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REVIEWS

Review

HRP2: Transforming Malaria Diagnosis, but with Caveats

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The major growth in point-of-care malaria diagnosis over the past decade has been based on immunochromatographic malaria rapid diagnostic tests (mRDTs), which generally detect *Plasmodium falciparum* via its abundant histidine-rich protein 2 (HRP2). Here, we review the discovery and biology of HRP2, as well as the strengths and weaknesses of HRP2-based diagnosis compared with alternative antigens. We highlight recent studies describing HRP2 deletion in Latin America, Eritrea, and possibly other regions, and the methodological challenges of confirming deletion of the *pfhrp2* gene. We also discuss the mechanism of persistent HRP2 positivity after effective antimalarial treatment, along with other emerging HRP2-based applications, including detection of submicroscopic malaria and diagnosis of severe malaria.

Improving Malaria Diagnosis via HRP2-Based Rapid Tests

In resource-poor settings, the diagnosis of malaria has tended to be made clinically without parasitological confirmation, leading to administration of antimalarials to febrile children without malaria, neglect of other diagnoses and treatments, wasted antimalarial courses, and unnecessary adverse effects [1]. The problem has been worst in sub-Saharan Africa, where, in 2006, most countries confirmed malaria in <20% of suspected cases[i]. To improve this situation, in 2010, the World Health Organisation (WHO) recommended prompt laboratory confirmation for all patients with suspected malaria, before treatment, and that children under 5-years old in high transmission areas should no longer receive treatment based on clinical features alone[ii]. However, microscopic diagnosis, the established method for laboratory confirmation of malaria, requires resources that are challenging to maintain in peripheral facilities[iii].

The subsequent deployment of immunochromatographic mRDTs has transformed the diagnostic landscape for malaria. According to WHO's *2018 World Malaria Report*[iv], in sub-Saharan Africa, from 2010 to 2017, the annual number of diagnostic malaria tests performed increased from 55 million to >223 million, with >75% of tests in 2017 being mRDTs. The percentage of suspected malaria cases tested in the public health sector rose from under 40% in 2010 to >80% in 2016. Of more than 1 billion mRDTs sold, most have involved detection of **HRP2** (see Glossary).

In this review, we examine the discovery and biology of HRP2 and how it took on such a central role in malaria diagnosis. We then highlight recent work on potential sources of inaccuracy with HRP2-based tests. Parasites with deletion of both ***pfhrp2*** and ***pfhrp3*** are now present in both Latin America and sub-Saharan Africa, and we discuss criteria for confirming that isolates have deletions. We also consider how such parasites can survive. We then examine why artemisinin-based treatments are associated with HRP2 persistence after antimalarial treatment, compromising specificity. These flaws are also considered in the context of alternative diagnostic antigens. Finally, we review other applications of HRP2 measurement related to malaria control, management of severe malaria, and *in vitro* growth studies. A comprehensive understanding of the strengths and weaknesses of HRP2-based malaria diagnosis should guide strategy on the future role of HRP2 detection in malaria control strategies, and define important areas for further research.

Biology of HRP2

Discovery of the HRPs of *Plasmodium falciparum*

The development of HRP2-based mRDTs can broadly be considered in three phases: an initial phase of pioneering bench science leading to the identification of several histidine-rich proteins; a development phase in which these findings were translated into a tool for malaria diagnosis; and a selection

Highlights

HRP2 detection forms the basis of the most sensitive mRDTs currently available, and HRP2-based mRDTs have been the central element in the scale-up of mRDTs over the last decade, allowing malaria diagnosis even in remote areas in sub-Saharan African. Over 1 billion mRDTs have been sold so far.

Parasites with HRP2 deletion were first reported from the Amazon region (Peru and neighbouring countries) in 2010 and, more recently, at high prevalence in Eritrea.

Persistence of HRP2 in the circulation for several weeks following successful antimalarial treatment is now understood to be due to splenic pitting, and HRP2 measurement can be used to predict postartesunate-delayed haemolysis.

The concentration of HRP2 can be used to define severe malaria (plasma) and quantify *in vitro* growth (laboratory culture).

There is increasing interest in ultrasensitive HRP2 detection to detect asymptomatic malaria in low-transmission areas, potentially allowing public health-level interventions.

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phase of rapid uptake of HRP2-based mRDTs driven by national malaria control programs and other healthcare providers supported by a range of global health agencies.

The discovery of HRP2 owes something to serendipity as well as to inventive and determined research. The first *Plasmodium* HRP was discovered during the 1970s by Araxie Kilejian at the Rockefeller Institute [2] while working on the avian parasite *Plasmodium lophurae*, which William Trager had chosen to study several decades earlier[v] because it could be maintained in domestic birds and grown for short periods *in vitro*. *P. lophurae* has characteristic cytoplasmic granules, from which Kilejian extracted a protein comprising >70% histidine residues.

Radiolabel studies to isolate analogous histidine-rich sequences from *P. falciparum* began soon after, following its successful laboratory cultivation by Trager and Jensen. These identified three HRPs, which became known as **knob-associated histidine-rich protein** (PfKAHRP or HRP1) [3,4], and PfHRP2 and PfHRP3, a pair of related sequences obtained via independent lines of investigation [5–7]. These early studies also showed that certain laboratory clones of *P. falciparum* lacked PfHRP2 (e.g., DD2) or PfHRP3 (e.g., HB3) [4,5,8]. **Membrane-associated histidine-rich protein (PfMAHRP)** was described later [9]. This review focuses on PfHRP2 and PfHRP3.

Genetic Structure and Primary Amino Acid Sequence

The genes encoding all *P. falciparum* HRPs are located within the highly labile subtelomeric chromosomal regions, alongside antigenically variant multigene families. Each contains two exons and two introns [10]. The first exon and start of the second exon encode signal and cleavage sequences. The main histidine-rich sequences of *pfhrp2* and *pfhrp3* are located within the second exon and are of low complexity (i.e., certain codons are over-represented) and encode mainly tri- or hexapeptide repeats (AHHAAD and AHHAAN, respectively). This amino acid content is distinct from that of most low-complexity sequences of *P. falciparum* [11] and indeed other organisms [12]. As with many *P. falciparum* low-complexity sequences, there is substantial indel polymorphism, leading to wide variation in number and type of repeat protein sequence [13].

