Original Article

Design and Validation of Real-Time PCR: Quantitative Diagnosis of Common *Leishmania* Species in Iran

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Abstract

Objective: Design and validation of Real-time PCR on the protected gene region ITS_2 to quantify the parasite load in common *leishmania* (L) species.

Materials and Methods: Probe and primer were designed from the ITS_2 region between the rRNA genes with minimum gene variation in three common *leishmania* species followed by a Real-time PCR using the Taq man probe method in the form of absolute quantification. A series of different concentrations of *leishmania* were analyzed. After the purified PCR product was successfully placed in a PTG19-T plasmid vector, specialized ITS_2 region was cloned in this plasmid. In the last phase, the cloned gene was transferred to the *Ecoli.*Top10F bacteria. The standard plasmid was provided in 10^7 to 10^1 copies/rxn concentrations. The specification and clinical sensitivity of the data was analyzed using inter and intra scales.

Results: The probe and primer were designed using three species, including *L. infantum*, *L. major*, and *L.tropica.* Seven concentrations of purified parasite in culture media showed that the selected region for quantifying the parasite is suitable. Clinical and analytical specificity and sensitivity were both 100%, respectively.

Conclusion: The Taq man method for the ITS_2 region in *leishmania* is one the most sensitive diagnostic test for identifying the parasite load and is suggested as a tool for fast identification and quantification of species.

Keywords: *Leishmania*, quantification, ribosome RNA

Cite this article as: Fekri Soofi Abadi M, Dabiri S, Fotouhi Ardakani R, Fani Malaki L, Amirpoor Rostami S, Ziasistani M, Dabiri D. Design and validation of real-time PCR: Quantitative diagnosis of common *leishmania* species in Iran. *Arch Iran Med.* 2016; **19(7):** 496 – 501.

Introduction

Depending on the species and the patient's response, there are three clinical sub-types of leishmaniasis, including: cutaneous, mucocutaneous, and visceral.[1,2] Depending on their life cycle, there are two forms of *leishmania*: amastigote and promastigote.[3,4] This infectious disease has become a problem in the past decades. Annually, around 1.5 – 2 million cutaneous *leishmania*, and 500 thousand visceral leishmaniasis are reported around the world.[4] Ninety percent of the cutaneous leishmaniasis are reported from Afghanistan, Pakistan, Iran, Syria, Saudi Arabia, Brazil, Peru and Algeria.[5]

Recently, leishmaniasis has been recognized as an emergency infectious disease in travelers and its treatment has proven to be a challenge for doctors in non-endemic areas.[1] Considering the living conditions and suburban life-style around cities, as well as the increase in the number of cases and geographical spread, new focal points of the disease have been developed.[2] The accurate diagnosis of *leishmania* species with Giemsa staining is not possible, due to the morphologic similarity of amastigote and promastigote of different species. Molecular techniques such as PCR and Real-time PCR can determine the species of the parasite with only a small amount of DNA from the infected tissue.[6–8] For molecular diagnosis of the *leishmania* parasite using the PCR method, species specific genetic markers such as ribosomal DNA, internal transcribed spacer or ITS,[9] gentubolin, *gp63* gene,[10] microsatellite DNA, extra chromosome DNA such as minicircles, kinetoplastic DNA (kDNA), are being used as targets.[11,12]

PCR is a diagnostic tool with a higher sensitivity compared to serologic methods in identifying parasite in tissue. However, the protocol of a normal PCR does not allow us to quantify the parasite load in clinical samples. The quantitative PCR method is able to define the parasite load for different species and conditions under normal and standard circumstances.[13]

The focus of the study was on presenting an analysis based on the ITS_2 gene region in common species of *leishmania* with a comprehensive bio-informatics background to reduce the errors by variation of the parasite genome in quantifying parasite load.

