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Novel Nested Direct PCR Technique for Malaria Diagnosis Using Filter Paper Samples[▽]

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The use of direct nested PCR enables the detection of *Plasmodium* spp. from blood samples collected on filter papers without requiring the time-consuming procedures associated with DNA extraction. Direct PCR provides a rapid, highly sensitive, and cost-effective alternative to diagnosing malaria using filter paper samples and standard nested PCR.

Malaria remains a major global health burden with an estimated death toll of almost 900,000 every year (11). Recent reports of newly emerging artemisinin resistance and the emergence of endemic populations of a number of "new," potentially human-pathogenic *Plasmodium* species, such as *Plasmodium knowlesi*, as well as a variety of *Plasmodium ovale* parasites, in Asia indicate that there is an urgent need for new techniques to provide rapid and highly accurate diagnoses to adequately treat and control malaria (3, 5, 9).

The use of direct PCR allows for PCR amplifications without any prior DNA extraction and purification steps. The Phusion blood DNA polymerase used in the assay is reported to lead to a 25-fold-lower error rate than the common *Thermus aquaticus* polymerase (2).

The aim of this study was to adapt this novel technique for use in the rapid laboratory-based detection of *Plasmodium* spp. and to validate the sensitivity of this technique in comparison to that of conventional nested PCR and microscopy (6, 8).

Patient samples were collected between 2007 and 2009 at the MARIB (Malaria Research Initiative Bandarban) center in Bandarban, Chittagong Hill Tracts, Bangladesh, as part of a hospital- and field-based fever survey. Written informed consent was obtained from all study participants or their legal representatives, and the study protocol was approved by the appropriate ethical review committee.

From all participating patients aged 8 years and older, 100 μl venous blood was drawn. From patients younger than 8 years, 2 drops of blood obtained by finger prick was collected and transferred onto 903 filter paper (Schleicher & Schuell BioScience GmbH, Dassel, Germany) in duplicate. Filter papers were air dried at room temperature and stored under airtight conditions at 4°C until further processing. A total number of 140 filter paper samples was included in the evaluation.

Direct nested PCR. A blood spot 2 mm in diameter was punched out of each filter paper sample and washed with 30 μl double-distilled water at 50°C for 3 min. The water was removed, and the PCR mixture (Phusion blood direct PCR kit; Finnzymes Oy, Espoo, Finland) was added directly to the sample. A modified standard nested-PCR protocol was used for the evaluation of genus- and species-specific *Plasmodium* DNA within the highly conserved regions of the small-subunit (SSU) rRNA gene (6, 7, 8). The following primers were used: rPLU1/rPLU5 for the nest 1 reactions and rPLU3/rPLU4 for the genus-specific nest 2 amplifications. Whenever the genus-specific nest 2 PCR revealed positive results, the following species-specific nest 2 primers were used to determine the *Plasmodium* species: rFAL1/rFAL2 (*P. falciparum*), rVIV1/rVIV2 (*P. vivax*), rMAL1/rMAL2 (*P. malariae*), rOVA1/rPLU2 (*P. ovale*), and Pmk8/Pmkr9 (*P. knowlesi*). All oligonucleotide primers were obtained from Microsynth AG (Balgach, Switzerland).

A 50-μl nest 1 reaction mixture, which included 25 μl 2× Phusion blood PCR buffer (which included 200 μM deoxynucleoside triphosphates [dNTPs] and 3 mM $MgCl_2$), 1 μl (2 U) Phusion blood DNA polymerase, and 5 μl of each primer (rPLU1 and rPLU5, 10 μM), was made according to the manufacturer's manual (2). The DNA was denatured at 98°C for 4 min, followed by 25 cycles of amplification (annealing, 65°C for 2 min; extension, 72°C for 2 min; denaturation, 94°C for 1 min). After 25 cycles, the final extension was done at 72°C for 4 min using an Eppendorf Mastercycler Personal (Eppendorf AG, Hamburg, Germany). The annealing temperature was determined using the Tm calculator on the manufacturer's website (https://www.finnzymes.fi/tm_determination.html) (2). The resulting nest 1 PCR product was centrifuged at 1,000 × *g* for 3 min. Volumes of 2.5 μl of nest 1 products (same in standard nested PCR and direct nested PCR) were used in 25-μl nest 2 amplification mixtures (GoTaq PCR core system; Promega, Madison, WI).

Known positive-control samples and nuclease-free water as the negative control were run with each PCR amplification. Nest 2 PCR products were analyzed by gel electrophoresis with 2% agarose and ethidium bromide staining.

Standard nested PCR technique. A modified Chelex-based method using an InstaGene whole blood kit (Bio-Rad Laboratories, Hercules, CA) was used to extract DNA from blood spots on filter paper. A blood spot of 4 mm in diameter was punched out and soaked overnight in 100 μl of phosphate-

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TABLE 1. Comparison of malaria diagnoses by direct nested PCR, nested PCR, and microscopy, including all samples or only those negative by microscopy or with a parasitemia of ≤200/μl

Malaria parasite(s) detected[a]	No. of all samples (n = 140) with detection by:			No. of samples with parasitemia of ≤200/μl or negative by microscopy (n = 61) with detection by:		
	Direct nested PCR	Nested PCR	Microscopy	Direct nested PCR	Nested PCR	Microscopy
Neg	45	48	51	45	48	51
Pf	60	65	72	5	4	9
Pv	7	8	9	1	1	1
Pm	3	3	2	1	1	0
Po	5	4	3	2	1	0
Pf + *Pv*	11	8	3	4	4	0
Pf + *Pm*	5	2	0	2	1	0
Pf+ *Pv* + *Pm*	1	2	0	0	1	0
Pf + *Pv* + *Pm* + *Po*	1	0	0	1	0	0
Pv + *Pm*	2	0	0	0	0	0
Pk	0	0	0	0	0	0

[a] Abbreviations: Neg, negative; *Pf, Plasmodium falciparum*; *Pv, P. vivax*; *Pm, P. malariae*; *Po, P. ovale*; *Pk, P. knowlesi*.

buffered saline (PBS) at 4°C. DNA extraction was performed on the following day as described previously (1). All samples were purified twice with the InstaGene matrix and stored at −20°C until further processing.

