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Accurate and Sensitive Detection of *Plasmodium* Species in Humans by Use of the Dihydrofolate Reductase-Thymidylate Synthase Linker Region▽†

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A nested-PCR protocol based on the linker region of the *Plasmodium* dihydrofolate reductase-thymidylate synthase gene (*dhfr-ts*) was developed. This provides highly sensitive specific detection and identification of the five parasite species that infect humans.

Thymidylate synthase (TS) and dihydrofolate reductase (DHFR) are enzymes of the folate cycle that produce the 2′-deoxythymidine-5′-monophosphate (dTMP) required for DNA synthesis. As these proteins have an essential role in the cell cycle, they are relatively conserved between distantly related species. In several protozoans, the two enzymes are encoded in a single gene, with a linker region between the two domains. Although this linker region is thought to control the orientation of the TS and DHFR domains relative to each other (8), it displays significantly higher sequence diversity than the relatively conserved enzymatic domains (12). Sequence analysis of the published *Plasmodium dhfr-ts* sequences revealed that the different species are characterized by unique linker sequences (Fig. 1), which are highly conserved between different isolates of the same species. The sequences analyzed (GenBank accession numbers are in brackets) included 9 from *P. falciparum* isolates (EU046228 to EU046231, EU046233, J03772, J04643, M22159, and XM_001351443), 12 from *P. vivax* isolates (DQ517894 to DQ517900, DQ514918-DQ514921, and XM_001615032), 8 from *P. malariae* isolates (AY846633 and AY846634, EF188271 to EF188273, and EF198109 to EF198111), 1 from a *P. brasilianum* isolate (EF188274), 20 from *P. ovale* isolates (EU266601 to EU266618 and GQ250090 and GQ250091), and 4 from *P. knowlesi* isolates (GQ250087 to GQ250089 and XM_002258192). Sequence variations in the *Plasmodium dhfr* domain have been investigated extensively because nonsynonymous mutations at defined residues are associated with decreased susceptibility to antifolate antimalarial drugs (4–6, 9). The detection and identification to species level of malaria parasites in field samples are often carried out in separate PCR amplification reactions, generally based on the small-subunit rRNA (ssrRNA) genes. Amplification of the *dhfr-ts* linker has also been shown to be effective at detecting low numbers of *P. falciparum* parasites (13). We wished to ascertain whether PCR primers that target the *Plasmodium dhfr-ts* genes could equally serve as a means to detect and identify the five parasite species that infect humans with high sensitivity and accuracy.

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PF  TNNKMLNEQNCIKGEEKNNDMPLK----------NDDKDTCHMKKLTEFYKNVDKYK----------INYENDDDDEEEDDFVYFNFNKEKEEKNKNSIHPNDFQIYNSLKYKY
PV  VGGGV--DGGASNGSTAD--SASENCNALNCNAPKCSAPNCRSPNGGTAQQGE-----WGKGPAACP---WQK-------NNAEAEEDDLVYFSFNNKVGE--KNPEHLQDFKIYNSLKIKQ
PM  RKKALTQESLPHQSSGSDKGSNTSS--TISNGAMSSNTIR-GSTTSSS---GKG----------KGGGES----IFEREYNFMGDEEDDLVYFNFNNNKNE-YKNAENANDFKIYNSLKFKH
PO  KKKDTQNDGDSSEGSTTTTATA--TWPGIAGATSSMNIAE-TSTTYASSHHGKGSSKWWTKVCTKSGAEWWKNMTLTSQGKDNVDEEDDYMYFSFNNKKKK-SQIGKNAEDFEIYNTIKLKQ
PK  VGGEV--DGGASNGSTMDMDSGSENCENVKCGAPNCNTSGCISPNCTSTREEK-----WRKCSPACQ---WKK-------SNADVEEDDLVYFSFNNKVGE--KNPEHLQDFKIYNSVKIKQ
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FIG. 1. Alignment of the DHFR-TS amino acids for the linker region of *Plasmodium* species that infect humans. The degenerate primers for the primary amplification are located at the start of the *dhfr* and *ts* segments. The positions of the species-specific paired primers located within the linker region of each *Plasmodium* species are indicated by the black boxes, and amplification protocols are in Table S1 in the supplemental material. PF, *P. falciparum*; PV, *P. malariae*; PM, *P. vivax*; PO, *P. ovale*; PK, *P. knowlesi*.

