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Original Article

Comparison of Parasite Burden Using Real-Time Polymerase Chain Reaction Assay and Limiting Dilution Assay in *Leishmania major* Infected Mouse

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Abstract

Background: Limiting dilution assay is considered as the gold standard method for quantifying the number of parasites in the animal model of *Leishmania* infection. Nowadays, real-time PCR is being increasingly applied to quantify infectious agents. In the present study, a real-time PCR assay was developed to estimate parasite burdens in lymph nodes of *Leishmania major* infected BALB/C mice. Enumeration of parasites was also performed by limiting dilution assay and compared with the results of real-time PCR based quantification.

Methods: The SYBR Green based real- time PCR assay was performed to amplify a 75 bp fragment of superoxide dismutase B1 gene in the lymph nodes of *L. major* infected BALB/C mice 8 weeks post infection. Mice were infected subcutaneously at the base of their tail with 2×10^5 *L. major* promastigotes in the stationary phase of growth. To compare parasite burdens obtained by real-time PCR assay with those of limiting dilution assay, twelve 8-fold serial dilutions of the lymph node homogenates were prepared in the Schneider medium and incubated at 26°C.After 7 days, wells containing motile parasites were identified by direct observation under an inverted light microscope and the total number of parasites was estimated using the ELIDA software.

Results: Spearman's correlation coefficient of the parasite burdens between real-time PCR and limiting dilution assay was 0.72 (*P* value = 0.008).

Conclusion: Real-time PCR assay is an appropriate replacement to existing limiting dilution assay in quantifying parasite burden in the experimental model of *Leishmania* infection.

Introduction

Leishmaniasis is a parasitic infection caused by *Leishmania* species and covers a disease spectrum from cutaneous to visceral disorders. Unfortunately, at present, there is no effective vaccine against leishmaniasis.

Traditionally, evaluating the efficiency of a new vaccine against leishmaniasis has been performed by measuring the size of the lesions in the animal models; however, lack of correlation between the size and parasite's number within the lesion or other organs led to the development of techniques for quantitation *Leishmania* parasites in the infected tissues (1-3, 8).

In the first attempt, *Leishmania* Donovan Units (LDU) was introduced to equate the organ mass (g) multiplied by the number of amastigotes per 1000 macrophages nuclei in the tissue smears (2).

Later, a more sensitive method named limiting dilution assay (LDA) was developed, based on the in vitro culture of infected tissues (1). Although LDA is considered as the gold standard method in quantifying *Leishmania* parasites, it is a time consuming assay (1).

Recently, researchers have used real-time PCR to quantitate *Leishmania* parasites in infected tissues regarding its sensitivity, rapidity, reproducibility and feasibility (3- 5). Despite these advantages, there is not enough evidence to support the notion that real-time PCR is an appropriate replacement for LDA.

In this study, the parasite burden of lymph nodes in *L. major* infected BALB/C mice was compared using LDA and real-time PCR assay.

Materials and Methods

Cultivation of L. Major and infection of mice

L. major strain (MRHO/IR/74/ER) used in this study was obtained from the spleen of *L. major* infected BALB/C mouse by culturing in NNN medium and then sub-culturing in RPMI 1640 supplemented with 12% heat - inactivated fetal bovine serum (FBS) and 1% Penstrep.

Female BALB/C mice (6-8 weeks old) were obtained from the Pasteur Institute of Iran. The animal care and the experimental protocols were approved by the Institutional Animal Care and Research Advisory Committee of the Shahid Beheshti University of Medical Sciences. Mice were infected intra-dermally at the base of their tail with 2×10^5 *L. major* promastigotes in the stationary phase of growth. Eight weeks post infection, inguinal lymph nodes were isolated, placed into a 70 µm cell strainer (BD Falcon, Mexico) which was put into a sterile Petri dish containing RPMI 1640 and mashed gently using the plunger end of the syringe. Then, the cell suspension was collected and centrifuged. After centrifugation, cells were counted using a hemocytometer and a cell suspension containing 4 × 106 cells was stored at -70 °C until DNA extraction. DNA extraction was performed using a Spin column-based nucleic acid purification kit (Prime Prep Genomic DNA Isolation Kit, GeNet Bio, Daejeon, Korea) according to the manufacturer's protocol. Extracted DNA was used as the template for real- time PCR.

