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Evaluation of the RealArt Malaria LC Real-Time PCR Assay for Malaria Diagnosis

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PCR-based methods have advantages over traditional microscopic methods for the diagnosis of malaria, especially in cases of low parasitemia and mixed infections. However, current PCR-based assays are often labor-intensive and not readily quantifiable and have the potential for contamination due to a requirement for postamplification sample handling. Real-time PCR can address these limitations. This study evaluated the performance characteristics of a commercial malaria real-time PCR assay (RealArt Malaria LC Assay; Artus GmbH, Hamburg, Germany) on the LightCycler platform for the detection of malaria parasites in 259 febrile returned travelers. Compared to nested PCR as the reference standard, the real-time assay had a sensitivity of 99.5%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 99.6% for the detection of malaria. Our results indicate that the RealArt assay is a rapid (<45 min), sensitive, and specific method for the detection of malaria in returned travelers.

For over a century, malaria diagnosis has relied on the microscopic detection of *Plasmodium* spp. on Giemsa-stained blood smears, as no other reliable and relatively rapid method for the detection of infection and quantification of parasite burden has been available. Within the last decade, PCR-based diagnostic methods for malaria have been shown to surpass microscopic methods with respect to sensitivity and specificity but have failed to offer the same quantitative advantage (2, 8). Currently reported amplification-based methods for malaria diagnosis, particularly nested PCR-based methods (16), are sensitive and specific but are also labor intensive, with turnaround times that are generally too long for routine clinical application. Moreover, these are open systems that require considerable pre- and post-test sample handling, and therefore special efforts need to be employed in order to prevent false-positive assays.

Real-time quantitative PCR technology has the potential to overcome these limitations, offering a simple, time-effective, and quantitative diagnostic option. With DNA binding dyes, such as Sybr green, molecular probes, or hybrids labeled with fluorescent probes, real-time PCR detects and quantifies the amplicons after each PCR cycle and completes an entire assay in as little as 40 min. In-house real-time quantitative PCR techniques have been applied in malaria research settings and in animal models (1, 3, 4, 6, 15, 18). However, to date there has not been a standardized good manufacturing practices diagnostic method suitable for routine use in a clinical diagnostic laboratory.

Artus-Biotech has developed a commercially available real-time assay (Real Art Malaria LC PCR assay), a standardized ready-to use real-time quantitative PCR system for the detection of *Plasmodium* spp. with the LightCycler Instrument (Roche Diagnostics). The objective of the present study was to evaluate the performance characteristics of the RealArt Malaria LC PCR assay for the detection of *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* malaria in febrile returned travelers, with nested PCR as the reference standard.

MATERIALS AND METHODS

Patients. Patients presenting or referred to the Tropical Disease Unit of the Toronto General Hospital–University Health Network, Toronto, Canada, from July 1999 to January 2003 with a history of fever and travel to an area of endemic malaria were eligible for inclusion in the study. Consecutive patients with blood films positive for malaria parasites regardless of species were enrolled. In order to provide a comparable control group, febrile travelers diagnosed with an illness other than malaria (and with repeatedly negative blood films for malaria) during the first 2 months of this study were also enrolled. The prevalence of malaria in this population during the course of the study was 15%.

Whole blood samples (pretreatment) were collected from all patients for thick and thin blood film preparation, PCR, and complete blood counts. Microscopy, nested PCR, and real-time PCR amplification were performed independently and in a blinded fashion. This study was reviewed and approved by the Research Ethics Board of the University Health Network–Toronto General Hospital.

Microscopy. Smears were examined by an expert microscopist and were considered negative if no parasites were seen in 500 oil immersion fields (×1,000) on a thick blood film. Parasite concentration was calculated by determining the number of parasites per 200 white blood cells in a thick blood film. Baseline white blood cell counts were then used to calculate parasitemia as parasites per microliter.

