Research Note

Quantification of parasite density in 200 microscopic fields underestimates the parasitemia level in malaria patients

Alves-Junior, E.R.1, Gomes, L.T.1,2,3, Assis-Oliveira, F.B.1, Silvério-Silva, L.R.1, Nery, A.F.1,3 and Fontes, C.J.1,3
1Federal University of Mato Grosso, Av Fernando Correa da Costa 2367, CEP: 78060-900, Cuiabá (MT), Brazil
2Univag University Centre, Av Dom Orlando Chaves 2655, CEP: 78118-900, Varzea Grande (MT), Brazil
3Facimed Cacoal Faculty of Medicine , Av Cuiaba 3087, CEP: 76963-665, Cacoal (RO), Brazil
*Corresponding author email: corfontes@gmail.com
Received 3 November 2013; received in revised form 11 November 2013; accepted 12 November 2013

Abstract. The determination of parasitemia in the diagnosis of malaria is a routine practice because it assists the selection of treatment. The techniques used for estimating parasitemia are based on leukocyte counts or on a fixed volume of blood examined in a microscopic field. This study evaluated the concordance between parasitemia estimated by counting parasites in 200 microscopic fields and by counting parasites per 500 leukocytes using the automated leukocyte count as a reference. This study included 403 patients with acute malaria. The parasitemia levels obtained by each method varied greatly. A large discrepancy was observed between the 2 methods with respect to parasitemia results, with 17.6% and 82.4% of the values being overestimates and underestimates, respectively, compared to quantification by the automated leukocyte counts. Thus, these findings reveal the inaccuracy of this method and should be considered by health professionals involved in clinical management of the disease.

The determination of parasite density is a routine practice in the diagnosis of malaria. It assists in determining the seriousness of the disease, assessing the in vivo response to therapy, and monitoring and forecasting the efficacy of old and new antimalarial drugs (Ross & Thomson, 1910). The precision of such estimates varies depending on the method used; it is higher for methods involving the quantification of circulating Plasmodium DNA in a specific blood volume by real-time PCR (Hwang et al., 2011) and lower for those involving thin blood film microscopy. In the latter technique, the blood volume is inferred by determining the leukocyte count or by examining a microscopic field (World Health Organization, 2010).

Although microscopy is not the most accurate method for estimating Plasmodium parasitemia, it is still the most widely used method because of its low cost and ease of use. Microscopy estimation is performed using different methods: (i) microscopic count of parasitized erythrocytes and its ratio to the total number of erythrocytes of the patient; (ii) parasite count per 500 leukocytes using automated leukocyte counts as a reference for the volume of blood examined; (iii) parasite count per 500 leukocytes assuming a fixed standardized value of 8,000 cells/µL for the leukocyte count of the patient; and (iv) parasite count in 100 or 200 microscopic fields assuming a fixed volume of blood in the examined field. The last 2 methods are widely used in the field,
particularly when a patient’s leukocyte count is unavailable (World Health Organization, 2010).

In the case of estimation by examining microscopic fields, it is assumed that 200 microscopic fields of a standardized thick blood film at 1 000× magnification contain approximately 0.45 µL blood. Thus, by multiplying the number of parasites counted in that field by 2.2, the number of parasites/µL blood can be obtained (Dowling & Shute, 1966; Trape, 1985).

Considering that blood smear thickness varies greatly in laboratory practice (Alexander et al., 2010), it is assumed the estimation of parasitemia by automated leukocyte count is more accurate than that by microscopic field examination. Therefore, the present study aimed to evaluate the concordance between parasite quantification using parasite counts in an examined microscopic field and that using parasite counts per 500 leukocytes using automated leukocyte counting as a reference.

This study was conducted at the Laboratory of Malaria of the Júlio Muller University Hospital, Federal University of Mato Grosso, which is the reference center for the diagnosis and treatment of malaria in the metropolitan region of Cuiabá. This is a cross-sectional descriptive study of the laboratory characteristics of malaria patients. Exploratory analysis of data regarding Plasmodium parasite density was performed. Patients whose diagnosis of malaria was confirmed by thick blood smear parasitological examination and for whom hematological assessment was conducted by complete blood count were included in the present study.

A total of 403 patients were selected from the archive of slides and records of consultations between January 2001 and December 2012. The analysis was confined to parasitological tests positive for malaria and leukocyte counts performed by automated complete blood counts performed for some of the patients observed in the abovementioned outpatient service.

Two thick blood films from each patient were prepared using approximately 10 µL blood, which was uniformly distributed over 1.5 cm². After air-drying, the samples were stained according to the Walker technique. Hematozoa were searched for by microscopic slide examination at 1 000× magnification.

Parasite density in the thick blood films was determined according to the 2 methods recommended by the WHO: the enumeration of all blood stages of the parasite relative to the quantity of leukocytes determined by microscopy and relative to a limited microscopic field. In order to ensure high-quality microscopic evaluation, both counts were performed by 2 highly qualified microscopists, one of them being the first author of this article. The mean values of the parasite counts performed by the 2 microscopists were used in the final analysis. In case of a discrepancy in the values of parasitemia quantified by the microscopists, the percentage of the discrepancy was calculated using the following formula (Prudhomme O’Meara et al., 2006):

\[
\text{Percent discrepancy between counts} = \left(\frac{X' - X''}{X' + X''} \right) \times 100
\]

In which

\[ X': \text{ parasitemia quantified by the first microscopist} \]
\[ X'': \text{ parasitemia quantified by the second microscopist} \]

In cases in which the discrepancy percentage between the 2 counts exceeded 40% (Prudhomme O’Meara et al., 2006), a third microscopist was recruited; the average of the values provided by the 3 microscopists was used for analysis. All microscopists used identical microscopes (Eclipse E200, Nikon Instruments Inc., Tokyo, Japan) with lenses and objectives of the same type. The readings were blindly recorded on independent spreadsheets, i.e., neither microscopist had access to the results of the other until the counting was completed.

