Parasite Epidemiology and Control 12 (2019) e00107

Contents lists available at ScienceDirect

Parasite Epidemiology and Control

journal homepage: www.elsevier.com/locate/parepi

A focus on improving molecular diagnostic approaches to malaria control and elimination in low transmission settings: Review

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ARTICLE INFO

Article history:
Received 14 March 2018
Received in revised form 19 April 2019
Accepted 19 April 2019

Keywords:
Diagnosis
Malaria
Control
Elimination
Review

ABSTRACT

The malaria elimination goal is back to the global agenda. Understanding its epidemiology in low transmission settings is crucial to design reliable strategies to detect a large reservoir of individuals infected with sub-microscopic (and often asymptomatic) infections characterized by low-parasite densities and gametocyte carriage. Traditional diagnostic methods such a light microscopy is widely used mainly in developing countries and as a result, the true picture of malaria epidemiology is misrepresented. In the last few decades, the advancement of molecular diagnostic tools significantly improved our understanding of the epidemiology of the diseases. However, the detection capacity of different molecular assays is determined by different factors such as the sensitivity of the assay and the transmission and infection dynamics of the disease particularly when there is low parasitic density in reservoir hosts. Hence, in this review, the epidemiology of malaria in low transmission settings and the priority in addressing the malaria control and elimination goals are highlighted.

Contents

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https://doi.org/10.1016/j.parepi.2019.e00107

1. Introduction

Malaria is one of the most important parasitic diseases which cause a significant mortality and morbidity in the world in particular in Africa. Different studies showed that the prevalence of malaria parasite infection, including both symptomatic and asymptomatic has decreased since 2000 (World Health Organization, 2014).

Reports from 2018 showed that in the year 2017, around 219 million malaria cases were reported globally. This is relatively higher comparing to the cases reported in 2016 which is 217 million. Whereas a report from 2010 showed that around 239 million malaria cases were reported, and this shows a reduction in malaria cases over the period of7 years (World Health Organization, 2014). Most malaria cases in 2017 were reported in WHO African regions (92%) and followed by WHO South East Asian regions (5%), and 2% cases in WHO Eastern Mediterranean Region (World Health Organization, 2018a).

As many countries are approaching malaria elimination goals, increasing surveillance system can help every infection is detected using adequate diagnosis, treated and reported (World Health Organization, 2018b).

However, the malaria elimination strategy is still a global challenge due to different disease management, epidemiology, diagnosis and treatment factors. Confirmatory parasitological diagnosis is important to avoid overtreatment with artemisinin-based combination therapy (ACTs) combination therapies which may facilitate to the development of resistance and waste of resources. A treatment decision is determined by clinical diagnosis only for children's in high transmission areas (World Health Organization, 2015a).

The diagnosis of malaria using Rapid Diagnostic Test (RDT) and Light Microscopy (LM) have the advantage being cheap and widely used for clinical management of malaria (Bell et al., 2005), but they have disadvantages due to lack of sufficient sensitivity to detect parasitemias by parasite density.(Aydin-Schmidt et al., 2014). RDT and LM have a detection limit of 100 and 5–50 parasites/μL respectively (Moody et al., 2000). Although microscopy is considered as a 'gold standard' for the malaria diagnosis (World Health Organization, 1999), it has been frequently indicated to yield misleading results like false positive & negatives in many clinical trials (Perandin and Manca, 2004).

It was frequently reported that the detection limit of microscopy is in the range of 100 to 200 parasites per microliter. However, by expert microscopy, as low as 50 parasites can be detected per microliter (reviewed in (Lindsey et al., 2015)).

Polymerase Chain Reactions (PCR) assays like multiplex PCR, and real time PCR has improved the sensitivity of plasmodium detection (Chavalitshewinkoon-Petmitr, 2010). PCR is advantageous in studying drug resistance parasites, identifying mixed infections, and in the processing of large number of samples in research and samples can be easily collected on filter papers and stored at room temperature for years (Wangai et al., 2011). PCR has a limitation as it requires well trained staff, good laboratory infrastructure, and again a good quality assurance system (Jelinek et al., 1996). As a result, the sensitivity of different PCR methods varies (Berry et al., 2008). In addition, PCR requires expensive equipment and well-trained technicians and is not usually adequately implemented in developing countries (Mens et al., 2007).

