Feasibility of Flow Cytometry for Measurements of \textit{Plasmodium falciparum} Parasite Burden in Areas of Malaria Endemicity by Use of Bidimensional Assessment of YOYO-1 and Autofluorescence\footnote{Published ahead of print on 12 January 2011.}

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The detection and quantification of \textit{Plasmodium falciparum} in human infections and in animal models has gone largely unchanged since the introduction of Giemsa microscopy more than a century ago. The limitations and disadvantages of microscopy are broadly recognized. While rapid diagnostic tests (RDTs) have joined conventional microscopy for malaria diagnosis in Africa, microscopy remains the primary tool for the evaluation of malariometric endpoints in clinical trials (31). New, more-powerful techniques, such as flow cytometry and PCR-based methods, have been developed with greater sensitivity and specificity and some day may replace conventional microscopy. The demand is ever greater for accurate, high-throughput methods for the evaluation of malaria parasite burden.

Microscopy is the standard method for assessing parasite burden, but it is labor-intensive and requires highly trained microscopists. The continuous evaluation and training of field microscopists is needed to ensure the correctness of slide results (24). Error in microscopy results is common and stems from multiple sources of variation, including differences in reader technique, slide quality, and the distribution of parasite populations across selected reading fields (26). Furthermore, discrepancies between readers are higher at lower parasite densities or if reading methods (thick or thin film) differ (25). Methods to augment conventional microscopy have been developed, such as staining films with the DNA staining dye acridine orange or, more recently, automated slide readers that employ digital algorithms for counting parasites (18, 27). These methods still are not widely used, and conventional microscopy remains the dominant tool for parasite quantification in most field laboratories. More standardizable methods that lend themselves to less subjectivity and greater potential for comparison between studies would be an advantage over current methods. Microscopy-based methods may not be adaptable to these research needs.

Flow cytometry, on the other hand, comes with high-throughput capabilities and less subjectivity. Since mature human erythrocytes do not contain nucleic acids, DNA staining techniques can exploit the presence of parasite DNA in infected erythrocytes and allow for the rapid quantification of parasitized erythrocyte populations by cytometric profiling. A variety of DNA-targeting dyes have been tested for this application. The higher target specificity and greater fluorescence intensity of a dye allow for a better separation of cellular populations. These are characteristics of the DNA-selective dyes, such as Hoescht 33258 and 33342, which show great specificity in the detection of infected erythrocytes but are...
restrictive due to fluorescence excitation that can be achieved only using UV lasers. Barkan et al. found YOYO-1 to be a high-quality non-UV-based dye for differentiating parasitized erythrocytes in the mouse malaria model (3). YOYO-1 is a bis-intercalating cyanine dye that is virtually nonfluorescent in solution but highly fluorescent when in complex with double-stranded DNA (dsDNA) (28). It can be excited using a 488-nm laser, which is equipped on most standard cytometers, and emits at 510 nm (12). It is 500 times as sensitive as ethidium bromide in detecting dsDNA, demonstrates less variability than other intercalating dyes, such as propidium iodide, and displays superiority over Hoescht in detecting microbiota by flow cytometry (12, 21, 28).

A limitation in flow cytometry has been overcoming the high background fluorescence of nucleic acid-containing noninfected erythrocytes, such as reticulocytes (13, 23). The problem may be even more punctuated in human studies of populations in which malaria is endemic, where chronic malaria infection, among other diseases, may cause the high prevalence of reticulocytosis due to anemia. Recent improvements in parasite staining methods have identified means of excluding background from noninfected populations. The analysis of the emission in two different wavelengths of blood samples stained with a single dye allow for the greater characterization of infected and noninfected events by separating the infected erythrocytes from nucleic acid-containing noninfected erythrocytes (6, 9, 15, 16, 20, 32). This method exploits the difference in autofluorescent patterns of erythrocyte subpopulations to distinguish reticulocytes from mature erythrocytes. Infected reticulocytes also may be distinguished (16). In mice, bidimensional analyses of emission at 530 and 585 nm from Plasmodium-infected samples stained with YOYO-1 reduced background noise 1 to 2 orders of magnitude from unidimensional analysis and greatly increased the dynamic range of detection and quantification (16). Additionally, the bidimensional staining pattern of the parasitized erythrocyte population allows for the characterization of specific asexual stages. This pattern has been established in multiple mouse models of Plasmodia as well as P. falciparum, both from parasite cultures and in a humanized mouse model of malaria (2, 15, 16, 20). Similar approaches using different dyes, such as acidine orange, have demonstrated promise but as yet have not shown superiority to YOYO-1 and suffer from issues such as hemolytic activity (6, 9).

