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Original Article

DNA Load Analysis Using Real Time PCR In Comparison With Immunohistochemical Findings of Dry Type Cutaneous Leishmaniasis; Before and After Treatment by Imiquimod,Glucantime and Combination of Both Drugs

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ABSTRACT

Background and Objectives: **Kerman Province, especially city of Bam in the southeast part of Iran, is epidemics for dry type cutaneous leishmaniasis (DTCL). This study was conducted to compare the effect of different treatments on parasite DNA load following therapies using Real-Time PCR method.**

Materials and Methods: **Fifteen patients were divided into three groups under therapy with intralesional meglumine antimoniate, topical imiquimod and combination of both drugs. After obtaining consent from patients, punch biopsies were taken before and after treatment .To compare the amount of DNA load a relative quantitative Real-Time PCR method was designed and set up using *Leishmania tropica* ITS (internal transcribed spacer) gene. After doing PCR, the obtained results were analyzed using $2^{-\Delta\Delta C_t}$ method and relativity of DNA load before and after treatment**

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were calculated.

Results: **The highest falling of DNA load was for imiquimod (mean 4/7 cases), glucantime (mean 2/2 cases) and combination therapy (mean 2/4). From clinical point of view combination therapy had the best response. On the other hand, the overall IHC findings showed good response based on decreased CD1a epidermal, increased CD1a dermal, decreased CD68 macrophages and increased CD3 and CD20 of dermis.**

Conclusion: **We set up a new method to compare Leishmania DNA load using the stable human gene of beta actin for normalization. We concluded that imiquimod was immune modulator and had synergistic effects on the best parasitocidal drug of glucantime for better response.**

***Keywords: Leishmania tropica*, Real Time PCR, Glucantime, Imiquimod**

Introduction

Leishmania species are protozoa belonging to the family *Trypanosomatidae*. They are transmitted by sandflies belonging to the genera phlebotomus (1). The old world cutaneous leishmaniasis (C.L.), as a dry type caused by *L. tropica* is one of the most common parasitic diseases in Kerman Province, southeastern Iran(2).

The diagnosis of C.L. is made by detecting amastigotes in biopsies or direct smears taken from skin lesions. Due to scanty organisms in cutaneous lesions, especially in chronic leishmaniasis, these methods have low sensitivity (3). PCR is a molecular method with high sensitivity and specificity for diagnosis of leishmaniasis and Real Time PCR is a method to make quantification of parasite burden(4, 5).

There are different types of treatments for C.L. systemic treatment including parenteral (I.V. or I.M.) pentavalent antimonials and oral agents (fluconazol, zinc sulfate and azithromycin). Localized therapy includes intralesional injections of pentavalents, topical drugs (imiquimod and parmomycin) and physical methods (cryotherapy, CO2, laser, topical heat, photodynamic therapy and surgical excision of the lesion) (6, 7).

Meglumineantimoniate (glucantime) is a form of pentavalent antimonial group. They have been the main therapeutic agent for treatment of leishmaniasis since 70 years ago. The mechanism of action is unknown, but may involve inhibition of the parasite's glycolytic and fatty acid oxidative activity resulting in decreased synthesis of ATP (8).

Imiquimod (aldara) is a form of imidazoquinoline. It is used to treat skin malignancies (basal cell carcinoma, Bowen disease, superficial squamous cell carcinoma), viral lesions (such as HPV lesions and mollscumcontagiosum) and also in combination with glucantime for treatment of resistant C.L.(9).

Relative quantification method is used for calculation of gene expression or parasite DNA load in different stages of disease or treatment (10, 11).

In calculating the amount of gene expression or DNA load, one of the samples was calibrator (reference) and other sample compared with that. As in our study before treatment samples were considered as calibrator, and after treatment specimens were reported as fold changes (12).

The current study tried to evaluate DNA load changes before and after treatment of cutaneous leishmaniasis, with imiquimod, meglumine antimoniate and combination of both medications, using Real Time PCR.

Materials and Methods

After explanation the study details to patients their medical consent were obtained. Also their personal information and diagnosis were kept secret.

