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Development of Real-Time PCR-Based System that Detects Kinetoplast DNA for Diagnosis of Leishmania Infections

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Abstract

Objective: The aim of the study was to develop a real-time polymerase chain reaction-based system that is simple, rapid, and sensitive enough for the diagnosis of *Leishmania* infections by targeting kinetoplast DNA amplification.

Methods: In the study, a standard *Leishmania tropica* strain was obtained from the Republic of Turkey Ministry of Health Microbiology Reference Laboratory Culture Collection. The parasites were cultivated in a medium. Kinetoplast DNA sequences of *Leishmania tropica* and kinetoplast DNA sequences representing *Leishmania major*, *Leishmania Infantum*, and *Leishmania donovani* species were downloaded from GenBank and were aligned with the SeqMan program in DNAStar. The highest overlapping kDNA sequences were detected with BioEdit program and pan-primers were designed targeting relevant sequences. The assays were performed in three separate days as triplicates and melting curve analysis was performed for each *Leishmania tropica* dilutions. Kinetoplast DNA real-time polymerase chain reaction results were compared with the same method detecting *Leishmania tropica* genomic DNA.

Results: The lower detection limit of the developed test was found to be 10° parasite/100 µL and for all groups of dilutions, and intertest coefficient of variation value and regression value (R^2) were 0.98 and 0.9589, respectively. The melting temperature was 79° for all *Leishmania tropica* dilutions.

Conclusion: The developed protocol can be used as an alternative tool for quantitative detection of *Leishmania* species. It is also a useful test to obtain quantitative and accurate results to be used in future studies because of its simplicity, rapidness, sensitivity, and the ease of adaptation to any laboratory setting.

Keywords: Leishmania, leishmaniasis, kinetoplast, kinetoplast DNA, real-time polymerase chain reaction

Introduction

Leishmania spp. are zoonotic-anthroponotic parasites from the order Kinetoplastida belonging to Trypanosomatidae family. Leishmaniasis caused by *Leishmania* spp. has worldwide distribution and is endemic in Turkey, especially in Southeastern Anatolia. The parasite infects mammals, invades blood and tissues of humans, and the disease is transferred via the bite of *Leishmania*-infected female *Phlebotomus* spp. Zoonotic reservoir hosts such as rodents and canines also play an important role in the transmission of the disease. The disease, although rare, can be transmitted by the transplacental route, blood transfusion, and contaminated needles. According to General Directorate of Public Health, a total of 26 366 new cases were reported between 2008 and 2017 in Turkey; Şanlıurfa, Çukurova, and Diyarbakır were registered as the provinces with the highest case numbers.[1,2] There are 22 species of *Leishmania* that are human pathogens but of those, 3 species have been indicated to be more common than others. These species are *Leishmania tropica*, *L. infantum*, and *L. braziliensis*. There are several forms of leishmaniasis, the form of the disease depends on the type of *Leishmania* as well as host immunity.[1,3] Visceral leishmaniasis (VL), Cutaneous leishmaniasis (CL), and Mucocutaneous leishmaniasis (MCL) are the most common forms being detected. Visceral leishmaniasis is also known as "Kala-Azar" and is usually caused by *L. donovani* and its subtypes. If the disease is not treated, the fatality rate can reach up to 100% within 2 years.[4] *Leishmania tropica*, *L. major*, and *L. infantum* are usually causative agents of CL. *Leishmania infantum* is also the causative agent of canine leishmaniasis (CanL) that causes infection in dogs.[5] Cutaneous leishmaniasis is known as "Oriental sore" in our country and has been observed in Anatolia for centuries.[6] Despite the fact that leishmaniasis is a relatively common disease, no vaccine or medicine have been developed to date.[3]

There are 3 main risk factors related to *Leishmania* infections: Socioeconomic conditions, population mobility, and climate change. Inadequate shelter and domestic hygiene conditions may increase the breeding and resting areas of sandfly vectors and the rate of accessibility of sandflies to human hosts. Leishmaniasis outbreaks are often associated with population mobility, which is related to relocation of people to endemic regions where vectors reside. In this respect, leishmaniasis has regained importance for our country in recent years due to large human migrations. Also, uncontrolled animal movements at the borders affect the epidemiology and clinical manifestations.[1] Due to the aforementioned reasons and the emergence of different clinical presentations with different species have led to the need for a more rapid, sensitive, and quantitative

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test that can be used not only for diagnostic purposes in the field but also for drug efficiency and vaccine development research.