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TABLE 1. Detection of minority parasite species in artificial mixed infections[a]

Species (no. of parasite genomes/μl) in mixture	Minority species detected with:	
	ssrRNA gene PCR	*dhfr-ts* linker PCR
PM/PO/PK (1–10 of each)	PM PO PK	PM PO PK
PM/PO/PK (10^2 of each)	PM PO PK	PM PO PK
PF (10^5) + PM/PO/PK (1–10 of each)	PF PM PO PK	PF PM PO PK
PF (10^5) + PM/PO/PK (10^2 of each)	PF PM PO PK	PF PM PO PK
PV (10^4) + PM/PO/PK (1–10 of each)	PF PM PO PK	PF PM PO PK
PV (10^4) + PM/PO/PK (10^2 of each)	PF PM PO PK	PF PM PO PK

[a] PF, *P. falciparum*; PM, *P. malariae*; PV, *P. vivax*; PO, *P. ovale*; PK, *P. knowlesi.*

TABLE 2. Detection of parasite species in field samples

Species[a]	No. of isolates detected with:	
	ssrRNA gene PCR	*dhfr-ts* linker PCR
PF	14	14
PV	16	16
PM	13	13
PO	5	5
PK[b]	4	4
PF+PV	6	6
PF+PM	5	5
PV+PO	1	1

[a] PF, *P. falciparum*; PM, *P. malariae*; PV, *P. vivax*; PO, *P. ovale*; PK, *P. knowlesi.*
[b] The genomic DNA from these four isolates was obtained from MR4.

We designed degenerate primers specific to *Plasmodium* parasites that would yield a fragment (ca. 1 kb) spanning the *dhfr* and *ts* domains from any *Plasmodium* species in a primary amplification reaction and in a seminested secondary amplification (see Table S1 in the supplemental material) and a set of five primers pairs located in the linker region and specific to each of the five parasite species that infect humans. The *P. ovale* primers hybridize to sequences conserved between the two types *P. ovale curtisi* and *P. ovale wallikeri* (11). We then optimized the reaction conditions for amplification (see Table S1 in the supplemental material). In order to assess the sensitivity and specificity of these primer pairs, serial dilutions of genomic DNA from the five malaria parasite species that occur in humans were prepared to serve as templates for PCR amplification. For each species, two isolates were selected: *P. falciparum* 3D7 and K1, Thai isolates of *P. vivax*, *P. malariae*, and *P. ovale*, and two isolates of *P. knowlesi* (MRA456 and MRA457) obtained from the Malaria Research and Reference Reagent Resource Centre (MR4 [http://www.mr4.org/]). In parallel, the same templates were amplified using an established protocol based on the ssrRNA genes (10) that is now considered the "gold standard" for sensitivity and specificity (1, 2). In all cases, the primary amplification reactions were initiated with 1 μl of the template genomic DNA, and 1 μl of the resulting product was used to initiate the secondary amplification. All amplifications were carried out in a total volume of 20 μl, in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 250 nM each oligonucleotide primer, 125 μM deoxynucleoside triphosphates, and 0.4 units of *Taq* polymerase (Invitrogen, United States). The cycling parameters consisted of an initial denaturation step at 95°C for 5 min, annealing for 1 min at a temperature defined for each primer pair (58°C for the ssrRNA gene primers; see Table S1 in the supplemental material for the *dhfr-ts* primers), and extension at 72°C for 1 min, followed by denaturation at 94°C for 1 min. After a given number of cycles (30 for the primary and 35 for the secondary amplifications), a final cycle with a 5-min extension step was carried out before product visualization on ethidium bromide-stained 2% agarose gels (BRL, United States). Negative controls (no-DNA template or DNA from heterologous species) were used in each set of amplifications.

The sensitivity and specificity of the nested-PCR *dhfr-ts* linker protocol were identical to those obtained with the ssrRNA gene protocol (data not shown) in that both methods specifically detected the different parasite species in aliquots that contained down to 1 to 10 parasite genomes. In order to confirm that the assays were specific to *Plasmodium* species, the nested reactions (genus and species specific) were applied to DNA purified from blood samples collected on admission from a total of 45 febrile patients: 15 with active melioidosis, 15 with active leptospirosis, and 15 with active scrub typhus. No amplification was observed for any of these 45 samples or for purified human genomic DNA (data not shown).

One of the more important advantages of amplification-based methods is the ability to detect mixed-species infections, where one species often predominates numerically over the other, so we produced artificial mixtures where the DNA template concentration from one parasite species was present in a 100- to 10,000-fold excess over the DNA templates from a set of the other species. The minor species were detected in all cases (Table 1). Finally, the results from the two protocols were fully concordant when applied to templates purified from 60 samples collected from Thai patients infected by one or other of the four parasite species or carrying mixed-species infections and four *P. knowlesi* isolates obtained from MR4 (Table 2).

Accurate detection and identification of malaria parasites provides important epidemiological data that inform malaria control programs. The reliability and sensitivity of molecular techniques make them ideally suited to gather this information. We show that an amplification protocol based on primers that target the *dhfr-ts* linker region equals the sensitivity and specificity of *Plasmodium* detection protocols based on ssrRNA genes. There are two advantages that this protocol has over the classical ssrRNA-based protocols. First, the amplification efficiency and specificity are less prone to genetic variations within a given species or to cross-hybridization between species (3, 7). Second, for the investigation of antifolate resistance, the product of the primary *dhfr-ts* amplification can be used to determine the presence of point mutations associated with drug resistance in the samples that prove to be positive for *Plasmodium* using the protocol presented above.

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