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Molecular Innovations in Malaria Diagnostics: A Critical Review of Multiplex PCR Approaches for Human and Vector Surveillance

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Abstract

Molecular methods are transforming malaria diagnosis and surveillance by enabling highly sensitive and specific identification of *Plasmodium* species, including mixed infections that microscopy often misses. Multiplex PCR assays offer additional advantages by detecting several species simultaneously in a single reaction, reducing reagent use and processing time. This review synthesizes current advances in multiplex PCR detection of *Plasmodium falciparum*, *P. ovale*, and *P. malariae* in human and mosquito samples, drawing on published literature and recent assay development efforts. We highlight methodological strengths, including species-specific primer design, improved detection of mixed infections, and applicability to entomological surveillance. We also critically examine persistent challenges small validation sample sizes, lack of analytical sensitivity data, absence of quantified parasite loads, and limitations in mosquito specimen characterization. Finally, we propose a framework for future assay validation that includes parasite quantification, rigorous analytical performance metrics, tissue-specific mosquito sampling, and expanded epidemiological studies. This synthesis demonstrates that multiplex PCR holds strong promise for integrated malaria surveillance but requires standardized validation pipelines before widespread adoption.

Keywords

Malaria Diagnostics, Multiplex PCR, *Plasmodium* Species, Vector Surveillance, Assay Validation

1. Introduction

Malaria remains one of the most important parasitic diseases worldwide, with

more than 240 million cases and over 600,000 deaths reported annually, the vast majority occurring in sub-Saharan Africa [1]. Despite substantial progress over the past two decades, malaria transmission persists in many endemic regions, underscoring the need for accurate, sensitive, and context-appropriate diagnostic tools. Reliable diagnosis is essential not only for individual case management but also for surveillance, stratification of transmission intensity, and evaluation of control and elimination strategies.

Conventional diagnostic methods—light microscopy and rapid diagnostic tests (RDTs)—have played a central role in malaria control. However, both approaches suffer from well-recognised limitations. Microscopy requires skilled personnel, is time-consuming, and has limited sensitivity for low-density infections and mixed-species cases [2]. RDTs, while operationally simple and widely deployed, show variable sensitivity across settings, perform poorly for non-*Plasmodium falciparum* species, and may yield false-positive results due to persistent antigens following parasite clearance [3]. These shortcomings are particularly problematic in low-transmission or pre-elimination settings, where asymptomatic and low-parasitaemia infections contribute disproportionately to residual transmission.

To overcome these constraints, nucleic acid-based diagnostic approaches have gained increasing attention. Techniques such as nested PCR, quantitative PCR (qPCR), loop-mediated isothermal amplification (LAMP), and next-generation sequencing provide substantially higher sensitivity and specificity than conventional methods [4]-[6]. Among these, multiplex PCR—allowing the simultaneous detection of multiple *Plasmodium* species in a single reaction—offers a practical compromise between analytical performance, cost, and laboratory feasibility. This approach is especially relevant in regions where *P. falciparum*, *P. ovale*, and *P. malariae* co-circulate, and where accurate species identification is critical for appropriate treatment and surveillance.

Beyond clinical diagnosis, multiplex PCR has become increasingly valuable for entomological surveillance. Molecular detection of *Plasmodium* infections in mosquito vectors enables identification of low-intensity infections that are frequently missed by microscopy-based methods and provides insights into transmission dynamics, vector competence, and intervention impact [7] [8]. Such applications are particularly relevant for countries transitioning from control to elimination, where detecting residual transmission foci is a priority.

Despite its promise, the validation and standardisation of multiplex PCR assays remain inconsistent across published studies. Key challenges include the determination of limits of detection, assay reproducibility, and performance across diverse epidemiological settings. In addition, vector sampling strategies, mosquito infection rates, and laboratory capacity vary widely between malaria-endemic countries, influencing the operational utility of molecular tools. Consequently, validation frameworks developed in one context may not be directly transferable to another.

In this review, we synthesise current advances in multiplex PCR assays targeting

Plasmodium falciparum, *P. ovale*, and *P. malariae* in both human and mosquito samples. We highlight progress in species-specific primer design and applications to entomological surveillance, while critically examining remaining technical and operational gaps. Importantly, we contextualise multiplex PCR deployment within heterogeneous malaria control programmes, emphasising the need for locally adapted validation strategies before integration into national malaria control and elimination efforts.

2. Primer Design and Species Specificity

The success of multiplex PCR critically depends on careful primer design to ensure high specificity and avoid cross-reactivity. Padley *et al.* pioneered species-specific primer sets targeting the 18S rRNA gene, generating amplicons of distinct sizes for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* [9]. Key considerations include targeting conserved but species-discriminatory regions, designing primers with compatible melting temperatures, minimizing primer-primer interactions, and ensuring adequate separation of amplicon sizes for gel-based detection. Failure to meet these criteria often results in preferential amplification of dominant templates or loss of sensitivity for minority species. Given the heterogeneity in validation practices across multiplex PCR studies, we propose a standardized minimum reporting and validation checklist (Table 1).

