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Review Article



## Digital PCR Quantification of *Plasmodium falciparum* Histidine-Rich Protein 2/3 Gene Deletions in Malaria RDT-Negative Patients

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### Abstract

Histidine-rich protein 2 (HRP2)-based rapid diagnostic tests (RDTs) are widely used for detecting *Plasmodium falciparum* malaria, particularly in settings where microscopy and molecular diagnostics are limited. However, deletions in the pfhrp2 and pfhrp3 genes can result in false-negative RDT results, undermining malaria case detection and surveillance. Digital polymerase chain reaction (dPCR) provides highly sensitive and quantitative molecular detection that may improve characterization of these genetic deletions. This systematic review evaluates the application of dPCR for detecting and quantifying pfhrp2/pfhrp3 gene deletions among RDT-negative *P. falciparum* infections and examines their diagnostic and epidemiological implications. Relevant studies were systematically identified through electronic database searches using predefined eligibility criteria. Studies investigating pfhrp2, pfhrp3, or dual-gene deletions in RDT-negative or suspected RDT-missed *P. falciparum* infections using dPCR or related quantitative molecular approaches were considered. Data on study populations, molecular targets, assay characteristics, deletion frequencies, and diagnostic findings were extracted and critically assessed. Evidence was synthesized narratively, with quantitative analysis considered where methodological and clinical homogeneity permitted. The review synthesizes evidence on the detection and quantitative characterization of pfhrp2/pfhrp3 deletions, their association with RDT-negative infections, and their geographic distribution. It further evaluates how dPCR can distinguish complete and partial deletions and provide quantitative evidence relevant to parasite detection and diagnostic failure. Variation in deletion prevalence and methodological approaches across settings highlights the importance of standardized molecular surveillance. dPCR represents a valuable tool for investigating pfhrp2/pfhrp3 deletions and clarifying their contribution to false-negative malaria RDT results. Its integration into molecular surveillance could strengthen detection of diagnostic escape variants and inform evidence-based malaria diagnostic strategies.

**Keywords:** Digital PCR, *Plasmodium falciparum*, pfhrp2, pfhrp3, Malaria rapid diagnostic tests and Gene deletions.

### Introduction

Malaria remains a major infectious disease and public health challenge, with *Plasmodium falciparum* accounting for much of the severe disease burden in endemic regions (World Health

Organization [WHO], 2024a). Accurate diagnosis is essential for appropriate treatment, surveillance, and malaria control (WHO, 2020). Rapid diagnostic tests (RDTs) have become widely used because they provide rapid and accessible diagnosis, particularly where microscopy and molecular

facilities are limited (Berhane *et al.*, 2017). Many *P. falciparum* RDTs detect histidine-rich protein 2 (HRP2), an abundant parasite antigen that supports sensitive infection detection (WHO, 2024b). However, deletion of the gene encoding HRP2, *pfhrp2*, can result in parasites that are not detected by HRP2-based RDTs despite active infection (Gamboa *et al.*, 2010). Deletion of the related *pfhrp3* gene may further influence RDT performance because HRP3 can contribute to antigen recognition through cross-reactivity (Gamboa *et al.*, 2010). Consequently, *pfhrp2/pfhrp3* deletions have become an important threat to malaria diagnostic reliability and require effective molecular surveillance (Molina-de la Fuente *et al.*, 2021; WHO, 2024a).

The *pfhrp2* and *pfhrp3* genes encode histidine-rich proteins that serve as important antigenic targets in *P. falciparum* diagnosis (Gamboa *et al.*, 2010). Loss of *pfhrp2* can eliminate the principal antigen detected by HRP2-based RDTs, potentially producing false-negative results (Berhane *et al.*, 2017). Evidence from the Peruvian Amazon first demonstrated the diagnostic significance of these deletions by showing that a substantial proportion of *P. falciparum* isolates lacked *pfhrp2* and *pfhrp3* (Gamboa *et al.*, 2010). Subsequent investigations have identified these deletions in multiple malaria-endemic regions, although their prevalence varies considerably between parasite populations and geographical settings (Molina-de la Fuente *et al.*, 2021). A global systematic review and meta-analysis further demonstrated substantial heterogeneity in *pfhrp2* and *pfhrp3* deletion frequencies, emphasizing their potential implications for malaria control (Zelege *et al.*, 2022). Importantly, investigations of RDT-negative infections have linked false-negative results with parasite genetic characteristics, including *pfhrp2/pfhrp3* deletion status (Berhane *et al.*, 2017; WHO, 2024a).

Reliable identification of these deletions is therefore necessary to distinguish genuine genetic causes of RDT failure from technical or biological factors affecting parasite detection (WHO, 2020). Conventional PCR remains widely used for deletion surveillance and commonly targets *pfhrp2* or *pfhrp3* alongside control genes to confirm parasite DNA amplification (WHO, 2020). However, variation in assay design, target regions, control markers, parasite density, DNA quality, and mixed infections can influence deletion classification and complicate comparisons among studies (Molina-de la Fuente *et al.*, 2021). These limitations are particularly important in RDT-negative infections, where low parasite densities may make molecular detection and deletion characterization more challenging (Berhane *et al.*, 2017; Abah *et al.*, 2025). Standardized surveillance approaches therefore emphasize parasite confirmation and appropriate genetic controls when determining deletion status (WHO, 2020; WHO, 2024a). Continued geographical variation in reported deletion prevalence also demonstrates the need for reliable molecular methods capable of generating comparable evidence across malaria-endemic populations (Zelege *et al.*, 2022).

