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Leishmania tropica: The comparison of two frequently-used methods of parasite load assay in vaccinated mice

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ABSTRACT

Objective: To compare limiting dilution assay and real-time PCR methods in *Leishmania tropica* parasite load measurement in vaccinated mice.

Methods: BALB/c mice were vaccinated by *Leishmania tropica* soluble *Leishmania* antigen or recombinant *Leishmania tropica* stress-inducible protein-1 with/without adjuvant. After three vaccinations, mice were challenged by *Leishmania tropica* promastigotes. Two months after challenge, the draining lymph nodes of mice footpad were removed and parasite load was assayed by limiting dilution assay and real-time PCR methods. Limiting dilution assay was done by diluting tissue samples to extinction in a biphasic medium. For real-time PCR, DNA of the lymph nodes was extracted, equal dilutions of each sample were prepared and hot-start real-time PCR was done using appropriate primers. The data of the two methods were compared by appropriate statistical methods.

Results: Both methods were able to measure different levels of parasite load in vaccinated/unvaccinated mice. In addition, wherever parasite load of a group was estimated high (or low) by one method, the estimated parasite load by another method was the same, although statistically significant differences were found between some groups.

Conclusions: Both methods lead to approximately similar results in terms of differentiating parasite load of the experimental groups. However, due to the lower errors and faster process, the real-time PCR method is preferred.

KEYWORDS: *Leishmania tropica*; Vaccinated mice; Limiting dilution assay; Parasite load assay, Real-time PCR

1. Introduction

Leishmaniasis is an intracellular protozoan disease, with different forms, namely, cutaneous, mucocutaneous, and visceral leishmaniasis[1]. Cutaneous leishmaniasis (CL) in the Middle East is mainly caused by *Leishmania major* (*L. major*) and *Leishmania tropica* (*L. tropica*). In contrast to *L. major*, little studies have been done on *L. tropica*. Furthermore, these two species are different in many aspects and cause different forms of diseases in humans and animal models. It has been found that in comparison to *L. major*, *L. tropica* is heterogeneous genetically, leading to variable manifestations[2].

In inbred BALB/c mice which are the most studied animal models in leishmaniasis, compared with *L. major* which results in a severe lesion, *L. tropica* causes mild pathogenesis with small (or no) swelling in infection sites[3-8].

There is no approved and available vaccine for CL. However, there are numerous researches ongoing in this field. The assessment of *Leishmania* vaccine efficiency has been traditionally performed by measuring lesion size in the animal models. However, there is always no direct correlation between the lesion size and parasite's burden within the lesion or other organs[9,10]. Moreover, some

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Leishmania species (such as some isolates of *L. tropica*) do not cause measurable lesions in the most studied animal models including BALB/c mice[3,4,6,11]. These issues led to the development of reliable techniques for the quantification of *Leishmania* parasites load as a better criterion for the evaluation of vaccine efficiency.

There are two frequently-used methods for parasite load measurement: the older method of limiting dilution assay (LDA) and the newer method of real-time PCR. LDA is a sensitive method based on the *in vitro* culture of infected tissues[12,13]. LDA method has been evaluated many times and its suitability for *Leishmania* quantification has been shown for many *Leishmania* species. However, it has some limitations including being laborious, time-consuming, more prone to fungal and bacterial contaminations and also needs counting by technicians that may increase human errors[14,15]. Thus, the more recent technique of real-time PCR has been developed with some advantages including faster process and being less prone to human manipulation errors in comparison to LDA[14,16,17]. Real-time PCR has also been used for the clinical diagnosis of *Leishmania*[18]. Despite these advantages, there is no enough evidence to support the replacement of LDA by real-time PCR. Although there are studies that compared these two methods[14,16], no information is available for comparing these methods for measurement of *L. tropica* load which causes mild pathogenesis in BALB/c mice model. Furthermore, as far as we know, there is no report to compare these two methods in vaccinated/unvaccinated experimental groups. Therefore, in the present study, using different vaccinated/unvaccinated BALB/c mice groups, the two methods of LDA and real-time PCR were compared regarding the quantification of *L. tropica* parasite load.