Leishmania spp. have a circular kinetoplast DNA (kDNA) that is specific for the species of Kinetoplastida order. Unlike genomic DNA (gDNA), kDNA has two groups of DNA: 20-25 copies of maxicircles and 10.000-20.000 copies of minicircles per cell.[7] Therefore, kDNA can be a good target for molecular testing and detecting relatively low number of parasites. Moreover, the heterogenic feature of kDNA minicircles make them suitable for phylogenetic studies. The unique and conserved nature of kDNA minicircles also makes them good candidates for the detection as well as species-level differentiation through melting curve analysis and next-generation sequencing purposes.[8-13]

Therefore, the aim of this study was to contribute to developing an in-house molecular laboratory method and to amplify kDNA using real-time polymerase chain reaction (real-time PCR) technology, which can detect *Leishmania* spp. in a single session.

Methods

Cultivation and Quantification of *Leishmania tropica*

Leishmania tropica strains grown on Novy-MCNeal-Nicolle (NNN) medium were obtained from the Republic of Turkey Ministry of Health Microbiology Reference Laboratory culture collection. For rapid cultivation purposes and to facilitate counting, 50 µL of the *Leishmania* promastigotes containing medium cultivated into 150 µL AmnioMAX™-II (Thermo Fisher Scientific, ABD) medium and incubated inside screw cap tubes. The caps of the tubes were loosely closed to ensure airflow and incubated for 3 days in the darkroom at room temperature. Following 3 days of incubation, turbidity was observed as a result of parasite growth, 20 µL of the medium was transferred into a 0.5 mL tube, and 20 µL of 2% formaldehyde solution was added to halt the motility of parasites and facilitate counting.[14] Ten microliters of the mixture were taken out and counted on the Fast-Read 102 slide (Biosigma, Italy) which has 10 grids with 1 µL volume per square. On average, 10 *Leishmania* promastigotes were counted per square and 10^4 *Leishmania* promastigotes were counted in 100 µL and these steps were repeated before each experiment. The 100 µL medium that contains 10^4 parasites was diluted with Phosphate Buffered Saline (PBS) and dilutions were prepared into 1.5 mL tubes containing 10^0, 10^1, 10^2, 10^3, and 10^4 *Leishmania* promastigotes.

Nucleic Acid Extraction

Chelex DNA extraction solution (Seegene, Republic of Korea) was used to extract nucleic acid from *L. tropica*.[15] Samples of each dilution were centrifuged at 4000 rpm for 5 min. The supernatant was discarded after centrifugation and 200 µL of Chelex solution was added to each tube. The vortexed tubes were kept in boiling water for 10 min. After 10 min, the tubes were vortexed again, centrifuged at 4000 rpm for 10 min. The supernatant was collected to be used for real-time PCR.

Alignment of kDNA Sequences and Primer Designing

Kinetoplast DNA sequences of *L. tropica*, *L. major*, *L. infantum*, and *L. donovani* were downloaded from GenBank and kDNA sequences representing each species were aligned with the SeqMan program in DNAStar. The aligned base sequences were transferred to the BioEdit program and the highest overlapping DNA sequences in all species were designated as targets for amplification. The amplification primers that were used for the kDNA were designed in accordance with the general primer design guidelines. The ambiguous base coding system was used for nucleotides in regions that differed between species despite the highest level of compatibility. Accordingly, forward primer F 5′-CAG TTT CCC GCC YCG GAG CYS A-3′ and reverse primer R 5′-CTC CGG GTA GGG GCG TTC TGC-3 were designed to amplify kDNA. The primer stock solution was prepared by diluting the ordered primers to a concentration 100 pMol/mL. This stock was diluted 1/4 times and used for real-time PCR studies. Simultaneously, the *Leishmania* gDNA primers were used for the *Leishmania* small subunit ribosomal RNA (SSUrRNA) gene region 5′-GGT TCC TTT CCT GAT TTA CG-3′ and 5′-GGC CGG TAA AGG CCG AAT AG-3′.[16]

Real-Time PCR Using kDNA and gDNA

Real-time PCR reactions were performed with SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Mass, USA) in a final volume of 20 µL, using 5 µL of kDNA extraction. The real-time PCR reactions were performed in a Roche LightCycler 2.0 (Rotkreuz, Switzerland) instrument. The cycle of threshold (CT) value was set to 0.05. Following detection of kDNA by real-time PCR, melting curve analysis of different dilutions was performed to assess the limit of detection of the test. Real-time PCR reactions were performed with SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Mass, USA) in a final volume of 20 µL using 5 µL of gDNA. The temperature profile which is used for the amplification of kDNA is also used for gDNA real-time PCR reactions (data not shown).

