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Molecular Diagnostic Tools and Malaria Elimination: A Review on Solutions at Hand, Challenges Ahead and Breakthroughs Needed

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Abstract: Malaria is a serious public health problem worldwide. Globally concerted efforts are underway to control and eliminate it. Despite recent slowdown, substantial achievements have been recorded in the last 20 years. However, its eradication requires successful elimination of all *Plasmodium* parasites among symptomatic, asymptomatic, and sub-microscopic infections. This review is aimed at assessing the role of molecular diagnostic tools in malaria elimination. Quality assured malaria diagnosis is fundamental to control and elimination of malaria. High-throughput molecular diagnostic tools are important for the diagnosis, and monitoring of interventions to mitigate malaria. Molecular techniques such as real-time PCR, LAMP, nPCR, RT-PCR, multiplex-PCR, NASBA, and CLIP-PCR have been instrumental for malaria control and elimination. They enabled the detection and identification of symptomatic, asymptomatic, and sub-microscopic parasitemia. They are also important in the discovery, and development of drugs. Despite their tremendous contribution and immense potential, they are not readily available in malaria-endemic settings, fail to detect hypnozoites and infectious gametocytes as well as not sufficiently optimized for fieldwork. Those challenges might delay malaria elimination thereby threatening the quest to reach the goal of a malaria-free world by 2050. Therefore, we need novel tools fit for field application and for detecting hypnozoites, infectious gametocytes, and in vitro analysis of *Plasmodium vivax*.

Keywords: Malaria Elimination, Recurrence, Molecular Diagnostic Tools, Polymerase Chain Reaction, *Plasmodium*

1. Introduction

Malaria elimination is breaking the local transmission of a particular species of *Plasmodium* across a demarcated geographical area through thoughtful interventions [1-3]. It involves reducing the incidence of indigenous cases to zero [2, 3] and sustaining this achievement for at least three years [2]. Sustained interventions such as improved case management, robust surveillance, and vector control are fundamental to achieving and maintaining malaria elimination [2, 4, 5].

The World Health Organization in its 2015 Global Health Assembly has set a strategic framework structured with three major pillars to eliminate malaria. These pillars are: 1) ensuring universal access to malaria prevention, diagnosis, and treatment; 2) accelerating efforts towards elimination and attainment of malaria-free status; and 3) transforming malaria surveillance into a core intervention. The first pillar focuses on improving access and utilization of quality-assured management of malaria cases [6]. Interventions focusing on the *Plasmodium* parasite include early diagnosis and prompt treatment of cases and monitoring of their productivity by a robust surveillance system [4, 5, 7, 8]. It is equally important to monitor the productivity of interventions to ensure they meet the goals put in place [2, 6]. A strong surveillance system is central to ensure not only cases are declining, but also follow the effectiveness of interventions and early notification of potential resistance to interventions deployed [2, 9, 10].

A robust laboratory tool is fundamental to achieving malaria elimination by fine-tuning information obtained from clinical

diagnosis and monitoring of antimalarial interventions [11, 12]. In areas where malaria transmission is declining, more sensitive laboratory tools are required for the detection of cases, especially those with low-density parasitemia, asymptomatic infections, and individuals carrying gametocytes that can serve as potential human reservoirs of transmission [11-13]. Molecular diagnostic tools play an essential role in identifying the above-mentioned segments of the population. These tools revolutionized the control and elimination of malaria and underpinned the successes made in the last 20 years [13, 14]. Moreover, they have improved the detection limit for malaria infection from 50 parasite/μL to below 0.02 parasite/μL [15, 16].

Lack of a clear understanding of the ever-evolving parasite not only lags the achievement of the milestones set in the march toward a world free of malaria; but also sustains wastage of expendable resources that no longer works for the contemporary parasite. It seems like re-arranging the deckchairs on the Titanic. Precise and up-to-date data allows devising and implementation of timely intervention. The effectiveness of a given intervention and its implementation strategy at a given place during a given time does not guarantee its effectiveness for everyone-everywhere-forever. Moreover, in a worst-case scenario, the mere adoption of certain applauded interventions and their strategy from somewhere might exacerbate the crippling public health of an area. Failure to properly address a local situation might also put us in a position incompetent to deal with the possible risk of epidemic in particular and pandemic in general [17]. Alas, resistance to antimalarials and insecticides is expanding. This is a bold signal to intensify the search for at least a temporary replacement until a lasting solution is in place. This review is aimed at assessing the application of molecular diagnostic tools in malaria elimination and identifying their strength and weakness. It also covers the their contribution to the achievements made, and remaining works to fill the identified gaps as well as raises outstanding questions to drive future researches.

2. Role of Molecular Diagnostic Tools in Malaria Elimination

Molecular diagnostic tools are pivotal for the diagnosis of malaria and follow response to treatment. They are important to characterize transmission intensity and dynamics by determining the connectivity of the parasite population through the identification of the gene flow of a parasite strain [3, 18]. Besides, these tools detect and distinguish local and imported cases, which is an essential indicator for assessing the closeness of an area to declare elimination [2].

Molecular diagnostic tools help identify hot pops and hotspots, thereby revealing the local transmission chain and identifying foci of transmission which is vital for targeted intervention [18, 19]. These tests are essential for in vivo efficacy studies to detect treatment failure, molecular signatures responsible for drug resistance, and predict resistance by monitoring markers of negative selection [2, 20]. They also help detect histidine-rich protein 2/3 (HRP2/3) deletions affecting malaria rapid diagnostic test (RDT) efficacy to inform decision-makers in the selection of diagnostics. This is important to address concerns about the origin, spread, and impact of resistance to antimalarial interventions [3, 21]. Furthermore, molecular diagnostic tools are useful to evaluate the efficacy of malaria vaccine and to check the range of vaccines against which species and/or strain it confers protection from infection and/or severe disease [3, 22]. In the following sub-sections we will discuss some of these tools with their application, strength and weakness.

2.1. Real-Time PCR

Real-time polymerase chain reaction (PCR), usually denoted as quantitative PCR (qPCR), is characterized by continuous monitoring of the production of target amplicons from the parasite deoxyribonucleic acid (DNA). It is a molecular technique involving the collection of data on the target DNA as it occurs throughout the PCR process [23]. This technique uses DNA-binding fluorescent-labeled probes whose fluorescence emission rises in response to binding to a target DNA. The florescence is analyzed and graphically presented corresponding to the presence and quantity of the parasite DNA [23].

