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Malaria Surveillance Using PCR Pooling in the Pre-Elimination Era: A Scoping Review

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ABSTRACT

WHO aims to eliminate malaria by 2030. Most of the cases and deaths were in the African Region, followed by the South-East Asia Region. With the number of cases decrease annually, more sensitive methods are needed for pre-elimination malaria surveillance program in endemic areas. Polymerase Chain Reaction (PCR) offers higher sensitivity and specificity, and with the integration of pooling strategies have further reduced costs. This review evaluates the application of PCR on sample pooling to enhance malaria surveillance in low-prevalence settings. A systematic search across two electronic databases identified 16 eligible studies, encompassing regions in Africa, Southeast Asia, and South America. Sample sources included whole blood (WB), Packed Red Cell (PRC), Dried Blood Spots (DBS), and serum. Pooling sizes ranged from 3 to 50 samples per pool, with hierarchical and matrix-based approaches utilized. The studies showed that PCR pooling maintained high sensitivity and specificity, detecting infections missed by microscopy or Rapid Diagnostic Test (RDT). In regions with malaria prevalence below 10%, pooling reduced testing costs by up to 50%. These findings support PCR on sample pooling as a reliable and scalable tool for pre-elimination surveillance programs. Future research should focus on optimizing sample types, pooling sizes, and standardized protocols to ensure both consistency and efficiency in malaria detection.

Keywords: Malaria, Polymerase Chain Reaction, Sample Pooling, Surveillance.

INTRODUCTION

The World Health Organization (WHO) reported in 2022 that malaria cases caused by *Plasmodium vivax (P. vivax)* and *Plasmodium falciparum (P. falciparum)* decreased by 82%.[1] However, in 2023, an estimated 263 million malaria cases and 597,000 deaths were recorded globally. Alongside these efforts, the number of malaria endemic countries decreased from 85 in 2022 to 83.[2]

Microscopy remains the WHO-recommended gold standard for diagnosing human malaria. However, it has limitations, particularly in detecting low parasitemia cases (<100 parasites/mL) and requires highly trained personnel for accurate interpretation.[3] Rapid Diagnostic Tests (RDT) offer a faster and easier alternative but are also constrained by several drawbacks, such as false-positive results due to underlying autoimmune conditions, non-malarial infections (e.g., dengue, leishmaniasis, or trypanosomiasis, which can affect RDT specificity), the persistence of *P. falciparum* Histidine-Rich Protein 2 (HRP-2) antigen despite infection resolution, or false negatives resulting from deletions in the HRP-2 gene.[4–6] In areas where case numbers are very low, positive cases are often missed by microscopy or RDT, even though they contribute to ongoing transmission. Due to the limitations of microscopy and RDTs, enhanced surveillance of asymptomatic and submicroscopic malaria is essential in endemic areas (to reduce transmission) and in post-elimination areas (to prevent reintroduction and reemergence).[7,8] Molecular diagnostic methods based on genomic amplification, such as polymerase chain reaction (PCR), have demonstrated superior sensitivity and specificity compared to microscopy and RDT.[7] These methods are capable to detect sub-microscopic infections and mixed-species infections, which are critical for guiding therapeutic decisions and effective patient management.[8] However, the high costs associated with individual PCR testing pose challenges for large-scale surveillance, particularly

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in countries approaching malaria elimination, where the number of positive human cases is relatively low.[9–11] To address this, several studies have explored sample pooling strategies to reduce the cost of PCR reactions while maintaining diagnostic accuracy to detect human infection in large-scale surveillance. Sample pooling strategy refers to the practice of combining multiple individual samples into a single group for collective analysis.[12] By pooling multiple samples, surveillance programs can efficiently screen large populations, reduce the number of PCR reactions needed, and significantly lower costs while maintaining high diagnostic sensitivity.

This scoping review aims to compile information from previous studies that employed pooling methods for malaria detection using PCR in human populations. It explores the types of specimens suitable for pooling, the maximum storage duration that preserves testing sensitivity, and the optimal pool size for efficient detection. By addressing these aspects, this review seeks to provide valuable insights for optimizing malaria surveillance through sample pooling strategies, tailored to the specific needs and resources of different countries.

MATERIALS AND METHODS

Selection of relevant and reliable studies

Original peer-reviewed articles published in English between 1988 and 2024 were identified through systematic searches of two databases: PubMed and Scopus, conducted from November 2024 to January 2025. The search strategy included keywords such as *Plasmodium* detection, *Plasmodium* infection, Pooling, and PCR. The inclusion criteria were limited to primary studies investigating malaria in humans, in which sample pooling strategies were employed as part of the diagnostic approach.

