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SHORT COMMUNICATION

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Systematic comparison of two methods to measure parasite density from malaria blood smears

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Abstract This study was designed to directly compare the accuracy, reproducibility, and efficiency of three methods commonly used to measure blood-stage malaria parasite density from Giemsa-stained blood films. Parasites and white blood cells (WBCs) were counted in 154 thick films by two independent microscopists. Forty-six slides were read by counting parasitized red blood cells (RBCs) in the thin film. Using these same slides, parasites were again counted by two independent microscopists using an ocular grid. Overall, parasite densities were significantly lower and discrepancy between readers was higher when using the grid method compared to the WBC method, but there was no difference when compared to the RBC method. When one reader who had difficulty with the grid method was excluded, the discrepancy between readers was equivalent for the three methods. Densities and discrepancy between readers were indistinguishable when parasites were counted until 200 or 500 WBCs. Counting beyond 200 WBCs may not significantly improve parasite density measurements. Using an ocular grid directly measures parasites per volume rather than using a WBC per microliter conversion factor and eliminates the need to switch from the thick film to the thin film for high parasitemias. However, significant differences in densities measured by the grid method and the WBC method need to be evaluated.

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Introduction

Parasite density is quantified by concurrently counting parasites and white blood cells (WBCs) on the thick film of a Giemsa-stained slide. The density is reported as parasites per WBC or, more often, a conversion factor of 8,000 WBC/μl is used to convert parasites per WBC to parasites per microliter. WBC counts, particularly in malaria infected individuals, can vary by as much as tenfold between individuals (McKenzie et al. 2005). Therefore, accurate density determination requires that a WBC count be performed each time a blood smear is prepared. For very high parasitemias, counting parasites per WBC in the thick film is tedious and impractical and parasitized red blood cells (RBCs) in the thin film are counted as an alternative. A conversion factor of 4.0–4.5 million RBCs/μl is used to convert to a value of parasites per microliter. Unlike the thin film, the thick film is not fixed and it was observed that a large fraction of parasites and gametocytes can be lost from the thick film during staining (Dowling and Schute 1966). In addition, the conversion factors for RBC and WBC per microliter are arbitrary and unrelated, causing an artificial discontinuity between measurements made from the thin film and those from the thick film. One possible remedy for these problems is to use an ocular grid, which maps out a defined area of the film and allows parasites per area to be counted (Earle and Perez 1932). If the thick blood film is prepared with a known volume of blood in a known area, then the grid allows parasites per volume to be measured

directly without converting to microliters via a WBC count or arbitrary conversion factor. In addition, the grid provides delineation, which assists in counting, and smaller portions of the grid can be counted for very high densities allowing parasitemias of all densities from very low to very high to be evaluated on the thick film without switching to the thin film and without overburdening the reader. To directly compare these two techniques, we undertook a study in which slides were read by both methods.

Materials and methods

Counting by WBCs Parasites and WBCs were counted simultaneously on the thick film using a 100× oil-immersion objective and the number of parasites was recorded when 200 WBCs were counted. Counting continued until 500 WBCs were counted (an addition of 300 WBCs) and the number of parasites was again recorded. One hundred high power fields were examined before a slide was declared negative. Counting began when the first parasite was observed and the edges of the film were avoided by starting in a field with at least 8 WBCs and moving horizontally across the film.

Counting with the grid A 10×10-mm square grid divided into 100 smaller squares etched onto a glass circle was fitted into the eyepiece of a microscope (Klarmann Rulings, Litchfield, NH, USA). One hundred high power fields were viewed and parasites within the area of the grid were counted. Based on the volume of blood used to prepare the thick smear and the area of the smear, the total volume of blood counted per 100 grids was about 0.057 μl. Parasites in the field of view but not within the grid were not counted. Counting began with the first field rather than the first parasite. Based on the mean WBC count, 100 grids are equivalent to approximately 450 WBCs.

Counting by RBCs Forty-six slides were identified as having very high parasite densities based on prior readings. For these slides, parasitized RBCs per 2,000 total RBCs were counted in the thin smear using a 100× oil-immersion lens. Grid counts were taken from the thick smear but microscopists were asked to count only two of the ten rows of the 10×10 grid. The data for these slides were analyzed separately.

Slides Slides were prepared from EDTA-preserved venipuncture blood. All slides were prepared and stained within 72 h of sample collection. Two microliters of blood were smeared to produce a thin film and 10 μl of blood were spread in a circle with a 15-mm diameter to produce a uniform thick film. Slides were air dried for 10–20 min. The thin film was fixed by dipping only the end of the slide with the thin film briefly into absolute methanol and allowing it to air dry. The slides were stained in 3% Giemsa for 1 h and then rinsed. Two hundred slides were chosen and randomly grouped into sets of 20. A CBC was available for each slide. Every slide was read by two independent microscopists using the WBC method (A and B) and by two independent microscopists using the grid method (C and D) giving a total of four readings from each slide. Forty-six slides were identified as having high parasite density.