The conserved regions flanking the repeat domains show 85–90% homology at the DNA level, indicating that this pair of sequences originated by duplication and divergence from a common ancestral sequence. Orthologs of both *pfhrp2* and *pfhrp3* are found in the genomes[vi] of the hominid parasite *Plasmodium reichenowi*, indicating that duplication occurred before these two malaria species diverged several million years ago. Single orthologues are also found in more distantly related species of the *Laverania* subgenus[vi], but no equivalents to *pfhrp2* or *pfhrp3* are present in other human species of malaria or the widely researched murine malarias [14].

Higher Level Protein Structure

The low-complexity primary structure of PfHRP2 suggests a protein lacking classical secondary structure [12], and circular dichroism studies match predictions for a random coil structure [15]. However, PfHRP2 appears to adopt a 3_{10} alpha-helix structure upon binding of other molecules, such as haem at pH 7.0 [16,17] and actin [18], consistent with the central importance of dynamic structure in disordered protein domains of functional importance [12]. There is also evidence for cysteine-dependent dimerisation of PfHRP2 [16].

Timing and Quantity of Expression

PfHRP2 and PfHRP3 are expressed at high levels early during the asexual cycle, with a rapid increase in concentration in the ring-stage and slower accumulation in the trophozoite and schizont stages (Figure 1), which contain ~10 fg per parasite [4,7,19,20]. PfHRP2 is the more abundant, and most subsequent functional and localisation studies have focussed on it, with relatively little direct work on PfHRP3. PfHRP2 is present in the first two gametocyte stages [21], but transcription is very low in subsequent sexual stages [22]. Studies of laboratory lines indicate substantial asexual stage variation in the expression of PfHRP2 and PfHRP3 among different isolates [23].

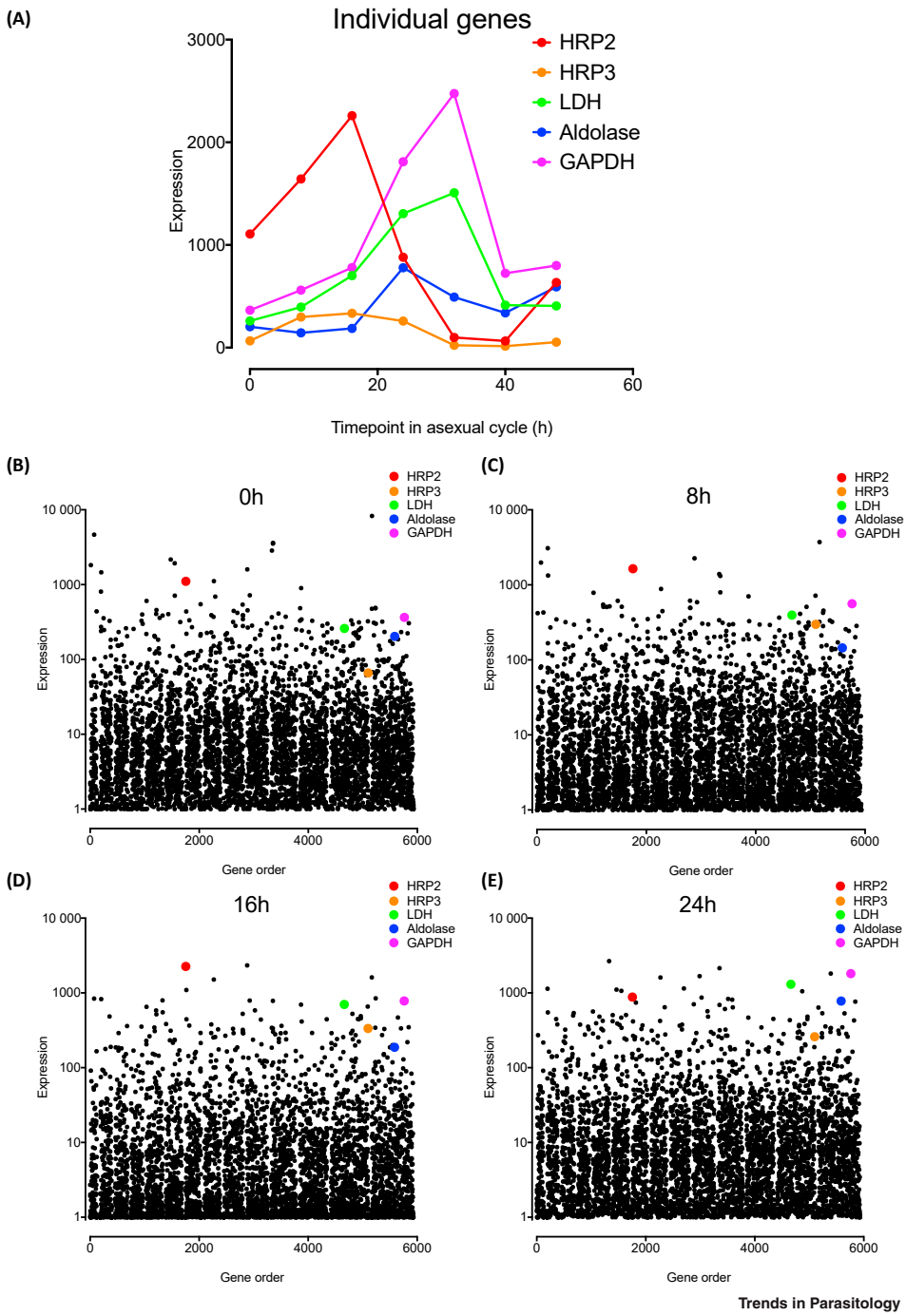

Figure 1. Asexual Stage Transcriptomic Data for *pfhrp2*, *pfhrp3*, and Three Other Diagnostic Antigen Genes during the Asexual Lifecycle.