The fluorescence labels include double-stranded DNA (dsDNA) intercalating dyes such as SYBR Green, and sequence-specific oligonucleotide probes such as TaqMan probe. However, the SYBR Green probe binds to the double-stranded PCR product and emits light that allows monitoring the total amount of the amplicon without distinguishing between different sequences. On the other hand, the TaqMan probe system involves incorporation of sequences complementary to the target DNA thereby making the probes specific to ensure the amplification of only target DNA sequences and result in fluorescence [23]. The TaqMan probe system enables the production of fluorescence directly proportional to the amount of the specific target sequence that has been amplified as displayed by the figure below [24]. (Figure 1).

Application

Real-time PCR is a central molecular tool with multiple application for various malaria elimination works [24]. It is an ideal tool for active case detection in epidemiological and clinical studies, mainly asymptomatic and sub-microscopic infections [24, 25]. It can also be used for monitoring therapeutic efficacy and assessing genes liable for resistance to drugs and insecticides [24].

Strength

Real-time PCR carries numerous advantages beyond a limited number of laboratory room requirements. It allows simultaneous amplification, detection, and quantification of the parasite in a single step by eliminating the need for gel electrophoresis. Besides, avoiding post-amplification manipulation reduces the risk of contamination and quick availability of results [13, 26]. It allows multiplexing; as well as can help detect genes responsible for resistance to antimicrobials [26, 27]. According to the report by Haanshuus *et al*. (2019), a genus-specific conventional

cytochrome b (cytb) SYBR real-time PCR has shown high sensitivity in field studies by detecting 70% sub-microscopic parasitemia. Such a highly sensitive, specific, and user-friendly real-time qPCR can be useful in both epidemiological and clinical studies [28].

This test provides direct information on the resistance status of the parasite and reveals the spread of drug resistance species and/or strain, both of which serve as crucial evidence to modify policies in malaria control and elimination [29]. Furthermore, combining, the Real-time PCR with High-Resolution Melt (HRM) showed a promising result to identify recrudescent *P. falciparum* in treated malaria patients. This test is good in terms of sensitivity, specificity, simplicity, speed, low risk of contamination, reasonable cost, and easy result interpretation [26, 29, 30].

Weakness

Real-time PCR cannot show the expression of a particular gene. Therefore, results from Real-time PCR should be augmented by biochemical assays and phenotypic tests including HRM to help a strong evidence-based decision [26, 31]. Sometimes real-time PCR may persist positive after treatment or recovery rendering overestimation of parasite prevalence after treatment [32].

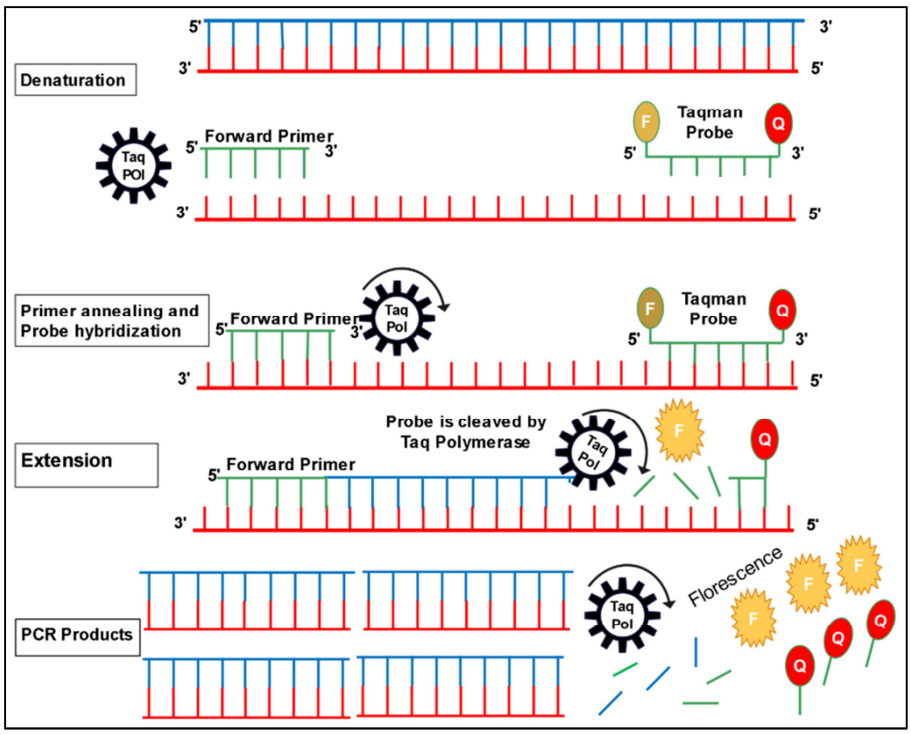

Figure 1. *Graphical display of real-time PCR using sequence-specific oligonucleotide probe (TaqMan System).*

The TaqMan System real-time PCR involves the use of probe sequence extending 18 to 25 nucleotides that is labelled at the five prime end with a floresent reporter dye (F) and at the three prime end with a quencher dye (Q). During the strand displacement step of the PCR, the five prime to three prime exonuclease activity of Taq Polymerase cleaves the florescent reporter from the probe sequence. This causes emission of floresence signal that is measured at every cycle of the amplification process. In intact probes, floresence of the reporter quenched by close presence of the quencher. Probes and the complementary DNA strand are hybridized and the reporter's floresence is still quenched. During extension step of the PCR, the probe is degraded by Taq Polymerase and the floresence reporter is released resulting in emission of floresence, which is an essental information to detect and quantify the target DNA. *PCR*: Polymerase Chain Reaction; *Taq Pol*: Taq Polymerase; *Q*: Quencher; *F*: Fluorophore

2.2. Nested PCR

Nested PCR is a type of PCR that involves two sets of primers used in two sequential runs of PCR. It is a technique whereby the first PCR generates a mix of all *Plasmodium* species DNA products, which can be used in the second PCR run with primers internal (nested) to the first pair of primers. The first set of primers binds outside of the flanking region of target DNA sequence thus called outer primer, and results in amplification of larger fragments. The second set of primers binds to the inner sequence of the first run reaction thus called inner primer, and results in amplification of smaller fragments [33]. The first amplification allows detection of genus-specific genes of the *Plasmodium*. Products of samples tested positive in the first reaction will then be subjected to a further four independent reactions (for four species of *Plasmodium*) to determine the species composition [33-36].

The nPCR assay genotypes malaria parasites by targeting genus-level-marker genes for 18S rRNA, followed by species-level-marker genes for the second round of four independent reactions meant for specie identification [33]. Merozoite surface proteins (MSP1 and MSP2), and gene of the Glutamate-rich Protein (GLURP) are highly polymorphic repetitive regions on different genes of the *Plasmodium*

species that are commonly used as markers for genotyping by nPCR. Detection of the amplicons from nPCR is usually done by agarose gel electrophoresis [37]. (Figure 2).