In this study, we aim to assess the feasibility of P. falciparum quantification in natural infections of children in an area of Mozambique in which malaria is endemic by flow cytometry using the bidimensional YOYO-1,530/585. This method was chosen due to its superior staining pattern and ease of protocol. However, the validation of the YOYO-1,530/585 technique for clinical studies comes with many challenges due to the practical difficulties and specific endpoints used in field studies. The YOYO-1 method has proven extremely valuable in routine drug candidate evaluation in the mouse models of malaria (14). The establishment of such a high-throughput tool with low interrater variability in malaria field studies and phase I/IIb or phase III drug and vaccine trials would improve research capacity in research centers in areas where malaria is endemic.

**MATERIALS AND METHODS**

**Informed consent and institutional review board approval.** All participants invited to enroll in this study gave signed informed consent before performing any study procedures. An explanation of the study and study procedures was given to the parents or guardians of participants, and they were informed that the extra blood sample taken during their clinical visit would be used to compare different methods to diagnose malaria and anemia. The study was approved by the Comité Nacional de Bioética para Saude (CNBS) of Mozambique and the Comité Ético de Investigación Clínica (CEIC) of Barcelona, Spain.

**Study population.** The study presented here is an ancillary component to a pilot hospital-based surveillance system for the detection of severe malaria disease and other serious morbidities in children aged 2 months to 4 years to support the implementation of the phase III multicenter efficacy trial of GSK Biological’s candidate malaria vaccine, RTS, S/AS01E. All acute medical admissions of children between 2 months and 4 years of age to the pediatric ward of the Manhiça District Hospital, in the rural town of Manhiça, Mozambique, were eligible for inclusion in the study until a maximum sample size of 100 children was reached.

For this ancillary study, a small blood sample was taken at admission by finger-prick blood draw. A few drops of blood were collected into a BD Microtainer tube with K3 EDTA (Becton Dickinson, Franklin Lakes, NJ) for parasite quantification by flow cytometry and complete blood count (CBC) hematological analysis using a Sysmex KX21 automated hematometry analyzer (Sysmex Corporation, Ronar, South Africa). Two thick- and thin-ﬁlm blood slides were prepared to assess the density by microscopy. Five or six 20-μl blood spots were collected on Whatman filter paper and stored in sealed plastic bags with desiccant at 4°C for the subsequent conﬁrmation of parasitemia by quantitative real-time PCR (qRT-PCR).

**Parasite quantification by flow cytometry.** Staining blood for the quantification of infected erythrocytes using the bidimensional assessment of YOYO-1 fluorescence was performed as previously described (16). Briefly, 2 μl of blood taken from the EDTA-coated microtainer tube was fixed in 200 μl of 0.025% glutaraldehyde (Sigma-Aldrich, Madrid, Spain) in duplicate wells of a 96-well V-bottom plate and stored for a minimum of 18 h at 4°C. On the day of staining, 40 μl of fixed cells was washed with phosphate-buffered saline (PBS) in duplicate, permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, Madrid, Spain) for 5 min, and treated with 0.5 mg/ml RNase A (Sigma-Aldrich, Madrid, Spain) for 30 min. Permeabilized cells were stained with 0.5 μM YOYO-1 (Molecular Probes, Invitrogen, El Prat de Llobregat, Spain) for 30 min sheltered from light and subsequently acquired on a FACScalibur flow cytometer, equipped with a 15-mW, 488-nm air-cooled argon ion laser using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). As a positive control to establish the gate for infected erythrocyte events, infected blood with high parasitemia from a donor at the Manhiça District Hospital was identified by standard patient care diagnostics (malaria blood film, scored as positive on an escalating scale of 1 to 6 plusses). The exact quantification of parasitemia was not necessary, as the control only served as a gating reference and not a standard. The blood sample was fixed and frozen in parasite cryopreservation solution (57% glycerol USP, 16 g/liter of sodium lactate, 300 mg/liter of KCl USP, and 25 mM sodium phosphate, pH 6.8, filtered sterilized) at −70°C; initially, 1/3 pellet volume was added dropwise while mixing and allowed to stand for 5 min before adding 1× pellet volume, making aliquots, and freezing them. Frozen aliquots of P. falciparum-infected blood taken from an infected donor were thawed each day and fixed in a separate positive control 96-well plate. New aliquots were fixed each day that test samples were fixed.

