Malaria diagnosis for malaria elimination

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Purpose of review
Limitations of blood smear microscopy contributed to failure of the 1950–1960s WHO Global Programme to Eliminate Malaria. All diagnostic methods encounter limits of detection (LOD) beyond which it will not be possible to identify infected individuals. When this occurs, it becomes difficult to continue evaluating progress of malaria elimination. The purpose of this review is to compare available diagnostic technologies, factors that underlie their LOD, and their potential roles related to the goal of elimination.

Recent findings
Parasite-containing cells, parasite proteins, hemozoin, nucleic acids, and parasite-specific human antibodies are targets of diagnosis. Many studies report advantages of technologies to detect these diagnostic targets. Nucleic acid amplification tests and strategies for enriching capture of malaria diagnostic targets have consistently identified a parasite reservoir not detected by methods focused on the other biological targets. Exploiting magnetic properties of hemozoin may open new strategies for noninvasive malaria diagnosis.

Summary
Microscopy and rapid diagnostic tests provide effective surveillance for malaria control. Strategies that detect a reservoir of submicroscopic infection must be developed and standardized to guide malaria elimination.

Keywords
diagnosis, elimination, limits of detection, malaria

INTRODUCTION

Five *Plasmodium* species cause human malaria infections, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (with subspecies *curtisi* and *wallikeri* [1]), and *P. knowlesi* (zoonotic – Southeast Asian macaques and humans [2]) and constitute targets of malaria diagnosis. As all diagnostic methods have limits of detection (LOD), this review will consider the status of diagnostic methods to detect these species to produce the data that will help National Malaria Control Programmes (NMCPs) reach elimination. It bears further mention that there are two parallel diagnostic objectives within the malaria context: first, point-of-care diagnosis for symptomatic patients presenting at clinics; and second, quantifying the parasite reservoir represented by asymptomatic submicroscopic infections (SMI) that stands as a barrier to achieving elimination. As this review considers diagnosis for elimination, we are focused on the second diagnostic objective of SMI. Malaria diagnosis must be strengthened given the importance of continuing progress against this disease and the global investment in malaria elimination. Methods for detecting human as opposed to mosquito [3,4] or nonhuman primate malaria [5] infections will be favored here. Aspects of malaria diagnosis beyond the focus of this review include monitoring antimalarial drug resistance markers and developing products to assess glucose-6-phosphate dehydrogenase deficiency (G6PDd) to improve safety of primaquine (and ultimately tafenoquine) treatment of *P. vivax* and *P. ovale* hypnozoites. The Worldwide Antimalarial Resistance Network (http://www.wwarn.org) and recent publications by Domingo *et al.* [6] and Satyagraha *et al.* [7] on testing for G6PDd provide excellent material on these topics.

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KEY POINTS

- Diagnosis is of central importance to malaria elimination.
- Although microscopy and rapid diagnostic tests help to achieve goals of malaria control, malaria elimination requires more sensitive infection detection strategies to prevent transmission.
- The nucleic acid amplification tests are solely responsible for calling attention to submicroscopic infections, a low-density, asymptomatic reservoir that has potential to confound progress in malaria elimination.
- Malaria diagnosis is currently limited by inefficiencies in analyte preservation outside the laboratory and reliance on invasive sample collection and insufficient standardization required to eliminate uncertainty when mapping elimination progress.

MALARIA ELIMINATION CHALLENGES MALARIA DIAGNOSIS

Malaria elimination aims at zero incidence of transmitted infection in a defined geographical area. Thus, elimination is focused on the capacity of regional and national malaria programs and awareness that climate, ecology, and socioeconomic factors can impact transmission of this disease [8,9]. The WHO annual World Malaria Reports (see the WHO WMR 2014 [10▪▪]) monitor country-specific progress with estimates of populations at risk of infection, clinical cases, and malaria mortality. These reports also summarize NMCP data on financial support to access the three basic components of a malaria control toolkit: long-lasting insecticide-treated bed nets (LLIN) to protect against exposure to mosquito transmission; fast-acting artemisinin combination therapies (ACTs); and rapid diagnostic tests (RDTs) capable of detecting malaria parasite proteins in the blood of infected people.

