



Detection and Species Identification of Malaria Parasites by Isothermal tHDA Amplification Directly from Human Blood without Sample Preparation

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Accepted for publication
 May 15, 2013.

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We report the clinical and analytical performance of an isothermal thermophilic helicase-dependent amplification assay for blood *Plasmodium* parasite detection and species-level identification. The assay amplifies the 18S rRNA gene fragment of all *Plasmodium* species and uses a species-specific probe and a pan-malarial probe to definitively identify *Plasmodium falciparum* from other infectious *Plasmodium* species. Amplicon-probe hybridization products are detected with a disposable dipstick enclosed in a cassette. With a pan-malarial—positive and *P. falciparum*—negative result, an additional test is performed to detect if the pan-malarial—positive band was the result of the presence of *Plasmodium vivax*. The assay uses only 2 μ L of human whole blood directly for a 50- μ L amplification reaction, without any pre-amplification processing. The clinical performance of the assay was validated using 88 samples from New York patients suspected of malaria or babesiosis. The overall sensitivity of the assay was 96.6% (95% CI, 87.3% to 99.4%), and the specificity was 100% (95% CI, 85.4% to 100%), compared with gold standard microscopy and a laboratory-developed molecular assay, respectively. The analytical sensitivity was 50 copies of DNA per assay or 200 parasites per microliter of blood, and the assay can detect samples with parasitemia levels <1%. This novel molecular diagnostic assay requires minimal laboratory instrumentation and uses un-processed blood as input; it can be readily performed in the field. (*J Mol Diagn* 2013, 15: 634–641; <http://dx.doi.org/10.1016/j.jmoldx.2013.05.005>)

Malaria is a mosquito-borne disease caused by intraerythrocytic protozoa of the genus *Plasmodium*. Five *Plasmodium* species are known to infect human beings: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*.¹ People infected by the malaria parasite experience fever, chills, and a flu-like illness that, if left untreated, may develop into severe complications, potentially leading to death. The World Health Organization reports that, in 2010, approximately 216 million people were infected with malaria, and 655,000 died as a result of their infection (World Malaria Report 2011, http://www.who.int/malaria/world_malaria_report_2011/en, last accessed June 24, 2013). Most fatalities (91%) occur in Africa, and are due to infection by *P. falciparum*. Although relatively less

severe than *P. falciparum*, *P. vivax* and *P. ovale* can enter a dormant stage as hypnozoites in the liver of the host and cause relapses by invading the bloodstream weeks, or even years, later (Centers for Disease Control and Prevention, <http://www.cdc.gov/malaria/about/facts.html>, last accessed June 24, 2013). Because *P. vivax* and *P. ovale* present a health risk for travelers to the Middle East, Asia, South America, and Central America, *P. falciparum* and *P. vivax* have different

Supported by National Institute of Allergy and Infectious Diseases award R43AI088787.

Disclosures: Y.L., B.L., and H.K. are employees of BioHelix Corporation, which supplied the tHDA amplification reagents.

drug-resistance profiles²; thus, species-specific identification is prudent if patients come from regions of the globe where more than one species is present.

Although microscopy of stained thick or thin blood film smears remains the gold standard for diagnosis of malaria infection, properly identifying the species of *Plasmodium* in a patient's blood is difficult for western clinical laboratories.³ The rapid lateral-flow antigen detection tests target the histidine-rich protein of *P. falciparum* and a pan-malarial lactose dehydrogenase protein; however, the persistence of histidine-rich protein of *P. falciparum* in patients receiving therapy renders these rapid lateral-flow antigen detection tests unsuitable for monitoring clearance of the pathogen.⁴ Moreover, recent reports on the deletion of the *HRP2* gene from the *Plasmodium* population in certain endemic areas have demonstrated that this deletion results in significantly higher false-negative diagnoses.^{5,6} Rapid lateral-flow antigen detection tests have good sensitivity for *P. falciparum* but lack sensitivity for the other species using the pan-malarial test line.^{7–9} Therefore, western laboratories often perform a laboratory-developed nucleic acid amplification test to detect and further identify the species of *Plasmodium* present in a patient's blood.^{10,11}

Several isothermal nucleic acid amplification tests have been developed to detect *Plasmodium* using loop-mediated isothermal amplification technology (LAMP).^{12–18} Some of these tests do not offer species discrimination,¹⁶ whereas others do distinguish between the species.^{15,18} Some target the multicopy rRNA genes,^{12,17} whereas others target other sequences also present in multiple copies in each cell.^{13,16} LAMP is viewed as a good platform for the detection of malaria in the field because it can be performed using a constant temperature incubator or water bath,¹⁹ and it allows for the detection of amplification products using the naked eye.²⁰ However, the complexity of the LAMP reaction, and its reliance on the use of six primer pairs, makes LAMP assays more difficult to manufacture than PCR tests, and complicates assay design.²¹ In addition, the LAMP-based assays still require dilution and heating of the blood sample before isothermal amplification,¹⁶ which potentially increases the complexity of assay workflow.

