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Review Article

The Use of Highly Sensitive Detection Methods for Eradication of *Plasmodium*

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Abstract

The key to a successful malaria eradication program is highly efficient detection of Plasmodium infected people followed by appropriate treatment to avoid spreading of the parasite. We will discuss some of the demands that such a detection method needs to fulfill and review some of the advantages and disadvantages of currently available detection methods.

ABBREVIATIONS

LAMP: Loop Mediated Isothermal Amplification; NAAT: Nucleic Acid Amplification Techniques; PCR: Polymerase Chain Reaction; Ptopoi: *Plasmodium*topoisomerase I; REEAD: Rolling Circle-Enhanced Enzyme Activity Detection

INTRODUCTION

An international effort against malaria has managed to reduce the number of deaths caused by malaria to30% in a five-year period from 2010 to 2015 [1]. Despite this promising development, almost half a million people still die from malaria every year. Most of these are children below 5 years of age [1]. In Africa alone, the economic burden of malaria is estimated to be around 12 billion USD in lost gross domestic product. The serious consequences of such loss for society as well as for the individual are enhanced by malaria being predominant in some of the poorest countries in the world [2,3]. It is therefore clear that a continued and optimized strategy to fight malaria is highly needed. Malaria is an infectious disease caused by infection with a parasite of the *Plasmodium* genus. Five members of this genus (*P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*) are known to cause malaria in humans. All five species are transferred by mosquitoes belonging to the *Anopheles* genus which is the host for part of the parasitic life cycle [4]. In areas where malaria is endemic, a large fraction (up to 40%) of the population will carry the *Plasmodium* parasite in their blood even if they are not displaying symptoms of malaria [5-8]. In fact, 92% of the *Plasmodium* infections found in a Kenyan screening study were asymptomatic [7]. The high prevalence of asymptomatic *Plasmodium* infections is true even for areas with a low *Plasmodium* burden [6]. Therefore, for eradication purposes very sensitive methods for detection of *Plasmodium* infections are required for finding and treating people unknowingly infected with the *Plasmodium* parasite. Apart from sensitivity, there are also a number of other demands which should be met by the optimal detection methodology. In surveillance projects a large number of asymptomatic individuals may be screened. The ideal method for use in surveillance and eradication should therefore be cost efficient, easy to use, and suitable for high throughput screening and, preferably, allow the use of non-invasive sample types such as saliva.

CURRENT METHODS FOR DETECTION OF PLASMODIUM INFECTIONS

Blood smear microscopy

The gold standard within malaria detection is blood smear microscopy [9]. While this method carries the benefit of direct observation of the *Plasmodium* parasite, microscopy is not suitable for high throughput screening and is relatively time consuming. Moreover, both specificity and sensitivity will depend on the experience of the microscopist. The detection level in a typical setting is50-100parasites per µL blood [10] which is sufficient for diagnosis in most cases but not necessary low enough to facilitate eradication of the *Plasmodium* parasite.

Rapid diagnostic tests

Antibody based rapid diagnostic tests (RDTs) have gained much popularity for diagnosis in endemic areas [11]. RDT's are easy to use and can be used with limited or no training. Moreover, they carry the advantage of being usable in low resource settings and as point-of care tests [11-13]. Unfortunately, the detection limits of RDTs are relatively high and RDTs are therefore not optimal for eradication studies as they show limited sensitivity for samples with low parasitemia [11]. Furthermore, mutations in the biomarker have been reported to lead to false negatives [14,15].

PCR

In recent years methods for detection of *Plasmodium* infections using nucleic acid amplification techniques (NAAT) have emerged [16]. The best established of these are PCR (polymerase chain reaction). PCR is an extremely sensitive method for detection of specific DNA sequences. When used for malaria diagnosis a part of the *Plasmodium* genome such as the 18S small-subunit RNA genes is used as a template and detected by PCR amplification [17,18]. The PCR protocols may be designed to detect only a single *Plasmodium* species or it may be designed to detect all members of the genus depending on the needs in a given situation [19, 20]. Most protocols use blood as sample type but also non-invasive sample types such as saliva and urine has been used. As mentioned, such non-invasive sample types can be used with benefit if a high number of asymptomatic persons are to be tested as part of a surveillance study [21-23]. Theoretically, as little as a single target DNA molecule can be detected using PCR making it an interesting methodology for eradication studies. In concert with the unsurpassable theoretical sensitivity of PCR, PCR protocols for detection of *Plasmodium* parasites has generally reported very high sensitivities and detection limits well below 1 parasite per µL blood [16,24,25]. Unfortunately, the ability of detecting a few target molecules and the exponential nature of PCR amplification also inevitably leads to a risk of false positives and reduced specificity. Another inherent risk is false negatives due to mutations in the *Plasmodium* target DNA. Indeed, as the burden of *Plasmodium* infection in a region is lowered and the attempts to eradicate the parasite are intensified, there will be a greatly increased evolutionary pressure on the *Plasmodium* parasite to mutate in order to render itself undetectable for the commonly used detection methods. Finally, it should be mentioned that PCR sample preparation and analysis requires trained personnel as well as specialized and expensive equipment, which may not be available to local laboratories in the relevant countries.