Data are plotted directly from Supplementary Table 3 of [49]. (A) Data for the five genes only over the entire asexual cycle. Genome-wide data (ordered by location across the 14 chromosomes) are shown for (B) 0, (C) 8, (D) 16, and (E) 24 h timepoints. GAPDH is not detected by current malaria rapid diagnostic tests (mRDTs), but has been proposed to be superior to lactate dehydrogenase (LDH) in quantitative terms [97].

Glossary

Histidine-rich protein 2 (HRP2): general term used when discussing applications (and research studies) in which it is not relevant (or possible) to differentiate between PfHRP2 and PfHRP3 (including RDTs), where both sequences contribute to the overall signal. This convention is also followed in many studies and WHO reports.

Knob-associated histidine-rich protein (PfKAHRP): a protein critical for knob formation. with one histidine-rich region of ~50 amino acids length, as well as other repeat elements that are not histidine-rich.

Membrane-associated histidine-rich protein (PfMAHRP): a protein targeted to the Maurer's cleft of *P. falciparum* with a histidine-rich C-terminal domain.

Pitting: mechanical process in which dead ring-stage parasites are removed from red cells in the spleen, with the return of once-infected red cells to the circulation.

***Plasmodium falciparum* histidine-rich protein 2 (PfHRP2)**: an antigenic protein expressed in the asexual stage of *P. falciparum* that comprises mainly histidine-rich repeats. The gene *pfhrp2* (PF3D7_0831800) is located on *P. falciparum* chromosome 8.

***Plasmodium falciparum* histidine-rich protein 3 (PfHRP3)**: a protein related to PfHRP2 with similar sequence organisation and somewhat lower levels of expression. The gene *pfhrp3* (PF3D7_1372200) is located on *P. falciparum* chromosome 13.

Localisation

A range of studies have localised PfHRP2 via monoclonal and polyclonal antibodies and tagging methodology. Given the cross-reactivity between PfHRP2 and PfHRP3 with some antibodies, it is likely that both proteins were labelled in many studies. However, the use of antibodies specific to PfHRP2 [7,24] or PfHRP3 [5] and of laboratory strains lacking one or the other protein [25] has controlled for this issue to some extent.

In ring stages, PfHRP2 is found both within the parasite and in the infected erythrocyte [5,7,24–27], appearing as a 'necklace' of punctate staining over the parasite cytoplasm as well as more diffusely across the erythrocyte cytoplasm (Figure 2, Key Figure). As parasites mature, more HRP2 accumulates as concentrated packets under the erythrocyte membrane [25,28], consistent with the presence of an N-terminal signal endoplasmic reticulum (ER) secretion sequence as well as a PEXEL-HT motif for erythrocyte export [29,30]; indeed, HRP2 has been a useful tool for deciphering the molecular content of the PEXEL-HT motif [29,30] and its processing [31,32]. The small proportion remaining within mature parasites concentrates in the digestive vacuole, possibly after uptake from the erythrocyte cytosol [25,28]. PfHRP2 appears in culture supernatants [7], mostly after the mature schizont ruptures to release new merozoites [19,33].

Function

Oddly for a protein that has been studied for so long, the precise functional role of PfHRP2 remains unresolved, reflecting the absence of homologues in other species and its nonglobular structure. Histidine binding to transition metal ions mediates HRP function in other contexts [34], and PfHRP2 was postulated to be involved in the detoxification of iron-containing haem groups resulting from the digestion of host haemoglobin (a process targeted by several antimalarials). *In vitro*, PfHRP2 binds large numbers of haem molecules and can catalyse haem crystallization to form haemozoin via a scaffold-like function [17,25], fitting with a proportion of PfHRP2 being localised to the digestive vacuole [25,28]. However, *P. falciparum* clones lacking *pfhrp2* and *pfhrp3*, and non-*P. falciparum* species, still produce haemozoin [25], suggesting that the situation *in vivo* is different, or that there is considerable redundancy. PfHRP2 may also neutralize haem in the erythrocyte cytoplasm and remove it from the erythrocyte membrane [35] (where most PfHRP2 is found in mature parasite stages).

The hallmark of *falciparum* malaria, and the pivotal event in severe malaria pathogenesis, is sequestration of infected red cells in postcapillary venules, a process that allows the parasite to avoid filtration of mature parasite stages by the spleen [36]. PfHRP2 may be involved in changes in the erythrocyte cytoskeleton [18]. PfHRP2 may also interrupt the integrity of the blood–brain barrier via inflammasome activation [37], perhaps causing increased expression of cytoadherence molecules on the endothelial surface, promoting cytoadherence and thus avoidance of splenic clearance. A further function might relate to evasion of the host immune system [38], since plasma PfHRP2 has been found to suppress proliferation of B and T lymphocytes, and inhibit the release of interferon (IFN)-γ by T lymphocytes [39].

Malaria RDTs

Development of HRP2-Based mRDTs

Work towards an HRP2-based immunochromatographic mRDT began during the early 1990s. The 1993 *WHO Global Plan of Action for Malaria Control*[vii] made no specific reference to mRDTs, but emphasised the importance of parasitological diagnosis at the peripheral and community level. HRP2 was known to be an abundant blood-stage protein with sequence repeats (hence, multiple epitopes, Figure 3) against which it had already proved straightforward to develop antibodies [7,27]. These properties led to HRP2 being used in a variety of clinical and research applications (Figure 2).

