

Recent Methods in Antimalarial Susceptibility Testing

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Abstract: As malaria continues to be a worldwide problem due to increasing drug resistance, several drug susceptibility techniques have been reported in the literature. A particularly confounding problem is the lack of standardization between methods that result in differences in sensitivities. In this review, we report on the types of antimalarial drug susceptibility assays available to clinical and research investigators. Techniques based on enzyme-linked immunosorbent assays (ELISAs), fluorescence, molecular assays, and optical methods will be outlined. Strengths and weaknesses, as well as field applicability, will be discussed. Furthermore, assay and culture conditions, particularly for the fluorescence-based assays, will also be detailed.

Keywords: Antimalarials, drug susceptibility, ELISA, fluorescence, molecular assays, real-time polymerase chain reaction, optical imaging, *Plasmodium*.

INTRODUCTION

Malaria is a severe global problem that affects approximately half of the world's population. This malaria burden is most acute in endemic regions, particularly in sub-Saharan Africa. Initiatives by the World Health Organization (WHO), the Gates Foundation, the Global Fund, and the World Bank for reduction and eradication of this disease are occurring with great success. Nevertheless, malaria cases still number in the millions (93 million), with 863,000 deaths in 2008 [1]. Furthermore, the WHO predicts the spread of insecticide-resistant mosquitoes and drug-resistant parasites that will serve as major threats to the goal of eradication. The emergence of multidrug-resistant *Plasmodium* species, particularly *Plasmodium falciparum* (*P. falciparum*) makes decisions regarding malaria chemoprophylaxis and treatment more complicated. Resistant parasites often have a local focus, requiring increased surveillance measures to quickly identify emergence of resistance. Moreover, the WHO noted that this spread is also directly related to weak surveillance programs in countries where resistant parasites are prevalent [1].

As with other pathogens, several methods are available to measure drug susceptibility. Assay ease of use often depends on the organism, and may range from relatively easy to extremely complex. For example, aerobically growing bacteria such as *Staphylococcus aureus* and *Escherichia coli* are relatively easy to manipulate since they are free-living and have very modest growth requirements. For *Plasmodium* species, assay development is particularly complicated given the complex life cycle of the parasite and the requirement for human blood and plasma for media. Regardless of the multitude of life cycles and the parasite's complex growth requirements, many different types of assays have been developed to measure drug resistance. Often, these methods en-

compass an array of disciplines ranging from basic biology to biochemistry to genomics. The WHO has published a manual on field applicability of *in vitro* parasite sensitivity assays. Although they describe the value of these assays as research and surveillance tools, they also indicate issues with the standardization of protocols [2]. In order for these standardized protocols to be developed, the malarial scientific community needs a consensus on a gold standard, which will provide reliable and reproducible results. Currently, therapeutic efficacy or *in vivo* tests are the accepted gold standard for measuring antimalarial drug susceptibility [2].

In this review, we present several methods for measuring drug resistance. Each technique will be described, along with their inherent strengths and weaknesses. Their utility in resource-limited and field environments will also be discussed.

ENZYME-LINKED IMMUNOSORBENT ASSAYS

Initial use of antibodies for detection of infectious diseases required the use of radioactivity [3]. Although effective, a safer alternative was sought and the use of color-changing substrates was established. Two methods were developed. One uses a direct immunoassay with which an antibody specific to a particular antigen is conjugated to an enzyme that can catalyze a substrate to change color. The second method utilizes a primary antigen-specific antibody with a secondary antibody that is specific to the previous one. Many variations of this technique are currently in use. Today, many rapid assays utilize this technology and enzyme-linked immunosorbent assays (ELISAs) are now a mainstay in clinical laboratories, diagnosing infectious diseases such as HIV, West Nile virus, and *Clostridium difficile*-induced enterocolitis [4].

Rapid methods using ELISA for the detection of malaria parasites in blood are available. These techniques have been adapted to measure antimalarial drug susceptibility and have several advantages over currently gold standard methods, such as the ³H-hypoxanthine assay. The assays are non-radioactive, highly sensitive, and easy to perform, while the

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equipment and reagents are relatively inexpensive, rendering them suitable for field applications. However, disadvantages of ELISAs for field use include the storage requirements of some reagents (i.e. antibodies), multiple washing steps, and lengthy incubation times. In this section, we discuss the two assays developed for specific antigens in measuring drug susceptibility.

Lactate Dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is an enzyme found among many species and utilized during energy production. Levels of *P. falciparum* LDH are distinct from human LDH, and can be correlated to parasite multiplication [5].

Plasmodial LDH has been used as a marker for parasite identification and detection. ELISA assays based on *P. falciparum* LDH have been adapted from initial enzymatic assays described by Makler *et al.* [6] and Delhaes *et al.* [7]. The modification of the LDH assay from an enzymatic-based assay to an ELISA-based assay significantly increased the sensitivity as the enzymatic assays could only be used if parasite density was greater than 1% [6, 8]. Furthermore, successful ELISAs have now been reported using an initial parasitemia of 0.005% [9].

The success of ELISA for diagnostics has resulted in the production of rapid diagnostic tests (RDTs). These tests measuring malaria LDH are recommended for use in areas where microscopy is not available [10]. Additionally, these tests have been suggested for use in drug efficacy monitoring given that, after treatment, LDH is removed rather rapidly from the bloodstream since it is produced only by viable parasites [11-14]. However, some cases have shown that the assay has poor sensitivity in patients with low parasitemia. In a study by Fogg, they compared two LDH tests to Giemsa staining and found significantly lower sensitivities at parasite loads less than 100 parasites/ μ l. The authors conclude that while the possibility of these patients experiencing severe clinical episodes is minimal, the low level parasitemia may still perpetuate parasite transmission [11]. Useful in diagnostics, ELISAs measuring *Plasmodium* LDH [15, 16] have also been adapted for drug susceptibility testing [17, 18].

