Clinical Infectious Diseases

Viewpoint

Title: The role of ultra-sensitive molecular methods for detecting malaria – the broader perspective.

Authors: Claire Kamaliddin[1], Colin J. Sutherland[2], Sandrine Houze[3,4], Gilles Cottrell[3], Valerie Briand[5], Daniel Castaneda Mogollon[1], Dylan R. Pillai[1]

Affiliations:

1. Department of Microbiology, Immunology, and Infectious Disease, Cumming School of Medicine, University of Calgary, AB, Canada
2. Department of Infection Biology, Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel St, London WC1E 7HT, UK
3. UMR261 – MERIT, IRD, Faculté de Pharmacie, Université de Paris, Paris, Île-de-France, FR 75006
4. Centre National de Référence pour le Paludisme, Bichat-Claude Bernard Hospital, 75018 Paris, France
5. Institut de Recherche pour le Développement (IRD), Inserm, UMR 1219, University of Bordeaux, Bordeaux, France

Summary (35/40 words) :

Ultra-sensitive malaria molecular diagnostics are providing insights not captured previously using traditional diagnostics methods. Identification and treatment of low-level malaria infections identified by molecular tools may benefit asymptomatic individuals, malaria in pregnancy, and elimination campaigns.

Abstract:

Ultra-sensitive molecular diagnostics are lowering the limit of detection for malaria parasites in the blood and providing insights not captured by conventional diagnostics such as microscopy and rapid antigen tests. Low-level malaria infections identified by molecular tools may influence clinical outcomes, transmission events, and elimination efforts. While many ultra-sensitive molecular methods require well-equipped laboratories, technologies such as loop-mediated isothermal amplification provide more portable and analytically sensitive solutions. These tools may benefit asymptomatic patient screening, antenatal care, and elimination campaigns. We review the recent evidence, offer our perspective on the impact of these new tests and identify future research priorities.

Main text (total word count 2104/ 3000 max):

In 2019, 228 million malaria cases occurred worldwide, with most cases (93%) and malaria-related deaths (94%) in the WHO Africa region [1]. Malaria diagnosis conventionally relies on the identification of asexual parasites in peripheral blood smears. Preparation and microscopic examination of smears requires expertise and delivers a moderate limit of detection (LOD) at 50-200 parasites/µL. The introduction of antigen based rapid diagnostic tests (RDT) in the 2000's and then more recently simplified molecular diagnostics such as loop-mediated isothermal amplification (LAMP) are providing new insights on malaria burden. However, the usefulness, relevance, and impact of such highly sensitive tests are tightly linked to the clinical and epidemiological context where those tests are implemented. In the following, we consider ultra-sensitive testing for molecular methods that detect DNA or RNA at or below an LOD of 0.1 parasites/µL.

A recent study investigated the relevance of "low-density" *P. falciparum* infection in febrile children in a moderately endemic area of Tanzania [2]. The authors provide important findings in terms of clinical

outcomes for febrile children at the primary care setting based on three cohorts defined by the diagnostic test result. Only patients from the "high-density" (rapid diagnostic test- positive) group were specifically treated for *P. falciparum* infection during the study. The authors concluded that, based *on post hoc* detection of malaria by ultra-sensitive methods (qPCR detection of the multigenic target *var*ATS, which has a LOD < 0.1 parasites/µL [3]),), conventional diagnostic tools are sufficient for the management of febrile children. The study did not observe differences in terms of outcomes between children diagnosed with no *Plasmodium* infection and those with a low-density *Plasmodium* infection. Importantly, children presenting with a high-density infection were more likely to have a hospital re-admission compared to low-density infection children. A second area that is noteworthy is that the study outcomes monitored were relatively severe in nature: proportion of clinical failures, secondary hospitalization, conversion to high-density malaria infection, and death in the 28 days following the study inclusion. These results support a previous study of school-aged children in Benin [4], showing that sensitive methods such as conventional PCR (LOD ~ 0.5-1 parasites/µL) would not bring benefit to the management of febrile malaria cases. However, as the authors specified, more studies are needed to confirm those findings in larger cohorts and different levels of endemicity.