Fifteen patients underwent treatment for five weeks and were randomly assigned to three different treatment groups: I) Imiquimod (seven cases) II) Imiquimod and meglumine antimoniate (four cases) and III) meglumine antimoniate (two cases). Imiquimod was used as a topical cream was administrated nightly for five weeks and intralesional meglumine antimoniate (0.5-1 ml/cm2) on a weekly basis. After taking consent from patients, punch biopsies were taken before and after treatment. The biopsies were fixed in formalin and embedded in paraffin blocks. Histological and immunohistochemical studies were performed (13).

The QIAamp DNA FFPE kit was used for DNA extraction. Subsequent steps were carried out according to manufacturerer's instructions. At first we cut up 8 sections 10 µm thick using microtome. Then usedxylen (2 ml) and ethanol (1ml) 2-3 times for removing paraffin from samples. The pellets were mixed with 180 µl buffer ATL and 20 µl Proteinase K, and incubated at 56°C for one hour, and then incubated overnight at 90 °C. They were mixed with 200 µl buffer AL 200 µl ethanol. After centrifuging the entire lysate, we washed them with 500 µl buffer AW1 and 500 µl buffer AW2. At last 50 µl buffer ATE were applied to the tubes. After incubating tubes at room temperature for 5 minutes, the DNA extraction was completed and they were reserved for next processes.

For identifying the *Leishmania* species a conventional PCR method was set up using a pair of primers (5' TCGCAGAACGCCCCTACC 3') and (5'-AGGGGTTGGTGTAAAATAGGC 3') specific for conserved sequences of kDNA of *Leishmania*. The agarose gel (1%)electrophoresis is used for running of the PCR products (14).

To compare *Leishmania* DNA load before and after treatment, at first the standard strain of *L. tropica* (MHOM'Sudan'58'OD) was cultured and four dilution (rate, one to ten) were prepared by neubauer chamber cell counting. After DNA extraction these samples were evaluated by absolute quantitative real time PCR and standard curve was obtained. After optimization of primers and probe a relative quantification real time method was set up. The ITS (internal transcribed spacer) gene of *L. tropica* was chosen for designing primers and probe (GenBank Accession number :FJ948464.1) (15). Forward primer (L.ITS.R: 5'-TTTAATAATCCTGGTCACAGCC-3') and reverse primer (L.ITS.F: 5'-CAAATACACGCATGCACTCTC-3') targeting the conserved region of the *Leishmania* ITS gene was designed. The fluorogenic probe of *L. tropica* (L.ITS.P: 5'-AGCGTCGAAACTCCTCTCTGGTGC-3') was synthesized using a 6-carboxy-fluorscein (FAM) receptor dye molecule attached to the 5' end, and a 6-carboxytetramethylrhodamine (TAMRA) quencher linked to the 3' end. For normalization the human gene of Beta actin was chosen and primes and probe were designed as housekeeping gene (NCBI Reference Sequence: NM_001101.3) (16). Beta Actin primers were (BAF: 5'- ACCACCTTCAACTCCATCATG-3') and reverse (BAR: 5'-CTCCTTCTGCATCCTGTCG-3') and its probe was (BAP: 5'-ACATCCGCAAAGACCTGTACGCC-3') modificated with 5' JOE and 3' TAMRA (Bioneer). These primers and probes were designed

by the Gene Runner software.

Real time PCR was performed in a 25µl reaction volume including 12.5µl of Tag Man Master Mix (Jena Bioscience) 300 nM of each primer, 200 nM of each fluorogenic probe and 50 ng of DNA. The thermal cycle conditions consisted of a 10 min initial incubation at 25 °C, followed by 4 min denaturation at 95 °C 50 cycles at 95 °C for 20 s and 60 °C for 1 min each.

Analysis of results by $\Delta\Delta C_T$ calculation:

Before treatment samples were considered as calibrator, and after treatment specimens were reported as fold change.

Calculation formula was as follows:

• Formula: $2^{-\Delta\Delta C_t}$

$\Delta\Delta C_t = (C_{t,test1} - C_{t,ref1}) - (C_{t,test2} - C_{t,ref2})$

• $C_{t,test1}$: ITS gene C_t in after treatment samples.