Application

Nested PCR is an important molecular tool to characterize recurrent *Plasmodium* infections, detect sub-microscopic parasitemia, and monitor the therapeutic efficacy of malaria drugs. Besides, its good sensitivity and specificity make nPCR suitable for diagnostic, research and epidemiological purposes [34, 38]. Furthermore, in areas with known species and low prevalence of malaria it can also be applied for epidemiological surveys, as there will be few samples for the second run [33].

Strength

Nested PCR is an ideal tool to assess the therapeutic efficacy of malaria drugs thereby signaling for the lurking potential drug resistance [39]. Its high sensitivity helps to detect asymptomatic and sub-microscopic parasitemia. It also helps to differentiate between recrudescent and reinfections due to *P. falciparum* [10, 40, 41]. This assay is not easily affected by DNA template quality and has reduced susceptibility to minor variations in the amplification conditions [33]. By involving the use of two sets of primers, nPCR reduces the risk of non-specific binding thereby enhancing the specificity of the PCR reaction [33, 42].

Weakness

Despite its remarkable benefits, nPCR is prone to contamination due to its high sensitivity, and the involvement of sample manipulation during and post-amplification [33, 34]. Besides, it cannot differentiate between relapse, recrudescence, and reinfection as *P. vivax* infection might involve parasites with similar or different genotypes to the parasite found in an initial infection [30, 36]. It is a resource-demanding technique, particularly time and extra primers [30]. Due to its high cost, nPCR is not widely available in developing countries suffering from a high burden of malaria. Even the cost will radically rise tests repeated due to contamination [36]. In the case of therapeutic efficacy study, nPCR may potentially over-estimate treatment failures resulting from poor absorption, inadequate biotransformation of pro-drugs, and rapid elimination due to diarrhea and/or vomit that is arising from inter-individual variations [29].

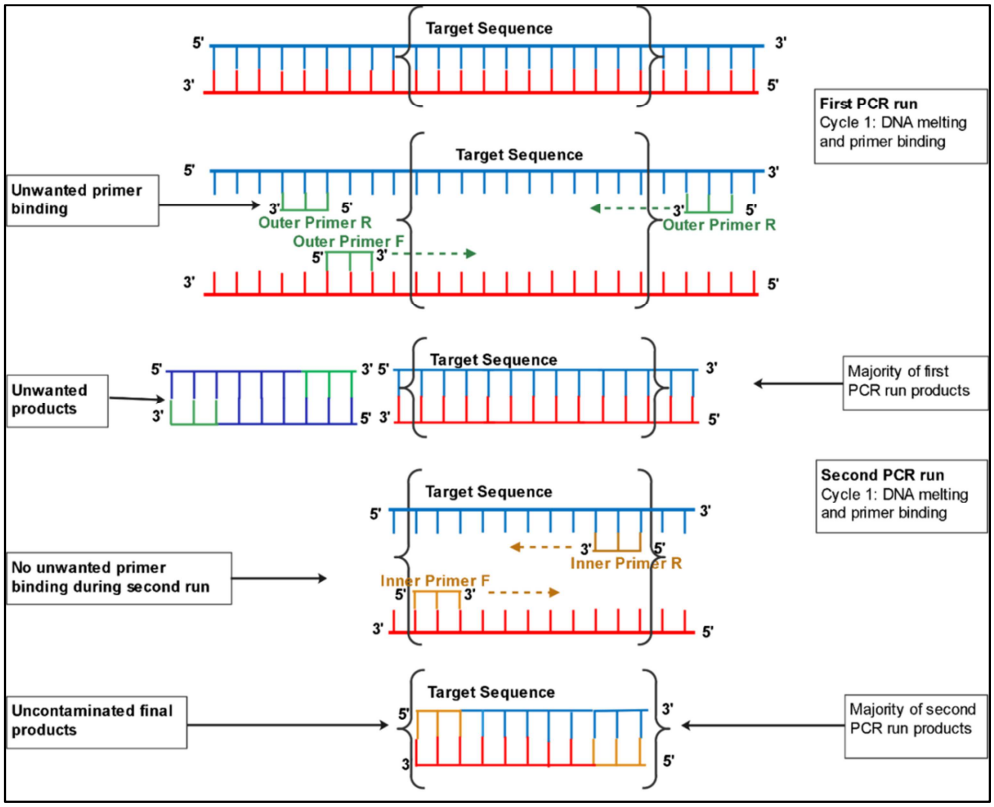

Figure 2. *Schematic diagram of nested PCR.*

Nested PCR technique involves a two-stage amplification of a target DNA of malaria parasites by using two sets of primers. These primers target markers at the genus-level and species-level of the *Plasmodium*. The outer primer sets target genus-level marker: 18S rRNA, whereas the inner primer sets (nested) target species-level markers. The first run may produce wanted and unwanted products, but no unwanted product amplification in the second run. A portion of the amplicons from the first run is used as a template in each of the subsequent four separate runs to provide uncontaminated species-level final products. *nPCR*: nested PCR; *F*: Forward; *R*: Reverse

2.3. Multiplex PCR

Multiplex PCR is a simultaneous detection of multiple targets within a single reaction using different pairs of primers designed for each target [27]. A multiplex assay is a type of assay allowing the simultaneous analysis of several

analyte in a single run of the assay. In malaria context, it enables detecting the presence of multiple species of *Plasmodium* in a particular sample with the help of specific primers designed to detect a universal malaria marker and species-specific markers [43]. Recent developments of a Multiplex Malaria Sample Ready PCR showed a promising result in Sierra Leone, where it showed twice and four times increased sensitivity compared to RDT and microscopy, respectively [44]. (Figure 3)

Application

Multiplex PCR is used for diagnosis of malaria, and to characterize recurrence showing its application in malaria control endeavors [45]. It plays an important role in malaria elimination since it can be used to monitor diversity of parasites after transmission-blocking intervention [44].

Strength

Multiplex PCR provides more information with fewer samples in a reasonably short time. It allows the detection of many species of *Plasmodium* from a single sample with a single run. Multiplexing is a good cost-saving option, with particular implications for resource-limited areas [46]. Moreover, by avoiding the sample manipulation compared to nested PCR, multiplex PCR is less prone to contamination [30].

