Quantitative Detection of *Plasmodium falciparum* DNA in Saliva, Blood, and Urine

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(See the editorial commentary by Sutherland and Hallett, on pages 1561–3.)

**Background.** Current methods for detecting malaria parasites are invasive and associated with poor compliance when repeated sampling is required. New methods to detect and quantify parasites in a less-invasive manner would greatly enhance the potential for longitudinal surveillance in clinical trials.

**Methods.** Saliva, urine, and blood samples from 386 Gambian outpatients with suspected malaria infections were analyzed by nested polymerase chain reaction (nPCR) to detect infection and to evaluate diagnostic accuracy in comparison to expert microscopy. The amount of parasite DNA in malaria-positive samples was estimated using real-time quantitative PCR (qPCR).

**Results.** Blood parasite density as estimated by qPCR correlated well with parasite counts established by microscopy ($r = 0.94$; $P < .001$). qPCR results for saliva had a significant correlation with microscopy counts ($r = 0.58$; $P < .001$), whereas qPCR results for urine had a positive but poor correlation with microscopy counts ($r = 0.20$; $P = .117$). The mean amounts of parasite DNA quantified in blood were greater than the mean amounts quantified in saliva and urine samples obtained concurrently from the same individual, by ~600-fold and ~2500-fold, respectively. When nPCR results were compared with microscopy results, nPCR of saliva had a sensitivity of 73% and a specificity of 97%; its sensitivity increased to 82% in samples with a parasite density of $\geq 1000$ parasites/$\mu$L. nPCR of urine had a sensitivity of 32% and a specificity of 98%.

**Conclusion.** Saliva sampling is a promising less-invasive approach for detecting malaria infection.

The deployment of an improved diagnostic test for malaria that required minimal infrastructure and was able to achieve 95% specificity and sensitivity could potentially avert $>100,000$ malaria-related deaths each year and save nearly $200$ million that is spent on unnecessary treatments annually [1]. Thick blood film microscopy, which is up to 10 times more sensitive than thin blood film microscopy [2], is the standard method for detecting malaria parasitemia and estimating its level. Thick blood film examination by an experienced microscopist should reliably detect parasites at a density of $>50$ parasites/$\mu$L [3], although routine laboratory diagnosis generally achieves a much lower detection sensitivity of $\sim 50$ parasites/$\mu$L [4].

Polymerase chain reaction (PCR)–based assays are available for the detection of malaria parasites in the blood and may be more accurate than microscopy for estimation of parasite density [5–9]. PCR methods are extremely sensitive, with a detection limit of $<10$ parasites/$\mu$L; they are also able to unambiguously identify the species of the malaria parasite and have the potential for high throughput processing of samples. Although these methods are not currently suitable for routine point-of-care diagnosis in malaria-endemic regions because of high initial setup costs and their use of specialized, temperature-labile consumables, their application in clinical trials is promising, and they are increasingly...
used for routine diagnosis in industrialized countries [10]. The potential of quantitative real-time PCR (qPCR) for endpoint determination in clinical trials of antimalarial interventions and follow-up of patients in drug resistance studies has been noted [8, 11–14], but widespread application has not yet been implemented.

Parasite density in peripheral blood undergoes frequent fluctuations over the course of an infection, sometimes decreasing to undetectable levels because of factors such as sequestration of parasites into the capillaries of the brain, spleen and other organs; the immune responses of the host; and competition between clones in multiclonal infections [13, 15–17]. Therefore, single time-point sampling to assess malaria status frequently underestimates both malaria prevalence and parasite load [16, 18, 19]. Assessment of the efficacy of antimalarial interventions would be improved by methods that allow repeated sampling of study cohorts during follow-up, because this would generate accurate data to evaluate intervention efficacy in different study arms. It has recently been shown that it is possible to detect malaria parasite DNA in samples of urine and saliva from some patients with malaria [20]. However, it is not known whether the amount of parasite DNA present in saliva and urine during a malaria episode can be quantified or whether it correlates with the parasite load in the blood. In the present study, we assess the diagnostic performance of parasite DNA detection in saliva and urine samples and investigate the correlation between the amounts of parasite DNA detected in these less-invasively obtained samples and parasite counts established by microscopy.

