DIAGNOSIS OF CUTANEOUS LEISHMANIASIS IN GUATEMALA USING A REAL-TIME POLYMERASE CHAIN REACTION ASSAY AND THE SMARTCYCLER®

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Abstract. The polymerase chain reaction (PCR) assay has been reported for the diagnosis of cutaneous leishmaniasis (CL). Real-time (RT) PCR offers several advantages over traditional PCR, including faster processing time and decreased risk of contamination. Enhanced portability is another benefit that expands the applicability of the assay. A portable RT-PCR assay was transported from the United States to Guatemala for comparison with traditional diagnostic modalities. With the clinical diagnosis of CL as the gold standard, RT-PCR was positive in 86% (37 of 43) versus 53% (20 of 38) for microscopy and 72% (28 of 39) for culture. Negative RT-PCR samples (6) were also negative by traditional diagnostic methods (although subsequently determined to be positive by a nested kDNA PCR). Sixty-four percent (9 of 14) of cases tested and negative by microscopy and/or culture were positive by RT-PCR. This study demonstrates that a RT-PCR assay can be successfully deployed to offer enhanced sensitivity for the diagnosis of CL.

INTRODUCTION

Cutaneous leishmaniasis (CL) is a significant health concern in many areas of the world. Infection can result in chronic, disfiguring skin lesions with potential economic and health consequences. CL is endemic in northern Guatemala and is caused by Leishmania (Leishmania) mexicana (L. (L.), mexicana) and Leishmania (Viannia) braziliensis (L. (V.) braziliensis).1 Traditional diagnosis rests primarily on tissue examination for the microscopic identification of Leishmania amastigotes or the propagation of promastigotes from infected tissues in culture. These classic methods can be time consuming, are limited by access to specialized laboratories and microscopic expertise, and have a reported sensitivity of 50–70%.2

Alternative methods of diagnosing CL have been reported, with many investigators reporting success with the traditional polymerase chain reaction (PCR) assay.3–6 Traditional PCR uses agarose gels for detection of PCR amplification at the end-point of the PCR reaction, whereas real-time (RT) PCR allows for detection of PCR amplification during the early phases of the reaction. The technology uses primer sets coupled with a fluorescent probe that hybridizes downstream from one of the primers. After successful hybridization of the probe to the target, polymerase cleaves the hybridized probe, separating a fluorescent dye-labeled “reporter.” The fluorescent signal increases in proportion to the number of amplicons generated during log-linear amplification. The presence of amplified product is confirmed when the fluorescent signal exceeds an automatic noise-based defined threshold. Benefits of this technology include rapid turn-around time and decreased risk of contamination (the PCR tubes remain closed and are then discarded, so amplified product is not released into the laboratory). A portable RT-PCR platform has been developed that weighs ≈ 35 kg (including thermocycler, computer, and wheeled carrying case), which is relatively easily maneuvered, similar to a large luggage bag. The assay can be set up within minutes and can be operated anywhere there is electrical power. Using a fluorogenic RT-PCR assay developed in our laboratory,7 we transported a portable RT-PCR platform from the United States to Guatemala for use in a prospective, feasibility study.

METHODS

Patients. The study was performed under an approved human-use protocol in a Ministry of Health clinic in Poptun in the Peten region of northern Guatemala. The guidelines for human experimentation of the US Department of Health and Human Services and those of the authors’ institutions were followed in conducting the clinical research, and informed consent was obtained from all patients. Adults presenting to the clinic with lesions consistent with CL were solicited for the study. A medical doctor and laboratory technician experienced in the diagnosis and treatment of CL interviewed and sampled the research subjects. A questionnaire was used to determine the medical history of the patient and any potential alternative diagnoses. Each subject was assigned a study code number to protect his or her identity. Forty-three subjects enrolled in this study, and one of the study investigators performed evaluations for clinical determination of CL. A description of the number, size, and age of the lesions was recorded. After the administration of a local anesthetic (2% lidocaine), tissue specimens were obtained by scraping the border of the lesion with a scalpel; the material was placed on a slide for histopathologic examination, in biphasic media for culture and in an Eppendorf tube containing 90% ethanol for later DNA extraction and PCR analysis. All patients received treatment with a 20-day course of intramuscular meglumine antimonate on the basis of clinical diagnosis.