DNA extraction and Real-time PCR

The specific primers were designed to amplify a 75 bp fragment of the SODB1 gene of *L. major* that is located on chromosome 32 using Beacon Designer software (ver. 8). The sequences of the primers were as 5'-TGGTGGACATCATCAAGT-3' for the forward primer and 5'-AGAAGAAGTCGTGGTTGTA-3' for the reverse primer. The 25 µl reaction mixture contained 12.5 µl of 2X Maxima SYBR Green q-PCR Master Mix (Thermo Fisher Scientific, Waltham, U.S.), 1 µl of each 10 pM primer, 5 µl of the template (148 ng per reaction) and the Dnase, Rnase free water (5.5 µl). A real-time hot start PCR was performed on the Rotor-Gene 6000 real- time PCR machine (Qiagen, Germany).The temperatures and times in the cycling program were as follows: initially, 10 min incubation at

95 °C followed by 40 cycles, including 30 s (30 s) at 95 °C, then 55 °C for 30 s and 60 s at 72 °C. The analysis of melting curve was performed in the temperature range of 65 to 95 °C at the end of each run. A non-template control (NTC) consisting of 5 µl water in place of template DNA was used in each run. All assays were done in duplicates.

In order to construct a standard curve, DNA extraction of 5 × 10^6 *Leishmania* promastigotes was carried out using a PrimePrep Genomic DNA Isolation Kit (GeNet Bio, Daejeon, Korea) according to the manufacturer's protocol and a 10-fold serial dilutions of *L. major* DNA, corresponding to 5 × 10^6 parasites to 0.5 parasites per reaction were prepared. The average cycle threshold (CT) of duplicates in each dilution was plotted against the number of parasites (4- 6). Reproducibility of the assay was determined by the assessment of inter-assay and intra-assay coefficients of variation of the CT values. The number of parasites per 4×10^6 cells of lymph nodes was calculated by interpolating cycle threshold (CT) of samples in standard curve.

Limiting dilution assay

LDA was performed on draining lymph nodes of the lesion site at 8 weeks post infection (1). In brief, after counting the cells of lymph nodes suspension, twelve 8-fold serial dilutions were prepared in Schneider medium supplemented with 12% heat-inactivated fetal bovine serum (FBS) and 1% Penstrep. Overall, 100 µl of each dilution was transferred into wells of microtiter plate (eight wells for each dilution). The control plates contained serial dilutions of a known number of *in* vitro-cultured *L. major* promastigotes. After 7 days of the incubation at 26 °C, the number of positive wells (presence of motile parasites) and negative wells (absence of motile parasites) was identified by direct observation under an inverted light microscope and the total number of parasites in two lymph nodes were estimated, as described by Taswell using the ELIDA software. The number of cells in two lymph nodes, the number of negative wells in each dilution as well as the number of cells in that dilution was inserted in the software (7).

Statistical analysis

Statistical analyses were carried out using statistical software, SPSS (v. 17; SPSS, Chicago, IL, USA). The correlation coefficient of data was determined by Spearman's test.

Results

Sensitivity and reproducibility of real-time PCR assay

To detect non-specific double-stranded reaction products in real-time PCR assay, melting curve analysis was performed at the end of each run. The melting curve of the specific PCR product showed a single peak with the melting temperature of 82.5°C, which indicating lack of any non-specific products such as primer dimmer (Fig. 1a). The results of melting curve analysis were confirmed using electrophoresis of PCR products on 2.5%, agarose gel (Fig. 1b). The Single peak at 82.5°C indicates the purity of the PCR product and lack of any primer-dimer (Figure 1a), Lanes 1 and 2 are representative of 50 bp ladder and 75 bp amplified product of SODB1 gene by real-time PCR, respectively (Fig. 2b)

To perform absolute quantification of *Leishmania* parasites and define the limit of detection, the standard curve was prepared using 10-fold serial dilutions of *L. major* DNA. The standard curve was linear over at least six serial dilutions of the parasitic DNA with the correlation coefficient (R^2) value of 0.99 and amplification efficiency of 0.91 (Fig. 2). The detection limit of the test was as little as 50 parasites per reaction.