Microscopists Eight microscopists who were trained during a week-long microscopy training workshop immediately before the study were invited to participate. The training session included 1 day of lecture and lab practice with the grid method of counting. Aside from this limited exposure, only two of the microscopists had any prior experience with the grid method. Slides were randomly assigned to each of the microscopists who were asked to read them using either the WBC method or the grid method. Not all of the microscopists participated equally—some performed as many as 200 readings, some as few as 60 readings. Reader A and reader B for the WBC method were never the same individual and likewise for reader C and reader D, although reader A may be the same as reader C for some slides.

Statistical analysis Data analysis was performed with Microsoft Excel and statistical tests were done using the Analyze-It package for Excel.

Results

WBC counting vs grid counting

Counts per 200 or 500 WBCs were multiplied by the WBCs per microliter as measured by CBC at the time of slide preparation. Actual WBC counts were used rather

Table 1 Median density and discrepancy between microscopists (difference between two reads divided by the mean of two reads) when using the WBC technique, the RBC technique, and the grid counting technique

	Median density (parasites/μl)	Median discrepancy	Correlation between density and discrepancy
Low to moderate density			
Grid	1,003*	0.719**	Positive (p=0.006)
200 WBC	2,080	0.455	Negative (p=0.019)
500 WBC	2,050	0.517	Negative (p=0.0002)
High density			
Grid (2 rows)	56,000	0.548	Not significant
RBC	49,000	0.623	Not significant

*Significantly different than 200 or 500 WBC technique, p=0.005
**Significantly different than 200 or 500 WBC technique, p<0.0001

than using the standard approximation of 8,000 WBC/μl, thereby revealing the true relationship between density and reader discrepancy. Although the mean WBC count was 7,900 WBC/μl, there was a strong positive correlation between density and WBC count (Spearman $p<0.0001$), indicating that high density parasitemias would be overestimated and low density parasitemias underestimated when using the 8,000 WBC/μl approximation. Scaled discrepancy between readings was calculated as the difference between the density reported by reader A and reader B (or reader C and reader D) divided by the mean density of reader A plus reader B (or reader C plus reader D). Scaling the discrepancy by the mean allows discrepancies across all densities to be compared.

Densities measured using the grid method were significantly lower than those measured by the WBC method (Table 1; Mann–Whitney $p=0.005$) but discrepancies measured by the grid method were significantly higher (Mann–Whitney $p<0.0001$). Both density and discrepancy measured by 200 or 500 WBCs were statistically indistinguishable (Mann–Whitney $p=0.88$ and 0.719, respectively).

Discrepancy between readings is expected to be greatest at very low parasite densities and to decrease as density increases. As expected, there was a negative correlation between density and discrepancy for the 200 and 500 WBC readings (Spearman $p=0.019$ and 0.0002, respectively), which agrees with previous studies (Prudhomme O'Meara et al. 2005). The positive correlation (Spearman $p=0.0059$) between density and discrepancy observed with the grid method was unexpected and is contrary to previous studies (Kilian et al. 2000).

RBC counting vs grid counting

RBCs per microliter were measured by CBC at the time of slide preparation. Actual RBC counts were used rather than using the standard approximation of 4×10^6 RBC/μl for the reason stated above. There was no significant difference in the densities or discrepancies measured by counting against RBCs vs counting with the grid method (Table 1; Mann–Whitney $p=0.35$ and 0.27, respectively). Only 46 slides were read for this analysis, thus, further readings might be required to resolve whether differences exist. There was no significant correlation between density and discrepancy for either the RBC or the grid method for this subset of slides.

Reader performance

The unexpected positive correlation between density and discrepancy with the grid method prompted a more detailed analysis of individual reader differences. We used the median discrepancy per reader as a measure of how much each reader differed from his/her peers. Most readers showed similar discrepancy when using either the grid method or the WBC technique. Median discrepancies ranged from 32.5% to 100% (Table 2). Microscopist 2 showed similar agreement with other readers when using the WBC method, but microscopist 2's discrepancy with others doubled when using the grid technique. Even though microscopist 5 also showed high levels of discrepancy with the grid, the level was equivalent to microscopist 5's discrepancy with the standard WBC method, indicating poor agreement with other microscopists, but not specific to the counting technique. When microscopist 2's reads are eliminated from the analysis of both techniques, the discrepancy associated with the grid technique is comparable to and slightly lower than the WBC technique, but differences cannot be resolved statistically with so little data (Table 3). Eliminating microscopist 2's data reduced the sample size by 50% for the grid data and 30% for the WBC data. The expected inverse relationship between density and discrepancy becomes evident, but the correlation is not significant.

