Field Evaluation of a Polymerase Chain Reaction–Based Nonisotopic Liquid Hybridization Assay for Malaria Diagnosis

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In a blind field evaluation of a nonisotopic liquid hybridization assay for detection of malaria parasites, 100 blood samples were tested from an area in which malaria is endemic; light microscopy was used as the reference test. Sensitivity, specificity, and positive and negative predictive values of the hybridization assay were 100%. One sample that was microscopy-negative and hybridization-positive was positive when reexamined. Another sample that was microscopy-positive and hybridization-negative was negative at reexamination. The detection limit of the test was ~0.0005% parasitemia. Four samples with mixed infections were misdiagnosed by microscopy as single-species infections. Four samples diagnosed as mixed infections by microscopy and single infection by the hybridization test had no evidence of a second *Plasmodium* species upon reexamination. The polymerase chain reaction–based nonisotopic liquid hybridization assay was better than conventional light microscopy in detecting low-grade parasite infection and offers an exceptional advantage for detecting mixed infections.

A definite diagnosis of malaria on the basis of clinical symptoms is very difficult [1]. For the past century, laboratory diagnosis of malaria has depended on visualization of the parasite by light microscopy. This procedure is simple and inexpensive but time and labor intensive and requires expert knowledge of the morphologic differentiation of *Plasmodium* species [2]. In addition, detection of mixed infections is a problem even to well-trained microscopists because 1 species can dominate the course of an infection [3]. Tests of alternatives to microscopy for malaria diagnosis have had varying specificities and sensitivities, and most do not match microscopy results [4–6].

Because of the limitations of the various procedures for malaria diagnosis, new approaches are warranted and badly needed. We sought to develop a fast, reliable, specific, sensitive, and field-usable malaria diagnosis assay. Our efforts resulted in a polymerase chain reaction (PCR)–based nonisotopic liquid hybridization assay [7]. Here we report the results of a blinded evaluation of this assay under field conditions that assessed the sensitivity, specificity, and predicted values of positive and negative tests compared with those of light microscopy.

Materials and Methods

Subjects. We evaluated 100 3- to 16-year-old students (55% were girls) who attended three schools in the Assembo Bay area of Kisumu, western Kenya, where malaria is endemic.

Microscopic diagnosis. Thick and thin blood films were prepared by fingerprick during the collection process. Slides were stained with Giemsa and analyzed for the presence of parasites and for parasite species. Analyses were done by expert microscopists from the Kenya Medical Research Institute (KEMRI) and the
Division of Parasitic Diseases (CDC). Parasites were quantified by counting the number of infected red blood cells (RBCs) per 300 white blood cells (WBCs). Parasitemia was calculated assuming $8 \times 10^3$ WBCs and $5 \times 10^6$ RBCs/mL of blood [8]. Samples were considered to be negative after 100 microscopic thick fields were examined with an oil immersion lens at $\times 1000$ magnification.

**Oligonucleotide primers and probes.** Sequence, specificity, and synthesis of amplification primers and revealing probes have been described [7].

**DNA preparation and amplification.** Venous blood samples (200 $\mu$L) were collected in tubes containing EDTA. Blood was centrifuged at 600 $g$ for 5 min at room temperature, and plasma and buffy coat containing WBCs were removed. The RBC pellet was used for DNA extraction using the QIAamp Blood Kit (QIAGEN, Chatsworth, CA) as described by the manufacturer. Extracted DNA was amplified as previously described [7], and negative controls were processed in each experiment.

**Detection of PCR products.** Amplified PCR products were detected by a nonisotopic liquid hybridization technique as described [7]. PCR products were also detected by running 10 $\mu$L of the PCR reaction mixture on a standard 1.2% ethidium bromide-stained electrophoretic agarose gel, which was visualized under UV light.

**Results**

Blood samples from 100 subjects were tested blindly by microscopy and by a PCR-based liquid hybridization assay for the presence of malaria [7]. The code was broken after the experiments were completed.