Weakness

Multiplex PCR suffers from process complexity, variability in efficiency for different templates, and poor universality. When multiplex PCR was used to replace nPCR for *Plasmodium* species, the sensitivity gets compromised due to competition among different amplified fragments for limited supplies available in the reaction well. Multiplex PCR suffers from self-priming among a diverse set of primers, and a lack of equal amplification efficiency on various templates [46, 47].

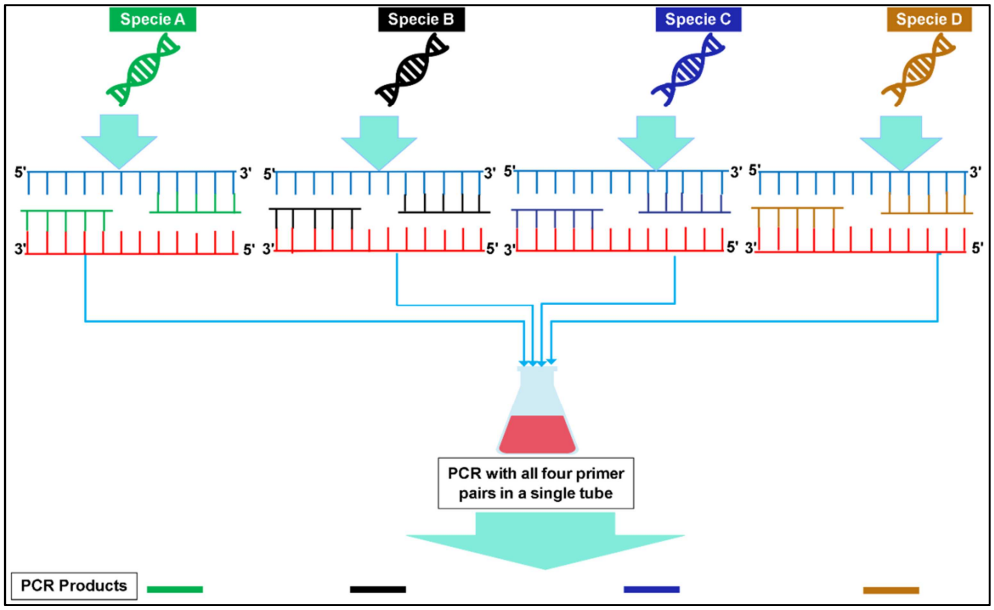

Figure 3. *Diagram showing multiplex PCR.*

Multiplex PCR is type of PCR whereby multiple target sequences are simultaneously amplified in a single reaction well. It involves use specific primers that can specifically combine with their corresponding DNA template, hence allow amplification of more than one DNA fragment from a single and/or multiple samples.

2.4. Reverse Transcription-PCR

Reverse transcription-PCR is a type of PCR characterized by the synthesis of complementary deoxyribonucleic acid (cDNA) from single-stranded ribonucleic acid (RNA) templates by reverse transcriptase followed by amplification of the cDNA by PCR [48]. Nowadays, RT-PCR has generally become a real-time RT-PCR (qRT-PCR) assay that utilizes a well-established three-step protocol. These steps are 1) a reverse transcription step that converts RNA into cDNA; 2) amplification of the cDNA using a heat-stable DNA polymerase known as Taq polymerase, and 3) detection and quantification of the amplified products in real-time [49].

Concerning malaria, RT-PCR indirectly assesses the parasite gene expression by targeting the translated RNA expressed in the nucleic acid sequence [13]. Gametocytes of *P. falciparum* and *P. vivax* are detected and quantified by qRT-PCRs targeting the two orthologous Pfs25 and Pvs25 transcripts using specific primers and probes. For male gametocyte detection and quantification, specific primers and probes will target Pfs230p [50, 51]. (Figure 4).

Application

An RT-PCR is an ideal tool for epidemiological study due to its application to determine gametocyte carriage, which is important to assess transmission dynamics [48, 52]. Besides, its high sensitivity makes an RT-PCR a good choice to assess asymptomatic and sub-microscopic infections [53, 54]. This qRT-PCR is a powerful tool to measure gene expression [49]. The capacity of RT-PCR to accurately quantify the

gametocyte sex ratio and density makes it vital for elimination phase as it can be applied to identify human infectious reservoirs of transmission. Furthermore, this tool helps to evaluate efficacy of transmission-blocking interventions [55].

Strength

As RNAs are more abundant than the corresponding gene, RT-PCR bears better sensitivity than PCR making them suitable for detecting sub-microscopic and asymptomatic *Plasmodium* infection [15, 56, 57]. These tools can be used to study the parasite at different stages, particularly at gametocyte stage [53, 54]. According to Schnieder *et al*., the Pfs25 and Pfs230p gametocyte-specific RT-qPCR have lower detection limit of 0.3 female and 1.8 male mature gametocytes per microliter of blood, respectively [55]. In general, RT-PCR method, targeting 18S rRNA of the parasite provides a service with high sensitivity that can reach up to 2-20/mL [13].

Weakness

An RNA-based test is usually less applicable in clinical settings due to inherent limitations. These tests are technically demanding due to their reliance on RNA purification and reverse transcription. Furthermore, RNA is susceptible to quick degradation [13]. Consequently, RT-PCR is less applicable for large-scale use in field surveys [58].

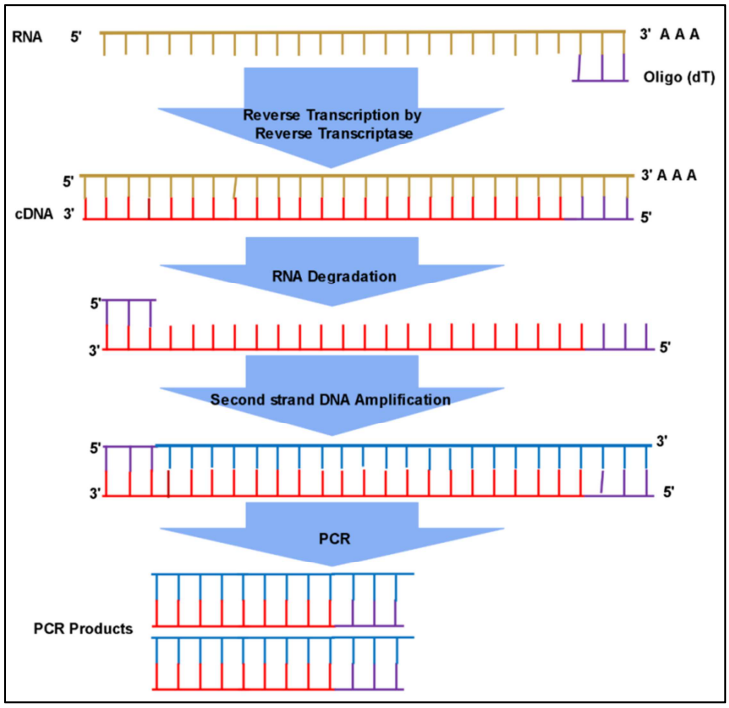

Figure 4. *Schematic display of RT-PCR.*

Reverse transcription-PCR (RT-PCR) involves conversion of RNA into compementary DNA (cDNA) by reverse transcriptase followed by amplification of the cDNA by PCR.

