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Molecular Diagnostic Techniques for Malaria Infection - A Review

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Abstract

Malaria is the most threatening disease protozoal and a major health problem worldwide especially in developing countries. WHO recommended that for every case of suspected malaria, diagnostic test must be done to confirm the disease. A more advanced Diagnostic Techniques were developed to overcome the problem of conventional microscopy. These techniques are known as Molecular diagnostic techniques, and they detect specific sequence in DNA, RNA and proteins to provide clinical information for human pathogens including malaria parasites. There are several techniques involve in molecular diagnostics, some are however discussed in this review. They include Polymerase Chain Reaction (PCR), Loop-mediated isothermal amplification (LAMP), Flow Cytometric technique (FCM), Nucleic acid based sequence amplification (NASBA), and Luminex xMax technology. Among these techniques; LAMP technique is the best techniques that can be deployed in the field settings (clinical and rural settings) because of its simplicity, reliability, stability, detection method as well as point-of-care and confirmatory ability. On the other hand, PCR-based technique is more suitable for research purposes because it can be used to identify drug resistance, follow-up therapeutic response, and detect asymptomatic malaria carriers who may be targeted for treatment. Hence, molecular diagnostic techniques are most innovative science and technical implementations that can be used to diagnose malaria infection and to overcome the limitations.

Key Words: Malaria, Polymerase chain reaction, Flow cytometry, NASBA, LAMP

INTRODUCTION

WHO initiated 'The T3 (Test, Treat, Track)' in 2012 to encourage diagnostic testing, treatment and surveillance for malaria in malaria-endemic countries, donors, and the global malaria community. This initiative makes malaria diagnosis an essential component of malaria control strategies (WHO, 2015b).Malaria diagnosis face challenge especially in poor malaria endemic areas, diagnosis in those areas are often based on clinical symptoms which is non-specific and may overlap with symptoms of other infectious diseases, this may result in development of drug resistance (Murphy *et al.,* 2013) or over-treatment of malaria or non-treatment of other diseases in malaria endemic areas (Tangpukdee, *et al.,* 2009)which can lead to misdiagnosis, thus, the primary function of laboratory diagnosis is to support clinical care (Singh *et al.,* 2010). Therefore, reliable, accurate and rapid diagnosis of malaria is very important in both treatment and control strategies of the disease; it decreases the chance of transmission and ease suffering. Many diagnostic techniques have been developed to confirm the presence of malaria parasite in blood cells, these techniques include; microscopy, serologic analysis, molecular diagnostics etc. Nevertheless, not all the tests are appropriate for every clinical diagnosis, some are suitable for research purposes.

Molrcular Diagnostic Techniques

Molecular Diagnostic assays have increased the analytical sensitivity of assays for human pathogens including malaria parasites. It uses >65 primer sets with at least five molecular targets that can be used to test as many as five human malaria species; *P. falciparum, P. ovale, P. vivax, P. malariae, P. knowleski* (Murphy *et al.,* 2013). Molecular diagnoses of malaria are new laboratory diagnostic techniques developed to detect, display and analyze high sensitivity and high specificity without subjective variation and extensive characterization of the malaria parasite (Tangpukdee *et al.,* 2009). The first detection methods by molecular diagnosis were published in 1980s, since then; there have been a tremendous amount of progress in the field; DNA extraction, simplified detection protocols,

specie-specific identification, quantification of parasite loads and rapid processing of large amount of samples (Steenkeste *et al.,* 2009).

These molecular biological techniques include; Polymerase Chain Reaction PCR (single-step PCR, nested PCR, real-time PCR), Loop-mediated Isothermal Amplification (LAMP), Microarray, Mass Spectrometry (MS), Flow Cytometric (FCM) assay techniques, Real-time SYBR, Nucleic Acid-based Sequence Amplification (NASBA), and Strand displacement amplification technique (SDA). This assays greatly improved detection and the ability to provide species identification using species-specific primers or probes and to precisely quantify parasites by comparison to standard curve materials (Murphy *et al.,* 2013). Some of these molecular techniques will be reviewed in this paper.