Table 2 Median discrepancy (disagreement) between each microscopist and peers using the grid technique or the WBC technique

Microscopist	Median discrepancy: 200 WBC method	Median discrepancy: grid method
1	0.434	0.531
2	0.488	1.065
3	0.475	0.478
4	0.495	0.495
5	1.025	0.929
6	0.694	0.798
7	NA	0.668
8	0.510	0.325

Table 3 Median density and discrepancy between microscopists when using the WBC technique or the grid counting technique when microscopist 2's results are excluded

	Median discrepancy	Correlation between density and discrepancy
Grid	0.452	Negative, but not significant ($p=0.3$)
200 WBC	0.471	Negative
500 WBC	0.473	Negative

Reader feedback

In a written survey at the conclusion of the study, two of the eight readers commented that the grid method was faster, less tedious, and more accurate than the WBC method. No readers made negative comments about the grid method.

Positive/negative disagreement

For the grid method and the WBC method, there were an equivalent number of disagreements (5) between readers about the presence or absence of parasites (false positive or false negative).

Conclusions

Accurate parasite density determination is critical to epidemiological studies, clinical trials, and patient care. In highly endemic areas, a clinical case of malaria is often defined according to a threshold parasitemia (i.e., Schellenberg et al. 1994; Beadle et al. 1995; Mwangi et al. 2005). If quantification is not accurate, cases may be miscategorized and incorrectly treated, leading to the misinterpretation of a field trial and poor patient outcomes.

To our knowledge, this is the first study that provides a side-by-side comparison of the WBC and the grid counting technique using slides generated in field trial conditions according to a detailed protocol. A previous study (Greenwood and Armstrong 1991) examined a smaller number of slides and compared the WBC counting technique and a high power field counting technique to the RBC counting technique when a single microscopist used all three techniques on each slide, rather than evaluating the discrepancy between microscopists for various techniques.

The grid method may eliminate the need for switching to the thin film for high parasite densities and performing CBC counts but requires that smears be prepared with a known volume of blood spread in a known area. In our study, the microscopists adapted quickly to the new technique and gave positive feedback. Our initial results showed a higher median discrepancy for the grid method compared to the WBC method. However, when the results of one microscopist were excluded, the discrepancies were indistinguishable. Seven out of eight readers with little or no prior experience with the grid showed no measurable difficulty in adopting this method and performed just as well with the grid as with their usual method of WBC counting. However, readers should be evaluated, as is demonstrated by the aberrant results of microscopist 2. This example highlights the possibility that with training and familiarity, the between-reader discrepancy using the grid may show further improvement.

Our results showed that continuing the count up to 500 WBCs does not significantly decrease discrepancy between readers compared to stopping the count at 200 WBC. The time involved in counting up to 500 WBC may not add to the precision of the measurement and may be unnecessarily taxing for the microscopists.

The difference in median density measurements obtained with the grid and with the WBC method is significant and should be investigated further. When using the WBC technique, the slide is scanned until a parasite is found and then parasites and WBCs are counted in each field until 200 WBCs (or 500) were counted. If a parasite is observed in the 99th field, counting of parasites and WBCs begins at the 99th field and continues until 200 WBCs. If no other parasites are seen, the density is reported as 1 parasite per 200 WBCs, but the actual density is 1 parasite in 99 fields plus 200 WBCs. When using the grid technique, 100 fields are counted and counting may begin with the first field rather than the first parasite. A parasite may be observed in the 99th field, then one more field is scanned and the density is reported as 1 parasite in 100 fields. The latter is closer to the true density but may be significantly lower than the former. If this explanation is correct, then the difference in density measurements between the two techniques should be highest at very low densities. This difference did, in fact, decline significantly with increasing density (Spearman p=0.002). Two of the eight microscopists considered the first field as "1" regardless of the presence of parasites whereas the other six microscopists began tallying fields only when the first parasite was observed. Another factor that could contribute to the difference is loss of parasites from the thick film during staining (Dowling and Schute 1966), which would lead to an underestimation of parasite density with the grid method. If WBCs are also lost during staining, then the ratio of WBCs to parasites would be maintained closer to their original value, leading to a discrepancy between the grid and the WBC counting methods.

Further experimental evidence is needed to determine which parasite counting technique is most accurate, reproducible, and efficient. Our results indicate that the grid method may be a less labor-intensive alternative to the WBC and RBC techniques, although the apparent systematic lower estimate of parasitemias should first be understood. Exact details of the counting protocol should always be reported in study reports when parasite densities are an important endpoint.

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