A template of 5 μl was used in a 50-μl nest 1 reaction mixture (GoTaq PCR core system; Promega, Madison, WI) under the following conditions: 5 μl of each primer (10 μM), 125 μM each dNTP, 2 mM $MgCl_2$, and 1 U of GoTaq DNA polymerase.

Nest 2 reactions and further procedures (with the exception of the centrifugation step of the direct PCR nest 1 product) were identical to the standard- and direct nested-PCR techniques discussed above.

Microscopy. Thick and thin smears were prepared in duplicate using each patient's blood and stained with Giemsa (Merck KGaA, Darmstadt, Germany). Each slide was examined by two expert microscopists blinded to each other's results. In thick films, 200 oil immersion fields were evaluated before a sample was declared negative and to rule out mixed infections. In thin films, the parasite count was established per 2,000 red blood cells.

The level of detection was determined in double-blinded fashion (each step blinded to the results of the others: microscopy, DNA extraction, PCRs, and gel electrophoresis) using filter papers with 100-μl blood spots with known parasitemia obtained from the K1 (1 parasite/μl to 250,000 parasites/μl) and 3D7 (1 parasite/μl to 290,000 parasites/μl) *Plasmodium falciparum* strains, as well as a *Plasmodium vivax* isolate (1 parasite/μl to 30,000 parasites/μl). The lowest parasitemias reliably resulting in positive results were 3 parasites/μl for the *Plasmodium vivax* isolate and the K1 strain isolate and 5 parasites/μl for the 3D7 laboratory strain.

Using direct nested PCR, 95 of 140 field isolates gave positive results with genus-specific primers, compared to 92 of 140 using standard nested PCR and 89 of 140 using microscopic determination (Table 1). Based on a total of 640 nest 2 PCRs (genus and species), a sensitivity of 99.8%, a specificity of 96%, a positive predictive value (PPV) of 90.9%, and a negative predictive value (NPV) of 99.7% were calculated in comparison to those of the standard nested PCR (Table 2). All field isolates giving positive results for malaria parasites by microscopy remained positive in direct nested PCR. The limitations

TABLE 2. Comparison of *Plasmodium* sp. detections by standard nested PCR and by direct nested PCR[a]

Direct PCR detection	No. of samples with standard nested PCR detection									Total no. of samples
	Neg	*Pf*	*Pv*	*Pm*	*Po*	*Pk*	*Pf* + *Pv*	*Pf* + *Pm*	*Pf* + *Pv* + *Pm*	
Neg	45[b]									45
Pf		59					1			60
Pv	1		6							7
Pm	1			2						3
Po	1				4					5
Pk						0				0
Pf + *Pv*		4					7			11
Pf + *Pm*		1		2				2		5
Pv + *Pm*			2							2
Pf + *Pv* + *Pm*									1	1
Pf + *Pv* + *Pm* + *Po*									1	1
Total	48	64	8	4	4	0	8	2	2	140

[a] Abbreviations: Neg, negative; *Pf*, *Plasmodium falciparum*; *Pv*, *P. vivax*; *Pm*, *P. malariae*; *Po*, *P. ovale*; *Pk*, *P. knowlesi.*

[b] This value includes two samples negative by standard nested PCR which gave positive results in genus direct nested PCR but remained negative in the species direct nested PCRs and after the genus direct nested PCRs were repeated.

in terms of specificity of the primers in the detection of *P. ovale* and *P. knowlesi* have previously been discussed (4, 9).

Although microscopy remains the gold standard for malaria diagnosis, the limits of detection may significantly differ between microscopists and have previously been estimated to range from 50 to 100 parasites/μl under field conditions (10). Despite their known limitations, microscopy and/or rapid diagnostic tests (RDTs) remain the primary techniques of malaria diagnosis. However, in past decades, the improvements in molecular diagnostic tools (e.g., PCR and real-time PCR) have resulted in the availability of far more sensitive tools.

With a limit of detection of only 3 parasites/μl, the novel assay is likely to be slightly more sensitive than standard nested PCR, with its limit of detection of 6 parasites/μl (6). The calculated values for the specificity (96%) and the PPV (90.9%) relative to those of the standard PCR (PCR-corrected microscopy) may possibly under- or overestimate the true specificity, as the higher proportion of positive samples found by direct PCR could possibly also be the result of the higher sensitivity of the new assay.

Certainly the biggest advantage of direct PCR is the fact that the extraction and purification of DNA from filter paper can be omitted, resulting in an overall saving in time of approximately 2 h, as well as the overnight DNA extraction step, which in our eyes justifies the slightly higher price of each single direct nest 1 PCR (~$2.10) in comparison to that of the standard nest 1 PCR ($1.70) for the DNA extraction and nest 1 reaction of one sample. At the same time, the collection of filter papers is a practical way of sampling, storing, and transporting diagnostic blood samples. This technique is not limited to screening for malaria parasite species; it might also be employed for genotyping, drug resistance research, and the diagnosis of other blood pathogens. We therefore conclude that direct PCR in combination with the collection of blood samples on filter paper provides a rapid, highly sensitive, and cost-effective alternative for malaria diagnosis.

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