Interestingly, the presence of HRP2 in plasma was also considered relevant, with the idea that it might allow detection of sequestered parasites [27,40]. However, there is no evidence that diagnosis of uncomplicated malaria depends on the detection of plasma HRP2, since most circulating HRP2 at diagnosis is within infected red cells (which are lysed in rapid tests releasing internal antigen). Plasma

Key Figure

Clinical Applications of Histidine-Rich Protein 2 (HRP2) Detection

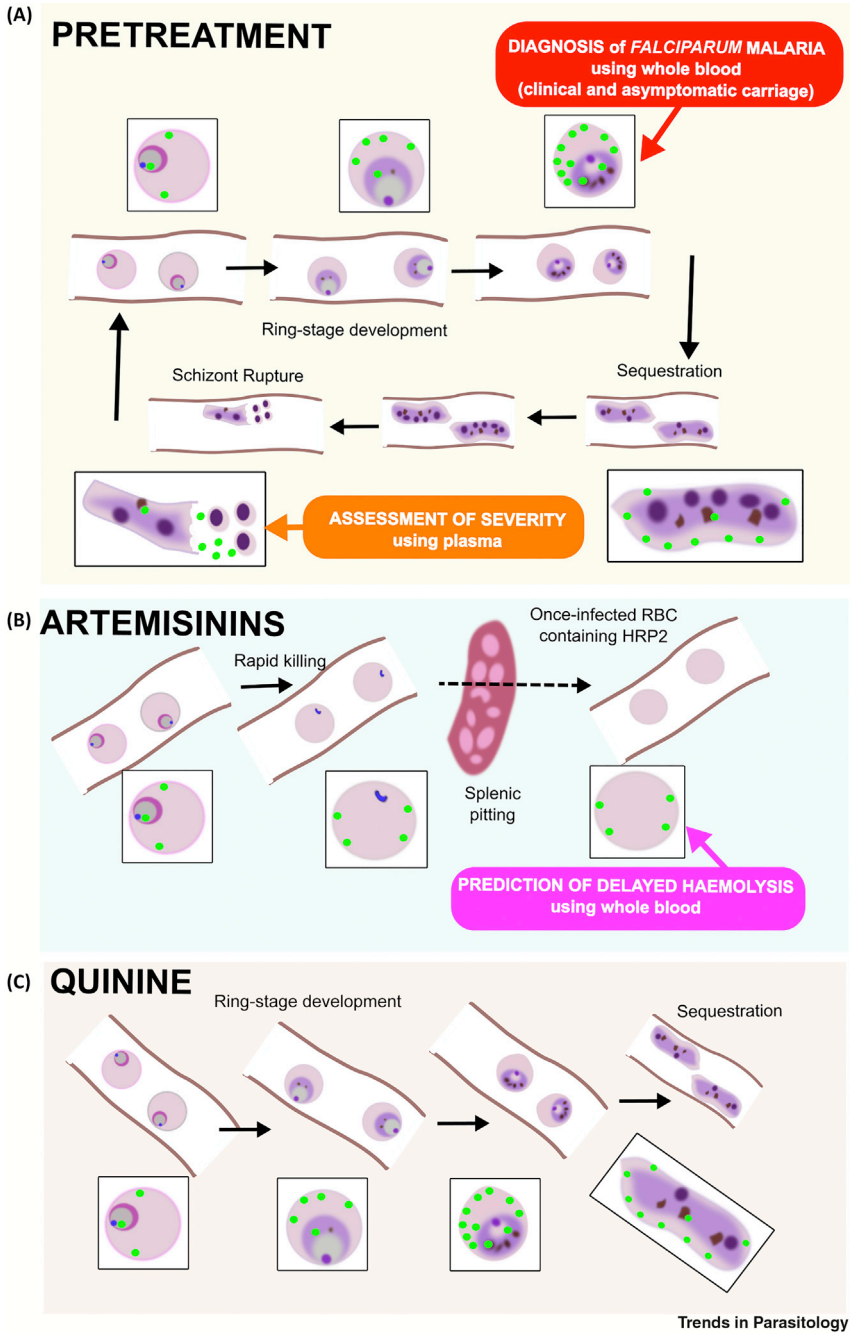

Figure360▷ **Figure 2.** For a Figure360 author presentation of Figure 2, see the figure legend at https://doi.org/10.1016/j.pt.2019.12.004.

(Figure legend continued at the bottom of the next page.)

HRP2 has instead turned out to be an accurate marker of the sequestered parasite biomass in severe malaria [41] (see later).

The first mRDT (*Para*Sight®-F) used a standard dipstick approach (Figure 3) involving a capture line of bound monoclonal antibody against the amino acid sequence $[AHH(AHHAAD)_2]_{10}$ (a peptide section of HRP2) and colorimetric detection via polyclonal rabbit antibodies to PfHRP2 conjugated to liposomes containing pink dye. These, and later tests, showed high levels of sensitivity when asexual parasitaemia was >100 parasites/μl of blood [42,43]. While the *Para*Sight®-F test required several manual steps, later kits were simpler, requiring only the addition of anticoagulated blood followed by a few drops of buffer solution [44]. A cassette format was also developed.

mRDTs Based on Other Antigens

Given that HRP2 is restricted to *P. falciparum*, mRDTs identifying other malaria species are needed, particularly in areas where non-*falciparum* malaria is common. mRDTs detecting the glycolytic enzymes *Plasmodium* lactate dehydrogenase (pLDH) and aldolase began to appear during the late 1990s. These proteins are present in all malaria species and have conserved amino acid sequences; thus, according to the antibody used, it is possible to detect individual species and/or the *Plasmodium* genus as a whole [45]. In reality, species-specific diagnosis of non-*falciparum* malaria via RDT can be challenging, particularly for *Plasmodium knowlesi* [46].

Initial work suggested that pLDH-based diagnosis of *P. falciparum* was as sensitive as HRP2 detection [47], but a meta-analysis indicated that HRP2-based mRDTs are more sensitive [48] for several possible reasons. First, HRP2 is present at higher levels in circulating ring stages (0–24 h of the asexual cycle), with *pfhrp2* mRNA peaking at 16 h compared with 24–32 h for glycolytic enzymes [49] (protein levels lag behind mRNA by several hours [50]). Second, antigen–antibody binding kinetics appear to be superior for HRP2. Finally, the repeat sequences of HRP2 may amplify signal by binding more than one secondary antibody (Figure 3).