The number of gametocytes in the peripheral blood much lower than the asexual for of the parasite and individuals do not show symptoms but can be a source of transmission and sometimes asymptomatic infections can remain residual even after anti-malarial treatment (Ouédraogo et al., 2010). The degree of gametocyte carriage among asymptomatic infection is less known in low transmission settings. So, it is essential to accurately quantify the human reservoir (Jessica et al., 2014). Low parasitemias has a potential for a transmission and it is a threat for elimination goals (The malERA Consultative Group on Diagnoses and Diagnostics, 2011). Hence, to achieve elimination, appropriate diagnostic tools for the detection of low density parasitemias are mandatory. Current methods of detection in epidemiological studies using qPCR collects 50 μL of blood in filter paper, of which 1 punch (approximately 7.8 μL blood) is used for extraction and this method adequately detects infection in high parasite density but it is less sensitive during low parasite densities. Higher blood volume increases sensitivity and allows detection of parasites from asymptomatic reservoirs (World Health Organization, 2018b; Canier et al., 2015).

2. The hidden malaria challenge: epidemiology of malaria in low transmission settings

Elimination is attaining malaria transmission at zero level in each geographic region and elimination in all regions leads to malaria eradication which is the permanent reduction to zero incidence globally (World Health Organization, 2018a). The context of malaria elimination goals paves a way for changing diagnostic tools and since the prevalence of malaria is declining, its management is focused on subclinical infections and on geographically as well as demographically concentrated human reservoirs (Tietje et al., 2014). There is no universal definition for asymptomatic parasitemias but most of the definitions involve detection of sexual or asexual parasites in the absence of symptom of malaria (mostly fever) and it excludes the dormant liver stage (Lindblade et al., 2013).

Symptomatic, asymptomatic, microscopic and sub microscopic infections involve the circulating gametocytes at low density, and it contributes to transmission of the disease (Bousema et al., 2014). Transmissions from humans to mosquitoes and to other human host is only carried out by the sexual gametocytes and routine microscopic examination is not sensitive enough for the detection of those low number of gametocytes (gametocytes constitutes <5% of total *P. falciparum* infections). The success of transmission depends on the number of matured gametocytes in the human peripheral blood (Bousema and Drakeley, 2011).

In high transmission settings (Parasite prevalence of ≥75% by microscopy), the proportion of sub microscopic infection is approximately 20%. Whereas, in low transmission settings (at <10% of parasite prevalence by microscopy), it increases to 70–80% (Okell et al., 2011), reviewed in Bousema et al. (2014). Depending on the transmission settings, both sub microscopic gametocytes

and microscopically detectable gametocyte densities can cause mosquito infection (Bousema et al., 2014). In low transmission settings, sub microscopic infections might signify an important fraction of infection and occurs at all transmission levels and the proportion depends on the transmission intensity, age distribution and level of immunity (Okell et al., 2009). The duration of submicroscopic infection varies but is often several months. In more seasonal malaria environments and in the absence of treatment, sub microscopic infection during the first low transmission season might be infectious to the vector during the following rainy season (Bousema and Drakeley, 2011).

To determine the importance of asymptomatic infections for transmission in areas of low transmission settings, the infection dynamics should be adequately identified using highly sensitive diagnostic methods (Felger et al., 2012). Table 1 briefly explains the detection limit of parasites by traditional and high throughput PCR assays.

3. Current diagnostic tools: challenges and limitations in low transmission settings

3.1. Light microscopy

The definitive diagnosis of malaria has been historically based on the confirmation of the plasmodium parasites in the blood particularly observing parasites in the red blood cells using thick & thin blood films. Conventional light microscopy is considered as a "gold standard" for malaria parasite identification and confirmation. Examination of stained blood films is widely used for clinical case management. However, in order to achieve adequate sensitivities and specificities, it requires trained and skilled microscopists, good supervisory personnel and adequate reagents (World Health Organization, 2012). An accurate laboratory diagnosis by good microscopists and the use of digital microscopy is important to avoid false positive and false negative results that may lead to mistreatment of the patients (World Health Organization, 2009). Moreover, quality-assured microscopy requires highly trained microscopists and a good quality control and quality assurance scheme.