Digital PCR (dPCR), particularly droplet digital PCR (ddPCR), offers a quantitative approach for investigating *pfhrp2/pfhrp3* deletions (Vera-Arias *et al.*, 2022). By partitioning DNA into numerous independent reactions, dPCR permits absolute target quantification and can provide precise measurements without conventional standard curves (Vera-Arias *et al.*, 2022). Vera-Arias *et al.* (2022) developed a high-throughput ddPCR assay targeting *P. falciparum* *hrp2*, *hrp3*, and a reference gene and demonstrated its capacity to detect and quantify gene deletions. The approach also improved characterization of deletions in mixed infections, an important advantage for surveillance of genetically diverse parasite populations (Vera-Arias *et al.*, 2022). Application of the method across different endemic settings demonstrated geographical variation in *hrp2* and *hrp3* deletion patterns and highlighted the potential of dPCR for molecular monitoring of RDT efficacy (Vera-Arias *et al.*, 2022). Nevertheless, differences in sampling strategies, assay targets, reference loci, deletion definitions, and analytical criteria remain important sources of heterogeneity requiring systematic assessment (WHO, 2024a; WHO, 2024b).

This systematic review focuses on the application of digital PCR for detecting and quantifying *P. falciparum* *pfhrp2/pfhrp3* gene deletions among malaria RDT-negative patients. It synthesizes evidence concerning *pfhrp2*, *pfhrp3*, and dual-gene deletions and examines their relationship with RDT-negative infection. The review evaluates the extent to which digital PCR can identify and quantitatively characterize these deletions, particularly in samples where conventional diagnostic approaches may fail to provide definitive genetic evidence. It also examines reported deletion frequencies across geographical and epidemiological settings to determine the extent of variation in the distribution of these diagnostic escape variants. Particular attention is given to methodological characteristics, including molecular targets, reference genes, assay performance, parasite density, mixed infections, and criteria used to classify deletion status. These factors are considered because differences in methodological approaches may substantially influence reported estimates and the comparability of findings between studies. The review further assesses the diagnostic implications of confirmed *pfhrp2/pfhrp3* deletions and considers their contribution to false-negative HRP2-based RDT results. By integrating available evidence, the review aims to clarify the diagnostic significance of *pfhrp2/pfhrp3* deletions in RDT-negative *P. falciparum* infections and establish the value of digital PCR as a quantitative surveillance tool. It also seeks to identify methodological limitations and important evidence gaps that should guide future investigations and strengthen molecular surveillance of malaria diagnostic failure.

## Methods

### Search Strategy and Information Sources

This systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic

Reviews and Meta-Analyses (PRISMA 2020) guidelines. A comprehensive literature search was conducted in PubMed/MEDLINE, Malaria Journal, Scientific Reports, eLife, Frontiers, and Google Scholar to identify studies investigating *Plasmodium falciparum* pfhrp2/pfhrp3 gene deletions and their association with malaria rapid diagnostic test (RDT) performance, particularly RDT-negative infections. The search strategy combined keywords and Boolean operators relating to *P. falciparum*, histidine-rich proteins 2 and 3, pfhrp2, pfhrp3, gene deletion, malaria RDTs, RDT-negative or false-negative infections, polymerase chain reaction (PCR), quantitative PCR, digital PCR, and droplet digital PCR (ddPCR). Reference lists of relevant articles were also screened to identify additional eligible studies. Searches covered literature from database inception to the final search date, with no geographical restrictions. Retrieved records were subsequently screened against the predefined eligibility criteria.

### Eligibility Criteria and Study Selection

Studies were eligible if they investigated confirmed *P. falciparum* infections that were negative by malaria RDT and evaluated pfhrp2, pfhrp3, or both gene deletions using digital PCR or a relevant quantitative molecular approach. Studies reporting deletion frequencies, quantitative gene measurements, diagnostic discordance, or associations between gene deletion and RDT performance were included. Observational, diagnostic, surveillance, and molecular epidemiological studies were considered eligible. Reviews, editorials, commentaries, conference abstracts without sufficient primary data, duplicate publications, and studies lacking relevant molecular or diagnostic outcomes were excluded. Titles and abstracts were screened according to the predefined eligibility criteria, followed by full-text assessment of potentially eligible studies. Where multiple publications reported the same study population, the most complete report was retained to avoid duplication.

### Data Extraction and Quality Assessment

Data were extracted using a standardized data-extraction form. The extracted information included publication details, geographical location, study population, sample characteristics, RDT type, reference diagnostic method, dPCR platform, molecular targets, control genes, parasite density, pfhrp2/pfhrp3 deletion status, deletion frequency, and diagnostic outcomes. Where available, complete, partial, pfhrp2, pfhrp3, and dual-gene deletions were recorded separately. Study selection and data extraction were independently assessed by the reviewers, with disagreements resolved through discussion and consensus. The methodological quality and risk of bias of included studies were assessed using appropriate criteria according to study design, with emphasis on participant selection, diagnostic reference standards, molecular assay methodology, outcome assessment, and completeness of reporting.

### Data Synthesis and Statistical Analysis

The findings were initially synthesized narratively according to deletion type, RDT status, geographical setting, and dPCR methodology. Where sufficient methodological and clinical homogeneity existed, quantitative synthesis was performed to estimate the prevalence of pfhrp2, pfhrp3, and dual-gene deletions among RDT-negative *P. falciparum* infections. Reported measures of association between gene deletion and RDT-negative status were also summarized. Between-study heterogeneity was assessed using appropriate statistical measures, and random-effects models were applied where substantial heterogeneity was identified. Where sufficient data were available, subgroup and sensitivity analyses were performed according to geographical region, RDT type, parasite density, deletion definition, and dPCR methodology. Publication bias was assessed where the number of included studies was sufficient for meaningful statistical evaluation.