In this study, we report the development of an alternative isothermal nucleic acid amplification platform that can detect all five species of *Plasmodium*, and can specifically differentiate *P. falciparum* and *P. vivax* parasites using a simple, handheld, disposable detection device. The assay uses thermophilic helicase-dependent amplification (tHDA)^{22,23} chemistry to amplify the 18S rRNA genes²⁴ of all *Plasmodium* species, followed by a species-specific, probe-based detection of *P. falciparum* versus the other *Plasmodium* species. A reflex test, using one species-specific primer, is performed to resolve *P. vivax* from *P. ovale*, *P. malariae*, and *P. knowlesi*. Because tHDA can be performed using a small amount (2 μ L) of unprocessed blood and a dry heat block, this assay has the potential of being used as a point-of-care device. The sensitivity and specificity of this assay were evaluated with a panel of 88 blood samples collected from either

symptomatic travelers returning from malaria-endemic countries to the New York City area or patients from eastern Long Island with a diagnosis of possible *Babesia* infection.

Materials and Methods

Control DNA

Genomic DNA of *P. falciparum* (3D7 strain) was purified from laboratory culture-derived parasites by the Chelex method.^{25,26} Briefly, cultured 3D7 parasites were mixed with red blood cells and normal human serum to achieve a final concentration of 50% hematocrit and 50% normal human serum; then, they were spotted on Whatman filter paper and allowed to dry at room temperature overnight. The dried filter paper was soaked in sterile 0.5% saponin in PBS for 30 minutes at 37°C. The filter paper was washed with sterile PBS and treated with 200 μ L of 5% Chelex 100 (Bio-Rad, Hercules, CA) (preheated at 65°C for 10 minutes) at 100°C. The supernatant obtained after centrifugation (2 minutes at 10,000 \times g) was respun to ensure complete removal of Chelex. The resulting supernatant was stored at –20°C until further use. Dried blood spots of *P. vivax*, *P. ovale*, and *P. malariae* on filter papers were obtained from the Malaria Research and Reference Reagent Resource Center (Manassas, VA). DNA was extracted by the Chelex method, as previously described. The total DNA concentration was assessed by OD₂₆₀ measurement. The OD₂₆₀/OD₂₈₀ ratio was also measured, and the values were approximately 2.0, indicating acceptable purity of the extracted DNA.

Plasmids carrying partial sequences of the 18S rRNA of *P. malariae*, *P. knowlesi*, *P. ovale*, *P. vivax*, or the internal control (IC) sequences were synthesized by BlueHeron (Bothell, WA) by cloning the desired fragment of 200 to 400 bp into pUC vector.

Clinical Samples

Fresh negative human whole blood used in spiking experiments was purchased from Research Blood Components (Boston, MA). EDTA whole blood samples ($n = 88$) collected from 55 patients between October 2009 and January 2012 for routine diagnostic purposes, from either symptomatic travelers returning from malaria-endemic countries to the New York City area or patients from eastern Long Island with a diagnosis of possible *Babesia* infection, were used for this clinical evaluation. At sample collection, EDTA whole blood samples were tested by thin-and-thick blood film microscopy and a nucleic acid sequence-based amplification malaria test (NASBAMT) developed by a laboratory^{10,11} (North Shore–LIJ Health System Laboratories, Lake Success, NY). Thirty-nine samples from 20 patients contained *P. falciparum*, 16 samples from nine patients contained *P. vivax*, three samples from two patients contained *P. malariae*, one sample from one patient contained *P. ovale*, 15 samples from 10 patients contained *Babesia* species, and 14 samples from 13 patients were negative for both *Plasmodium* and *Babesia*.