Cytometer voltages were set with an unstained sample. The positive-control infected blood was acquired to adjust compensation settings and establish the infected erythrocytes gate. All samples were acquired until at least 100 events were recorded in the infected erythrocyte gate, 1×10^5 events were recorded in the total cells gate, or until the entire sample was acquired.

FlowJo software (Tree Star, Inc., Ashland, OR) was used for the postacquisition analysis of dot plots. Gates for total cells and infected erythrocytes were reestablished. Additionally, a gate for leukocytes was drawn in the top right corner of the logarithmic dot plots where the leukocyte population is located due to the staining of higher DNA content. Due to slight differences in cellular morphology depending on the time of fixation, infected erythrocytes gates were adjusted individually for samples with sufficient events (greater than 100) to more precisely score the populations. Independent researchers (J.A. Campo and A.J. Nhambomba) analyzed each sample to calculate parasitemia. Parasite densities were calculated using the parasitemia returned from the analysis of samples stained with YOYO-1, measuring emission at 530/30 nm (FL1, accounting mostly for nucleic acid content in cells) and 585/42 nm (FL2, accounting for autofluor-
rescence) in bivariate dot plots. The number of erythrocytes stained and read on the cytometer could not be estimated by assuming a fixed hematocrit, as the erythrocyte concentration ranged from 1 to 6 million erythrocytes/ml of blood, not including one outlier with a high erythrocyte concentration. Parasitemia was multiplied by the concentration of erythrocytes in whole blood returned by CBC using the following equation: [number of infected erythrocytes/(number of total cells — number of leukocytes)] × (erythrocytes/µl).

Conventional microscopy, Thick- and thin-film blood slides were stained with Giemsa’s azure eosin methylene blue (Merck & Co., Inc., Whitehouse Station, NJ) and read using standard methods at the Centro de Investigação em Saúde de Manhica (CISM). Briefly, thick smears were read by two independent microscopists trained at the CISM and who perform routine diagnostic microscopy for P. falciparum infection. The numbers of parasitized erythrocytes and leukocytes per field were counted. In thick films, where the number of infected erythrocytes was too high to accurately count, thin-film blood slides were assessed. The numbers of parasitized erythrocytes and total erythrocytes from the first field of vision were counted. Subsequently, parasite density was calculated for thick- and thin-films blood smears using hematological data from CBC for the absolute concentration of leukocytes/µl or erythrocytes/µl whole blood, respectively. The mean results of the two readers were used to calculate a final density. In the case of discordant results, a third reader was employed, and the means of the two most concordant readers were used.

Quantitative real-time PCR. A 20-µl blood spot was cut from Whatman filter papers for each sample. DNA was extracted from the filter paper blood using a QiAamp DNA blood mini kit (Qiagen Iberia, Madrid, Spain). DNA was eluted in 150 µl elution buffer. In parallel, a 20-µl filter paper blood spot was prepared from an ongoing culture of P. falciparum strain 3D7 at approximately 24,000 parasites/µl (50% hematocrit). DNA was extracted using the same method and frozen in aliquots for use as a standard curve.