Sample processing. Samples were kept at −20°C and were processed without knowledge of culture or histopathologic results. DNA was extracted from each sample by use of a commercial kit in accordance with the manufacturer’s instructions (QIAamp Tissue Kit; Qiagen, Chatsworth, CA).

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**Real-Time PCR.** PCR reactions were conducted in a 25-μL volume containing 2.0 μL of DNA template, 1 PCR bead (PE Biosystems, Foster City, CA), 8 mM MgCl₂, 100 pM of each oligonucleotide primer, and 10 pM of an oligonucleotide fluorogenic probe. The primer/probe set “Genus” (forward primer 5'-AAGTGCTTTTCCATCGCAACT-3', reverse primer 5'-GACGCATAAACCCCTTCGAA-3', and probe 5'-CGGTGGGTGTTGTGCGGC-3') has been shown to amplify a segment of the *Leishmania* 16S rRNA gene conserved among *Leishmania.*

PCR amplification and detection were performed with the SmartCycler® (Cepheid, Sunnyvale, CA), with preincubation for 95°C for 2 minutes, followed by 40 cycles of 2-step incubations at 95°C for 15 seconds and 60°C for 30 seconds. The presence of amplified product was confirmed when the fluorescent signal exceeded an automatic noise-based defined threshold, and values were recorded as the second derivative of the primary signal, which is the point of greatest change along the growth curve. Stringent measures to control for contamination included performing sham DNA extractions and negative controls (PCR reactions without DNA) with each PCR assay (1 and 2 samples, respectively, for every 10 samples). All PCR-negative samples were subsequently tested with a PCR assay containing primers for the human β-actin gene to detect possible PCR inhibition.

The limit of detection for this assay was defined as the lowest concentration of DNA at which the assay detected three out of three replicates. Using this definition, the limit of detection was determined to be 5.6 pg, which is equivalent to ∼165.4 genome copies.

**Statistical analysis.** McNemar’s test for post-hoc pairwise comparison was used to calculate differences among the three diagnostic assays.

**RESULTS**

**Subjects.** A total of 43 adult patients presenting to the Ministry of Health leishmaniasis diagnostic clinic in Poptun, in the Peten region of northern Guatemala, volunteered for this study.

**Lesions.** The mean number of lesions in this group of patients was 1.7 (range, 1–7). The mean duration of symptoms was 104 days (range, 8–1460).

**Conventional diagnoses.** Of 43 volunteers enrolled into the study, 38 had tissue biopsies processed for microscopy, and 39 for culture. Fifty-three percent (20 of 38) of samples were positive by microscopy, and 72% (28 of 39) by culture.

Thirty-five had both microscopy and culture performed, and one sample had neither. Of the 35 samples that had both modalities performed, 57% (20 of 35) samples were positive for both, while 0% (0 of 35) were positive only for histology and 20% (7 of 35) were positive only for culture. Twenty-three percent (8 of 35) of samples were negative by both techniques.

**RT-PCR results.** RT-PCR using the “Genus” primers/probe was positive for 86% (37 of 43) of the patients with a clinical diagnosis of CL. There was a significant difference between the sensitivity of the RT-PCR and that of microscopy (N = 38; P < 0.0005) and culture (N = 39; P = 0.016). All 6 samples that were negative by RT-PCR were also negative by histopathology and culture. RT-PCR assay using primers for human β-actin were positive in all 6 samples, negating PCR inhibition as an etiology for the negative results. Subsequent testing of these 6 samples with a nested PCR designed to amplify a segment of kDNA was positive for the presence of *Leishmania.* RT-PCR was positive in 100% (28 of 28) of samples diagnosed by conventional diagnostic methods (microscopy and/or culture). Of 14 samples that were tested and negative by at least one conventional diagnostic method, 64% (9 of 14) were positive by PCR.

