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Real-time *Leishmania* genus master mix: a platform compatibility and stability study

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ABSTRACT

Performing diagnostics and vector-pathogen surveillance in austere environments is challenging. On-site diagnostic/detection mitigates vector-borne disease complications during military or humanitarian deployments to disease endemic locals. The mobile molecular diagnostic platform, Joint Biological Agent Identification and Diagnostic System (JBAIDS; BioFire Diagnostics Inc., Salt Lake City, UT, USA), rapidly identifies biothreat pathogens. Although ideal for remote diagnostics, the platform was validated for specific pathogens of insignificant epidemiological consequence. Recognizing the JBAIDS's remote diagnostic/detection versatility, we tested a *Leishmania* genus real-time PCR master mix validated for use on the SmartCycler® (Cepheid, Sunnyvale, CA, USA) for concomitant use on the JBAIDS. We evaluated assay sensitivity, precision, and specificity of one or more *Leishmania* spp. on the JBAIDS and found that the JBAIDS produces superior detection sensitivity and specificity compared to the SmartCycler®. We also examined the storage stability of a bulk lot preparation of the *Leishmania* genus real-time PCR master mix on the SmartCycler® to ensure that long periods of frozen storage that would translate to a field environment with the JBAIDS were not detrimental to the reagent. We found that the bulk master mix maintains its stability over a 13-month time period. Overall, these studies confirm JBAIDS's versatility and demonstrate a streamlined assay development approach where reagents are compatible with both platforms.

1. Introduction

Leishmaniasis, transmitted by infected sand flies of the species, *Phlebotomus* or *Lutzomyia*, is a spectrum of diseases caused by species in the *Leishmania* genus. Leishmaniasis is endemic in 88 countries (WHO, 2013a, 2013b). Three clinically recognized significant diseases, cutaneous, mucosal, and visceral leishmaniasis, represent the majority of cases (Antinori et al., 2013; Weina et al., 2004; WHO, 2013a, 2013b). The most common cutaneous form results in ulcerated sores near the original bite site, whereas the mucosal and visceral forms develop when the parasite invades tissue and/or vital organs. The Center for Disease Control and Prevention estimates that there are approximately 1.5 million new human cases of cutaneous and 400,000 cases of visceral leishmaniasis annually (CDC, 2013). Although leishmaniasis is considered a neglected tropical disease (Antinori et al., 2013; CDC, 2013), there have been human cases reported as far north as Germany (Antinori et al., 2013), and a few cases of human cutaneous leishmaniasis have even been reported in the United States (CDC, 2013). Leishmaniasis is primarily a problem in the developing world and is endemic to areas of poor sanitation, which allows sand flies to flourish. Due to military deployments into endemic areas, leishmaniasis has garnered attention as a significant vector-borne pathogen of consequence to human health.

Visualization and culture of amastigotes is the gold standard for diagnosing leishmaniasis (Alves et al., 2013); however, due to increased sensitivity, PCR-based assays are used to clinically diagnose leishmaniasis (Weina et al., 2004; Wortmann et al., 2007) and detect *Leishmania* in sand flies extracted from insect traps (McAvin et al., 2012). Currently, no Food and Drug Administration (FDA)–cleared, commercially available PCR diagnostic assay is available (Antinori et al., 2013); however, the Walter Reed Army Institute of Research and its collaborators have developed a wet chemistry–based real-time PCR assay for diagnosing leishmaniasis in humans (Wortmann et al., 2001). This assay has been validated according to the College of American Pathologists (CAP) standards for use on the SmartCycler® real-time PCR instrument (Cepheid, Sunnyvale, CA, USA).