To apply the eligibility criteria, three reviewers with a background in parasitology independently screened the articles. The first step involved title and abstract screening to exclude irrelevant studies (duplicate publications, systematic reviews, or meta-analyses) using Rayyan AI. The second step involved full-text screening by all authors to ensure compliance with the inclusion criteria. In cases where consensus could not be reached, second opinion was sought from other authors to resolve disagreements. This process resulted in the inclusion of 16 studies, as illustrated in the flow diagram (Figure 1.).

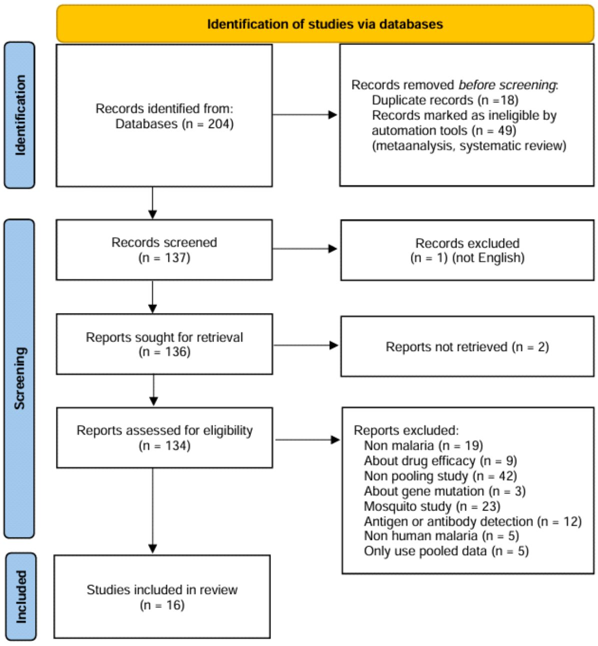

Figure 1. Flow chart of studies identification and selection process

These studies provide the foundation for analyzing the use of pooling strategies in Malaria detection via PCR methods.

Data Extraction and Synthesis

A structured data extraction process was carried out to collect relevant information from each included study. Key data extracted consist of study location, types of specimens, PCR methods, sample pooling strategies, pool sizes, limits of detection (LOD) and cost-effectiveness. The extracted data were organized into a summary table to facilitate comparison. Key findings were then synthesized narratively based on predefined themes to map existing evidence and identify trends across studies.

RESULT

Study Locations

Based on total 16 studies, 8 studies (50%) were conducted in Africa: Kenya, Uganda, Zanzibar

(Tanzania), Malawi, and Congo.[7,10,11,13–17] In Asia: Myanmar and Thailand, 5 studies (31,25%) were conducted.[9,18–21] Myanmar was highlighted as a key focus due to its high malaria transmission within the Greater Mekong Subregion.[9,21] Lastly, Latin America: Peru and Brazil was represented by 3 studies.[22–24]

Types of Specimens Used in PCR with Sample Pooling

Sixteen studies evaluated various types of specimens for PCR-based malaria detection (Figure 2). Dried blood spots (DBS) were the most frequently used sample type, appearing in 12 out of 16 studies. DBS offers ease of collection, transport, and storage, especially in resource-limited settings. [11,17] DBS showed high sensitivity when stored at room temperature (20–22°C) for 4 weeks to 8 months, and up to 2 years at –80°C.[11,16,17] PCR testing may yield false negative results when performed on DBS samples stored for more than two years, likely due to DNA degradation over prolonged storage.[16] Adding desiccants in DBS storage bags improved sample integrity during storage.[7,21] An interesting finding came from Hergott et al, where DBS samples were self-collected by participants after proper training and resulted in 96% of participants successfully collecting sufficient blood volumes (40-60 µL) on DBS. This study successfully identifying 11 *P. falciparum* positive from 121 microscopy negative subjects.[11]

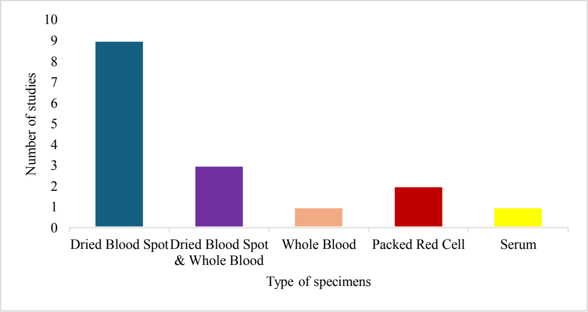

Figure 2. Type of sample included in this scoping review.