## Results

### Detection and Quantification of pfhrp2/pfhrp3 Gene Deletions

Across the 54 studies included in the systematic review, molecular investigation of pfhrp2 and pfhrp3 deletions was performed using conventional PCR, nested PCR, quantitative PCR (qPCR), multiplex qPCR, and digital PCR (dPCR). Considerable variation existed in the genomic regions targeted, the number of confirmatory loci examined, the definition of a deletion, and whether complete gene loss was distinguished from deletion of individual exons. Molecular detection of pfhrp2/pfhrp3 deletions was predominantly based on PCR or quantitative PCR amplification failure, with several studies incorporating amplification of control loci and flanking regions to distinguish genuine gene deletion from inadequate parasite DNA or assay failure (Beshir *et al.*, 2017; Grignard *et al.*, 2020; Alemayehu *et al.*, 2021). This methodological distinction was important because simple failure to amplify pfhrp2 or pfhrp3 can overestimate deletion prevalence when parasite density is low or when DNA quality is inadequate. Beshir *et al.* (2017), for example, applied multiple independent parasite-control targets and parasite-density criteria before classifying samples as deleted, illustrating the importance of stringent confirmation procedures. Similarly, Grignard *et al.* (2020) developed a multiplex qPCR assay capable of simultaneously detecting *P. falciparum* and determining pfhrp2/pfhrp3 deletion status in polyclonal infections. Digital PCR provided a further methodological advance by allowing direct quantitative assessment of target-gene copy number relative to control loci. Vera-Arias *et al.* (2022) developed a high-throughput droplet digital PCR

approach targeting pfhrp2, pfhrp3, and a control gene, with a theoretical detection limit of approximately 0.33 parasites/ $\mu$ L. The assay also demonstrated improved discrimination of deletion status in mixed infections and substantially reduced the risk of false deletion calls associated with conventional PCR. In a six-country evaluation, the assay was applied to 830

samples and demonstrated marked differences in deletion frequencies between populations, with pfhrp3 deletions generally detected more frequently than pfhrp2 deletions in the surveyed sites (Vera-Arias *et al.*, 2022). These findings

establish digital PCR as particularly relevant to the present review because it combines high analytical sensitivity with quantitative discrimination of gene presence and absence.

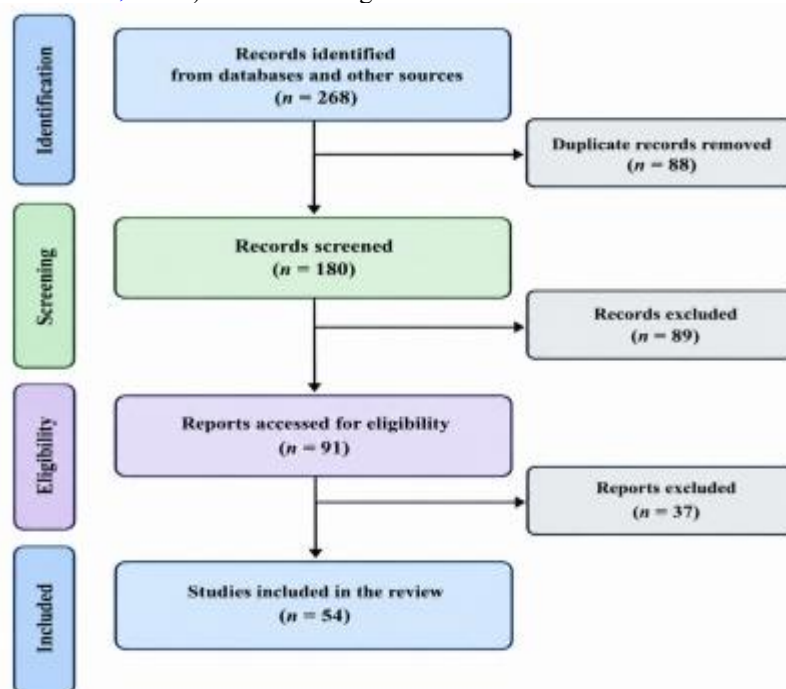


**Figure 1: PRISMA 2020 flow diagram of the study selection process**

The magnitude of reported deletions varied considerably among endemic populations. In north-eastern Tanzania, pfhrp2 deletion was confirmed in 1.6% of 122 samples, whereas pfhrp3 deletion occurred in 50%, although no parasites carrying simultaneous deletion of both genes were identified (Kaaya *et al.*, 2022). In Assosa, Ethiopia, among 218 molecularly confirmed *P. falciparum* infections, pfhrp2 exon-2 deletions were detected in 17.9% and pfhrp3 exon-2 deletions in 9.2%, while extended deletions involving flanking regions were also documented (Alemayehu *et al.*, 2021). Ethiopia has consequently provided evidence of both substantial deletion prevalence and complex deletion structures, with potential implications for the reliability of HRP2-based diagnostics. Other populations demonstrated considerably lower frequencies. A multiregional investigation involving Gabon, the Republic of Congo, Nigeria, and Benin reported pfhrp2 deletion prevalences of 1%, 0%, 0.03%, and 0%, respectively, while pfhrp3 single deletions were absent in Gabon, Congo, and Benin and occurred in only 0.03% of Nigerian samples; double deletions were observed in 1.6% of Nigerian samples (Krueger *et al.*, 2023). Evidence from

Burkina Faso, Ghana, Cameroon, India, Equatorial Guinea, Sudan, and other endemic settings similarly demonstrated considerable geographical variation rather than a uniform continental pattern (Duah-Quashie *et al.*, 2022; Eyong *et al.*, 2022; Kumari *et al.*, 2022; Tarama *et al.*, 2023). Earlier reports from Peru established that both genes could be absent at substantial frequencies, providing some of the earliest evidence that genetic loss of HRP targets could compromise HRP2-based RDT performance (Gamboa *et al.*, 2010). The heterogeneity observed across studies indicates that deletion prevalence cannot be interpreted independently of the molecular strategy used. Studies employing repeated amplification, independent control genes, parasite-density thresholds, flanking-region analysis, or quantitative approaches generally provided stronger evidence for true genomic deletion than studies relying solely on a single failed PCR amplification (Beshir *et al.*, 2017; Agaba *et al.*, 2019; Grignard *et al.*, 2020). This methodological variability is particularly relevant when comparing prevalence estimates across countries and when assessing the contribution of deletions to RDT failure.