When compared to the standard isotopic microtest, the LDH ELISA has been used to evaluate malaria drug susceptibility to at least nine antimalarial drugs in field isolates including several artemisinin combination therapy (ACT) component drugs [9, 18, 19]. In some studies, 50% inhibitory concentrations (IC_{50} s) determined by LDH ELISAs are higher than those obtained by the isotopic assay, although the global values often show parallel trends [9, 18, 19]. These differences have been attributed to the different metabolic pathways used to measure growth inhibition (hypoxanthine incorporation versus protein production [9]). In addition, the LDH ELISA has been used to determine IC_{50} cut-offs defining phenotypic resistance for the drugs monodesethylamodiaquine (80 nM), lumefantrine (150 nM), and dihydroartemisinin (10 nM), while the isotopic assay has only defined a cut-off IC_{50} for chloroquine (100 nM) [19-22].

Experiments utilizing the LDH ELISA have demonstrated the importance of drug shelf-life in relation to IC_{50} values. Kaddouri *et al.* observed significantly higher IC_{50} s

when testing pre-dosed plates of dihydroartemisinin and lumefantrine past the drug expiration dates [19]. These results emphasized the requirement to strictly follow standard operating procedures to avoid false positives that indicate drug resistance.

Histidine-Rich Protein II (HRP2)

HRP2 is a histidine- and alanine-rich protein found in the cytoplasm of all strains of *P. falciparum* [23]. It is a secreted water-soluble protein that has been recovered from plasma and culture supernatants. HRP2 can also be found in concentrated packets in the host erythrocyte cytoplasm as well as located on the infected erythrocyte membrane [24].

Used in RDTs for falciparum malaria [25, 26], HRP2 is quite stable and protein levels found in culture samples are proportional to parasite development and multiplication [27]. Due to their relatively low cost and high sensitivity, these RDTs are recommended for use in *P. falciparum* endemic areas [28] where their use can minimize inappropriate treatment of non-malarial febrile illnesses [29]. The stability of HRP2, which allows these diagnostic tests to be highly sensitive, also renders them less specific since the HRP2 antigen can persist long after effective treatment [30]. Evaluations into the accuracy of these tests reveal high false positive rates and variable positive predictive values [29, 31]. Regardless, the advantages to this technique are three-fold. First, the assay is capable of detecting parasites in cases of placental malaria [32]. Another advantage is detection of drug resistance in patients with low levels of parasitemia due to therapeutic failure [9]. Finally, the HRP2 assay can also measure current and recent numbers of parasites during malaria intervention trials [33].

In one diagnostic comparison to microscopy, Kifude *et al.* demonstrated the value of the HRP2 ELISA for parasite quantification in clinical samples, including whole blood and serum [33]. In their studies, assay conditions were optimized, such as washing away secreted HRP2 and identifying the maximum number of freeze-thaw cycles (four) for HRP2 control antigen. A recognized technical issue in malarial quantification is that more than one detector antibody may bind to a single protein, rendering reconciliation with clinical peripheral ring-stage parasitemia on blood films impractical. However, the precision measurements and repeatability of the HRP2 ELISA are within the industry standard, while the microscopy in this analysis yielded mixed results. Thus, the authors propose the utility of the HRP2 ELISA as an adjunct to microscopy with potential applications during malaria intervention trials.

Recently, the LDH and HRP2 assays have been applied in combination using standardized conditions to test spiked and clinical blood samples. These studies show that resulting sensitivity levels are equal to or better than microscopy and RDTs [34]. This 'unified' ELISA protocol overcomes limitations found in individual assays by allowing real-time examination of both past and present infection as well as parasite burden from the same sample, thus proving useful for clinical management of the patient. This approach could be especially useful in pregnant patients where microscopy can be unreliable in detecting sequestered parasites [32].

The first reported use of the HRP2 ELISA for drug susceptibility testing utilized a commercial HRP2 capture ELISA kit requiring an incubation time from 48 to 72 hours [35]. This assay was modified for field use by simplifying the handling and culturing of the parasites through reagent substitutions as well as omission and reduction of some centrifugation and washing steps. Using fresh isolates, this HRP2-based field test yielded similar results to those obtained with a modified WHO schizont maturation assay [36]. Overall, the HRP2 ELISA has been used successfully in malaria drug susceptibility. Specifically, the assay was utilized in drug combination studies of amodiaquine plus artesunate or sulfadoxine-pyrimethamine [37] and future treatment regimens (artesunate and pyronaridine [38]).

FLUORESCENCE-BASED ASSAYS

Taking advantage of the lack of RNA and DNA in mature erythrocytes, microtiter plate assays using fluorescent nucleic acid intercalating dyes such as SYBR green I, PicoGreen, and YOYO-1 have been developed to measure malaria drug resistance [39-45]. Fluorescence-based assays are accurate, reliable, less expensive than isotopic substrates and antibodies and easy to perform; many involve only one step in one plate to obtain results. These assays are highly sensitive, and compared to isotopic methods, SYBR green I has been shown to detect wider ranges of parasitemias when using cultured strains [40, 46]. Initial experiments using fluorescent dyes required complex, multistep protocols [47, 48], however, these assays have been notably improved and are now amenable to high-throughput screening [40, 49]. Furthermore, LED-based light sources have emerged [50, 51], which will simplify the use of these assays and reduce the associated costs [52].

Fluorometer Methods

This new round of fluorescence-based assays has been extensively investigated alongside standard isotopic and ELISA assays. In these studies, known antimalarial drugs have been tested against malaria culture strains to determine their IC₅₀s. Similar IC₅₀ values have been obtained from fluorescence-based assays when compared with those obtained from radioactive assays and/or microscopic examination [41, 43, 53]. For example, in our further validation of the malaria SYBR green I-based fluorescence (MSF) assay, we obtained similar drug sensitivity profiles after testing an extensive drug panel including antibiotics and antifolates and comparing these results to the ³H-hypoxanthine assay [49]. Additionally, the SYBR green I assay has been used successfully to determine the IC₅₀s of clinical isolates in comparison with the HRP2 ELISA [54] and the ³H-hypoxanthine assay [55].

