

Review

Why Continue Studying HRP2 and HRP3 in Malaria?

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Abstract: The main malaria control strategy is rapid and accurate diagnosis followed by effective treatment. The quality of the diagnosis is relevant, since an erroneous diagnosis can generate an increase in morbidity and mortality significantly. The World Health Organization (WHO) has recommended that all patients with suspected malaria be diagnosed by microscopy or a Rapid Diagnostic Test (RDT) before treatment. Microscopy and RDT are the main options for diagnosing malaria in the field and in remote areas of endemic countries. This is in line with pillar 1 of the elimination strategy "Achieve universal access to malaria prevention, diagnosis and treatment". Most of the RDTs available on the market detect an antigen from the parasite produced throughout the life cycle of *P. falciparum*; this corresponds to the Histidine-Rich Protein 2 (HRP2). In general, HRP2-based RDTs are more sensitive and stable than those based on other *Plasmodium* antigens, making them the best choice in most endemic countries where *P. falciparum* malaria predominates. In this review we present evidence on the genetic variability of PfHRP2 and 3 and its potential effect on the efficacy of rapid tests routinely used to diagnose malaria and as main methodological tool elimination programs worldwide.

Keywords: Malaria, *Plasmodium*, Diagnostic Test, Immunologic Tests

Introduction

In Colombia, malaria is considered a public health problem, it is estimated that at least 70% of the territory has adequate ecological conditions for the transmission of the disease; and in the Urabá - Bajo Cauca - Alto San Jorge; the Pacific coast and in the Orinoquia-Amazonia regions reported the highest index of the disease (Osorio *et al.*, 2004; Padilla *et al.*, 2011; 2015; Moreno *et al.*, 2017).

Table 1 presents the situation of malaria worldwide, in the Americas region and in Colombia.

Once the suspicion of malaria infection is established, it is essential to carry out a rapid, accurate and reliable diagnosis. In this way, the best treatment can be found and administered as soon as possible to avoid aggravation of the patient's symptoms.

The (World Health Organization, 2018) recommends Rapid Diagnostic Tests (RDTs) as a good alternative malaria-diagnosis method in remote parts and as part of the elimination strategy worldwide. The majority of commercial RDTs currently available detect the *Plasmodium falciparum* Histidine-Rich Proteins (HRPs).

HRP2-based tests have been preferred in areas where *P. falciparum* is predominant, due to a higher reported sensitivity (Houzé *et al.*, 2009).

Rapid Diagnostic Tests (RDTs)

RDTs for malaria are lateral flow immunochromatographic tests that detect specific circulating antigens produced by malaria parasites (Moody, 2002). There are at least 25 commercial RDT products on the market, some of which detect only *Plasmodium falciparum*, while others detect *P. falciparum* plus 1 or more other species of human *Plasmodium*. The most commonly targeted antigens are histidine-rich protein 2 (HRP2), which is specific for *Plasmodium falciparum*, *Plasmodium* Lactate Dehydrogenase (pLDH), which is present in all *Plasmodium* species. Currently available tests detect one or both antigens simultaneously (Houzé *et al.*, 2009) and there is also some evidence that RDTs that detect HRP or HRP/LDH are more heat stable than tests based on pLDH (Chiodini *et al.*, 2007) and aldolase only (Baker *et al.*, 2005).

