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REVIEW

Molecular tools are crucial for malaria elimination

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Abstract
The eradication of *Plasmodium* parasites, responsible for malaria, is a daunting global public health task. It requires a comprehensive approach that addresses symptomatic, asymptomatic, and submicroscopic cases. Overcoming this challenge relies on harnessing the power of molecular diagnostic tools, as traditional methods like microscopy and rapid diagnostic tests fall short in detecting low parasitaemia, contributing to the persistence of malaria transmission. By precisely identifying patients of all types and effectively characterizing malaria parasites, molecular tools may emerge as indispensable allies in the pursuit of malaria elimination. Furthermore, molecular tools can also provide valuable insights into parasite diversity, drug resistance patterns, and transmission dynamics, aiding in the implementation of targeted interventions and surveillance strategies. In this review, we explore the significance of molecular tools in the pursuit of malaria elimination, shedding light on their key contributions and potential impact on public health.

Keywords Malaria · Elimination · Molecular tools · Asymptomatic malaria · Genomics

Introduction

According to the World Health Organization (WHO), the global malaria-related death toll in 2021 reached an estimated 619,000, alongside 247 million estimated cases. The WHO African region had the highest number of cases, while the WHO South-East Asia region accounted for 2% of the total burden [1]. During the period of 2020–2021, the South-East Asia region experienced a significant increase of 400,000 malaria cases, with over 50% originating from Myanmar. India continued to bear the highest disease burden in the region, representing 79% of malaria cases in 2021 [1]. Despite the persistently high incidence of cases and fatalities in the region, progress has been made in reducing the malaria burden over the past decade. The region witnessed a remarkable 76% decrease in malaria cases, declining from 23 million in 2000 to approximately 5 million in 2021 [1].

The transmission of the malaria parasite to humans occurs through the bites of infected female *Anopheles* mosquitoes. During their blood meals, these mosquitoes inject *Plasmodium* sporozoites into human subcutaneous tissues, thereby initiating the malarial life cycle within the host. Malaria in humans is caused by five species of *Plasmodium*: *P. vivax* (*Pv*), *P. falciparum* (*Pf*), *P. knowlesi* (*Pk*), *P. malariae* (*Pm*), and *P. ovale* (*Po*). Among these species, *Pf* is responsible for the most severe form of malaria and, together with *Pv*, accounts for the majority of cases worldwide [1].

In regions where malaria is prevalent, individuals can exhibit different presentations upon infection, ranging from symptomatic to asymptomatic carriers [2]. Symptomatic cases, as classified by the WHO, can be further categorized into severe and uncomplicated malaria [3]. Severe malaria (SM) caused by *Pf* is characterized by the sequestration of infected erythrocytes (iEs) in the host's microvasculature [4, 5]. This sequestration, coupled with an excessive production of inflammatory mediators, leads to dysfunction in peripheral organs, either individually or in combination, resulting in severe complications [2, 6]. These complications

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may include acute respiratory distress syndrome (ARDS) affecting the lungs, jaundice impacting the liver, acute kidney injury (AKI) affecting the kidneys, or cerebral malaria (CM) affecting the brain [2, 4]. The situation is further complicated when traditional diagnostic tools like microscopy and rapid diagnostic tests (RDTs) fail to detect patients with asymptomatic malaria (AM) and parasitaemia at submicroscopic levels [7].

Asymptomatic malaria refers to malaria in individuals who lack apparent clinical symptoms and do not seek treatment. These individuals often go unnoticed by passive surveillance systems but serve as important reservoirs for gametocytes, contributing to the persistence of malaria transmission [8]. Previously, AM was considered relatively benign, but studies have shown associations with recurrent episodes of symptomatic parasitaemia, chronic anaemia, maternal and neonatal mortality, co-infection with invasive bacterial diseases, cognitive impairment, and continuous transmission [9]. Therefore, malaria elimination programs must include tactics to target these subclinical reservoirs that can sustain transmission and adapt surveillance as well as response strategies tailored to local contexts.

Molecular tools show promising potential to detect multiple *Plasmodium* species, AM patients, and parasitaemia at submicroscopic levels [10]. Furthermore, molecular techniques provide a means to detect the emergence of mutations that confer resistance to antimalarial drugs [11, 12]. They can also identify deletions that impact the sensitivity of *Pf* histidine-rich protein 2 (*pfhrp2*)-based RDTs [7, 13, 14], posing a threat to malaria control strategies [7]. By employing genomic scans [15, 16], these molecular tools can pinpoint adaptations in the parasite caused by single nucleotide polymorphisms (SNPs) and structural variations (gene copy number), which may necessitate a coordinated response. To gain insight into the ongoing transmission of malaria and distinguish between cases that originate locally and those imported from other regions in the context of elimination efforts, metrics like identity by descent (IBD) can be employed to assess the relatedness of parasites [17]. In areas approaching elimination, these metrics can help identify transmission foci [18] and differentiate between indigenous and imported cases [19]. Notably, the implementation of control and elimination measures has been shown to reduce the genetic diversity of *Pf* parasites and promote increased genetic similarity through inbreeding and recent common ancestry [20]. These pieces of evidence may play a crucial role in designing targeted interventions and developing surveillance strategies tailored to the specific circumstances of the local context. In this review, we explore the significance of molecular tools in the pursuit of malaria elimination, shedding light on their key contributions and potential impact on public health.