Growth in HRP2-Based mRDT Use and Product Evaluation

In 2004, <1 million malaria mRDTs had been used; by 2013 >300 million were being sold each year (Figure 4). This exponential growth was associated with the widespread deployment of artemisinin-combination therapies (ACTs), a context in which parasitological diagnosis became relatively cost-effective (compared with the previous practice of empirical treatment with inexpensive chloroquine) [46].

Reports of variable performance of mRDTs led the WHO to develop a Malaria RDT Evaluation Programme, including prepurchase performance evaluation (product testing, the basis of WHO recommendations for RDT procurement) and predistribution quality control (lot testing). The Programme has also allowed assessment of heat stability, batch variability, labelling, and clarity of instructions. Products are evaluated against geographically diverse, cryopreserved *P. falciparum* clinical samples diluted to 200 and 2000 parasites/μl, with defined concentration ranges of HRP2, pLDH, and aldolase. More than 200 different mRDTs have been assessed (many on multiple occasions), with most using HRP2 to detect *P. falciparum*[viii]. Recent results[viii] show that kits using PfLDH to detect *P. falciparum* are of lower sensitivity, consistent with earlier findings [48].

HRP2 is depicted as green dots within enlarged images of parasite stages (shown in boxes). (A) When patients present with untreated *falciparum* malaria, HRP2-based malaria rapid diagnostic tests (mRDTs; red box) detect circulating ring stages, which have increasing concentrations of HRP2 as they develop. The level of HRP2 in plasma at clinical presentation (orange box) reflects the sequestered biomass as HRP2 is released into plasma at schizont rupture. (B) Treatment with an artemisinin derivative results in rapid killing of ring-stage parasites, removal of dead parasites via pitting in the spleen, and recirculation of once-infected red blood cells (RBCs). These cells, which have a shortened lifespan, contain HRP2 and, thus, the level of whole-blood HRP2 after parasite clearance (purple box) is predictive of delayed haemolysis. (C) With more slowly acting antimalarials, such as quinine, the main mechanism of clearance is sequestration and, hence, removal from the circulation of infected RBCs in their entirety, along with their HRP2.

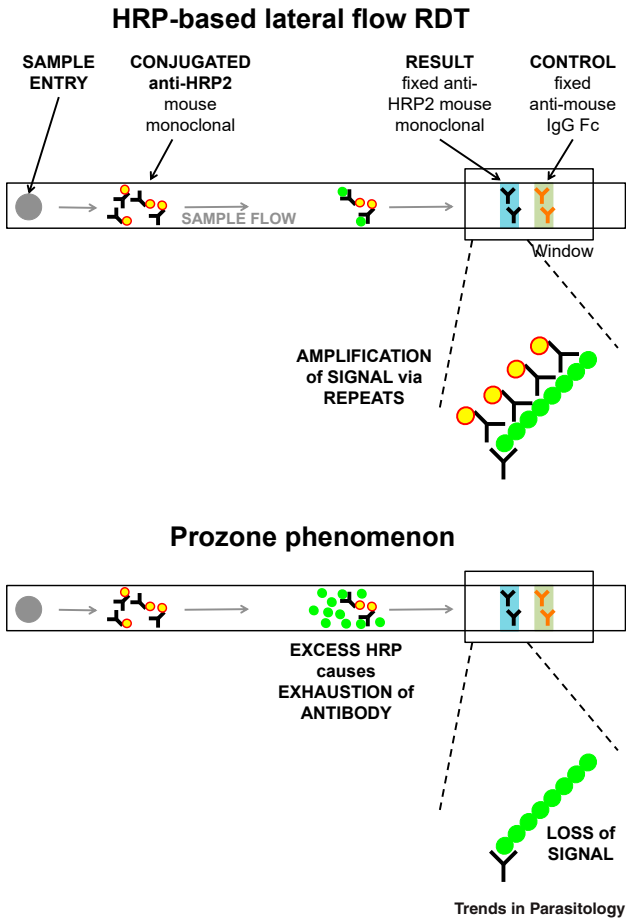

Figure 3. Histidine-Rich Protein 2 (HRP2)-Based Malaria Rapid Diagnostic Tests (mRDTs): Normal Operation and the Prozone Phenomenon.

(A) Illustration of an HRP2-based mRDT showing how signal from one HRP2 molecule can be amplified by the presence of repeats. (B) Illustration of how excess antigen can produce false-negatives through exhaustion of the mobile anti-HRP2 antibody (prozone phenomenon).

Given the range of sequence repeat numbers and types, it was logical to look for a relationship between *pfhrp2* sequence and sensitivity of HRP2-based mRDTs. An initial large study found a correlation between numbers of certain HRP2 repeat types and mRDT sensitivity [13], but a subsequent global study was unable to validate this finding [51]. Defining such relationships is challenging, given numerous confounders that vary across samples, including parasite stage and mRNA expression. PfHRP3 sequence is also a factor because it shares repeat motifs with PfHRP2 [13], and anti-HRP2 antibodies can cross-react with HRP3 [14]. Incidentally, this can provide a form of 'safety net' in cases of *pfhrp2* deletion (see later).

Ultrasensitive HRP2-Based mRDTs for Use in Malaria Control

There is increasing interest in the identification of cases of malaria where parasitaemia is below the level of detection of microscopy and standard mRDTs (50–200 parasites/μl [46]), since, in low transmission settings, such infections have been found to be proportionally more common than symptomatic cases and, hence, may contribute to transmission [52]. 'Ultrasensitive' HRP2-based mRDTs (uRDTs) with enhanced signal detection methodology have a detection threshold of <10