Current progress against malaria follows a plan similar to the WHO's Global Programme to Eliminate Malaria [11,12], and includes Control (Scaling Up for Impact and Sustaining Control), Preelimination, Elimination, and Prevention of Reintroduction [11] stages. Progress through these phases requires regions to define areas of coverage, establish administration of service, and program evaluation mechanisms, provide assurances to confirm training of central program to community-based workers and document continuous availability of LLIN, ACTs, and RDTs. Underscoring the importance of diagnosis, progress toward elimination is documented by reduction of annual parasite incidence (API) (Control areas: API $\geq$5 cases/1000 population; Preelimination: API $<$ 5/1000; Elimination: API $<$ 1/1000; Prevention of Reintroduction: zero locally acquired cases; WHO Certification after three years of sustained zero local transmission [10▪▪,13▪▪]). The current status of the 106 malarious countries is summarized below [10▪▪] (WHO regions: AFRO, African Regional Office; AMRO, Region of the Americas; EMRO, Eastern Mediterranean Regional Office; SEARO, South-East Asia Regional Office; WPRO, Western Pacific Regional Office).

(1) Preelimination ($n = 10$): AFRO – Cabo Verde; AMRO – Belize, Costa Rica, Ecuador, El Salvador, Mexico, Paraguay; SEARO – Bhutan, North Korea; WPRO – Malaysia.
(2) Elimination ($n = 9$): AFRO – Algeria; AMRO – Argentina; EMRO – Iran, Saudi Arabia; EURO – Azerbaijan, Tajikistan, Turkey; SEARO – Sri Lanka; WPRO – South Korea.
(3) Prevention of reintroduction ($n = 6$): EMRO – Egypt, Iraq, Oman; EURO – Georgia, Kyrgyzstan, Uzbekistan.
(4) WHO certified malaria free since 2000 ($n = 4$): EMRO – Morocco (2010), United Arab Emirates (2007); EURO – Turkmenistan (2010), Armenia (2011).
(5) Malaria Control ($n = 77$): [10▪▪].

As countries make progress against malaria, LOD are reached for microscopy and RDTs before parasites are cleared from endemic populations. These SMI have been validated and summarized in numerous excellent reviews [2,14▪▪,15,16,17▪▪,18▪▪]. Furthermore, regional and national studies regularly report SMI in many malaria-endemic regions when diagnoses by microscopy/RDTs have been coupled with more sensitive and specific nucleic acid amplification (NAA) strategies (14 manuscripts in 6 months of 2015 alone: AFRO – 6 [19–24]; EMRO – 1 [25]; SEARO – 2 [26,27]; WPRO – 4 [20,28,29▪,30▪]; AMRO – 1 [31]). Although microscopy and RDTs help to achieve goals of malaria control, and RDTs have helped to significantly reduce widespread overtreatment with antimalarial drugs [32], these surveys indicate that malaria elimination (the absence of all parasites) requires more sensitive infection detection strategies to prevent transmission [17▪▪]. With recognition that malaria elimination must integrate research [11], diagnostic technology advances must continuously optimize stringent barriers (treatment and mosquito exposure) against malaria transmission. Therefore, with currently reported evidence of SMI in five of six

