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Increasing the Sensitivity of *Leishmania* RNA Virus 2 (LRV2) Detection with a Modification in cDNA Synthesis

Leishmania RNA Virüs 2 (LRV2) Saptanmasında cDNA Sentezindeki Bir Modifikasyon ile Hassasiyetin Artırılması

Muhammed Nalçacı[1], Mehmet Karakuş[2], Yusuf Özbel[3], Ahmet Özbilgin[4], Seray Töz[3]

[1]Ege University Graduate School of Natural and Applied Sciences, Department of Biology, İzmir, Turkey
[2]University of Health Sciences Turkey Hamidiye Faculty of Medicine, Department of Medical Microbiology, İstanbul, Turkey
[3]Ege University Faculty of Medicine, Department of Parasitology, İzmir, Turkey
[4]Celal Bayar University Faculty of Medicine, Department of Parasitology, Manisa, Turkey

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ABSTRACT

Objective: *Leishmania* RNA virus was detected the first time in the New World *Leishmania* species. Recent studies were also showed the presence of *Leishmania* RNA virus 2 (LRV2) in Old Word *Leishmania* species including Turkish *L. major* and *L. tropica* isolates. This study aimed to increase the sensitivity of qPCR with a modification in the denaturation step of cDNA preparation protocol.

Methods: In this study, LRV2+ three *L. major*, two *L. tropica* strains and *L. major* control strain (MHOM/SU/73/5-ASKH) were included. Total RNA isolation was done using different numbers of *Leishmania* promastigotes (10^8, 10^5 and 10^3). Before cDNA synthesis, samples were denatured at 95 °C for 2 min, as a modification of the kit procedure. qPCR was undertaken using 0.5 mM primers (LRV F-HR/LRV R-HR) diluted in SYBR Green Master mix.

Results: We observed lower Ct values in amplicons with the modified version than with the classical kit protocol for cDNA synthesis, in all of the strains used in the study. The addition of pre-denaturation step at 95 °C showed lower Ct values meaning the sensitivity increased. Different parasite dilutions showed similar results.

Conclusion: It is important to increase the sensitivity especially with the aim for detecting LRV in clinical samples obtained from patients probably have less number of parasites. The presence and burden of the virus can help to understand the relationship between the clinical findings and the pathogenicity of the parasite which may lead to changes in the course of treatment.

Keywords: *Leishmania*, *Leishmania* RNA virus 2, LRV2, cDNA, qPCR

ÖZ

Amaç: *Leishmania* RNA virüsü, ilk olarak yeni dünya *Leishmania* türlerinde tespit edilmiştir. Son çalışmalar, Türkiye'deki *L. major*, *L. tropica* izolatları dahil olmak üzere, eski dünya *Leishmania* türlerinde de *Leishmania* RNA virüsü 2'nin (LRV2) varlığını göstermiştir. Bu çalışmada LRV2'nin tespiti için cDNA hazırlama protokolünün denatürasyon aşamasında yapılan bir modifikasyon ile qPCR'nin duyarlılığının artırılması amaçlanmıştır.

Yöntemler: Bu çalışmada, LRV2+ 3 *L. major*, 2 *L. tropica* ve kontrol olarak *L. major* (MHOM/SU/73/5- ASKH) suşları kullanılmıştır. Total RNA izolasyonu 10^8, 10^5 ve 10^3 sayılarına ulaşan *Leishmania* promastigotları kullanılmıştır. cDNA sentezinden önce numuneler, kit prosedüründen farklı olarak 95 °C'de 2 dakika denatüre edilmiştir. qPCR, SYBR Green Master karışımında seyreltilmiş 0,5 mM primerler (LRV F-HR/LRV R-HR) kullanılarak yapılmıştır.

Bulgular: Klasik kit prosedüründen farklı olarak 95 °C'de ön denatürasyon adımının eklenmesi, duyarlılığın arttığını gösteren daha düşük Ct değerleri ortaya çıkarmıştır. Farklı parazit dilüsyonları ile de benzer sonuçlar gözlenmiştir.