2. Materials and methods

2.1. Parasites

L. tropica strain MHOM/AF/88/KK27 which has been isolated from Afghanistan and kindly donated by Dr. D. Sacks (Laboratory of Parasitic Diseases, National Institute of Health, Bethesda, Maryland, USA) and *L. major* strain MRHO/IR/75/ER as a gift from M. Mohebali (School of Public Health, Tehran University of Medical Sciences, Tehran, Iran), were used in this study. All procedures of maintaining and species confirmation, parasite culture and preservation of their virulence were done as stated in our previous reports[11,19].

2.2. Antigens and adjuvants

Whole-cell soluble *Leishmania* antigen (SLA) of *L. tropica* and recombinant *L. tropica* stress-inducible protein-1 (rLtSTI1) were used as antigens in this study. SLA antigen was prepared as previously reported[3] and rLtSTI1 was cloned and expressed in *Escherichia coli* expression system[20]. Two adjuvants were used: R848 (resiquimod) and monophosphoryl lipid A (MPL) which were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Invivogen (Toulouse, France), respectively.

2.3. Animals, immunization, and infection

BALB/c mice, female, 5-7 weeks old were purchased from Pasteur Institute of Iran (Tehran, Iran). Mice were divided into seven groups (10 mice/group) and named as follows: S (vaccinated by *L. tropica* SLA), SM (vaccinated by *L. tropica* SLA+MPL), SR (vaccinated by *L. tropica* SLA+R848), L (vaccinated by rLtSTI1), LM (vaccinated by rLtSTI1+MPL), LR (vaccinated by rLtSTI1+R848), and phosphate buffered saline (PBS) (vaccinated by PBS as negative control). A naive unvaccinated group was challenged by *L. major* (indicated by *L. m*) to compare the swelling pattern of *L. tropica* with *L. major*.

Vaccination and challenge programs were done as previously reported[21]. Briefly, vaccines were injected subcutaneously in the hind footpad of mice three times with two weeks interval. Six weeks after the last vaccination, mice were challenged subcutaneously with 2×10^6 stationary promastigotes with ~30% morphologically metacyclic as assessed by Ficoll enrichment[22].

2.4. Ethical statement

The whole study was approved by the Ethics Committee of the Pasteur Institute of Iran (license number 95/0201/20704). All procedures involving mice followed the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23) and Pasteur Institute of Iran guidelines for animal care.

2.5. Footpad swelling measurement

Footpad swelling development was monitored biweekly using a dial-gauge caliper (Mitutoyo, Kawasaki, Kanagawa, Japan). The swelling values were obtained using the following formula: Swelling value = T_{ui} - T_i, where T_{ui} is the values of thickness of uninfected footpad and T_i is the values of thickness of contralateral infected footpad.

2.6. Parasite load assay

Two methods were used for parasite load assessment. The draining lymph nodes of mice were removed from the infected footpad, homogenized, and divided into two parts. One part was applied for

parasite load assay by LDA; another part was used for parasite load assay by real-time PCR. LDA was done by diluting tissue samples to extinction in a biphasic medium, as previously reported[12]. Briefly, the lymph node cell suspensions were serially diluted (ten-fold) in culture medium (RPMI-1640) and added to Novy MacNeal Nicolle (NNN) medium in 96-well plates in triplicate to the extinction of parasite growth. The highest dilution, at which parasites can be seen by microscopic inspection, was recorded as lymph node parasite load. The geometric mean of individual titers was used to compare the parasite load of different experimental groups.

Parasite load measurement by real-time PCR was done as previously described[12,23]. Briefly, DNA of the lymph nodes was extracted using DNeasy Blood & Tissue Kit (Qiagen, USA). Equal dilutions (10 ng/μL) of each sample were prepared and hot-start real-time PCR was done using SYBR green master mix in final volumes of 25 μL, using the following primers which target the conserved region of the kinetoplast DNA: Forward, 5'- GGGTAGGGGCGTTCTGC-3'; Reverse, 5'- TACACCAACCCCCAGTTTGC-3'.

The program of thermocycling was as follows: 95℃ (4 min), 42 cycles of 95℃ (10 s) and 60℃ (35 s) followed by melting analysis. In order to quantify the parasites, using different dilutions of 10^8 to 100 copies of *L. tropica* genomic DNA, a standard curve was generated. The number of parasites DNA copies as a representative parasite load of each sample was calculated automatically by the apparatus software by interpolating cycle threshold (CT) of each sample in the standard curve. Two negative controls were used in each run: 1) DNA from the lymph node of naive mice (negative control), 2) distilled water (non-template control).