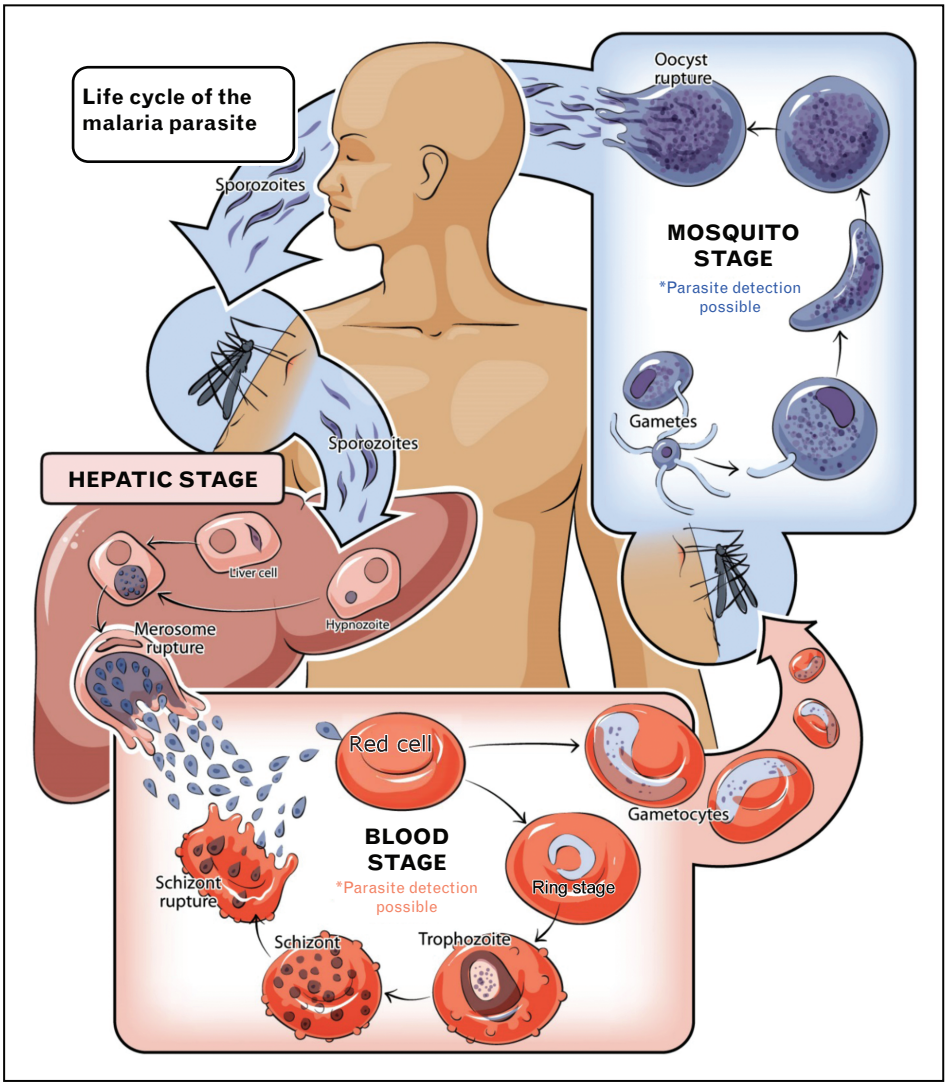


FIGURE 1. Malaria life cycle impact on diagnosis. Quantitative differences among the five malaria species [*P. falciparum* (*Pf*), *P. vivax* (*Pv*), *P. malariae* (*Pm*), *P. ovale* (*Po*), *P. knowlesi* (*Pk*)] during pre-erythrocytic and erythrocytic stages affect the composition of infected blood samples. Numbers of merozoites from infected hepatocytes can range from 2000 in *Pm* to 30 000–40 000 in *Pf*. Erythroid target cells (approximately, 5×10^6 RBCs/μl) of *Pf* and *Pk* include all RBCs. *Pm* preferentially infects mature RBCs. *Pv* and *Po* infect reticulocytes (0.5–1.5% of the RBC population). The RBC infection cycle is 24 h for *Pk*, 48 h for *Pf*, *Pv*, and *Po*, and 72 h for *Pm*. Target-cell availability and duration of the RBC infection can influence average blood stage parasitemia [*Pf* = 20 000–500 000 infected red blood cells (iRBC)/μl; *Pv* = 20 000 iRBC/μl; *Pm* = 6000 iRBC/μl; *Pk* – 600–10 000 iRBC/μl].

WHO regions, review of the WHO's API thresholds for elimination must be considered. Furthermore, NMCP decisions to treat must be reviewed because of the potential for SMI to contribute to sustained malaria transmission [33].