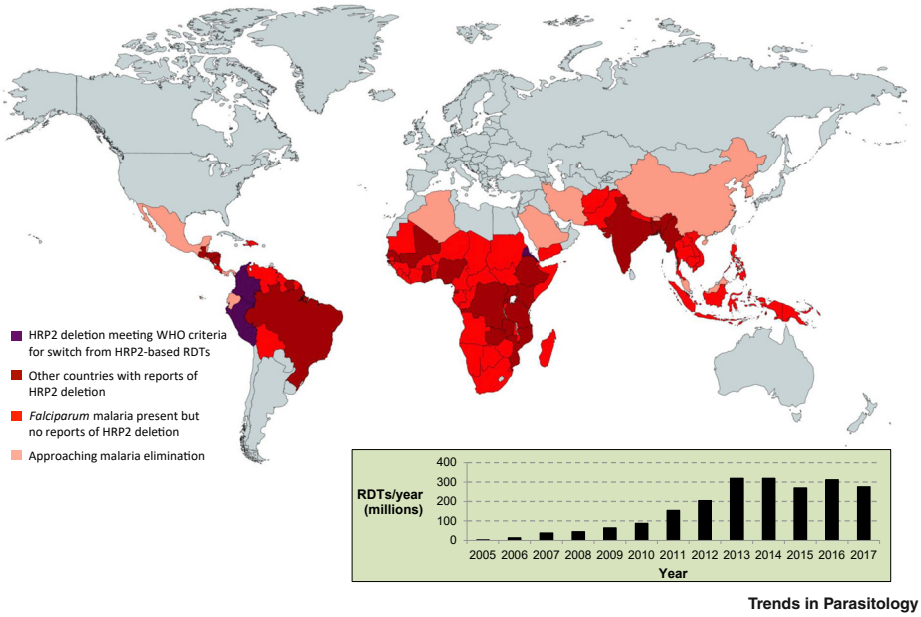

Figure 4. Deployment of Malaria Rapid Diagnostic Tests (mRDTs): Reports of Histidine-Rich Protein 2 (HRP2) Deletion and Overall Supply of mRDTs Per Year.

The map shows the global distribution of *falciparum* malaria (identifying countries nearing malaria elimination) and illustrates countries with reports of HRP2 deletion[viii], including Peru, Columbia, and Eritrea, where World Health Organisation (WHO) criteria for switching from HRP2-based mRDTs have been met. The map was created with mapchart.net. The inset graph shows the annual supply of all mRDTs from 2005 to 2017 in millions (data from WHO Malaria Reports[i,iv]).

parasites/μl [53], translating into significantly increased detection of asymptomatic cases in lower transmission areas [54,55] (but not of symptomatic cases in higher transmission settings [56]). Knowing the prevalence of low-density asymptomatic infection could guide mass drug administration interventions [57].

The recent development of bead-based detection of HRP2 [58] and other malaria diagnostic antigens [59] provides an extremely sensitive (>100 times more than ELISA) reference method for external validation of mRDT accuracy.

Disappearing Genes

From the earliest studies of HRP2, it was known that laboratory isolates lacking *pfhrp2* and/or *pfhrp3* could reproduce in asexual stage cultures [5,8,60–62], but it was not until 2008 that field isolates lacking the HRP2 antigen were identified. *P. falciparum* isolates from the Amazon region of Peru tested negative with HRP2-based kits because of deletion of both *pfhrp2* and *pfhrp3* [63], along with their respective flanking genes. A prevalence survey in the region indicated that 41% and 70% of 148 samples lacked *pfhrp2* or *pfhrp3*, respectively, with >20% lacking both [63]. Subsequent work showed that the problem has worsened over time [64] and is likely to extend to contiguous areas in Colombia and Brazil [65–67], indicating the need to switch from using exclusively HRP2-based mRDTs for detecting *P. falciparum* (Figure 4). A case presenting in France after travel to Brazil illustrated the impact of a falsely negative HRP2-based RDT result (caused by double deletion of *pfhrp2* and *pfhrp3*) on clinical management [68].

Robust evidence was recently obtained for a similar problem in Eritrea, where most *P. falciparum* infections lack *pfhrp2* and *pfhrp3* [69], selected for by use of HRP2-based tests. Microscopy mRDTs detecting other parasite antigens (e.g., pLDH) have had to be deployed.

The Paradox of HRP2 Deletion

How can parasites survive without such highly expressed and ancient genes? The answer probably involves changing evolutionary forces, such as falling immunity [70] and multiplicity of infection, or possibly chloroquine resistance [62,71] (Box 1).

HRP2 Deletion Surveys

Given these concerning findings, investigators in many malaria-endemic regions have looked for evidence of *pfhrp2* and *pfhrp3* deletions in local parasite isolates. These studies provide reassurance that, in most settings, HRP2-based mRDTs remain appropriate. However, a significant number of studies have reported low prevalence of parasites with combined *pfhrp2/3* deletions (Figure 4; also see the Malaria Threats Map[ix]). The methodology across these studies has been variable [72], a common issue being the tendency to analyse retrospective samples via PCR for *pfhrp2* and *pfhrp3* with inconsistent reference to RDT results, level of parasitaemia, or presence of symptoms. Absence of PCR products can simply reflect low levels or poor quality of DNA in the sample, particularly given the varying sensitivity across the range of primers and conditions described for *pfhrp2/3* PCRs [73] and the intrinsic stochasticity of PCR. Therefore, it is appropriate to await further regional surveys targeting symptomatic patients before recommending changes of RDT in these areas.

Procedures for Confirming HRP2 Deletion

These developments led to a set of WHO-recommended procedures for confirming that parasites lack HRP2 [72] (Box 2), while also avoiding unsubstantiated reports of parasites lacking *pfhrp2*, which

Box 1. The Paradox of Parasite Survival without HRP2

Genetic evidence suggests that *pfhrp2* deletions in Peru have appeared on several separate occasions, indicating that the deletion event itself is common. Yet, both *pfhrp2* and *pfhrp3* have been maintained in the genomes of the hominid malaria lineage since the last common ancestor of *Plasmodium falciparum* and *Plasmodium reichenowi*. Therefore, their loss in field isolates is likely to reflect a recent change in selective pressures due to the following reasons.