As previously mentioned, a concern with the SYBR green I assay is the lack of standardized conditions for parasite growth. Bacon *et al.* examined the potential effects of several assay conditions in their comparison of the SYBR green I assay and the HRP2 ELISA [54]. In their work, 25 blood samples were analyzed by both assays in the presence of chloroquine and mefloquine, and the IC₅₀ and IC₉₀s were determined. Both methods were tested with an incubation time of 72 hours, while the SYBR green method was also

used to test the samples at 48 hours. Ten of the 25 samples were successfully analyzed by both methods after 72 h of incubation; however, only 7 of these were successful in the SYBR green assay at 48 h. In addition, the authors investigated the effect of phenol red in the growth medium during the SYBR green assay. While no significant difference was observed in the IC₅₀ values between samples with and without dye, the parasites grew better in medium containing phenol red. Other variable growth conditions that were identified include the storage of fresh clinical samples and the hematocrit. Since storage of isolates for more than 24 h has been shown to considerably affect IC₅₀ values [56], isolates were stored at room temperature for no more than 12 h. Basco *et al.* have shown that increased hematocrit resulted in increased IC₅₀s, thus in their study they adjusted the hematocrit to 1.5% to compensate for the increase [56]. In another study, however, Sharrock *et al.* used a hematocrit of 2% while comparing chloroquine sensitivity of *P. vivax* and *P. falciparum* in ring versus trophozoite stage parasites [57]. Initially, it has been shown that oxygen levels during parasite cultivation may affect parasite growth [58]. In our experiments, we observed only minor differences between parasites grown in high (approximately 21%) versus low (5%) oxygen environments, Fig. (1), as determined by the testing of 1,280 compounds in the Library of Pharmacologically Active Compounds (LOPAC) chemical set (Sigma-Aldrich). However, humidity does affect parasite growth as cultures incubated in less than 95% humidity resulted in significant plate edge effects (unpublished data).

Additionally, conditions during the performance of the SYBR green assay have been addressed and need to be standardized. For instance, some investigators have found that freezing plates after incubation and before performing the SYBR green assay generated more reliable results [54]. Although the removal of host white blood cells (WBCs) appears to result in greater accuracy [46], some studies have shown that this step may not be necessary. Any WBC contribution to background fluorescence can be removed or left in the final IC₅₀ calculation without significantly affecting the final IC₅₀ value determined [54]. Also, the addition of a lysis step can reduce SYBR green incubation time from 1 hour to 5 minutes while the IC₅₀ values remain similar [54]. Moreover, through changing the concentrations of lysis buffer components to those outlined by Plouffe *et al.*, one may increase sensitivity and lower the detection limit [59, 60]. Furthermore, through the use of a higher SYBR green I concentration and overnight incubation with dye as initially described by Plouffe *et al.*, we have demonstrated enhanced sensitivity in the MSF assay. The sensitivity of *P. falciparum* strain D6, as well as others (data not shown), to chloroquine was investigated and IC₅₀ values were reproducibly calculated from parasitemias as low as 0.0375% Fig. (2).

The presence of hemoglobin may also affect fluorescence-based assays due to its wide absorption spectrum which can interfere with the emission of both PicoGreen and SYBR green I [41, 46]. Additionally, detergents found in lysis solutions can have a negative effect on DNA detection by fluorophores [41]. To overcome these potential complications, Moneriz *et al.* used saponin to disrupt parasite infected erythrocytes, washed them several times to remove hemoglobin and detergent, and then used detergent-free fluores-

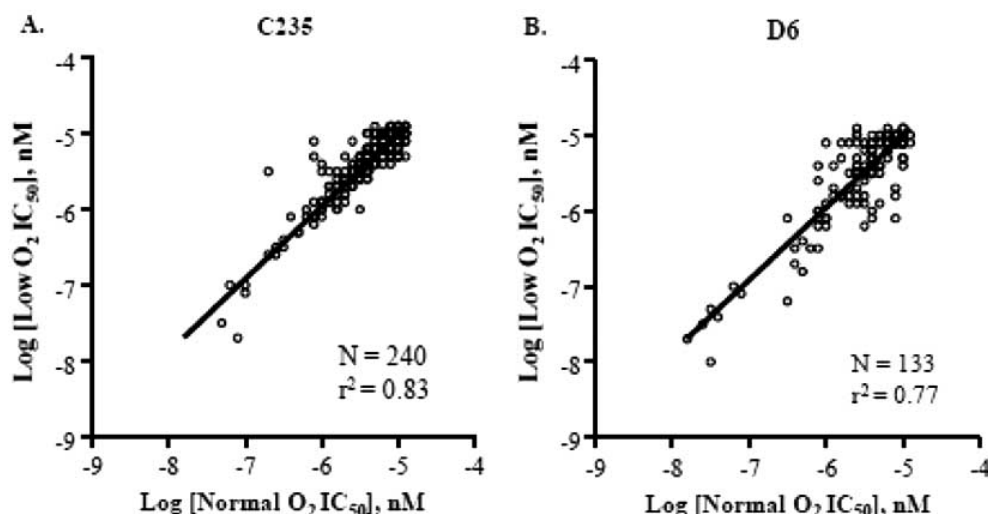


Fig. (1). *P. falciparum* strains (A) C235 (multidrug resistant) and (B) D6 (drug sensitive) were screened against the Library of Pharmacologically Active Compounds (LOPAC) under high (approximately 21%) or low (5%) oxygen conditions. N is the number active antimalarial compounds tested within LOPAC.

cent dye [46]. The authors did observe, however, that the presence of hemoglobin and detergents as well as their choice of fluorescent dye (PicoGreen vs. SYBR green I) did not affect IC_{50} values and suggest that low sensitivity is not critical for IC_{50} calculation except in situations where microscopic methods are not effective such as pregnancy or early stages of disease.