Footnote: from 16 studies, 9 studies used sample from Dried Blood Spot (DBS); 3 studies used sample from DBS and Whole Blood (WB); 2 studies used sample from Packed Red Cell (PRC); 1 study used sample from Whole Blood; and 1 study used sample from Serum

Chang et al., demonstrates that qRT-PCR for DBS samples performs comparably to WB (liquid sample) across a concentration range of 0,5 to 40.000 parasites/µL, with a strong correlation ($r^2 > 0.96$) indicating excellent assay linearity for DBS. qRT-PCR amplification in this study detected as low as 28 parasites/µl of blood with *P. falciparum*.[17] In the study by Nyunt et al., nested PCR from WB samples had a LOD 2 parasites/µL for *P. falciparum* and 0.2 parasites/µL for *P. vivax*.[20] Lima's study utilized blood pellet stored at -20^{0}C reported sensitivity 86,7% in malaria detection using both qPCR and nested PCR compared to microscopic positive sample.[22] In a subsequent study, Lima et al. utilizing packed red cells (PRC) derived from *P. falciparum* culture dilutions demonstrated that molecular assays using PET (Photo-Induced Electron Transfer) genus PCR achieved a LOD 3.5 parasites/µL, while the Lima-genus PCR further improved sensitivity to 0.35 parasites/µL. Bharti et al, employed a matrix pooling strategy for serum samples to detect malaria at known prevalence rates of 1% and 5%, with samples stored at –20°C for 6 weeks and at –80°C for 4.5 years. This study reported a LOD >20 parasites/µL.[24] In matrix pooling method, samples are arranged in a grid where each appears once in a row and column, allowing positive cases to be identified by intersecting coordinates. Although initial testing required twice the number of reactions compared to individual testing, subsequent steps reduced the total number of reactions, especially when positive samples were few.[17,24] Chang's study used GUI (Graphical User Interface) program to simulate three-stage hierarchical pooling for 1,000 samples. Larger initial pools (7–25 samples), followed by smaller ones (4–5), were optimal for very low prevalence (<2%), while smaller initial pools (4–6) followed by 2–3 samples were more efficient at 5–10% prevalence.[17] Hsiang's study using pooled sample and nested PCR detected 3.2% asymptomatic infections in children, compared to only 0.8% by microscopy.[16] Moreover, Zhou et al. reported that nested PCR applied to DBS and placental blood samples exhibited equal sensitivity (96%) and specificity (99.1%) in detecting *P. falciparum*, outperforming traditional histology methods (49.7%).[15]

Primers and Types of PCR-Based Malaria Detection Studies

Among the 16 reviewed studies, various PCR methods were employed: nested PCR, qRT-PCR, and real-time PCR. A common approach was targeting the 18S rRNA gene, particularly in genus-specific PCR used as a preliminary screening before species-level identification.[17,21] The PCR method utilized in some studies included are detailed in Table 1.

Table 1. PCR Methods, Pooling Strategies, and Detection Limits in Malaria Detection Studies

Author/ Year of Publication	Country of Study	PCR method	Samples in one pool	Limit of detection
Chang et al., 2021[17]	Tanzania	qRT-PCR	3-28	28 parasites/µl for *P. falciparum*
Grossenbacher et al, 2020[14]	Tanzania	Real time PCR	5	1,12 parasites/µL for *P. falciparum*
Lima et al., 2016[23]	Brazil	Real time PCR, Nested PCR	10	3,5 parasites/µL (PET-PCR), 0,35 parasites/µL (Lima-genus PCR) for *P. falciparum*
Nyunt et al., 2016[20]	Myanmar	Nested PCR	10	2 parasites/µL (*P. falciparum*), 0,2 parasites/µL (*P. vivax*)
Hsiang et al., 2010[16]	Uganda	Nested PCR, Real time PCR	10	10 parasites/µL (*P. falciparum*)
Taylor et al., 2010[7]	Congo	Real time PCR	4	4 parasites/µL for *P.falciparum*
Bharti et al, 2009[24]	Peru	PCR	10	>20 parasites/µL for *P. vivax*

