Sensitivity of malaria diagnosis in blood samples by PCR assay: A comparison with microscopy

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Abstract

This research was undertaken to compare the accuracy of Polymerase Chain Reaction, simple and nested assays, in detecting the DNA of the malaria parasite *Plasmodium falciparum*, to that of expert microscopy, the most commonly used method. Microscopy must detect the whole parasite, whereas PCR can detect only the presence of the parasite’s DNA, which is useful when the parasite is in the dormant stage. DNA was extracted from whole blood samples (collected on IsoStyx™) and amplified in a thermocycler. The amplified DNA was separated using 2% agarose gel electrophoresis. The products were then differentiated by fragment size, depending on the target product. Primers rPLU5, rPLU6, FAL 1, FAL 2, *Plasmodium*-specific, PL 1473F18, PL 1679R18, and *P. falciparum*-specific were successful in detecting *P. falciparum* DNA. Primers UNR, PLF, PLF, FAR, and *P. falciparum*-specific were also successful, but at lower parasitemia levels. Overall, PCR was found to be as accurate, and, in the case of PLF, far more accurate than microscopy.

Introduction

Microscopy has been the principal technique used in the diagnosis of malaria, by default at first, later by convenience and availability. In diagnosing malaria, microscopy has been referred to as the “gold” standard. More modern techniques include the Rapid Diagnosis Test, Immunofluorescent Assay, Enzyme-Linked Immunosorbent assay, and Polymerase Chain Reaction assay. Microscopy has been around for a long time, it is relatively low-priced and most clinical and hospital staff are trained in using it, but it has limits and drawbacks. Malaria is a serious disease caused by a parasite, and if left untreated, can be fatal. It is caused by the protozoal parasite *Plasmodium* (CDC, 2005). Only four of the known species of *Plasmodium* are able to infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The parasite is transmitted to humans by the female of the mosquito species *Anopheles*, injecting the *Plasmodium* sporozoites as it feeds on human blood. According to Roll Back Malaria, an arm of the World Health Organization estimates, as many as 300-500 million people are affected by malaria each year (2005). Over one million of those infected die from the disease. The disease is limited geographically by environmental factors. The conditions must be such that allow the parasite to reproduce in the vector. Therefore, malaria is restricted to the tropical and sub-tropical regions of the world, and it is also restricted by altitude; typically malaria won’t be found at altitudes above 1,500 meters. Temperature is the most critical factor in the parasite’s ability to survive. At temperatures below 20° C, *P. falciparum* is unable to complete its growth cycle. The symptoms of malaria include
high fevers, shaking, chills, and flu-like illness. Because the symptoms are so familiar, they are often misdiagnosed. This is true particularly of frequent overseas travelers, who upon infection, return home to the United States, where a doctor might not recognize the disease unless aware of the patient’s travel. The symptoms can progress quickly and result in death in as little as 24 hours. *P. falciparum* is the most commonly found of the four species, and accounts for up to 80% of the infections on a global scale. It is also responsible for most of the deaths: at least 1 million annually. *P. vivax* is the second most common of the *Plasmodium*. It affects approximately 75 million people each year, *P. malariae* and *P. ovale*, although geographically widespread, are not nearly as common as the first two.

A study by Nandwani et al. (2005) evaluated polymerase chain reaction analysis in the diagnosis of malaria caused by *P. falciparum*, a protozoan parasite. They compared PCR to microscopy, the gold standard, and found that PCR had a higher sensitivity, especially at low parasitemia (number of parasites per microliter) levels. According to their study, PCR is able to detect parasite levels as low as 1 parasite per microliter. Using microscopy as the only means of diagnosing malaria can lead to false negatives, because even a seasoned laboratory worker can miss the presence of the parasite if the level of parasitemia falls below 500 parasites per microliter. Their study offered some excellent material for supporting my hypothesis that PCR is a more accurate and more sensitive method of diagnosing malaria.