Sonuç: Özellikle hastalardan elde edilen klinik örneklerde muhtemelen daha az sayıda parazit bulunması nedeniyle, LRV'nin saptanabilmesi amacıyla duyarlılığın artırılması önemlidir. Virüsün varlığı ve yükü, klinik bulgular ile parazitin patojenliği arasındaki ilişkiyi anlamaya yardımcı olabilecek ve bu da tedavi aşamasında değişikliklere yol açabilecektir.

Anahtar Kelimeler: *Leishmania*, *Leishmania* RNA virus 2, LRV2, cDNA, qPCR

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Address for Correspondence/Yazar Adresi: Muhammed Nalçacı, Ege University Graduate School of Natural and Applied Sciences, Department of Biology, İzmir, Turkey
Phone/Tel: +90 555 677 48 39 **E-mail/E-Posta:** muhammednalcaci@gmail.com **ORCID ID:** orcid.org/0000-0002-9265-2887

Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Leishmaniasis is a vector-borne disease and endemic in more than 98 countries with the main clinical forms as cutaneous, mucocutaneous and visceral leishmaniasis (1,2). As an estimation, 350 million people are at risk of leishmaniasis globally (2). As agents, more than 20 *Leishmania* spp. protozoan parasites in *Leishmania* genus are transmitted by infected female sand flies (3,4). Cutaneous leishmaniasis (CL), the most common clinical form, is endemic in New and Old World countries, with an estimated one million new cases each year. CL is mainly caused by *L. tropica* and *L. major,* and less frequently by *L. infantum* and *L. aethiopica* in the Old World. Also, localized CL cases caused by *L. donovani* have been reported (5,6). Turkey is one of the 12 countries including Syria, and Iran, as being high-burden (>2.500/year cases) for CL in 2016 according to World Health Organization (4,7).

In Turkey, CL is endemic in Southeast Anatolia and the East part of the Mediterranean region, and most of the cases (96%) were reported from Şanlıurfa, Adana, Osmaniye, Hatay, Diyarbakır, İçel and Kahramanmaraş. In recent years, CL cases were also reported in the areas where known as non-endemic possibly due to migrated refugees and non-permanent workers. *L. tropica* is the most reported agent of CL and followed by *L. infantum* and *L .major* in Turkey. Recently, the presence of *L. donovani* as a causative agent of CL has been also shown in the country (8,9). *L. tropica* is known to cause anthroponotic CL with a slow course (12-18 mo) and late ulceration. However, *L. major* is known as zoonotic CL and lesions show a severe inflammation and recover in fast course (2-8 mo). Frequently, *L. major* can cause big, ulcerative and usually wet lesions or dysfunctional scarring (8). The disease progresses to chronic form in 5-10% of total CL cases which does not improve spontaneously or with treatment in two years in Turkey (10).

Aggressive symptomatic outcomes of CL has been implemented to a strong correlation with parasite species due to the presence of parasite-intrinsic virulence factors (11). *Leishmania* RNA virus (LRV) belongs to a double-stranded RNA virus genus, in Totiviridae family. Because of differences in gene regulation and sequences, LRV differentiated as LRV1 and LRV2 that seen in the New and Old World respectively. LRV1 has been detected in *L. guyanensis, L. braziliensis* and *L. shawi* in the New World (12-15) while LRV2 has been detected in *L. major, L. aethiopica, L. infantum,* and *L. tropica* in the Old World (16-19). Recently, the role of LRV on the development of infectious metastases in the New World has been shown in some studies in both human and animal models and the presence of LRV1 predisposing to metastatic development indicates a relationship between the virus and metastatic complications of the disease, such as mucocutaneous leishmaniasis (20). For this reason, the detection of the LRV became important for understanding different clinical outcomes of CL. For the detection of LRV, different molecular and serological methods are used with different sensitivities.