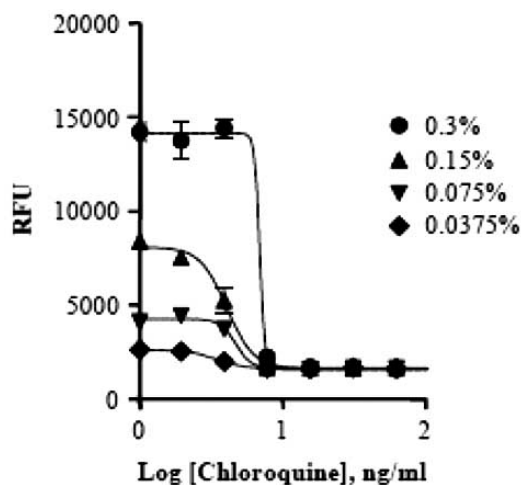


Fig. (2). The sensitivity of *P. falciparum* strain D6 to chloroquine was investigated using the MSF assay [49] and a 10X concentration of SYBR green I, followed by an overnight incubation at room temperature before relative fluorescent unit (RFU) measurement. IC_{50} values were calculated using initial assay parasitemia between 0.0375 – 0.3%.

Recently, the MSF assay has been used to discern between single and mixed *P. falciparum* populations as well as to examine current antimalarial combination therapies in a high-throughput screening format [59, 61]. Using the method adapted from Oduola *et al.*, total population and individual sub-population IC_{50} s were calculated from a mixed *P. falciparum* population and yielded similar results to Oduola *et*

al.'s findings using 3H -hypoxanthine [59]. However, one limitation was identified when testing sub-populations with similar sensitivities to the same drug; the MSF assay was less accurate in identifying the sub-populations. Co *et al.* recommends testing with multiple drugs to further interrogate the levels of drug susceptibility found in mixed populations [59]. This assay may have a profound impact in clinical testing and provide a useful tool to assess the efficacy of combining drugs [62].

Although IC_{50} values are necessary to determine parasite susceptibility disposition, they may not be clinically relevant. Some studies have shown that depending on the resistance phenotype and drug mechanism of action, dosing based on IC_{50} values may not be appropriate due to insufficient amounts of drug. Cabrera *et al.* have recently reported on a method that measures the kill concentration required as opposed to IC_{50} determinations [63]. In their studies on chloroquine accumulation in live malarial parasites, they found that chloroquine resistance was not a result of reduced accumulation. This finding was made by quantifying resistance using cytotoxic activity and examining drug accumulation at clinically relevant 50% lethal doses (LD_{50}). This technique has a profound impact on measuring drug susceptibility because instead of making a subjective susceptible/resistant judgment on the parasites' disposition, the medical provider can base drug dosing on the results of the assay. Furthermore, this necessitates the need to study drug resistance mechanisms so that appropriate drug doses can be administered and consequent treatment failure averted.

Flow Cytometry

Flow cytometry provides a sensitive and simple assay for detection of malaria that provides objective data quickly with minimal expenditure of time and effort. Each cell that is interrogated by flow cytometry yields information on forward scatter, which is relative to size, and side scatter, which is relative to granularity, and fluorescence. The use of inexpensive DNA or RNA binding dyes such as acridine orange,

thiazole orange, Hoechst dyes, or YOYO avoids the use of isotopes such as ^3H -hypoxanthine and provides a definitive measurement of the parasitemia associated with each cell. In contrast, isotopic methods such as ^3H -hypoxanthine provide a population measurement of parasitemia. When conducting a flow cytometry assay for malaria, forward scatter is plotted versus fluorescence to determine not only the presence of malaria parasites but also the relative proportions of rings, late trophozoites, merozoites, and schizonts [64]. The sensitivity and specificity of flow cytometry has been shown as low as 0.002 – 0.003% parasitemia, and in the same study [64], flow cytometry was also shown to be a reliable means to conduct drug sensitivity studies. In this study, IC_{50} values of chloroquine and pyrimethamine by flow cytometry and microscopy showed no statistical difference ($P < 0.05$), however, a small statistically significant difference was noted when comparing either flow cytometry or microscopy to ^3H -hypoxanthine. In a larger study conducted using 453 clinical samples submitted for testing, flow cytometry showed sensitivity of 91.26%, specificity 86.28% and accuracy 87.42% when compared to microscopy and a detection limit of 0.05 – 0.01% parasitemia [65].

In addition, flow cytometry provides a sensitive and specific method of quantitating white blood cells that have engulfed infected RBCs containing hemozoin. During the digestion of hemoglobin by *Plasmodium* parasites, heme is detoxified into a crystalline pigment, hemozoin. When the parasites are released into the blood, the hemozoin crystals are also released and removed from the blood by monocytes, tissue resident macrophages, or granulocytes. Accurate assays of hemozoin containing leukocytes may serve as a prognostic marker for disease severity and progression, and a number of studies have shown that increased quantities of hemozoin containing white blood cells correlates with severity of disease [66, 67]. Specifically, higher numbers of pigment containing neutrophils have been shown to correlate best with severe cases of malaria ($P < 0.0001$) [68]. Traditionally, the presence of pigmented white cells has been determined by microscopy; however, this method has disadvantages with regards to accuracy and observer variance. In one recent study, flow cytometry identified 5.8% Hz-containing monocytes and 1.8% Hz-containing neutrophils while microscopic examination yielded between 10 – 13% of Hz-containing monocytes, as well as 0 – 0.5% of Hz-containing neutrophils [69]. While many flow cytometers are quite expensive and are not portable, innovations in equipment design of the microcapillary Guavacyte flow cytometer (Millipore) show that simple, compact, low-cost flow cytometers are available today that are practical for field use.