Isolation

If HRP2 promotes parasite fitness, it will be maintained when there is a high multiplicity of *P. falciparum* infection. As transmission falls, single-clone infections begin to predominate and parasites with relatively low fitness can survive. The same applies to isolated laboratory cultures. Direct evidence that *pfhrp2* deletion causes reduced fitness is lacking, since there has never been an isogenic *pfhrp2* knockout or knockdown. Some evidence for a role of HRPs in parasite fitness comes from a classical genetic cross in which there was inheritance bias towards maintenance of *pfhrp3* [64].

Falling Immunity

HRP2 may enhance parasite survival in the face of host immune and clearance mechanisms. As transmission falls, these mechanisms weaken, and the ratio between survival benefit and resource expenditure also falls, potentially increasing the prevalence of parasites with deletions.

Chloroquine Resistance

Most *P. falciparum* parasites around the world today differ from ancestral ones in being chloroquine resistant. All isolates found to have deletion of both *pfhrp2* and *pfhrp3* have been chloroquine resistant [60,62] or developed in regions with established chloroquine resistance [63,69]. Chloroquine acts on the haemozoin formation pathway, inhibits interactions between HRP2 and haem, and mutations in *pfcrt* affect digestive vacuole physiology [73]. Hence, the effect of HRP2 deletion on parasite fitness may be attenuated in chloroquine-resistant parasites.

Use of HRP2-Based RDTs

Routine usage of HRP2-based mRDTs for malaria diagnosis produces further positive selection of HRP2-deleted parasites already selected by the above mechanisms.

Box 2. Recommended Approach for Accurate Reporting of *pfhrp2* Deletion Derived from [72] and Further Guidance[ix]

Obtain Initial Evidence

- Microscopy positive for *Plasmodium falciparum* (two independent microscopists and slide archived)
- No HRP2 band on two quality-assured HRP2-detecting RDTs
- Positive mRDT with pan-LDH or PfLDH band
- Sample confirmed as *P. falciparum* by PCR and negative for other *Plasmodium* species by PCR

Obtain Confirmatory Evidence in Samples with Initial Evidence of Deletion

Gene Deletion Analysis

- PCR fails to amplify the full length of *pfhrp2* exon 2 and/or the region across exon 1 and exon 2
- PCR amplifies at least two single copy genes from the same sample (not ribosomal genes, which are multicopy)
- PCR amplifies none or only one of the genes flanking *pfhrp2*.

Antigen Analysis

- Negative for HRP2 on second brand of quality HRP2-detecting RDT or negative HRP2 ELISA

Establish Prevalence

- Surveys in the specific geographical region to determine the prevalence of parasites carrying gene deletions

could trigger a change of RDT product and damage user confidence in mRDTs. Subsequent guidance[x] has further emphasised the importance of studying samples from patients with a positive blood film interpreted by a qualified microscopist, or a positive result with a quality-assured RDT targeting a different *falciparum*-specific malaria antigen (e.g., PfLDH). In parallel, a WHO protocol for malaria control programs[xi] aims to identify provinces with a 5% or higher prevalence of false-negative mRDT results caused by *pfhrp2/3* deletion (a suggested threshold for switching from HRP2-based mRDTs to detect *P. falciparum*).

Relatively few studies have systematically examined the sensitivity of HRP2-based mRDTs in samples with a deletion of *pfhrp2* only, but the last round of WHO product testing[viii] and separate research [53] included both 'double-deleted' *pfhrp2*$^{-}$/*pfhrp3*$^{-}$ and 'single-deleted' *pfhrp2*$^{-}$/*pfhrp3*$^{+}$ parasites. Some HRP2-based mRDTs clearly retain reasonable sensitivity for single-deleted parasites[viii] [13,53], but others do not, reflecting the variable degree to which their component antibodies cross-react with PfHRP3.

Other Sources of Inaccuracy

Persistence Following Parasite Clearance

Since the first clinical studies of HRP2-based mRDTs, it has been evident that a significant proportion of patients remain persistently mRDT positive for several weeks after parasite treatment [40,42,74]. Clearly, this is problematic in terms of interpretation for individual patients, particularly in high transmission settings, although the information could be used at a public health level [75].

For many years, the mechanism of persistence remained unclear, but it is now clear that the issue reflects splenic **pitting**, the main mechanism of parasite clearance following artemisinins (Figure 2). After pitting, once-infected red cells still containing HRP2 (exported to the erythrocyte before pitting, see earlier) return to the circulation [76,77], where they persist for several weeks, corresponding to the period of persisting mRDT positivity. This explains a previously reported finding of artemisinin-based treatment being associated with rapid parasite clearance but slower HRP2 clearance than the antifols [78], which, similar to other slower acting drugs [79], allow parasites to mature and sequester leading to more rapid HRP2 clearance [78] (Figure 2).

Other Causes of False Negatives and False Positives

With very high levels of HRP2, the mobile (conjugated) antibody may become saturated and unbound HRP2 binds to the target line, generating a false-negative result (the 'prozone' phenomenon) (Figure 3) [80]. Uncommonly, false-positive mRDT results have been reported in patients with autoantibodies such as rheumatoid factor [81], or chronic infections typically encountered in malaria endemic areas [46].

Other Applications

Plasma HRP2 Concentration to Define Severe Malaria

Severe malaria results from the sequestration of mature (pigmented) asexual *P. falciparum* stages in the capillaries and venules of vital organs; therefore, peripheral blood parasitaemia is an inaccurate marker of severe malaria [82]. HRP2 is released into plasma at schizont rupture and, hence, reflects the sequestered parasite biomass [82] (Figure 2). Plasma HRP2 correlates with malaria severity and prognosis in both African children [41,82,83] and adults in Papua New Guinea [84], and can identify Malawian children with histologically confirmed or retinopathy-positive cerebral malaria with high accuracy [area under the receiver operating characteristics (AUROCs) of 0.98 and 0.9, respectively] [85]. As a practical consequence, plasma HRP2 level can be used to differentiate 'true' severe malaria from alternative severe febrile illnesses with coincidental parasitaemia, a common diagnostic challenge in severely ill children in high transmission settings in Africa [41,85–87]. Semiquantitative plasma measurement can be undertaken using a standard HRP2-detecting RDT [88], although this approach awaits application at scale. Plasma HRP2 concentration also predicts progression to severe malaria in African children with uncomplicated malaria [89].

