Short Report: Large Variation in Detection of Histidine-Rich Protein 2 in *Plasmodium falciparum* Isolates from Colombia

Zuleima Pava,* Diego F. Echeverry, Gustavo Díaz, and Claribel Murillo*

Centro Internacional de Entrenamiento e Investigaciones Médicas, Cali, Colombia

**Abstract.** Most rapid diagnostic tests (RDTs) available use histidine-rich protein 2 (HRP2) as a target. However, it has been reported that sequence variations of this protein affects its sensitivity. Currently, there is insufficient evidence for HRP2 variability in *Plasmodium falciparum* isolates from Colombia and its relationship with RDT performance. To determine possible geographic differences and their effects on the performance of RDTs, 22 blood samples from patients with *P. falciparum* malaria from Tumaco and Buenaventura, Colombia were assessed by measurement of HRP2 concentration by an HRP2 enzyme-linked immunosorbent assay, RDTs, and thick blood smear. Statistical analysis showed an association between RDT performance and HRP2 concentrations. No significant difference was found between locations. A large variation of antigen concentration in samples was found at same parasitemia. In contrast to previously reports, there was no correlation between initial parasitemia and HRP2 concentration. Our results indicate that antigen quantity should be studied more carefully because the sensitivity of the RDT is affected more by antigen concentration than by parasitemia.

In Colombia, more than half of the population is at risk for malaria. In 2007, 110,389 malaria cases were reported, 19 of them fatal. Most of the fatal cases occurred because of complications caused by delays in diagnosis and treatment. Therefore, one of the principal objectives of malaria control strategies recommended by the World Health Organization (WHO) is to improve access to diagnosis and effective treatment.

Currently, the gold standard diagnosis method for malaria is based on identification of the parasite in Giemsa-stained blood smears by using microscopy. Its use in remote malaria-endemic areas is restricted by a lack of qualified personal and basic infrastructure. Such drawbacks have led to development of simpler diagnostic strategies, including rapid diagnostic tests (RDTs). Currently, malaria RDTs are widely used in countries in Africa, and their use is spreading in countries in South America, such as Peru, where much progress has been made in malaria control.

The RDTs are immunochromatographic tests that detect specific parasite antigens through antibody capture. The main antigens detected by RDTs are lactate dehydrogenase, aldolase (panspecific, which is present in all *Plasmodium* species), and histidine-rich protein 2 (HRP2), which is specific for *Plasmodium falciparum*. Most RDTs available use HRP2 as a target. It has been reported that sequence variations of this antigen, particularly the type and number of specific amino acid repeats, can affect the sensitivity of HRP2-based RDTs at low parasitaemias. Currently, there is insufficient evidence about HRP2 variability in isolates from Colombia and its relationship with RDT performance.

The purpose of this study was to evaluate the amount of HRP2 in *P. falciparum* isolates from two malaria-endemic cities on the Pacific coast of Colombia to determine changes between locations and their effects on RDT performance. Symptomatic patients with a *P. falciparum*-positive thick smear were enrolled in the study if they had a parasitemia ≥ 3,000 parasites/μL, had not taken antimalarial drugs in the last two months (negative by the Saker-Solomon test), and provided a signed informed consent that was approved by the Centro Internacional de Entrenamiento e Investigaciones Médicas (Cali, Colombia) institutional review board.

We evaluated *P. falciparum* blood samples from Buenaventura (Valle Department) and Tumaco (Nariño Department), which are cities located on the Pacific coast of Colombia. The 12 samples collected in Buenaventura were independently analyzed and reported as part of a larger multi-center collaboration between the Foundation for Innovative New Diagnostics and WHO. Differences in protein expression levels were determined by measurement of HRP2 concentration using an enzyme-linked immunosorbent assay (ELISA) for HRP2. Statistical analyses were conducted to determine the association between geographic origin and parasitemia with HRP2 concentrations. Additionally, malaria RDTs were performed with a dilution of the patient blood to determine sensitivity.

Five milliliters of venous blood was collected into tubes containing EDTA, and two thick blood smears were prepared. Parasitemia was determined by two expert microscopists at Centro Internacional de Entrenamiento e Investigaciones Médicas, and the mean value was calculated. A third microscopist was consulted if the variation coefficient between counting was > 20%. Blood was mixed for thirty minutes, and each sample was diluted to a parasitemia of 200 parasites/μL using blood from healthy donors, according to the WHO–West Pacific Regional Office protocol for preparing quality-control samples of malaria RDTs. The HRP2 ELISA and RDT were used for testing the dilutions containing 200 parasites/μL. This parasite density enables interpretable and comparable antigen quantification from all samples, and is close to the RDT detection limit of 100 parasites/μL recommended by past WHO consultations in 1999 and 2003. Parasitemias of the dilutions were corroborated by using the Earle-Pérez thick blood film method and thin blood films.

The RDT (Parahit® Span Diagnostics Ltd., Surat, India) was performed immediately after dilutions were made according to the manufacturer’s specifications. The RDT results were classified into three groups based on intensities of the test lines and using the control line intensity as reference: one cross, two crosses, and three crosses if the test line intensity was weaker.
the same, or stronger than the control line, respectively. The RDT results were considered negative if only the control line was seen, and they were invalidated and repeated when no line was shown. After RDT reading, the dilutions were stored at \(-20^\circ C\) until ELISA was conducted.