Materials and Methods

Collecting data

A total of 30 samples from lesions suspected of leishmaniasis and three standard samples of *L.major* (MRHO/IR/64/Nadim.1Strain), *L.tropica* (MHOM/SUDAN/58/ODStrain) and *L.infantum* (MCAN/98/LLM.877) were collected from patients referred to clinics of Kerman University of Medical Sciences, Iran. All clinical samples were acquired with the patient's consent and finally 300 positive, and 300 negative controls of clinical diagnosed for leishmaniasis were used for verification.

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Accepted for publication: 18 May 2016

First a smear from the lesion was made and direct smear was stained with giemsa. The stained sample was observed under inverted microscope and examined for *leishmania* amastigote bodies inside and outside of macrophages. For *in vitro* culturing, two media were used, a modified NNN (Nicole. Novy. MacNeal) for survival, and an enriched RPMI 1640 for mass reproduction. Finally, the cultured samples were transferred to a 22°C incubator (Figure 1).

DNA extraction

DNA extraction was carried out, using the QiaAmp DNA mini kit. Determining the concentration was done by the Nano drop device (2000 thermo) in a wavelength of 260 nm and the ratio of absorbed DNA of a wavelength of 260 nm compared to 280 nm (A260/A280) was acquired.

PCR with the aim of placing the K DNA gene

To identify the species of *leishmania,* the cinnagen qualitative PCR detection kit was used. The mentioned substances in Table 1 were poured into a 0.2 mL micro tube and after a spin they were placed inside the thermo cycler device (C1000 touch bio Rad) and PCR was carried out according to the procedure below:

Initial denaturation 180 seconds 95°C, annealing 30 seconds 63°C, extension 60 seconds 70°C, denaturation 40 seconds 93°C, annealing 40 seconds 63°C, and extension 60 seconds 72°C (the last three steps are repeated for 35 cycles).

Analyzing the PCR product

Electrophoresis of PCR products was done next to the 100pb marker ((Fermentase, Cat. No.: 3SMO323) on 2% agarose gel with TBE 0.5X as buffer (trise, boric acid and 0.5M EDTA) and the colorant syber safe 1.0 mg/mL. To observe the length of the PCR product bands, the Bio. Rad model of the Gel DOC device was used under UV light (Figure 2).

Creating recombinant plasmid and preparing for quantification

Part of the ITS_2 gene region from the common species of *leishmania*, part of the GAPDH gene from the normal human genome and part of the Beta actin gene as an internal control were made separately with quantifiable primers specified for humans. Then, the bands from the agarose gel were purified using the Roche kit (Cat. No.: 11732676001) and ligation process was carried out according to the instructions of the PTG19-T PCR cloning vector kit (Cat. No.: TAolovivantis) in the PTG19-T vector. Three plasmids were separately transformed into the Top10F strain of the *E.coli* bacteria. After culturing the mentioned transformed bacteria and developing a colony, plasmid extraction was carried out using the Accuprep Nano. Plus plasmid Mini extraction kit (Cat. No.: k.3030.1), the verification of creating the recombinant plasmid was done by restriction enzymes and the Real-time PCR method using specified primers. Then, the ITS_2 plasmids with concentrations ranging from 10^7 to10^1 copies/rxn and the GAPDH plasmids with concentrations ranging from 10^7 to10^1 copies/rxn were prepared. Also, the internal control plasmid was diluted, so that one suitable copy was experimentally obtained in which the CT = 34 ± 3 could be acquired and added to the sample whose DNA was to be extracted from.

Designing the probe and primer

This analysis aims to compare and synchronize the genetic sequence of the different species of the *leishmania* parasite by emphasizing on the ITS_2 gene region and using bioinformatics software such as Gene Runner, CLC Genomics workbench, Allele ID, and the Internet software. BLAST on the NCBI Website and the Gene bank data bank, through finding similar areas on the gene and creating probes and primers such as:

· ITS-P: 5'- FAM –AGCGTCGAAACTCCTCTCTGGTGC- BHQ1-3'
· ITS-R: 5'- TTTAATAATCCTGGTCACAGCC-3'
· ITS-F: 5'-CAAATACACGCATGCACTCTC- 3'