If assays that use less-invasively obtained samples prove to have high diagnostic accuracy, malaria could be diagnosed more accurately and parasite density estimated more precisely, because samples could be obtained more frequently with minimal inconvenience to patients. This would be particularly relevant to longitudinal surveillance of cohorts in antimalarial intervention trials in which repeated sampling of healthy individuals is required.

METHODS

Patient enrollment and sampling. From October through December 2006, patients aged ≥10 years with suspected malaria infections and referred for blood film microscopy at the outpatient clinic facility of the Medical Research Council, Fajara, The Gambia, were prospectively enrolled before determination of malaria status. Subject enrollment, sample collection, and laboratory analyses were performed as outlined in figure 1. All patients gave informed written consent, and consent from a parent or guardian was obtained for participants aged ≤18 years prior to enrollment. The study was jointly reviewed and approved by the Gambian Government–Medical Research Council Laboratories Ethics Committee and the Western Institutional Review Board.

Matched samples of blood, urine, and saliva were collected from each patient. Approximately 250 μL of blood, obtained via finger prick, was collected into tubes that used ethylenediaminetetraacetic acid as an anticoagulant; a 2-mL saliva sample and a 5-mL urine sample were collected into separate aseptic Sterilin bottles (Barloworld Scientific). All samples were kept at 4°C–8°C until DNA was extracted, usually within 2 h of sampling.

Microscopy. Thick blood smears were prepared with 10 μL of blood and stained with Giemsa stain in accordance with a standard protocol. Each slide was evaluated independently by 2 experienced microscopists who were blinded to each other’s result; the microscopists examined 500 high-power fields (hpf) under oil immersion (1000×) before the sample was declared negative for parasites. Reading of 500 hpf from a thick film is approximately equal to screening 1 μL of blood [18, 21, 22]. Parasite density was estimated by counting the number of parasites in 100 hpf and converting the value to parasites per microliter of blood by using a multiplication factor of 5. Discrepancies of >1 log unit between the 2 slide readings were resolved by a third experienced microscopist.

Preparation of qPCR DNA standards. Laboratory culture of *Plasmodium falciparum* clone 3D7 was grown to 8%–11% parasitemia (i.e., 8%–11% of red blood cells in the culture medium were infected) and centrifuged in a Percoll gradient to isolate ring-stage parasites, the stage of the life cycle normally detected in peripheral blood. Serial 10-fold dilutions to 1:10⁶ were prepared with uninfected whole blood, and red blood cell (RBC) counts were determined using a Coulter counter. Parasite density was estimated by counting 50,000 RBCs in a thin film at the 1:100 dilution; it was confirmed by counting 500 hpf of duplicate thick film and converted to parasites per microliter by using the actual RBC count of 3.9 × 10⁶ cells/μL. The parasite densities for the rest of the dilution series were extrapolated from the value obtained for the 1:100 dilution.

DNA extraction and nested PCR (nPCR) assays. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), in accordance with the manufacturer’s protocol. Qualitative detection of parasite DNA was based on nPCR amplification of the multicopy 18s rRNA gene in a reaction that used 1 μL of extracted DNA in a total volume of 15 μL, as described elsewhere [5]. Additionally, all samples were screened for the presence of the other human malaria species, *Plasmodium malariae*, *Plasmodium vivax*, and *Plasmodium ovale*, using species-specific primers (table 1, which appears only in the electronic version of the journal). Positive controls consisting of parasite DNA and a negative control consisting of DNA extracted from malaria-negative blood were included in each PCR run. nPCR results were scored as categorical variables (presence vs. absence of amplification) and summarized in a contingency table.

qPCR analysis. qPCR was used to analyze concurrently obtained blood, urine, and saliva samples from 81 patients who tested positive for *P. falciparum* by either microscopy or
nPCR of blood, saliva, or urine and samples from 5 P. falciparum–negative patients. Quantification of parasite density was done with an Opticon 2 Real-Time PCR Detection System using the Opticon Monitor software (version 3.1; Bio-Rad Laboratories). Two quantitation methods, the TaqMan assay—which uses a fluorogenic probe to detect a specific PCR product [6]—and SYBR Green 1 detection were used.