In this study, we aimed to increase the sensitivity of quantitative real-time polymerase chain reaction (qPCR) based detection of LRV with by adding a modification step in cDNA synthesis using *L. major* and *L. tropica* strains which are found to be LRV2-1 positive previously (19).

METHODS

Parasite Cultures

Previously isolated and cryopreserved, three *L. major* and two *L. tropica* LRV2 positive strains (19) and a control strain (MHOM/SU/73/5- ASKH, *L. major*) which was also LRV2 positive were included Table 1.

Mass cultivation of *Leishmania* promastigotes were performed in RPMI 1640 medium supplemented with 10% fetal calf serum at 24-25 °C. Different promastigote dilutions ($1x10^8$, $1x10^5$ and $1x10^3$) were prepared for comparison.

RNA Isolation and Complementary DNA (cDNA) Synthesis

Total RNA was isolated with different promastigote dilutions using trizol reagent (Roche) as performed previously (19). The quantity of the extracted RNAs was analysed using a NanoDrop spectrometer 100 ng RNA was added per reaction. Synthesis of cDNA was performed using a cDNA Synthesis Kit (EvoScript Universal cDNA Master, Roche) with random hexamer primers according to the protocol provided by the manufacturer (15 min at 42 °C, 5 min 85 °C, 15 min at 65 °C and cool to 4 °C with an unlimited hold time). The modification was done prior to cDNA synthesis, the samples were denatured at 95 °C for 2 min, as a modification of the kit procedure.

qPCR

Quantitative real-time PCR was performed with a reaction solution of 0.5 mM primers in SYBR Green Master mix (Roche). The reaction performed with an initial denaturation (95 °C for 5 min) followed by 40 amplification cycles (10 s at 95 °C, 10 s at 55 °C, 10 s at 72 °C) and a fluorescence detection step (70 °C to 95 °C) with quantification of amplified DNA after each cycle. The DNA oligonucleotides (Microsynth, Switzerland) targeted LRV 2-1 RNA Dependent RNA polymerase (*RdRp*) gene were used (18,19). Molecular grade water was used as negative control.

Table 1. LRV positive strains used in the study

Strain WHO code	Clinical type	*Leishmania* species	LRV code	LRV accession number
MHOM/TR/93/EP1	VL	*L. tropica*	LRV2-Ltr-EP1	MK24753
MHOM/TR/04/EP92	CL	*L. tropica*	LRV2-Ltr-EP92	MK24757
MHOM/TR/2012/CBU18	CL	*L. major*	LRV2-Lmj-CBU18	MK24760
MHOM/TR/2014/CBU33	CL	*L. major*	LRV2-Lmj-CBU33	MK24761
MHOM/TR/2013/CBUALA17	CL	*L. major*	LRV2-Lmj-CBUALA17	MK24762
MHOM/SU/73/5ASKH	International control strain	*L. major* (positive control)	LRV2-Lmj-5-ASKH	

WHO: World Health Organization, CL: Cutaneous leishmaniasis, VL: Visceral leishmaniasis

Table 2. Sybr green real-time PCR assays for the detection of LRV2 and Ct values of different dilutions respectively. This relative abundance which gives a corresponding measure of the viral load per parasite may be one of the factors associated with the pathogenesis of *Leishmania*

Strain WHO code	Clinical type	*Leishmania* species	$1x10^8$ pre-denaturation (Ct value)	$1x10^5$ pre-denaturation (Ct value)	$1x10^3$ pre-denaturation (Ct value)	$1x10^8$ non-denaturation (Ct value)	$1x10^5$ non-denaturation (Ct value)	$1x10^3$ non-denaturation (Ct value)
MHOM/TR/93/EP1	VL	*L. tropica*	31,22	36,84	37,55	36,28	-	-
MHOM/TR/04/EP92	CL	*L. tropica*	17,97	31,64	36,45	20,45	29,91	39,94
MHOM/TR/2012/CBU18	CL	*L. major*	2.33	6.67	7.20	4.67	9.05	11,02
MHOM/TR/2014/CBU33	CL	*L. major*	26,17	32,67	35,28	28,72	33,19	39,94
MHOM/TR/2013/CBUALA17	CL	*L. major*	30,08	30,25	31,37	32,48	34,37	38,08
MHOM/SU/73/5ASKH	International control strain	*L. major* (Positive control)	7.78	8.37	9.96	10,81	11,09	11,70