MOLECULAR METHODS

Molecular tools, such as polymerase chain reaction (PCR), are currently being used in the detection and diagnosis of various pathogens, including bacteria. Since its discovery in the 1980s, PCR has been a mainstay in molecular biology and currently available molecular assays rely heavily on this technology. However, it was only recently that these methods have been utilized in tandem to detect and diagnose antibacterial resistance. Many bacterial resistance markers have been fully elucidated and are now being used diagnostically in clinical labs to guide antibiotic treatment. One ex-

ample is the *mecA* gene in Methicillin-resistant *Staphylococcus aureus* (MRSA), which is responsible for wound and skin infections [70]. Although culture methods are available, they are time consuming (24 – 48 hour turn-around time) and ultimately delay appropriate treatment of the infection. In contrast, molecular diagnostics can be performed in as short as 2 hours [70].

In addition to diagnostics, molecular tools have also been used in the surveillance of resistance genotypes. Epidemiological data have been gathered on whether bacterial strains are being spread in a hospital-acquired fashion. For example, bacteria that produce extended beta-spectrum lactamases (ESBLs) are of great concern in the infection control field, due to their increased resistance to virtually all beta-lactam antibiotics [71]. To determine the relatedness of separate strains isolated in a hospital setting, pulse-field gel electrophoresis has been used. Although not mainly used in diagnostics, it has served as a reliable research and epidemiological tool in comparing a large number of samples.

Although a variety of molecular methods are available for detecting various microorganisms, identifying markers for resistance and utilizing them for detection poses a challenge. The problem is threefold. One is the difficulty in identifying drug targets, particularly for *Plasmodia* where many of the drug mechanisms of action have yet to be elucidated. In addition, some of the drugs also act on multiple targets and thus may have multiple mechanisms. For example, although in use for many years, it has been shown that chloroquine does not exclusively act on hemozoin formation [72]. Another confounding issue is the conflicting correlation between identified nucleotide polymorphisms (SNPs) in candidate genes and clinical outcomes. A third issue is that some resistance phenotypes are a result of altered gene expression. For example, some chloroquine-resistant *Plasmodia* do not possess SNPs in their *pfmdr* genes, but amplification of *pfmdr* results in increased chloroquine IC_{50} [73]. Finally, the multiplicity of genetic changes in identified drug targets makes development of an easy-to-use assay difficult. Nevertheless, continued studies on resistance markers will allow for development of better pharmacophores for rational drug design and strengthening of structure-activity relationships, as well as predicting SNPs that can result in drug resistance.

The *Plasmodial* molecular markers for resistance to selected drugs are shown in Table 1. Although not an exhaustive list, they show the multitude of genes associated with antimalarial drug resistance. The drug mechanisms of action toward these gene products are similar, resulting in either decreased accumulation or binding affinity to the target. There have been recent reports regarding disagreements on the mechanism of resistance to some antimalarial drugs, [63] which adds to the complexity of using molecular markers for detection.

In this section, we will describe the current and commonly used molecular methods for the detection of antimalarial resistance. A brief description of methodology, along with advantages and drawbacks of each method will be discussed.

Direct Sequencing

Of the most commonly described molecular techniques for the detection of SNPs and mutations, direct sequencing is

Table 1. Summary of Molecular Markers Associated with Antimalarial Resistance^a

Drug	Genes	References
Chloroquine	<i>pfcr1, pfmdr</i>	[85, 91, 95, 97, 100, 101, 107]
Pyrimethamine-Sulfadoxine	<i>dhfr, dhps</i>	[71, 84, 87, 92, 104]
Proguanil-Atovaquone	<i>cyt b</i>	[77-81, 88]

^aThis is a representative list rather than a comprehensive one.

the most definitive. Utilizing either radiolabeled- or dye-terminator (Sanger Method) sequencing, useful information such as predominant SNPs and microsatellites can be identified. Many labs have used this method for surveillance and detection of novel SNPs [74, 75]. With this method, it is possible to perform up to 384 sequencing reactions, for up to as many as 24 runs a day [76]. A variation of this method is the single nucleotide primer extension, where polymorphic sites are PCR-amplified using standard methods and an oligonucleotide probe one base short of the SNP is hybridized. A typical sequencing reaction is carried out and as dideoxynucleotides are added, dye termination occurs and sequence is read by the unique emission wavelength using typical sequencing instrumentation [77, 78]. A third variation of this method is pyrosequencing, where instead of terminal dideoxynucleotides, the pyrophosphate release during nucleotide incorporation is detected using chemiluminescence. Commercial services are available for pyrosequencing and it has been used in the detection of SNPs [74, 79, 80] and mixed infection populations [61]. Among the sequencing strategies mentioned, pyrosequencing is described as the most sensitive and most amenable to a high-throughput format [80].

One of the challenges for sequencing is the poor quality sequence found in the first 15 – 40 bases and the deteriorating quality of signal after 700 – 900 bases. Although base-calling software is available to reasonably identify poor quality signals in a chromatogram, it is more prudent to perform PCR-amplification of the succeeding DNA sequence, essentially “walking” down the gene and reassembling all the sequences into the open reading frame. Another issue with DNA sequencing is the cost. Although methods such as high-throughput sequencing are available and the cost of individual sequencing reactions is not high, the basic equipment required are quite expensive. Even for a modest system, a genetic analyzer cost could reach upwards of \$60,000. This is primarily the reason why many DNA sequencing functions are carried out at facilities with laboratory core capabilities such as an academic institute or a commercial genomics center. Thus, DNA sequencing cannot be practically performed in a limited resource setting, such as field sites and even in general hospitals. With the actual sequence itself, depending on the method, the chromatogram is subject to operator error, resulting in mistakes in analysis. With regards to *Plasmodia* themselves, this method cannot distinguish between phenotypes stemming from genetic changes and decreased gene expression. In spite of its problems, sequencing remains the most definitive of all the methods de-

scribed and is commonly used in surveillance and research studies [81-85].

