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Passive gravitational sedimentation of peripheral blood increases the sensitivity of microscopic detection of malaria

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ABSTRACT

Objective: To determine if passive gravitational sedimentation of blood samples, followed by buffy coat thin smear preparation could increase the sensitivity of malaria diagnosis when compared to conventional thin smear preparation without the additional cost of centrifuges or molecular diagnostics. **Methods:** Blood samples were collected from 205 patients. Each patient sample was analyzed using all three methods of sample preparation. **Results:** Buffy coat analysis of centrifuged blood samples greatly increased the sensitivity of malaria diagnosis when compared to standard thin smear techniques. Sensitivity between mechanically centrifuged samples and gravitationally sedimented samples showed equal improvement in sensitivity when compared to standard thin smear preparation. **Conclusions:** Passive gravitational sedimentation of red blood cells followed by buffy coat analysis dramatically improves the sensitivity of malaria diagnosis without the additional costs associated with centrifugation.

1. Introduction

Infection with malaria is one of the greatest single causes of mortality and morbidity in the world. 300-500 million people are thought to be currently infected with malaria, with annual deaths estimated at 1-3 million[1]. Public health programs in several developed countries have efficiently eliminated reservoirs of the disease. This achievement has come about through a variety of measures ranging from vector control to identification and treatment of plasmodium-infected individuals. However, in many malaria endemic areas, the intensive screening and treatment of individuals needed to reduce reservoirs of infection is complicated by lack of funds for accurate diagnoses and treatment of infected individuals.

Effective, economical, and prudent treatment of malaria is facilitated by sensitive, accurate, and economically feasible diagnosis[2]. Currently, many diagnostic tests are available, ranging from molecular-based assays to simple microscopic examination of blood films[1]. Although molecular and antibody-based rapid diagnostic techniques are commonly employed in malaria diagnosis, false positive and false negative results are common[3]. Microscopic examination of blood films and identification of parasitized red blood cells is highly specific, but this method is also prone to false negative results, especially in cases of low parasitemia.

The lack of sensitivity of many diagnostic techniques leads to a failure to diagnose and treat some patients. These patients may then serve as reservoirs of malaria infection. Although many molecular, biochemical and antibody based diagnostic techniques exist, examination of blood films remains the gold standard in malaria diagnosis[4, 5]. However, the challenges of accurate microscopic detection of plasmodium when low levels of parasitemia are present complicates the effective diagnosis of malaria and, consequently, the distribution of limited antimalarial medication in malaria endemic regions.

In this report we describe a simple, economically-feasible technique to concentrate *Plasmodium*-infected red blood cells. Using this method in two clinical laboratories in Mozambique, we demonstrate a significant increase in the

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sensitivity of microscopic examination of blood films to diagnose malaria. Concentration of *Plasmodium*-infected RBCs through centrifugation, to increase the sensitivity of malaria diagnosis has been reported in the past[6-14]. In this report we demonstrate that passive sedimentation of blood samples works equally well as centrifuging blood samples, without the additional cost of a centrifuge or the need for electricity. The simple, modified method described here adds minimal cost to the conventional procedure, yet dramatically increases assay sensitivity when compared to traditional thin smear preparation.

Accurate diagnosis of plasmodium infection is essential for the providential distribution of antimalarial medication. In addition, increased sensitivity of diagnosis facilitates the identification and treatment of subclinical carriers who, although not debilitated by the disease, continue to serve as important reservoirs of infection.

2. Materials and methods

2.1. Study location and participant selection

This study was conducted in two sites in Maputo, Mozambique, in August 2010. The first site was the central hospital (Hospital Central de Maputo), where 139 patient samples were collected and analyzed in triplicate. The second site was a smaller clinic (A Clínica de Saúde de Alto Maé) where 66 patient samples were collected and analyzed in triplicate. All blood samples were collected from patients who had come to the hospital or clinic for reasons requiring a blood draw and no additional blood collection was performed in connection with this study. All blood samples used in this study were coded upon collection to ensure patient anonymity.

2.2. Preparing and staining blood smears

Blood samples were drawn in 5 mL tubes with anticoagulant. Each tube was then mixed well and divided into three subgroups. In group 1, a conventional thin blood smear was made. In group 2, ~40 μL of blood from the original 5 mL tube was loaded into a plastic heparinized hematocrit tube. One end of the tube was then sealed with Critoseal® capillary tube sealant. Blood within this tube was then centrifuged at high speed in a micro centrifuge for 2 minutes. Following centrifugation, the plastic hematocrit tube was cut (~2 mm) below the red blood cell (RBC)/serum interface. A thin smear was then made using the RBCs collected from near the buffy coat. In group 3, blood was collected in the capillary tube as described above for group 2. However, rather than centrifuging the blood sample, the blood filled hematocrit tube was placed on end and allowed to undergo passive gravitational sedimentation for 4 hours. Following this sedimentation, the tube was cut and a blood smear made as described above. Following smear preparation, all samples were stained with Giemsa stain. The presence of malaria-infected red blood cells was determined and enumerated by resident hospital or clinic technicians.