LAMP

Another NAAT method that has attracted much attention is LAMP (Loop Mediated isothermal amplification) [16,26,27]. As in the case of PCR the LAMP technology depends on primer based DNA target amplification. This gives LAMP the same advantages as PCR when it comes to flexibility in assay design and allow either specie specific or genus specific detection of *Plasmodium* [28-30]. However, the risk of mutations giving rise to false negatives is also a problem for LAMP. In contrast to PCR, LAMP benefits from the amplification step being isothermal and the technique is hence more usable for low resource settings than PCR. The detection limit of LAMP protocols are down to 1-2 parasite per µL blood and when compared to PCR, the sensitivity often exceeds95% [16,30,31]. The detection limit of LAMP is thus better than for blood smear microscopy but may still not be good enough for eradication studies.

REEAD

A fundamentally different nucleic acid amplification based detection method, REEAD (rolling circle enhanced enzyme activity detection), has recently been described [32,33]. As implied by the name REEAD allows the detection of *Plasmodium* through detection of the activity of a *Plasmodium* encoded enzyme, topoisomerase I (pTopoI). The core of the REEAD technology is pTopoI mediated conversion of a specific DNA substrate into a DNA circle which is amplified using rolling circle amplification and detected [32,33]. Topoisomerases are found in high copy numbers in all living cells from bacteria to humans and their activity is essential to cell survival [34]. When using pTopoI activity as biomarker, the risk of getting false negatives due to mutations is therefore very low. Neither neither the pTopoI reaction nor the rolling circle amplification step used for amplification of the pTopoI generated signals require heating or specialized equipment. Furthermore, the read-out, which is currently based on fluorescence microscopy, can easily be adapted for user-friendly and high throughput use. Such adaptation can also render the REEAD assay well suited for use in low-resource settings and even for use completely without electricity by exploding e.g. a horseradish peroxidase mediated color reaction for visualization (Hede et al. unpublished data).The detection limit of REEAD was found to be as low as 0.06 parasites per µL blood. Together with the fact that REEAD allows detection of the *Plasmodium* parasite using saliva as test material the low detection limit makes it a very promising emerging technology for malaria eradication purposes [33].

DISCUSSION AND CONCLUSION

In conclusion, malaria elimination projects present a unique set of challenges to detection methods. First of all, the detection method should detect the presence of *Plasmodium* parasite directly, it should be suitable for high throughput screenings, and be sensitive enough to detect even very low concentrations of *Plasmodium*. Secondly the method should be easy to operate in a low technological setting and not be susceptible to false negatives due to mutations in the biomarker. Lastly the detection method should ideally allow testing of non-invasive sample sets such as urea or saliva. As summarized in Table 1, none of the currently used malaria diagnostic tests meet all these criteria. Microscopy, RDT's, PCR, and LAMP all face inherent issues in

Table 1: Comparison of malaria detection methods - indicates "not possible", + indicates "possible"; (+) indicates potentially possible.

	Microscopy	RDT	PCR	LAMP	REEAD
Adaptable for high throughput screening	-	+	+	+	+
Detection limit below 1 parasite/µL	-	-	+	(+)	+
Adaptable for use without electricity	-	+	-	(+)	+
Low cost	+	(+)	+	+	+
Vulnerable to mutations	-	+	+	+	-
non-invasive sample types	-	-	+	+	+

terms of sensitivity or liability to mutations that will be difficult if not impossible to circumvent. Hopefully, further developments on NAAT techniques based on detection of essential enzymatic activities such as REEAD will be able to circumvent these difficulties and pave the way for better, more efficient eradication programs.

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