Malaria diagnosis

Traditionally, the gold standard for malaria diagnosis has been the light microscopy method. Expert microscopists, with proper training, can detect as few as 5 parasites/µl blood, whereas regular laboratory staff usually require concentrations of 50 to 100 parasites/µl of blood to identify a positive blood smear [21]. Good microscopy results rely on properly maintained equipment, a consistent supply of high-quality reagents, well-trained personnel, and effective monitoring and supervisory systems, which may not be available in all settings. Additionally, microscopy can lead to misdiagnosis in cases of low parasitaemia and mixed infections [22], and it can be time-consuming. RDTs are widely used in malaria case management and elimination programs, especially in remote areas where microscopy facilities are unavailable [7]. Traditional RDTs have a limit of detection (LOD) of approximately 800 pg/mL and show decreased sensitivity to detect low-density *Pf* infections (< 100 parasites/µl) [7]. To detect low-density parasitaemia, a new generation of highly sensitive RDTs (hsRDTs) has been developed [14]. hsRDTs with a LOD of 80 pg/mL enable the detection of low-density *Pf* malaria infections (3–10 parasites/µL). These hsRDTs offer a 10-fold lower LOD compared to conventional RDTs [7, 23]. However, the emergence and spread of deletions in the *pfhrp2* gene, a crucial target antigen for detecting *Pf* in commercially available RDTs, can have detrimental effects on affected individuals due to false-negative diagnoses, resulting in delayed or no treatment [7]. Other limitations of RDTs include lower sensitivity compared to microscopy, especially in cases of low parasite density. While RDTs excel at detecting *Pf*, they may miss other malaria species such as *Pv*, *Po*, and *Pm* due to lower sensitivity towards them, leading to potential misdiagnoses. Interpreting RDT results can be tricky due to subjective visual assessment, leading to errors with faint or unclear lines [24].

Molecular tools, such as PCR-based assays (Fig. 1), have demonstrated high sensitivity and specificity in detecting malaria parasites [25]. PCR is a high-throughput technique capable of processing multiple samples simultaneously with a LOD of 1 to 5 parasites/µl of blood. In a single test, it can differentiate between multiple *Plasmodium* species, thereby reducing time, manpower, and other resources while improving efficiency in detecting malaria parasites. Furthermore, with advancing technology, the cost of PCR-based tests has decreased, making them readily available for diagnosis in many endemic areas, although not all, due to the need for specialized equipment and trained personnel. Several molecular assays to detect asymptomatic and submicroscopic infections depend on the 18S small subunit ribosomal RNA (18S rRNA) gene, such as quantitative reverse transcription PCR (qRT-PCR) [26] and nucleic