Table 1: Situation of malaria worldwide, in the Americas region and in Colombia

Variable	Malaria in the world	Malaria in the Americas	Malaria in Colombia
Countries and regions affected by malaria	It affects more than 100 countries in the world and 40% of the world population in risk of contracting it. 86% of the world's cases are in sub-Saharan Africa	Endemic-epidemic in 21 countries. Most of the cases in Venezuela, Brasil, Colombia, Perú, Haiti y Guyana (Organización Mundial de la Salud, 2019).	Most of the cases occur in the departments of Chocó, Nariño, Córdoba, Antioquia, Norte de Santander (Instituto Nacional de Salud, 2019).
People at risk	4 billion people at risk	132 million people at risk (Organización Mundial de la Salud, 2019).	10 million people at risk (Organización Mundial de la Salud, 2019).
Number of cases on average by population and by <i>Plasmodium</i> species	2010-2018: Number of cases were reported on average (Organización Mundial de la Salud, 2019): Suspected 386,368,994 Indigenous: <i>P. vivax</i> 290,112 Indigenous: <i>P. falciparum</i> 75,990 Indigenous: Mixed 11,927 Indigenous: Other species 68 Total: <i>P. vivax</i> 1,742,687 Total: <i>P. falciparum</i> 35,513,717 Total: Mixed cases 310,815 Total: Other species 53,100	75% belongs to <i>P. vivax</i> malaria. 2010-2018: Were reported on average (Organización Mundial de la Salud, 2019): Suspected 7,152,884 Indigenous: <i>P. vivax</i> 287,554 Indigenous: <i>P. falciparum</i> 70,495 Indigenous: <i>P. falciparum</i> 70,495 Indigenous: Mixed 11,899 Indigenous: Other species 55 Total: <i>P. vivax</i> 9,184 Total: <i>P. falciparum</i> 36,992 Total: Mixed cases 2,471 Total: Other species 78	2019: 77,172 cases of uncomplicated malaria and 1,341 of complicated malaria (Instituto Nacional de Salud, 2019). <i>P. vivax</i> 48,5% <i>P. falciparum</i> 50,9% mixed cases 0,9% Population most affected by uncomplicated malaria Afro-Colombians (41%); Indigenous (24%); Miners (7%); foreigners (3%), while Afro-Colombians (21%) and Indigenous are the populations most affected by complicated malaria (Ministerio de Salud y Protección Social, 2020).
Total number of reported cases	2010-2018: An average of 230 million cases were reported (Organización Mundial de la Salud, 2019).	2010-2018: 686,000 cases were reported on average (Organización Mundial de la Salud, 2019).	2010-2019: Between 40,000 and 118,000 cases are reported per year (Instituto Nacional de Salud, 2017).
Average of Deaths	2010-2018: An average of 442 thousand deaths were reported (Organización Mundial de la Salud, 2019).	2010-2018: an average of 441 deaths were reported (Organización Mundial de la Salud, 2019).	Deaths: 2015 (15)/2016 (36) /2017 (19)/2018 (9)/2019 (3) (Instituto Nacional de Salud, 2019; Instituto Nacional de Salud, 2017).

It is relevant that the RDTs are stable, especially for regions in which there are high temperature and relative humidity conditions, which are typical conditions in many of the regions where there is malaria, so that these tests can be used and obtained reliable results. Moreover, compared with RDTs that detect pLDH, in general HRP2-detecting RDTs are more sensitive and are less susceptible to degradation from heat and humidity during transport and storage (World Health Organization, 2015b).

Many of these *P. falciparum* RDTs have been field-tested and different levels of sensitivity have been reported reporting that some RDTs have similar sensitivities to those commonly obtained by microscopy (~100 parasites/ μ L) (Beadle *et al.*, 1994; Garcia *et al.*, 1996; Palmer *et al.*, 1998). Furthermore, other studies have shown sensitivities well below the level required for operational use (Forney *et al.*, 2001; 2003; Gaye *et al.*, 1998; Huong *et al.*, 2002; Iqbal *et al.*, 2001; Jelinek *et al.*, 1999; Mason *et al.*, 2002; Rubio *et al.*, 2001; Stow *et al.*, 1999; Wongsrichanalai *et al.*, 1999). Similarly, there are

several studies that indicate little or low consistency in the results obtained for individual products or for the same product tested in different regions (Beadle *et al.*, 1994; Forney *et al.*, 2001; Gaye *et al.*, 1998; Jelinek *et al.*, 1999; Rubio *et al.*, 2001; Stow *et al.*, 1999; Wongsrichanalai *et al.*, 1999; Banchongaksorn *et al.*, 1997; Craig *et al.*, 2002; Mharakurwa *et al.*, 1997). Although there are reports of malaria RDTs that do not detect infections with high-level parasitaemia (Gaye *et al.*, 1998; Iqbal *et al.*, 2001; Stow *et al.*, 1999; Wongsrichanalai *et al.*, 1999; Birku *et al.*, 1999; Playford and Walker, 2002), most of the reported variation has been from relatively low-level parasitaemia (100-500 parasites/ μ L) (Beadle *et al.*, 1994; Huong *et al.*, 2002; Stow *et al.*, 1999; Bechem *et al.*, 1999; Iqbal *et al.*, 2002; Mills *et al.*, 1999; Playford and Walker, 2002; Proux *et al.*, 2001; Singh and Valecha, 2000), which generally results in clinical malaria in non-immune individuals, the causes of such variation being unknown (Baker *et al.*, 2005).