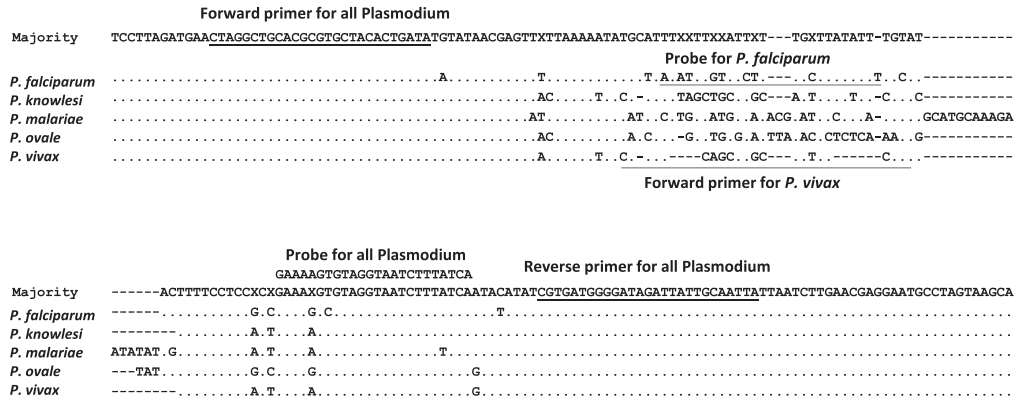


Figure 1 Alignment of *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale*, and *P. vivax* 18S rRNA sequences. The sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide>; accession numbers AL010278, L07560, AB489196, AJ001527, and U93233) for each species, respectively. Only the region surrounding the tHDA amplification product is shown. The primers and probes used in the two tHDA tests are indicated on the alignment.

Samples from the same patients were collected on different days or at different times on the same day. Samples were stored frozen at -80°C until shipment to BioHelix Corporation (Beverly, MA) for the tHDA-based molecular tests. Whole blood samples were de-identified in accordance with a protocol approved by the Feinstein Institute for Medical Research Institutional Review Board (Manhasset, NY). All samples provided to BioHelix Corporation were stripped of all patient identifiers and provided with a reference number. Samples used for initial assay verification contained *Plasmodium* or *Babesia* or were negative for both. The initial panel of samples ($n = 20$) was provided with microscopy and NASBAMT results. A second panel of samples ($n = 68$) containing various types of *Plasmodium* and *Babesia* and samples negative for both blood parasites were provided blinded (without microscopy and NASBAMT results) to BioHelix Corporation.

Design of Primers, Probes, and ICs for tHDA Amplification

The tHDA-based malaria detection and typing assay consisted of two separate tests. The first test (test 1) detects all five *Plasmodium* species, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*, known to infect humans. In addition, it selectively identifies the presence of *P. falciparum*. Primers for tHDA amplification in test 1 were designed using Primer3 software version 0.4.0,²⁷ following the specifications described in the IsoAmp III tHDA amplification kit (BioHelix Corporation). Primer pairs were evaluated by real-time tHDA amplification in the presence of EvaGreen fluorescent dye (Biotium, Hayward, CA). The tHDA reaction conditions were then optimized using the best-performing primer pairs. Asymmetric tHDA amplification conditions were evaluated with one of the primers

Table 1 Primer, Probe, and IC Sequences

Test no.	Primer/probe	Sequence	Label	Concentration (nmol/L)
1	Forward primer	5'-CTAGGCTGCACGCGTGTACACTGATA-3'	None	40
	Reverse primer	5'-TAATTGCAATAATCTATCCCCATCAGC-3'	5' Biotin	120
	Probe for all 5 <i>Plasmodium</i> species	5'-GAAAAGTGTAGGTAATCTTTATCA-3'	3' DIG	50
	Probe for <i>P. falciparum</i>	5'-ATATTTGTATCTTTGCTTATATT-3'	3' FAM	30
	Probe for IC	5'-CCGCTATTTTCATAGTAAGTC-3'	3' DNP	30
	IC 1	5'-CTAGGCTGCACGCGTGTACACTGATACAACATTGACAACCTA AAACCGCTATTTTCATAGTAAGTCCTTCGCTCTATGACTATCTGT GCCAAATCATCTAGCAAACCTGCTAATCCAATTTATTTTCATCAA ATCGTGATGGGGATAGATTATTGCAATTA-3'	None	500 Copies
2	Forward primer	5'-CGATTCAGCTTGCTGTTTCGTA-3'	None	50
	Reverse primer	Same as in test 1	None	100
	Probe for <i>P. vivax</i>	Same as in test 1	3' DIG	30
	Probe for IC	Same as in test 1	3' DNP	30
	IC 2	5'-CGATTCAGCTTGCTGTTTCGTAATTTTCTCAACATTGACAA CTAAAACCGCTATTTTCATAGTAAGTCCTTCGCTCTATGACTATC TGTGCCAAATCATCTAGCAAACCTGCTAATCCAATTTATTTTCAT CAAATCGTGATGGGGATAGATTATTGCAATTA-3'	None	2000 Copies

Forward and reverse primer-binding regions are underlined.