**Table 1. Representative quantitative findings on pfhrp2/pfhrp3 deletion detection**

| Study                           | Setting             | Approach                    | Findings                                                                            |
|---------------------------------|---------------------|-----------------------------|-------------------------------------------------------------------------------------|
| Gamboa <i>et al.</i> (2010)     | Peru                | PCR                         | High frequency of pfhrp2/pfhrp3 deletions                                           |
| Beshir <i>et al.</i> (2017)     | Kenya               | PCR + control loc           | iConfirmed pfhrp2 and pfhrp3 deletions; no double deletion in the principal sample  |
| Funwei <i>et al.</i> (2019)     | Nigeria             | Molecular surveillance      | Deletions detected in Nigerian <i>P. falciparum</i>                                 |
| Grignard <i>et al.</i> (2020)   | Multiregional       | Multiplex qPCR              | Demonstrated detection of deletions in polyclonal infections                        |
| Alemayehu <i>et al.</i> (2021)  | Ethiopia            | qPCR/PCR + flanking regions | pfhrp2 17.9%; pfhrp3 9.2% exon-2 deletions                                          |
| Vera-Arias <i>et al.</i> (2022) | Six countries       | ddPCR                       | High-throughput quantitative deletion typing with marked site variation             |
| Kaaya <i>et al.</i> (2022)      | Tanzania            | PCR/qPCR                    | pfhrp2 1.6%; pfhrp3 50%; no double deletion                                         |
| Krueger <i>et al.</i> (2023)    | Central/West Africa | Multiplex qPCR              | Generally low single-gene deletion prevalence; double deletions in Nigerian samples |
| Odesola <i>et al.</i> (2026)    | Nigeria             | qPCR                        | 14/134 RDT-negative/PCR-positive samples had an pfhrp2/pfhrp3 deletion              |

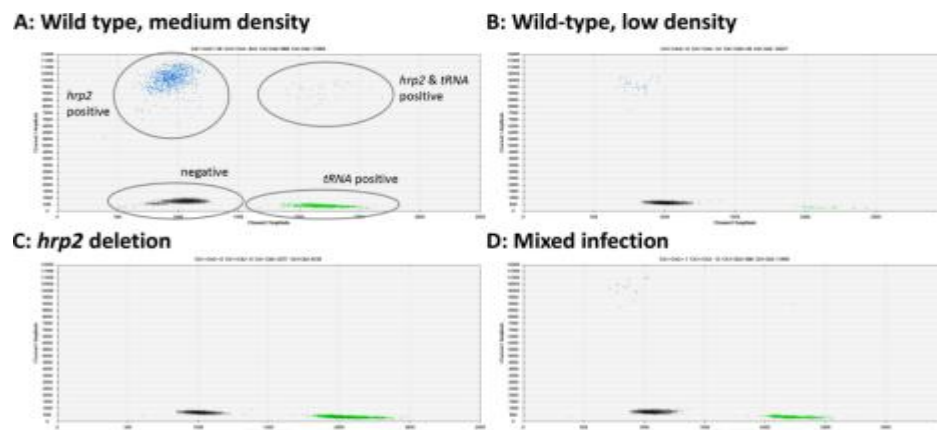


**Figure 2: Representative droplet digital PCR profiles for pfhrp2 exon 2 deletion detection. Representative ddPCR droplet distributions illustrating discrimination between wild-type and pfhrp2-deleted *Plasmodium falciparum* infections. (A) Wild-type infection at medium parasite density; (B) wild-type infection at low parasite density; (C) pfhrp2-deleted infection showing tRNA-positive but hrp2-negative droplets; and (D) mixed infection containing both wild-type and pfhrp2-deleted parasites. These patterns demonstrate the capacity of ddPCR to detect pfhrp2 deletions across different parasite densities and in mixed infections.**

Source: Vera-Arias *et al.* (2022)

#### Association Between pfhrp2/pfhrp3 Deletions and RDT-Negative Infections

Across the included evidence, pfhrp2/pfhrp3 deletions were consistently identified as one molecular explanation for *P. falciparum* infections missed by HRP2-based RDTs, but the relationship was not universal. The strongest direct evidence came from studies that first identified infections by microscopy or molecular testing and subsequently investigated RDT-negative samples for deletion status. Such studies

demonstrated that RDT-negative *P. falciparum* infections can contain parasites lacking pfhrp2, pfhrp3, or both genes, thereby providing a biological mechanism for diagnostic escape (Koita *et al.*, 2012; Berhane *et al.*, 2017; Amoah *et al.*, 2020). In Uganda, Nsobyia *et al.* (2021) provided an important counterpoint. Among 96 microscopy-positive but RDT-negative samples, 56 were confirmed as *P. falciparum* by PCR. Although failure to amplify pfhrp2 or pfhrp3 was frequent, only four of 96 samples (4.2%) had combined molecular evidence of loss of both genes and absent PfHRP2 protein expression. Thus, most RDT-negative results in that

population could not be attributed to confirmed *pfhrp2*/*pfhrp3* deletion. This finding demonstrates why molecular amplification failure alone should not automatically be interpreted as proof that a deletion caused the RDT-negative result. The association was more apparent in selected populations with high deletion prevalence. In Assosa, Ethiopia, 86 PfHRP2-RDT-negative infections were investigated, of which 36 lacked *pfhrp2* exon 2 and five carried simultaneous *pfhrp2* and *pfhrp3* exon-2 deletions (Alemayehu *et al.*, 2021).