Table 1. The quantity and type of material used in PCR experiment

Type of material	Values (µL)
Master mix	20
Taq DNA polymerase	0.3
DNA	5
Total volume	**25**

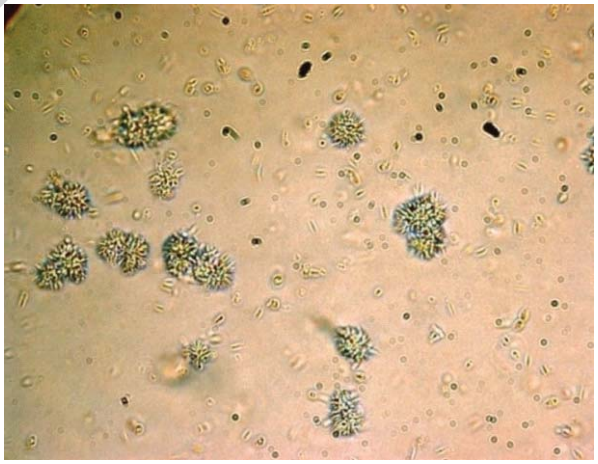

Figure 1. Parasite growth in culture media was seen by an inverted microscope.

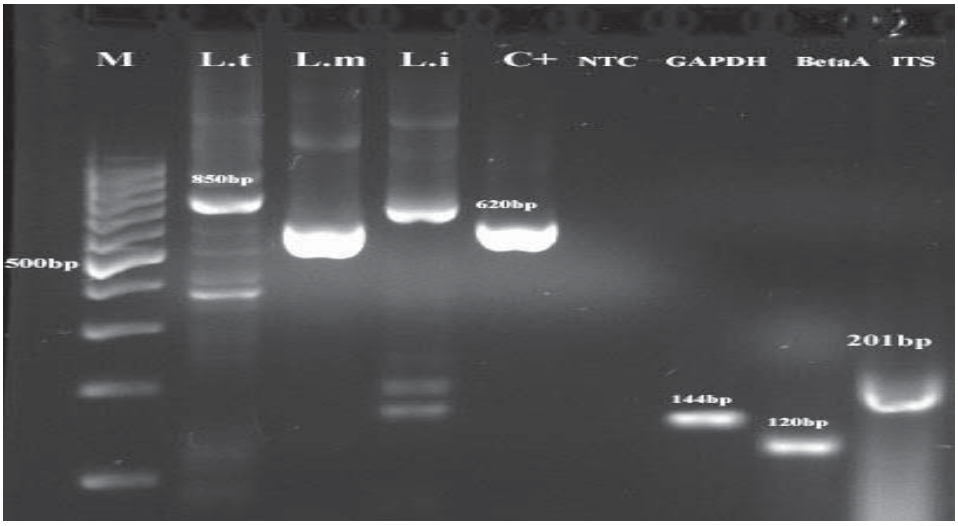

Figure 2. Electrophoresis of kDNA showed gene PCR product in the common species of *leishmania* on the 2% agarose gel; from left to right, column 1: molecular marker 100bp; column 2: fragment length for *L.tropica* 850bp; column 3: fragment length for *L.major* 620bp; column 4: fragment length for *L.infantum* 800bp; column 5: positive control of *L.major*; column 6: contamination control.

With a length about 200 bp to enable quantifying the parasite load, using the Real-time PCR method. The second series of probe and primer were designed on the Beta actin gene to be used an internal extraction control, which include:

· ACTB-F: 5'-ACCACCTTCAACTCCATCATG-3'
· ACTB-P: 5'-VIC - ACATCCGCAAAGACCTGTACGCC -TAMRA- 3'
· ACTB-R: 5' - CTCCTTCTGCATCCTGTCG - 3'

with a band size of 120bp.

This process enables controlling the separation process of DNA and the possible deterrence of PCR.

The third set of primer and probe was made on GAPDH from the human genome as an endogenous reference to normalize any changes, which include:

· GAPDH-F: 5'- CCCATGTTCGTCATGGGTGT- 3'
· GAPDH-P: 5'- FAM- CTGCACCACCAACTGCTTAGCACCCC-BHQ1- 3'
· GAPDH-R: 5'-TGGTCATGAGTCCTTCCACGATA - 3'

with a band size of 144bp.