Table 1. Primers, amplification protocols, and expected amplicon sizes for nested and quantitative polymerase chain reaction (PCR) assays used to evaluate samples for the presence of malaria parasites.

The table is available in its entirety in the online edition of The Journal of Infectious Diseases.

Figure 1. Flowchart showing patient enrollment, samples obtained, and laboratory analyses performed on samples.
using the quantitation standards and found that a cutoff threshold of 0.02 fluorescence units robustly detected the threshold cycle for the lowest dilution and was in the log-linear phase of the amplification.

**Statistical analysis.** Diagnostic performance was evaluated using standard measures of diagnostic accuracy: sensitivity, specificity, positive predictive value, and negative predictive value. The $\kappa$ coefficient, which estimates agreement between different measurements on a scale of 0–1 after correcting for agreement due to chance [23], was used to assess the concordance between microscopy results and detection by nPCR and the concordance between 2 independent microscopy slide readings. Parasite densities were determined per microliter of sample. Because of the skewed distribution of parasite count data, logarithmic transformation was performed, and the correlation of qPCR estimates of parasite density with microscopy results for blood were analyzed using scatter plots and correlation coefficients. Overall agreement between different estimates of parasite density was evaluated with Bland-Altman plots [24]. All statistical analyses were performed with Stata (version 9.3; Stata) and Prism (version 4.0; GraphPad).

**RESULTS**

**Subject enrollment and sample processing.** There were 386 patients enrolled in the study. The mean age was 25 years (range, 10–45 years; median, 24 years); 244 patients were female, and 142 were male. All study subjects provided blood, urine, and saliva samples, except for 2 individuals who were unable to produce a urine sample. High-molecular-weight DNA was consistently obtained from blood samples and from most saliva samples, although a few of the latter produced a smear of DNA bands with molecular weights indicative of degradation. The amount of DNA in the urine samples was not detectable on an agarose gel. All parasite-positive samples showed *P. falciparum* infection only; none of the other species of human malaria parasites were detected.

**Comparison of microscopy and nPCR results.** Two experienced microscopists independently examined 500 hpf for each sample; malaria parasites were detected in 54 (14%) of 386 slides read by the first microscopist and 62 (16%) of 386 slides read by the second microscopist. Discordant results were reported mainly for samples from patients with low-grade infections (17 of 23) with parasite density of <100 parasites/µL. Parasite prevalence as determined by concordant microscopy results was 13% (51 of 386), compared with 18% (68 of 386) by nPCR of blood, 12% (47 of 386) by nPCR of saliva, and 6% (22 of 384) by nPCR of urine (table 2).

nPCR of blood had sensitivity of 98% (i.e., the percentage of microscopy-positive samples correctly identified by nPCR), and specificity of 95% (i.e., the percentage of microscopy-negative samples correctly identified by nPCR). nPCR of saliva had sensitivity and specificity of 73% and 97%, respectively, whereas nPCR of urine had sensitivity and specificity of 32% and 98%, respectively (table 3). When a parasite density threshold of 1000 parasites/µL was used as a proxy for clinical malaria, the sensitivity of nPCR of saliva increased to 82%, with a slight reduction in specificity to 95%. nPCR of urine had sensitivity and specificity of 42% and 98%, respectively. The combination of microscopy and nPCR of blood as a composite “reference standard” for the presence of parasites even at very low levels resulted in estimated sensitivity of 74% (51 of 69 samples correctly identified) for microscopy, compared with 99% (68 of 69) for nPCR of blood, 57% (39 of 69) for nPCR of saliva, and 23% (16 of 69) for nPCR of urine. nPCR of blood detected 18 additional malaria-positive samples that were not detected by microscopy; in contrast, microscopy identified parasites in 1 sample that had tested negative with the standard nPCR assay. The possibility that PCR inhibitors were present was excluded by successful quantification of parasite DNA that was added to the PCR-negative sample. Parasite DNA was detected in 10 saliva and 6 urine samples obtained from individuals who tested negative for parasites by microscopy and nPCR of blood.