qRT-PCR for the detection of P. falciparum parasitemia was performed as described previously (1, 10). Briefly, primer and probe sequences from the 18S rRNA subunit were used for amplification (forward primer, 5’ GGA ATG ATA GGA ATT TAC AGG GT 3’; reverse primer, 5’ TCA ACT ACG AAC GTC TTA ACT GCA AC 3’). A TaqMan probe (5’ TCC CAG CCG CCG TAA TTC 3’) labeled with a TAMRA (6-carboxytetramethyl-rhodamine) quencher and a FAM (6-carboxyfluorescein) reporter was used to detect P. falciparum-positive samples. Duplicate PCR were performed in MicroAmp optical 96-well reaction plates (Applied Biosystems, Madrid, Spain) at a final volume of 20 µl with 5 µl of extracted DNA, 300 nM forward primer, 300 nM reverse primer, 150 nM TaqMan probe, and 10 µl of TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain). DNA from the positive-control standard was run in triplicate reaction mixtures in six dilutions of a 1:10 dilution series, starting at the frozen aliquot concentration. The amplification and detection of parasite DNA were performed on an ABI Prism 7500 (Applied Biosystems, Madrid, Spain) using the following conditions: stage 1 at 50°C for 2 min, stage 2 at 95°C for 10 min, and 40 repetitions of stage 3 at 95°C for 15 s, followed by 60°C for 1 min. Samples were considered positive if reporter emissions from the range of highest and lowest standard curve dilutions were considered within the quantifiable range of detection. The range of quantification with the reported positive standard was 24,000 to 2,400 parasites/µl blood times the dilution factor of the test sample. The final dilution of the standard curve crossed the positive threshold within 40 thermal cycles (cycles falling within the range of highest and lowest standard curve dilutions were considered within the quantifiable range of detection). The range of quantification with the reported positive standard was 24,000 to 2,400 parasites/µl blood times the dilution factor of the test sample. The final dilution of the standard curve crossed the positive threshold between 37 and 39 C_T. Samples falling above the quantifiable range were diluted 1:100 and repeated, and samples falling below the quantifiable range were considered positive but unquantifiable. Parasite densities calculated by qRT-PCR were not compared to densities calculated by flow cytometry or microscopy.

Data analyses. qRT-PCR is considered the gold standard for determining positive or negative parasitemia. Mean difference dot plots were constructed to illustrate interrater agreement for the quantification of parasitemia by flow cytometry as well as agreement between parasite density by YOYO-1/530/585 and conventional microscopy (7). These include Pitman’s test of difference in variance, 95% confidence intervals (CI) for mean difference, and a linear regression fit with a Student’s t test on individual independent variables. In cases where log transformation did not correct for heteroskedasticity, and where the mean difference and variance were not constant, we modeled absolute residuals against the mean to use as a proxy for the calculation of the standard deviations and construction of nonlinear limits of agreement, as proposed by Bland and Altman (8). For the easier visualization of log-transformed data, mean difference dot plots for interrater agreement and agreement between YOYO-1/530/585 and microscopy have been returned to the arithmetic scale and are represented as relative differences (ratios) against geometric mean parasitemia or parasite density, respectively. Compared to that of qRT-PCR, the sensitivity and specificity of flow cytometry were calculated using receiver operating characteristic (ROC) curves by examining the area under the curve, with standard errors and 95% confidence intervals, and comparing results to those of microscopy sensitivity and specificity (with 95% confidence intervals). All analyses were performed using STATA statistical software, version 11.0 (College Station, TX).

RESULTS AND DISCUSSION

Study population and clinical results. This study aimed to test the feasibility of a flow cytometry-based technique for the quantification of P. falciparum infection in an field setting in which malaria is endemic. A total of 99 children aged 2 months to 4 years were enrolled to determine clinical algorithms of severe malaria and other serious morbidities to support the implementation of the phase III multicenter efficacy trial of the candidate malaria vaccine RTS, S/AS01E (ClinicalTrials.gov identifier: NCT00866619). The study population presented to the pediatric ward with a range of severe morbidities, including P. falciparum malaria, sepsis, acute respiratory infections, meningitis, and diarrheal diseases, and included children with concurrent morbidities (clinical data not shown). Thick- and thin-film blood slides were taken and examined for all children; 91 children were successfully screened by the YOYO-1/530/585 method for parasitemia (8 individuals were acquired under faulty voltage settings), and filter paper blood spots were successfully screened by qRT-PCR for 95 children.

Interrater reliability.

In practice at a field site, performing the YOYO-1/530/585 assay and setting compensation to establish the parasite staining pattern was much the same as in the mouse model, although P. falciparum parasites recovered in peripheral blood lacked advanced stages of development that could readily be identified in the mouse model and P. falciparum cultures, such as late trophozoites and schizonts (2). Figure 1 illustrates the gating and establishing of a region for infected erythrocytes after correct compensation.

