Microscopic determination of malaria parasite load: role of image analysis

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Quantitation of malaria burden provides prognostic and disease monitoring information, vital for proper clinical management of patients. It may also be required to assess response to malaria vaccines or drugs in clinical trials. Accurate microscopic malaria diagnosis requires training and experience, and is therefore an obvious target for automation using digital image processing and analysis systems. Most of the literature in this field has focused on detecting, identifying, and quantifying malaria parasites on thin blood films. To date it is clear that routine automatic microscopy for malaria infection detection and species identification is still a distant prospect, but quantitation of malaria burden is a more realistic target. A method to reliably count malaria parasites on conventional thick blood films, using readily available equipment and software, is described. Good correlation between manual and digital counts was achieved in a proof-of-principle study.

Keywords malaria; microscopic diagnosis; quantitation; digital image analysis

1. Introduction

Malaria is caused by intracellular parasites belonging to the genus \textit{Plasmodium}; 5 species are recognized as pathogens of humans, namely, \textit{P. falciparum}, \textit{P. vivax}, \textit{P. ovale}, \textit{P. malariae}, and \textit{P. knowlesi}. Depending on the intensity of transmission and the parasite species involved, the clinical and public health impact of malaria is geographically variable. Most serious illness and mortality from malaria in the world is caused by \textit{P. falciparum}. It has been estimated that half of the world’s population (3.5 billion people) will live in malaria transmission areas in 2010.\cite{1} The profound effect of malaria on much of sub-Saharan Africa, in particular, is well known, and most of the estimated 1 million deaths caused annually by malaria occur in Africa.\cite{2}

2. Rationale for malaria parasite load estimation

\textit{Plasmodium falciparum}, unlike the other human malaria species, has the capacity for nearly unlimited replication in the human host, and very high parasitaemias (>50% of erythrocytes infected) are possible in falciparum infections. Assessment of the parasite burden provides a useful indicator of severity of infection, particularly in non-immune patients, and the level of parasitaemia (parasite burden or load) correlates generally with clinical features and prognosis. Thus, 4 or 5% or more parasitaemia, or more than 100 000 parasites/µl, are commonly regarded as indicators of risk of severe malaria in a low-transmission setting.\cite{3} Parasite load estimation is also an objective measure of response to treatment; an aid to clinical decision-making about the likely cause of febrile illness in highly endemic areas;\cite{4} and as an end-point in clinical trials of antimalarial drugs or vaccines, at a pre-determined parasite density threshold.\cite{5} However, there are some caveats to parasite burden estimation: the peripheral blood parasitaemia may well not accurately reflect the total body burden of parasites, because of sequestration of parasitized erythrocytes in the capillaries and venules of the deep circulation. As an alternative, the proportion of malaria pigment-containing neutrophils has been used as an indirect measure of recent parasite replication.

3. Methods for estimating parasite load

There are two acceptable methods of expressing the parasite load: either as the percentage of infected erythrocytes as counted on a stained thin blood film (e.g. 1% parasitaemia), or the number of parasites per unit volume of blood (e.g. 5000 parasites/µl). The latter is usually assessed on a stained thick film by counting parasites against leukocytes (100, 200 or more), then multiplying by either the patient’s own leukocyte count if available, or a standard count of 8000/µl.\cite{6} When the parasite count is either very high or very low, thick film and thin film counts, respectively, are inaccurate. Ideally, therefore, both methods should be in a good malaria microscopist’s repertoire. Semiquantitation (parasite density graded as + through ++++), although often used in laboratories in developing countries, is inherently imprecise and often incorrectly applied, and is less satisfactory than the other methods. It is no longer recommended for routine use.\cite{6}
Whilst trained technologists readily recognize and count parasitised erythrocytes (the numerator) in an adequately stained thin smear, estimating the denominator (total number of erythrocytes) of the required fraction is the source of most error. The use of a simple microscope eyepiece graticule (Miller squares: Graticules Ltd, Tonbridge, UK) markedly improves accuracy and reproducibility of parasitaemia estimates. This technique was originally used for reticulocyte counts, where it was shown to produce smaller standard errors than the conventional method. The technique cannot be applied to parasite load quantitation on most thick smears because of the typical locally uneven dispersion of parasites. It is also rather slow if a large number of fields are required to be counted, as when the parasitaemia is low. Another approach is to use an average number of erythrocytes per field to establish the denominator, but the accuracy of this method is limited by the ability of the microscopist to choose areas of comparable density both within and between thin blood films.

Another method is to count parasites in an assumed volume of blood per high power field on the thick film.[7] A variation of this method was described by Planche et al.[8] In this technique, a known volume of blood is spread over a standard area on the slide. Taking into account the diameter of the microscopic field, the average number of fields per microlitre of blood can be calculated by comparison with the known white cell count in a calibrator blood specimen. It has been pointed out that counts done on standard thick blood films consistently underestimate parasitaemia (as compared to quantitative PCR) because of loss of parasites during staining.[9] Nevertheless, parasite density estimation on thick blood films is recommended by the World Health Organization.[6]

There is a striking lack of evidence to support widely-held assumptions about the accuracy and consistency of malaria microscopy.[5,9] Studies have shown substantial intra- and inter-observer inconsistencies in density quantitation.[5,10] Programmatic proficiency testing of quantitative malaria laboratory diagnosis in African medical laboratories over the past 4 years has yielded highly variable and overall, disappointing results, with typically very wide dispersions around the true value (Fig. 1), that are generally skewed towards overestimation (B Poonsamy, J Frean: unpublished data).