Evaluation of the Performance of the Developed Method

In order to evaluate the performance of the developed test based on detecting kDNA, parasites were diluted with PBS to contain 10^0-10^4 parasites in 100 µL. Following nucleic acid extraction, real-time PCR test was performed with known numbers of nucleic acid. Tests were performed on 3 different days in 3 groups (triplicates) per each dilution according to the validation–verification procedures to determine the analytical sensitivity and stability of the developed test.[17]

Statistical Analysis

The regression (R^2) analysis was performed with the Microsoft-Excel-automated spreadsheet in order to determine the consistency between the dilutions studied for 3 different days in accordance with verification rules.[17,18]

Results

Comparison of Real-Time kDNA PCR with Real-Time Genomic DNA PCR Results

Initially, the results of real-time PCR targeting kDNA and gDNA were compared for the assessment of the sensitivity of the assay. As a result of this comparison, the CT values were found to be 31.56 and 40.38 for kDNA and gDNA, respectively (Figure 1). The difference between the two targets delta CT (ΔCT) was found to be 8.82. Considering that the target doubled in each PCR cycle, the ΔΔCT value would indicate the robustness of kDNA detection compared to gDNA and this value was calculated to be approximately 450.

Evaluating the Performance of the kDNA Assay

For the determination of the dynamic range of real-time PCR, amplification runs were performed on parasitic dilutions, consisting of 10^4, 10^3, 10^2, 10^1, and 10^0 *Leishmania* promastigotes. In order to specify the accuracy and to detect the precision limit of the assay, PCR runs were performed on 3 different days as triplicates.

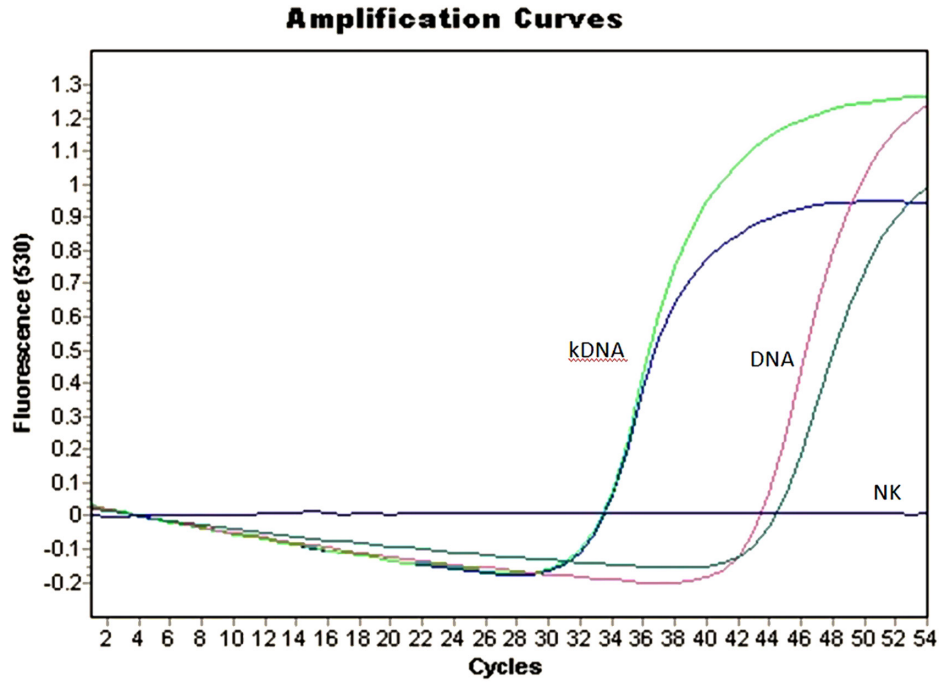

Figure 1. Comparison of kDNA and gDNA real-time PCR. kDNA, kinetoplast DNA; gDNA, genomic DNA.

The amplification and melting curve analysis of the test runs are shown in Figure 2 and the intra-assay reliability findings are shown in Table 1. The inter-assay CTs and mean values per dilutions were as follows: 25.74 ± 0.61 for non-diluted (ND); 29.17 ± 0.70 for dilution 1 (D1); 32.79 ± 0.37 for D2; 35.62 ± 0.50 for D3; and 37.63 ± 0.86 for D4.

According to these results, R^2 was performed using Microsoft Excel program. Thus, the samples containing the same number of parasites amplified on different days with the same study method, the results were similar and R^2 value was detected to be 0.9589 (Figure 3). Besides real-time PCR experiment, we also performed melting curve analysis for the standard *L. tropica* obtained from the reference laboratory. As a result of this, *L. tropica* melting temperature (Tm) value was 79° for all dilutions (Figure 1).