HRP2 and HRP3 are proteins routinely used in detection by Rapid Diagnostic Tests (RDT), test considered to be the most sensitive currently available and useful for the diagnosis of this disease in remote areas of endemic countries. The HRP2 concentration can be used to define severe malaria (plasma) and to quantify in vitro growth (laboratory culture) as well as to detect asymptomatic malaria in areas of low transmission, potentially allowing interventions at the public health level however, there have also been recent reports of *pfhrp2* gene deletions being found in parasites collected from several African countries, decreasing the effectiveness of these diagnostic tests (Poti *et al.*, 2020).

In vivo, the two antigens (HRP2 and pLDH) are cleared with different speeds, which appears to affect their specificities (notably, their ability to detect current infection) depending on the context. An evaluation in both high- and low-transmission areas in Uganda showed that median time for an HRP2 test to become negative after an effective treatment was 35-42 days, but that the median time to become negative for a pLDH test was only 2 days (Grandesso *et al.*, 2016). This likely explained the higher specificity of the pLDH test (94%) compared to HRP2 test (80%) in the high-transmission area, while specificities were similarly high (~99%) in the low-transmission area. The higher specificity of pLDH tests was also seen in hospitalized children in Burkina Faso (Maltha *et al.*, 2014). These findings have important implications in high-transmission environments, where interpretation of HRP2 tests can be difficult in the peak malaria season, leading to false positive results and hence unnecessary treatments (Coldiron *et al.*, 2019; Arróspide *et al.*, 2004; Moody, 2002).

Since microscopy is the best diagnostic alternative, RDTs should be considered as a second option, for endemic areas with fewer resources since, among others, they are very stable tests and their operation is not affected by high temperature or humidity conditions, conditions typical of the tropics, where there is a high prevalence of malaria. The ability to discern the *Plasmodium* species, in addition to being able to quantify the degree of parasitaemia make microscopy the most effective method, however, the simplicity and speed of RDTs are far from this gold standard: In just a few minutes, a staff with basic training can deposit the patient's blood drop in the device and make their diagnosis, on the other hand, to carry out microscopic techniques complex equipment, electricity and qualified personnel are required, which is not always easy in endemic third world countries (Barbadillo Ruiz, 2019).

HRP2 and HRP3 Proteins

HRP2 is a protein produced exclusively by *P. falciparum*; the gene that codes for this protein consists

of a single subtelomeric copy located on chromosome 8 (Scherf and Mattei, 1992; Fontecha *et al.*, 2019) of the *P. falciparum* genome, encoding an amino acid sequence containing 34% histidine, 37% alanine and 10% aspartic acid. PfHRP2 is a water-soluble of 60-105 kDa protein (the gene has a 1073 pb), synthesized and present throughout the asexual life cycle and identified as a surface protein. This protein is exported by the parasite into the Red Blood Cells (RBC) cytosol and is also found circulating in the peripheral blood of infected individuals (Howard *et al.*, 1986; Baker *et al.*, 2010). As parasites rupture from the host cell, RBC cytosolic components, including HRP2, are released into the bloodstream. In plasma, HRP2 can reach 100 µg/ml. Since its discovery (Wellems and Howard, 1986), many functions have been attributed to it, including hemozoin crystallization, actin formation, T cell suppression, glycosaminoglycan binding and procoagulation (Pal *et al.*, 2016; Sullivan *et al.*, 1996; Mashima *et al.*, 2002; Benedetti *et al.*, 2003; Ndonwi *et al.*, 2011).