The mean difference of parasitemia estimates between analyzers was 0.42% with a standard deviation of 1.95%. Parasitemia were Ln transformed before constructing mean difference plots between analyzers (Fig. 2) to display interrater agreement. As expected, greater variability between analyzers is observed at lower parasitemia, most notably below 0.2%, above which the mean difference in parasitemia approaches zero. Mean difference plots were not constructed for microscopy, because a third slide reader was used to control for nonconcordance, which does not allow a fair comparison, and the primary objective was not the assessment of microscopy. It is worth noting that of the 99 patients assessed for parasitemia by conventional microscopy, 19 of these required a reading by a third reader due to nonconcordance. In one study, disagreement between parasite density measurements by microscopy were nearly 10-fold in either direction (19). In this analysis, YOYO-1/530/585 demonstrates tighter limits of agreement than previous reports of microscopy and thus very good concordance of results when two trained readers establish gating independently (19). Additionally, the acquired data can be reassessed easily and does not lose integrity over time, and a faulty acquisition of data only necessitates a repeat of the assay using the remaining fixed sample.

Sensitivity and specificity. To assess the sensitivity and specificity of microscopy and the YOYO-1/530/585 method, qRT-PCR was used as a gold standard to determine true positives.

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and true negatives. qRT-PCR results confirmed parasitemia in 63 samples, two of which were positive but below the range of qRT-PCR quantification. The sensitivity of microscopy was 79.4% (95% CI, 67.3 to 88.5%), and specificity was 96.9% (95% CI, 83.8 to 99.9%).

Since confirmed parasite-free blood was not available to establish a positive threshold for the YOYO-1\textsubscript{530/585} method, data were analyzed by ROC tables and curves to assess sensitivity and specificity at incremental cutoff thresholds (Fig. 3). With specificity as high as that of microscopy (96.55% in the ROC table), both raters of the YOYO-1\textsubscript{530/585} method achieved a sensitivity of 79.3% (data not shown). The positive thresholds to establish this level of sensitivity and specificity are 0.282 and 0.393% for raters 1 and 2, respectively. For comparison, microscopy data were plotted on the ROC. The area under the ROC, a measure of closeness to the gold standard, is approximately 0.90 for both flow cytometry and microscopy, which is acceptably close to the true disease status. Taking these results together, the YOYO-1\textsubscript{530/585} method performs similarly to conventional microscopy in sensitivity and specificity (Table 1).

**Estimation of parasite density by YOYO-1\textsubscript{530/585} and complete blood count.** Parasite density was estimated by multiplying the frequency of infected erythrocytes from total erythrocytes returned from the YOYO-1\textsubscript{530/585} method by absolute concentration of erythrocytes in whole blood by CBC. The estimated parasite densities then were compared against those returned by conventional microscopy in mean difference dot plots using Ln-transformed data to assess agreement (Fig. 4). The mean difference of YOYO-1\textsubscript{530/585} versus microscopy corresponds to a 2.3-fold higher estimation of parasite density from the YOYO-1\textsubscript{530/585} method than that of conventional microscopy (mean difference ratio, 2.3; 95% CI, 1.456 to 3.597). The limits of agreement show a range of 10-fold lower estimation to 53-fold higher estimation of YOYO-1\textsubscript{530/585} compared to that of microscopy. The regression of these data points shows a trend toward greater difference between estimations at lower parasitemias, although the fitted line was significant for only one of the two raters (rater 2; not shown).

In our analyses, parasite density by flow cytometry using an algorithm of parasitemia and density of red blood cells by CBC resulted in estimates that generally were much higher than those returned by conventional microscopy. While a higher estimation of parasite density could be expected, as observed in a comparison of qRT-PCR and microscopy (5), several factors may explain the high deviations observed. The background that remained with each sample when calculating parasite density, while initially a small percentage of total events, is amplified by factors of 10\textsuperscript{6} to 10\textsuperscript{8} when adjusting for the concentration of erythrocytes per microliter of blood. The exploratory adjusted analysis we performed here does not serve as a replacement for a proper negative control to set the baseline from a day-to-day basis. Thus, both batch variation and individual variation are likely to be a source of error that is amplified through this algorithm. Hemolysis during the fixing and staining process also may lead to an overestimation of parasitemia, which again will be amplified in the conversion to parasites per microliter (32). The future development of this assay for field studies must account for these sources of error.