**Comparison of microscopy and qPCR results.** Malaria parasite DNA was successfully quantified in concurrently ob-
tained saliva, urine, and blood samples from 47 patients. The geometric mean parasite density detected by microscopy was 1785 parasites/μL (95% confidence interval [CI], 695–4588 parasites/μL), whereas the equivalent estimates by quantitation of the amount of parasite DNA in blood, saliva, and urine were 694 parasites/μL (95% CI, 291–1658 parasites/μL), 1.123 parasites/μL (95% CI, 0.55 –2.294 parasites/μL), and 0.275 parasites/μL (95% CI, 0.154–0.492 parasites/μL). The amount of parasite DNA quantified in peripheral blood samples from infected patients was ~600-fold greater than that in saliva samples and ~2500-fold greater than that in urine samples from the same individual. Similar estimates of the amounts of parasite DNA were obtained with both methods of qPCR analysis (SYBR Green and TaqMan).

A scatter plot matrix of parasite densities showed a linear relationship between counts established by microscopy and qPCR results for blood and, to a lesser degree, for saliva, but not for urine—(figure 2). Spearman’s rank correlation coefficient for microscopy results and TaqMan qPCR of blood (ρ = 0.94; P < .001) was slightly higher than the correlation between independent microscopy readings of the same slide (ρ = 0.93). The correlation between parasite count as established by microscopy and qPCR of parasite DNA in saliva was moderate but highly significant (r = 0.58; P < .001), which suggests a concentration-dependent release of parasite material into the saliva. The correlation between microscopy and qPCR estimates of parasite DNA in urine was not significant (ρ = 0.2; P = .117).

Agreement between parasite density estimates. The agreement between microscopy results and qPCR estimates of parasite density was similar across the whole range of parasitemia levels (figure 3, which appears only in the electronic version of the Journal). The mean difference and 95% limits of agreement were −0.275 to 1.4 log units, detecting only 32% of microscopy-positive samples. However, nPCR of saliva correctly identified 73% of microscopy-positive samples, and its sensitivity increased to 82% for samples with parasite density ≥1000 parasites/μL, a level of parasitemia seen in most patients with malaria in The Gambia and other malaria-endemic areas.

The sensitivity of malaria parasite detection is mainly dependent on the volume of sample that is examined [18]. Parasite prevalence in malaria surveys has been shown to vary relative to the number of microscopy fields screened, with detection of malaria-positive samples increasing by 5%–10% if the number of microscopy fields examined is doubled [22, 26]. It was expected that the sensitivity of nPCR for saliva would depend on the sample volume that was screened in a single assay. Because of the ease with which large volumes of sample could be obtained and the possibility of optimizing PCR protocols for saliva to screen larger volumes of sample than were examined in this study, a considerable increase in assay sensitivity should be achievable. Modifications to the PCR protocol, such as the targeting of parasite sequences with higher copy numbers than the commonly used 18s rRNA gene [25] and the use of the SYBR Green detection system, which has >200 times the sensitivity of ethidium bromide staining [27], would considerably improve the sensitivity of detection for parasite DNA in less-invasively obtained samples. However, in its present form, the lower sensitivity and longer turnaround time for PCR of saliva would

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Positive predictive value, %</th>
<th>Negative predictive value, %</th>
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<tbody>
<tr>
<td>Microscopy</td>
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<tr>
<td>Microscopist 1</td>
<td>96 (87–100)</td>
<td>99 (97–100)</td>
<td>91 (80–97)</td>
<td>99 (98–100)</td>
<td>0.92</td>
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<tr>
<td>Microscopist 2</td>
<td>98 (90–100)</td>
<td>96 (94–98)</td>
<td>81 (69–90)</td>
<td>100 (98–100)</td>
<td>0.87</td>
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<tr>
<td>Nested PCR</td>
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<td>Blood</td>
<td>98 (90–100)</td>
<td>95 (92–97)</td>
<td>74 (61–83)</td>
<td>100 (98–100)</td>
<td>0.81</td>
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<tr>
<td>Saliva</td>
<td>73 (58–84)</td>
<td>97 (95–99)</td>
<td>79 (64–89)</td>
<td>96 (93–98)</td>
<td>0.72</td>
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<tr>
<td>Urine</td>
<td>32 (20–47)</td>
<td>98 (96–99)</td>
<td>73 (50–89)</td>
<td>91 (87–93)</td>
<td>0.4</td>
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NOTE. Consensus expert microscopy, as defined in table 2, was used as the reference standard. CI, confidence interval.
make the assay unsuitable for clinical evaluation of suspected malaria cases for treatment.