Predicting Postartesunate Delayed Haemolysis

A proportion of patients with severe malaria who are treated with artesunate are affected by haemolytic episodes occurring well after parasite clearance (postartesunate delayed haemolysis; PADH) [90]. Recent work showed that PADH is caused by the destruction of once-infected circulating red cells from which parasites have been removed by pitting [91] (see earlier). These once-infected red cells have reduced survival and their subsequent removal and haemolysis explains the drop in haemoglobin 1 week or more after treatment with artesunate. Pitting is more prominent after artesunate compared with quinine treatment (Figure 2), although clinically significant delayed haemolysis after parenteral artesunate is uncommon in African children [92].

As discussed earlier, HRP2 persists after parasite clearance precisely because once-infected red cells carry HRP2 [76] (Figure 2). Put another way, HRP2 persistence and PADH are manifestations of the same process. In line with this, the concentration of whole-blood HRP2 (measured after parasite clearance, via a semiquantitative approach using RDTs) can predict subsequent risk of PADH, with 89% sensitivity and 73% specificity in the first prospective study of this application [76].

In Vitro Growth Measurement

HRP2 is a useful marker of parasite growth for *in vitro* drug sensitivity testing [93], with production that peaks somewhat earlier than other growth markers (pLDH or SYBR green).

HRP2 in Other Samples

During malaria infection, the greatest concentrations of HRP2 are found in red cells and plasma. HRP2 can also be measured in cerebrospinal fluid [20,94], urine [95], and saliva [96] although such measurements have not yet found clinical application.

Concluding Remarks

We have reviewed the discovery and basic biology of HRP2, noting that HRP2 was discovered somewhat serendipitously and chosen as a suitable antigen for mRDTs before the availability of the now vast genomic and proteomic data on *P. falciparum*, which allow the rational identification of diagnostic antigens. The subsequent rollout of HRP2-based mRDTs has been a major success, allowing

parasitological confirmation of *P. falciparum* infection to extend into settings where malaria diagnosis was previously impossible, on a global scale. We also described the increasing interest in the use of 'ultrasensitive' uRDTs to detect submicroscopic malaria infections thought to have a significant role in maintaining malaria in low transmission areas. A more complete understanding of HRP2 biology has also helped to develop other areas where its measurement may guide clinical care: defining severe malaria (based on plasma concentration) and predicting PADH (based on persistence in once-infected red cells). Scale-up of these other applications has yet to take place because they require semi-quantitative assessment of HRP2 level, and involve fewer patients than mRDTs.

Unfortunately, all HRP2-related applications are potentially threatened by the emergence of parasites with deletions of *pfhrp2* and *pfhrp3* and consequent false-negative mRDTs; in the Amazon region of Peru (and contiguous areas) and Eritrea, such parasites are common and mRDTs based on alternative antigens (associated with lower sensitivity) have had to be introduced. Evidence that this problem exists in other areas is less robust, given the methodological challenges when confirming deletions. Important basic knowledge gaps remain: PCR primers and conditions have not been optimised, and the sensitivity of the full range of certified HRP2-based mRDTs for 'single-deleted' *pfhrp2*$^{-}$/*pfhrp3*$^{+}$ parasites has not been well studied (see Outstanding Questions). Appropriate design of prospective studies, ideally with consortium-level support from WHO, will be vital for decision-making in terms of continued use of HRP2-based mRDTs in a given area compared with the introduction of mRDTs detecting multiple antigens. A coordinated approach would also help to answer important wider questions regarding the long-term use of HRP2-based RDTs.

Resources

[i] https://apps.who.int/iris/bitstream/handle/10665/43939/9789241563697_eng.pdf

[ii] http://apps.who.int/medicinedocs/documents/s19105en/s19105en.pdf

[iii] http://documents.worldbank.org/curated/en/933151468740676854/pdf/multi-page.pdf

[iv] https://apps.who.int/iris/bitstream/handle/10665/275867/9789241565653-eng.pdf

[v] www.nasonline.org/publications/biographical-memoirs/memoir-pdfs/trager-william.pdf

[vi] https://plasmodb.org

[vii] https://apps.who.int/iris/bitstream/handle/10665/37106/WHO_TRS_839_eng.pdf

[viii] https://apps.who.int/iris/bitstream/handle/10665/276190/9789241514965-eng.pdf

[ix] http://apps.who.int/malaria/maps/threats/

[x] https://apps.who.int/iris/bitstream/handle/10665/258972/WHO-HTM-GMP-2017.18-eng.pdf

[xi] https://apps.who.int/iris/bitstream/handle/10665/260140/WHO-CDS-GMP-2018.03-eng.pdf

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Outstanding Questions

What is the current global extent of parasites with HRP2 deletion?

What are the optimal PCR conditions to detect *pfhrp2* and *pfhrp3*?

How do current RDTs perform on 'single-deleted' *pfhrp2*$^{-}$/*pfhrp3*$^{+}$ parasites? In areas where such parasites are prevalent, does the expression of *pfhrp3* provide an adequate safety net to allow continued use of HRP2-based RDTs?

At what level of HRP2 deletion should countries switch to mRDTs using alternative diagnostic antigens?

Are there alternative antigens that would be better than those currently available, with sufficient concentrations to allow sensitive diagnosis of all symptomatic cases and/or not affected by issues of gene deletion and antigen persistence?

How can other diagnostic approaches, such as DNA and bead-based antigen detection, contribute to diagnosis in peripheral settings?

What is the function of HRP2, and what forces promote deletion of *pfhrp2* and *pfhrp3*?

Can uRDT data help to target public health interventions in low transmission areas and, hence, contribute to elimination?

Can semiquantitative measurement of plasma HRP2, to distinguish severe malaria from severe febrile illness with coincidental peripheral blood parasitaemia, improve the care of severely ill African children?

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