• $C_{t,ref1}$: Beta actin gene C_t in after treatment samples.

• $C_{t,test2}$: ITS gene C_t in before treatment samples.

• $C_{t,ref2}$; Beta actin gene C_t in before treatment samples.

The relative quantitative real-time PCR method that we used to analyze our data is also used to evaluate gene expression in various stages of disease or treatment (17).

Results

Thirty paraffin blocks were used and useful DNAs were successfully extracted from twenty six of them. Four of them were failed to extract DNA. As expected; all samples were *L. tropica,* identifying by conventional PCR (Fig.1).

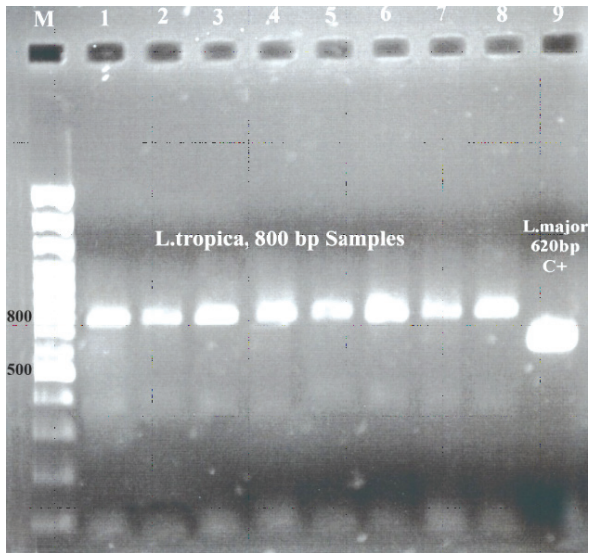

Fig. 1: Identification of *Leishmania* spp. isolated from skin biopsies by conventional PCR based on specific primer for conserved sequences of kDNA. Line 9 is positive control of *L. major* (620 bp) and Line 1 to 8 are *L. tropica* (800 bp)

After absolute quantification of the parasites, we plotted the Ct values against the different dilutions. Results show that quantification was linear over serial dilutions. Cure obtained from different samples had slope of -3.471(Fig. 2).

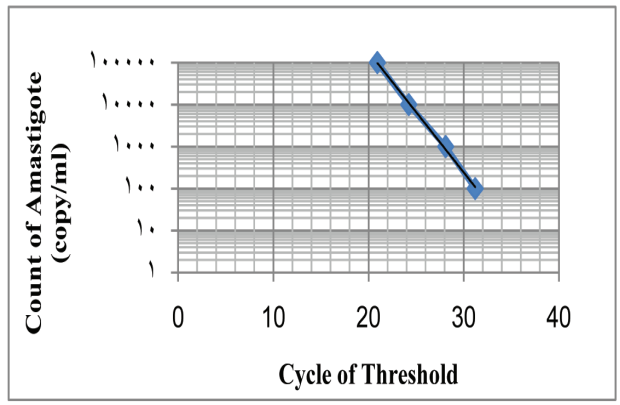

Fig. 2: Standard curve obtained from serial dilution of *L. tropica* DNA expressed as the number of parasites per ml (R^2 = 0.9983-Slope= -3.472)

Afterwards; *Leishmania* DNA load was estimated by relative quantitative real-time PCR. The value

of ΔCt was calculated for each sample before and after treatment by subtracting Beta actin Ct from ITS Ct. then the ΔΔCt was obtained for each sample by subtracting before treatment ΔCt from after treatment ΔCt. Then the value of 2^-ΔΔCt was calculated which showed the ratio of DNA load after or before treatment (Table 1). For imiquimod four cases of seven have decreased DNA load (57% of patients). In glucantim therapy group, in both cases the *Leishmania* DNA load had been decreased (1/8192 & 1/4096). In combination therapy group, two of four cases showed reduction in DNA load (50% of patients) (Table 1).