Tham et al. (1999) compared the ability to detect the malaria parasite in clinical environments by PCR, ParaSight-F, microscopy, and ICT Malaria Pf. They state that although microscopy is low-cost and simple, it does require much labor by personnel who have been well-trained, and they found that microscopy was able to detect infection as well as PCR, as long as parasitemia levels were high. They also showed that microscopy missed a mixed infection diagnosis, which was only treated for *P. falciparum*; whereas PCR initially diagnosed as both *P. falciparum*, and *P. vivax*, and the patient was later treated for an infection of *P. vivax*. Overall, PCR was very effective at detecting the disease at extremely low parasitemia levels, as few as 4 parasites per microliter. Their findings are important to my hypothesis because they support my assertion that microscopy is a more accurate, more specific technique of diagnosing malaria, specifically mixed infections of malaria.

Quintana et al. (1998) compared the efficiency of dipstick assay versus thick film microscopy. The dipstick assay was designed to detect *P. falciparum* specifically, and at the same time, all *Plasmodia* infections. Microscopy in this test was thick film; Giemsa (stain specifically designed to show DNA’s phosphate groups) stained. They showed that dipstick assay was as sensitive as thick film microscopy in the diagnosis of malaria, as long as the parasitemia was higher than 88 parasites per microliter. This study is relevant to my hypothesis because Quintana et al. (1999) used PCR as the means to analyze discordant results between dipstick assay and microscopy, due to the proven efficiency and sensitivity of PCR in detecting both the presence, and species of malaria infection.

In another study, Mangold et al. (2005) compared real time PCR (a process that differs from simple PCR by the use of a dye, such as ethidium bromide, a camera, and an ultraviolet light source, to examine the accumulation of product as it occurs (Genomic, 2005), with thick- film, Giemsa, microscopy. They concluded real time PCR
was superior to microscopy in the diagnosis of malaria, and more importantly, mixed infections of the disease. They also make mention of the time, preparation, and training that accompany microscopy; drawbacks, according to them, when compared to PCR. This is important for my proposal because it reinforces my position that PCR has higher sensitivity than microscopy.

Singh, *et al.* (1999) in a study that was developed specifically to test nested PCR assay’s ability to detect both genus and species of malaria, used specific primers for identification of the most common Plasmodium found in human infections: *P. falciparum, P. vivax, P. vivale,* and *P. malariae.* Their study showed that microscopy missed many of the simple infections, and all of the mixed infections. By comparison, nested PCR assay was able to detect parasite concentrations as low as 1 parasite per microliter, and mixed infections of two, and even three different species, but the authors conclude that it is only reliable at 3 or more parasites per microliter. They assert that microscopy’s failure to detect mixed infections might be due to disparate proportions of parasite species, in other words, it would be easy to miss 10 *P. vivax,* among 1000 *P. falciparum.* These findings are important to my hypothesis because I maintain that PCR has greater accuracy because it does not rely on visual observation of the parasites.

Current diagnostic methods for malaria include clinical diagnosis, microscopy, serology, molecular diagnosis, and antibody detection (Tham *et al.*, 1999). Clinical diagnosis of malaria is difficult at best, because the symptoms are, for the most part flu-like, and can include any and all of the common flu symptom such as fever, chills, headache, weakness, vomiting, and diarrhea. Clinical diagnosis is also complicated by the stage of the infection, the infecting species, skill of the microscope technician, and even the immune system status of the patient (CDC 2005).

Microscopy has been the standard for clinical diagnosis for malaria but also has limitations (Singh *et al.*, 1999). Microscopy consists of examination of a stained (usually with Giemsa) blood smear, at high magnification power, usually 100X. The criteria used to differentiate between the particular species are all visual, and are not always reliable because morphologic characteristics can overlap between species, making specific *Plasmodium* type detection difficult for poorly trained personnel. Well-trained laboratory personnel are not common in rural areas of developing countries, and to compound the problem, often the materials used are substandard as well.