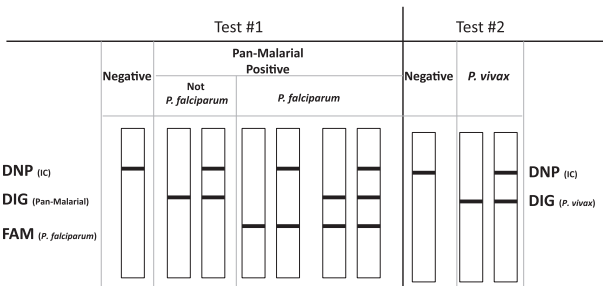


Figure 2 Schematic diagram of the tHDA-based malaria assay results. There are three antibody lines on the lateral-flow test strip: anti-DNP, anti-DIG, and anti-FAM (from top to bottom). In test 1, if only the DNP line is present, the test result is negative for *Plasmodium*. If the FAM line is present (the other two lines can be present or absent), the test result is positive for *P. falciparum*. If the FAM line is absent, but the DIG line is present (the DNP line can be either present or absent), the result is positive for *Plasmodium* but negative for *P. falciparum*. In test 2, if only the DNP line is present, the test result is negative for *P. vivax*; and if the DIG line is present (the DNP line can be either present or absent), the test is positive for *P. vivax*. The FAM line should always be absent for test 2.

labeled with biotin at 5' and in excess to the other unlabeled primer.

A digoxigenin (DIG)-labeled probe was designed to anneal to a conserved region in the tHDA amplification product among all five *Plasmodium* species. A second probe labeled with FAM was designed to anneal specifically only to *P. falciparum* but not the other four species. After amplification, the reactions were applied to BEST cassettes (BioHelix Corporation). The probes annealed to the DNA strand were generated by the biotin-labeled primer at room temperature and were captured by anti-DIG or anti-FAM antibody lines on a lateral-flow test strip in the BEST cassettes. Detection of the probe-amplicon complex was achieved using streptavidin-coated color latex particles.²⁸

An IC sequence was designed to be amplified by the same primer pairs as the target amplicon, with a random sequence in the middle between the primers. The IC sequence was cloned into a pUC plasmid and included in the tHDA amplification reaction. A probe labeled with 2,4-dinitrophenyl (DNP) was designed to anneal to the IC sequence. The amplification products were detected on the lateral-flow strip by an anti-DNP antibody.

The second test (test 2), which serves as a reflex test, detects the presence of *P. vivax*. Amplification of *P. vivax* in test 2 used the same reverse primer as in test 1. The forward primer was designed to be specific for *P. vivax* and would not amplify other *Plasmodium* species. The same DIG-labeled detection probe in test 1 was also used in test 2. After tHDA amplification, the product was detected on the lateral-flow strip through anti-DIG antibodies. An IC sequence for this test was designed similar to test 1. The IC sequence for test 2 was the same as that for test 1, except for the forward primer-binding region, which only binds the forward primer for *P. vivax*. This IC sequence was also cloned into a pUC plasmid and included in the reaction, and its amplification

product was detected on the lateral-flow strip through binding to the same DNP-labeled probe as in test 1.

The DNP-labeled probe for the ICs was synthesized and high-performance liquid chromatography purified by Tri-Link (San Diego, CA). All other primers and probes were synthesized and high-performance liquid chromatography purified by Eurofins MWG Operon (Huntsville, AL).

Assay Procedure

Both tHDA tests 1 and 2 were performed in a 50- μ L reaction volume. Test 1 contained 1 \times annealing buffer II (BioHelix Corporation), 4 mmol/L MgSO₄, 40 mmol/L NaCl, 3.6 μ L IsoAmp II dNTP mix (BioHelix Corporation), 40 nmol/L DIG-labeled probe for all *Plasmodium* species, 30 nmol/L FAM-labeled *P. falciparum*-specific probe, 30 nmol/L DNP-labeled IC probe, 5 U of DdeI (New England Biolabs, Ipswich, MA), 150 ng extreme thermostable single-stranded DNA binding protein (BioHelix Corporation), 500 copies of the IC 1 IC plasmid, 2 μ L IsoAmp III Enzyme Mix (BioHelix Corporation), and input template, as indicated in the figures and text. Test 2 was performed in similar conditions as test 1, except for the primers/probes and IC, which contained 50 nmol/L of the *P. vivax*-specific forward primer, 100 nmol/L reverse primer, 30 nmol/L DIG-labeled probe, 30 nmol/L DNP-labeled IC probe, and 2000 copies of IC 2 IC plasmid. The reaction mixture was allowed to incubate at 64°C for 90