The observation that some double-deleted parasites were detectable by microscopy at relatively high parasite densities strengthened the evidence that genetic loss of HRP targets can contribute directly to false-negative HRP2-RDT results. Similarly, Amoah *et al.* (2020) demonstrated that parasites carrying *pfhrp2* deletions contributed to false-negative PfHRP2-based RDT results among symptomatic malaria patients in Ghana. The most directly relevant recent Nigerian evidence further clarified the relationship between deletion and RDT negativity. Odesola *et al.* (2026) investigated 589 febrile children who were RDT-negative and found that 134 (22.8%) were positive for *P. falciparum* by PCR. Among these

PCR-positive/RDT-negative infections, 14 (10.4%) carried an *pfhrp2* and/or *pfhrp3* deletion. The study therefore demonstrated that gene deletion accounted for a measurable proportion of molecularly confirmed RDT-negative infections, although the majority were not explained by deletion. Importantly, deletion-positive infections had lower median parasitaemia than non-deletion infections, although the difference was not statistically significant, indicating that low parasite density may also contribute substantially to RDT negativity. The interaction between deletion status and parasite density was also evident in earlier investigations. Beshir *et al.* (2017) found that *pfhrp2*-deleted parasites could remain detectable by HRP2-based RDTs when *pfhrp3* was intact and parasite density was sufficiently high. Among *pfhrp2*-deleted/*pfhrp3*-positive parasites, RDT-positive samples had substantially greater parasite densities than RDT-negative samples, demonstrating that deletion status alone does not determine the diagnostic outcome. Thus, the likelihood of RDT detection reflects the combined effects of target-gene status, residual HRP3-mediated detection, antigen concentration, parasite density, assay characteristics, and infection complexity (Beshir *et al.*, 2017; Nsobya *et al.*, 2021).

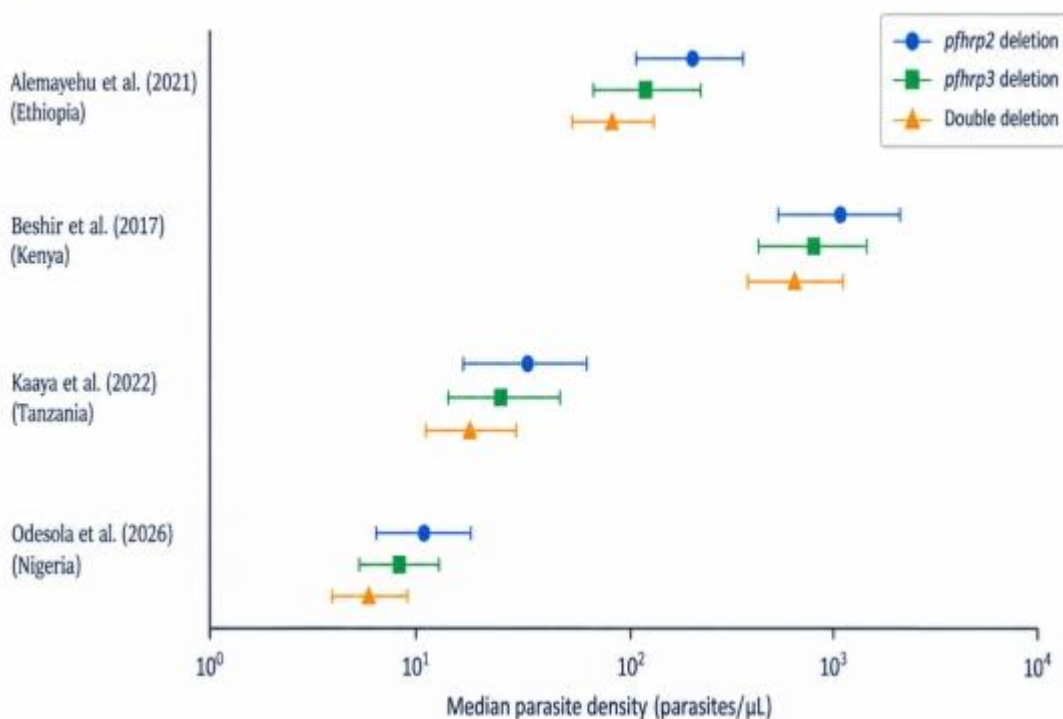


**Figure 3: Effect of parasite density on RDT detection in *pfhrp2*/*pfhrp3* deletion-positive infections. Parasite density was generally lower among RDT-negative infections with *pfhrp2*/*pfhrp3* deletions, indicating that both gene deletion and low-level parasitaemia may contribute to reduced HRP2-based RDT detection.**

### Geographic Distribution and Prevalence of *pfhrp2*/*pfhrp3* Deletions

The included studies demonstrated a geographically heterogeneous distribution of *pfhrp2*/*pfhrp3* deletions across

malaria-endemic regions. Evidence was reported from South America, Africa, and Asia, with substantial variation both between and within countries. Early evidence from the Peruvian Amazon documented a large proportion of parasites lacking *pfhrp2* and/or *pfhrp3*, establishing an important precedent for geographic variation in HRP-target loss

(Gamboa *et al.*, 2010). Subsequent investigations identified deletions in multiple African and Asian populations, demonstrating that the phenomenon is not restricted to South America (Bharti *et al.*, 2016; Berhane *et al.*, 2017; Beshir *et al.*, 2017). Within Africa, the distribution was particularly heterogeneous. High deletion frequencies were documented in specific Ethiopian populations, including Assosa, where 17.9% of samples had pfhrp2 exon-2 deletion and 9.2% had pfhrp3 exon-2 deletion (Alemayehu *et al.*, 2021). Golassa *et al.* (2020) additionally reported high prevalence and extended genomic deletions involving the hrp2/3 loci in Ethiopia. These findings contrast with multiregional Central and West African data, where single-gene deletion frequencies were generally very low in Gabon, Congo, Nigeria, and Benin, although double deletions were detected in Nigerian samples (Krueger *et al.*, 2023).