3.2. Rapid diagnostic test (RDT)

RDT is an immune chromatographic test which detects parasite antigens in blood. Malaria RDTs come in to action in the mid-1990s (Palmer et al., 1998). The two most common and currently in use target proteins are Histidine Rich Protein-2 (HRP-2) and Lactose Dehydrogenase (LDH). PLDH- Parasite Lactate Dehydrogenase or Aldolase is also a common malaria antigen used during RDT diagnosis The main advantages of RDTs are: i) it is simple to use and needs a little expertise to interpret ii) it is easily movable and it does not require electric source, iii) it is suitable for field works and for travellers to manage, iv) result can be obtained very quickly in few minutes, v) RDTs are important for qualitative results in endemic areas for screening of many clinical cases in short time (Aydin-Schmidt et al., 2014). In addition, it has a limitation because it is difficult to read and interpret faint bands, persistence of HRP2, HRP2 deleted parasites, health workers difficult to deal with negative RDTs etc.

Rapid diagnostic tests (RDTs) can detect 100 parasites per microliter, equivalent to parasitemias of 0.002%. RDTs have a sensitivity of minimum 95% compared to microscopy and a specificity of >90% for all plasmodium species (World Health Organization/United States Agency for International Development, 2000). Therefore, studies have shown a high sensitivity & specificity of RDTs. For example, in a study that included 84 evaluations of HRP-2 RDTs showed an average sensitivity and specificity of 95.0% and 95.2%, respectively (Abba et al., 2011). RDTs has also an advantage over light microscopy since it detects the circulating antigens because if can detect *P. falciparum* infections in conditions when the parasites are sequestered in deep vascular compartments and at this time those parasites are not detectable by microscopic examination of peripheral blood smears (World Health Organization/United States Agency for International Development, 2000). One of the limitations of RDTs is a prozone effect of hyperparasitemia or antigen overload as it indirectly detects the presence of antigen and this leads to leading to false-negative results (Luchavez et al., 2011).

3.3. Molecular diagnostic methods

The PCR techniques which are common for the diagnosis of malaria includes conventional PCR nested PCR, qPCR, and multiplex PCR. Other less widely used nucleic acid-based amplification techniques which do not require thermal cycler is the LAMP and nucleic acid sequence-based amplification (NASBA) (Hopkins et al., 2013).

Real-time quantitative PCR is important for quantifying parasite density. Molecular techniques are significantly more sensitive than the traditional diagnostic methods like microscopy and RDTs and can be able to detect low parasitemias. The detection limits of PCR is in general <5 parasites per microliter (Cordray and Richards-Kortum, 2012) or 2 parasites per microliter or fewer. The volume of blood and the extraction methods are important factors in defining the detection limits in very low transmission settings and low parasitemias. Molecular methods have a proving track of detecting mixed infections in addition to the detection of sub microscopic or subclinical infections. However, it has its limitation since it is not fast, needs advanced equipment, expensive reagents, experienced personnel and is not applicable in most field conditions (Mens et al., 2007). Most of the mass screening studies use a small volume of around 5–30 μL of blood and this is one of the main limitations of molecular diagnostics since it compromises the real parasite burden in areas of low transmission settings and to overcome such problems higher blood volumes are recommended (Canier et al., 2015). Recently, simple, fast, specific and sensitive detection of method called nucleic acid lateral flow immunoassay or NALFIA is developed. It is shown to have a lower detection limit of 0.3–3 parasites per microliter which is 10-fold higher than gel electrophoresis analysis. In addition, the same study revealed that NALFIA is more sensitive than

Table 1
Operational features and performance of molecular methods.