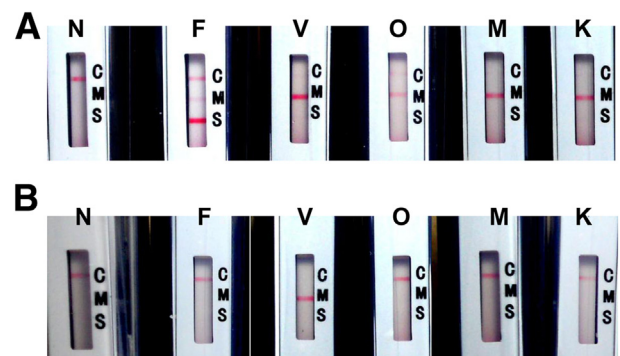


Figure 3 Amplification and detection of all five *Plasmodium* species and identification of *P. falciparum* and *P. vivax* by tHDA and lateral-flow cassettes. **A:** Test 1. Genomic DNA of *P. falciparum* (lane F) or plasmids containing the 18S rRNA fragment of the other *Plasmodium* species (lanes V, O, M, and K) were added to 50 μ L of tHDA test 1 amplification reaction to a final amount of 50 or 500 copies. **B:** Test 2. Plasmid DNA containing the 18S rRNA fragment of *P. vivax* was added to tHDA test 2 at 50 copies per reaction (lane V). Plasmid containing *P. ovale*, *P. malariae*, or *P. knowlesi* DNA fragment or genomic DNA of *P. falciparum* was added to tHDA test 2 at 1000 \times limit of detection (final 50,000 copies) per reaction (lanes F, O, M, and K). In both **A** and **B**, the reactions were incubated at 64°C for 90 minutes before the tubes were applied to BEST cassettes. Negative reactions without input template (NTC) were also performed (lane N). Each reaction was performed in triplicate. In **A**, only NTC and reactions containing 50 copies of the input DNA template were shown. Only one of the triplicates was shown for each reaction. Label C on cassettes indicates the DNP line for IC; M, DIG line for *Plasmodium*; and S, FAM line for *P. falciparum*.

Table 2 Parasitemia Level of the Malaria-Positive Clinical Samples

Sample	No. of samples	Range of parasitemia (%)	Mean parasitemia (%)	Median parasitemia (%)
<i>P. falciparum</i>	39	0.01–36.9	4.17	0.7
Others	20	0.03–2	0.55	0.39
All positives	59	0.01–36.9	2.98	0.5

(without blood) or 120 (with blood) minutes. The reaction tubes were applied to BEST cassettes after incubation, and images were obtained 15 minutes later.

Determination of Analytical Sensitivity and Specificity of the tHDA Assay

Genomic DNA from *P. falciparum* was extracted from laboratory culture–derived parasites. The concentration of the DNA was determined by OD₂₆₀ measurement. Tenfold serial dilutions (from 10⁴ to 10 copies per μ L) of the *P. falciparum* genomic DNA were used to determine the limit of detection for the tHDA-based malaria test 1. Because parasite cultures of the other four *Plasmodium* species could not be obtained, plasmids containing a fragment of the 18S rRNA region for *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* were constructed, serially diluted (from 10⁴ to 10 copies per μ L), and used as templates to determine the analytical sensitivity of test 1 for each of these four species and that of test 2 for *P. vivax*. Each dilution was tested in triplicate. High copy numbers (50,000) of either genomic DNA from *P. falciparum* or plasmid DNA containing a partial sequence of *P. ovale*, *P. malariae*, and *P. knowlesi* were used to assess the specificities of test 2.

Amplification by tHDA in the Presence of Unprocessed Human Whole Blood

To test the effects of human blood on tHDA amplification, 2 to 5 μ L of fresh human whole blood (clinical test input volume) was added to each 50- μ L tHDA reaction test 1 containing 50 or 500 copies of *P. falciparum* genomic DNA. Amplification in the presence of human blood was detected using BEST cassettes (BioHelix Corporation).