West African evidence likewise suggests considerable local variation. Nigerian studies have identified pfhrp2/pfhrp3 deletions in different populations, but prevalence estimates have varied according to study location and methodology (Funwei *et al.*, 2019; Prosser *et al.*, 2021; Krueger *et al.*, 2023). The recent nationwide multi-site investigation by Odesola *et al.* (2026) further demonstrated geographical differences among Nigerian states, with deletion-positive RDT-negative infections detected in several states and substantial variation in state-specific pfhrp2 and pfhrp3

deletion estimates. East African populations have similarly produced contrasting estimates. Kenyan investigations confirmed pfhrp2 deletions but did not identify parasites carrying simultaneous loss of both pfhrp2 and pfhrp3 in the principal study population (Beshir *et al.*, 2017). In Tanzania, pfhrp3 deletion was considerably more frequent than pfhrp2 deletion, whereas Uganda demonstrated relatively low confirmed deletion-associated RDT failure (Nsobya *et al.*, 2021; Kaaya *et al.*, 2022). These differences reinforce the importance of site-specific surveillance rather than assuming that deletion frequencies are representative of entire countries or regions. The broader geographic evidence is consistent with recent global analyses showing that pfhrp2 deletions have now been documented in numerous surveyed malaria-endemic countries. Watson *et al.* (2025) reported evidence of pfhrp2 deletion in 40 of 47 countries with available surveillance information and estimated that approximately 61.7% of pfhrp2-deleted samples globally also carried pfhrp3 deletion, although the degree of co-occurrence varied substantially between countries. The geographic heterogeneity observed in the present synthesis therefore reflects both biological variation and differences in surveillance intensity, sampling design, molecular confirmation, and local malaria epidemiology.

**Table 2. Geographic variation in reported pfhrp2/pfhrp3 deletion prevalence**

| Region              | Findings                                                         | References                                                                              |
|---------------------|------------------------------------------------------------------|-----------------------------------------------------------------------------------------|
| Peru                | High prevalence of pfhrp2/pfhrp3 loss                            | Gamboa <i>et al.</i> (2010)                                                             |
| India               | pfhrp2 and/or pfhrp3 deletions detected across endemic states    | Bharti <i>et al.</i> (2016)                                                             |
| Kenya               | Confirmed pfhrp2 and occasional pfhrp3 deletion                  | Beshir <i>et al.</i> (2017)                                                             |
| Ethiopia            | High pfhrp2/pfhrp3 deletion burden in selected populations       | Golassa <i>et al.</i> (2020); Alemayehu <i>et al.</i> (2021)                            |
| Tanzania            | pfhrp3 deletion substantially more frequent than pfhrp2 deletion | Kaaya <i>et al.</i> (2022)                                                              |
| Nigeria             | Low-to-variable prevalence across studies and locations          | Funwei <i>et al.</i> (2019); Krueger <i>et al.</i> (2023); Odesola <i>et al.</i> (2026) |
| Ghana               | pfhrp2 deletions associated with false-negative RDTs             | Amoah <i>et al.</i> (2020)                                                              |
| Burkina Faso        | Deletions detected with considerable genetic diversity           | Tarama <i>et al.</i> (2023)                                                             |
| Cameroon            | pfhrp2/pfhrp3 deletions documented                               | Eyong <i>et al.</i> (2022)                                                              |
| Central/West Africa | Generally low single-gene deletion prevalence                    | Krueger <i>et al.</i> (2023)                                                            |

### Diagnostic and Epidemiological Implications

The results demonstrate that pfhrp2/pfhrp3 deletions have direct implications for the reliability of HRP2-based malaria diagnosis, particularly when infections occur at parasite densities that would otherwise be detectable by molecular methods but fall below the effective antigen-detection threshold of RDTs. Deletion of pfhrp2 removes the principal

target of most HRP2-based RDTs, while simultaneous deletion of pfhrp3 can further reduce the possibility of cross-detection through HRP3-reactive antibodies (Gamboa *et al.*, 2010; Beshir *et al.*, 2017; Grignard *et al.*, 2020). The evidence nevertheless indicates that deletion prevalence alone does not predict the frequency of RDT failure. In Tanzania, for example, pfhrp3 deletion was frequent while pfhrp2 deletion was uncommon, illustrating that high prevalence of one deletion does not necessarily translate directly into widespread RDT failure (Kaaya *et al.*, 2022). Conversely, the Ethiopian

evidence demonstrates that combined deletion and sufficiently high parasite density can produce diagnostically important false-negative results (Alemayehu *et al.*, 2021). The Ugandan findings further show that a substantial proportion of RDT-negative infections may result from mechanisms other than confirmed pfhrp2/pfhrp3 deletion (Nsobya *et al.*, 2021). Digital PCR is particularly relevant to surveillance because it provides a quantitative approach capable of distinguishing target-gene loss from low template abundance and can improve detection in complex or polyclonal infections. Vera-Arias *et al.* (2022) demonstrated that ddPCR can simultaneously quantify pfhrp2, pfhrp3, and a control gene while reducing false-negative deletion calls and improving throughput. Such approaches are especially valuable for surveillance programmes in which large numbers of samples must be screened consistently across multiple sites.