Pulse-Field Gel Electrophoresis/Restriction Fragment Length Polymorphism

Pulse-field gel electrophoresis (PFGE) and restriction fragment length polymorphism (RFLP) both utilize restriction endonucleases, which are enzymes that cut double-stranded or single stranded DNA at specific nucleotide sequences. Once fragmented, the DNA is then separated using gel electrophoresis. Individual samples can then be compared for relatedness by the pattern observed, with the more different the pattern, the more divergent the genes or species being compared. Both are used mainly for estimating relatedness between genes (RFLP) or between species (PFGE).

As mentioned previously, the RFLP method involves the use of restriction endonucleases to cleave DNA at specific restriction sites. Specific genes, such as 16S or 18S ribosomal RNA genes, are PCR-amplified and subjected to restriction analysis. In comparison, pulse-field gel electrophoresis (PFGE) is RFLP on a larger scale. Commonly used in bacteria and some parasites, this method does not require PCR-amplification since it involves restriction digestion of the whole organism genome. The organism(s) is grown at high concentrations to achieve sufficient DNA for digestion. As many as 20 restriction enzymes can be used to cleave the genome. The other main difference between the two methods is the relative size of fragments being resolved by electrophoresis. When resolved using standard gel electrophoresis, larger fragments migrate at an extremely slower rate than smaller fragments and, thus, identical larger fragments are not readily distinguishable. With PFGE, the voltage is switched between three directions and pulse times are altered to allow for better DNA fragment separation. The obtained restriction pattern is compared to a standard, which is also subjected to the same digest.

This method is amenable to field or resource-limited conditions due to the ease of material acquisition and modest requirements to perform the analysis. Both methods require restriction enzymes, an electrophoresis apparatus, and a method to visualize the DNA (via ethidium bromide staining). Even though some RFLP methods require a thermocycler, smaller laboratories can perform their own DNA amplification and analysis, without relying on a core laboratory for sequencing. However, as with other techniques, there are limitations to these methods. One drawback is the possibility of missing rarely seen SNPs, as well as some crossover events where nucleotide ratios are similar. This is particu-

larly challenging in *P. falciparum* where the genomic AT content is approximately 80% [86]. Another potential problem is the turn-around time required for PFGE, requiring a minimum of 90 to a maximum of 155 hours to run due to the size of the fragments. Nevertheless, some studies have successfully utilized this method in genotyping [87] and identifying resistance SNPs [88-92].

A modification of this technique is the heteroduplex tracking assay (HTA), which also uses gel electrophoresis but not restriction analysis. For this assay, the gene of interest is PCR-amplified and is incubated with a radiolabeled probe. This probe contains a sequence complementary to the SNP of interest. The reaction mixture is subsequently electrophoresed and visualized [93]. Three types of complexes should form: a probe-probe homoduplex, PCR amplicon-probe heteroduplex, and an amplicon-amplicon homoduplex. The type of pattern observed upon electrophoresis is dependent on the complementation between the sample amplicon and probe. The higher the complementation, the faster the migration is of the complex. Thus, the assay can differentiate between wild type and SNPs. Furthermore, the HTA has been shown to be more sensitive to minority variants and is less prone to cross-contamination [93]. Kwiek *et al.*'s study also indicates that the method is qualitative and is capable of detecting subpopulation parasitemias as little as 1% [94]. However, the major disadvantage to this method is the requirement for radioactivity. Moreover, it also suffers from the same problems seen with RFLP where similar sequences could run in the same gel position, limiting its resolution. Nevertheless, this method has been tested on field isolates from Malawi, China, and Peru and is capable of detecting novel *dhps* and *pfert* polymorphisms [95, 96].

Real-Time Polymerase Chain Reaction (RT-PCR)

One of the most commonly used methods in research and diagnostics, real-time PCR has gained prominence because of its ease of use and rapid turn-around time. Briefly, this technology depends on a DNA sequence specific probe, which contains a fluorescent reporter and a quencher. The fluorescent signal is suppressed by the quencher. Upon binding to a complementary DNA sequence and after subsequent PCR, the fluorescent probe is released and a signal is generated. Since PCR is an amplifying process, more and more probes are released with an increase in fluorescent signal, and the threshold cycle (C_T) is determined using computational algorithms. Most RT-PCR methods are quantitative (qRT-PCR). Variations of this method include melting curve analysis-fluorescence resonance energy transfer (FRET-MCA) and high-resolution DNA melting (HRM). The major differences between these methods are the number of fluorophores used and the temperatures at which the PCR is carried out. Nevertheless, outputs are similar and these methods have been used in the detection of prevalent SNPs [97-101].

RT-PCR is the most convenient of all the molecular methods currently described because of its relative cost and time required to perform the assay. The process does require some sample pre-processing like the previously described methods, but when taken together, the whole procedure can be performed in as short as 4 hours. In the past few years, several investigators have utilized this method for surveil-

lance of field isolates [102-107], also resulting in the discovery of novel mutations [108]. Furthermore, RT-PCR has been shown to be capable of measuring transcript levels of select genes, for both mixed infection detection [109, 110], and drug resistance [111]. In addition, a recent study shows application of this method to saliva samples. Reports by Nwakanma *et al.* indicated that the RT-PCR method had a sensitivity of 73% and specificity of 97% when compared to microscopy as a reference standard [112]. Although they focused on quantitative detection of *P. falciparum*, conceivably this assay could be tailored to detect resistance SNPs.

In terms of cost and throughput, the assay is relatively inexpensive to run and is amenable to a high throughput setup. Various DNA and RNA extraction kits are available in the market and primer/probes sets can be acquired from established vendors such as Invitrogen or Qiagen. In terms of the instrumentation, the 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems) is capable of performing 96 reactions in a 96-well plate format. In fact, this instrument was recently FDA-approved for the detection of H1N1 influenza, with sensitivity and specificity superior to that of rapid antigen tests.