Discussion

Evaluating the accuracy of a test under its own laboratory conditions in addition to the precision is crucially important. It is necessary to know the test performance to be used in the laboratory and to report the experimental test results. Moreover, the lower limit of detection for qualitative tests and the dynamic range for quantitative tests need to be determined. In order to verify the developed test under the laboratory conditions, the test results should be compared with previous reports compared to a reference test method.[19]

For the assessment of the accuracy of a recently developed test, current literature recommends that two series of high and low concentrations of reference material should be tested 2-5 times, and under particular laboratory conditions, all the dilutions used should give the same results. For the evaluation of repeatability and reproducibility, the sensitivity between consecutive tests is to be determined. For quantitative tests, the results of 2 separate samples should be observed for 20 days, or alternatively, 2 different concentrations in triplicate runs should be tested over 5 days. Thus, in a 5-day period, 20 data points are analyzed for low concentration samples; the dynamic detection range, the number of copies, or units and quantitative lowest detection limit should be determined. Therefore, starting from concentrations close to the lowest detection limit, 5-7 concentrations are tested as duplicates.[17]

To ensure the requirements indicated above, *L. tropica* promastigotes were cultivated and 5 different concentrations were prepared as 10^0, 10^1, 10^2, 10^3, and 10^4 in 100 µL and tested in 3 different days as triplicates for each concentration. The obtained test results were determined to be consistent with each other (R^2 = 0.9589). In addition, the recently developed test was compared with a real-time PCR that detects *Leishmania* gDNA which was previously reported to observe the sensitivity.

The precision of a test is correlated with repeatability, replicability, and reproducibility. Repeatability indicates the stability of a test that is independently measured multiple times under the same conditions such as same observers, same laboratory, and same equipment using the same method. Reproducibility on the other hand denotes the stability of a test that is independently measured multiple times under different conditions such as different laboratories or different performers using the same method. As for replicability, the assay must be performed under the same conditions by different observers.[20] For the detection of repeatability, replicability, and reproducibility, variation coefficient and standard deviation of the assay must be analyzed correlated with aforementioned validation and verification protocols.[17] All the necessary verification and sensitivity analyses have also been performed for this currently developed method.

In addition, the linear range of quantification has to be evaluated for quantitative tests. In order to determine the linear range, linear regression analysis should be performed due to inter- and intra-assay findings. The closer the linear range is to $y = 2x$, the greater the repeatability of the results of the test.[17,21]

Based on this information, the assay was designed to detect a low parasite load with high sensitivity as well as high reproducibility and repeatability. Since the linear regression analysis of the assay is close to the $y = 2x$ curve for real-time PCR tests, the developed test results show that reliable quantitative results can be obtained in the dynamic range between 10^0 and 10^4 parasites/100 µL. These