Determination of Clinical Sample Sensitivity and Specificity

The 88 frozen EDTA whole blood samples were thawed at room temperature just before the tHDA tests. To each of the 50- μ L tHDA reactions in test 1, 2 μ L of the blood sample was added directly without heat denaturation or nucleic acid extraction. Samples being identified by the tHDA test as positive for *Plasmodium* (DIG-line positive) but negative for *P. falciparum* (FAM-line negative) were subjected to test 2 to identify the presence or absence of *P. vivax*. The samples

were tested in tHDA assays either not blinded ($n = 20$) or blinded ($n = 68$). Results were compared with microscopy and NASBAMT. The analytical sensitivity of the tHDA assay from human whole blood samples was evaluated by serial dilutions of the frozen blood samples with known parasitemia levels into fresh blood. Four *P. falciparum* samples with parasitemia levels ranging from 0.3% to 8.8% were diluted up to 2000-fold. The final concentrations of the diluted samples were between 100 and 250 parasites per μ L of blood. Each dilution was tested by tHDA test 1 in duplicates.

Results

Assay Overview

The tHDA-based malaria detection and typing assay consisted of two separate tests. The first test (test 1) detects all five *Plasmodium* species, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*, known to infect humans. In addition, it selectively identifies the presence of *P. falciparum*. The second test (test 2), which serves as a reflex test, detects the presence of *P. vivax*. Both tests 1 and 2

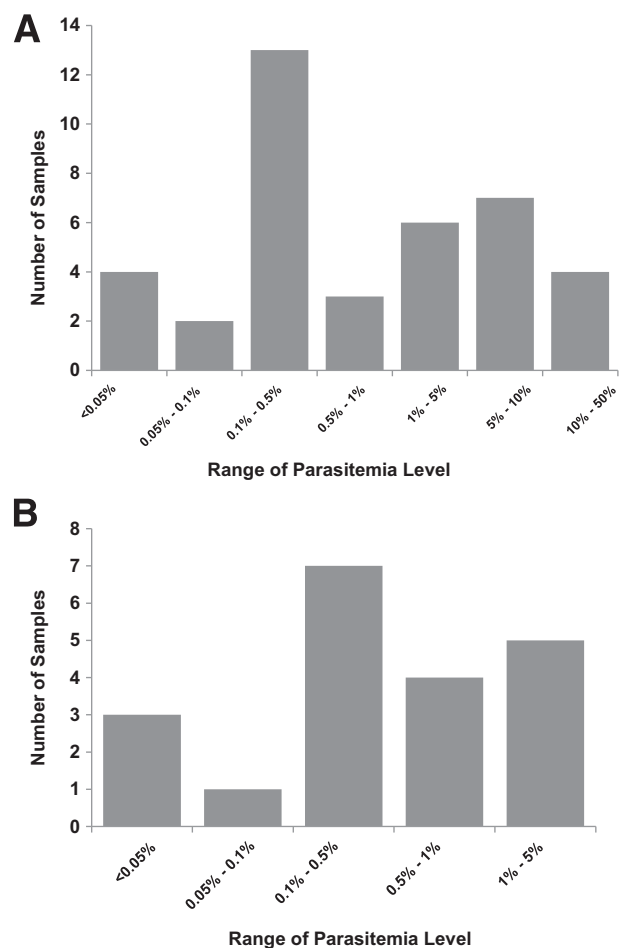


Figure 4 Histogram of the parasitemia levels of the positive clinical samples. **A:** *P. falciparum*-positive samples. **B:** All other *Plasmodium*-positive samples.

Table 3 Clinical Performance of the tHDA Assay Compared with Microscopy/NASBAMT

		Microscopy/NASBAMA		
		Positive	Negative	Total
<i>P. falciparum</i>				
HDA	Positive	38	0	38
	Negative	1	49	50
Total		39	49	88
		95% CI		
Sensitivity (%)		97.4	84.9–99.9	
Specificity (%)		100.0	90.9–100.0	
<i>P. vivax</i>				
HDA	Positive	16	0	16
	Negative	0	72	72
Total		16	71	88
		95% CI		
Sensitivity (%)		100.0	75.9–100.0	
Specificity (%)		100.0	93.7–100.0	
Other <i>Plasmodium</i>				
HDA	Positive	3	0	3
	Negative	1	84	85
Total		4	84	88
		95% CI		
Sensitivity (%)		75.0	21.9–98.7	
Specificity (%)		100.0	94.6–100.0	
Overall				
HDA	Positive	57	0	57
	Negative	2	29	31
Total		59	29	88
		95% CI		
Sensitivity (%)		96.6	87.3–99.4	
Specificity (%)		100.0	85.4–100.0	