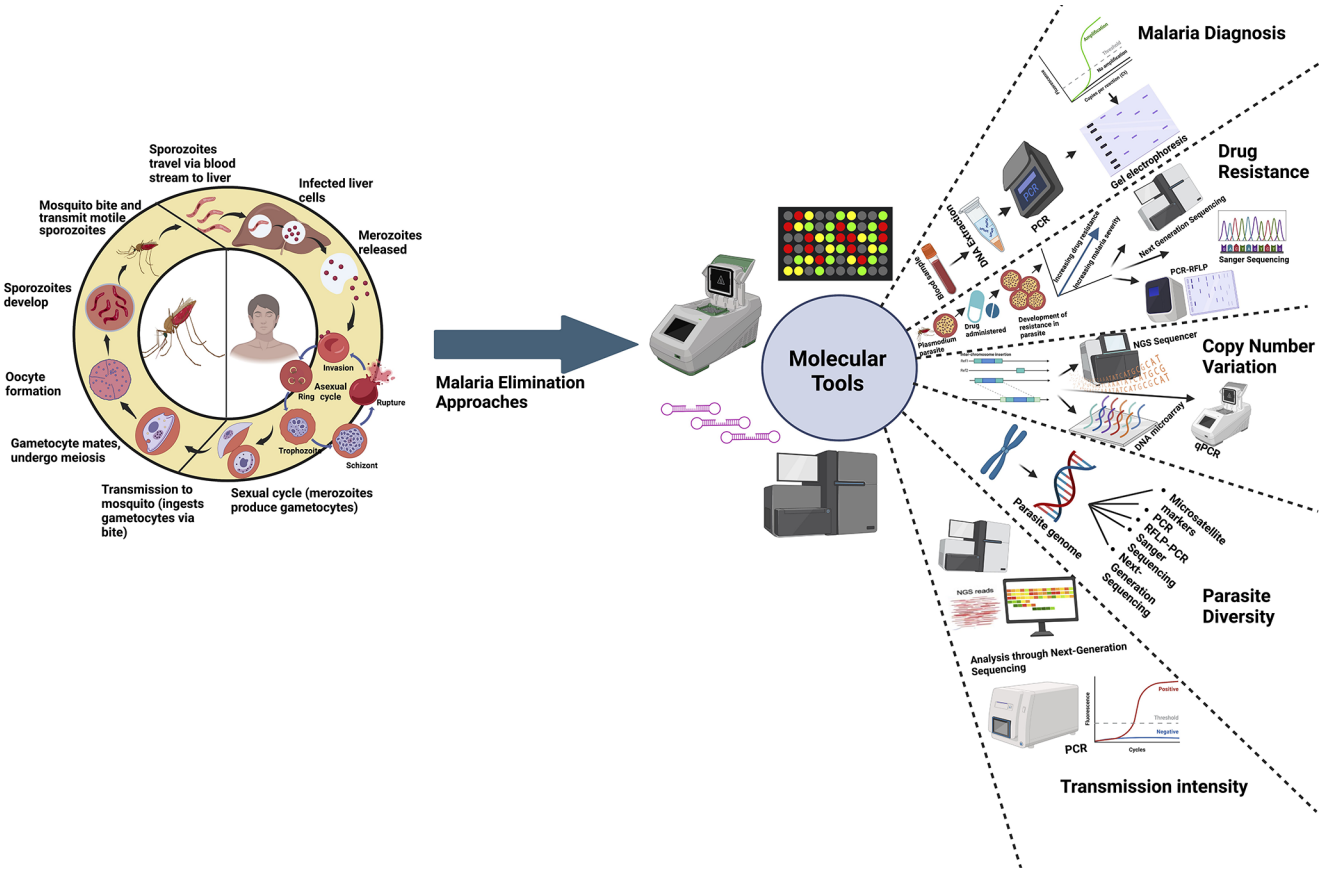

Fig. 1 Potential role of molecular tools in different aspects of malaria disease and its elimination

acid sequence-based amplification (NASBA) [27]. The 18S rRNA gene is present in 5–8 copies per genome, varying with the parasite strain [28], thus enhancing the assay's sensitivity compared to genes with a single copy per genome. However, due to the RNA's unstable nature, these assays require specific and controlled sample collection and storage, limiting their application in field settings.

DNA-based techniques are generally more adaptable to field conditions for detecting asymptomatic and submicroscopic infections. Examples include nested PCR [29], quantitative PCR (qPCR) [30], loop-mediated isothermal amplification (LAMP) [31], isothermal recombinase polymerase amplification (RPA) [32], and alternative PCR-based detection methods including fluorescence resonance energy transfer (FRET) real-time PCR [33], PCR-nucleic acid lateral flow immuno-assay [34], photo-induced electron transfer (PET) fluorogenic primers based PCR [35], and capture and ligation probe-PCR (CLIP-PCR) [36]. LAMP, among these tools, demonstrates better accuracy, particularly in resource-limited settings [25]. Overall, DNA-based approaches offer greater versatility compared to RNA-based assays due to the stable nature of DNA molecules.

Novel molecular methods that rely on repetitive genome targets have shown the ability to detect a range of 0.03 to 0.15 parasites/µl blood, exhibiting 10 times higher sensitivity compared to standard 18S rRNA qPCR [37]. Hofmann et al. (2015) discovered 250 copies of telomere-associated repetitive element 2 and 59 copies of the *var* gene acidic terminal sequence in the *Pf* genome. They developed an ultra-sensitive assay utilizing a combination of repetitive genome targets and real-time PCR to detect *Pf* parasites [37], particularly in patients with low parasitaemia. Similarly, Demas et al. (2011) identified 14–41 copies of the Pvr47 and Pfr364 sequences specific to *Pv* and *Pf* parasites, respectively, and demonstrated their utility in a single-step PCR assay with higher sensitivity than 18S rRNA [38]. In a separate study, Lucchi et al. (2012) detected 7 copies of the Pkr140 sequence in the *Pk* genome [39]. Gupta et al. (2016) further expanded on this approach by identifying PfMLS152 and PvMLS110 sequences, which were found to be 44 and 34 times more abundant in the genomes of *Pf* and *Pv*, respectively [40]. By employing multi-copy DNA sequences present throughout the genomes of malaria parasites as amplification targets in molecular assays like LAMP, the sensitivity of these assays for detecting different *Plasmodium* species in patients with low parasite levels can be significantly improved. Furthermore, such assays can be conveniently deployed in field settings.

Recently, a Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) diagnostic method has emerged [41]. This innovative approach combines recombinase polymerase pre-amplification, CRISPR-RNA base-pairing, and LwCas13a activity for the detection of nucleic acids. SHERLOCK assays have shown their effectiveness in detecting all *Plasmodium* species, with sensitivity levels ranging from 2.5 to 18.8 parasites per reaction when tested against laboratory strain genomic DNA. In a study involving 123 clinical samples, the *Pf*-based SHERLOCK assay achieved an impressive 94% sensitivity and 94% specificity when compared to a real-time PCR-based assay for *Pf* detection [41]. However, it is worth noting that this robust diagnostic assay is currently in the developmental phase and requires further optimization before it can be deployed in the field.

Drug resistance surveillance

Over the past few decades, various molecular genotyping techniques have been developed and applied for the purpose of monitoring drug resistance in clinical isolates (Fig. 1). Traditional methods such as polymerase chain restriction fragment length polymorphism (PCR-RFLP) analysis, real-time PCR, molecular beacons, single-nucleotide primer extension, and dot blot probe hybridization have been employed [42]. Furthermore, high-throughput methods have emerged, including high-resolution DNA melting (HRM), a TaqMan allelic discrimination assay, mass spectrometry-based SNP genotyping, a SNP-based custom genotyping assay, a ligase detection reaction fluorescent microsphere (LDR-FM) assay, and LAMP assay have been used for molecular genotyping [42]. Each of these methods possesses its own advantages and disadvantages, allowing researchers to choose an appropriate technique based on their specific requirements, available facilities, and resources. Among these methods, PCR-RFLP is frequently employed due to its relative ease, affordability, and speed in comparison to other molecular approaches [43]. One limitation commonly shared by these techniques is that although they enable the identification of known resistance alleles in a specific gene, they do not facilitate the identification of novel genetic polymorphisms that might contribute to drug resistance. Additionally, some of these techniques have lower sensitivity and may yield results that may not be comparable across studies [42, 44].