MALARIA LIFE CYCLE AND GEOGRAPHY IMPACT DIAGNOSIS

Figure 1 and Table 1 summarize the complex malaria life cycle. *Anopheles* mosquitoes inject sporozoites to initiate infection of human hepatocytes (pre-erythrocytic infection – approximately, 2 weeks). Hepatocytes rupture to release merozoites that will initiate erythrocytic infection; this stage results in malaria symptoms. Blood stage parasites can cycle many times back through red blood cells (RBCs), or the parasites can differentiate into male and female gametocytes ready for sexual reproduction after being ingested into the mosquito midgut. The resulting ookinete migrates through the midgut wall and initiates asexual replication to form a new brood of sporozoites that will become positioned in the mosquito salivary glands ready for release into another human host. Parasites are detectable during infection of RBCs and mosquitoes. Sporozoites through liver stages are refractory to detection. Life

Table 1. Life cycle biology influencing human malaria diagnosis

Characteristic	*Plasmodium falciparum*	*Plasmodium vivax*	*Plasmodium malariae*	*Plasmodium ovale*	*Plasmodium knowlesi*
Merozoites/hepatocyte	40 000	10 000	2000	15 000	10 000
Dormant hypnozoites	No	Yes	No	Yes	No
Erythrocytic cycle (hours)	48	48	72	48	24
Erythrocyte target cells	All RBC	Reticulocytes	Mature RBC	Reticulocytes	All RBC
Merozoites/iRBC					
Minimum (ring)[a]	1[b]	1	1	1	1
Maximum (schizont)	32–36	18–20	10–12	12–18	14–16
Parasitemia/μl					
Average/range	50 000–500,000	10 000–20 000	6000	9000	
Maximum	2 500 000	100 000	20 000	30 000	800 000
Submicroscopic infections	Yes	Common	Common lasting years	Common	Yes[c]
Geographic range	Global/Tropics	Global/Temperate	Global/Temperate	Global/Temperate	SE Asia/zoonosis[d]

iRBC, infected RBC; RBC, red blood cells.
[a]iRBC may include occurance of multiple ring stage parasites.
[b]Sequestration of trophozoites through schizonts.
[c]Morphology with *P. malariae* is similar, parasitemia can increase rapidly.
[d]Zoonosis involving humans and macaques (long-tailed and pig-tailed) found in Southeast Asia – see [2].

cycle and geographic characteristics of *Plasmodium* species that further influence malaria diagnosis are as follows:

(1) *P. vivax* and *P. ovale* produce dormant liver stages, hypnozoites, that initiate new RBC infections independent of mosquito transmission. Hypnozoites are currently invisible to diagnosis.
(2) All malaria parasites digest hemoglobin and crystalize bioreactive heme into hemozoin, a paramagnetic substance that aggregates during development inside infected RBCs (iRBCs) [34].
(3) *P. falciparum* trophozoites through schizonts (≥75% of iRBCs) sequester in the peripheral vasculature [35]. Sequestration of other human malaria species is poorly understood.
(4) Variable numbers of merozoites are present in iRBCs [36–38].
(5) *P. falciparum* and *P. vivax* are often coendemic globally [39,40], whereas *P. knowlesi* is a zoonotic infection of humans and macaques in Southeast Asia [41]. Although travelers have become infected there are no reports of human-to-human transmission beyond this region [42▪].

Plasmodium species-specific features can influence the cellular and molecular content available for malaria diagnosis. Furthermore, coinfections of multiple species complicate malaria diagnosis in rural field settings and in very well resourced laboratories.