Table 1. Validation checklist for multiplex PCR assays for *Plasmodium* detection in human and mosquito samples.

Validation domain	Key item	What to report/minimum requirement	Why it matters
Assay design	Target selection	Gene target(s) (e.g., 18S rRNA), rationale, accession(s) for reference sequences	Ensures comparability and biological relevance
Assay design	Primer/probe sequences	Full sequences, expected amplicon sizes, Tm/GC%, in silico specificity checks	Prevents cross-reactivity and mis-priming
Assay design	Multiplex compatibility	Primer-dimer assessment; optimization strategy (primer ratios, $MgCl_2$, cycling)	Reduces competition and dropouts in multiplexing
Controls	Positive controls	Species-specific positive controls for each target (Pf, Pm, Po, Pv if included)	Confirms each channel is functional
Controls	Negative controls	No-template control + extraction blanks + uninfected human/mosquito DNA	Detects contamination and non-specific amplification
Controls	Internal control	Host gene or exogenous spike-in (when feasible)	Flags inhibition and extraction failure
Analytical validation	Limit of detection (LOD)	Serial dilutions of quantified material; LOD95 (or defined threshold) per species	Defines sensitivity, especially for low-density infections

Continued

Analytical validation	Analytical sensitivity/specificity	Sensitivity and specificity vs. reference method with 95% CI	Enables objective performance comparison
Analytical validation	Dynamic range	Range of detectable concentrations; saturation effects	Supports interpretation across parasitemia levels
Analytical validation	Repeatability/reproducibility	Intra-run and inter-run metrics (≥2 operators/ ≥2 days, when possible)	Demonstrates robustness
DNA input & quality	DNA quantity/purity	DNA concentration (ng/μL) and purity (A260/280 or equivalent)	Improves reproducibility across labs
DNA input & quality	Inhibition testing	Dilution/cleanup strategy; inhibition control outcomes	Avoids false negatives from inhibitors
Clinical validation	Sample size	Adequate n per species and negatives; specify inclusion/exclusion criteria	Prevents overclaiming from small datasets
Clinical validation	Parasite density	Microscopy parasite density or qPCR estimate; stratify by density	Links detection to biologically meaningful thresholds
Clinical validation	Mixed infections	Validate on artificial and/or natural mixed infections	Confirms multiplex advantage and avoids masking
Entomological validation	Mosquito species ID	Morphological + molecular confirmation when needed	Ensures epidemiologic relevance
Entomological validation	Collection context	Where/how collected (indoor/outdoor, resting/landing, field/colony), season/site	Affects infection prevalence interpretation
Entomological validation	Tissue processed	Whole body vs midgut vs salivary glands; justify choice	Distinguishes infection from transmission potential
Entomological validation	Stage-specific interpretation	Clarify what PCR positivity means (DNA signal vs viable parasites)	Avoids overinterpreting "infectiousness"
Reporting & transparency	Full protocol details	Cycling conditions, reagent brands, gel %/run settings (or qPCR chemistry)	Enables replication
Reporting & transparency	Data reporting	Handling of weak bands/indeterminate calls; failed amplifications; missing data	Prevents inflated accuracy estimates
Reporting & transparency	Reference standards	Comparator assay (nested PCR/qPCR) and microscopy definition; blinding if used	Strengthens credibility

3. Advantages and Limitations of Multiplex PCR

Multiplex PCR reduces reagent use and processing time, improves throughput, and enhances detection of mixed infections that are frequently underestimated by microscopy [10]. These features make it attractive for large-scale surveillance in

resource-limited settings. However, multiplexing introduces technical challenges, including primer competition, reduced analytical sensitivity compared with nested PCR or qPCR, and difficulties in balancing amplification efficiency across targets [11]. Importantly, many studies fail to report critical analytical metrics such as limits of detection (LOD), DNA input concentrations, or assay reproducibility, limiting cross-study comparison and standardization.

4. Gaps in Current Evidence

A recurring limitation across the literature is the use of small sample sizes, often involving fewer than 20 clinical samples or a handful of mosquito specimens, which undermines statistical robustness [12]. Parasite densities are rarely quantified prior to molecular testing, despite their strong influence on detection probability. In entomological studies, mosquito species identification, collection methods, tissue specificity (midgut versus salivary glands), and parasite stage are frequently omitted, constraining interpretation of transmission relevance [13]. Additionally, the exclusion of *P. vivax* from many multiplex panels substantially reduces global applicability, given its major contribution to malaria burden outside Africa [14].

5. Conclusion

Multiplex PCR represents a powerful diagnostic and surveillance tool for malaria, with clear advantages in sensitivity, species discrimination, and detection of mixed infections. However, most published assays—including recent triplex PCR systems—lack rigorous analytical and epidemiological validation. Establishing standardized guidelines for sample size, parasite quantification, LOD determination, and vector sampling is essential before multiplex PCR can be reliably integrated into national malaria surveillance and elimination programs.

Consent for Publication

All authors' consent to publication.

Availability of Data and Materials

Available upon reasonable request.

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Authors' Contributions

DFDSH and EB wrote the first draft of the manuscript. All authors read and ap-

proved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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