Sanger sequencing, a newer and widely accepted method, serves as the gold standard for the molecular genotyping. It also enables the discovery of novel genetic polymorphisms. However, its application in large-scale surveillance is hindered by low throughput and higher costs associated with reagents, which increase with the number of samples genotyped, as well as the inability to detect minor frequency polymorphisms, particularly in areas with high disease transmission [42, 44, 45].

Next Generation Sequencing (NGS) has greatly improved our understanding of antimalarial resistance [42, 46–48]. NGS offers several advantages over traditional Sanger sequencing. It is more sensitive, allowing for the detection of low-frequency drug resistance mutations that may be missed by Sanger sequencing. Studies have shown that NGS can detect a higher number of drug resistance mutations compared to Sanger sequencing [49]. While it requires trained staff, like other molecular techniques, NGS has the potential to detect novel mutations and minor variant genotypes in mixed infections, as well as accurately quantify allele frequencies in mixed genotypes. This is particularly important since mixed genotypes are often classified as mutant, leading to the underestimation of wild-type parasites [47]. The study by Hendenstrom et al. (2019) used deep amplicon sequencing (AmpSeq) to enhance sensitivity and reliability in detecting minority clones in multi-clonal infections, providing a highly reproducible and robust characterization of clone dynamics during trial follow-up [50]. Similarly, Rao et al. (2016) demonstrated the potential of AmpSeq to discriminate between single and multiclonal infections [42].

This improved sensitivity to detect antimalarial resistance markers can help in identifying patients at risk of treatment failure and guide appropriate treatment choices. By sequencing the entire parasite genome, NGS can identify novel resistance markers and track their emergence and spread in different populations. However, it is important to note that the clinical significance of all detected drug resistance mutations identified by NGS, especially those present at low frequencies, is still being investigated. Further research is needed to determine the impact of these mutations on treatment outcomes and patient care. In summary, NGS holds great promise in the detection of malaria drug resistance mutations.

Recently developed SHERLOCK-based assays have undergone testing to detect the dihydropteroate synthetase gene SNP (A581G), associated with sulfadoxine resistance [41]. In comparison with AmpSeq, the SHERLOCK assay demonstrated 73% sensitivity and 100% specificity when applied to a panel of 43 clinical samples. Notably, false-negative results were primarily observed at lower parasite densities [41], indicating the promising potential of this new technology for identifying malaria drug resistance alleles. Nevertheless, further extensive research involving a larger sample size is necessary before considering its use in the field.

Surveillance of gene deletion and duplication

Copy number variations (CNVs) are structural genomic alterations that encompass a significant portion of genetic variations, namely gene deletions and duplications. Numerous studies [51, 52] have established associations between CNVs and various diseases. Additionally, CNVs contribute to the great diversity in the *Plasmodium* genome and confer drug resistance. To identify and characterize CNVs, a range of techniques such as quantitative PCRs and microarrays have been employed (Fig. 1); nevertheless, these methods have limitations.

Microarrays enable high-throughput examination of DNA sequences, facilitating comprehensive CNV assessment in malaria research. Labelled DNA fragments on arrays, probed for distinct genomic segments, reveal CNVs. Hybridization signal intensity indicates associated copy numbers [53]. Microarrays identify amplified/deleted sequences, detecting DNA variations from SNPs to large CNVs [53, 54].

In Dharia et al.'s study (2009), a microarray-based methodology proficiently detected occurrences of gene deletions and amplifications. This underscores the significance of microarrays in CNV detection across the malarial parasite genome. The approach involved a methodical probe selection, specifically targeting alignment with the 3D7 reference genome to ensure robust data. For systematic gene deletion detection, the authors employed a match-only integral distribution (MOID) algorithm, effectively eliminating extraneous signals and noise [55]. Similarly, Cheeseman et al. (2016) demonstrated the presence of 134 high-confidence CNVs across the parasite exome, including 33 deletions and 102 amplifications, using microarrays [56]. In another study, Simam et al. (2018) identified 94 CNV loci using microarrays in 183 fresh field isolates from three populations in Eastern Africa with different malaria transmission intensities [16].

In malaria research, a microarray aids in identifying prospective genes, expediting large-scale investigations, revealing adaptive evolutionary patterns, advancing comprehension of genetic diversity, and expanding knowledge of CNVs [53, 54, 57], despite concurrent consideration of newer technologies such as NGS. However, the accuracy of microarray-based CNV detection relies on probe quality, inherent resolution limitations for identifying subtle CNVs and distinguishing closely related sequences, and the requisite use of specialized bioinformatics tools to ensure meticulous data analysis.

Another technique, quantitative-PCR (qPCR), is a potent tool for amplifying and quantifying specific DNA sequences. It can be harnessed to ascertain and measure CNVs within genes or genomic segments linked to antimalarial resistance, virulence factors, or other attributes pertinent to the disease's pathogenesis. The process involves amplifying an unidentified copy number locus alongside a reference locus possessing a known copy number. Through the comparison of relative copy numbers of distinct genetic elements across diverse samples, researchers can attain insights into the potential impact of CNVs on malaria transmission, disease severity, and the effectiveness of treatment interventions.