HRP2 has a high degree of stability and it has been verified that there is a positive correlation between its concentration in blood and the biomass of the parasite. Three histidine-rich proteins have been identified, the most relevant for the diagnostic field being PfHRP-2 and PfHRP-3. The first one is present in all infected red blood cells, while the second one has in its sequence repeated regions rich in histidine that are recognized by monoclonal antibodies to RDT that detect PfHRP-2 (Barbadillo Ruiz, 2019; Muñoz *et al.*, 2015; Organización Mundial de la Salud, 2006).

PfHRP-2 is found in the digestive vacuole or in the cytoplasm of the parasite and plays a key role in the metabolism of *P. falciparum*. This protein actively facilitates the polymerization of the heme toxic group, resulting from the breakdown of the host hemoglobin, to form a malaria pigment, hemozoin, which is no longer toxic (Sullivan *et al.*, 1996; Barbadillo Ruiz, 2019).

PfHRP-2 is expressed in gametocytes and all the erythrocytic stages of *P. falciparum*. The protein is released after the breakdown of the schizont and is therefore found in the blood of individuals infected with these parasites. In this way, even if the sequestered forms of *P. falciparum* cannot be found by microscopy, the presence of PfHRP2 can be detected in blood and this will contribute to establishing a positive diagnosis (Hayward *et al.*, 2000).

HRP2 has been used as a biomarker for *P. falciparum* infection and forms the basis of many current rapid diagnostic tests (Dondorp *et al.*, 2005; Parra *et al.*, 1991). On postmortem analyses, HRP-2 has been observed to line the endothelial walls of blood vessels (Aikawa *et al.*, 1990). Several correlative studies showed an association between plasma HRP2 levels and disease severity or development of Cerebral Malaria (CM) (Dondorp *et al.*,

2005; Hendriksen *et al.*, 2013; Seydel *et al.*, 2012; Hendriksen *et al.*, 2012; Kariuki *et al.*, 2014). Natural populations of HRP2-deficient *Plasmodium falciparum* parasites exist, though these tend to be in areas of low CM incidence (Pal *et al.*, 2016; Gamboa *et al.*, 2010; Koita *et al.*, 2012; Kumar *et al.*, 2013).

The PfHRP-3 gene (Histidine-Rich Protein 3), encodes the Histidine-3-Rich Protein (HRP3), which is also known as the small histidine-rich protein, the gene is located within the non-telomeric region of chromosome 13. PfHRP3 is a 30-35 kDa polypeptide, containing 30% histidine and 29% alanine. In the main protein-coding region there are two regions that have tandem-repeating units, these two regions are separated by a 72-nucleotide region that does not contain repeating units. It is not clear what the role of this protein is in the survival of the parasite (Sharma, 1988).

PfHRP-3 shares many similarities in structure and in function with PfHRP-2. Both contain a peptide that functions as a signal in exon 1, exon 2 encodes 75-90 nucleotide sequences of the histidine-rich amino acid. Although the composition in histidine in PfHRP-3 is slightly less than that of PfHRP-2 (28% Vs. 34%), both proteins share many repeats rich in histidine and alanine. These similarities suggest that these two genes are related and could come from duplication of an ancestral gene. The sequence of PfHRP-2 and PfHRP-3 is highly variable, so could give rise to a significant variation in the presence and frequency of epitopes recognized by monoclonal antibodies and therefore the impact on detection sensitivity (Baker *et al.*, 2010).

Repeated histidine-rich repeats that are recognized by RDT monoclonal antibodies intended to detect PfHRP2 (Mouatcho and Goldring, 2013), are therefore useful for this purpose. However, immunochromatography devices that detect PfHRP2 have a number of limitations, for example, they are not capable of determining infections by other *Plasmodium* species, which limits the diagnostic possibilities. Second, RDTs that detect PfHRP2 are not helpful in monitoring antimalarial treatment. This is due to the persistence of the antigen in the peripheral blood circulation even after the elimination of the parasite, which can lead to false positives (Mayxay *et al.*, 2001; Barbadillo Ruiz, 2019).

Another additional problem is the fact that there are worldwide-distributed *P. falciparum* strains that express genetic variation in the amino acid sequence of the protein.