Another diagnostic tool is Rapid Diagnostic Tests, or RDTs, also known as dipstick assays (Quintana *et al.*, 1998). RDTs test for the presence of antigens in the blood. RDTs are simple to use, but do require correct interpretation of the results by a trained professional. One of the disadvantages of RDT’s is the appearance of false positives, as well as false negatives. A false positive indicates the presence of the antigen, but does not prove the presence of the parasite. A false negative indicates the absence of the antigens, but does not rule out the presence of the parasite. Another disadvantage of the RDTs is the lack of differentiation ability. Some of the tests are species specific, that is, they may not detect the presence of *P. vivax* if they were designed for *P. falciparum,* or one of the other species. With serology, which uses immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA), it is possible to detect the antibodies produced by the immune system, however, this only shows past infection, not necessarily presence of the parasite in the bloodstream.
Molecular diagnosis consists of using polymerase chain reaction, or PCR, to detect the presence of certain parasite nucleic acids in blood samples. Because specific primers are used, each of the *Plasmodium* species can be detected by itself, or in mixed infections. PCR’s accuracy is higher than that of any of the other methods, and the cost of PCR is in most cases comparable to RDTs. In malaria diagnosis, microscopy is the most widely used method (Tham *et al* 1999). Microscopy is adequate when the number of parasites present in the blood is high, 100-1000 parasites per microliter. PCR can detect the presence of parasites as low as 2-3 parasites per microliter. Finally, the appearance of false positives and/or false negatives is also a disadvantage of microscopy. According to Nandwani et. al (2005) microscopy alone is not enough to accurately diagnose the presence of malaria because microscopy misses many mixed infections, and is only accurate at parasitemia levels over 500 parasites per microliter, Tham *et al* (1999) also state that microscopy is only accurate at high parasitemia levels and is labor intensive, and Singh et al (1999) found that microscopy missed all mixed infections and many simple infections. A more accurate method of diagnosing malaria in a clinical setting is needed. I believe that polymerase chain reaction will be more accurate in the detection of the malaria parasite at low parasitemia levels than microscopy.

**Methods**

After review of the applicable literature, I decided upon the selection of the following sets of primers for my research. Set 1, UNR (5‘-GACGGTATCTGATCGTCTTT-3'), PLF (5‘-AGTTGATCATCAATCGAGTTT-3'), and FAR (5‘-AGTTCCCCCTAGAATAGTTACA-3') a *Plasmodium* universal primer set for the first assay. (Rubio, 2005) Set 2, PL 1473F18 (5‘-TAA CgA ACg AgA TCT TAA-3') and PL 1679R18 (5‘-gTT CCT CTA AgA AgC TTT-3') a *P. falciparum* specific set (Mangold, 2005). Set 3, rPLU5 (5‘-CCT GTTGTTGCCTAAA-CTTC-3'), rPLU6 (5‘TTAAAATTTGTGCCAGTAAAACG3'), and FAL1 (5‘TTAAACTGGT TTGGGAA AACCAAATATT3') and FAL2 (5‘ACA CAATGAACACTCAATGACTACCCGT C 3’) also a *P. falciparum* specific set (Perandin, 2004).

Blood samples which tested positive by expert microscopy for *P. falciparum* were collected from patients in Honduras, these were used as positive controls. Negative blood samples were collected from non-endemic area for malaria without any risk of infection for malaria, samples were tested negative by expert microscopy test. DNase and RNase free de-ionized water was used for negative controls. For DNA extraction, blood samples were collected using IsoCode™ Stix by the U.S. Army’s Center for Health Prevention and Promotion. Blood samples were placed into 1.5ml microcentrifuge tubes by breaking off one of the four available samples on the card. Using the cap of the tube as a means to hold the sample as it breaks, minimizes contamination. Samples were washed with 500µl of de-ionized (dH2O) water. Samples were pulse vortexed 3x each, for a total of 5 seconds. Excess dH2O was extracted with micropipette and discarded. 100 µl of DNase-RNase free dH2O was added to samples. Care was taken to ensure that samples were completely immersed in the DNase-RNase dH2O. Samples were pulse vortexed 60x each. 45 µl of the eluent containing the DNA was placed in clearly labeled 1.5 ml microcentrifuge tubes. A second set of identical tubes were prepared, to be used as backup in case of contamination. DNA
extract stored at 20° C until needed. Master mix amounts were calculated for each of the primer sets. 10µl of DNA extract were added to each PCR tube. 25µl of DNA template (negative and positive where appropriate) were added to their respective tubes. Tubes were centrifuged for 20 seconds and then placed in the thermocycler