It should be mentioned that the ITS_2 probe and primer did not amplify any genes from parasite species and the synthesis of these probes and primers were carried out by the Korean company, Bioneer.

Real-time PCR reaction

The Real-time PCR reaction was carried out by the Biosystem step one (ABI) device. The volume of 25 µL of reaction mixture contained 12.5 µL of 2x Quantifast probe PCR kit (Qiagen), 2 µL of forward primer, 2 µL of reverse primer, 1.5 µL of probe, 2 µL of H_2o and 5 µL of extracted DNA. The temperature of the protocol was 95°C over 2 minutes, 45 cycles in 20 seconds at 95°C, 30 seconds at 60°C.

In all the experiments, seven standards with the dilution serial from 10^7 to 10^1 copies/rxn, both for the ITS_2 region of the gene from the *leishmania* parasite and for the GAPDH, were separately proliferated and the standard curve for each of them was drawn as CT against the different parasite concentration. All the test samples, standards, negative controls and positive controls were used in three sets and analyzed using the same device. Using CT, the difference in reaching the threshold line was analyzed. The slope was the gradient of the line. The slope was "a", the gradient of the line.

Statistical analysis

Analysis of variance (ANOWA), mean, standard deviation, coefficient of variation and Pearson correlation were performed, using the SPSS V.15 program to compare and depict the correlation of variables. $P < 0.05$ was considered significant in the study.

Results

Determination of analytical specificity and sensitivity

To determine the analytical sensitivity, a series of different concentration of plasmid from 10^7 to 10^1 were provided. By carrying out a test with 10 repetitions for each serial dilution of *leishmania* plasmid of three species for 15 consecutive days, the Limit of Detection (LOD) was determined between 1 and 10 copies/rxn with 95% Confidence Interval. The analytical specificity was determined by designing and choosing suitable probes and primers, as well as accurate reaction conditions. It was proven that this set of ITS_2 probes and primers do not amplify any other parasite genes.

Linear range

By doing some analysis, some of the concentrations of standard ITS_2 plasmid in the range of 10^7 to 10^1 copies/rxn were determined, but the results showed that the linear range surely covered the minimum concentration of 10^7 to 10^1 copies/rxn and is quantifiable. The standard curve according to the diagram slope is -3.23 and R_2 is 0.997 and efficiency is 103.657, which showed a suitable standard curve for quantifying the parasite load (Figures 3 and 4).

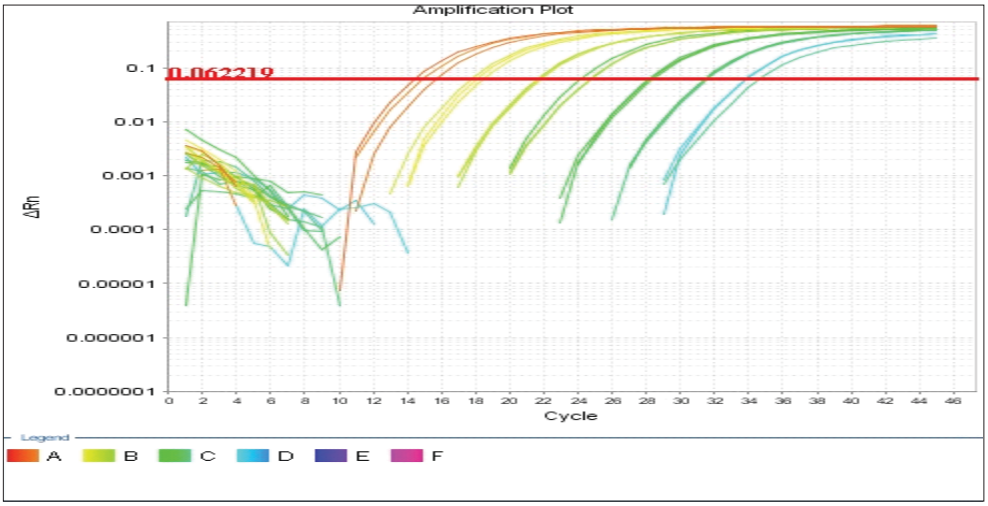

Figure 3. Amplification curve for parasite plasmid in a serial dilution of (10^7-10^1copies/rxn) in which the cycle threshold was shown.