2.7. Statistical analysis

Multiple comparisons were done by the analysis of variance (ANOVA) and the two-way comparisons between the groups were done by student's *t*-test using GraphPad Prism software (version 6.0). The correlation coefficient of data was found out by Spearman's test. *P*-value < 0.05 was assumed to be significant. In order to express the data, the mean value ± standard deviation (SD) was applied.

3. Results

3.1. Swelling measurement

The mice's footpad thicknesses were measured up to 16 weeks after the challenge. No (or little) swelling was observed in all experimental groups challenged by *L. tropica* (Figure 1). Conversely, unvaccinated mice challenged by *L. major* showed significant lesion diameters up to 10 weeks after challenge when the measurement was discontinued due to necrosis in the footpad.

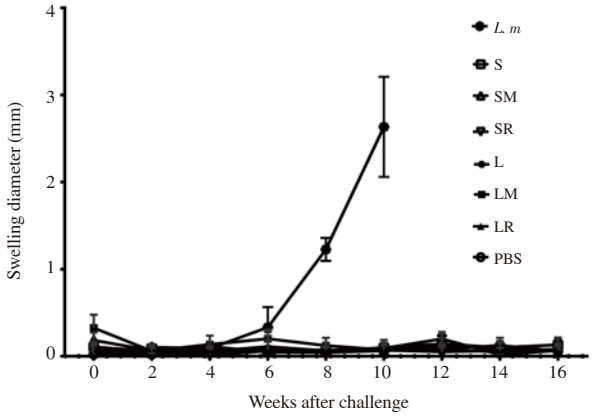

Figure 1. Footpad swelling measurement. Each bar demonstrates mean ±SD of swellings. The measurement was discontinued for *L. m* 10 weeks after challenge due to necrosis. S: SLA, SM: SLA+MPL, SR: SLA+R848, L: rLtSTI1, LM: rLtSTI1+MPL, LR: rLtSTI1+R848, *L. m*: A naive group challenged by *Leishmania major*. SLA: soluble *Leishmania* antigen, rLtSTI1: recombinant *Leishmania tropica* stress-inducible protein-1, MPL: monophosphoryl lipid A.

3.2. Real-time PCR setting

At the end of each run, melting curve analysis was performed to detect non-specific double-stranded reaction products. A single peak with the melting temperature of ~84℃ was observed for each sample, indicating a specific PCR product (Figure 2). Moreover, single bands of ~150 bp corresponding to target amplicon of kDNA were observed for all real-time PCR products running on 2% agarose gel which re-confirmed the melting analysis results.

The standard curve was made using 10-fold serial dilutions of 10^8 to 100 copies of *L. tropica* genomic DNA to perform quantification of *Leishmania* parasites. The suitability of the standard curve was shown by the amplification efficiency of 0.92 and the correlation coefficient (R^2) value of 0.98 (Figure 3).

3.3. Parasite load assay

Two months after challenge, parasite load was assayed in all groups by LDA and real-time PCR methods. Both LDA and real-time PCR assays showed approximately similar results. According to results of both assays, groups SM, SR, LM, and LR had significant differences with the control (PBS) group. In addition, wherever parasite load of a group is estimated high (or low) by one method, the estimated parasite load by another method is the same, although the statistically significant differences were only seen between some groups. In both methods, the mice which received MPL + rLtSTI1 showed lower and mice which received R848 + SLA showed higher parasite load than others (Figure 4).