**Field adaptation of YOYO-1\textsubscript{530/585} method.** The YOYO-1\textsubscript{530/585} method was optimized previously in murine drug-screening studies, and several adaptations were made for employment in nonexperimental human studies. Whereas mice may be infected for a continuous source of a confirmed positive control, such a source of fresh infected blood may not be possible in human field studies. Acquiring fresh blood samples from a malaria-positive patient for each assay would require

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**FIG. 1.** Gating population of infected erythrocytes in bidimensional dot plots of YOYO-1 and autofluorescence. (A) Region of total cells in logarithmic forward-scatter versus side-scatter dot plots. (B) Region of infection events after compensation of the FL1 from the FL2 channel (autofluorescence), established by a positive reference sample.
elaborate human use protocols, and positive blood may be difficult to obtain during low-transmission or off-season periods. To produce a reliable source of infected human erythrocytes for establishing compensation settings, blood taken from an infected adult donor was cryopreserved in one-time-use aliquots each assay day. Of note, changes in erythrocyte morphology and background due to freeze/thaw is irrelevant in the positive control. A positive reference had to be fixed on each day of sampling, since longer periods in glutaraldehyde fixation shift the positive population incrementally lower on the staining axis, presumably due to the partial degradation of parasite DNA. This provided a reliable source of reference for establishing the population pattern for infected erythrocytes in test samples.

Likewise, it is difficult to procure reliable malaria-negative blood in endemic populations to set baseline cutoffs. Blood from patients on antimalarial prophylaxis or blood treated with antimalarials are poor options, because remnants of drug-treated parasites produce unique staining patterns (I. Angulo-Barturen and M. B. Jiménez-Díaz, unpublished data), and resistant strains could introduce problems. The option to use frozen aliquots of blood from a nonexposed donor was rejected, since the thawing process alters the morphology of erythrocytes and produces higher background noise levels. No negative control was included in this study, since no confirmed negative samples could be sourced without performing advanced diagnostic techniques (PCR based) at the time that samples were collected. The further adaptation of the technique must find a source of negative-control samples from which to remove background noise on a daily basis. A possible solution is to combine negative blood with a red blood cell-stabilizing reagent, similar to those used for blood bags to preserve cellular viability and maintain active metabolism (11, 22).

Whereas an animal model provides physiologically similar or even genetically identical patients, field studies cannot be controlled as such, and patients present with a multitude of morbidities and heterogeneities that may influence the cellular morphology that is measured in flow cytometry-based techniques. In Manhiça District, Mozambique, bacterial bloodstream infections are common, comprising up to 8% of pediatric hospital admissions (30), and bacteremia among severe malaria patients also is frequent (4). Previous studies of blood transfusion screening have demonstrated flow cytometry to be a reliable tool for identifying bloodstream bacteria using largely the same principles as those of the current method being tested (17, 29). It is difficult to compare techniques from such different studies, but it is possible that the cytometric

![FIG. 2. Interrater agreement of the YOYO-1530/585 method, represented as the relative ratio against geometric mean parasitemia estimated between raters. The interrater difference ratio displays significant heteroskedasticity (test for heteroskedasticity; $P = 0.0001$), even after Ln transformation, and shows a directional tendency ($P = 0.0003$). The solid green line displays the dynamic mean difference with respect to parasitemia and crosses the line of no difference (interrater ratio of 1). The limits of agreement (gray region) show the 95% confidence interval of the regression line and illustrate that differences are greater at lower parasitemia levels, as supported by the observation of the greater fanning out of data points in the low-parasitemia range.](image1)

![FIG. 3. ROC curve of sensitivity and specificity for the YOYO-1530/585 method compared to that of qRT-PCR. Sensitivity and specificity values are calculated (table not shown) for incremental cutoff thresholds for the YOYO-1530/585 baseline signal (only rater 1 is shown).](image2)
The staining profile of bloodstream bacteria overlaps with that of parasitized erythrocytes. The patients surveyed in this study were comprised of very ill children, and two out of six children that had confirmed sepsis also had concurrent malaria. In an area of highly prevalent bacteremia and mixed infections, such as Manhiça District, the detection of bacteria by flow cytometry may contribute to skewed parasitemias or background signal and reduce the reliability of the method. Further studies must characterize the contribution of different pathogens to parasitemia estimates or identify a separate strategy for increasing the specificity of infected erythrocyte events.