For instance, in cases where a specific gene is replicated within a parasite strain in contrast to a reference strain, the qPCR assay would exhibit an elevated fluorescence signal attributed to the increased gene copy number [58]. An increased copy number of *plasmepsin* 2 (*pfpm2*) has been associated with piperaquine resistance, measurable using qPCR-based assays [58, 59]. Furthermore, an increased *pfmdr1* copy number has been associated with resistance to mefloquine and partially to lumefantrine [11, 60]. Additionally, Srisutham et al. successfully assessed CNVs of *pfpm2*, *pfmdr1*, and *pfgch1* using the Droplet digital PCR (ddPCR) assays [61]. These findings suggest that qPCR-based assays serve as versatile and potent tools for understanding the genetic basis of parasite characteristics and their implications in malaria disease. Nonetheless, the identification of CNVs using qPCR is notably constrained by the relatively small number of genes that can be simultaneously assessed. Moreover, the choice of appropriate endogenous controls, such as housekeeping genes, becomes crucial, as improper selection can introduce bias into the outcomes [62]. Technological progressions that enable comprehensive whole-genome sequencing, such as NGS, empower the execution of more intricate analyses concerning genomic variations.

The application of NGS technology has revolutionized the genetic analysis, propelling genomics beyond laboratory confines and into practical field settings (Fig. 1). By swiftly generating millions of short nucleotide sequences, NGS enables a high-throughput method to accurately assess the frequency of specific genetic variants in *Pf*. CNVs can be determined based on the mean depth of coverage of individual amplicons between the reference sample (3D7, without CNVs) and test sample. Notably, studies that have harnessed NGS technology have effectively reported the presence of *pfhrp2/3* deletions. These studies have found varying frequencies of *pfhrp2/3* gene deletions, with some regions showing higher prevalence than others [63, 64]. In a study by Flannery et al. (2015), NGS technology was employed to successfully identify the copy number of drug-resistance associated genes in Peruvian isolates [65]. Similarly, Beghain et al. (2016) utilized NGS technology to estimate the copy number of several *Plasmodium* genes. To facilitate this process, they devised an algorithm called PlasmoCNVScan [66]. Based on the evidence, it appears that the application of NGS technology shows significant

potential in detecting CNVs, which can further supplement the efforts of malaria elimination. However, further research is needed to improve the technology, making it more accessible and easier to use.

Parasite diversity

Gaining insights into the genetic diversity and population structure of persistent malaria parasite populations is vital as we strive for malaria elimination. These insights reveal resilience, spatial distribution patterns, and help evaluate malaria control strategies and antimalarial interventions. They also aid in targeting malaria reservoirs, covering symptomatic and asymptomatic carriers [67]. Additionally, they illuminate human migration patterns related to parasite movement, enhancing our understanding of parasite flux dynamics [68]. Primarily, the genetic diversity in *Pf* parasites results from recombination among distinct clones, complemented by SNPs within clones. These intra-clone variations include gene duplications, chromosomal deletions, variations in repeat sequences, and point mutations across various genetic loci [69]. Furthermore, data pertaining to parasite diversity and population structure carry substantial significance in the context of malaria epidemiology and parasite virulence [70]. Characterizing the parasite's population structure helps elucidate malaria transmission intensity fluctuations across diverse ecological zones and even within the same zone at different times [71]. Indeed, evidence suggests that *Pf* genetic diversity indicates the parasites' ability to adapt to hosts, favouring advantageous traits including drug resistance and antigenic variability [72].

Assessing *Pf* genetic diversity involves genotyping antigenic markers such as *msp1*, *msp2*, and *glurp* [73]. An alternative method employs multi-locus genotyping with non-antigenic markers—SNPs and microsatellites [73]. Microsatellite markers are highly polymorphic repetitive sequences (with tandem repeats of 2–6 bp) present in the parasite's genome. They are typically considered to have neutral evolutionary significance, but they may exhibit functional relevance when located near drug resistance genes. These markers are highly prevalent in the parasite genome [74] and play a crucial role in assessing the diversity and distribution of different parasite genotypes in various transmission settings [73, 74]. Parasite diversity increases with marker count, but some markers are more polymorphic than others. Therefore, utilizing a few highly diverse markers may yield a comparable level of complexity compared to using a larger number of markers with lower to moderate diversity.

Traditionally, PCR, RFLP-PCR, and DNA sequencing-based assays (Fig. 1) have been used to analyse the multiplicity of infection (MOI) of the malaria parasite and determine the number of distinct strains within an individual [73, 75]. Through the application of molecular genotyping approaches, various factors influencing the *Pf* population structure and diversity can be effectively characterized. These factors include geographic isolation, inbreeding, gene flow, the introduction of foreign parasites, and epidemic population expansion. Various genetic metrics, such as the number of alleles, linkage disequilibrium, heterozygosity, MOI, proportion of polymorphic loci, and F-statistics, are employed to assess the factors influencing the population structure of malaria parasites [73]. Investigating genetic diversity and dynamics through molecular tools informs the risk of parasite importation and the impact of intensified malaria interventions [73]. This suggests that molecular techniques are essential for comprehending parasite populations and optimizing malaria management.