In order to compare real-time PCR and LDA, a statistical Spearman's correlation coefficient analysis was performed. The results showed the correlation coefficient was 0.659 for the parasite

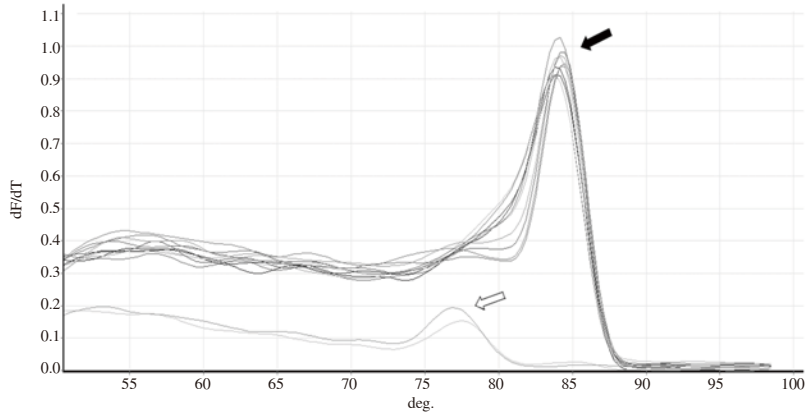

Figure 2. Melting curve and 2% gel electrophoresis of the amplified fragment of kDNA gene. A single peak at ~84 ℃ of melting curve (shown by black arrow) indicates the purity of the real-time PCR product and lack of any non-specific primer dimer. Both non-template and negative controls show a peak at ~75 ℃ corresponding to non-specific primer dimer (shown by white arrow).

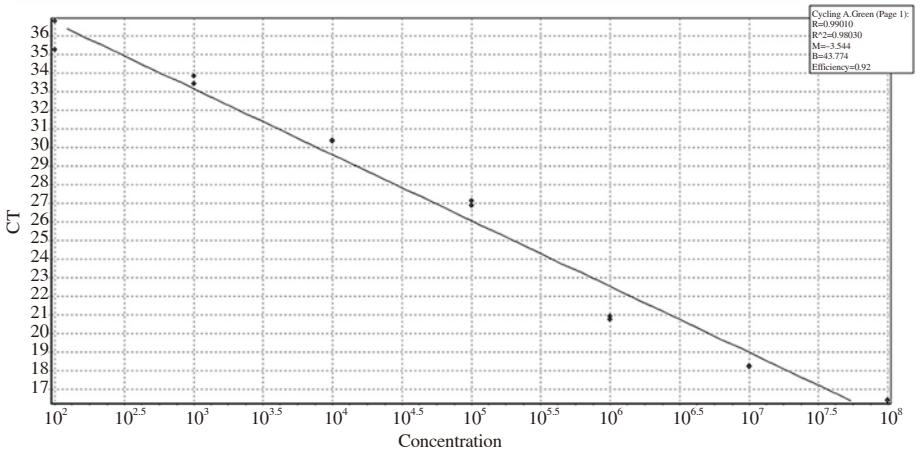

Figure 3. Standard curve of real-time PCR. The plot demonstrated the mean CT values ± SD from two replicates against the number of parasites. The amplification efficiency and the correlation coefficient (R^2) values of the standard curve were 0.92 and 0.98, respectively.

burden between two methods with statistically significant difference (P = 0.001), confirming that real-time PCR results were correlated with LDA results and could be an appropriate alternative for it.

4. Discussion

As shown in our study and the previous reports[3,4,6,7], *L. tropica* causes mild pathogenesis in BALB/c mice with a small or no detectable swelling in the injection site. Consequently, the assessment of its pathogenesis by swelling development is not promising and the parasite load in animal tissues can be a more reliable criterion for this purpose.

Two quantification methods were used for determining the parasite load of the lymph node of vaccinated/unvaccinated animal models, namely, LDA and real-time PCR method. The results indicated that both methods can produce approximately similar results in terms of showing relationship between parasite load of different experimental groups. In other words, if the parasite load of a group is higher than another group, both methods can show this difference. However, there are some advantages and limitations of both methods. It is noteworthy that the number of parasites counted by real-time PCR was not the same as that estimated by LDA. This can be explained by different counting bases used by each technique or by the fact that LDA only quantifies live parasites instead of both live and dead parasites that are counted in the real-time PCR method.

LDA is more prone to errors due to more manipulations required for relatively long time cultures and counting errors by eye-inspection[14,15]. In LDA, the parasite load is usually counted 10 days after seeding homogenized tissue into culture plates[12]. However, it seems that for *L. tropica* longer time is needed, since in a study by Anderson *et al*., the parasite load was counted 15-30 days after primary culture[5]. In the present study, in some cases, especially where parasite load was low, we hardly observed live parasites in the highest dilutions that need more eye-inspections for longer times (especially when many samples were examined). These conditions may in turn increase visual errors. To test the possible longer time of cultivation for *L. tropica* parasite detection in LDA assay, we inspect some culture plates 25 days after primary culture, but they showed similar results as 10 days-inspection detections and no improvement was seen which leaves the issue unsolved.