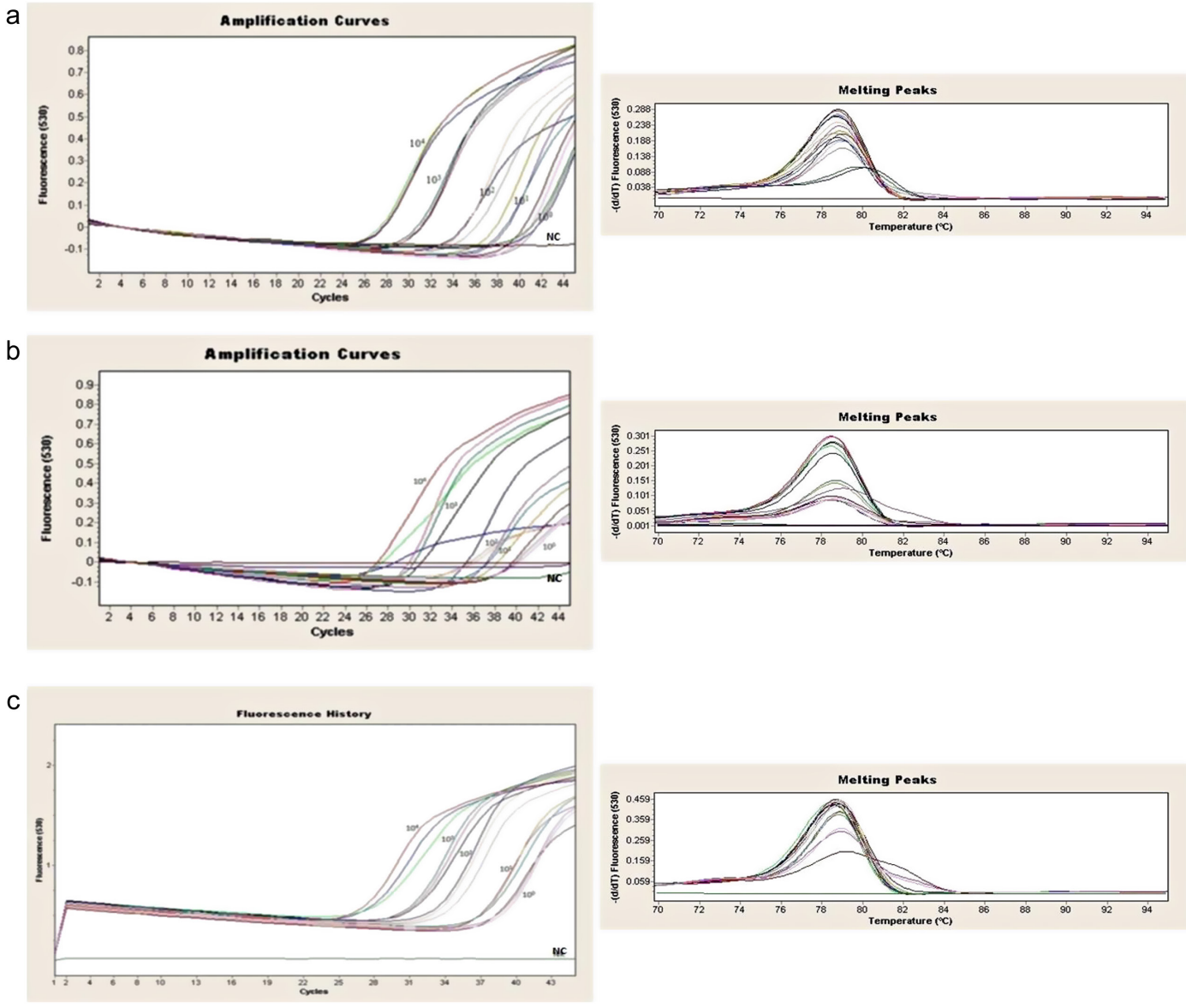

Figure 2. Real-time PCR for kDNA quantitation and melting curve analysis of dilutions studied in triplicate on 3 different days. (a) Day 1, (b) Day 2, (c) Day 3. Real-Time PCR.

findings are obtained by the following validation–verification protocols in accordance with the quality control procedures.[17]

Turkey, which is an important country for the epidemiology of *Leishmania* has different ecological and climatic conditions. Thus, both CL and VL cases are common and still important public health problems.[22] Leishmaniasis outbreaks are constantly associated with population mobility, migration, and relocation to places in the transmission cycle. In this regard, *Leishmania* is gaining importance for our country and its epidemiology and clinical manifestations change in recent years due to the large human migrations.[1]

The sensitivity of microscopy and culture- and serology-based methods is high. However, species-level differentiation

Table 1. Test Reliability Due to Average CT Values and Mean Values of 3 Triplicates per experiment.

	Average CT's and Mean Values of Each Triplicate					
	ND (10^4)	D1 (10^3)	D2 (10^2)	D3 (10^1)	D4 (10^0)	NC
First run	25.85 ± 0.11	29.22 ± 0.16	33.31 ± 0.74	36.22 ± 0.41	38.85 ± 0.53	>40.00
Second run	24.94 ±0.10	28.28 ± 0.47	32.60 ± 0.39	35.00 ± 0.41	37.13 ± 0.30	>40.00
Third run	26.43 ± 0.65	30.01 ± 0.21	32.46 ± 0.37	35.64 ± 0.30	36.92 ± 0.46	>40.00

ND, non-diluted; D1, dilution 1; D2, dilution 2; D3, dilution 3; D4, dilution 4; NC, negative control. **Mean values were calculated for triplicates of each dilution