The emergence of NGS technology has started a new era for studies on population genetics, diversity, and MOI. Notably, remarkable progress has been made in the interpretation of NGS data and the estimation of MOI, facilitated by the development of diverse bioinformatic tools.

NGS exhibits heightened sensitivity by producing numerous reads per sample, enabling the detection of minor alleles with precision. Utilizing NGS, the mitochondrial genome region (3.4 kb) revealed 69 unique *P. falciparum* haplotypes in phylogenetic analyses, 62 of which were previously unreported. Furthermore, analysis of the conserved cytochrome b (*cytb*) gene identified multiple parasites, with up to four parasites detected, including non-falciparum species in 10% of the total 437 samples collected from Cameroon [76]. By employing short amplicon (117 bp) deep sequencing of *pvmsp1*, Lin et al. discovered 67 distinct haplotypes in 78 Cambodian *Pv* samples, revealing an average of 3.6 MOI per individual [77]. For increased sensitivity in detecting minor alleles, three *Pf* amplicons—321 bp (*pfcsp*), 305 bp (*pfama1*), and 306 bp (*pfk13*)—underwent ultra-deep sequencing, capable of quantitatively identifying unique haplotypes present in as little as 2% of polyclonal infections [78]. The highly polymorphic conserved *Plasmodium* membrane protein (*cpmp*) was identified as a reliable marker, with a 100% sensitivity assay to detect clones above 1% frequency [79]. However, distinguishing true minority haplotypes from PCR and sequencing errors was found challenging. To overcome this challenge, several packages and algorithms have been developed to analyse NGS data, allowing the detection of low-frequency variants. Notable examples include HaplotypR [79], SeekDeep [80], and estMOI [81]. Lerch et al. (2019) compared NGS and length-polymorphism (traditional) based genotyping assays for their performance in detecting minority clones in longitudinal samples from Papua New Guinea. NGS results,

utilizing the HaplotypR algorithm, outperformed the length-polymorphic *msp2* marker in detecting minority clones (sensitivity NGS: 95%, *msp2*: 85%). The MOI by NGS was 2.32 compared to 1.73 for *msp2* (traditional assay) [82]. Evidence suggests that the utilization of NGS technology holds significant potential for detecting parasite diversity, further complementing malaria elimination efforts. Nonetheless, additional research is needed to enhance the technological framework, making NGS more accessible and user-friendly.

Transmission intensity

To effectively reduce the global malaria burden, accurate estimates of malaria transmission intensity are a key element for monitoring changes in transmission and assessing the impact of anti-malaria interventions. This measure is intricately connected to factors such as the prevalence of infected hosts and mosquito vectors, the distribution of infection outcomes, and the genetic diversity of the parasite population.

Efficiently monitoring malaria transmission intensity relies on the availability of tools that can generate reliable estimates across diverse transmission settings [83]. Historically, malaria transmission intensity and its associated dynamics have been evaluated through methodologies such as entomological inoculation rates, spleen rates, and parasite prevalence [84]. These approaches contributed to the creation of maps illustrating mortality rates, prevalence, and the incidence of malaria, enabling a comprehensive assessment of the global malaria burden [85]. However, these methodologies have encountered limitations in terms of their sensitivity, particularly in geographical regions characterized by exceedingly low transmission rates. Due to their intrinsic limitations, these approaches are incapable of identifying genetic markers and the structural composition of parasite populations that signify altered transmission patterns in response to interventions [85]. Studies have shown that the genetic diversity of parasites changes in response to variations in transmission intensity, with higher parasite diversity observed in hyperendemic regions compared to areas with lower endemicity [86]. This confirms that the genetic diversity of parasites could serve as a reliable indicator of the fluctuations in transmission intensity. An alternative approach for gauging the intensity of malaria transmission involves assessing the prevalence of individuals harbouring the parasite, denoted as parasite prevalence. This metric can be ascertained through cross-sectional surveys employing techniques such as microscopy, RDTs, and PCR methods for detecting infections within individuals [87]. Furthermore, the use of serological data to monitor malaria transmission intensity is increasing, proving particularly effective in regions with low transmission rates. Nevertheless, this method has its own set of limitations, as it requires the imposition of a predetermined threshold to differentiate between seropositive and seronegative individuals. Additionally, this approach overlooks the continuous spectrum of antibody levels, which could potentially lead to a decrease in the accuracy of the estimation [87].

Advances in NGS technologies have significantly contributed to the understanding of malaria transmission intensity. NGS offers a high-throughput analytical approach for investigating parasite genomes, facilitating comprehensive insights into genetic diversity, mixed infections, and spatial-temporal dynamics. This transformative capability has led to a paradigm shift in the exploration of malaria transmission intensity, encompassing both local and population levels, and has greatly informed the development of strategies for controlling and eradicating the disease [85].

Field applicability of molecular tools

In regions where malaria is endemic, the successful elimination of the disease requires the identification and treatment of not only symptomatic cases but also asymptomatic carriers. Therefore, timely and precise diagnosis, along with prompt and effective treatment, plays a crucial role in implementing malaria elimination strategies in areas with varying transmission intensities that are plagued by malaria.