are based on isothermal amplification of the 18S rRNA gene (Figure 1) by tHDA platform.^{22,23} In both tests, 2 μ L of human whole blood is added directly to the tHDA reaction mix for DNA amplification. No nucleic acid extraction or any other type of sample preparation is necessary. The reactions are incubated at a constant temperature of 64°C for 2 hours. During incubation, thermal stable UvrD helicase unwinds double-stranded DNA template for hybridization to sequence specific primers, which are subsequently extended by thermal stable DNA polymerase.^{22,23} During amplification, probes labeled with either FAM or DIG hybridize specifically to the target amplicon (Table 1 and Figure 1). The end product is then detected using a disposable lateral-flow strip by binding to anti-FAM or anti-DIG antibodies.²⁸ In both tests, an IC plasmid was included in the reaction mix to monitor the amplification and was detected through a DNP-labeled probe on the lateral-flow strip (Table 1 and Figure 2).

Analytical Sensitivity and Specificity

For all five species, the tHDA-based malaria assay test 1 was able to detect as low as 50 copies of input template DNA

(genomic DNA for *P. falciparum* and constructed plasmid DNA containing sequences of the other four species) per reaction (Figure 3A). This test also successfully identified the presence of as low as 50 copies of *P. falciparum* per reaction (Figure 3A). The specificity of the *P. falciparum* probe was demonstrated by the absence of the FAM line in the tests using any of the other four *Plasmodium* species as input template (Figure 3A).

Similarly, test 2 detected 50 copies of the plasmid containing the *P. vivax* 18S rRNA fragment at 50 copies per reaction (Figure 3B). The amplification was specific for *P. vivax*. The test result was negative (Figure 3B) when 50,000 copies, which was 1000 \times the limit of detection of this test, of *P. falciparum*, *P. ovale*, *P. malariae*, or *P. knowlesi* were used as input template.

Although purified genomic DNA from *Plasmodium* species other than *P. falciparum* could not be obtained, total DNA from dried blood papers containing *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* was used to further verify the ability of test 1 to detect genomic DNA from these species. Different amounts (from 1 to 0.05 μ L) of each extracted sample were added to the 50- μ L test 1 reaction mix. The test was able to detect all four species using as low as 0.05 μ L of sample input (data not shown).

Effect of Human Whole Blood on tHDA Amplification

The effect of human whole blood on tHDA amplification was evaluated using test 1. Our results demonstrated that the presence of 2 μ L of human whole blood did not inhibit tHDA amplification. When 2 μ L of whole blood was added to the 50- μ L reaction mix of test 1, the analytical sensitivity of 50 copies per reaction was not compromised (data not shown). When the volume of the blood increased to 5 μ L per reaction, we started to observe failure of amplification and detection when the target input was close to the limit of detection of 50 copies (data not shown).

Clinical Sensitivity and Specificity

Among the 88 EDTA whole blood samples, 59 were identified as positive for malaria by microscopy/NASBAMT. These 59 samples contained various types of *Plasmodium*, with 39 *P. falciparum*, 16 *P. vivax*, 3 *P. malariae*, and 1 *P. ovale*. The parasitemia levels range from 0.01% to 36.9% (median, 0.5%), with most ($n = 37$) of the 59 positive

Table 4 Analytical Sensitivity of the tHDA Malaria Assay

Sample no.	Parasitemia (%)	Fold of dilution	Final concentration (parasites/ μ L)*	tHDA test 1 results (positive/total)
NS-046	2.3	1000	115	1/2
NS-060	8.8	2000	220	1/2
NS-109	0.3	100	150	2/2
NS-110	1.1	333.3	165	1/2

*The parasite concentration in blood was calculated using the following formulation: Number of Parasites/ μ L = % Parasitemia \times (5×10^6 Red Blood Cells/mL)/(100 \times Fold of Dilution).

samples being <1%. Ten of them were <0.1% (Table 2 and Figure 4). The five lowest parasitemia levels were 0.01%, 0.03%, 0.03%, 0.05%, and 0.05%.