TARGETS OF MALARIA DIAGNOSIS

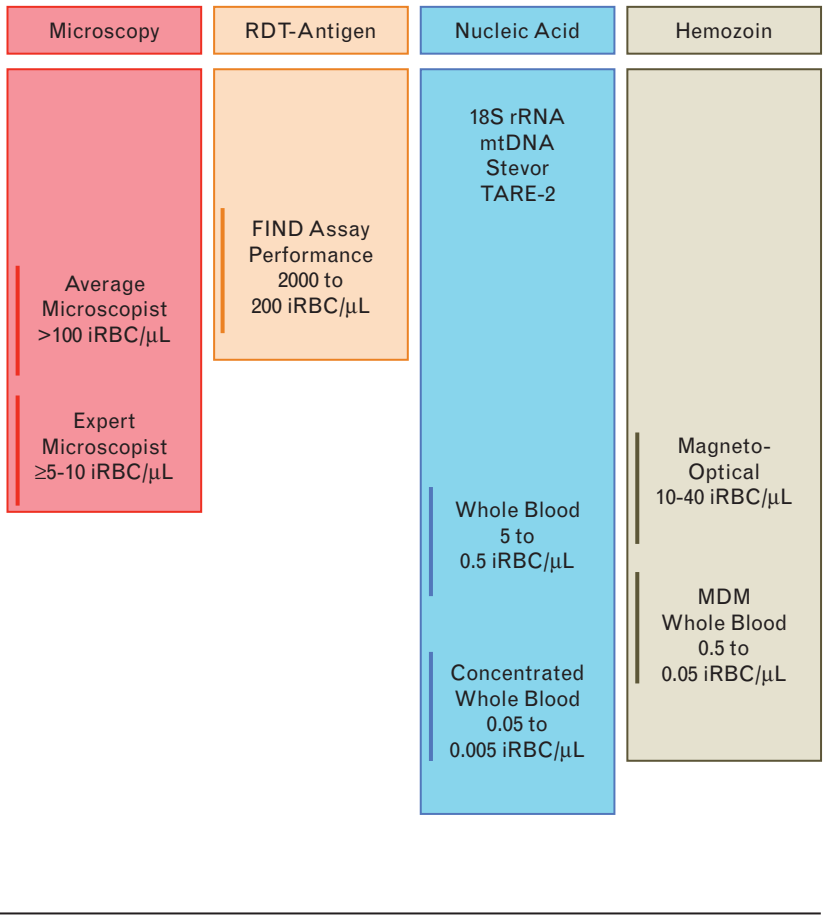
The cellular targets of malaria diagnosis in humans, therefore, include iRBCs or leukocytes that have ingested parasites. Detectable analytes (chemical constituents) include nucleic acids, antigens, and hemozoin. Additional analytes include human anti-parasite antibodies. For elimination, it is important to identify and treat malaria-positive individuals before parasites can be transmitted [17▪▪,43], ideally before symptoms and gametocyte production. Diagnostic targets of infection that linger beyond the duration of infection (parasite antigens or parasite-specific human antibodies) would contribute to false-positives and potential treatment of uninfected people. Conversely, analytes that decrease significantly during infection (missing low parasitemia or sequestered *P. falciparum*) would contribute to false negatives and fail to identify parasites ready to sustain transmission. Markers of greatest utility to malaria elimination are those that accurately reflect the biomass, species composition, and transmissibility of parasites comprising individual infections [17▪▪]. Parasite polymorphism in any molecular assay targets would alter diagnosis.

MALARIA DIAGNOSTIC TECHNOLOGIES AND LIMITS OF DETECTION

LOD vary inherently across technologies (Table 2), but factors that further influence detection capacity include functioning reagents/materials across all sample collection, storage, processing and diagnostic assay steps, operator

Table 2. Limit of detection of a single infected red blood cell (5×10^6 RBC/μl; 8×10^3 WBC/μl)

iRBC/μL	Parasitemia
50,000	1.00000000%
5,000	0.10000000%
500	0.01000000%
50	0.00100000%
5	0.00010000%
0.5	0.00001000%
0.05	0.00000100%
0.005	0.00000010%
0.0005	0.00000001%



iRBC, infected RBC; RBC, red blood cells; RDT, rapid diagnostic test.

proficiency, sample quality, and sample volume. The blood volume analyzed constrains diagnosis significantly; 5 μl (RDT sample) to 200 μl (DNA sample) are extremely small portions of the average adult blood volume (5–6l).