2.5. Quantitative Nucleic Acid Sequence-Based Amplification (QT-NASBA)

Nucleic acid sequence-based amplification (NASBA) is an isothermal (~41°C) nucleic acid amplification method involving RNA target. The method uses a combination of three enzymes (reverse transcriptase, T7 RNA polymerase, and RNase H). These enzymes act in collaboration to bring about a rapid amplification of target sequences without the involvement of thermal cycling to yield a single-stranded RNA from original RNA template [13, 59, 60]. (Figure 5).

Application

Nucleic acid sequence-based amplification technique can be applied to amplify and produce several copies of a specific segment of RNA/DNA. These amplified RNA and DNA molecules can be used for genotyping, sequencing, and detection of pathogens [59, 61]. It is also an important tool in the detection of sub-microscopic and asymptomatic *Plasmodium* infection [59, 61-63]. The QT-NASBA successfully enabled determining gametocyte carriage that can be utilized for fieldwork [59-62]. Application of gametocyte QT-NASBA can optimize understanding the biology and epidemiology of malaria transmission [60, 63]. This tool plays a pivotal role in malaria elimination efforts to characterize malaria transmission by detecting sub-microscopic gametocytemia, which is critical to guide targeted intervention [60, 64]. A QT-NASBA can serve as an ideal tool to monitor drug resistance and therapeutic efficacy in malaria [65, 66].

Strength

The use of NASBA transformed RNA detection through its quick one-step isothermal process. It avoids the need for an expensive thermo-cycler as the reaction occurs isothermally, but it can also involve both isothermal and non-

isothermal amplification [67]. The high sensitivity of NASBA makes it appropriate to detect sub-microscopic and asymptomatic infections [68]. Real-time NASBA is more convenient than real-time PCR for quantification of *P. falciparum* [59]. Quantification of various developmental stages of *P. falciparum* can be done by QT-NASBA [60]. Since the reaction does not require a DNA denaturation step, it avoids genomic DNA amplification, thus helping mRNA detection with no risk of DNA contamination [59, 61].

Weakness

With due consideration of equipment and supplies, NASBA is estimated to have a high cost compared to other types of PCR [69]. In addition to that, NASBA is less reproducible than qRT-PCR in case of low density gametocytemia [70].

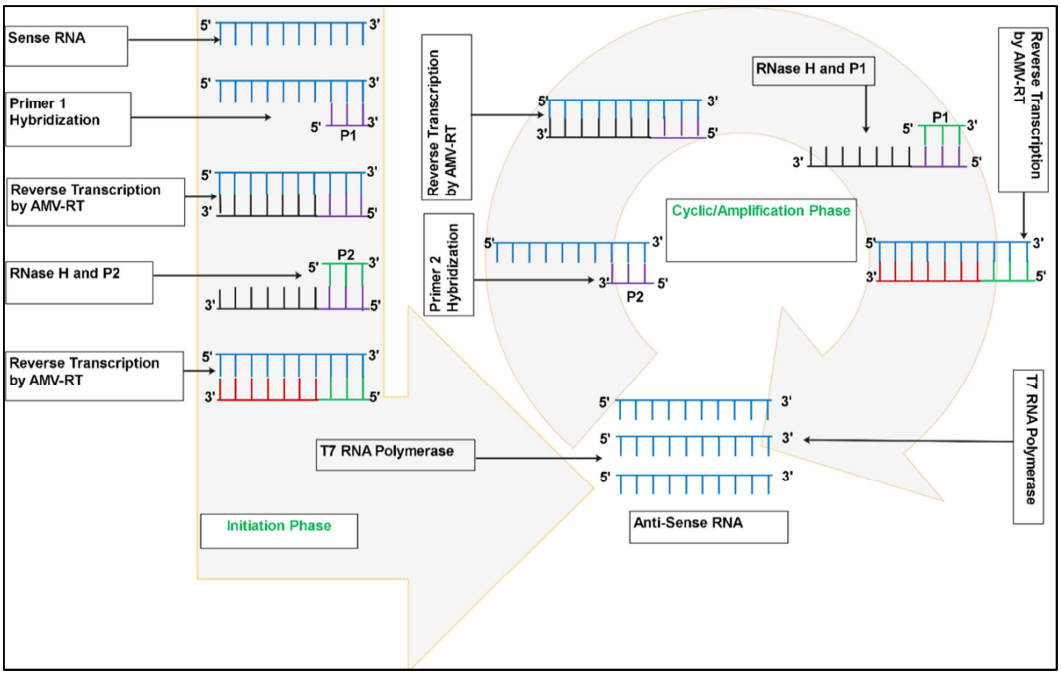

Figure 5. *Schematic representation of NASBA.*

Nucleic acid sequence-based amplification method is a two-step molecular biology method to produce multiple copies of single stranded RNA. It takes RNA molecule and aneals it with specifically designed primers to amplify the target by making use of an enzyme cocktail. This enzyme cocktail, which is composed of Avian Myeloblastosis Reverse Transcriptase (AMV-RT), RNase H, and RNA polymerase, helps for successful amplification. An AMV-RT synthesises a cDNA strand from the RNA template once the primer is arranged. RNase H hten degrades the RNA template and the other primer binds to the cDNA to form double stranded DNA, which RNA Polymerase uses as a baseline to synthesize copies of RNA. cDNA: Complementary DNA; RNA: Ribonucleic acid; P1: Primer 1; P2: Primer 2.

2.6. Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a one-step amplification reaction of nucleic acids that employs self-recurring strand-displacement synthesis at a single temperature. This tool is primed by a specially designed set of primers. It uses four to six different primers specifically designed to recognize the corresponding four to six particular regions by binding to distinct sequences on the target gene. These distinct regions in the target gene are F3C, F2C, and F1C regions at the 3' end, and R1, R2, and R3 regions at the 5' end [13, 69, 71]. This technique uses a DNA polymerase extracted from *Bacillus stearothermophilus*, which has strand displacement activity and can perform isothermal amplification. The amplification product can be detected visually under UV fluorescence or via densitometry and turbidimetry [72, 73]. (Figure 6).