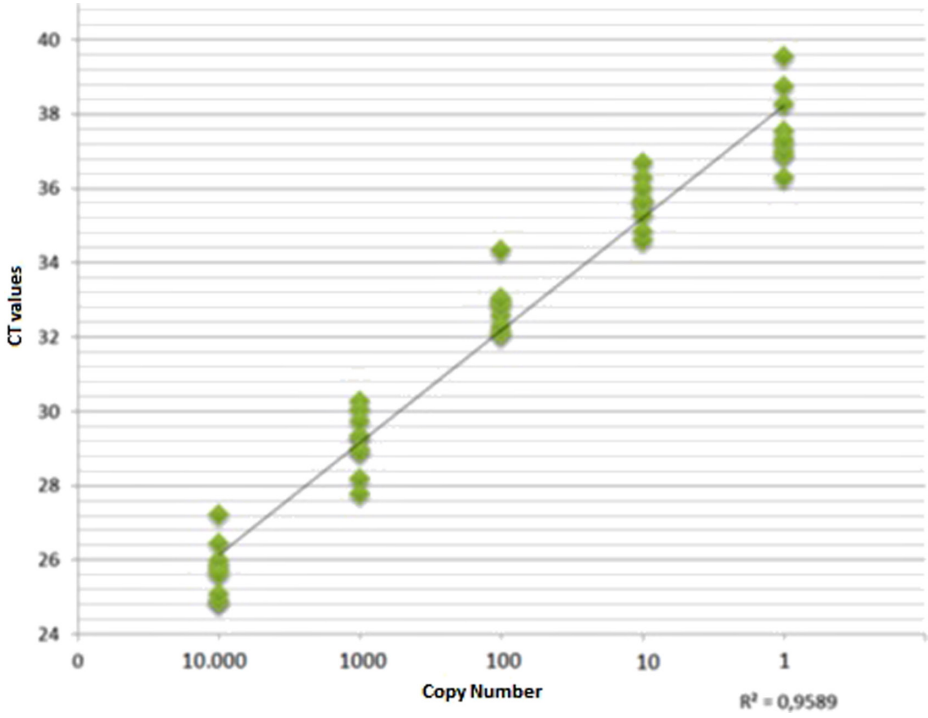

Figure 3. Comparison of study data for different dates; CT values and regression analysis (R^2 = 0.9589). CT, cycle of threshold.

from each other is not possible with these techniques, and cross-reactions with antibodies produced against other pathogens may occur. Furthermore, clinical samples with low parasite loads can't be detected by using these methods.[23] Belinchón-Lorenzo et al[24] reported that the kDNA-based real-time PCR tests have high sensitivity and specificity for blood samples taken from seropositive symptomatic and asymptomatic canine cerumen samples. Accurate diagnosis, as well as determination of the species, is important in terms of prognosis and appropriate treatment of the disease.[25] Diagnosis of leishmaniasis by direct tissue examination and culture has significant limitations.[26] Despite the fact that direct examinations are cheap and easy to perform, sensitivity decreases when the amount of parasite in the tissue is low and requires an experienced technician.[10,27] During in vitro cultures, *Leishmania* promastigotes can aggregate with each other which are called "rosette form."[28] This formation causes the promastigotes to not be counted precisely with microscope examination and causes deficiencies in circumstances that require quantitative results such as drug efficiency and vaccine development research. In this respect, the main objectives of this study were to develop a rapid, easy-to-use, sensitive, and comprehensive test method for the diagnosis of *Leishmania* infections that can be used in the field and also standardize quantitative *Leishmania* detection for follow-up studies.

Leishmania kDNA is an ideal target for molecular testing due to its highly conserved structure. Since a single *Leishmania* cell contains thousands of these highly conserved kDNA minicircles, targeting kDNA for quantitative molecular amplification as well as kDNA minicircle-based phylogenetic studies of *Leishmania* are trending in recent years. Therefore, kDNA-PCR method is a sensitive and highly reproducible diagnostic approach as stated by authors before.[7,10,11] Since kDNA is considered to be the most appropriate target region due to its high sensitivity, kDNA real-time amplification has gained importance to eliminate the disadvantages associated with low parasitic load.[27] kDNA real-time PCR-based method can also be used to detect parasitic loads rapidly and easily in different tissue samples for the detection of mucosal, cutaneous, and visceral leishmaniasis.[10] Additionally, the sensitivity of tests for clinical cases varies in parallel with *Leishmania* amastigote load in the lesion area, such as ulcer-intact tissue border, ulcer base, and complete mid-point of the ulcer.[29] In some cases, it is difficult to detect amastigotes residing by microscopy in chronic CL due to having low parasite numbers. For the diagnosis of leishmaniasis with direct microscopy, an experienced laboratory worker is required. Where the experienced laboratory worker is not present, it is difficult to diagnose infections with low parasitic load directly and false-negative results can be reported.[27] For this reason, it is significant to detect parasitic load by using molecular methods for those cases in particular. Quantitative results were detected with kDNA real-time PCR method with high sensitivity even if the lower parasitic load of the clinical sample.[10] There are several papers that are targeting *Leishmania* kDNA detection among different clinical samples.[8,10-12,23,29,30] All the abovementioned literatures indicate that kDNA real-time PCR method is a sensitive and an ideal approach for the quantitative detection of *Leishmania* infections. Moreover, provides a suitable platform for vaccine and drug researches as well as monitoring in vivo drug response in patients with VL.[31]