Genetic Variation of HRP Genes

Baker *et al.* (2010), studied possible genetic variations in HRP in 75 *P. falciparum* isolates from 19 countries. When performing the translation of amino acid sequences, PfHRP2 was observed that these sequences had consisted of variable numbers of repeats

of 9, 6 and 3 amino acids, identifying a total of 14 different amino acid repeats from the PfHRP2 sequences. The composition, number and order of repeat types in PfHRP2 differed between individual parasites (Anders *et al.*, 1986). 56 unique PfHRP2 sequences were detected; 43 were reported only once, while the remaining 13 sequences were seen in more than one isolated parasite; Nine of these 13 sequences were shared by isolates from the same country, while 4 were shared by isolates from different countries. All the different PfHRP2 sequences started with the type 1 repeat (AHHAHHVVD) and ended with the type 12 repeat (AHHAAAHEAATH). A relatively conserved repeat motif of types 7, 8, 2 and 7 described in this study was present in the central region of the sequence in 87.8% (65/74) of the sequenced isolates. Of the 9 isolates that did not have this motif, 7 (9.5%) lacked only the first or last repetition of type 7 (Baker *et al.*, 2005; Barbadillo Ruiz, 2019).

Nderu *et al.* (2019a), study 244 isolates collected in Kenya. The diversity of PfHRP2 and PfHRP3 was described as a function of frequency, occurrence and organization of the types of amino acid repeats in histidine-rich proteins. In this study, a unique distribution pattern of amino acid repeats was observed in both PfHRP-2 and PfHRP-3, identifying 228 and 124 different amino acid sequences of PfHRP2 and 3, respectively. Of these, 214 (94%) PfHRP2 sequences and 81 (65%) of PfHRP3 occurred only once. Thirty-nine new repeats were identified for PfHRP2 and 20 for PfHRP3 and PfHRP-3 was found structurally to have a lower degree of genetic variability than PfHRP2 (Nderu *et al.*, 2019a; 2019b; Barbadillo Ruiz, 2019). Figure 1 shows the structure, subcellular location and possible repeats found in the PfHRP genes.

Accordingly, genetic variability in histidine-rich proteins can lead to false negatives in diagnostic results with PfHRP-2 RDT.

This discovery is the result of studies carried out in the Amazon jungle of Peru and in India, which reveals the absence of the PfHRP-2 and 3 genes in the parasites of those regions (Barbadillo Ruiz, 2019; Gamboa *et al.*, 2010; Kumar *et al.*, 2013).

On the other hand, a study with 158 blood samples infected with *P. falciparum* was carried out in Honduras, Nicaragua and Guatemala. 25.8% of the isolates did not present the partial coding region between exon 1 and 2 (intron 1) of PfHRP2 and 91.4% of the isolates lacked the homologous region of PfHRP3. Parasites from all three countries showed deletions from one or both of the genetic regions that code for these proteins. The highest proportion of exon 1-2 deletions in PfHRP2 was found in samples from Nicaragua (30.9%), while isolates from Guatemala revealed the lowest number of deletions (14.3%). Parasites collected in Honduras showed the

highest proportion of exon 1-2 clearance in PfHRP3 (96.2%). A relevant result was the finding of 27 (21%) double negative isolates (negative PfHRP2 and negative PfHRP3). Most of the double negative parasites were detected in Honduras 13/52 (25%), followed by Nicaragua 11/55 (20%) and Guatemala 3/21 (14.3%) (Fontecha *et al.*, 2019; Barbadillo Ruiz, 2019). In another way, the presence of pfHRP-2/3 deletions in Tanzania and Uganda, along with reports of pfhrp2/3-deleted parasites in neighboring countries, reinforces the need for systematic surveillance to monitor the reliability of RDTs in malaria-endemic countries.