<table>
<thead>
<tr>
<th>Program for thermocycler:</th>
<th>Temperature (° C)</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature</td>
<td>94</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denature</td>
<td>94</td>
<td>20 seconds</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
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<td>10 seconds</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 seconds</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
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<td>7 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>Indefinite</td>
<td>1</td>
</tr>
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</table>

Preparation of the agarose gel beds was as follows: 2% agarose gel in 1X TBE buffer was prepared by heating in microwave. 5µl of Ethidium bromide for each 100 ml of 2% agarose gel was added, mixture was then swirled gently. The 2 % agarose gel mixture was poured onto a clean and dry gel tray containing a comb at one end. Mixture was allowed to polymerize for 20-30 minutes. The gel-containing tray was loaded onto an electrophoresis chamber. Sufficient volume of 1X TBE buffer was added to the chamber to just cover the gel. The comb was removed. Preparation of samples for developing in the electrophoresis chamber was next, 10µl of PCR product were mixed with 3µl of loading solution, and loaded into each well in the gel. 4µl of 100 bp DNA markers, mixed with 3µl of loading solution and loaded into the 1st well in the gel chamber. Electrophoresis chamber was covered, leads were connected. Electrophoresis chamber was activated for 30 minutes at 100 volts.

Developed gel beds were examined using a short-wave ultraviolet illuminator. Photos of the developed gel beds were taken using a camera adaptor. Data was analyzed first by examination of the appearance or lack of the appropriate size band in the developed gel beds. Accuracy was differentiated by the appearance of the appropriate size band in the wells filled with the diluted amplified product. The results were then compared to the expert microscopy results.

For the electrophoresis chambers, 2% agarose gel in 1X TBE buffer was prepared by heating in microwave. 5µl of Ethidium bromide for each 100 ml of 2% agarose gel was added and the mixture was then swirled gently. The 2 % agarose gel mixture was poured onto a clean and dry gel tray containing a comb at one end. Mixture was allowed to polymerize for 20-30 minutes. The gel-containing tray was loaded onto an electrophoresis chamber. Sufficient volume of 1X TBE buffer was added to the chamber to just cover the gel. The comb was removed.

For sample preparation, 10µl of PCR product were mixed with 3µl of loading solution, and loaded into each well in the gel. 4µl of 100 bp DNA marker, mixed with 3µl of loading solution and loaded into the 1st well in the gel chamber. Electrophoresis chamber was covered, and the leads were connected. The electrophoresis chamber was activated for 30 minutes at 100 volt.
Developed gel beds were removed from the electrophoresis chambers, and then were examined using a Kodak short-wave (254nm) ultraviolet illuminator to detect the presence of the target bands in the appropriate band sizes.

Results

I conducted Polymerase Chain Reaction (PCR) assays on negative, positive and unknown human blood samples to evaluate the performance of the following primer sets in the detection of malaria parasite *Plasmodium falciparum*. I used simple as well as nested reactions. I noted the primer sets used on the appropriate figures. I developed the resulting amplified product in agarose gel beds and used electrophoresis to separate the product. I used 100 base pair markers in all the beds. I noted the target sequences on the appropriate figures. Table 1 shows the results of all three PCR’s side by side for comparison. Primer sets UNR, PLF, PLF, FAR used on PCR 1, first amplification showed sensitivity to a dilution of 1:1000 and on the second amplification showed sensitivity to a dilution of 1:1000. Primer sets PL 1473F18, PL 1679R18 used on PCR 2, a single amplification, showed a sensitivity to 1:1000 in the first run, and 1:1000 on the second run. Primer sets rPLU5, rPLU6, FAL 1, FAL 2, used in PCR 3, showed sensitivity to a dilution of 1:1,000,000 in the first amplification, and 1:1000 in the second amplification.