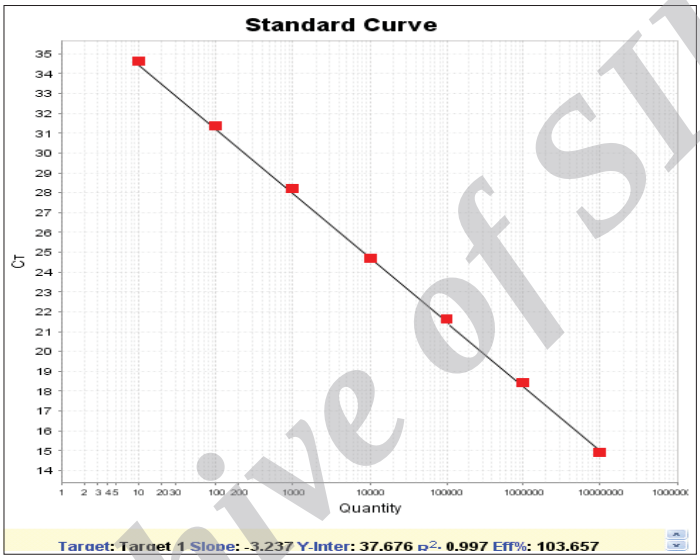

Figure 4. The standard curve by use of a serial dilution of *leishmania* plasmid (10^7–10^1 copies/rxn). The X-axis indicates the cycle threshold (CT) the Y-axis indicates the quantity. Each point on the curve was done as triplicate test. Efficiency is 103.657%, slope is -3.237 and R_2 is 0.997.

Intra and inter assay

By using the accuracy data for each concentration of the ITS_2 region for common species of *leishmania*, coefficient of variation (CV) for CT values which were acquired by different user and devices from the same laboratory were determined in a single experiment on average to be maximum 0.88% and 2.89% for intra and inter assay for the three species of *leishmania*. The acquired values were used to determine standard deviation, variance and variation constant for each of the standard concentrations used which are shown in Table 2.

Analytical specificity and sensitivity

To carry out diagnostic evaluation, clinical sensitivity and specificity was analyzed. For clinical sensitivity, 300 samples, which were verified as positive, were examined and were all proven to be positive. For clinical specificity, 300 negative samples were examined and again they were all verified to be negative. Therefore, the clinical sensitivity and specificity of the *leishmania* species was 100%.

Determination of accuracy

The method was compared with standard curve gene region of ITS_2 and standard curve, namely kinetoplast gene *leishmania* cytochrome-b (genesing standard kit HB 10.04.07) as a gold standard to show its accuracy. Data showed a close correlation in a wide range of concentrations, as well as results of positive and negative control sample tests.

Discussion

In recent decades, using techniques that depend on Nucleic Acid Test (NAT) in the field of diagnosing infectious diseases has become common. The increase in use of these diagnostic tests in laboratories requires a strong validation. Considering this, an important responsibility burdens the laboratories to verify and validate the domestic test before they can be used routinely in analyzing and diagnosing infections. Therefore, economic

Table 2. The average of intra-inter assay data in the three common species of *leishmania* based on standard dilution of plasmid (10^7 – 10^1 copies/rxn). Each standard dilution of the parasite's sample was performed ten times.