Application

Its high sensitivity made LAMP a good tool to detect asymptomatic malaria and placental malaria from peripheral blood [74]. Also, its temperature range made it suitable for field surveillance of both symptomatic, asymptomatic and sub-microscopic parasitemia [13, 69, 71]. LAMP can serve as a point-of-care NAAT-based diagnostic test [75]. Furthermore, LAMP has been extensively applied to various operational researches on malaria [76].

Strength

Loop-mediated isothermal amplification method is technically easy to operate and has excellent sensitivity and specificity at a relatively low cost compared to its return [30, 74, 77]. Eyes, gel electrophoresis, and turbidimeter can be used to visualize the amplified DNA product. The reaction takes place at a single temperature without the need for expensive equipment [13, 69, 71]. The use of *Bacillus stearothermophilus* DNA polymerase made LAMP more resistance to inhibitors such as heme than TAQ polymerase-based PCR [73, 76].

Weakness

Loop-mediated isothermal amplification method has limited amenability to multiplexing and involves a complex approach to designing its primers [30]. It is still relatively

more expensive than the conventional techniques, thus not readily available in malaria endemic settings [76].

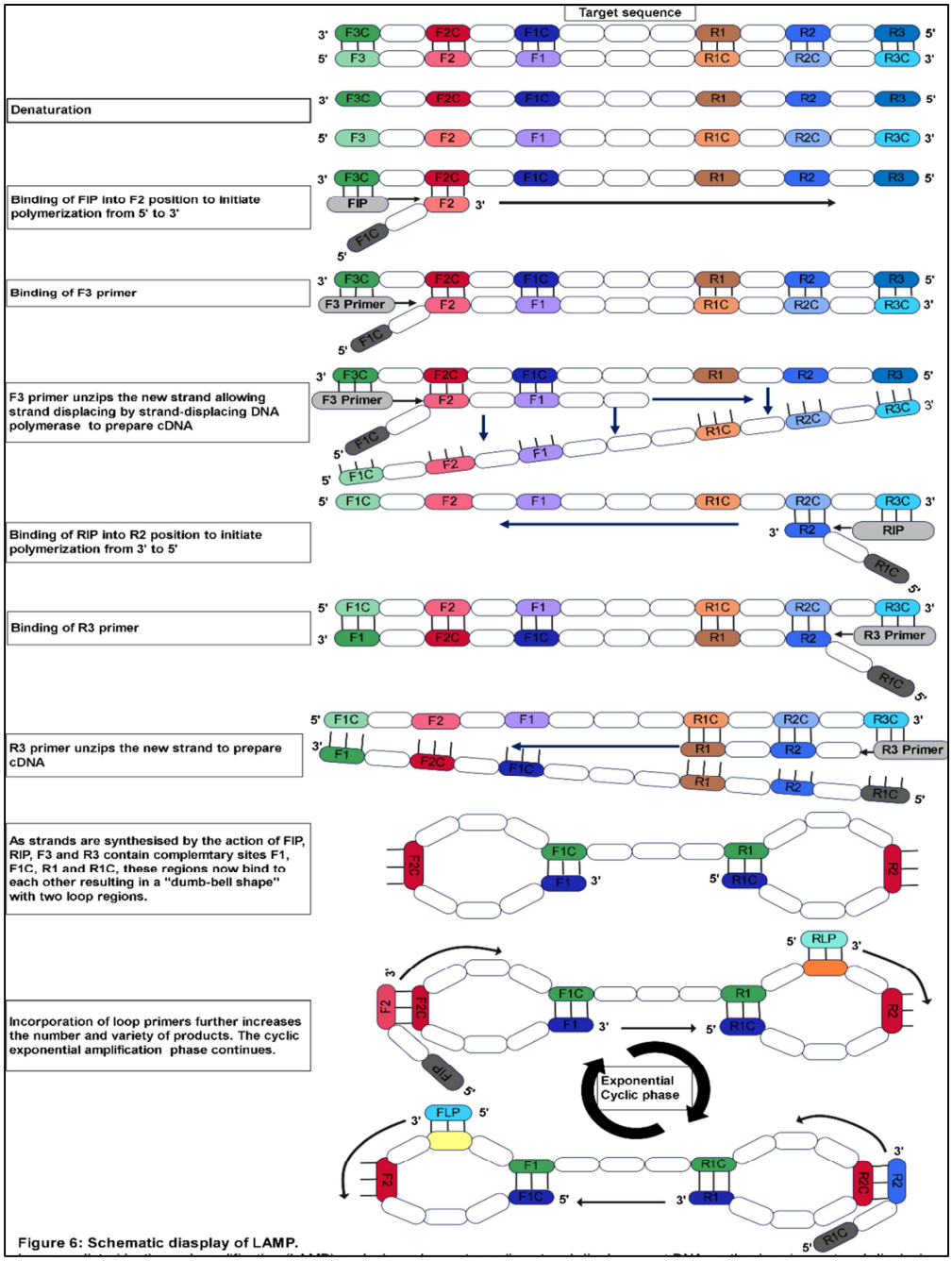

Figure 6. *Schematic display of LAMP.*

Loop-mediated isothermal amplification (LAMP) works based on auto-cycling strand displacement DNA synthesis using strand displacing DNA polymerase and two-pairs of target-specific primers. A strand displacing DNA polymerase initiates the synthesis and two of the primers form loop structure to facilitae the subsequent rounds of amplification. The single stranded DNA forms stem-loop structure (dumb bell appearance) through self-annealing to the corresponding equence and therefore, acts as a template for cyclic exponental amplification. Forward loop primers and reverse loop primers help to increase the detection efficiency by accelerating the amplification reaction. *FLP*: Forward Loop Primer; *RLP*: Reverse Loop Primer; *FIP*: Forward Inner Primer; *RIP*: Reverse Inner Primer: *R3*: Reverse Outer Primer: *F1C*: Complemetary to Forward 1; *R1C*: Complemetary to Reverse 1.

2.7. Capture and Ligation Probe PCR

Capture and ligation probe-PCR (CLIP-PCR) is a molecular method involving isolation of 18S rRNA of the *Plasmodium* from the blood that will be captured onto 96-well plates and quantified by the amount of ligated probes continually binding to it [16]. (Figure 7).

Application

Due to its high sensitivity and high-throughput approach, CLIP-PCR is ideal to detect asymptomatic and sub-microscopic infections. Furthermore, as it allows the

incorporation of pooling strategy, it is good for large-scale surveillance of malaria in elimination areas [16, 30].