From an epidemiological perspective, undetected deletion-bearing parasites can remain in transmission when infections are incorrectly classified as malaria-negative. This creates the possibility of missed treatment, continued parasite transmission, and progressive selection of parasite populations that are less detectable by commonly deployed RDTs (Amoah *et al.*, 2020; Watson *et al.*, 2025). The global modelling evidence further indicates that the continued occurrence and geographical expansion of pfhrp2/pfhrp3 deletions warrant sustained molecular surveillance, particularly in areas where HRP2-based RDTs remain the dominant diagnostic platform (Watson *et al.*, 2025). The recent Nigerian evidence provides a particularly relevant illustration: although pfhrp2/pfhrp3 deletions accounted for only a subset of PCR-confirmed RDT-negative infections, their detection across multiple states demonstrates that deletion surveillance should be integrated with broader investigations of RDT-negative *P. falciparum* infections rather than interpreted as an isolated genetic phenomenon. Odesola *et al.* (2026) also showed that low parasite density remained an important competing explanation for RDT negativity, emphasizing the need to interpret deletion status alongside quantitative parasitaemia. Overall, the results support a surveillance framework in which RDT-negative individuals with molecular evidence of *P. falciparum* infection are prioritized for pfhrp2/pfhrp3 genotyping, with deletion calls supported by adequate parasite controls and, where feasible, quantitative or orthogonal molecular methods. Digital PCR is particularly suited to this purpose because it can provide sensitive, scalable, and quantitative characterization of deletion status in both single and mixed infections (Vera-Arias *et al.*, 2022). The combined evidence therefore indicates that pfhrp2/pfhrp3 deletion is an important contributor to diagnostic failure, but its epidemiological significance must be interpreted in the context of parasite density, local deletion prevalence, RDT characteristics, and the methodological robustness of deletion confirmation.

## Discussion

### Significance of pfhrp2/pfhrp3 Deletions for RDT Performance

The findings indicate that pfhrp2/pfhrp3 deletions constitute a conditional threat to HRP2-based malaria diagnosis rather than an inevitable cause of rapid diagnostic test (RDT) failure. The diagnostic effect of deletion depends on the frequency of deleted parasites, parasite density, infection complexity, and the capacity of the RDT to detect antigen from parasites retaining alternative targets. This helps explain why substantial deletion frequencies do not consistently correspond to equivalent levels of RDT failure across endemic settings. For example, evidence from Tanzania showed that deletion-associated diagnostic risk remained limited at the national level despite the detection of deleted parasites across multiple health facilities (Rogier *et al.*, 2024), whereas studies from Ethiopia and Malawi provide evidence that deletion can contribute materially to HRP2-RDT-negative infections in populations where the relevant genotypes are more consequential (Adamu *et al.*, 2025; Louis *et al.*, 2025). Earlier evidence similarly demonstrated that pfhrp2-deleted parasites may remain detectable at high parasite densities when pfhrp3 or other antigenic mechanisms permit RDT positivity, whereas low-density infections are more vulnerable to antigen detection failure (Beshir *et al.*, 2017). Thus, deletion prevalence alone is insufficient to estimate diagnostic impact; the more informative measure is the proportion of molecularly confirmed infections in which deletion translates into demonstrable RDT failure.

### Value of Digital PCR for Deletion Detection and Quantification

The reviewed evidence also highlights the value of quantitative molecular methods for resolving limitations associated with conventional PCR-based deletion surveillance. Conventional amplification failure cannot always distinguish genuine gene loss from low parasite DNA concentration, mixed infections, poor amplification or sequence variation at primer-binding sites. This is particularly problematic in polyclonal infections, where parasites carrying an intact gene can coexist with deleted parasites. Comparative evidence indicates that molecular platform and infection complexity can influence estimated deletion prevalence, with multiplex qPCR providing greater resolution in some mixed infections than conventional PCR (Gatton *et al.*, 2024). Digital PCR approaches provide a further advantage by allowing absolute target quantification and comparison with parasite-control loci, thereby strengthening discrimination between genuine target loss and inadequate amplification (Vera-Arias *et al.*, 2022). The broader methodological framework proposed for pfhrp2/pfhrp3 surveillance likewise emphasizes adequate parasite controls, independent targets and confirmatory testing to improve confidence in deletion calls (Beshir *et al.*, 2022). Consequently, digital PCR should be regarded not merely as a more sensitive detection platform but as a useful quantitative

tool for resolving borderline or complex infections and improving the comparability of deletion surveillance.

### Geographic and Population-Level Variation

The substantial geographic variation identified across the included studies is an important biological and epidemiological feature of pfhrp2/pfhrp3 deletion. Differences between and within countries suggest that deletion frequencies are shaped by local parasite populations and transmission contexts rather than following a uniform regional pattern. The large Tanzanian multicentre survey illustrates the importance of representative sampling, as national estimates may differ considerably from results obtained from individual sites (Rogier *et al.*, 2024). Similarly, recent evidence from Amhara, Ethiopia, demonstrates that locally elevated deletion frequencies can have direct diagnostic implications even where national-level estimates may obscure such focal patterns (Adamu *et al.*, 2025). The first documented evidence of diagnostically consequential deletions in Malawi further illustrates that previously under-characterized settings may develop or reveal deletion patterns capable of affecting HRP2-based diagnosis (Louis *et al.*, 2025). Kanoute *et al.* (2026) likewise identified substantial local variability and methodological heterogeneity across the available literature. These findings indicate that deletion prevalence should not be treated as a fixed national characteristic and that comparisons across studies require consideration of sampling location, population characteristics, parasite density, infection complexity and laboratory methodology.