	Intra-assay CT values			Inter-assay CT values		
No. of copies	**Mean**	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
10^7	15.07	0.133	0.883	14.906	0.431	2.891
10^6	18.31	0.146	0.802	17.993	0.483	2.686
10^5	21.544	0.179	0.833	21.410	0.418	1.957
10^4	25.055	0.192	0.767	24.693	0.551	2.233
10^3	28.546	0.176	0.619	28.073	0.563	2.007
10^2	32.047	0.166	0.520	31.29	0.862	2.754
10	35.493	0.166	0.469	34.673	0.821	2.371

CV: Coefficient of variation.

analysis under expensive standardizing is necessary for each NATs, before using the kit.[14,15] The minimal needs for verifying and standardizing the quantity tests in infectious diseases, such as *leishmania* parasite include correctness, sensitivity, specificity, and accuracy of the linear serial concentrations under inter and intra assay.[16,17] The majority of molecular tests carried out in laboratories are FDA approved test, which are produced commercially. However, sometimes these tests are made, tested and verified in certain laboratory settings. These tests are only used by the laboratory, which they are produced in and are not distributed to other laboratories or sold to them.[18] In order to identify and quantify *leishmania* in tissue, different genetic targets and proliferation techniques have been used.[19] Different molecular approaches for identifying *leishmania* have been comprehended until now. For example, the PCR method with kDNA minicirlces as targets for separating the parasite has a high sensitivity as there are many minicircles in each kinetoplast.[20] The polymorphism, which exists with a high rate in the sequences of the minicircles prevents identification of different species, using the protocols dependent on the PCR of kDNA. The rRNA target gene has a certain benefit to other genes and can be used for detecting *leishmania* species using high sensitivity approaches.[18] Furthermore, if it is needed to detect *leishmania* species in diagnostic laboratories, in molecular approaches different primers will be required. Although, a strategy with this potential has been made, the pollution in the area does not allow for this to happen. Schonian, et al. were able to identify different species of *leishmania* in different clinical samples with the aim of placing the ITS_1 gene and determining its sequence.[21] Al-Jawabreh, et al. used the ITS_1-PCR method in Palestine to identify different species of *leishmania*, 75% were *L.major* and 43% were *L.tropica*.[22] Laurent, et al. analyzed a kinetoplastic gene in patients infected with *leishmania* sensitive and resistant treatment using the PCR method.[23] Others verified the use of the ITS_2 region as a suitable method of identification of the different *leishmania* species and suggested that this region be used as a diagnostic tool in reference laboratories on a molecular scale for identification of this pathogen.[24] Using the sybrgreen method on the tri-pardoxin peroxidase gene, Khosravi, et al. were able to differentiate between two common species of *L. tropica* and Major and emphasize the necessity of the Real-time PCR method.[25]

Quantifying the *leishmania* parasite load is dependent on the protected ITS_2 gene region with the least amount of variation in its sequence. In order to achieve this, chains of ordered trinucleic acid sequences from the three common species of *leishmania* were compared using the genome information bank. The probe and primer designed for the ITS_2 region, which was used in this study were the same for all three species *L.major*, *L.tropica* and *L.infantum* and it was efficient in determining their quantity. The ITS_2 region is between 5/8s and 28s of the RNA and in terms of repeatable genetic sequence and amount of polymorphism for the species, it is suitable and easily amplified.[14] Besides the ITS_2 gene region, a probe and primer were chosen as exogenous and endogenous control. The internal extraction control checks the DNA extraction stages and the endogenous reference control, which controls the personnel and device, as well as the PCR placement conditions in the reaction. To identify the species, the Cinnagen kit was used and the desired gene was kinetoplast (kDNA), the PCR was placed and then the size of the bands was measured on the gel. The developed analysis in the current study showed the average CV to be 0.88% and 2.89% respectively for the intra and inter assay, which showed the repeatable quantity of *leishmania* plasmid in a dynamic range. Until now, there is no study regarding the validity on probe and primer, which can examine common *leishmania* species quantity with the Real-time RCR method in Iran. This method can be used for general laboratory and research applications and at least we can have a self-sufficiency of internal laboratories dependence on imported laboratory products with a lower cost in this field.

Acknowledgments

The authors would like to thank all friends and colleagues of Pathology and Stem Cell Research Center and the Pathology Department of Kerman Medical Sciences University for their relentless hard work and efforts.

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