WHO: World Health Organization, CL: Cutaneous leishmaniasis, VL: Visceral leishmaniasis, Ct: Cycle treshold, PCR: Polymerase chain reaction

Statistical Analysis

Statistical analysis has not been made in this study.

RESULTS

Effect of pre-cDNA Modification on Cycle Treshold (Ct) Values

The differences were determined after the modification was applied. After the amplification of LRV2-1 specific RNA dependent RNA polymerase (RdRp) region, we observed lower Ct values in amplicons with modified version than classical kit protocol for cDNA synthesis in all of the strains used in the study (Only one strain at the 10^5 dilutions showed discordance with other results). This is showing that the sensitivity of the quantitative real time PCR (qPCR) is increasing by modification in cDNA synthesis protocol (Table 2).

Results with Limiting Dilution

Whether this difference in the sensitivity of qPCR performed with the products of the modified and classical cDNA synthesis is effected with the limitation of the parasite numbers is also examined. With this aim modified and classical protocols were performed to three different dilutions as 10^8-10^5 and 10^3 to all strains included in the study and than PCR results were compared. Lower Ct values in $1x10^8$ and $1x10^5$ parasite numbers than $1x10^3$ in all strains. When the test conducted using 10^3 parasites, Ct values increased to 37-38 depending on different strains (Table 2). These values can be considered as weak reactions and in some cases it can be said that they are inadequate to give clear results. In the melting curve analyzes, there were not any non-specific products. All of our products were higher than 80 °C showing that they are not primer dimers but PCR products (Figure 1).

DISCUSSION

Studies have shown LRV positivity in *Leishmania* species/strains is one of the important factor in the worsening of prognosis of CL lesion and drug resistance of the causative agent (21). Previously, LRV was found to be positive in some *L. major* and *L. tropica* strains isolated from Turkey (19) after it is reported in the *L. major, L. infantum* and *L. aethiopica* strains from Old World countries (17,18). In this study, we aimed to increase the sensitivity of PCR by the modification of cDNA synthesis due to double stranded character of LRV. SYBR green qPCR was performed in order to compare the sensitivity of the modified method. In the literature, we could not find any application for LRV similar to our modification step (17,22). However, we have observed in several publications that for different double-stranded viruses a additional step is added before cDNA synthesis (23-25). In two studies, on Bursal disease virus seen in young chickens and Magnaporthe oryzae virus, which causes disease in rice plants, denaturation process was applied before cDNA synthesis. No information about the purpose of pre-denaturing was given in the studies (23,25). In recent studies on Pseudomonas virus and Saccharomyces cerevisiae virus, it has been reported that DMSO is used together with the temperature factor before cDNA synthesis. So far, successful results have been obtained in the denaturation of dsRNA. However, more research is needed on this subject (26,27).