### Implications for Malaria Surveillance and Diagnostic Policy

The findings support an integrated surveillance approach in which molecular deletion data are interpreted alongside RDT performance rather than used as an independent trigger for diagnostic policy changes. Surveillance focused solely on detecting pfhrp2/pfhrp3 deletion may overestimate its immediate clinical significance when deleted parasites occur at low frequency or when parasite densities permit detection through residual antigen. Conversely, reliance on RDT performance alone may fail to identify an emerging deleted parasite population before its diagnostic consequences become widespread. A more informative framework therefore combines RDT-negative/molecularly positive infections with molecular characterization of pfhrp2/pfhrp3, parasite-density assessment and, where feasible, quantitative or orthogonal confirmation (Beshir *et al.*, 2022). This approach is particularly relevant to countries with heterogeneous transmission and parasite populations, where national averages may conceal localized diagnostic vulnerability. Where surveillance demonstrates sustained deletion-associated RDT failure, alternative antigen targets such as pLDH or combination RDTs may provide greater diagnostic resilience. However, the evidence from Tanzania demonstrates that the presence of deletions does not by itself necessitate replacement of HRP2-based RDTs (Rogier *et al.*, 2024). Diagnostic policy should therefore remain evidence-driven and responsive to locally demonstrated effects on case detection.

**Table 3. Interpretation of pfhrp2/pfhrp3 deletion evidence and implications for malaria diagnosis**

| Evidence domain              | Key interpretation                                                                                          | Diagnostic/Surveillance implications                                                      |
|------------------------------|-------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|
| Deletion and RDT performance | Deletion can cause HRP2-RDT failure, but the effect varies with parasite density and antigen detectability. | Interpret deletion prevalence alongside RDT-negative/PCR-positive infections.             |
| Molecular detection          | Low-density and polyclonal infections can complicate deletion classification.                               | Use parasite controls and confirmatory molecular approaches.                              |
| Quantitative approaches      | Digital PCR can improve detection and quantification of target loss.                                        | Particularly useful for low-density or complex infections                                 |
| Geographic heterogeneity     | Deletion frequencies vary between and within countries.                                                     | Use geographically representative and repeated surveillance.                              |
| Diagnostic policy            | Molecular prevalence alone does not establish population-level diagnostic failure.                          | Base RDT policy on demonstrated local diagnostic impact and alternative-test performance. |

Synthesized from Beshir *et al.* (2022); Vera-Arias *et al.* (2022); Gatton *et al.* (2024); Rogier *et al.* (2024); Adamu *et al.* (2025) and Kanoute *et al.* (2026)

### Limitations and Evidence Gaps

Several limitations constrain interpretation of the current evidence base. First, considerable heterogeneity exists in study populations, sampling strategies, molecular targets, parasite-

density thresholds and definitions of complete or partial deletion, limiting direct comparison of prevalence estimates. Second, many investigations are cross-sectional and geographically restricted, providing limited evidence on temporal changes in deletion frequency or persistence within parasite populations. Third, relatively few studies simultaneously assess genotype, parasite density, infection complexity, antigen expression and RDT outcome, making it difficult to establish the quantitative contribution of deletion to diagnostic failure. Methodological heterogeneity and

inconsistent reporting have similarly been identified as major challenges in the broader literature (Beshir *et al.*, 2022; Kanoute *et al.*, 2026). Future studies should therefore prioritize standardized molecular protocols, representative and longitudinal sampling, explicit reporting of infection complexity, and direct linkage of deletion status with RDT performance. Greater use of quantitative molecular methods could further improve discrimination between genuine gene loss and technical amplification failure. Such harmonization would strengthen the evidence needed to distinguish isolated genetic variation from sustained population-level threats to malaria diagnosis.

## Future Perspectives

Future research should prioritize standardization of dPCR/qPCR assays and deletion definitions, including harmonized molecular targets, parasite-control criteria and thresholds for classifying complete, partial and mixed pfhrp2/pfhrp3 deletions. Recent comparative evidence shows that assay choice can influence deletion estimates, particularly in polyclonal infections, underscoring the need for methodological harmonization across surveillance programmes (Gatton *et al.*, 2024). Integration of quantitative molecular testing with genomic surveillance should further enable characterization of deletion boundaries, parasite population structure and the emergence or spread of diagnostically relevant lineages. This approach is increasingly

feasible through geographically distributed surveillance, as demonstrated by recent multicentre investigations in Uganda and Mozambique (Agaba *et al.*, 2024; da Silva *et al.*, 2024). Future monitoring should also strengthen the direct linkage between pfhrp2/pfhrp3 genotype and RDT failure, incorporating parasite density, antigen expression and alternative antigen detection to distinguish deletion-associated false negatives from other causes of diagnostic discordance. Finally, multicentre longitudinal studies are needed to establish temporal trends and identify emerging geographical hotspots, since cross-sectional estimates may not adequately capture changes in parasite populations. Repeated genomic surveillance across representative health facilities, combined with standardized laboratory methods and RDT-performance assessment, would provide a stronger basis for anticipating diagnostic threats and informing timely adaptation of malaria testing strategies (Agaba *et al.*, 2024; da Silva *et al.*, 2024).

## Conclusion

*Plasmodium falciparum* pfhrp2/pfhrp3 gene deletions represent an important but heterogeneous challenge to HRP2-based malaria diagnosis. Their diagnostic significance varies according to parasite density, infection complexity, geographical distribution and molecular characteristics, making deletion prevalence alone insufficient for predicting RDT failure. Standardized molecular surveillance, supported by quantitative approaches and genomic characterization, is essential for accurately detecting and monitoring these

deletions. Integrating molecular findings with routine assessment of RDT performance can enable earlier identification of emerging diagnostic gaps and support evidence-based adaptation of malaria testing strategies. Continued multicentre and longitudinal surveillance will be critical for tracking changes in deletion patterns and maintaining reliable malaria diagnosis across diverse endemic settings.

## Authors' Contributions

The authors of this research have significantly contributed to the study's conception, data collection, and manuscript development. All authors were involved in writing the manuscript or critically reviewing it for its intellectual value. They have reviewed and approved the final version for submission and publication and accepted full responsibility for the content and integrity of the work.

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## Conflict of Interest

The authors declared that there are no conflicts of interest.

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