The tHDA malaria assay test 1 identified 57 of the 59 microscopy/NASBAMT-positive malaria samples as positive for *Plasmodium*, of which 38 were identified as *P. falciparum* by tHDA. One sample, identified as *P. falciparum* by microscopy/NASBAMT with a parasitemia level of 0.05%, was negative by tHDA. Among the 20 non-*P. falciparum* malaria samples, 19 were identified by tHDA as *Plasmodium* positive but *P. falciparum* negative (16 *P. vivax*, 2 *P. malariae*, and 1 *P. ovale* by microscopy/NASBAMT). These samples were subjected to test 2, which identified the 16 *P. vivax* samples as positive for *P. vivax*. The two *P. malariae* samples and one *P. ovale* sample were identified by tHDA test 2 as negative for *P. vivax*. One sample was identified by tHDA as malaria negative but by NASBAMT as *P. malariae*. This sample was also negative by microscopy. The 14 negative and 15 *Babesia* samples were all identified by tHDA assay as negative for *Plasmodium*. Overall, compared with combined microscopy and NASBAMT results, the tHDA malaria assay resulted in a sensitivity of 96.6% (95% CI, 87.3% to 99.4%) and specificity of 100% (95% CI, 85.4% to 100%) (Table 3).

The analytical sensitivity of the tHDA assay from human whole blood samples was evaluated by serial dilutions of the frozen blood samples with a known parasitemia level into fresh blood. Four *P. falciparum* samples with parasitemia levels ranging from 0.3% to 8.8% were diluted up to 2000-fold. The final concentrations of the diluted samples were between 100 and 250 parasites per μL of blood. Each dilution was tested by tHDA test 1 in duplicate. All four samples were positive by tHDA test for at least one of the duplicates (Table 4). These results suggested that the sensitivity of the tHDA assay was approximately 200 parasites per μL of blood.

Discussion

The tHDA-based malaria assay demonstrated the capability of detecting the five malaria causing *Plasmodium* species using only 2 μL of unprocessed human whole blood without the need for nucleic acid extraction. The tHDA amplifications were performed under isothermal conditions at a constant temperature of 64°C, which can be performed in a simple dry heat block or water bath. The detection of the target-specific amplification products was achieved by single-use disposable lateral-flow-based cassettes.

The tHDA-based malaria assay was able to identify the two most common *Plasmodium* species, *P. falciparum* and *P. vivax*. *P. falciparum* causes approximately 81% of total malaria worldwide (World Malaria Report 2011), with *P. vivax* making up the rest.²⁹ *P. falciparum* is the most dangerous cause of malaria, with the highest mortality rate (World Malaria Report 2011). *P. vivax* is often nonfatal, but more widespread, than *P. falciparum* (World Malaria Report

2011). It is believed to account for 70 to 80 million cases annually (ie, 34% of the global incidence and 56% of malaria cases outside of Africa).²⁹ Moreover, *P. vivax* can cause relapses years after the initial infection if not completely eradicated (Centers for Disease Control and Prevention, <http://www.cdc.gov/malaria/about/facts.html>). In contrast, the incidence of the other species of *Plasmodium* is seldom discussed in the literature. *P. falciparum* and *P. vivax* also have different drug-resistance profiles.² Therefore, the ability of the tHDA assay for species-specific identification would help determine the proper treatment in a timely manner, especially in regions where more than one species is present.

The analytical sensitivity of the tHDA-based assay was 50 copies of *Plasmodium* genomic DNA or plasmid, in the presence of 2 μL of human whole blood spiked into the 50- μL reaction. Compared with the analytical sensitivity using pure DNA with spiked blood, the analytical sensitivity using patient samples was approximately 5 to 10 times lower (approximately 200 parasites per μL of blood). This could be because of the fact that, after long-term storage and repeated freeze and thaw, the parasite DNA might have partially degraded or the quality of the blood may have caused some inhibition to tHDA reactions. These possibilities can be tested in future studies by using fresh blood samples as a template for tHDA amplification.

In summary, the combination of the two tHDA tests offers a simple, cost-effective way to detect *Plasmodium* species and identify *P. falciparum* and *P. vivax* in unpressed blood without a sample preparation step. The work flow renders the assay essentially instrument free, eliminating the need for costly thermocyclers required for molecular diagnostic assays based on PCR. The tests can also be performed in a random access manner, allowing for on-demand testing that would be particularly beneficial for the rapid diagnosis of malaria and other blood-borne parasites, such as *Babesia*, in persons seen in both emergency departments and urgent care centers. Potentially, this assay can be developed as a low-cost and fast turnaround point-of-care assay performed in the field by people with minimal training.

Acknowledgment

We thank the Malaria Research and Reference Reagent Resource Center for providing dry blood filter papers containing *P. vivax*, *P. ovale*, and *P. malariae* blood samples.

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