2.3. Evaluating blood smears

All blood samples were coded by research personnel to ensure unbiased analysis and evaluation by the clinical staff. Each sample was first evaluated by clinic/hospital technicians by performing a one minute scan of the slide. If the technician did not observe any malarial parasites during this one minute scan, the sample was scored as negative. If a *Plasmodium* parasitized red blood cell was observed during this one minute scan, the technician immediately initiated an evaluation of 1000 RBCs and the number of parasitized red blood cells was recorded. Statistical differences in the number of parasites counted by each method were determined by a paired student's t-test. P values of <0.01 were considered statistically significant.

3. Results

Previous research has demonstrated that malaria infected RBCs differ in density compared to normal RBCs and are enriched near the buffy coat. Furthermore, the microscopic analysis of red blood cells from this fraction can increase the sensitivity of microscopic detection of *Plasmodium* infected RBCs[6-14].

In an effort to enhance the sensitivity of microscopic analysis of blood films for the detection of malarial parasites, we compared three methods of blood film preparation. In the first method, blood films were made from whole blood samples; in the second method, blood samples were centrifuged in plastic micro-hematocrit tubes as described in *Materials and methods*. In the third method, blood was allowed to gravitationally sediment in plastic micro-hematocrit tubes. In methods two and three the blood smear was prepared from cells isolated near the buffy coat.

Results demonstrate that during the initial one minute diagnostic scans of blood films, 25.37% of traditional blood smears were identified as positive for the presence of plasmodium infected RBCs. In samples where cells were centrifuged or gravitationally sedimented, 30.24% of patient samples were identified as positive for infected RBCs (Figure 1). These results indicate that there is a statistically significant increase in the sensitivity of malaria detection in blood samples where parasitized cells have been enriched through centrifugation or sedimentation. Importantly, the observed increase in sensitivity was identical, regardless of if the patient samples had been centrifuged or subjected to passive gravitational sedimentation.

Results confirmed that centrifugation of blood samples leads to a significant increase in assay sensitivity and that

passive gravitational sedimentation results in an equally efficient detection rate. We next sought to determine the percent parasitemia in samples prepared by each of the three different methods of microscopic malaria detection. Data indicate that centrifugation as well as sedimentation of the blood samples resulted in a statistically significant increase in the number of parasites counted per 1000 RBCs when compared to traditional blood smear preparation ($P<0.01$). A statistically significant difference in the number of parasites observed in the centrifuged samples compared to sedimented samples was also seen, representing a 1.4% difference in percent parasitemia (Figure 2).

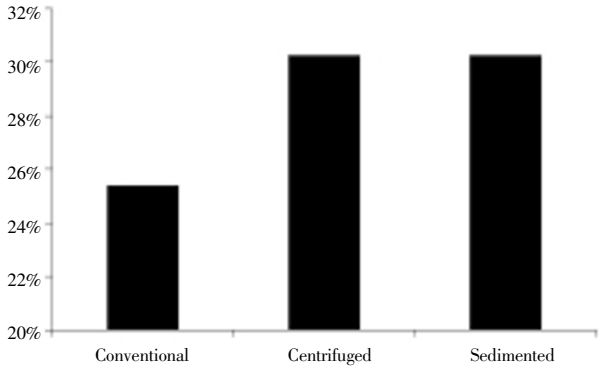

Figure 1. Mechanical centrifugation and passive gravitational sedimentation increases the sensitivity of malaria detection.
Microscopic detection of malaria was performed on patient samples prepared either by conventional thin smear, centrifuged buffy coat thin smear or passively sedimented buffy coat thin smear, as indicated in x-axis. The y-axis represents the percent of patients testing positive for malaria using each technique.