Variability in the conditions of patients may affect the cytometric pattern that would be expected from healthy individuals; reticulocytosis is a highly prevalent condition accompanying anemia, which makes the proper gating of infected erythrocytes difficult. At high levels of anemia, *P. falciparum* may infect reticulocytes and form a separate population of infected events. Additionally, patients with severe anemia may have dead and destroyed erythrocytes in the blood that lend to higher levels of background. Although not assessed in this study, other blood abnormalities and hemoglobinopathies, such as thalassemias, may result in atypical cytometric profiles and difficulty in correctly establishing infected and noninfected populations.

**Conclusions.** Flow cytometry is an appealing approach to detecting and estimating parasite density in field epidemiology and intervention studies of *P. falciparum*. The YOYO-1530/585 method can be at least as sensitive and specific as conventional microscopy, as estimated by ROC curve analysis, and is a higher-throughput assay with low interrater variability in the hands of trained personnel. A cost/benefit analysis was not done during this study but will be assessed once a validated assay is established. Before the YOYO-1530/585 method can be implemented, several assay conditions must be improved. Additionally, the technique must be validated for field studies, setting defined assay criteria and reducing the level of nonspecific signal. A method of estimating density within the flow cytometry apparatus, such as acquisition with BD Trucount tubes, could be assessed to check agreement with the current algorithm and agreement with microscopy. Taking the limitations of the assay into consideration, the YOYO-1530/585 method remains a technique of great potential for implemen-

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**TABLE 1. Sensitivity analysis of microscopy and flow cytometry relative to qRT-PCR**

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Likelihood ratio</th>
</tr>
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<tbody>
<tr>
<td>Microscopy</td>
<td>79.4 (67.3–88.5)</td>
<td>96.9 (83.8–99.9)</td>
<td>98.0 (89.6–100)</td>
<td>70.5 (54.8–83.2)</td>
<td>25.40 0.21</td>
</tr>
<tr>
<td>YOYO-1530/585 (both raters)</td>
<td>79.3 (66.6–88.8)</td>
<td>96.6 (82.2–99.9)</td>
<td>97.9 (88.7–99.9)</td>
<td>70.0 (53.5–83.4)</td>
<td>23.00 0.21</td>
</tr>
</tbody>
</table>

* Diagnostic characteristics for microscopy (n = 95) and YOYO-1530/585 (n = 87) using cutoff values of 0.282 and 0.393% for raters 1 and 2, respectively. PPV, positive predictive value; NPV, negative predictive value.

![FIG. 4. Agreement between parasite density estimations by YOYO-1530/585 and microscopy algorithms, constructed from Ln-transformed data and represented as the relative difference against geometric mean parasitemia. Crude parasite density is the mean difference (solid red line) corresponding to a 2.3-fold higher estimate by YOYO-1530/585 than that by microscopy (CI, 1.46 to 3.60) and limits of agreement (dashed red lines) between 10-fold-lower and 53-fold-higher estimates. An outlier on the high range of parasite density (3.9 × 10⁵ infected erythrocytes/µL) is explained most likely by a highly elevated CBC count (3.75 × 10⁸ erythrocytes/µL), which may be a technical error in the Sysmex reading. Only rater 1 is shown. |
tation in field studies, but we do not currently recommend it. The future work of our group aims to optimize and validate the technique for the quantification of \textit{P. falciparum} in settings in which it is endemic.

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J.J.C. and A.N. performed the data acquisition and flow cytometry analysis for the YOYO-1\textsubscript{530/585} method; I.A.B. and M.B.J.D. developed the YOYO-1\textsubscript{530/585} method, transferred the technique to field site analysis and interpretation and drafted the manuscript. We all critically formed qRT-PCR; J.J.C., J.J.A., and C.D. were involved in data analysis for the YOYO-1\textsubscript{530/585} method; I.A.B. and M.B.J.D. developed the clinical data collection and interpretation; C.D., I.A.B., and J.J.A. conceived and designed the project; J.J.C. performed qRT-PCR; J.J.C., J.J.A., and C.D. were involved in data analysis and interpretation and drafted the manuscript. We all critically revised and approved the manuscript.

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