Although designed to be portable, the SmartCycler® is not an optimal, transportable real-time PCR platform. The Joint Biological Agent Identification and Diagnostic System (JBAIDS; BioFire Diagnostics Inc., Salt Lake City, UT, USA) (US Department of Defense's standard biothreat diagnostic/detection system or the Ruggedized Advanced Pathogen Identification Device [commercially available equivalent]) is designed for use in mobile laboratories or field

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hospitals and can deliver results in 30 minutes for up to 32 samples. The JBAIDS is intended for use by medical personnel to quickly identify biological agents in both clinical specimens and environmental samples (Joint Operation Requirements Document, 2003). The JBAIDS instrument typically uses commercial lyophilized master mixes that can be stored up for a year. It is compatible with wet reagents; however, these require cold chain shipping and storage, which is not preferred for field use. FDA-cleared diagnostic assays available as lyophilized reagents for the JBAIDS include *Yersinia pestis*, *Bacillus anthracis*, *Francisella tularensis*, and avian influenza A & B (BioFire Diagnostics Inc, 2013a, 2013b). Developing additional lyophilized assays for more common infectious diseases has garnered support, and there is no real-time PCR *Leishmania* diagnostic PCR assay (wet or lyophilized) on the JBAIDS instrument.

In maintaining a CAP-certified clinical laboratory, critical reagents for assays must be identified with specific storage and lot performance testing criteria. The *Leishmania* genus PCR master mix, a selected critical reagent for the SmartCycler® platform, was evaluated for compatibility with the JBAIDS instrument. Using the same freshly prepared master mix and target *Leishmania* spp., the JBAIDS excels the SmartCycler® in sensitivity and specificity in a side by side comparison. Additionally, a bulk lot of the *Leishmania* genus real-time PCR master mix was prepared, frozen and stored over approximately 1 year, and tested for storage stability on the SmartCycler® with results indicating that lengthy frozen storage of wet reagents for use on the JBAIDS is acceptable. These results demonstrate simplicity in transfer of real-time assays from instrument to instrument, which forgoes lengthy validation studies.

2. Materials and methods

2.1. Sample preparation

Ten different cell culture grown *Leishmania* spp. (Table 1), selected based on endemic locale and disease type (i.e., visceral versus cutaneous), were extracted with the QIAamp DNA Mini Kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions. The extracted DNA was serially diluted in Molecular Biology Grade (MBG) water (Sigma-Aldrich, St Louis, MO, USA) to 400 pg/μL and sequentially diluted to 40 pg/μL, 4 pg/μL, 0.4 pg/μL, 0.04 pg/μL, and 0.004 pg/μL (depending on the study described below). In addition, 3 *Trypanosoma* spp. (*Trypanosoma cruzi*, *Trypanosoma rangeli*, and *Trypanosoma lewisi lincicome*) and 1 *Crithidia* (*Crithidia fasciculata*) specie were extracted as described above and analyzed on separate real-time PCR platforms described below at the following concentrations: 14.65 ng/μL, 35.59 ng/μL, 95.00 ng/μL, and 93.46 ng/μL, respectively.

Table 1
Leishmania spp. used in the sensitivity study.

Name	Strain(s)	Type	Country of origin	Storage concentration (ng/μL)
L. tropica[a]	EP139	Cutaneous	Turkey	36.77
L. (v) naiffi	WR2301	Cutaneous	Belize	13.08
L. aethipoica	WR2790 LEM144	Cutaneous	Ethiopia	19.57
L. achibaldi	LEM3463	Cutaneous	Sudan	30.48
L. infantum	WR2794 LEM75	Visceral	India	20.63
L. donovani chagasi	ATCC30881	Visceral	Honduras	42.16
L. braziliensis	PAB3980	Cutaneous	Peru	21.87
L. major	ATCC50122	Cutaneous	Israel	21.23
L. mexicana	UA2236	Cutaneous	Colombia	10.95
L. panamensis	WR2792 LEM702	Cutaneous	Panama	30.30
L. tropica	WR2122	Visceral	Kuwait-Iraq	26.16

[a] Positive control template DNA.

2.2. Real-time PCR

The SmartCycler® (Cepheid, Sunnyvale, CA, USA) used 2 μL of extracted *Leishmania* spp. or near neighbors at various concentrations in 24 μL of master mix, and the JBAIDS (BioFire Diagnostics Inc., Salt Lake City, UT, USA) used 2 μL of the same in 18 μL of master mix. Negative controls contained MBG water as a sample substitute with master mix commensurate with the instrument.