Footnote: PCR, polymerase chain reaction; PET-PCR, photo-induced electron transfer polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction

The rPLU primers (e.g., rPLU1, rPLU3, rPLU4, rPLU5, and rPLU6) were used in seven studies as part of a nested PCR protocol to detect the *Plasmodium* genus. These primers were often combined with species-specific primers targeting *P. falciparum*, *P. vivax*, *Plasmodium malariae (P. malariae)*, and *Plasmodium ovale (P. ovale)*.[9,15,16,20–22] Liu et al. identified 277 infections among 1.991 volunteers in Myanmar, significantly outperforming microscopy (14 infections) and RDTs (103 infections).[9] Additionally, Zhou et al. showed that nested PCR achieved 96% sensitivity for *P. falciparum* detection in pooled samples of both peripheral and placental blood.[15] Primers targeting the *cytochrome b* gene combined with Chelex extraction, as used by Hsiang et al., achieved 93% sensitivity for *P. falciparum* at 10 parasites/µL in pools of 10 samples and 100% for *P. vivax* at 640 parasites/µL in pools of 25 samples.[16]

Real-time PCR proved effective in pooled sample testing, with Taylor et al. detecting 35 positives among 1.092 microscopy-negative cases in pooled DBS samples, with LOD 4 parasites/µL.[7] Hergott et al. applied qRT-PCR with both *P. falciparum*-specific and pan-*Plasmodium* primers, successfully identifying 11 submicroscopy-negative cases from 121 subjects through pooling sample. [11] In Chang's study, qRT-PCR was also tested using quantitative standards, such as armored RNA containing *P. falciparum* 18S rRNA, to ensure consistent and reliable results in low-resource settings.[17] The LOD from several studies is summarized in Table 1. Aside from the chosen PCR method, pre-PCR preparation significantly affects PCR performance. Grossenbacher et al. found the direct pre-PCR method more effective than Chelex and boil-and-spin extraction, detecting *P. falciparum* at 0.1–0.05 parasites/µL in 3 of 4 replicates with minimal variation. It also improved pooled sample sensitivity, achieving a LOD 1.12 parasites/µL using five pooled samples and 3 µL of DNA.[14]

Cost Efficiency in PCR-Based Sample Pooling for Malaria Detection

A total of seven studies included in this review discussed the cost-effectiveness of PCR-based sample pooling compared to individual testing for malaria diagnosis. The most recent study by Hergott et al. evaluated 3.569 dried blood spot (DBS) samples using pools of 2–6 samples, resulting in 1.831 PCR reactions. The estimated cost per pooled test was USD 11 compared to USD 10,70 for individual tests, leading to an overall savings of approximately USD 15.800 in reagents and USD 9.550 in labor.[11] Taylor et al. analyzed 1.092 samples by grouping four samples per pool, forming 293 pools. A total of 586 PCR reactions were performed, detecting 35 positive cases. If all samples had been tested individually in duplicate, 2.184 PCR reactions would have been needed. At a reagent cost of USD 1.25 per reaction, the pooling strategy cost USD 1.553, whereas individual testing would have cost USD 3.170, resulting in a 51% cost reduction.[7] Chang et al, tested 78 DBS samples pooled in sets of three, which resulted in 41 PCR reactions and the detection of five *Plasmodium* positive samples. Sample pooling effectively reducing the number of PCR reactions by nearly half.[17] Liu et al. applied a three-sample pooling strategy to 1.778 samples, followed by species-specific PCR on positive pools. This approach required a total of 2.170 PCR reactions, compared to 4.050 reactions if all samples had been tested individually.[9] Rogawski et al, pooled 187 samples (four per pool) and conducted 67 PCR reactions. With reagent costs at 37,5 baht (USD 1,25) per reaction, the pooling strategy saved approximately 4.500 baht from total cost of 7.012 baht.[19] Wang et al. pooled 1.537 samples into 385 pools and performed 1.018 nested and real-time PCR reactions, compared to 3.074 reactions for individual testing. Finally, Hsiang et al. implemented a three-stage hierarchical pooling strategy. Initially, 4.028 RDT-negative DBS samples were grouped into 162 pools of 25 samples each. Two positive pools were subsequently divided into ten sub-pools of five samples. Two positive sub-pools were then tested individually. In total, 182 PCR reactions were conducted at a cost of USD 291 (USD 1,55 per test), substantially lower than the estimated cost of USD 6.248 for individual testing.[10] These findings consistently demonstrate that PCR-based pooling significantly reduces testing costs and laboratory workload, particularly in large-scale surveillance efforts or low-prevalence settings.