Polymerase Chain Reaction (PCR) Technique

Polymerase chain reaction technique is a sophisticated technique that is use for DNA and RNA analysis (Parija, 2010), it performs selective amplification from complex genome (Tavares *et al.,* 2011), and the target is genus specific small subunit 18s ribosomal ribonucleic acid (rRNA) gene of the parasite (Ahmed, 2013).PCR have the ability to detect low level parasitaemia and allows accurate identification of specie, therefore it can be used for the diagnosis of malaria when conventional techniques show negative result, and hence, it can be used as confirmatory test. It has a detection limit of one parasite per microliter, therefore it has the ability to detect mixed infection (Tangpukdee *et al.,* 2009), and also it can detect asymptomatic malaria carriers who may be targeted for treatment (Oriero *et al.,* 2014).

However, PCR molecular assays are not feasible for field settings as it can be contaminated easily (Dhama *et al.,* 2014), and it also requires post amplification protocols like electrophoresis and it takes 3-4 hours in order to know the result (Dhama *et al.,* 2014). Thus, Real-time PCR was developed, which eventually replaced the conventional nested and semi-nested PCR techniques due to technical difficulty, time consumption and contamination. It is quick to perform, simple and reduce the risk of contamination (Ahmed, 2013), it allows simultaneous detection and quantification of parasites and efficient technique for applications with large numbers of samples (Lima *et al.,* 2011). Another added advantage of Real-time PCR is, while DNA-based PCR techniques (conventional nested and semi-nested) are sufficiently sensitive when a large extraction of blood volume is used, RNA-based PCR (Real-time) techniques are more sensitive using a small volume of blood because it use the parasite 18S rRNA (Murphy *et al.,* 2013).

Numerous studies have shown that PCR technique is more sensitive and specific than conventional microscopy (Echeverry *et al.,* 2016, Tangpukdee *et al.,* 2009).Recently, field PCR units and portable real-time PCR systems with freeze-dried reagents have been developed for mobile analytical laboratories and field hospitals for detecting parasites (Oriero *et al.,* 2014).

Loop-Mediated Isothermal Amplification (LAMP) Technique

LAMP was first described in the year 2000 (Oriero *et al.,* 2014). This technique detects the conserved 18s RNA gene of *P. falciparum,* although other studies indicated detection on *P. vivax, P. ovale, P. malariae* (Tangpukdee, *et al.,* 2009) and *P. knowleski* (Oriero *et al.,* 2014). In this technique, DNA amplification is achieved by the use of DNA polymerase with strand-displacing activity under isothermal condition, it requires four to six different primers that are designated specifically to recognize six to eight specific gene sequences (Drapala and Kordalewska, ND), in the process pyrophosphates are formed causing turbidity that facilitates visualization in a more effective way than PCR (Dhama *et al.,* 2014).

Moreover, real-time detection can be performed with a Loopamp real-time turbidimeter (Sattabongkot *et al.,* 2014). LAMP results can also be visualized by fluorescence under a blue light emitting diode light (Polley *et al.,* 2013) or by microcrystalline wax dye capsule containing the DNA fluorescence dye SYBR green I (Tao *et al.,* 2011).

As a molecular diagnostic tool, LAMP has the potential to combine both the effect of high sensitivity with the possibility of performing the test under field condition with limited technical resources (Paris *et al.,* 2007), and it can be used for Point-of-care testing in both developing and developed countries and also be used as confirmatory assay in place of a PCR-based assay (Sattabongkot *et al.,* 2014).

Paris *et al.,* (2007) conducted a research where they compared LAMP technique to RDT and microscopy, they concluded that, the technique lack sufficient accuracy due to method used to extract the DNA (heating) which deteriorated the specificity, However, in a studies conducted by Sattabongkot *et al.,* (2014),

they used same method to prepare template DNA which was efficient comparable to the referenced Saponin/Chelex method, in contrast to the former, the method proved to be simple, fast and suitable for use in the field.

LAMP have certain advantages compared to PCR in the diagnosis of malaria because; it have the ability to perform the reaction and read the result without opening the tubes, it also has potential application for clinical diagnosis together with surveillance of infectious diseases without the need for sophisticated equipment and skilled personnel in developing countries. Moreover, a genus-specific and species-specific diagnostic method was developed using heat-treated clinical samples which simplified the DNA extraction method and suitability for use in the field (Sattabongkot *et al.,* 2014).