Strength

Capture and ligation PCR allows the use of dried blood spots with a matrix-pooling strategy providing a service with remarkable sensitivity and throughput [13, 16]. Besides, if pooling is done on 96 well plate, this method can cost-effectively detect asymptomatic and sub-microscopic infections [16, 30].

Weakness

This method is relatively complex requiring for optimal training of laboratory personnel [30, 78]. CLIP-PCR is relatively expensive for dealing with individual samples [30].

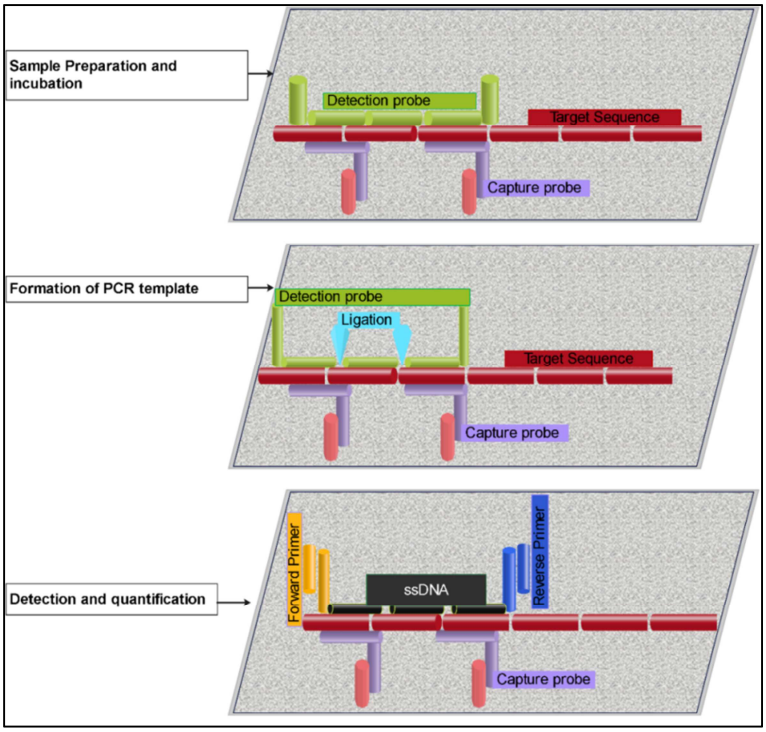

Figure 7. *Schematic illustration of CLIP-PCR.*

Capture and ligation probe-PCR (CLIP-PCR) involves isolation and capture of a target sequence by capture probes immobilized into 96-well plates and quantification by amount of ligated probes continually binding to it. Basically, CLIP-PCR involves three steps:

1) *Sample processing*: Samples (often DBS) are lysed to release 18S rRNA.

2) *Formation of PCR template*: During an overnight incubation of sample lysate, capture probes, and detection probes bind to a contigous part of the highly conserved region in 18S rRNA of the *Plasmodium*. The capture probe incorporates two regions: one for target binding and one for anchoring the target to a solid surface by hybridizing with probes on the solid surface. Following the removal of the unbound probes by washing, detection probes that bind adjacent to one another, are ligated to form a longer ssDNA. Besides, the detection probes located at both ends contain additional region that serve as a universal primer binding site.

3)*Detection and quantification*: The newly ligated ssDNA, whose quantity is proportional to target RNA, is quantified by qPCR with a universal primer set and SYBR green approach.

3. Achievements Made by Leveraging Molecular Diagnostic Tools into Mitigation of Malaria

The introduction of molecular techniques substantially contributed in multiple ways to the control and elimination of malaria [79, 80]. Advances in molecular biology helped to unlock the gametocyte biology that led to progress in diagnostic and therapeutic approaches [81-84]. These tools were essential for the recently approved RTS, S/AS01 vaccine for use in under-five children which marks a key milestone in the tenacious search for malaria vaccine [14].

Molecular techniques transformed diagnostic tests by detecting asymptomatic and sub-microscopic infections [9, 85]. It also helped to monitor therapeutic efficacy and characterize recurrent infections with *P. falciparum* [9, 79]. Sequencing technology has enhanced our understanding of the mechanisms of action of current and new antimalarials through drug-resistant parasite selection in vitro [86]. In addition, sequencing technologies showed promises for characterizing genes and their products conferring resistance to antimalarial drugs [87-89]. Molecular diagnostic tools also enabled the use of non-invasive PCR-based assays to detect *Plasmodium* parasites in saliva, [90, 91], though efforts continued to make it suitable for routine diagnosis [92].

4. Remaining Works

Despite our major triumphs in the last 20 years, there are areas to work on to optimize these gains and safeguard our

effective interventions. We need tools to study *P. vivax* gametocyte development; hypnozoite aspect; and ookinete biology [80]. Moreover, we need a robust molecular tool to diagnose hypnozoites of *P. vivax* [14, 93-95]. Hypnozoites can give rise to malaria recurrence and sustain onward transmission thereby jeopardizing elimination efforts [80, 94]. Malaria recurrence raises the efficiency, likelihood, and longevity of transmission [2, 18, 80, 96].

Basic science and operational researches are needed to optimize understanding and guide application. Lack of dependable studies supporting the efficient application of gene-editing technologies such as CRISPR/Cas-9 [80]. We need diagnostic tools that can help diagnose G-6-PD deficiency in fetuses and children under six months old for extensive use of primaquine as a radical cure [97, 98]. The need for effective therapeutic and diagnostic tools to unlock the puzzles of *P. vivax* remained widely open [99, 100]. To minimize the complexity and resource demand, assays for in vivo detection of infectious gametocytes are needed [99].

On the other hand, a big knowledge gap exists to characterize parasite resistome and mitigation strategies to improve the efficiency of therapeutics and protect them from premature removal by unproven resistance [80, 101]. Molecular markers for drug resistance are often assessed in research only, but not thoroughly monitored in clinical work and surveillance by national malaria control programs [21]. Generally, it is fundamental that molecular diagnostic tools are efficient enough to address these problems to break transmission and thus, eliminate malaria [2, 18, 80, 96]. Summary of remaining works, their inter-relations, public health importance and potential research questions are diagrammatically displayed below. (Figure 8).

