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Simple Histidine-Rich Protein 2 Double-Site Sandwich Enzyme-Linked Immunosorbent Assay for Use in Malaria Drug Sensitivity Testing

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A simple double-site sandwich enzyme-linked immunosorbent assay (ELISA) for *Plasmodium falciparum* in vitro drug sensitivity tests based on measuring histidine-rich protein 2 (HRP2) is presented. The ELISA uses two commercial monoclonal antibodies and provides a drastically cheaper alternative to the test kits previously used in the HRP2 drug sensitivity test. The assay is simple to establish and perform. The sensitivity is comparable and the drug sensitivity results very closely match those obtained with the commercial ELISA kits (R^2 = 0.979; P < 0.001; mean log difference at the 50% inhibitory concentration = 0.07).

Malaria in vitro drug sensitivity assays are largely based on measuring parasite growth in cultures exposed to different antimalarial drug concentrations. Enzyme-linked immunosorbent assays (ELISAs) can provide very sensitive measures of parasite growth by quantifying biomolecules produced during parasite development (1), such as histidine-rich protein 2 (HRP2) or parasite lactate dehydrogenase (3, 4). Although very convenient and user friendly, commercial test kits are relatively expensive. We therefore sought to develop a generic antigen capture HRP2 ELISA in order to drastically reduce the cost of the HRP2 malaria drug sensitivity assay.

Study samples. The *Plasmodium falciparum* culture samples used for validating the new ELISA originated from 18 symptomatic adult outpatients at the malaria clinics in Mae Sot, So Oh, and Chedi Koh in Tak province, western Thailand. The study protocols were approved by the appropriate ethical review boards, and written informed consent was obtained from all study participants. The parasite densities ranged from 0.01 to 0.95% of infected red blood cells (geometric mean, 0.27%).

Culture. The culturing was performed as previously described (5). In brief, the fresh *P. falciparum* parasite isolates were cultured in the presence of serial dilutions of antimalarial drugs (dihydroartemisinin [DHA], mefloquine [MEF], quinine [QNN], and chloroquine [CQ]) at 1.5% hematocrit in RPMI 1640 with 0.5% Albumax I (Gibco, Bangkok, Thailand) without freezing, washing, diluting, adding serum, or preculturing. After 72 h of culturing, the plates were frozen and stored at −20°C.

Antibodies. Two commercial monoclonal antibodies (Immunology Consultants Laboratory Inc., Newberg, OR) directed against *P. falciparum*-specific HRP2 were employed: MPFM-45A (MPFM-55A), an immunoglobulin M (IgM) antibody used as the capture antibody, and MPFG-45P (MPFG-55P), a horseradish peroxidase-conjugated IgG antibody used as the indicator antibody.

HRP2 double-site antigen capture ELISA. High-binding 96-well ELISA plates (Costar 3590; Corning Inc., NY) were coated with 100 μl/well of a 1.0-μg/ml solution of anti-HRP2 IgM antibody solution (MPFM-45A) in phosphate-buffered saline (PBS). Then, the plates were sealed and incubated overnight at 4°C. The supernatant was discarded, and plates were saturated for 2 h with 200 μl/well of a 2% bovine serum albumin (Sigma-Aldrich, A9647) solution in PBS. The well supernatant was again discarded, and plates were washed three times with 200 μl/well of PBS-Tween washing solution (0.05% Tween 20 in PBS). The plates were then sealed and stored at or below −20°C.

The cultured samples were hemolyzed by freeze-thawing at least twice, and samples with initial parasite densities of >0.1% were diluted directly on the precoated ELISA plates with distilled water to equivalents of approximately 0.05% starting parasitemia (potential testing range for the ELISA, <0.01 to approximately 0.1%). This was done by first adding distilled water to the ELISA plates and then transferring and carefully mixing the hemolyzed cell-medium mixture in each well using a multichannel pipette. The ELISA plates were then incubated

TABLE 1. Individual IC_{50}s and IC_{90}s for DHA, MEF, QNN, and CQ in ng/ml measured using the new HRP2 ELISA and the CELISA test kits

Test	No. of samples	Result for:							
		DHA		MEF		QNN		CQ	
		IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
New ELISA	18	1.03	2.79	24.94	77.33	120.51	321.24	79.84	210.45
CELISA	18	1.18	2.86	31.33	81.45	138.03	325.07	89.93	217.84
Difference[a]		NS	NS	NS	NS	NS	NS	NS	NS

[a] Significances by Mann-Whitney U tests are given for the differences in inhibitory concentrations measured with the two ELISAs (NS, not significant; $P > 0.05$).