One of the limitations of this method is the assay's sensitivity and specificity may be severely affected if the amplified gene is highly polymorphic [113, 114]. This problem is especially acute in malaria differentiation assays, where the 18S gene is used as a marker. A brief study by Bialasiewicz *et al.* showed that sequence variation in the target gene affects amplification, particularly in a mixed species environment [113]. Although their studies focused on differentiation of *P. falciparum* and *P. vivax*, their findings have a profound effect on SNP occurrence because RT-PCR has a tendency to amplify genes that occur at a higher concentration. This may result in false positive results, depending on the prevalence of the target gene in the population. They concluded with the recommendation that existing probes and primers be evaluated and carefully designed to increase assay sensitivity [113]. This observation is echoed by Liu *et al.* with their studies on mixed populations. They found RT-PCR and pyrosequencing can underestimate the presence of a minor subpopulation, resulting in false negative results [61].

Other Molecular Methods

Since their introduction in the late 1980s [115], microarrays have been instrumental in the detection of gene expression changes [116, 117] and SNPs [118]. The most basic microarray experiment involves hybridization of the PCR-amplified sample gene onto a fixed probe, which is affixed on a "chip". Relative fluorescence increases depending on the degree of complementation between sample gene and probe. Utilizing a solid surface, such as a glass, plastic or a silicon biochip, thousand of probes encompassing an organism's genome can be "spotted".

Microarray technology is ideal for antimalarial resistance detection due to the higher number of genes that can be screened simultaneously [118]. Cramer *et al.* have reported on a genotyping array called ResMalChip that can monitor 34 SNPs in the five *P. falciparum* genes that confer resistance to antimalarial drugs [119]. More recently, another

array setup called the FlexChip package was evaluated because it is more customizable compared to the ResMalChip and is used with its own dedicated software (R software). The investigators have found high agreement between the two chip sets, and that the FlexChip array has a sensitivity of 95.88% and specificity of 97.68% when compared to sequencing as the reference standard. They also found high concordance between the chip's results and reference laboratories [120]. However, as with other methods, the authors emphasize that only validated SNPs are detectable using this assay. On the other hand, it is possible to detect novel resistance conferring SNPs using this technology if used in a genome-wide approach. Dharia *et al.* also used microarray technology to survey entire genomes for novel SNPs and gene copy number variations [118]. Using their methods, they were also able to track generation of *de novo* mutations when parasites are subjected to drug pressure. However, a drawback to this technology is the high technical skill required to analyze the microarray output and to produce the microarray chips. In addition, chip-spotting technology is also cost-prohibitive, limiting its field-worthiness. As a result, uses of this method may be limited to "centers of excellence" or core facilities that can house and afford large pieces of instrumentation.

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy has been used recently in the detection of pyrimethamine resistance in *P. falciparum*. Initially used for identifying proteins and other biomolecules, it has been shown to be capable of differentiating wild type and mutant *dhfr* alleles [121]. A detailed description of this method is described by Pusch *et al.* [122], but briefly it involves elongation of a sample PCR-amplified target gene with an oligonucleotide probe adjacent to the SNP. Dideoxynucleotides are added and incorporated onto the growing double-stranded DNA chain. The mixture is purified and added onto a soluble matrix, after which it is subjected to laser excitation and subsequent mass measurements using mass spectroscopy. The major advantage of this method is the possibility of increased throughput. Conceivably, up to 96 samples could be tested in one run. However, this method is not as field amenable as the other methods described due to the reagents and instrumentation required. As with microarray technology, this method is better suited to be conducted at a large core facility.

OPTICAL METHODS

Hematology Analyzers

Many modern hematology analyzers produced today are equipped with advanced detectors capable of performing full blood counts with five part differentials that delineate the neutrophil, basophil, eosinophil, monocyte, and lymphocyte white blood cells. This technology also provides an added benefit of being able to detect the malaria pigment, hemozoin, in neutrophils and monocytes that have engulfed red blood cells infected with malaria.

The CellDyn 3000, 3500 and 4000, for example, are analyzers equipped with the multiple-angle polarization scatter separation detectors capable of detecting polymorphonuclear and mononuclear white cells that have engulfed red cells containing hemozoin. Hemozoin has the unusual property of

depolarizing light at 90 degrees to the original light path while hemoglobin or free heme does not. In the CellDyn analyzers, 90 degree depolarized light (normally used to quantitate eosinophils) can be used to detect depolarized light arising from the neutrophil and monocyte white blood cells that contain hemozoin from phagocytosed malaria-infected red blood cells. In one study, 831 blood samples were tested for white blood cells bearing hemozoin. Of these 831 samples, 417 tested negative for malaria, 64 were shown to be from patients convalescing from malaria, and 350 were shown to be positive for malaria. To correlate the hematology results, confirmatory results were obtained from thin/thick film microscopy, rapid malaria diagnostic testing, polymerase chain reaction analysis and clinical history. The specificity and positive predictive values for malaria (active and convalescent) were high (97.4 and 96.8%, respectively), while sensitivity and negative predictive values were 80.0 and 83.0% respectively [123].

The advanced Coulter GENS and LH750 analyzers have also been used to detect malaria through a different approach. In samples from patients suffering from malaria, there were changes in both the white blood cell scattergram and white blood cell volume distribution curves provided by these two instruments. To provide an objective means of interpreting these changes, statistical analysis of the volume, conductivity, and scatter data from each cell was performed. The standard deviation of lymphocyte and monocyte cell volumes in these patient samples was found to correlate well with *Plasmodium* infection. A discriminator combining the two parameters showed a sensitivity of 96.9% and a specificity of 82.5% for detection of the presence of *Plasmodia* after testing 89 patients with unexplained fever [124]. In a similar study, 275 patient samples with symptoms of unexplained fever were tested using the Coulter GENS. The results of this study showed a sensitivity of 98% and specificity of 94% for the detection of malaria [125].