The plot is representative of the mean CT values ± SD from two replicates against the number of parasites. Inter-assay coefficients of variation of CT values for six 10-fold serial dilutions of *L. major* DNA corresponding to 5 × 10^6 parasites to 50 parasites in three different runs were 11.9, 7.9, 6.9, 5.9, 2.0 and 3.3%.

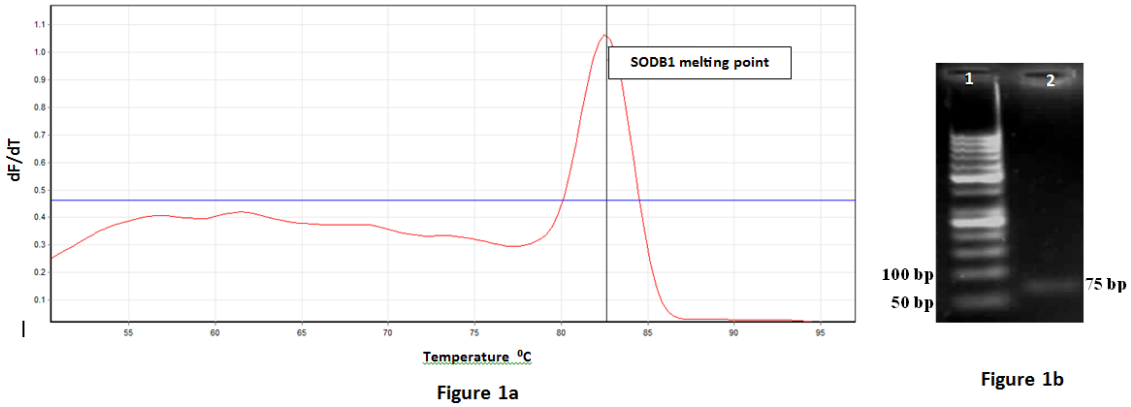

Figure 1a

Figure 1b

Fig.1 Melting curve and gel electrophoresis of the amplified fragment of SODB1 gene.

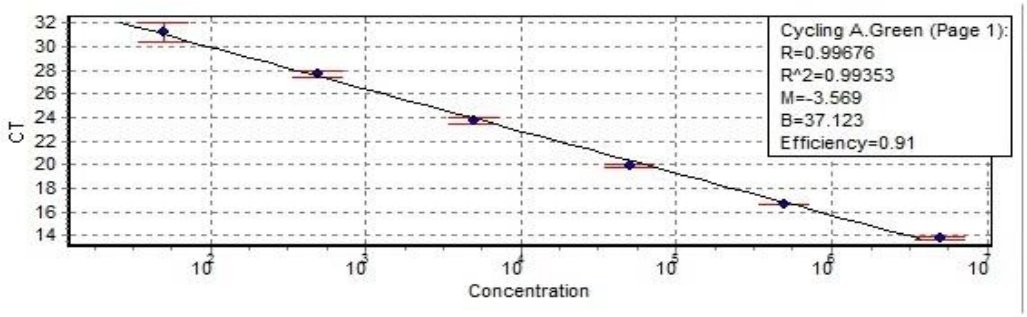

Fig. 2: Standard curve obtained from the amplification of *L. major* SODB1 gene over six 10-fold serial dilutions of the parasitic DNA, ranging from 5 × 10^6 parasites to 50 parasites per reaction

The intra- assay coefficients of variation between replicates in a single run were 0.7, 0.4, 0.5, 0.14, 1.6 and 3.8%, respectively.

Correlation between LDA and real-time PCR

The number of parasites per 4×10^6 cells of lymph nodes was calculated using real-time PCR by interpolating cycle threshold (CT) of samples in a standard curve. Details of CT values and the number of parasites in twelve mice are shown in Table 1. The parasite burden of two lymph nodes for each mouse was also estimated using LDA. The results can be seen in Table 1.