PCR. PCR detection and malaria species identification were performed as previously described (14, 16). Briefly, genomic DNA was extracted from 200 μl of whole blood on Qiagen columns (Qiagen, Chatsworth, Calif.) according to the manufacturer's instructions. A 5-μl aliquot of the DNA extract was used in a nested PCR assay to amplify a segment of the *Plasmodium* 18S rRNA gene of all four human malarial species. The resulting PCR product was analyzed on a 2% agarose gel stained with ethidium bromide.

Real-time PCR. The RealArt Malaria PCR assay (Artus GmbH, Hamburg, Germany) was used with a Lightcycler (Roche Diagnostics) platform. It contains reagents and enzymes for the specific amplification and detection of a species-

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TABLE 1. Results of the RealArt malaria assay compared with nested PCR as the reference standard for the diagnosis of malaria in 259 febrile returned travelers

Real-time PCR result	No. of samples with indicated nested PCR result					
	Positive					Negative
	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*	Mixed	
Positive	100	90	9	3	6	0
Negative	1	0	0	0	0	50
Total	101	90	9	3	6	50

TABLE 2. Parasite burden ascertained by real-time PCR quantification versus microscopy

Plasmodium species	Quantification range (copies/μl)	Parasitemia range (parasites/μl)
P. falciparum[a]	$0.45–2.7 \times 10^6$	$16–1.2 \times 10^5$
P. vivax[b]	$1.10–2.5 \times 10^5$	$40–3.5 \times 10^4$
P. ovale	$57–4.8 \times 10^4$	$50–1.2 \times 10^4$
P. malariae	$1.0–1.1 \times 10^4$	$150–2.1 \times 10^3$
Mixed infections	$911–9.3 \times 10^4$	$520–6.5 \times 10^4$

[a] Significantly correlated with parasitemia ($P = 0.05$).
[b] Significantly correlated with parasitemia ($P = 0.01$).

conserved 140-bp region of the *Plasmodium* 18S rRNA gene of all four human malaria species. The multicopy 18S (small subunit) rRNA genes of *Plasmodium* spp. that infect humans have been demonstrated to be highly stable and conserved, and assays to detect them have displayed no cross reactions to human DNA or other human pathogen DNA or RNA, including non-human *Plasmodium* spp. (16, 17).

In addition to the hybridization probe used for amplicon detection, the RealArt Malaria assay contains a second heterologous amplification system to identify potential PCR inhibition in samples. Four quantification standards with known concentrations of gene copies (equivalent to 70 to 70,000 genome copies per μl) are used as known positive controls and to generate a standard curve to assess parasite burden. The current assay version is designed to diagnose malaria infection versus no infection, i.e., to detect all four human *Plasmodium* spp. but not to differentiate between them. The assay was carried out according to the manufacturer's instructions. Briefly, a 5-μl aliquot of the same DNA extract used in the nested PCRs and 15 μl of master mix solution were added per capillary. Water (5 μl) was used as an internal negative control, and four quantification standards (5 μl) served as positive controls. Additional known negative and positive samples for all species of human malaria were included in each run.

RESULTS

During the study period, 259 consecutive individuals who presented with fever after travel to an area of endemic malaria were enrolled. The ratio of male to female patients was 1.6 (63.5% males), with a mean age of 32 ± 1.1 years (range, 4 months to 71 years). Travel destinations included Africa (58.1%), the Indian subcontinent (17%), Latin America (17%), Oceania (3.8%), Southeast Asia (2.9%), and the Middle East (0.9%). Whole blood samples from 101 of these individuals were confirmed by nested PCR to be positive for *P. falciparum* malaria, 90 were PCR-confirmed *P. vivax* infections, 9 were *P. ovale*, 3 were *P. malariae*, 6 were mixed infections, and 50 were known negative controls (i.e. diagnosis of febrile illness other than malaria).