Because *P. falciparum* isolates negative for PfHRP2 and PfHRP-3 have been reported in the neighboring regions of the Peruvian and Brazilian Amazon, a study was conducted in Colombia where 100 samples collected from six departments between 1999 and 2009 were included to detect the presence of PfHRP2, PfHRP3 and their flanking genes. Additionally, seven neutral microsatellites were used to determine the genetic background of these parasites. In total, 18/100 parasite isolates were found to have deleted PfHRP-2, most of

which (14/18) were collected from the Department of Amazonas, which borders Peru and Brazil. Deletions of pfhrp3 were found in 52/100 samples collected from all regions of the country. The PfHRP2 PF3D7_0831900 and PF3D7_0831700 flanking genes were deleted in 22/100 and 1/100 samples, respectively. PfHRP3 flanking PF3D7_1372100 and PF3D7_1372400 genes were missing in 55/100 and 57/100 samples. Analysis of the structure of the microsatellite data indicated that the Colombian samples analyzed in this study belonged to four groups and were segregated mainly according to their geographical region. Most of the parasites with deletions in PfHRP2 were assigned to a single group and originated in the Amazonas Department, although some PfHRP2-negative parasites originated in the other three groups (Cordoba, Nariño and Valle). The presence of a high proportion of negative PfHRP2 isolates in the Colombian Amazon may have implications for the use of PfHRP2-based RDTs in the region and may explain the inconsistencies observed when conducting tests and trials based on PfHRP2 (Murillo Solano *et al.*, 2015).

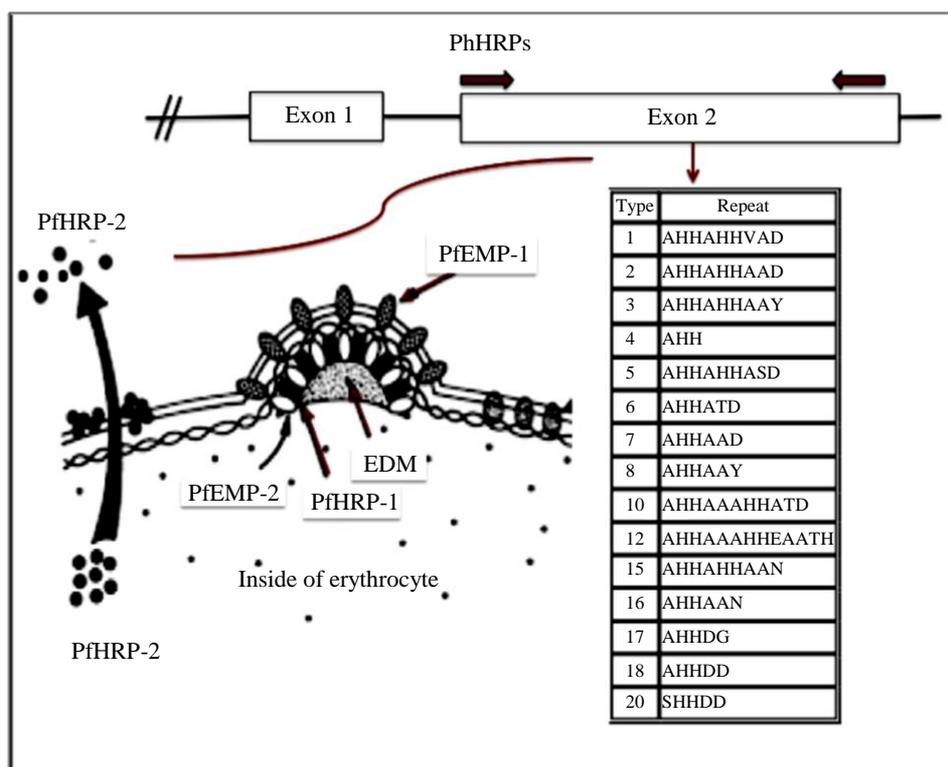


Fig. 1: Subcellular location and possible repeats found in the PfHRP genes. The scheme of a red blood cell and some of the proteins expressed by *Plasmodium falciparum* are presented (Modified of <https://encrypted-tbn0.gstatic.com/images?q=tbn%3AANd9GcR-1AYefV7yWDyfBF2h2xWLhbKjVZBAh7Gg&usqp=CAU>). Also included the 20 repeat sequences found in exon 2 of the PfHRP-2 and 3 genes. PfEMP-1 = *Plasmodium falciparum* Erythrocyte Membrane Protein 1; PfEMP-2 = *Plasmodium falciparum* Erythrocyte Membrane Protein 2; PfHRP-1 = *Plasmodium falciparum* Histidine-Rich Protein 1; PfHRP-2 = *Plasmodium falciparum* Histidine-Rich Protein 2; EDM = Electrodense Material

In a pilot study we carried out in Tarapacá (unpublished data), we found a 25% positivity using RDT for *P. falciparum*, while by PCR we obtained 39% positivity and were able to detect mixed infections in 7.14% of the samples, which were also confirmed by nucleic acid sequencing.