DISCUSSION

Studies conducted in Africa, Asia and Latin America reflect a growing interest in improving malaria surveillance and detection techniques in areas where malaria transmission remains endemic. The populations studied across all regions shared common characteristics and included high-risk groups (pregnant women, migrant workers, and rubber plantation labourers). Many studies targeted asymptomatic individuals as reservoirs of infection, reflecting the importance of detecting subclinical malaria for control and elimination programs.[9,15,21,24]

Some studies have highlighted the potential of PRC and WB samples for detecting extremely low parasitemia, while serum showed lower sensitivity compared to PRC, DBS, or WB.[23,24] The use of whole blood or packed red cells offers a substantial advantage over dried blood spots (DBS) in molecular malaria surveillance, particularly for detecting low-density and asymptomatic infections. A recent systematic comparison demonstrated that DNA recovery from whole blood was 5 to 10 fold higher than from DBS, resulting in a 2 to 3 fold lower limit of detection (LOD) in qPCR assays. In many molecular surveillance studies, DNA extraction from dried blood spots (DBS) is performed using only a small amount of blood (often less than 50 µL). However, the elution or final volume of buffer used to extract the DNA is typically much larger, around 100–200 µL. This causes the DNA to become highly diluted. In contrast, when using whole blood, a larger volume of blood (such as 200 µL) is concentrated into a smaller elution volume (usually 50–100 µL), resulting in more concentrated DNA. As a result, DNA extracted from DBS is often more diluted and may be less sensitive for detecting low-level infections compared to DNA from whole blood.[25] Another reason for the lower DNA yield from DBS compared to whole blood is that during the lysis phase of DNA extraction, partial retention of nucleic acids may occur within the filter paper matrix, thereby reducing the overall recovery of nucleic acids relative to liquid whole blood samples.[26] This difference in DNA recovery is critical when targeting submicroscopic parasitemia, which is commonly observed in pre-elimination or low-transmission settings, as reduced DNA yield may compromise the sensitivity of molecular diagnostic methods such as PCR.[25]

In terms of sample collection, the use of self-collected dried blood spots (DBS) by Hergott et al highlighting the feasibility of this strategy in detecting submicroscopic infections, particularly in remote or resource-limited settings, where sample collection by trained personnel may be logistically challenging.[15] About pooling strategies, some studies highlighted the importance of methods such as matrix pooling and random pooling to optimize resource utilization. Although pool sizes varied across studies, in regions with malaria prevalence below 10%, pooling 3 to 6 samples has proven efficient, potentially reducing costs by up to 50%.[7,9,11,17,19,21] Other modifications, such as increasing template volume in the direct pre-PCR method, have been reported to increase sensitivity by 8,6 fold for low-density infections.[14] Performing sample dilution using known parasite concentrations helps determine the lower detection threshold of *Plasmodium*, supporting the evaluation of detection methods in pooled clinical samples.[17]

Overall, sample pooling strategies in PCR-based malaria detection not only reduce the number of tests required but also significantly lower costs, making them an invaluable tool for malaria control and elimination programs, particularly in resource-limited settings. However, this review has several limitations. The relatively small number of studies included (n=16) may restrict the generalizability of the findings. Methodological heterogeneity includes variations in PCR techniques, pooling approaches, and sample types complicate direct comparison and the ability to draw definitive conclusions. Additionally, operational and logistical challenges associated with implementing pooling strategies in real-world settings are also not extensively discussed.

CONCLUSION

This review provides an overview of pooling strategies and PCR methods for malaria detection, synthesizing evidence from 16 studies conducted in various geographical regions. DBS emerged as a practical and reliable option for field-based studies, while WB and PRC provided enhanced sensitivity for low-parasitemia settings. Advantages of self-collected DBS further improved feasibility and efficiency, enabling large-scale surveillance programs. Pooling strategies consistently demonstrated high sensitivity and specificity, even for sub microscopic infections, while addressing cost constraints in low-transmission and pre-elimination settings. Further research should incorporate larger datasets, broader geographical coverage, refined pooling protocols, optimized sample types, and standardized PCR methodologies to enhance the applicability of this approach in malaria surveillance and support global elimination effort. More information regarding the pooling strategy employed in this scoping review is detailed in Supplementary table 1.

Conflict of Interest

The authors declare no conflict of interest.

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