Results of conventional light microscopy and hybridization tests are summarized in table 1. Both methods yielded 74 positive and 24 negative samples. One sample was hybridization-positive and microscopy-negative; another was microscopy-positive at the 0.001% parasitemia level and hybridization-negative. We used microscopy as our reference test. Thus, these preliminary data resulted in hybridization sensitivity and specificity and positive and negative predictive values of 98.6%, 96.0%, 98.6%, and 96.0%, respectively. The 2 samples with contradictory results were blindly reevaluated by both assays. When the slide that was initially microscopy-negative and hybridization-positive was reexamined by 3 different microscopists, 2 examiners considered it positive with a parasitemia level of 0.0005%. The slide that was positive by microscopy and negative by hybridization was reexamined by 2 expert microscopists who found no parasites. The reexamination thus demonstrated that the “low” specificity and negative predictive value and the “low” sensitivity and positive predictive value of the hybridization test were due to a lack of sensitivity and specificity of microscopic evaluation, respectively. On the basis of these data, when the sensitivity, specificity, and positive and negative predictive values of the hybridization assay were recalculated, they were 100%. There were no false-positive or false-negative results by the PCR assay.

Table 2 shows mixed infections detected by microscopy and hybridization. Among the 12 samples that tested positive for >1 species by either assay, 4 had similar results by the 2 procedures. In the remaining 8 samples, 4 were positive for mixed infection by microscopy and negative by hybridization, and 4 were positive by hybridization and negative by microscopy. When these slides were reexamined, microscopy led to misdiagnoses in 7 of 8 samples. No conclusion could be reached for 1 sample, which was poorly stained. Most of the mixed infections were due to a *Plasmodium falciparum*–*Plasmodium malariae* association. One sample showed a *P. falciparum*–*Plasmodium ovale* mixed infection.

Parasite densities detected by hybridization were 0.0005%–0.64%. These results indicate that the assay could detect $\geq 0.0005\%$ parasitemia, the lowest level of parasitemia found among the 100 samples.

Detection of PCR-amplified products by the nonisotopic liquid hybridization technique was compared with detection by standard 1.2% ethidium bromide–stained agarose gel electrophoresis. The agarose gel failed to detect 15 of 75 positive samples, yielding 20% false-negatives.

**Discussion**

We developed a PCR-based nonisotopic liquid hybridization technique to detect human malaria parasites [7]. In laboratory-induced malaria infections in monkeys, this test was specific for the 4 human *Plasmodium* species. The assay detected 300 fg of DNA and $\geq 0.0001\%$ parasitemia. Our strategy for the PCR amplification uses a genus-conserved oligonucleotide primer pair yielding a 678-bp fragment of the small subunit ribosomal RNA. Variable sequences in the center of this region were exploited to design species-specific oligonucleotide

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**Table 1. Number of malaria cases detected by microscopy and by PCR-based nonisotopic liquid hybridization.**

<table>
<thead>
<tr>
<th>Hybridization</th>
<th>Initial microscopy</th>
<th>Microscopy after reexamination of sample results</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>Positive</td>
<td>74</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>24</td>
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<tr>
<td>Total</td>
<td>75</td>
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probes. This approach is an alternative to using probes against chromosomal DNA targets and offers a distinct advantage for species- and stage-specific rRNA expression.

Here we report a field evaluation of this test. In a blinded study, blood samples were tested by the hybridization assay and results were compared with microscopy (the reference standard). Although the reference standard was assumed to have a sensitivity and specificity of 100%, several studies have shown this assumption to be false [9, 10]. The lack of sensitivity and specificity of microscopy is even more critical for detection of mixed infections [11, 12]. Nevertheless, microscopy remains the cornerstone to which other diagnostic tests are compared.

Initial hybridization and microscopy results were 98% in agreement; 2 of 100 samples yielded contradictory results. One of these, a hybridization-positive and microscopy-negative sample, could have resulted from a hybridization false-positive, an incorrect microscopic identification, or the lower limit of detection of the hybridization method. The other contradictory sample (hybridization-negative and microscopy-positive) could have been a hybridization false-negative or an incorrect microscopic identification. To address these possibilities, the samples were reevaluated by both tests. While hybridization results remained consistent with the original evaluation, the microscopy reexamination did not agree with the initial thick blood smear reading. In the subsequent examination, a false-positive and a false-negative result originally attributed to hybridization were found to be microscopy errors. The reevaluation of the slides at a higher sensitivity rendered hybridization at least as sensitive and specific as microscopy: Sensitivity was 100% in detecting malaria parasites in all of the true positive cases, and specificity was 100% in all of the true negative cases.