These results indicate the need to use microscopy and others diagnostic methods and RDT that detect different antigens.

Furthermore, it has been determined that the sensitivity of PfHRP-2 RDT is influenced by the presence or not of rheumatoid factor, since they seem to cause a cross reaction. There are studies that have tested how rheumatoid factor patients but not malaria patients tested positive for these RDTs. However, when rheumatoid factor was absorbed and the test was repeated, the result was negative. Some authors suggest that this fact is related to the type of antibody used in RDT. Thus, there is a cross reaction and false positive if IgG is used, but this does not occur if IgM is used, which does not bind to rheumatoid factor (Iqbal *et al.*, 2001; Mishra *et al.*, 1999; Barbadillo Ruiz, 2019).

The relationship between the detection of PfHRP2 and the prozone effect has also been described, which consists of obtaining false negatives in immunological tests, due to an excess of antigens or antibodies. In the case of RDT-PfHRP-2, it has been confirmed that elevated *P. falciparum* parasitemias can cause the aforementioned effect leading to false negatives (Gillet *et al.*, 2009).

Despite these drawbacks, detection of HRP2 is the most common method of RDT, since *Plasmodium falciparum* is the most lethal species and is the only significant species in large parts of Africa. In addition, methods based on detection of HRP-2 have a sensitivity between 77 and 100% and a specificity between 83 and 93% with parasitemias >100 parasites/ μ L, making it in many cases a method comparable to that of microscopy to diagnose *P. falciparum* malaria (Zuluaga and Trujillo, 2010).

There are other diagnostic techniques, such as multiple PCR, which allows the detection of genomic DNA from all species. Conventional PCR amplification that allows detecting up to 3-4 parasites/ μ l and determining mixed infections, but it is a reserved technique to validate the results of the other techniques. On the other hand, there is the immunodiagnostic, which detects anti-*P. falciparum* antibodies in serum by immunofluorescence or enzyme immunoassay, which has low sensitivity and is used when microscopy is negative. However, the use of these techniques is less frequent since they are expensive and not available to all laboratories (Turrientes and López-Vélez, 2000; Torrús *et al.*, 2015; Wilson, 2013).

The sensitivity and specificity of the conventional RDTs performed in the routine health facility consults

and survey exit interviews were acceptable and there was no evidence of PfHRP2- and PfHRP3-deleted parasites. Mono-infections with non-falciparum malaria species comprised <1% of the total malaria infections. Nearly all malaria antigen-positive patients had detectable PfHRP-2, confirming that this antigen remains an appropriate malaria diagnostic target in the surveyed provinces (Plucinski *et al.*, 2019).

The pfHRP2 gene amino acid sequence and repeats have been shown to vary substantially across different geographic regions (Ramutton *et al.*, 2012). Genomic sequencing of exons and flanking regions would provide more information on sequence diversity among these samples; similar pfHRP2-positive samples may have genetic diversity with implications for RDT detection. Although diversity in the pfHRP-2 gene has not been found to affect RDT affinity in samples with parasite densities of clinical significance (Bharti *et al.*, 2016; Baker *et al.*, 2010), it has been shown to affect RDT results at densities <200 parasites/ μ L (Baker *et al.*, 2005; Thomson *et al.*, 2019).