Another difference between LDA and real-time PCR methods is

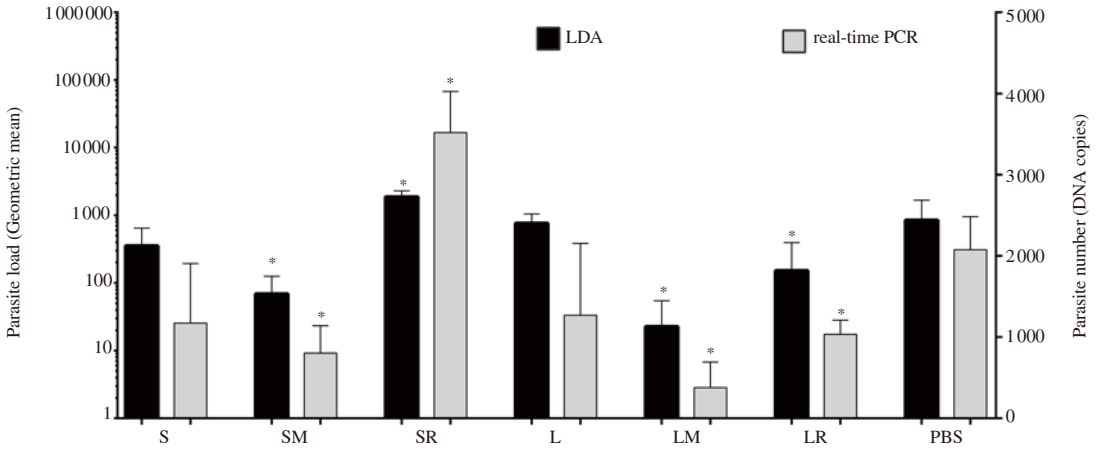

Figure 4. Parasite load of the lymph nodes in mice determined by LDA and real-time PCR assays. Significant differences between each group and PBS were indicated by "*". Left and right Y-axes show units of LDA and real-time PCR, respectively. S: SLA, SM: SLA+MPL, SR: SLA+R848, L: rLtSTI1, LM: rLtSTI1+MPL, LR: rLtSTI1+R848. PBS: phosphate buffered saline, SLA: soluble *Leishmania* antigen, rLtSTI1: recombinant *Leishmania tropica* stress-inducible protein-1, MPL: monophosphoryl lipid A.

the SD values of parasite burden between similar samples (*e.g.* two similar mice of one group) and also between replicates with each other which is much higher in LDA in comparison to real-time PCR, suggesting that real-time PCR is a more efficient method. These differences may be due to the error-prone process of LDA from the culturing process to technician eye-inspection of the parasites. The SD values of LDA in our results are shown on log10 scale and much higher than the SD values depicted for real-time PCR method which are shown on linear scale.

On the other hand, LDA has some advantages which make it a frequently-used method. In contrast to real-time PCR method which counts all live or dead parasites (because it counts DNA without discrimination between DNA of live or dead parasites), LDA only detects live parasites. Furthermore, LDA is cheaper due to no need for expensive devices such as a real-time PCR apparatus.

The present and previous studies[10,14,16,17,23,24] showed the efficiency of real-time PCR to quantify *Leishmania* species and revealed that real-time PCR could be a good alternative for LDA with advantages such as reproducibility, sensitivity, rapidity, and feasibility. Thus, the overall results of the present study indicate that in order to quantify the parasite load of vaccinated/unvaccinated animal models for *L. tropica*, both LDA and real-time PCR methods can be used. However, due to lower errors and faster process, the real-time PCR method is preferred.

Conflict of interest statement

The authors declare that there are no conflicts of interests.

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Authors' contributions

MR and HMN contributed to the study design. Material preparation, data collection and analysis were performed by FNZ, MR and HMN. The first draft of the manuscript was written by MR, AA, and HMN. All authors read and approved the final manuscript.

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