Compared to nested PCR as the reference standard, the RealArt malaria assay was 99.5% sensitive and 100% specific for the detection of malaria parasites in returned travelers (Table 1). Of note, the RealArt assay also detected two *P. ovale* samples with a variant 18S rRNA sequence that had been reported to be difficult to detect with previous 18S rRNA PCR-based assays (10). Based on a 15% disease prevalence in the study population, the corresponding positive and negative predictive values were 100% and 99.6%, respectively. Based on nested PCR and microscopy, there were no false positives but one false-negative result for a *P. falciparum* sample with low parasitemia (80 parasites/μl). Table 2 indicates the real-time PCR quantification ranges in comparison to parasitemia levels for each *Plasmodium* species. There was a significant correlation between parasitemia as determined by microscopy and gene copy number for *P. vivax* ($r = 0.4$; $P = 0.01$ [Spearman]) and *P. falciparum* ($r = 0.2$; $P = 0.05$), but the correlation was low to moderate.

DISCUSSION

This study represents an evaluation of the first commercially available standardized real-time PCR assay for the diagnosis of malaria. Our results indicate that the RealArt Malaria LC PCR assay is rapid (assay time, <45 min), sensitive (99.5%), and specific (100%) for the detection of malaria parasites in febrile returned travelers.

PCR, in particular nested PCR, has proven to be a more sensitive diagnostic method for malaria than smears, particularly in cases of low parasitemia and mixed infections. (2, 5, 7, 8, 9, 11, 12, 13, 14, 16). However, despite their sensitivity, current PCR assays are relatively labor intensive and may have slow turn-around times, requiring up to 8 h for nested PCR assays; furthermore, the results are not readily quantifiable. In contrast, the RealArt malaria PCR assay can generate quantitative results in less than an hour from a 5-μl DNA sample following a simple and standardized protocol.

Real-time PCR assays have closed amplification vessels during thermocycling and amplicon monitoring and detection. This minimizes the need for excessive handling of samples, particularly compared to nested reactions, and eliminates additional post-PCR sample-handling steps normally required for amplicon detection. These features significantly reduce the potential for sample contamination. The DNA extraction quality and the PCR run quality are simultaneously monitored with the provided internal control, allowing the user to easily monitor for PCR inhibition. When the real-time assay is combined with the available robotic sample preparation and nucleic acid extraction modules, the process may be automated. This, combined with their ease of use and rapid turnaround times, makes these assays well suited to routine diagnostic laboratories.

The quantitation feature of real-time PCR platforms is a clear benefit over traditional PCR assays. The RealArt malaria assay quantifies each sample based on a standard curve generated from four quantitation standards with known concentrations of cloned PCR product. Direct correlations between parasitemia, as determined by microscopy, and gene copy number, as determined from the standard curve, are somewhat confounded by the multicopy nature of the rRNA genes, by the variable numbers of these genes within each species, and by the presence of multinucleate schizont stages. Together, these two approaches represent alternative methods of quantifying par-

asite burden within a sample, and additional clinical trials will be required to determine which method is more predictive of clinical outcome.

One limitation of the present assay, as is the case with a number of rapid diagnostic assays for malaria, is its inability to differentiate among the four *Plasmodium* species. At present, a positive assay would need to be accompanied by a malaria smear or nested PCR in order to ascertain the *Plasmodium* sp. involved. Since therapy is dependent on the infecting species, future versions of this assay would need to include a species identification component, although this may not be as important if the assay is used primarily as a screening test or to exclude malaria in returned travelers or in blood products. Another potential limitation of this approach is the current limited availability of real-time PCR platforms. However, these platforms are becoming much more common and affordable and are replacing traditional PCR methods, especially when combined with automated DNA extraction. Although the Artus RealArt assay was very specific in this study population (100%; 95% confidence limits, 97.4 to 100%), additional studies in low-prevalence populations will also be required to fully evaluate its performance characteristics.

In summary, compared to nested PCR and microscopy, the RealArt malaria assay is a rapid, sensitive, and specific method for the detection of malaria in febrile returned travelers. Diagnostic laboratories may find that the rapid turnaround time, quantitative results, high sensitivity, and excellent negative predictive value (allowing the exclusion of malaria in an ill returned traveler) have potential impact on patient care.

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