**DISCUSSION**

Standard methods for diagnosing CL are histopathology and culture. In the current study, we hand-carried a portable RT-PCR thermocycler (the SmartCycler®) and supplies from Washington, DC, to Guatemala to compare the diagnostic sensitivity of RT-PCR with conventional methods. When compared with positive conventional diagnostic assays (histopathology and/or culture), the sensitivity of the RT-PCR was 100%. Using an expert clinician’s (B.A.A.) diagnostic acumen as the “gold standard” and assuming that all patients enrolled in the study had CL, an overall sensitivity of 86% for PCR versus 53% for microscopy and 72% for culture was determined.

Determining a “gold standard” for the diagnosis of CL is difficult, as conventional diagnostic methods are known to be insensitive. The validity of our decision to use the clinical diagnosis of an experienced physician as the “gold standard” is buttressed by the further testing and clinical outcome of six samples that were clinically diagnosed as CL but that were negative by all diagnostic testing modalities (RT-PCR, microscopy, and culture). These samples were later tested and found to be positive with a nested PCR designed to amplify a portion of kDNA. In addition, all patients responded to a course of treatment with meglumine antimonate.

The ability of nested PCR to detect *Leishmania* DNA that was not detected by the RT-PCR assay is likely related to the greater starting copy number of kDNA in *Leishmania* as compared with the rRNA gene (which is the target of amplification for the RT-PCR assay), as well as to the overall enhanced sensitivity of nested PCR. With our RT-PCR assay, kDNA was not chosen as a target for amplification for technical reasons. In designing a RT-PCR to detect all strains of *Leishmania* (a “genus-level” assay), a conserved region of DNA for the annealing of primers and a probe must be determined. In addition, the primers in a RT-PCR assay are recommended to be designed to amplify as short an amplicon as possible. Although regions of kDNA have been reported to be conserved among *Leishmania,* we were unable to determine a segment of kDNA that fit the requirements (primers and probe sequences conserved among all *Leishmania* and production of a short amplicon) for use in our assay. Although the nested PCR detected *Leishmania* DNA in six clinical samples that were negative by RT-PCR, nested PCR is time-consuming to perform and carries a greater risk of laboratory contamination.

Given the diagnostic accuracy demonstrated by an experienced clinician in this current study, one may argue that new technologies for the diagnosis of CL are not needed. However, the pre-test probability of CL in our research cohort is unlikely to be duplicated in clinical practice, and the clinician in our study has several decades of experience with the pre-
sentation of the disease. For many physicians, the differential diagnosis of a non-healing skin lesion would include fungal and mycobacterial infections, as well as cutaneous malignancies, and thus a rapid, sensitive, and specific assay for CL would be beneficial.

Although the use of conventional PCR as a diagnostic tool has been well-reported for several infectious diseases (including leishmaniasis), the advent of rapid, RT-PCR is a more practical modality for clinical diagnosis. With a turn-around time of 40 minutes for our PCR assay (and much faster in other published assays), a physician would potentially have a confirmed diagnosis within hours of obtaining the biopsy. A manual DNA extraction step was used in the current study (which added ~2 hours to the processing time), but recent advances in the field have automated this step, and one manufacturer advertises a 30-minute time from unprocessed sample to real-time PCR result.

Recent data suggests that polymorphism-specific PCR is as reliable as multilocus enzyme electrophoresis for identifying Leishmania species, and our laboratory has presented data demonstrating the usefulness of this concept when applied to a RT-PCR platform. The advent of portability adds further possibilities to this technology, and one can envision point-of-care diagnostics that would allow providers to prescribe therapy on the basis of the infecting species (this would be especially useful in locales such as Guatemala, where both L. mexicana and L. (V.) braziliensis are present). Finally, rapid identification of Leishmania to the species level may eventually prove helpful when performing therapeutic intervention trials, as various species of Leishmania respond differently to various compounds that can interfere with the interpretation of clinical response. Issues with cost (the real-time PCR platform used in this study cost ~US $30,000), technician training, and the potential risk of cross-contamination are still stumbling blocks to wide application of this technology, but these should be overcome as the technology advances.

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