In addition to the detection of *Leishmania* and the parasite load in clinical samples; kDNA real-time PCR can amplify multiple *Leishmania* species and species-level identification is possible by high-resolution melting curve analysis with high sensitivity and specificity.[8,9,11,12] Heterogenic and unique features of kDNA minicircles make them useful tools for phylogenetic studies.[13] In light of this information, we performed melting curve analysis along with real-time PCR with our reference *L. tropica* strain. Weirather et al[9] conducted an assay similar to ours. They used serial parasitic dilutions PCR assay for detection, species discrimination, and quantification of *Leishmania* spp. in clinical samples.[9] Our results are concordant with this literature in terms of *L. tropica* kDNA melting temperature values. Additionally, through melting curve analysis, species-level detection of *L. tropica* was

successful in this study. Although we aimed to distinguish four different *Leishmania* species with melting curve analysis using different primer sets we had to proceed with only *L. tropica*, and clinical specimens couldn't be used due to some unexpected budget restrictions, which has been a drawback for our current work. This method can be suitable for accurate quantitative detection of *Leishmania* spp. for research purposes and highlights to develop an in-house diagnosis technique. As an advantage, melting curve analysis can be a good choice to differentiate by subtypes as well as by Kinetoplastida order. In order to use the test for the routine diagnosis of infections, controlled studies with clinical samples should be tested and the results must be compared with standard methods as well as with the current literature.