Various diagnostic methods are available for identifying *Plasmodium* species, including light and fluorescence microscopy, immunochromatographic lateral flow assays (RDTs), serology, and nucleic acid amplification techniques (NATs) such as PCR and isothermal amplification. While light microscopy is cost-effective and efficient, its sensitivity is limited in low transmission settings and for detecting asymptomatic cases. This limitation can lead to an underestimation of disease prevalence when compared to PCR-based assays [88]. Even the most sensitive RDTs, which rely on specific antibodies to detect malaria antigens such as histidine-rich protein 2 (HRP-2) and lactate dehydrogenase (LDH), encounter similar limitations to microscopy [7]. RDTs have been demonstrated to lack sufficient sensitivity for the community screening of asymptomatic carriers of *Pf* [89]. Additionally, RDTs designed for HRP-2 should not be employed to monitor the response to therapy, as this antigen remains detectable in the blood from a few days to several months after parasite clearance [7]. Furthermore, the use of RDTs for detecting *Pv* poses challenges due to the low parasitaemia often observed in vivax patients and the instability of LDH at higher temperatures. Consequently, there is a need for diagnostic tools with improved analytical sensitivity to effectively detect the low-level parasitaemia commonly associated with asymptomatic carriage of *Plasmodium* species.

At present, NATs stand as the most sensitive diagnostic method available, and various assays have been developed to accurately identify and distinguish all human *Plasmodium* parasite species. A comprehensive review was recently conducted to evaluate the accuracy and performance of these assays [25]. Nested PCR and real-time quantitative PCR represent additional advancements in molecular diagnostic methods. These techniques exhibit high sensitivity, capable of detecting as few as 0.03 to 5 parasites/µl of blood (which falls well below the detection threshold of microscopy and RDTs), and they offer improved specificity for identifying mixed infections. Despite their high sensitivity and specificity, the implementation of nested PCR and real-time quantitative PCR in field clinics remains limited in several malaria-endemic regions. This limitation is primarily attributed to the requirement for relatively expensive equipment and advanced training, which hinders their widespread adoption in such settings [37, 40]. Efforts aimed at enhancing the field applicability of NATs have led to the development of various assays, including PCR-enzyme-linked immunosorbent assays (PCR-ELISA), nucleic acid lateral flow immunoassay (NALFIA), nested PCR-high-resolution melting analysis (nPCR-HRM), PCR-ligase detection reaction assays (PCR-LDR), and adaptations of PCR-LDR to LDR-fluorescent microsphere assay (LDR-FMA) [90]. Cordray et al. (2012) reviewed these assays and demonstrated good analytical sensitivity; however, their practical implementation in the field is constrained by the necessity of a PCR amplification step [90].

Isothermal molecular diagnostic techniques, including LAMP, NASBA, thermophilic helicase-dependent amplification (tHDA), and RPA, present viable and practical alternatives. LAMP, a highly efficient approach for detecting DNA, is a relatively simple and field-adaptable technique [91]. In contrast to PCR, LAMP requires less complex equipment and is significantly faster. This makes LAMP a promising molecular diagnostic tool for point-of-care (POC) testing in various settings, including both developed and developing nations. LAMP has already demonstrated its efficacy in diagnosing a range of infectious diseases, including malaria [92]. LAMP performs both amplification and detection of target DNA in a single isothermal step, employing a *Bacillus stearothermophilus* DNA polymerase with strand displacement activity and a set of four specially designed primers capable of identifying six distinct regions of the target DNA [91]. Auto-cycling strand displacement DNA synthesis enables the amplification of a few initial DNA copies to an impressive quantity of 10^9 copies in less than an hour. The amplified products, comprising a series of stem-loop DNA structures with different lengths, can be easily detected through visual inspection of the turbidity caused by magnesium pyrophosphate. This by-product of DNA synthesis is generated in correlation with the quantity of amplified DNA, providing a straightforward means of detection [91, 93].

Remarkably, a genus- and species-specific LAMP diagnostic method was successfully employed for malaria diagnosis at a field clinic located in a malaria-endemic area of Thailand [94]. When tested with clinical samples, LAMP exhibited a sensitivity of 98.3% and a specificity of 100% compared to microscopy [94]. Similarly, another study demonstrated that LAMP exhibits a sensitivity of 98.3% and a specificity of 100% in comparison to microscopy for detecting *Pv* infections. Furthermore, LAMP results closely agreed with findings from a nested polymerase chain reaction method [95]. Hence, the LAMP method holds significant promise as a field-usable molecular tool for malaria diagnosis. It provides an alternative to microscopy, particularly in resource-limited settings where operators may have limited experience. Furthermore, LAMP can serve as a viable substitute for standard PCR-based analysis in clinical and operational programs conducted in the field to detect asymptomatic and sub-microscopic infections.

Another possible approach for malaria surveillance is the utilization of serological tests. These tests enable the identification of exposed individuals without relying on typical blood-stage diagnostics. They consist of screening individuals for antibodies highly specific to chosen malaria parasite protein antigens, rather than screening for the presence of the parasite DNA, thus enabling malaria exposure history to be captured [96]. These serology tools have been developed to detect *Plasmodium* infections by targeting different parasite antigens [97]. Serological methods offer advantages, including being less labour-intensive, faster, less expensive, and simpler than gold-standard microscopy. Additionally, serological tests require only one blood spot sample per individual to estimate longitudinal and recent exposures for multiple *Plasmodium* species, thereby saving resources compared to other methods [98]. Nevertheless, serological methods also exhibit certain limitations, including cross-species reactivity, the challenge of selecting appropriate antigens and antibodies, a lack of standardized methods, and varying applicability across different epidemiological settings [98].