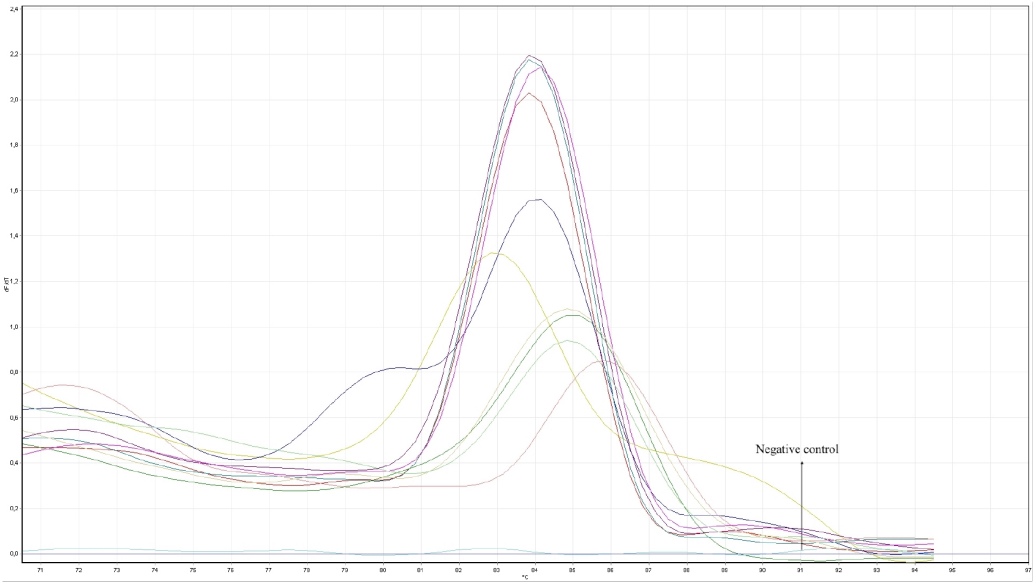

Figure 1. SYBR green real-time PCR melt curve analysis. Melting analysis was performed to determine the presence of non-specific conditions such as primary dimer. The dissociation curves provide a melting temperature of the amplified PCR products thus confirming its specificity. The changes observed in the melting curves are due to differences in the virus genome
PCR: Polymerase chain reaction

The modification in this study was applied in order to separate the chains of the double stranded virus, LRV2, allowed us to obtain different Ct values on the same strain. We observed lower Ct values in amplicons with the modified version for cDNA synthesis in the strains included in the study except one (MHOM/TR/04/EP92). We observed incompatibility in only this strain at the 10^5 dilutions, probably due to a manipulation in the lab. This is showing that the sensitivity of the qPCR increase with our modification in cDNA synthesis protocol. The importance of the modification was also showed with the limiting dilution of the parasites (Table 2). Higher positivity rates were obtained by molecular methods than either agarose gel method or serological methods in LRV studies (28). In our previous study, LRV2 could detected in three *L. major* strains by agarose gel method and PCR while seven *L. tropica* strains were found to be LRV2 positive only by PCR (19). Higher sensitivity is especially important for the LRV detection in patient's clinical samples in order to determine the prognosis of the disease.

The present study was performed using laboratory strains isolated before, therefore we did not have the clinical records. In Turkey, approximately 10% of CL cases were reported as chronic CL patients with a survey longer than two years. It is reported that, some CL cases caused by *L. major* were showing a rapid progressing course with severe inflammation and ulceration. These cases had frequently numerous large lesions with a bad appearance or scatrises which is causing functional disorders. Usually *L. tropica* causes small and dry lesions with a long course. However, some *L. tropica* cases have lesions continuing many years, resistant to therapy and especially spreading to the other parts of the face (8,10).

CONCLUSION

The sensitivity of qPCR was increased with a modification in pre cDNA synthesis protocol. It is important especially for detecting LRV in clinical samples obtained from patients which probably have less number of parasites especially to understand the relationship between LRV and pathogenecity.

*Ethics

Ethics Committee Approval: No studies were conducted on humans or animals in this study. Therefore, there was no need for approval from the local ethics committee.

Informed Consent: Patient consent not applicable for this study.

Peer-review: Externally peer-reviewed.

*Authorship Contributions

Concept: M.N., M.K., Y.Ö., S.T., Design: M.N., M.K., Y.Ö., S.T., Data Collection or Processing: M.N., A.Ö., S.T., Analysis or Interpretation: M.N., M.K., Y.Ö., A.Ö., S.T., Literature Search: M.N., S.T., Writing: M.N., Y.Ö., S.T.

Conflict of Interest: All authors declared that there is no conflict of interest.

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