Diagnostic method	Operational features	Performance	Advantage	Disadvantage	Throughput	Optimal setting for field use
Nested PCR	• Two sets of primers used in successive reactions; therefore, more expense, time and potential contamination than single-step PCR	• Limit of detection: at least 6 parasites/μL for blood spots • More sensitive than single-step PCR for the four main *Plasmodium* species • Hands-on time to result: 3 h; total time: 10 h	• Simple, it reduces the degree of non-specific binding, • The specificity of the PCR reaction is enhanced by reducing the non--specific binding with the help of the two sets of primers	• Time consuming, • Needs more reagents such as extra set of primers, high chance of contamination	High	Field applicable
Multiplex PCR	• Simultaneous, multiplex PCR to detect the presence of multiple *Plasmodium* species	• Limit of detection: 0.2–5 parasites/μL • Hands-on time to result: 2 h; total time: 4.5 h	• More information with less sample, cost effective, time saving, high accuracy, less pipetting errors, less contamination.	• Low amplification efficiency, complex, variability in efficiency in different templates and poor universality	High	Field applicable
Quantitative PCR	• Rapid amplification, simultaneous detection and quantification of target DNA by use of specific fluorophore probes	• Limit of detection: 0.02 parasites/μL for genus-level identification, 1.22 parasites/μL for *P. falciparum* detection • Hands-on time to result: 1 h; total time: 2.5 h	• Fast, efficient, and gives a qualitative result	• It is not cost effective and complex due to simultaneous thermal cycling and fluorescence detection.	High	Field applicable
Nucleic acid sequence--based amplification	• Assay includes a reverse transcriptase step, less inhibition than PCR. Isothermal method. Can be used to quantify gametocytes. Detects all four *Plasmodium* species, targeting 18S rRNA. Result by fluorescence	• Limit of detection: 0.01–0.1 parasites/μL per 50-μl sample • Result within 90 min (not including extraction time of about an additional 90 min)	• A major advantage of NASBA is the production of single stranded RNA amplicons that can be used directly in another round of amplification. • It supports the detection of human mRNA sequences without the risk of DNA contamination • It helps in better RT-PCR reaction as it offers faster amplification kinetics.	• Expensive thermocycling equipment is not needed as the reaction occurs isothermally at 41 °C.	High	Field applicable
CLIP-PCR	• Highly sensitive method rRNA of the plasmodium parasite can be released from the blood and then captured onto 96-well plates. Finally, quantified through the number of ligated probes which bounds to it.	• Capture and ligation probe PCR (CLIP-PCR) which enables to detect the parasite density in blood as low as 0.01 parasites per microliter of blood.	• CLIP-PCR is highly sensitive, and can detect malaria concentrations as low as 0.01 parasitized cells/-microliter of blood	• Expensive, and complex	High	Field applicable
LAMP	• Boil-and-spin extraction can be used, with amplification by isothermal method. Result determined by turbidity or fluorescence. Sensitivity increases by including mitochondrial targets. Genus-level targets, *P. falciparum* and *P. vivax*.	• Limit of detection: 0.2–2 parasites/μL • Results within 30 min with a tube scanner	• LAMP is cost-effective and requires minimal capital equipment investment	• Restricted availability of reagents and instruments, no multiplex capability and limitations related with primer design. • Does not allow the inclusion of an internal PCR inhibition control (IC)	High	Field applicable
HtLAMP	• A high-throughput LAMP (HtLAMP) platform amplifying mitochondrial targets using a 96-well microtitre plate platform. The HtLAMP assay proved to be a simple method generating a visually-detectable blue and purple colour change that could be objectively confirmed in a spectrophotometer at a wave length of 600 nm.	• Limit of detection is 2.5 parasites/μL	• Simple, highly sensitive, and more specific. When compared with PCR, overall HtLAMP-Pg had a sensitivity of 98%	• LAMP based assays are in general expensive	High	Field applicable

Table 2
Summary on malaria elimination plan of WHO by 2030.

Goals	Milestones		Targets
	2020	2025	2030
1. Global reduction of mortality rate compared comparing with 2015	≥40%	≥75%	≥90%
2. Global reduction of malaria case incidence comparing with 2015.	≥40%	≥75%	≥90%
3. Eliminate malaria from countries in which malaria was transmitted in 2015	Minimum 10 countries	Minimum 20 countries	Minimum 35 countries
4. Prevent re-establishment of malaria in all countries that are malaria-free	*Re*-establishment prevent	Re-establishment prevent	Re-establishment prevent

microscopy for detection of plasmodium parasites (Mens et al., 2008). Table 1 shows the molecular methods with a detection limit of parasites per microliter under optimal circumstances (World Health Organization, 2008).