Discussion

Overall, PCR was successful in detecting the presence of *Plasmodium falciparum* in human blood samples as expected. The sensitivity ranged from medium (detection of parasite in 1:1000 dilution) to very high (detection of parasite in 1:1,000,000 dilution). Primer sets UNR, PLF, FAR in nested assay worked adequately. Primer sets PL 1473F18, PL 1679R18 also worked adequately, but it was primer sets rPLU5, rPLU6, FAL1, FAL2 that worked the best, even though I expected all primer sets to work equally well based on the research. I found some non-specific bands (PCR3b, lanes 7-10, 15-17) that are probably due to a high concentration of primers, these could probably have been avoided by dilution of the amplified DNA. I did not find any false negatives with any of the primer sets. PCR was successful in that respect because there were no infections that were missed. I also did not have any false positives with any of the primer sets, this is important because a false positive would indicate an infection were there is none. The difference in the sensitivity of the primer sets was unexpected. The range is greater than I thought there would be. Since all experiments were carried out under similar conditions, by the same person, I conclude that primer sets UNR, PLF, FAR and sets PL 1473F18, PL 1679R18 are not as sensitive as primer sets rPLU5, rPLU6, FAL1, FAL2. Some of the discrepancies might be due to the relatively small number of samples involved in the study. A larger study might generate different results. I have no data on the specificity of the primer sets because *P. vivax* samples were not available for the study. Overall my study adds validity to the claim that PCR is more accurate and sensitive in the detection of the malaria parasite in human blood samples. In the future, lowered PCR costs will make it the new standard in malaria detection.
<table>
<thead>
<tr>
<th>Sample description</th>
<th>Primer Set</th>
<th>PCR #</th>
<th>Positive</th>
<th>Negative</th>
<th>False Positive</th>
<th>False Negative</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNR, PLF</td>
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<td>22</td>
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</tr>
<tr>
<td>Negative from Non-endemic area</td>
<td>PL 1473F18, PL 1679R18</td>
<td>2</td>
<td>0</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
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<td>3</td>
<td>0</td>
<td>22</td>
<td>0</td>
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<tr>
<td>Positive Control: Plasmodium falciparum</td>
<td>UNR, PLF</td>
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<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1 in 1000 parts</td>
</tr>
<tr>
<td>PLF, FAR</td>
<td>PLF, FAR</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1 in 1000 parts</td>
</tr>
<tr>
<td>Negative from Non-endemic area</td>
<td>PL 1473F18, PL 1679R18</td>
<td>2</td>
<td>9</td>
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<td>UNR, PLF</td>
<td>FAL 1, FAL 2</td>
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<td>7</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1 in 1,000,000 parts</td>
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<tr>
<td>Positive Control: Plasmodium falciparum</td>
<td>UNR, PLF</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1 in 100 parts</td>
</tr>
</tbody>
</table>
Figure 1. PCR 1. First Amplification. Target sequence 783-821 bp.

Gel Electrophoresis as viewed under ultraviolet light source. Negative except for nonspecific results on lane 4, 14.

Fig. 2 PCR 1  Second Amplification. Target: 395 bp.

Gel Electrophoresis as viewed under ultraviolet light source. Positive results at 395 bp on lanes 26-29 and 34-36 with nonspecific results on lane 46.

Fig 3. PCR 2a Target Sequence 206 bp
Gel Electrophoresis from PCR as seen under ultraviolet light. The arrow clearly indicates a positive result at 206 bp.

Fig 4. PCR 2b Target sequence 206 bp

Gel Electrophoresis from PCR as seen under ultraviolet light. Positive results at 206 bp in 8-12 and 16-17.
Fig. 5 PCR 2c. Target Sequence 206 bp.

Gel Electrophoresis as seen in ultraviolet light. Positive results at 206 bp in 42-43.

Fig. 6 PCR 3a Target sequence 1.2 kbp

Gel electrophoresis as seen under ultraviolet light. Negative.
Fig. 7 PCR 3b Target Sequence 205 bp.

Gel Electrophoresis from PCR as seen under ultraviolet light. Positive results in 6-12, 15-17. Non-specific product on 26.

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