Image Analysis

Image analysis of Giemsa stained smears provides a means of combining the traditional staining techniques used for malaria testing with high-tech automation. One approach utilizes the software program MalariaCount, which automatically generates parasitemia percentages from images of Giemsa stained blood smears. In this study analyzing this software, images were acquired manually using Giemsa stained smears of cultured *P. falciparum*. The MalariaCount software was tested by comparing manual counts done in duplicate to the parasitemia percentages obtained by automatically evaluating 200 images of a thin blood smear (approximately 15,000 RBCs per smear). There was good correlation of the two methods ($n = 20$ slides; $R^2 = 0.982$; $P < 0.0001$). In addition, a further test of imaging chloroquine and febrifugine-treated parasites was conducted to examine how well the software would perform when imaging parasites whose visual characteristics may be altered by drug treatment. MalariaCount also showed an excellent correlation for drug assays with chloroquine ($n = 28$ slides; $R^2 = 0.958$; $P < 0.0001$) and febrifugine ($n = 28$ slides; $R^2 = 0.928$; $P < 0.05$) when compared with manual counts. This is an interesting proof of concept for this method and may provide a means to automate the laborious task of reading ma-

laria slides. This also provides the objectivity of automated, rule-based analysis [126]. In another study of semi-automated microscopy, images of Giemsa stained thin blood smears were acquired using an automated microscope and processed using software to divide the red blood cells on the slide into two categories, either healthy or infected in a specific life stage, based on their internal structure but with no explicit parasite detection. Automatic identification of infected erythrocytes using this method showed a specificity of 99.7% and a sensitivity of 94%. The infection stage was determined with an average sensitivity of 78.8% and average specificity of 91.2% [127].

Optical Tweezers and THG Microscopy

Optical tweezers use continuous infrared lasers focused on a target such as a moving stream of cells. As the laser is of only moderate power with only small absorption by biological material, the biological molecules are not affected. The focused laser beam generates light pressure and gradient forces to move cells toward the laser focus and they are fixed in the focus as they would be if grabbed by micromechanical tweezers. Normal red blood cells suspended in hypertonic buffer rotate by themselves when trapped by a conventional optical tweezers. In contrast, under the same experimental conditions, the RBC infected with malarial parasite does not rotate. The difference in rotational speeds of the cells when trapped by optical tweezers could be exploited for the high throughput diagnosis of malaria [128].

Third Harmonic Generation imaging is an intrinsic, material dependent property of matter that produces blue shifted images. Hemozoin produces a very strong THG signal after excitation by an infrared femtosecond pulsed laser light source, and this emission can be readily used to image malaria-infected red blood cells and to differentiate between infected and uninfected cells. In this study, the blue-shifted THG signal was confirmed to be derived from malaria infected red blood cells by overlaying the THG image onto the image obtained from Giemsa stain [129]. Multiphoton images of the hemozoin THG signal can be superimposed on the red signal derived from red blood cells [130]. THG images could be the foundation of an image-analysis system or this technology could be used to create a THG flow cytometer that would provide automated counts of infected cells. THG microscopy systems are currently in their infancy, and no automated tools for malaria detection have yet been created. This is similar to the development of flow cytometry tools; bulky and difficult to use initially, and over time, more compact, usable and portable instrumentation was created.

CONCLUDING REMARKS

In this review, we presented several methods for the detection of drug resistance in *Plasmodial* species to include ELISAs, fluorescence methods, molecular assays, and cell imaging methods. We also expanded on the WHO's initial report on *in vitro* methods to include culture conditions on the SYBR Green I fluorescence assay, which has been indicated to vary across different laboratories. The vast array of possible techniques for measuring malaria drug resistance we have described shows how far the field has advanced since the introduction of the macrotest (1960) and ³H-

hypoxanthine methods (1980) for measuring malaria drug susceptibility [2]. Many of these methods are cheaper, more portable and practical in a field environment.

Although our review focused on *Plasmodia*, all these methods have applicability for measuring drug resistance in other agents of infectious disease. ELISA and fluorescent techniques are applicable to many organisms due to availability of antibodies and reagents. Molecular methods are often used in the detection of drug-resistant bacterial species, but are applicable to other systems due to the universality of DNA. Finally, microscopy techniques have the advantage of providing drug resistance determinations in real time and without culture. Although each organism has its own unique life cycle and growth requirements, many of these techniques can be tailored to the specific needs of the investigator.

One such disease is Leishmaniasis, which occurs in cutaneous and visceral forms, and affects 12 million people in 88 countries [130]. Rapid assays for the detection of cutaneous leishmaniasis have been lacking. In order to diagnose this disease, relatively invasive methods are utilized to obtain the sample and require the expertise of a skilled clinical pathologist. Recent reports have indicated *in vitro* assays are available for antileishmanial susceptibility testing, but are hampered by poor infectivity of parasite for the macrophage host cell. Da Luz *et al.* mention the requirement for assay standardization, which is one of the main assertions of this review [131]. Specifications, such as culture conditions that have been determined from years of study on malaria can serve as a template and starting point for study of other infectious systems. Other diseases that these techniques can be adapted to are trypanosomiasis, cryptosporidiosis, and schistosomiasis among others.

Many of the techniques described here can also be used in tandem to improve detection of altered drug susceptibility. For example, Liu *et al.* used the SYBR green I assay in tandem with real-time PCR and pyrosequencing to correlate drug resistance phenotype to mixed infections [61]. Although in their hands, molecular methods were not as sensitive as the SYBR green I assay, they still recommend careful molecular marker selection to mitigate this loss in sensitivity.

As demonstrated here, each assay has unique advantages and disadvantages. The researcher has to make a determination on their requirements (sensitivity vs. specificity, field portability, turn-around time, etc.) and resources (trained personnel, cost, time, utilities required, etc.) to select which assay can fulfill their clinical and research needs.

ACKNOWLEDGMENTS

The opinions or assertions contained herein are our private views and are not to be construed as official or as reflecting views of the United States Department of the Army or the United States Department of Defense. Work was supported by the Military Infectious Disease Research Program funding.

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