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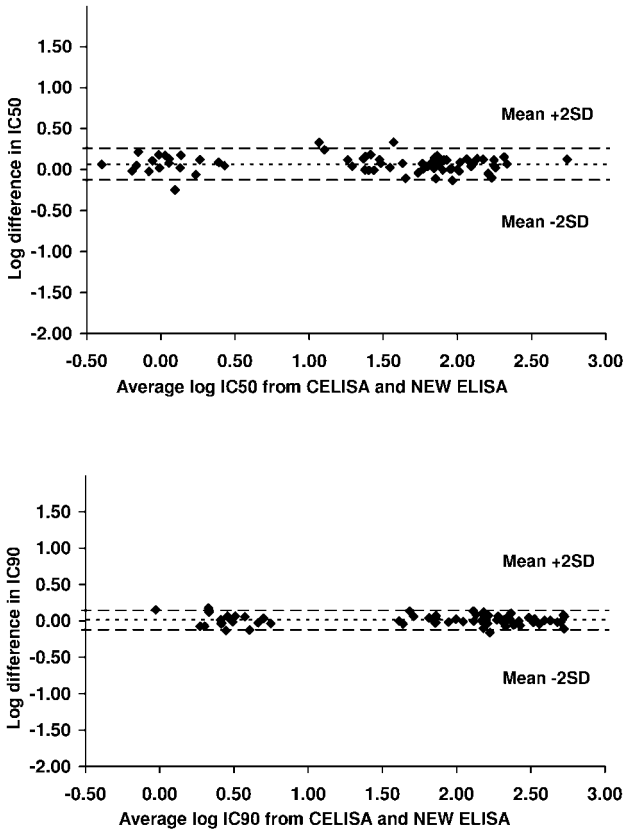

FIG. 1. Bland-Altman plots for the agreement of pooled 50% (upper panel) and 90% (lower panel) inhibitory concentrations for DHA, MEF, QNN, and CQ determined by the HRP2 drug sensitivity assay using the new HRP2 ELISA and CELISA kits (mean difference on the log scale at the IC_{50} was 0.07, with limits of agreement of −0.13 and 0.26; at the IC_{90}, the mean difference on the log scale was 0.01, with limits of agreement of −0.12 and 0.15). SD, standard deviation.

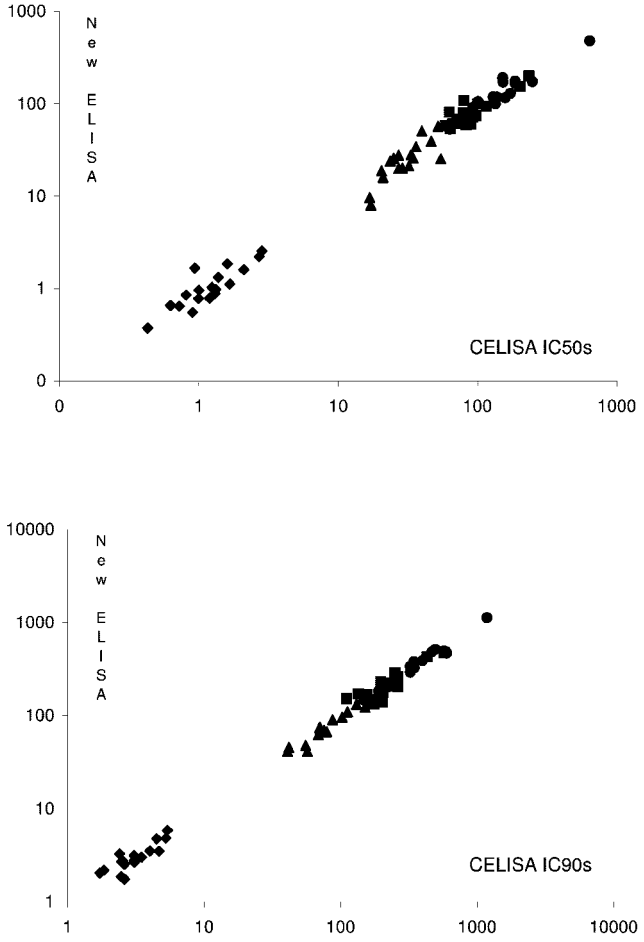

FIG. 2. Scatter plots for the linear associations of pooled 50% (upper panel) and 90% (lower panel) inhibitory concentrations in ng/ml for DHA (diamonds), MEF (triangles), QNN (circles), and CQ (squares) determined by the HRP2 drug sensitivity assay using the new HRP2 ELISA and the CELISA kits ($n = 72$; $R_{IC50} = 0.981$; $R_{IC90} = 0.985$; $P < 0.001$).

with the diluted samples for 1 h at room temperature and washed three times with PBS-Tween washing solution.