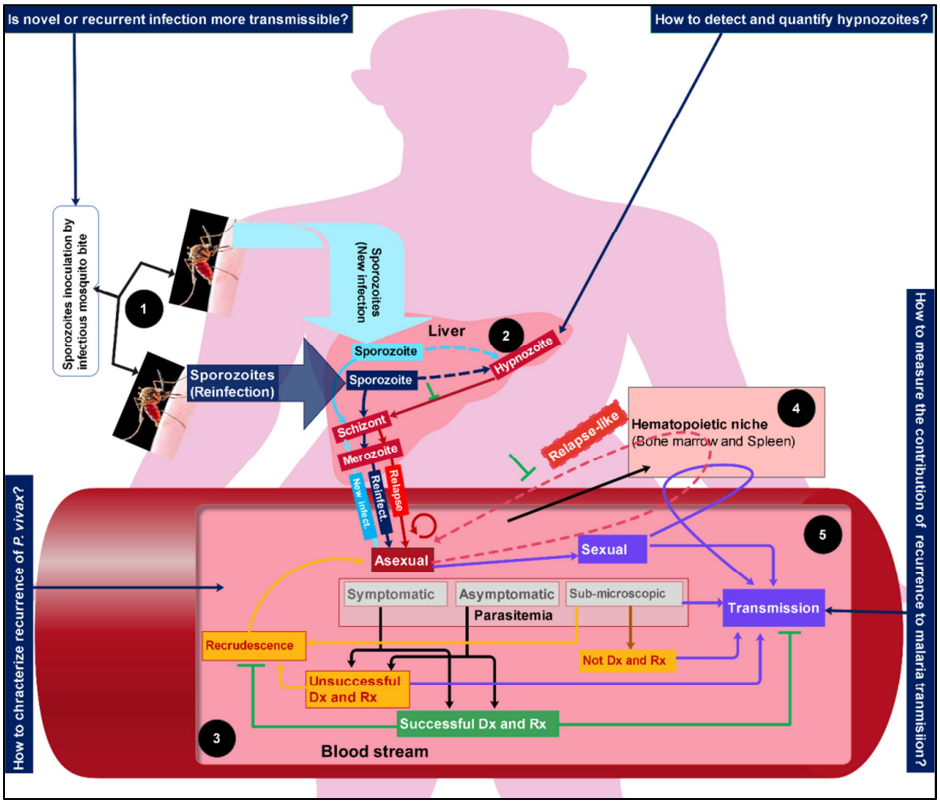

Figure 8. *Infection, recurrence, transmission, challenges and weak spots to intervene for malaria elimination.*

1) Human infection with Plasmodium occurs due to inoculation of sporozoites by a bite from infected female anopheles mosquito. Blue and pale blue solid lines show flow of malaria pathogenesis from new and reinfection following sporozoite inoculation.
2) The inoculated sporozoites after arriving in the liver, either transform into hepatic schizonts or directly become hypnozoites that later become schizonts (P. vivax and P. ovale) that give rise to merozoites.
3) The merozoites join the blood circulation to invade erythrocytes and produce asexual stages of the parasite. These asexual stages are responsible for signs and symptoms of malaria. New or recurrent infection with *Plasmodium* can cause symptomatic, asymptomatic and sub-microscopic malaria. Brown lines show the aftermath of sub-microscopic parasitemia left untreated. Red circular arrow show the erythrocytic cycle for the asexual reproduction of the parasite.
4) Purple boxes and lines show young gametocytes localizing into the hematopoietic niche and rejoining the circulation for maturation purpose.
5) Gametocytes may result from any untreated infection or unsuccessful treatment or after treatment with non-gametocytocidal drug. These gametocytes mediate the transmission of malaria.
6) Broken red lines show asexual parasites that periodically sequester in hematopoietic niche and return into the circulation resulting in relapse-like parasitemia. Black line indicate parasites sequestering in the hematopoietic niche. Greenish and yellow boxes and lines show the result of unsuccessful treatment and sub-microscopic parasitemia, both of which can lead to recrudescence and/or transmission. Green T-shaped lines indicate possible weak spots for intervention (such as early diagnosis and prompt treatment with efficacious radical cure and transmission-blocker) to tackle recurrence and/or transmission. The four questions inside blue boxes with long arrow at periphery show potential areas for research.

5. Conclusion and Recommendation

5.1. Conclusion

A quality-assured laboratory test provides accurate, reliable, and timely information that can guide clinical and public health decision-making. Molecular diagnostic tools, which keep advancing daily, carry a tremendous potential to help achieve local elimination and global eradication of malaria. They guide the evidence-based choice of the right drug and monitor its efficacy. They fine-tune a piece of information generated at the clinical setting, public health level, and researches level.

However, these molecular diagnostic tools are not readily available in areas carrying the largest share of the global malaria burden- the WHO African region. The sub-Saharan African countries largely depend on conventional diagnostic tools, such as RDT and light microscopy, despite carrying nearly 94% of the worldwide prevalence of malaria.

Despite the remarkable success in the last 20 years, the malaria burden is rising in recent years. Even though the COVID-19 pandemic is partly blamable, the growing spread of resistance to treatments, insecticides, and diagnostic tools makes achieving the ambitious goal of malaria-free questionable. Generally, although molecular tests have considerably revolutionized malaria mitigation, lots of work remains to optimize their yield in the fight against malaria.

5.2. Recommendation

1) It is urgent to put a concerted effort to make molecular diagnostic tools available in areas heavily affected by malaria. It is essential to make them user-friendly, durable, and cost-effective while maintaining their quality.
2) Stakeholders should attempt to avail field applicable molecular diagnostic tools to improve case detection, particularly for asymptomatic and sub-microscopic infections.
3) It is equally important to continue searching for new tools with better resolution capacity to improve molecular surveillance for monitoring the efficacy of interventions currently used.
4) Efforts should continue in the potential use of gene-editing technologies such as CRISPR/Cas-9 to render the parasite no longer troublesome for the human host.
5) Search should continue, mainly in metabolomics-based methods, to discover biomarkers that can serve as targets for drugs and diagnostics to accelerate efforts toward elimination.

Abbreviations

CLIP-PCR: Capture and Ligation Probe PCR; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; DNA: Deoxyribonucleic Acid; dsDNA: Double-stranded DNA; LAMP: Loop-mediated Isothermal Amplification; nPCR: Nested PCR; PCR: Polymerase Chain Reaction; QT-NASBA: Quantitative Nucleic acid Sequence-based Amplification; qPCR: Quantitative PCR; RDT: Rapid Diagnostic Test; RNA: Ribonucleic Acid; RT-PCR: Reverse Transcription-PCR.

Author Contributions

AA was involved in designing, searching for important literatures, write-up, and review of the manuscript. All the authors have read and approved the final manuscript.

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Availability of Data and Materials

The data used to support the findings of this study are all included in the manuscript.

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