Flow Cytometric Technique (FCM)

Flow cytrometric technique has been developed recently to detect and quantify *Plasmodium falciparum* in the laboratories, (Grimberg *et al.,* 2008), it is becoming more accessible equipment in malaria research laboratories, less expensive, more reliable and simple (Roobsoong *et al.,* 2014) and throughput procedure with less subjectivity (Campo *et al.,* 2011).

FCM is an analytical method that allows the rapid measurement of light scattered and fluorescence emission produced by suitably illuminated cells (fluorochromes) which bind to specific cell compounds such as proteins, nucleic acids and lipids, they can be conjugated to antibodies, depend on enzymatic activity or depend on cellular physiological parameters such as pH and membrane potential. Flow cytometry has been proposed as rapid diagnostic tool in the fight against infection caused by bacteria, viruses, fungi and parasites. It can be used to isolate microorganisms, identify them, and determine antibodies to a particular parasite or pathogen, in different stages of the disease and in direct detection of essential components such as nucleic acids and proteins in clinical specimens (Pieretti *et al* 2012). It could also count number of parasites and evaluate the malaria-infected red cells (Jang *et al.,* 2014). FCM is a very powerful tool in malaria research because it could identify various developmental stages of *P. falciparum* based solely on nucleic acid content without recourse to morphological changes (Chevally *et al.,* 2010). It offers higher precision and efficiency than Giemsa Stain Microscopy (Jang *et al.,* 2014). Gradually FCM method is being developed for field and clinical laboratory usage.

Flow cytometric assays use different staining methods to detect parasite infected cells, most of which use nucleic staining procedure because nucleic acids can be found only in malaria infected RBCs but not in normal RBCs (Jang *et al.,* 2014).There are numerous stains used in FCM, such as; Hoechst 33258 (Brown *et al.,* 1980), Acridine orange (Whaun *et al.,* 1987), Thiazole orange, (Makler *et al.,* 1983), Hydroethidine (Wyatt *et al.,* 1991), YOYO-1 (Barkan, *et al.,* 2000), SYBR Green I and CD235A (Pieretti *et al.,* 2012). These DNA-targeting dyes have different target specificity and fluorescence intensity which allows for better separation of cellular populations (Rye *et al.,* 1992) and they can be used simultaneously as in Jang *et al.* (2014).

However, the drawback of FCM assay is the contaminated white blood cells that are frequently found in blood samples of patients, and anaemia in chronic infections which can enhance reticulocytaemiain malaria patients. Therefore this complicates the use of nucleic acid stain-based methods especially in the detection of *P. vivax* (Roobsoong *et al.,* 2014). Hence, Roobsoong *et al.* (2014) developed an antibody-based staining method to overcome the complications of nucleic staining method.

Another alternative method is based on the principle of hemozoin detection, by depolarization of laser light as cells pass through a flow-cytometer channel. Hemozoin is produced when malaria parasites digest host hemoglobin; it released toxic heme, which will be crystalized in the acidic food vacuole (Tangpukdee *et al.,* 2009).

Nucleic Acid Sequence Based Amplification (NASBA)

NASBA was first described by Kievits *et al.* (1991) and then developed by Compton (1991a). It is a novel nucleic acid amplification method for the detection of RNA targets (mRNA, rRNA and genomic RNA). It is a homogenous (Oriero *et al.,* 2015), sensitive, isothermal and transcription-based amplification system that uses three specific enzymes and do not require expensive thermal-cycling equipments (Oriero *et al.,* 2015). These enzymes include avian myeloblastosis virus reverse transcriptase (AVM-RT), RNase H and T7 DNA dependent RNA polymerase (DdRp) (Fakruddin*et al.,* 2012). The end product is a single-stranded RNA antisense to the original RNA template (Compton, 1991b)

and it generates a high number of RNA copies per cycle, enabling it to generates detectable products in a shorter time frame than other amplification techniques (Compton, 1991c). NASBA have a detection limit of 0.02 parasites per microliter of blood and it allows for precise quantification of the parasite load over a range of 20-10^8 parasites per milliliter of blood (Schneider *et al.,* 2005). NASBA is also known as Self-sustained Sequence Replication(3SR) **(**Guatelli *et al.*, 1990**)** and Transcription Mediated Amplification (TMA) (Gill and Ghaemi, 2008).