In general, nucleic acid techniques are the most sensitive diagnostic approaches for plasmodium species identification. However, while easily accessible in reference laboratories, these techniques are not easily accessible in resource limited settings due to expensive reagents and infrastructures and technical experts required. Some efforts have been done to apply NATs in field conditions, and this resulted in the development of new techniques such as PCR-enzyme linked ELISA (Laoboonchai et al., 2001), nested PCR-HRM (nested PCR high resolution melting analysis) (Kipanga et al., 2014), PCR-LDR (PCR-Ligase detection reaction assay) (McNamara et al., 2004), and modification of PCR-LDR into LDR-FMA or LDR-Fluorescent microsphere assay (McNamara et al., 2006). In addition, RNA based assays were also used for detection parasites but generally considered as technically challenging for application in areas where resources are limited. However, recent studies showed that capture and ligation probe PCR or CLIP-PCR was used to purify RNA from samples and perform reverse transcription PCR. This technique is high throughput by virtue of its 96 well plate format and sample pooling. This approach can be performed from whole blood with LOD of 0.05 parasites per μL or from pooled DBS with LOD of 0.3 parasites per μL (Cheng et al., 2015).

Loop mediated isothermal amplification (LAMP) has been used widely to identify plasmodium species (Han et al., 2007). Nevertheless, there is a need for further optimization of LAMP techniques in order to process large samples as this might be needed to apply for malaria application (Labarre et al., 2010). Application of diagnostic devices should be focused on the simplicity of an assay to be implemented with minimum infrastructural requirements, for example reliable supply of electricity supply in the area and for example, non-experimental nucleic acid amplification assay or NINA (Sema et al., 2015) has been used to detect plasmodium species as LAMP-NINA assay, which needs exothermic chemical reaction to generate heat resources to perform LAMP (Hsiang et al., 2014).

4. Discussion and conclusion

The main and fundamental strategies to fight against malaria include prompt and adequate diagnosis followed by adequate treatment (World Health Organization, 2015a). With a vision of having world free malaria, WHO sets a strategic frame work of eliminating malaria by 2030. And Table 2 shows the plan of attaining this goal (World Health Organization, 2015b). A frame work on the malaria elimination strategies has been recently developed and includes the following major elements: Ensuring global access to prevention, diagnosis of malaria, attaining malaria free world by accelerating efforts towards elimination, transforming malaria surveillance to core intervention strategies, fostering innovation and expanding research, and capacity development. The introduction of molecular assays considerably improves the sensitivity for malaria diagnosis (Canier et al., 2015). One of the major limitations of PCR assays is the amount of blood sample collected for assessment which is usually in the range of 5–30 μL and this much volume cannot adequately detect parasites in situations where the prevalence of asymptomatic or low density parasitemias is high and during mixed infections. Hence, higher blood volume increases the sensitivity of detection from low parasite densities. The assessment of parasite infection using higher blood is not widely studied but according the published work by (Canier et al., 2015), using field samples the sensitivity of PCR assay from blood volumes of 5 μL to 1 ml was evaluated based on the dried blood spot extraction method (which is fast and less expensive extraction method which is widely used in large scale epidemiological investigations in malaria endemic areas) and venous bloods. The result showed that the degree of detection from the dried blood spot (5 μL) was lower than from the venous blood with a volume of 50 μL,200 μL, and 1 ml. Dried blood spot extraction protocol usually uses 5 mm punch which is equivalent to 7.8 μL and this is insufficient to detect and quantify the malaria parasite carriers and to address this issue a larger blood volume is recommended particularly in the context of malaria elimination. Therefore, prompt diagnosis and improving molecular diagnostic tools and their application in combination with control strategies such as MSAT and FSAT can lead to make elimination of malaria from global landscape a practical reality.

Authors' contributions

MT drafted, wrote, read, and approve the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data supporting this finding can be found from the corresponding author on request.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

Funding

Not applicable

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