Detecting Malaria Parasites outside the Blood

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(See the article by Nwakanma et al., on pages 1567–74.)

In 1880, Charles Laveran first visualized the protozoan parasite that causes malaria in a smear of blood under a microscope, and from the early decades of the 20th century to date, microscopy has been the mainstay of malaria detection. In laboratories throughout the world, routine diagnosis is carried out by microscopic examination of Giemsa-stained blood films for the intraerythrocytic asexual stages of the \textit{Plasmodium} parasite that are indicative of active malaria infection. Clinical trials of antimalarial interventions also rely on good-quality slide reading for endpoint determination. In recent years, molecular methods for detecting the DNA of \textit{Plasmodium} species that infect humans have been incorporated into some trial protocols to increase the sensitivity of detection (polymerase chain reaction [PCR] is typically 1–2 orders of magnitude more sensitive than microscopy), to distinguish genetically distinct parasite lineages, and to answer additional questions, such as the prevalence of mutations associated with drug resistance \cite{1, 2}.

A major constraint for such studies is the need to obtain blood samples repeatedly during posttreatment follow-up; these samples are often collected from infants, young children, and pregnant women, who bear the brunt of the malaria disease burden and are therefore the targets of most antimalarial interventions. Blood sampling, with its requirement for trained personnel, an attendant risk of infection and, in some countries, associated taboos can result in poor compliance when repeated testing is needed.

In this issue of the \textit{Journal}, Nwakanma et al. \cite{3} have comprehensively evaluated the potential of saliva and urine samples as alternative sources of parasite DNA from \textit{Plasmodium falciparum}, the species that causes the majority of malaria deaths worldwide and that predominates in sub-Saharan Africa. After obtaining blood, saliva, and urine samples from Gambian patients with suspected malaria infections, skilled microscopists carried out standard diagnosis and DNA was extracted from all samples for analysis by 2 methods: an established conventional nested PCR \cite{1} was used to provide a benchmark for diagnostic sensitivity and specificity, and a quantitative PCR (qPCR) was used to estimate the amount of parasite DNA in these biological fluids.

The study demonstrated that nested PCR, using DNA extracted from saliva samples, had encouragingly high sensitivity of 73% and specificity of 97%, compared with microscopic examination of peripheral blood samples obtained simultaneously. Moreover, a significant positive correlation was observed between parasite counts established by microscopy and estimates of parasite DNA in saliva by qPCR \((r = 0.58; P < .001)\). When DNA extracted from urine was used as a template, the sensitivity of nested PCR compared with microscopy of blood was much lower (32%), and the correlation between counts established by microscopy and qPCR results was not significant. This suggests that either insufficient parasite DNA is excreted in the urine for these samples to provide a useful amplification template or that novel approaches to isolation, purification, and concentration are needed for DNA obtained from urine samples.

In addition to diagnostic applications, the possibility that DNA from saliva samples could be used to distinguish variant parasite genotypes in samples from patients with malaria was raised by Mhara- kurwa et al. \cite{4}, who attempted to amplify a polymorphic region of the \textit{P. falciparum} \textit{msp2} gene from blood, saliva, and urine samples obtained from individuals in Zambia who tested positive for malaria by microscopy. These authors also found that urine rarely yielded a PCR product, whereas the \textit{msp2} genotypes amplified from saliva samples regularly matched those found in blood samples obtained from the same person. The chance of amplifying parasite DNA from saliva samples improved as parasite density in the blood...
increased, as might be expected, but success was also dependent on the DNA extraction method and PCR primer set used, providing some pointers as to how sensitivity may be increased. Mharakurwa et al. [4] observed that shorter PCR amplicons were more likely to be amplified from DNA extracts obtained from saliva, which suggests that parasite DNA may be very fragmented by the time it has reached the saliva. This result has previously been observed for amplified human DNA derived from frozen urine samples [5].