In malaria endemic countries, the evaluation and monitoring of pfHRP-2/3 deletions and their impact must be carried out effectively and efficiently, along with many other public health and malaria control priorities. The WHO recommends the use of a protocol to implement surveys designed to measure parasites with pfHRP2 deletions among malaria suspects. The WHO guidelines state that if the prevalence of deletions of the pfHRP2 gene that cause HRP2-based false negative RDT results in a representative sample is greater than 5% (this value corresponds to the prevalence in which the benefits of Non-HRP2-based diagnostics to detect parasites with pfHRP2 deletions exceed the reduced sensitivity of these tools to detect wild-type parasites), HRP2-based RDTs should be replaced with a new diagnostic tool. In these cases, RDTs that target other antigens, such as those that detect pan-LDH or Pf-pLDH, can be used although these are less sensitive and heat stable than HRP2-based RDTs and this should be considered before using these RDTs (Thomson *et al.*, 2019; World Health Organization, 2015a).

As mentioned before, the occurrence of pfHRP-2/3 gene deletions has a substantial risk to malaria control and could reverse the advances made through the rapid expansion of RDT uptake over the past decade. Prescribing adherence to test results, especially negative test results, has been a significant effort of RDT implementation. False-negative RDT results lead to underdiagnosis of malaria and if patients who are infected but test negative do not receive antimalarial treatment, severe disease and even death may result (Thomson *et al.*, 2019; Gatton *et al.*, 2017; Bruxvoort *et al.*, 2017; Burchett *et al.*, 2017).

But Why Continue Studying PfHRP2 and PfHRP3?

PfHRP-2 and PfHRP-3 are located in the subtelomeric region of the chromosome and because they are in this region have great variability in their sequences and are normally subject to changes during episodes of recombination (Baker *et al.*, 2010). The deletion of the PfHRP2 and PfHRP3 genes on chromosomes 8 and 13, respectively, in the *P. falciparum* parasites could be affecting the RDTs because the results could be false negatives in the absence of these genes. The target of RDT in parasites is a consequence of the deletion of these genes from the respective chromosomes. The deletion of these two genes has been previously reported in laboratory lines as Dd2 (deletion of PfHRP2), HB3 (deletion of PfHRP3) and 3BD5 with double deletion (Gamboa *et al.*, 2010).

Agree with this, the importance of knowing the presence of those genes would allow us to evaluate which would be the best way to diagnose malaria and also know how effective rapid tests are. It is important to know if these genes are found in the samples (parasites) studied and to determine if the use of rapid tests to diagnose malaria in the country is reliable. In Colombia, the studies that have evaluated the genetic variability of these genes and their correlation with the efficacy of RDTs are limited and, due to the number of samples used, they do not allow establishing or extrapolating the results for the entire country. On the other hand, countries working towards the elimination of malaria will have to take into account the availability of diagnostic methods that maximize progress on the path towards elimination. Accordingly, in the elimination framework, WHO stands out through its support element "Taking advantage of innovations and expanding research, recommends: Basic research to foster innovation and the creation of new and improved instruments, including improved diagnostic tests".

Performance of HRP2-based RDTs can be affected by factors including antigenic variability of the target protein, antigen persistence in the bloodstream following elimination of parasites and parasite density below the RDT threshold of detection. The existence of parasites lacking pfhrp2 would affect RDT accuracy in a broader range of malaria-endemic regions and would have significant implications for RDT implementation, clinical case management and malaria control efforts.

Conclusion

Currently, there is little evidence to document the true extent of HRP mutations worldwide. In malaria endemic countries, it is necessary to assess the genetic variability of the genes discussed in this review in order to determine which mutations are present in each country so as to improve the diagnosis and interpretation of the results obtained by rapid and molecular tests, which will

reduce the occurrence of false negatives and in the medium-long term, the improvement of malaria management programs and interventions.

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Author's Contributions

María Luz Gunturiz: Wrote the article, participated with substantial contributions to study design and conception of the project "Eliminación de la malaria: Un reto para Colombia" and collaborated in the critical proofreading of the article and gave the final approval of the version to be submitted.

Pablo Enrique Chaparro, Gabriel Carrasquilla and Mónica Marcela Jiménez: Participated with substantial contributions to study design and conception of the project "Eliminación de la malaria: Un reto para Colombia" and collaborated in the critical proofreading of the article and gave the final approval of the version to be submitted.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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