The classification of low-density and high-density infections is challenging. First, RDT performance (including the newer "ultra-sensitive RDTs") may be affected by histidine rich protein (HRP) 2/3 deletions, leading to false negative tests [5]. Furthermore, RDT detection of non-*falciparum* species is unreliable due to poor weak detection of the antigen, typically lactate dehydrogenase (LDH). For the two previously mentioned reasons, a negative RDT with overt malaria symptomatology and clinical signs such as thrombopenia should alert the provider of the possible malaria diagnosis. The interpretation of discrepancy between positive PCR results and negative RDT results remains controversial. Patients recently treated for malaria may have persistently positive PCR test results, the likelihood increasing with the sensitivity of the molecular test used [6,7]. Detection of parasite DNA may indicate residual *P.*

falciparum asexual parasites [7], gametocytaemia, or lingering parasite genetic material rather than active infection[8], and so interpretation of positive molecular tests should be related to the clinical context.

Finally, three factors influence the respective limits of detection of molecular methods: the volume of sample extracted and concentrated, the molecular target (DNA, RNA, and their respective copy number or expression), and the methodology itself (optimization, reagent mix, method of signal detection, usage of probes, as examples). Regarding the volume of sample, field-based sampling is usually limited by finger prick techniques to collect blood which yields approximately 5 µL of blood. Formal venipuncture can provide volumes in excess of 1mL and therefore permit greater input blood volumes for extraction methods. The simple change of input volume into a nucleic acid extraction pipeline can improve the sensitivity of a given technique [9]. Molecular methods can detect either RNA or DNA. While RNA methods have shown higher sensitivity of highly expressed genes such as 18SrRNA [10], multicopy genomic targets also provides analytically sensitive *Plasmodium* DNA detection [3]. Several published papers compared the different PCR methods' respective sensitivity for *Plasmodium* detection in certain epidemiological settings [10–13]. Lastly, the particular method's final readout approach can affect a given target's sensitivity: for example, fluorescence-based detection (qPCR, RT-qPCR and LAMP) has higher sensitivity than visual-based detection (conventional PCR or RT-PCR with agarose gel revelation of amplicons).

Notably, low-density infections may lead to chronic health issues in malaria-endemic areas and impact public health measures. Recurrent episodes of symptomatic malaria are observed in longitudinal follow-up studies and can occur over a longer time frame than 28 days [14,15]. Recurrence of high-density parasitemia may contribute to chronic anemia in infected individuals by peripheral destruction of the erythrocytes and impairment of erythropoiesis [16]. A common finding in individuals with asymptomatic malaria infections identified by ultra-sensitive molecular methods is a significantly higher risk of reduced hemoglobin levels [17]. In chronically infected individuals, splenomegaly occurs from constant splenic filtration of infected erythrocytes and parasitic debris [18]. Furthermore, it has been reported that

asymptomatic low-level infection impairs neutrophil function, increasing risk of severe systemic bacterial co-infections (particularly *Salmonella* infections) [18]. Low-level infections have also been associated with increased inflammation assessed with CRP level in a manner that correlates with parasite density [19]. Asymptomatic *P. falciparum* infections may also affect cognition and development in children, as shown in studies conducted in Uganda and Kenya [20,21]. In these studies, introduction of malaria chemoprevention in the form of intermittent treatment in children reduced school absenteeism and improved health and cognitive abilities in semi-immune children [20,21].

While ultra-sensitive molecular malaria diagnostic methods may not be feasible nor relevant in their current state of technology readiness at the primary care setting in LMIC, studies in this area have contributed significantly in three significant groups within at-risk communities: (i) asymptomatic low-level malaria infections, (ii) malaria in pregnancy (MiP) and (iii) malaria elimination settings. Low-level malaria infection usually exhibited in asymptomatic individuals is revealed using ultra-sensitive molecular methods but not traditional methods like microscopy or RDT [10,17]. The ability to detect these low-level malaria infections improves estimation of malaria prevalence in endemic areas, but can also contribute to transfusion and transplant safety. In non-endemic areas, screening of migrants entering from endemic settings for low—level infections may prevent introduction of parasites into local *Anopheles* spp. populations. Methods using ultrasensitive detection of the parasite RNA can identify a 4- or 5-fold greater number of low-level infections than traditional RDTs and microscopy in a high-transmission setting of Ethiopia[17]. Other studies reported a similar trend in asymptomatic adults in Ghana [22] and in India [23].