The route of Plasmodium DNA into the saliva is not defined at present. Whole saliva consists of secretions from the salivary glands, bronchial and nasal secretions, bacteria, viruses, fungi, and epithelial cells. Blood and blood derivatives enter the saliva via intraoral bleeding (serum and cells) and the gingival crevicular fluid (serum exudates and inflammatory cells) [6]. Plasmodium DNA from lysed parasites may be carried passively via the serum or within the phagosomes of macrophages.

As the most readily available and easily collected biological fluid, saliva samples are already used in the detection of many immunoglobulins, hormones, drug levels, and electrolytes. Oral fluid–based diagnostic tests for antibodies against viruses that cause infectious diseases, such as HIV and hepatitis viruses A, B, and C, are well established, as are saliva–based surveillance assays for monitoring immune status, for example against the measles, mumps, and rubella vaccine [7]. As a source of nucleic acids, saliva has been investigated for the direct detection of viruses (herpes simplex virus and varicella–zoster virus) [8] and bacteria (Helicobacter pylori) [9]. However, as Nwakanma et al. [3] note, PCR of saliva DNA would not be a useful method for clinical diagnosis of malaria in its current form; it is less sensitive than microscopy, and PCR requires greater time to obtain a result. Because molecular methods require a laboratory with electricity, specialized equipment, and trained staff, they are unsuitable for many settings in which malaria diagnosis is needed. Finally, the acceptability of saliva–based testing may be a problem—when people are sick and malaria is suspected, a blood test is expected.

So, when might a saliva–based PCR for malaria actually be employed? Antimalarial drug trials are one possibility. In such trials, repeated sampling is desirable during the days immediately after drug treatment to assess parasite clearance. This may be a particularly useful strategy for new efficacious artemisinin combination therapies, because delayed parasite clearance is predicted to be an important early sign of the development of drug tolerance [10]. The finding that the genotypes of parasites in the blood are reflected in the saliva is good news for studies of intrahost dynamics, in which single time–point sampling of peripheral blood reveals little about the complex fluctuations in parasite populations over the course of an infection. Malaria vaccine trials are another potential application. Current vaccine candidates are not expected to induce sterile immunity, and measuring the time to infection by PCR is a useful end point to assess the level of protection. Again, this requires that samples be obtained repeatedly from volunteers. Sentinel surveillance is yet another situation in which saliva–based PCR might be used. As we move towards programs for malaria elimination in some parts of the world, longitudinal surveillance will be required, and less-invasive sampling techniques would improve population coverage. Furthermore, assessment of the impact of other interventions, such as intermittent preventive treatment and insecticide–treated nets, would be enhanced if the prevalence of parasites in asymptomatic individuals could be measured in a less invasive and ethically acceptable way.

An improvement in the sensitivity of saliva–based PCR is needed before this method can become a tool with real utility. Nwakanma et al. [3] found that the sensitivity of their nested PCR increased to 82% when they restricted their analysis to samples with parasite densities ≥1000 parasites/μL. Although this may serve as a useful cutoff to define a clinical case of malaria, the potential applications outlined above would require the sensitivity of a saliva–based PCR to approach that of a blood–based method. This necessitates not only the design of PCR based around shorter amplicons [4, 5] but also stringent empirical evaluation of the impact of PCR inhibitors on candidate tests [11]. Further exploration of the time during infection at which parasite DNA appears in the saliva and its persistence after clearance of blood parasites is also essential.

The potential use of saliva for isolation of Plasmodium DNA comes alongside an increasing interest in bringing appropriate molecular methods to field settings. For example, a lateral flow immunoassay to detect PCR–amplified P. falciparum nucleic acids was tested recently in a clinical trial in Kenya and its results compared well with those of standard gel electrophoresis [12]. If this could be coupled with a simpler amplification method that does not require thermal cycling, such as nucleic acid sequence–based amplification or loop–activated amplification, it can be envisioned that high–throughput nucleic acid detection in the form of a dipstick might be available for future clinical trials, which would enable genotypic end points to be incorporated. It is hoped that developments such as these will lead toward more ambitious and informative studies and surveillance programs in which participants are not asked to undergo repeated needlesticks and analyses that previously required the shipment of samples to specialist laboratories and a delay in the production of data can be carried out quickly, on–site, and with larger populations than before.

References