NASBA has been used for the detection and semi-quantification of malaria parasites (Oriero*et al.,* 2015) with 97% sensitivity and 81% specificity compared to microscopy (Smits *et al.,* 1997) or a sensitivity of 100% and specificity of 94% (Shallig *et al.,* 2003). Prevalence and density of plasmodium gametocytes has also been determine using quantitate-NASBA(QT-NASBA), which indicate the ability to detect as low as 0.02-0.1 gametocytes per microliter of blood (Bousema *et al.,* 2010), and it is also used increasingly to detect both *P. falciparum* and *P. vivax*gametocytes (Wampler *et al.,* 2013).

As a POC technique, NASBA has merits and demerits; the former being that it has high degree of sensitivity and specificity, detection limit of 0.01 parasites per microliter of blood determined by serial dilution of clinical samples of known parasitemia, thus has lowest limit of detection of any of the investigated malaria diagnostic, and can produce results in an hour, while the latter being; prone to contamination and false-positive results, requires more extensive sample preparation than LAMP and it is expensive for practical POC use, therefore it is more suitable for use in regional or central health facilities. However, because of its most sensitivity to low-level infection, it has the potential to be used as screening tool despite its relatively high cost (Cordray and Richard-Kortum, 2012).

Luminex xMax Technology

Luminex technology is a technology that is bead-based flow-cytometry assay. It uses microsphere beads that allow the detection of various targets simultaneously by emitting unique fluorescent signals when excited by laser. Up to 100 microspheres are available that can bond covalently to antigens, antibodies or oligonucleotides that serve as probes. The assay could identify different genotypes of a particular organism or multiple organisms during the same reaction utilizing very low volume. This approach is very useful for the diagnosis of parasitic diseases. It was able to detect all the blood stages of the four human plasmodium species (McNamara *et al.,* 2006). Luminex technology can improve the speed, the accuracy and the reliability of other PCR methods because it eliminate the need for gel electrophoresis, and samples can be handled simultaneously and continuously through 96-well plate format from DNA extraction all through data analysis which is automated and therefore can be uniform (Ndao, 2009).

Conclusion

Although microscopy is the gold standard for malaria diagnosis, it however has its limitations. Molecular diagnostic techniques were developed to overcome these limitations. Molecular diagnostic techniques were designed to detect specific sequences in DNA, RNA and proteins of the parasites; it is enabled by genomic technologies and information where DNA, RNA, and Proteins are measured by PCR, Hybridization, microarray, antibodies and mass spectrometry. The advantages of molecular techniques over other diagnostic techniques are; high sensitivity and specificity, quantification across wide range of parasites densities, species identification, earlier detection of new infection and detection of asymptomatic carriers, potential for strain identification and high throughput. Nevertheless, it also has its advantages which include; time consumption, expensive equipment, requires extensive training, difficult to deploy in field or rural settings, can be easily contaminated, it is therefore unsuited for routine use in field or clinical laboratories but best suited for research laboratories.

Detection of Plasmodium parasites in both symptomatic patients and asymptomatic carriers to cure and prevent transmission of the parasites is essential in malaria control strategies, PCR-based techniques such as Real-Time PCR, nested-PCR and semi-nested PCR, were however developed to detect low level parasitaemia, allows species identification, detect mixed infection, therefore can be used as an excellent tool for diagnosis of malaria parasites when conventional techniques show negative results.

But it cannot be deployed in the field, it is expensive, it takes time and requires heavy equipment. Isothermal amplification techniques such as LAMP, NASBA, SDA etc. on the other hand were developed to overcome the problems of PCR-based techniques.

It therefore have the potential for field diagnosis of malaria infection due to several reasons, which include absence of costly and power-intensive thermocycler, produce results in a short time, can be used to process large number of samples, ability to detect <1 parasites per microliter of blood of both sexual and asexual stages. However, the specificity and sensitivity are both comparable with PCR-based techniques.

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