The antibody conjugate (MPFG-45P) was diluted in a solution of 2% bovine serum albumin and 1% Tween 20 in PBS to 0.05 μg/ml. Because the activity of the conjugate may vary from batch to batch, every new batch was titrated to establish the best concentrations (approximately 0.05 to 0.5 μg/ml). After the addition of 100 μl of the diluted conjugate to each well, the plates were incubated for another hour at room temperature and washed three times with PBS-Tween solution.

One hundred μl of 3,3′,5,5′-tetramethylbenzidine (tetramethylbenzidine single-solution chromogen; Zymed Lab. Inc., San Francisco, CA) was added to each well, and the plates were incubated in the dark for 5 to 10 min. The reaction was stopped with 50 μl of 1 M sulfuric acid. Spectrophotometric analysis was performed at 450 nm using an ELISA plate reader (SpectraMAX 340 Microplate spectrophotometer; Molecular Devices, Sunnyvale, CA).

HRP2 CELISA. The samples from the same culture plates were also tested with a commercial HRP2 ELISA test kit (Malaria Ag CELISA; Cellabs Pty. Ltd., Brookvale, New South Wales, Australia) to quantify the HRP2 in the culture samples as described previously (4, 5).

Individual 50 and 90% inhibitory concentrations (IC_{50}s and IC_{90}s) were calculated by nonlinear regression analysis using the lowest value in each test as the background. Standard operating procedures and updated information for the new HRP2 ELISA are available at http://malaria.farch.net.

Results. In order to assess the performance of this new ELISA for in vitro drug sensitivity testing, 18 fresh *P. falciparum* isolates were tested with the above-described HRP2 assay using the new ELISA, and the results were compared to results obtained when using commercial HRP2 ELISA test kits. The geometric mean inhibitory concentrations measured by both ELISAs are shown in Table 1. The IC_{50}s for the new ELISA were generally slightly lower than those obtained with the CELISA kits. In Mann-Whitney U tests, no significant differences were found (Table 1).

In nonparametric correlation analysis and Bland-Altman plots, the results for both ELISAs were closely related. The mean differences on a log scale for the IC_{50}s and IC_{90}s obtained using the two ELISAs were 0.07 (limits of agreement,

−0.13 and 0.26) and 0.01 (limits of agreement, −0.12 and 0.15), respectively (Fig. 1), suggesting excellent concordance. In correlation analysis, the pooled results obtained with the new ELISA showed distinct linear association with those obtained using the test kits ($n = 72$; R_{IC50} [correlation coefficient at IC_{50}] = 0.981; $R_{IC90} = 0.985$; $P < 0.001$) (Fig. 2).

Conclusions. The new ELISA provides a convenient and economical alternative to the previously used test kits. It takes little more effort or time, but, unlike the test kits, it may require basic knowledge in setting up an ELISA. The sensitivity of the new ELISA was comparable to that of the previously used CELISA kits permitting the testing of parasite densities as low as 0.002%. The HRP2 drug sensitivity assay, therefore, is sensitive enough to allow the use of virtually any fresh isolate without regard to parasite density and can be used in the HRP2 assay without requiring any changes to the previously published culture procedures (5).

In fact, the dynamic range (i.e., the ability to measure the antigen over a biologically relevant assay range) of the new ELISA was found to be somewhat wider than that of the test kits, thereby consistently leading to slightly flatter dose-response curves. This resulted in minimally lower IC_{50}s, even though the IC_{90}s were almost identical. However, none of the differences between IC_{50}s or IC_{90}s obtained using the two assays were significant at this sample size, either for individual drugs or for the pooled results.

From our experience, the new ELISA achieves our original goal of providing a cost-effective alternative to the commercial HRP2 ELISA kits, thereby reducing the cost of the ELISA for the HRP2 drug sensitivity assay by more than 80% to around $10 U.S. per plate. This development will make HRP2-based in vitro drug sensitivity testing more affordable to researchers in countries most affected by malaria and considerably cheaper than traditional radioisotope-based drug sensitivity assays (6, 2). Although it is slightly more labor-intensive than the test kits, we conclude that it provides a cost-effective and convenient alternative to commercially available test kits for use in the HRP2 assay without requiring any changes to the culture procedures.

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