Blood sampling for investigations

Blood sampling is a crucial aspect of malaria diagnosis and other investigations, including the study of parasite diversity, drug resistance patterns, CNVs, transmission dynamics, and sero-surveillance. Two common methods for blood sampling in malaria diagnosis and research involve the use of whole blood vials and filter papers.

Whole blood vials, both with and without anticoagulants, are typically employed to collect 2–5 ml blood samples. These samples are then separated into blood cells and either plasma or serum for laboratory-based assays such as microscopy, ELISA, PCR, quantitative bead-based array, microarray, NGS, and more. These methods enable the detection of malaria parasites, identification of their species, analysis of parasite diversity, investigation of drug resistance patterns, study of CNVs, understanding transmission dynamics, and assessment of seropositivity.

In contrast, filter papers, also known as blood spots, are used to collect blood samples in resource-limited or field-based settings. A small amount of blood is obtained by pricking the patient's finger or heel and then applied to a filter paper. The blood is allowed to dry on the filter paper, preserving the DNA of the malaria parasites. These filter papers can be easily transported, stored at room temperature, and utilized for similar investigations mentioned above.

Both whole blood vials and filter papers have their advantages and limitations. Whole blood vials provide a larger volume of blood, allowing for more accurate investigations, especially when dealing with low parasite densities. However, this method of blood sampling requires more resources, laboratory infrastructure, and trained personnel for processing.

Filter papers, on the other hand, are cost-effective, easy to transport, and do not require specialized laboratory equipment. They are particularly useful in remote or resource-limited areas. However, the sensitivity for laboratory investigations may be lower compared to whole blood vials, especially when dealing with low parasite densities.

Challenges and prospects ahead for molecular tools

The use of molecular tools for diagnosing malaria, detecting drug resistance, identifying copy number variations, and understanding parasite diversity has shown great promise in advancing our knowledge of the disease and supporting efforts to eliminate malaria. However, several challenges hinder their use in real-world settings. Molecular techniques such as PCR, LAMP, and NGS offer high sensitivity and specificity, but they can be expensive and require special equipment, trained staff, and suitable labs, limiting their feasibility in resource-limited areas. Technical expertise is vital for accurate interpretation, but limited access to skilled personnel can reduce effectiveness. Problems with preserving and transporting samples, especially in tropical regions, can affect reliability [99]. Limited access to electricity and well-equipped labs in remote areas restricts widespread use; solutions include portable diagnostic devices and mobile health technologies. Longer wait times compared to rapid tests can delay treatment; optimization is important. Sensitivity in detecting low levels of the malaria parasite is useful but might lead to false positives [99]; combining molecular results with clinical assessment and improving assay specificity is essential. Handling large amounts of data, especially with NGS, is challenging logistically and requires a good understanding of data interpretation and bioinformatics tools [100]. Maintaining quality control and standardization across labs is demanding. Overcoming these issues involves testing proficiency, following international guidelines, and navigating regulatory and ethical considerations. Despite NGS's potential for malaria research and elimination, regions with limited resources face barriers related to infrastructure, funding, and expertise. To address these challenges, it is necessary to provide equipment, build capacity, simplify protocols, and foster collaborations. Additionally, user-friendly bioinformatics tools are crucial for enhancing the accessibility of NGS. Collaborating among organizations, institutions, and governments is essential for optimizing NGS in routine malaria surveillance and accelerating progress towards elimination.

The future prospects of molecular tools in the realm of malaria elimination hold exceptional promise, offering the potential to drive transformative changes in our approach to eradicating this devastating disease. As technology continues to advance and become more accessible, molecular tools are positioned to fulfil a pivotal role across various domains. These include enhancing diagnostics, monitoring drug resistance, parasite diversity and importation, enabling personalized treatment, informing vector control strategies, vaccine development, facilitating real-time surveillance, empowering data-driven decision-making, and fostering global collaboration to track and coordinate elimination efforts.

Conclusions

Molecular tools have revolutionized our understanding of malaria, enabling more accurate diagnosis, tracking drug resistance, detecting copy number variations, and unravelling parasite diversity (Fig. 1). These advanced techniques have shown their potential to enhance malaria control and elimination efforts. Through molecular assays, we have gained deeper insights into the intricate aspects of malaria, from identifying parasite species and strains to monitoring drug resistance and assessing population diversity. These tools have proven invaluable in both research and clinical settings, offering high sensitivity, specificity, and the ability to detect minor variants that may be missed by traditional methods. They have also enabled us to uncover the complexities of asymptomatic carriers, submicroscopic infections, and the genetic variations that influence malaria

transmission and disease severity. Such insights are crucial for designing effective intervention strategies, tailoring treatments, and making informed decisions in the pursuit of malaria elimination. As these molecular tools continue to evolve and become more accessible, they hold the promise of further transforming our approach to tackling malaria. With ongoing research and technological advancements, we can anticipate even greater precision in diagnosis, a more comprehensive understanding of drug resistance mechanisms and parasite diversity, as well as improved strategies for monitoring and controlling malaria.

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Declarations

Ethics approval and consent to participate Not applicable.

Competing interests The authors declare no competing interests.

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