The HRP2 concentrations were measured by using the Malaria Ag CELISA\textsuperscript{a} Kit (Cellabs, Sydney, New South Wales, Australia).\textsuperscript{7} A standard curve was established with a recombinant protein (\textit{P. falciparum} HRP2-HB3) kindly provided by Dr. David Sullivan (Johns Hopkins School of Public Health, Baltimore, MD) after confirming its stock concentration by using the technique of Bradford.\textsuperscript{11}

Ten serial dilutions were prepared from the protein stock by using phosphate-buffered saline–0.05% Tween 20 as diluent. Each dilution was tested in triplicate, and the experiment repeated 11 times. The negative control value was established by measuring the optical density (OD) of five non-infected venous blood samples, testing in duplicate, and calculating the mean ± OD (0.093 ± 0.004, maximum = 0.098, minimum = 0.090). For each sample, the dilution of 200 parasites/μL was tested in triplicate, and the concentrations were determined by comparison with a recombinant HRP2 standard curve run in parallel. Data were analyzed by using Stata Statistical Software version 9.2 (StataCorp, College Station, TX). A value > 0.05 was considered statistically significant.

During January–November 2008, we obtained 22 samples, 10 from Tumaco and 12 from Buenaventura. There was no statistically significant difference between parasitemias observed in patients from these two cities (\(P = 0.990\); geometric means = \(8,915\) parasites/μL and \(8,569\) parasites/μL, respectively).

The HRP2 standard curve had a large linearity range between 0.49 ng/mL and 31.5 ng/mL of HRP2 protein, with a positive correlation of R = 0.948 \((P = 0.001)\), which showed a strong linear relationship between HRP2 concentration and OD. Large HRP2 concentration variations were found in the 22 samples assessed at a concentration of 200 parasites/μL; values ranged from 1.6 ng/mL to 52.7 ng/mL of HRP2 (Figure 1). Two samples were additionally diluted to 1:2 with phosphate-buffered saline to estimate the HRP2 concentration because their ODs at a concentration of 200 parasites/μL was above the detection limit.

Three samples showed negative results or could not be detected by one or both detection methods when diluted to a concentration of 200 parasites/μL (Figure 2). One of these three samples (RDT negative and ELISA result below the detection limit of 0.49 ng/mL) had a relatively high initial parasitemia of 14,327 parasites/μL (Figure 2B).

The HRP2 concentrations observed in dilutions of 200 parasites/μL in samples from Tumaco were higher than in samples from Buenaventura (geometric mean = \(13.12\) ng/mL and \(8.86\ ng/mL\), respectively), but this difference was not statistically significant \((P = 0.331)\). All 10 samples from Tumaco had positive RDT results at a dilution of 200 parasites/μL; 50% of these samples had an RDT intensity of two crosses. In contrast, 2 of the 12 samples from Buenaventura at the same dilution showed negative results (Figure 2).

The RDT results of dilutions of 200 parasites/μL showed a correlation with the concentration of HRP2 at the same dilution (Figure 2A). The highest intensities in the RDT were obtained for samples with higher HRP2 concentrations. Nevertheless, the initial parasitemia was not correlated with RDT results of the dilutions of 200 parasites/μL (Figure 2B). This finding indicates that the intensity of RDT results reflects the amount of HRP2, but not necessarily the initial parasitemia.

The most striking result of this study is that there is a large variation of antigen concentration in the samples, even though all dilutions were to the same parasite density of 200 parasites/μL of blood. There are various potential explanations for this finding. One possibility is that polymorphism in the HRP2 sequences lead to differences in the HRP2 detection signal by the ELISA, and consequently in antigen concentration. Lee and others showed that genetic variability of HRP2 affects sensitivity of techniques based on the detection of this antigen by monoclonal antibodies (malaria RDTs, ELISA kits).\textsuperscript{6} Baker and others reported differences between geographically widespread parasites originating from various countries.\textsuperscript{12} However, the possible HRP2 sequence variations between wild-type parasites from the same country remain unknown. It would be interesting to compare \textit{P. falciparum} \textit{hrp2} gene sequences and copy number variation of collected parasite samples and include samples from other areas of the same country.

![Figure 1. Histidine-rich protein 2 of \textit{Plasmodium falciparum} isolates from Tumaco and Buenaventura, Colombia, at a concentration of 200 parasites/μL with respect to the initial parasitemia of the patient infections. A poor association was found between the two variables (correlation coefficient = 0.503, 95% confidence interval = 0.09–0.77, \(P = 0.703\)). Two samples could not be analyzed by an enzyme-linked immunosorbent assay.](image-url)
Furthermore, antigen concentration can depend on several characteristics of the infection because antigen accumulates during infection and varies in expression levels during the parasite life cycle. We also observed no clear correlation between initial parasite density of infection and *P. falciparum* HRP2 content in the dilutions containing 200 parasites/μL. Similarly, we found no correlation between the initial parasite density of infection and pfHRP2 content in the whole sample. These results differ from previous findings in parasites isolated from Thailand, where a high correlation was observed between parasitemias and protein concentrations. Our study also indicated a slight variation in HRP2 levels between study sites, although without statistical significance. A larger study would be necessary to assess this hypothesis.

Our results indicate that pfHRP2 sequences and their expression levels should be studied more carefully in various malaria-endemic areas of Colombia. Variations in both findings could have significant consequences on the performance of malaria RDTs.

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Authors’ address: Zuleima Pava, Diego F. Echeverry, Gustavo Díaz, and Claribel Murillo, Centro Internacional de Entrenamiento e Investigaciones Médicas, Carrera 125 No. 19-225, Cali, Colombia.

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