Run conditions were slightly different for the SmartCycler® and JBAIDS instruments. Prior to amplification, the SmartCycler® was setup as previously described (Wortmann et al., 2001). The JBAIDS required sample pre-incubation at 95 °C for 2 minutes followed by 45 cycles of a 2-step incubation at 95 °C for 15 seconds and 60 °C for 30 seconds. Each JBAIDS reaction contained 18 μL of either freshly prepared or thawed bulk lot *Leishmania* genus master mix depending on the study (platform compatibility or stability) and 2 μL of template with *Leishmania tropica* EP139 serving as the positive control. The real-time PCR assay for *Leishmania* genus described here defines sample positives as having a quantification cycle (Cq) value greater than 39.9 (Lefever et al., 2009). One sample of each *Leishmania* spp. was initially tested at 0.04 pg/μL on the JBAIDS and SmartCycler®. If a negative result was obtained, the species was then tested in triplicate at 0.4 pg/μL and 4 pg/μL. For *Trypanosoma* and *Crithidia*, 1 sample of each species was examined in each run; however, a species was re-tested in triplicate if a positive result was achieved for either the JBAIDS or SmartCycler®.

The freshly prepared master mix was prepared per 1 reaction (25 μL) as follows: 1 illustra™ puRETaq Ready-To-Go PCR Bead (GE Healthcare, Waukesha, WI, USA), 2.6 mmol $MgCl_2$ (Sigma-Aldrich), 769 nmol of each primer (LEISL1 and LEISHU1) (Invitrogen, Grand Island, NY, USA), 115 nmol probe (LEISP1) (Invitrogen), and 15.1 μL MBG water were combined in a 1.5-mL micro-centrifuge tube. Ready-To-Go Beads contain deoxynucleoside triphosphate, Taq polymerase, 1.5 mmol $MgCl_2$, and buffer (Wortmann et al., 2005). This type of master mix was used for the platform compatibility study and validated in accordance with CAP standards for use on the SmartCycler® instrument.

The bulk lot of master mix was prepared using the same recipe as the freshly prepared master mix described above just on a much larger scale, which was then aliquoted and frozen for various periods of time. Specifically, twenty 50-mL conical tubes each containing 100 reactions per tube (approximately 2.5 mL) were used to prepare the bulk lot. The conical tubes were briefly vortexed and centrifuged with an Eppendorf 5810R before the contents of each of the 20 tubes were combined into one 50-mL conical tube. To ensure a homogenous solution, this 50-mL conical tube was vortexed and centrifuged briefly. Aliquots of 450 μL of the bulk lot master mix were dispensed into 0.5-mL O-ring micro-centrifuge tubes. The micro-centrifuge tubes were then divided into 2 groups, and each group was stored in a −20 °C (±2 °C) freezer with recorded temperature telemetry. One aliquot of the bulk lot master mix was not frozen. It was used in a comparison test to freshly prepared master mix to ensure the bulk lot master mix had the same functionality as the freshly prepared prior to being frozen and stored. This comparison was performed on each the SmartCycler® and JBAIDS platforms using extracted *L. tropica* EP139 DNA that was serially diluted in MBG water to 400 pg/μL, 40 pg/μL, 4 pg/μL, 0.4 pg/μL, and 0.04 pg/μL.

2.3. Platform comparison

A comprehensive evaluation of the JBAIDS instrument and a side-by-side comparison of the JBAIDS and SmartCycler® were performed with dilutions of *L. tropica* EP139 (400 pg/μL, 40 pg/μL, 4 pg/μL, 0.4 pg/μL, and/or 0.04 pg/μL) where each dilution was run in triplicate (intra-assay) and 3 real-time PCR runs were performed (inter-assay). This comparison was performed during the same time the bulk lot master mix stability study was ongoing but was performed using freshly prepared master