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References

1. *Şark Çıbanı İstatistik Verileri [Internet]*. Halk Salığı Genel Müdürlüğü; 2017. Accessed November 30, 2021. https://hsgm.saglik.gov.tr/tr/zoonotikvektorel-sarkcibani/istatistik.
2. Guerin PJ, Olliaro P, Sundar S, et al. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. 2002;2(8):494-501.
3. Centers for Disease Control and Prevention. Centers for disease control and prevention. *Indian J Pharmacol*. 2004;36:268-269.
4. *Leishmaniasis [Internet]*. World Health Organization; 2021 Accessed Nov 30, 2021. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.
5. Ribeiro RR, Michalick MSM, da Silva ME, Dos Santos CCP, Frézard FJG, da Silva SM. Canine leishmaniasis: an overview of the current status and strategies for control. *BioMed Res Int*. 2018;2018:3296893. [CrossRef]
6. Özbel Y, Özensoy Töz S. Leishmaniasis. In: Özcel MA, Özbel Y, Ak M, eds. *Özcel'in Tıbbi Parazit Hastalıkları*. Türkiye Parazitoloji Derneği Yayını; 2007:197-244.
7. Camacho E, Rastrojo A, Sanchiz Á, González-De La Fuente S, Aguado B, Requena JM. Leishmania mitochondrial genomes: maxicircle structure and heterogeneity of minicircles. *Genes*. 2019;10(10). [CrossRef]
8. Ahuja K, Vats A, Beg MA, et al. High resolution melting based method for rapid discriminatory diagnosis of co-infecting Leptomonas seymouri in Leishmania donovani-induced leishmaniasis. *Parasitol Int*. 2020;75:1-18.
9. Weirather JL, Jeronimo SMB, Gautam S, et al. Serial quantitative PCR assay for detection, species discrimination, and quantification of Leishmania spp. in human samples. *J Clin Microbiol*. 2011;49(11):3892-3904. [CrossRef]
10. Jara M, Adaui V, Valencia BM, et al. Real-time PCR assay for detection and quantification of Leishmania (Viannia) organisms in skin and mucosal lesions: exploratory study of parasite load and clinical parameters. *J Clin Microbiol*. 2013;51(6):1826-1833. [CrossRef]
11. Ceccarelli M, Galluzzi L, Migliazzo A, Magnani M. Detection and characterization of Leishmania (Leishmania) and Leishmania (Viannia) by SYBR Green-based real-time PCR and high resolution melt analysis targeting kinetoplast minicircle DNA. *PLoS ONE*. 2014;9(2):e88845. [CrossRef]
12. Diotallevi A, Buffi G, Ceccarelli M, et al. Real-time PCR to differentiate among Leishmania (Viannia) subgenus, Leishmania (Leishmania) infantum and Leishmania (Leishmania) amazonensis: application on Brazilian clinical samples. *Acta Trop*. 2020;201:105178. [CrossRef]
13. Kaufer A, Stark D, Ellis J. Evolutionary insight into the Trypanosomatidae using alignment-free phylogenomics of the kinetoplast. *Pathogens*. 2019;8(3). [CrossRef]
14. Mottram J. *Protocols for Handling and Working with Leishmania Species*. Mottram Laboratory; 2008.
15. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques*. 2013;54(3):506-513.
16. Cruz I, Acosta L, Gutiérrez MN, et al. A canine leishmaniasis pilot survey in an emerging focus of visceral leishmaniasis: Posadas (Misiones, Argentina). *BMC Infect Dis*. 2010;10:342. [CrossRef]
17. Rabenau HF, Kessler HH, Kortenbusch M, Steinhorst A, Raggam RB, Berger A. Verification and validation of diagnostic laboratory tests in clinical virology. *J Clin Virol*. 2007;40(2):93-98. [CrossRef]
18. Ercan M. *MS Excel'in İstatistik Fonksiyonları. Çeşitli Ya*. İzmit: T.C. Orman Bakanlığı Kavak ve Hızlı Gelişen Orman Ağaçları Araştırma Enstitüsü Müdürlüğü; 1997.
19. Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in vitro diagnostic medical devices and repealing Directive 98/79/EC and Commission Decision 2010/227/EU [Internet]. 2017 Accessed Nov 30, 2021. https://eur-lex.europa.eu/eli/reg/2017/746/oj.
20. Plesser HE. Reproducibility vs. replicability: a brief history of a confused terminology. *Front Neuroinform*. 2018;11:1-4.
21. Linnet K, Boyd J. Selection and analytical evaluation of methods—with statistical techniques. In: Burtis C, Ashwood E, Burns D, eds. *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*. 4th ed. St. Louis: Elsevier Saunders; 2006:353-407.
22. Özbilgin A, Harman M, Karakuş M, et al. Leishmaniasis in Turkey: visceral and cutaneous leishmaniasis caused by Leishmania donovani in Turkey. *Acta Trop*. 2017;173:90-96. [CrossRef]
23. Mohammadiha A, Mohebali M, Haghighi A, et al. Comparison of real-time PCR and conventional PCR with two DNA targets for detection of Leishmania (Leishmania) infantum infection in human and dog blood samples. *Exp Parasitol*. 2013;133(1):89-94. [CrossRef]
24. Belinchón-Lorenzo S, Parejo JC, Iniesta V, et al. First detection of Leishmania kDNA in canine cerumen samples by qPCR. *Vet Parasitol*. 2016;228:65-68. [CrossRef]
25. Blum J, Desjeux P, Schwartz E, Beck B, Hatz C. Treatment of cutaneous leishmaniasis among travellers. *J Antimicrob Chemother*. 2004;53(2):158-166. [CrossRef]
26. Deniau M, Cañavate C, Faraut-Gambarelli F, Marty P. The biological diagnosis of leishmaniasis in HIV-infected patients. *Ann Trop Med Parasitol*. 2003;97(suppl 1):115-133. [CrossRef]
27. Aghamolaei S, Behniafar H, Behravan M, Hajjaran H, Moin Vaziri V. Probability of false-negative results in microscopical detection of cutaneous leishmaniasis: more accurate screening by kDNA-PCR during epidemiological survey. *J Parasit Dis*. 2020;44(4):781-784. [CrossRef]
28. Iovannisci DM, Plested CP, Moe GR. Evidence for rosettes as an unrecognized stage in the life cycle of leishmania parasites. *J Eukaryot Microbiol*. 2010;57(5):405-414. [CrossRef]
29. Suárez M, Valencia BM, Jara M, et al. Quantification of leishmania (Viannia) kinetoplast DNA in ulcers of cutaneous leishmaniasis reveals inter-site and intersampling variability in parasite load. *PLoS Negl Trop Dis*. 2015;9(7):1-14.
30. De Paiva Cavalcanti M, Dantas-Torres F, Da Cunha Gonçalves de Albuquerque S, et al. Quantitative real time PCR assays for the detection of Leishmania (Viannia) braziliensis in animals and humans. *Mol Cell Probes*. 2013;27(3-4):122-128. [CrossRef]
31. Pourabbas B, Ghadimi Moghadam AG, Pouladfar G, Rezaee Z, Alborzi A. Quantification of Leishmania infantum kinetoplast DNA for monitoring the response to meglumine antimoniate therapy in visceral leishmaniasis. *Am J Trop Med Hyg*. 2013;88(5):868-871. [CrossRef]