Figure 1 Scatter plot of typical results of a proficiency survey of African malaria microscopists (n=152). Participants returned P. falciparum parasite counts for 11 challenges (numbered 1, 3, 4, etc on X-axis). The median of all counts per challenge was taken as the ‘true count’ and results plotted as % deviation from the median (Y-axis, where 0=target value). The linked triangles indicate the mean % deviation for all participants for each challenge.

4. Alternative methods for parasite load estimation

Microscopic examination of stained thick and thin blood films remains the ‘gold standard’ for routine malaria diagnosis,[6] notwithstanding development and introduction of techniques such as lateral-flow immunochromatography (rapid ‘dipstick’-type diagnostic tests) and nucleic acid-based detection techniques (PCR). Other advances in diagnostic science will no doubt emerge as potentially useful methods.[11] Accurate microscopy requires training and experience, however, and is prone to operator error; and therefore malaria diagnosis by microscopy is an obvious target for automation using digital image processing and analysis systems. Most of the literature has focused on detecting,
identifying, and quantifying malaria parasites on thin blood films. The intracellular location of malaria parasites, and the relatively low ratio of infected to uninfected erythrocytes that has to be catered for, as well as leukocytes, platelets, and stain, dust, microbial or other artefacts, leads to a number of interesting technical and statistical problems around image acquisition, processing, analysis, and pattern recognition.[12-15] Quantitation of malaria parasite burden by digital image analysis of thin stained blood films is necessarily constrained by these technical issues. Variability in appearance because of different ages of parasites, and in the application of the Giemsa staining technique, are but two of many problems. Another basic issue is acquisition of a sufficient number of focused images in a reasonable time, but the high cost of automated microscope stages and autofocusing for microscopes is a serious obstacle.

5. Practical application of image analysis to parasite load estimation

Fully automated malaria diagnosis and quantitation is clearly some time away from being a realistic replacement for the skill and experience of a trained human microscopist. The requirements of a proficiency testing scheme for malaria microscopy in African laboratories led to the development and proof-of-principle testing of a low-cost method of digital image analysis to count *P. falciparum* parasites on thick blood films.[16] Using open-access software and avoiding custom programming or any special operator intervention, accurate digital counts were obtained, particularly at high parasite densities that are difficult to count conventionally. The essential features of this method are summarised as follows:

5.1 Images (n = 20-30, mean 25) of each slide are captured by a standard commercial laboratory microscope and digital camera.

5.2 ImageJ (version 1.41)[17], an open-access Java-based image-processing programme, is used for image analysis. The software segments or classifies particles to be counted on the basis of their relative density (darkness) compared with the background, via a thresholding process. Particle size (area) and degree of roundness are other classification variables. Fine morphological and differential staining characteristics of parasites are ignored.

5.3 Standard ImageJ preprocessing and particle analysis commands provide a preliminary particle count, and the particle size frequency distributions are plotted by the software. At this point the results of segmentation and classification processes reveal that there can be considerable variation in appearance between slides made from different blood specimens, and this precludes application of a global algorithm for all slides (Fig. 2). Particle size frequency distributions can show wide variation in relative proportions of ‘signal’ (parasites) and ‘noise’ (artefacts) (Fig. 3).

![Figure 2](image_url)

*Figure 2* A. Giemsa-stained thick smear showing well-developed trophozoites, and resulting processed image (L. inset) that is relatively ‘noisy’. B. Giemsa-stained thick smear showing younger trophozoites and a cleaner processed image (R. inset). Note the difference in staining colour and intensity.
After testing several basic statistical parameters related to distribution shape (variance, standard deviation, etc), it was found that a correction factor based on the ratio of kurtosis to skewness of the distributions optimised the signal/noise ratios of images to provide reliable counts via a second (corrected) algorithm. Other statistical parameters with known relevance to size and shape of malaria parasites might also be used in objectively setting parameters in the algorithm for machine counting. The counts produced by the corrected algorithm correlated well with both manual counts done on the same images, and with conventional counts of the same specimens (Fig. 4). Slides with low counts produced lowest correlation coefficients, probably because of misclassification of artefacts as parasites; however, low counts are easier to do conventionally than very high ones, in which the image analysis process achieves high levels of accuracy. Overall, the accuracy of digital counts fell well within the WHO recommendations for malaria microscopy.[18]
A major constraint of digital image analysis as described here is the need to capture substantial numbers of digital images of the specimens, which is not difficult but is time-consuming. Computer-controlled motorized microscope stages and automatic focusing are solutions to this problem that are already available from some manufacturers, but would add to costs. In contrast, the subsequent digital counting process is fast, requiring a few minutes per specimen to complete.

In conclusion, this proof-of-principle study showed that it is possible to achieve high quality standards in thick film malaria parasite counts by digital image analysis. Free software and semi-automation of the counting process make this technique potentially widely accessible to many diagnostic laboratories. While it cannot replace the skill and training required of a malaria microscopist to detect the presence of parasites and make a parasite species identification, it has the potential to reduce some of the drudgery, and more importantly, improve the accuracy and reproducibility, of routine parasite quantitation.

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References