Blood safety in transfusion should rely on blood and tissue donors' screening [24,25]. All symptomatic patients are by default excluded from any blood donation procedure in any country. However, asymptomatic individuals may harbor *Plasmodium* parasites. Countries like the United Kingdom (UK) and France perform antibody-based screening of at-risk donors depending on the individual’s travel

history and birthplace. However, screening may be inefficient due to immune tolerance to malaria, as recently reported in France [24]. On the other hand, the implementation of extended deferral after travel to endemic areas and/or documented malaria episodes in donors (such as the policies implemented in Canada or the USA) restricts the population from contributing to blood donation efforts. A study in the UK found that a positive malaria PCR result in an antibody-negative patient can be associated with low-level asymptomatic parasitemia [26]. Low-level parasitemia puts the recipient at risk of transfusion-acquired malaria. Asymptomatic screening of at-risk individuals using ultrasensitive techniques could improve safety of the blood supply while allowing more individuals to be blood donors. This strategy of molecular-based screening could also apply to organ donors to avoid post-transplantation malaria.

Last, asymptomatic individual screening with ultra-sensitive molecular testing can benefit migrant populations. A recent screening of migrants from sub-Saharan Africa in Spain revealed that approximately 8% of the screened individuals retained low-level malaria parasitemia [27]. Another example is the temporary re-introduction of *P. vivax* in Greece with local outbreaks related to migration [28]. Furthermore, Middle East countries, among others that have previously achieved malaria elimination, remain at risk of malaria re-introduction. Migrant workers are a likely source of re-introduction in countries that achieved elimination but have a large immigrant work-force, such as Qatar [29] or Singapore [30].

Pregnant women can also benefit from ultra-sensitive molecular testing screening strategies. In pregnancy, *P. falciparum* placental sequestration results in low-level peripheral blood parasitemia (reviewed i [31]). MiP may be asymptomatic for the pregnant mother. However, *P. falciparum* in the placenta affects fetal development and birth outcome, leading to fetal loss, developmental delays and low birth weight of the newborn [31]. In 2019, the WHO reported that 29% of pregnancies in moderate to high malaria transmission areas are at risk of MiP, and that 16% of low birth weight newborns in these areas were a consequence of malaria infection, putting 822,000 neonates at risk [1]. The current strategy

for MiP prevention targets the vector (insecticide-treated bed nets) and the systematic administration of intermittent preventive treatment (IPT) which is the combination of sulfadoxine and pyrimethamine. However, IPT-based strategies present several limitations. The coverage of IPT remains low, with 34% of women attending ANC fulfilling the three IPT doses recommended by the WHO [1]. Additionally, sulfadoxine-pyrimethamine drug resistance is a concern in sub-Saharan Africa. IPT coverage is limited to the second and third trimester of pregnancy. However, infections acquired during the first trimester of pregnancy display "placental" phenotype through variant surface expression and are associated with adverse birth outcomes [32,33]. It is also known that infections acquired before pregnancy may persist at a low asymptomatic level, and clonal expansion occurs with the placental maturation, putting these women at risk of MiP [34,35]. To detect and treat these infections, molecular screening with ultra-sensitive molecular malaria diagnostics is a potential approach. Ultra-sensitive molecular testing and subsequent treatment could prevent adverse birth outcomes related to MiP [36]. The feasibility of this is, however, unproven in resource-limited settings.

Finally, low-density asymptomatic malaria cannot be ignored in elimination strategies. For instance, it has been determined that asymptomatic cases represent up to 75% of infections, and that it can contribute up to 50% of onwards transmission when parasites are present in their sexual form [37]. However, the role of these low-level infections that are revealed only by ultra-sensitive methods remains unclear. A recent study provides insights into the transmission potential of low-level infections based on the minimal gametocytemia necessary to infect a mosquito [38]. The authors showed that conventional detection methods might be sufficient for parasite detection at a level that matters to prevent onward direct transmission at the time of sampling. However, asymptomatic parasitemia and low-level gametocytemia may oscillate over time and this is only captured in longitudinal studies. Importantly, low-level parasitemia can contribute to malaria transmission. Indeed, at the end of the rainy season malaria parasites persist at a low level in individuals and allow the parasite to survive and resume transmission at the next rain season [39,40]. Tracking the low-level infected asymptomatic patient promotes malaria

control strategies. With malaria elimination on the road map, ultra-sensitive point of care molecular diagnostics for malaria are needed for rational treatment of individuals with low level infection and to reduce or interrupt transmission of the parasite.

To conclude, there is currently no consensus regarding the value of wide-scale implementation of ultra-sensitive malaria testing. Policies regarding the implementation of ultra-sensitive diagnostic test must be tailored to the epidemiological context and the public health policy of a given area. Targeting the right population is a key strategy for management of resources and rational allocation of testing to thosein malaria-endemic areas that would benefit the most: pregnant women, communities approaching elimination, and so-called “asymptomatic” carriers are leading candidates for such interventions.

Conflict of Interest Statement:

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