The hybridization technique could detect as low as 1 parasite per 300 WBCs, corresponding to 0.0005% parasitized RBCs. Since the test could detect the lowest level of parasitemia present in the 100 samples, it was not possible to precisely determine the assay’s lower limit of detection.

The hybridization assay was remarkably better than microscopy in detecting mixed infections. Twelve of 100 samples were either hybridization- or microscopy-positive for mixed infection (Table 2). Four of the 12 samples had similar results by both assays. When the 8 remaining samples were reexamined, 4 were false-positive and 3 were false-negative by microscopy. No conclusion could be reached for 1 sample, which was poorly stained. Failure to detect mixed infections by microscopy could lead to inadequate treatment of patients. The 3 patients infected only with P. falciparum, but misdiagnosed by microscopy as being infected with P. falciparum and P. ovale, would be treated unnecessarily with drugs to eradicate hypnozoites. Conversely, a patient with a mixed P. falciparum–P. ovale infection, who was diagnosed by microscopy as being infected only with P. falciparum, would not receive the proper treatment. In addition, the microscopic detection of only P. malariae in a sample that also contained P. falciparum could potentially harm the patient in areas of chloroquine resistance.

When we compared the detection efficacy of agarose gel versus liquid hybridization, we found that 15 of 75 hybridization-positive samples were not positive by the conventional agarose ethidium bromide–stained electrophoresis gel technique. This result confirms our previous finding that liquid hybridization was at least 1 log more sensitive than agarose gel for detecting amplified PCR fragments [7]. Not only is the agarose gel inadequate to reveal a few nanograms of DNA, the finding of a band of the expected size is not definite proof of the specificity of the amplification. Therefore, the assay that is based on a hybridization detection of the PCR fragment is far more sensitive and specific than other assays based on gel electrophoresis [13].

### Table 2. Detection of mixed human malaria (Plasmodium) infections by microscopy and hybridization.

<table>
<thead>
<tr>
<th>Hybridization</th>
<th>Microscopy</th>
<th>After reexamination of sample results</th>
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In addition to the field test reported here, the PCR-based liquid hybridization assay performed exceptionally well in a molecular epidemiologic study in a region of Brazil in which malaria is endemic (unpublished data). The epidemiologic study investigated the prevalence of *Plasmodium vivax* types 1 and 2 and a *P. vivax*-like parasite using *P. vivax* types 1 and 2 and *P. vivax*-like specific detection probes. The hybridization procedure was also used to monitor the presence of parasites in sporozoite-induced *P. vivax* infections in *Saimiri* monkeys (Grady KK, unpublished data). The results were compared with those obtained by examination of Giemsa-stained thick blood films. Parasitemia was detected earlier by the hybridization method, and some animals that were never parasitemic by blood-film examination had short periods of subpatent parasitemia determined by hybridization.

Taken together, the hybridization assay is highly reproducible, field-usable, and extremely specific and sensitive. Furthermore, the test is compatible with standard ELISA and other diagnostic laboratory equipment, permitting the processing of a large number of samples. In its present form, the test can be used in research-based epidemiologic surveillance, field assessment of potential vaccines, mass surveys, and the rational deployment of treatment and control programs.

In conclusion, we have shown that a PCR-based nonisotopic liquid hybridization technique for diagnosis of malaria can be used with a high degree of confidence. The evaluation of the test was difficult because it was potentially of greater sensitivity and specificity than the reference microscopy test. Our results demonstrate that nucleic acid–based approaches can provide improved diagnostic tests for malaria. The next step will be to adapt this technology into a simple-to-use slide or dipstick format so that field-based malaria clinicians and technicians can use it with confidence.

**Acknowledgment**

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**References**