Microscopy and cytometry

Conventional blood smear microscopy [44] typically evaluates a fraction of 1 μl of whole blood (thin smears 1.25×10^5 RBCs; thick smears 1.0×10^6 RBCs [45]) and encounters LOD between 5 and 200 iRBCs per μl depending on laboratory facilities and expertise (Table 2). Microscopy enables identification of individual species and developmental stages, however, as parasitemia decreases and/or an infection is comprised of multiple species, the accuracy of diagnosis can be challenging even to expert microscopists [46]. Manipulating blood after sample collection by exposure to magnetic fields enriches capture of iRBCs [47,48] resulting from the presence of hemozoin. By this approach, 100 μl of blood can be screened (5×10^8 RBCs) increasing the total capture of iRBCs from 10-fold to 100-fold [47] and magnetic capture begins to rival NAA sensitivities. Further, hemozoin-containing monocytes observed by microscopy may provide insight regarding the total body parasite burden, as these cells do not sequester in *P. falciparum* infections [49,50]. Flow cytometric devices may also contribute new approaches for malaria diagnosis with emphasis on field deployment, however, further development of instrumentation is required [51–55]; limitations of species-specific detection must be acknowledged for flow cytometry.

Antigen-based rapid diagnostic tests

Over 200 RDT products from over 30 different manufacturers have been evaluated by the WHO and Foundation for Innovative New Diagnostics (FIND) and other malaria elimination stakeholders [56▪▪]. Operational detection thresholds have been set at a low density of 200 parasites/μl and a higher density of 2000–5000 parasites/μl [56▪▪]; the approximate RDT LOD is equivalent to 200 iRBCs/μl of blood (Table 2). RDTs detect *P. falciparum* histidine-rich protein II (PfHRP2), *Plasmodium* lactate dehydrogenase and

aldolase [56▪▪]. Although they identify *P. falciparum*, specifically, RDTs have no species-specific capacity to identify all five malaria species and cannot provide information on developmental stages. As these methods have not been coupled with strategies for concentrating parasite protein from the blood prior to analysis, they are limited by the 5 μl sample volume applied to the RDT cartridge. Further complications linked to RDTs have arisen through reported false-positive and false-negative results systematically reviewed through the WHO–FIND collaboration [56▪▪]. False-positive results are associated with persistence of PfHRP2 in peripheral blood, cross reactivity against human rheumatoid factor, and other infectious diseases [56▪▪,57,58]. False-negative RDT results are associated with deletions of pfhrp2 and pfhrp3 genes [59]. Cheng *et al.* [60▪▪] reported, however, that PfHRP2-detecting RDTs are effective for routine clinical case management in most malaria-endemic regions. When PfHRP2 deletion prevalence is greater that 10% in a region PfHRP2-detecting RDT usage is discouraged [61].

Nucleic acid amplification tests

NAA tests [14▪▪,62,63], most often PCR-based, are able to identify individual species but not developmental stages. These methods are also considered to be lab-bound. Despite these limitations, NAA tests are now acknowledged to be more sensitive in detection of malaria parasites compared with microscopy and RDTs [64]. The NAA tests are solely responsible for calling attention to SMI [15,65], an asymptomatic reservoir that has potential to confound progress in malaria elimination [66]. Accumulating assessments now suggest that the proportion of SMI is, approximately, 20% of infections in areas of high transmission intensity (communities with parasite microscopy prevalence $\geq$75%) but increases to 70–80% of infections in areas of low transmission intensity (wherein microscopy prevalence is $<$10%) [15].

