay</td>
<td>High sensitivity(^\text{9})</td>
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</table>
CONCLUSION

Conclusively, the HRP II assay was found simple to operate, less labour intensive and result reading was comparatively faster. Besides being user friendly the sensitivity of this method was found to be better than WHO Mark III.

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Conflict of interest

The authors declare no conflict of interest.

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