Parasitemia was estimated from the leukocyte counts on the basis of the ratio between the average number of parasites counted by microscopy per 500 leukocytes and the total number of leukocytes obtained by automated counting using the following formula: \((\text{AL} \times \text{P}500)/\text{L}500\), where AL is the automated leukocyte count of the patient.
Table 1. *Plasmodium* spp. parasitemia estimated by the 2 quantification methods in 403 acute malaria patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Parasitemia (/µL blood)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Automated</td>
<td>Microscopic field</td>
</tr>
<tr>
<td></td>
<td></td>
<td>leukocyte count</td>
<td>examination</td>
</tr>
<tr>
<td>Minimum value</td>
<td></td>
<td>31</td>
<td>5.5</td>
</tr>
<tr>
<td>Maximum value</td>
<td></td>
<td>64 930</td>
<td>63 976</td>
</tr>
<tr>
<td>25th percentile</td>
<td></td>
<td>1 442</td>
<td>925</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>4 503</td>
<td>2 686</td>
</tr>
<tr>
<td>75th percentile</td>
<td></td>
<td>9 446</td>
<td>5 812</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>7 519</td>
<td>4 906</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td>9 527</td>
<td>7 587</td>
</tr>
<tr>
<td>Standard error</td>
<td></td>
<td>475</td>
<td>378</td>
</tr>
<tr>
<td>Mean LL (95% CI)</td>
<td></td>
<td>586</td>
<td>4 163</td>
</tr>
<tr>
<td>Mean UL (95% CI)</td>
<td></td>
<td>8 452</td>
<td>5 649</td>
</tr>
<tr>
<td>Geometric mean</td>
<td></td>
<td>3 404</td>
<td>2 037</td>
</tr>
<tr>
<td>Discrepancies</td>
<td></td>
<td>17.6%</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>82.3%</td>
<td></td>
</tr>
</tbody>
</table>

*Student's *t*-test; **Wilcoxon test; LL: lower limit; UL: upper limit; CI: confidence interval.
In the present study, *Plasmodium* parasitemia was estimated by 2 different routine methods: microscopic quantification of parasites in thick blood smears (i.e., automated leukocyte count) and that according to the volume of blood in an examined microscopic field (World Health Organization, 2010). As a consequence of the lower probability of error associated with measurement, estimation by automated leukocyte count was used as a reference, i.e., the actual parasitemia in the population of malaria patients (O’Meara et al., 2005; Jeremiah & Uko, 2007; Olliaro et al., 2011). It should be emphasized that parasites in the blood smears were quantified by 2 or 3 experienced microscopists, which minimized the possibility of systematic errors during counting.

Errors in the determination of *Plasmodium* parasitemia are a consequence of the density of parasites in blood; they generally occur due to the measurement technique, quality of the microscopic examination of the blood film, and statistical distribution of the count. Therefore, considering the methods of quantification used in the present study, the following sources of systematic errors are possible explanations for the observed discrepancies in the results: (i) loss of parasites and leukocytes during the staining process of the blood smears; (ii) degradation of parasites and leukocytes during slide storage; (iii) low accuracy of resources used in the microscopic quantification of parasites; and (iv) random distribution of parasites in blood smears (O’Meara et al., 2005).

Quantifying parasitemia in malaria on the basis of the quantification of parasites present in a limited microscopic field is a simple method; it represents a reasonable and more sensitive approximation of the actual number of circulating parasites, particularly in populations with highly variable leukocyte counts (Greenwood & Armstrong, 1991). However, in the present study, the quantification method using blood volume in an examined microscopic field underestimated parasitemia. Previous studies report similar findings and explain them as a result of technical problems associated with the test, such as a systematic loss of parasites during the preparation and staining of blood smears (Dowling & Shute, 1966; Bejon et al., 2006). Nevertheless, it is unlikely that such problems substantially contributed to the underestimation observed in the present study because of the high experience and qualification of the professionals who performed evaluations. Therefore, it is plausible that lower quantities of parasites were counted in the scanning of 200 fields as a consequence of low parasitemia, which has been demonstrated in other studies (Dowling & Shute, 1966; Petersen et al., 1996).

Another source of error in the quantification of parasitemia by microscopic field examination is the thickness of the thick blood smear, which is obviously associated with the quantity of parasites present in the sample. A volume between 5 and 10 µL blood results in a thick blood smear is adequate for the determination of parasitemia (Greenwood & Armstrong, 1991). Nevertheless, in laboratory practice, blood smears are expected to exhibit some variability in thickness, which implies different volumes of parasitized blood are examined in the same microscopic field (Alexander et al., 2010). Therefore, variation in the thickness of the smears examined in this study, especially if smears with less blood volume were predominant, may also explain the observed discrepancy.

In conclusion, the present study shows that the method for determining parasite density using an assumed blood volume in microscopic field examination as recommended by the WHO (World Health Organization, 2010) underestimates parasitemia in patients with acute malaria. This information should be considered by health professionals involved in clinical management of the disease who aim to assess the response to antimalarial treatment.

Acknowledgments. We thank CAPES and PRONEX-Malaria Network (FAPEMAT/CNPq) for the financial support.
REFERENCES