The LOD for NAA tests are at least two to four orders of magnitude lower than microscopy and RDTs (virtually all NAA strategies are able to detect 0.05–5 iRBCs/μl of blood; Table 2). Early NAA tests focused on detecting the 18S ribosomal RNA gene sequences (DNA template), present in 5–10 copies per *Plasmodium* genome. These sequences were either amplified using species-specific primers with products visualized following gel electrophoresis, or by genus-specific PCR amplification followed by post-PCR methods to distinguish species [67,68]. *Plasmodium* species NAA assays have expanded to target additional gene sequences [including the *P. falciparum stevor multigene* family [69], mitochondrial DNA (mtDNA) [70], and telomere-associated repetitive element 2 (TARE-2) [71] sequences and *P. vivax* Pvr64 sequences [72] and mtDNA [70]. Assays targeting stevor, TARE-2 and Pvr64 are limited by their single species focus; 18S rRNA gene and mtDNA [20] assays are developed to identify all human malaria species]. Assay development focused on these sequences theoretically improves infection detection beyond assay formats that target 18S rRNA gene sequence due to increased copy number (stevor, 30–40 copies/parasite genome [69]; mt cytb, 30–100 copies/iRBC [70]; TARE-2,250 copies/parasite genome [71]). With a 100-fold increase in target sequence it is possible that the LOD could reach 0.005–0.05 iRBCs/μl or 1–10 iRBCs/200 μl of blood (Table 2) [73] as this very small number of iRBCs would be releasing hundreds of copies of the target DNA sequence into the final 200 μl volume of purified genomic DNA and just one template sequence would be needed to drive the NAA reaction.

Additional NAA strategies that have been shown to improve sensitivity of molecular diagnosis include amplification of expressed nucleic acid sequence (RNA template) and concentration of RBC away from serum and WBC of whole blood. Expressed nucleic acid compared with genomic DNA benefits from parasite amplification of target sequence 1000 to 3500-fold. This increase in nucleic acid template concentration would push the LOD to 0.0005–0.005 iRBCs/μl or 1–10 iRBCs/2 ml of blood (Table 2). To fully implement this increased capacity for detection requires RBC concentration strategies. Concerns that discourage RNA-based infection detection include the lability of RNA compared with the durability of DNA. RBC concentration strategies have been implemented in the context of malaria diagnosis in field-based malaria elimination [74▪] and in the context of clinical trials [75▪]. Nucleic acid extraction performed on the RBC fraction from 1 ml of whole blood (approximately, 5×10^9 RBCs) would enable surveillance 0.2% of the adult blood volume. Increasing the availability of nucleic acid template has facilitated successful pooling of patient samples to accelerate sample processing [30▪,71,76,77] that will be called for as malaria elimination requires increasing surveillance. Finally, loop-mediated isothermal amplification exhibits potential to release NAA from the laboratory and enable highly sensitive, NAA malaria diagnosis in remote healthcare settings [72,78]. Additionally, recent advances with lab-on-chip [79] and noninstrumented nucleic acid amplification [23] strategies advance promise for NAA point-of-care testing.

EMERGING MALARIA DIAGNOSTICS

A number of recent studies have reported on strategies to exploit physical and/or electromagnetic

features of hemozoin crystals to detect infection by malaria parasites [54,80–86]. Generally, the LOD of these methods is between 1 and 30 iRBCs/μl and, therefore, not as sensitive as NAA strategies. However, as these methods claim to rely on inexpensive and portable technologies, there is potential that they may contribute to more efficient and sensitive point-of-care malaria diagnostic strategies with further optimization. Finally, more recent work from Lukianova-Hleb *et al.* [87,88▪▪] describes a noninvasive method for detecting hemozoin. These authors indicate that hemozoin specificity for malaria and the susceptibility of this nanocrystal to optical excitation by laser pulse generates expansion and collapse of a vapor nanobubble. The resulting pressure pulse is easily detected through the skin with an ultrasound sensor. Preliminary studies in mice, one human infection, and mosquitoes have provided results that demonstrate a noninvasive strategy for malaria diagnosis.

CONCLUSION

Malaria elimination will require very sensitive infection detection and capacity to process extremely large sample quantities. Malaria diagnosis is currently limited by inefficiencies in analyte preservation outside the laboratory and reliance on invasive sample collection and insufficient standardization [89] required to eliminate uncertainty when mapping elimination progress [90▪]. New technology would need to match or surpass the superior sensitivity of NAA methods.

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Conflicts of interest

There are no conflicts of interest.

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