Table 1: Estimation of the parasite's number of inguinal lymph nodes in *L. major* infected BALB/C mice at 8 weeks post infection using real-time PCR and LDA

Mice	Number of parasites / 4 × 10^6 cells of lymph node (real-time PCR)	Mean CT ± SD	Number of parasites/2 lymph nodes (LDA)
1	1479	26.61 ± 0.17	2.82×10^6
2	4257	24.97 ± 0.4	7.30×10^7
3	5606	24.55 ± 0.73	7.01×10^7
4	4578	24.86 ± 0.48	5.22×10^7
5	21112	22.49 ± 0.04	1.29×10^8
6	9133	23.79 ± 0.36	4.31×10^7
7	4067	25.05 ± 0.33	1.63×10^7
8	3051	25.49 ± 0.34	1.20×10^7
9	9945	23.66 ± 0.82	4.09×10^7
10	5309	24.63 ± 0.0	1.28×10^8
11	18177	22.72 ± 0.24	1.72×10^8
12	23562	22.32 ± 0.69	9.53×10^7

In order to find out whether real-time PCR is an appropriate replacement for LDA, statistical analyses was performed. Spearman's correlation coefficient of the parasite burden between two techniques was 0.72 and was statistically significant (*P* value = 0.008).

Discussion

In the present study, a real-time PCR is established based on the amplification of a 75 bp fragment of superoxide dismutase B1 (SODB1) gene of *L. major* to estimate the parasite burden of lymph nodes in the experimental model of leishmainasis and demonstrated that it is an appropriate replacement for LDA.

At present, the most common stringent technique for evaluating vaccines and drugs against *Leishmania* infection in in vivo experimental models is the determination of parasite burden by LDA.

LDA, introduced by Lima et al., is a culture-based technique in which in vitro incubation of infected tissues in culture medium leads to transformation of the amastigote forms to the promastigotes (1). The raw data was analyzed using the ELIDA software introduced by Taswell, to estimate the number of parasites in the desired organ (7). ELIDA utilizes a single-hit Poisson model and the minimum chi-squared iterative method for statistical determination of parasite burden in the organ (7). Although motility of the promastigotes allows easy identification of the viable parasites under inverted microscope, it takes long time for the development of promastigotes (at least 7 days) which raises the possibility of bacterial and fungal contamination (5).

Recently, real-time PCR has been developed to diagnose and quantify *Leishmania* parasites (3-5, 9). Real-time PCR provides the advantages of sensitivity, rapidity, reproducibility and feasibility for enumeration of parasites in any type of tissues (soft or hard, sterile or non-sterile) that gives the possibility of analyzing all types of clinical human specimens in addition to experimental samples.

Many of the studies that developed a real-time PCR for quantifying *Leishmania* parasites utilized fragments of Kinetoplastid DNA (kDNA) for amplification (4-6, 10). kDNA consists of mini-circles and maxi-circles, that have been concatenated within the mitochondria (11). Utilization of a fragment of mini-circles in real-time PCR renders it a highly sensitive target due to high copy number of mini-circles (up to 10^4 per cell). However, variability in copy number of mini circles among different *Leishmania* strains would make it an unsuitable target for the quantification (12, 13). Thus, in this study a fragment of a chromosomal gene with fixed copy number known as SODB1 was amplified using an intercalating-based real-time PCR assay (SYBR Green) and parasite burden of lymph nodes in *L. major* infected BALB/C mice was calculated.

SODB1 is a multi-copy number gene, which encodes an enzyme catalyzing the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide in *L. major* and other *Leishmania* species. SODB1 is highly immunogenic and antibody produced against this antigen is specifically raised in leishmaniasis patients (14).

Moreover, this study quantified parasite burden of lymph nodes in these animals using LDA. The results of this study showed that there was a significant correlation between parasite burdens obtained by LDA and real-time PCR. This result indicates that real-time PCR may be an appropriate replacement for LDA.

In line with the results of this study, Bretagne *et al.* showed a good correlation (R = 0.66) between TaqMan based real-time PCR and LDA for the quantification of *L. infantum* in the livers of infected mice (3).

In spite of the strong relationship between the parasite burdens obtained by the two techniques, the number of parasites estimated by real-time PCR was not the same as that calculated by LDA. This can be explained by differ-

ent bases used by each technique to calculate the number of parasites in the organ leading to different estimates of parasite burdens. With regard to these different bases in calculation of parasite burdens by each technique, the correlation between parasite's burdens obtained by both techniques is enough to show that real-time PCR is a suitable replacement for LDA. Considering that LDA is a culture-based assay that only quantifies live parasites may be the other explanation.

Conclusion

The results of this study highlighted the advantage of real-time PCR for quantifying parasite burden in mouse model, which is practically important, given intense use of this model to test drugs and vaccines against *L. major* infection.

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