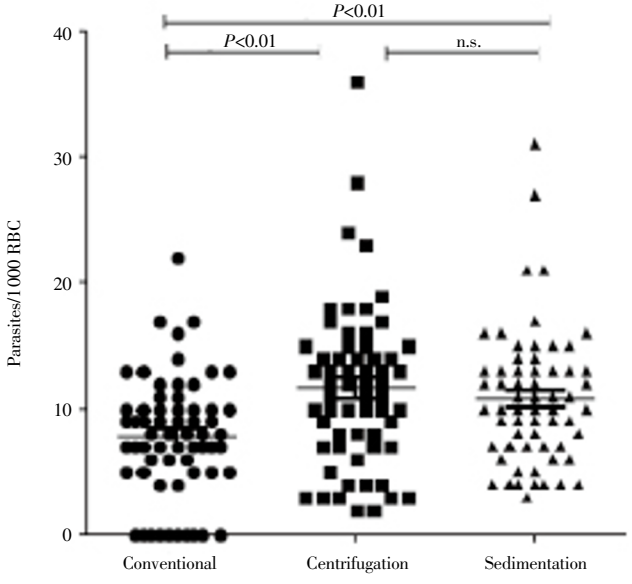

Figure 2. Mechanical centrifugation or passive gravitational sedimentation significantly increases the percent parasitemia detected in blood smears of malaria infected patients.
Microscopic enumeration of infected red blood cells was performed on patient samples prepared either by conventional thin smear, centrifuged buffy coat thin smear or passively sedimented buffy coat thin smear as indicated in the x-axis. The y-axis represents the percent parasitemia detected using each technique.

The microscopic detection of malaria infected red blood cells in patients with relatively high levels of parasitemia is straight forward and accurate. However, in patients who exhibit low levels of parasitemia, false negative results often occur[3]. The efficient identification and treatment of patients with low levels of parasitemia constitutes a significant challenge in decreasing disease transmission.

We next sought to better understand differences in the efficacy of these three methods of plasmodium detection in samples with low levels of parasitemia. In this analysis only samples with low levels of parasitemia were analyzed (<1.0% as determined by observation of the centrifuged sample.) In this analysis, blood samples which had been centrifuged were used as reference samples and samples from the same patient, which had been prepared using standard techniques or sedimented, were compared to this reference sample. All samples which exhibited low levels of parasitemia (as described above) are included in Table 1. Analysis indicates that samples with greater that 0.5% parasitemia were detected by all three methods of blood film preparation. However, patient samples with lower than 0.5% parasitemia (in centrifuged samples) were consistently scored as negative when read by technicians using conventional blood films. Conversely, there was 100% concordance between detection of parasitized samples which had been centrifuged or passively sedimented.

Table 1
Mechanical centrifugation and passive sedimentation greatly increase the sensitivity of microscopic detection of malaria in patients with low levels of parasitemia (Parasites/1000 RBCs).

Sample number	Standard	Centrifuged	Sedimented
2011	7	9	8
8601	6	9	8
4911	6	8	8
3601	6	8	7
15801	5	8	7
4311	5	7	7
5401	5	7	7
7901	5	7	7
11101	5	7	6
10801	4	6	6
10601	4	5	6
2211	–	4	4
14501	–	4	4
3411	–	3	4
5411	–	3	5
11001	–	3	4
14301	–	3	4
5201	–	3	4
7711	–	2	3
8801	–	2	4

4. Discussion

The accurate identification of malaria infected individuals is vital both to treating infected individuals and to effectively reducing the reservoirs of infection for malaria. Although many molecular and biochemical approaches have been successfully used to detect and diagnose malaria infected individuals, many of these techniques remain cost prohibitive in rural areas. In addition, due to the relatively high rate of false positive results from many of these techniques, microscopic confirmation of malaria infection is often performed and remains the gold standard of malaria diagnosis around the world[4, 5].

Previous research has shown that malaria infected cells are of a different density than uninfected cells and that through centrifugation infected cells can be concentrated, thus increasing the sensitivity of malaria diagnosis. Although increasing the sensitivity of microscopic examination through centrifugation has been shown to improve the accuracy of plasmodium detection, in many areas of the world the additional cost, maintenance, as well as the need for electricity, associated with centrifugation is sometimes prohibitive. In this study, experiments were designed to test the effectiveness of gravitational sedimentation of RBCs within a hematocrit tube followed by thin smear preparation compared to results from standard thin blood smears and patient blood samples concentrated through mechanical centrifugation.

Results demonstrate significant improvement in the detection of malaria using centrifuged and passively sedimented blood samples when compared to standard blood smear preparation. These results demonstrate that through the relatively easy and inexpensive technique of gravitational sedimentation, the sensitivity of microscopic malaria diagnosis can be significantly increased. This striking increase in sensitivity represents significant improvement in diagnostic accuracy without the need for electricity of additional equipment.

In addition to concentrating parasites and thus facilitating the accurate diagnosis of malaria, perhaps the most interesting finding from this study is the striking increase the ability to diagnose malaria in patients with very low levels of parasitemia without the use of a centrifuge. This study demonstrates that it is possible to increase the sensitivity of microscopic malaria detection up to 30% through passive gravitational sedimentation of blood samples. No diagnostic advantage to using mechanical centrifugation over passive sedimentation in diagnosing patients with very low levels of parasitemia was observed.

Conflict of interest statement

We declare that we have no conflict of interest.

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