PCR analysis of saliva and urine revealed a small number of samples that tested positive for parasite DNA but were negative for parasites by microscopy and nPCR of blood. This result implied that parasite DNA was present in these samples in the absence of detectable parasites in peripheral blood. Malaria parasite density in the peripheral blood fluctuates widely over the course of an infection, sometimes decreasing to undetectable levels because of mechanisms that include sequestration of infected erythrocytes, the immune responses of the host, and competition between clones in multiclonal infections [13, 15, 17]. A possible explanation for these results could be that blood sampling coincided with the stage in the parasitic life cycle during which parasites are mostly sequestered in body organs, resulting in their transient absence from peripheral blood. Because this was a cross-sectional study with no follow-up of patients, it was not possible to determine whether the presence of parasite DNA in urine and saliva preceded the presence of parasites in peripheral blood or persisted for some time after the clearance of parasites from the blood. Further studies will be required to investigate this as well as other challenges to less-invasive sampling methods, such as the feasibility of obtaining saliva samples from younger children, who are the major group at risk of malaria and thus constitute the ultimate target for antimalarial vaccine efficacy trials.

Using Bland-Altman plots to directly compare qPCR estimates of parasite density with microscopy counts, we found that
the results of qPCR of blood agreed closely with microscopy results; the qPCR results were comparable to independent examinations of the same blood film by experienced microscopists. It has recently been reported that microscopy underestimates parasite density relative to qPCR [11]; however, the results from quantifying serial dilutions of cultured parasite and a limited number of clinical samples showed that microscopy, despite being the less-sensitive method, consistently gave higher estimates of parasite density than qPCR [6, 9]. This result was attributed to the use of a multiplication factor to derive parasite density from a small amount of screened blood (usually the equivalent of about ~0.2 μL blood if 100 hpf were read) [21, 22]. In the present study, we also observed a small discrepancy between microscopy counts and qPCR parasite density estimates; microscopy gave a slightly higher estimate, consistent with previous reports [6, 9] and suggesting that microscopy readings may tend to result in slightly overestimated parasite counts.

The biological processes leading to the release of parasite DNA into saliva and urine are unclear. Although the concentrations of parasite DNA in peripheral blood and saliva are very different, the demonstration of a significant statistical correlation between blood parasite density and the amount of parasite DNA in saliva indicates that saliva sampling could be potentially useful for detecting malaria cases in longitudinal follow-up studies and for endpoint measurements in clinical trials.

In malaria-endemic areas, the microscopic detection of parasites—even with an accompanying febrile condition—is relatively nonspecific for malaria disease [28–30] owing to the acquisition of partial immunity and the high prevalence of asymptomatic parasite carriage in the population. Therefore, the use of parasite density thresholds (e.g., >1000, >3000, or >5000 parasites/μL) has been recommended for more specific clinical case definitions of malaria at different levels of endemicity [28, 31] and for end-point determination in vaccine trials in malaria-endemic areas [32, 33]. This is particularly relevant for assessing the efficacy of blood-stage vaccine candidates that would be expected to act by reducing parasite density to levels that prevent clinical disease but would not elicit sterile immunity [33, 34]. Thus, an assay with high specificity but lower sensitivity than microscopy might be adequate to measure vaccine efficacy in malaria-endemic populations by excluding clinically irrelevant parasitemia. Our results indicate that PCR of saliva has potential application for endpoint determination in clinical trials, and after further optimization of the assay protocol to improve sensitivity, it could serve as a complement to blood sampling for longitudinal surveillance in antimalarial intervention trials.

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