Table 1-ΔCt and $2^{-\Delta\Delta C_t}$ values in three treatment groups using ITS and Beta Actin genes:

-Treatment with Imiquimod reduced DNA load in four cases of seven patients.

-Treatment with Glucantime reduced DNA load in both cases.

-Treatment with combination of Imiquimod and Glucantime reduced DNA load in two cases of four patients.

Drug	No. of sample	After treatment ΔC_t	Before treatment ΔC_t	$2^{-\Delta\Delta C_t}$
Imiquimod	1	14	2	2^-12
	2	10	10	2^0
	3	5	-4	2^-9
	4	0	2	2^2
	5	12	6	2^-6
	6	29	8	2^-21
	7	10	9	2^1
Glucantime	1	7	-5	2^-12
	2	11	-2	2^-3
Combination of both drugs	1	14	5	2^-9
	2	8	10	2^2
	3	10	-6	2^-16
	4	1	2	2^1

Maximum decreased DNA load in all three regimens belongs to one case treated by imiquimod alone. In this case the value of $2^{-\Delta\Delta C_t}$ is 2^-21; it means that the ratio of DNA load in after treatment sample was 1/2097152 to before treatment sample. In Table 2, samples are sorted according to $2^{-\Delta\Delta C_t}$. Since this study was following the previous study done by Dr. Shamsi Meymandi *et al.* (13), we reevaluated their IHC findings and compared them with real-time PCR results (Table 2).

Table 2- The samples were arranged due to amount of 2^-ΔΔCt (Inc: Increase, Dec: Decrease)

	$2^{-\Delta\Delta C_t}$	Epi CD1a	Der CD1a	Der CD68	Der CD3	Der DC20	Clinical data
1 Aldara	2^-21=1/2097152	Dec	Inc	Dec	Inc	Inc	Cure
2 Combination	2^-16=1/65536	Dec	Inc	Dec	Dec	Dec	Partial cure
3 Aldara	2^-12=1/4096	Dec	Inc	Dec	Inc	Inc	Cure
3 Glucantime	2^-12=1/4096	Dec	Inc	Dec	Inc	Inc	Cure
4 Aldara	2^-9=1/512	Dec	Inc	Dec	Inc	Inc	Cure
4 Combination	2^-9=1/512	Dec	Inc	Dec	Inc	Inc	Partial cure
5 Aldara	2^-6=1/64	Dec	Inc	Dec	Inc	Inc	Cure
6 Glucantime	2^-3=1/8	Dec	Inc	Dec	Inc	Inc	Partial cure

Discussion

This was the first study comparing the therapeutic effect of glucantime, imiqimod and combination of both using real-time PCR and obtaining parasite DNA load. Other studies compared them by clinical manifestation or using histological and immunohistochemical methods. Our study revealed that imiquimod was the highest reducer of DNA load and by mean 57% of cases but mean of response for glucantime was 100% and for combination was 57%. Although it seems the glucantime was the best reducer of the DNA load but as we had only two patients treated by glucantime, it could not be considered as the best medication. In terms of IHC and clinical findings combination therapy was the best responder. It had shown that combination therapy is a better treatment than glucantime or imiquimod alone (18).

In another study "Arevalo" had tried combination of imiquimod and glucantime in 12 patients which did not respond to meglumine antimonite therapy, 90% of the patients were cured after 6 month (19).

Although glucantime is a very old drug, its exact mechanism is unknown (20). But several mechanisms have been proposed for the drug by recent studies. Sereno *et al.* have reported apoptosis in antimonial-treated amastigotes involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of the plasma membrane (21). As regards our study the *Leishmania* DNA load was markedly decreased after treatment of glucantime and it emphasized the theory of DNA fragmentation.

Imiquimod is an immunresponse modifying agent (22). It activates immune cells, through stimulation of Toll-Like Receptors (TLRs), localized on the surface of antigen presenting cells. These immune cells release several endogenous pro-inflammatory cytokines such as INF- α, TNF- α, IL6 and IL12; resulting in upregulation immune system activity. Imiquimod also releases nitric oxide which can activate macrophages to kill *Leishmania* amastigotes (9, 22).

Imiquimod did not demonstrate direct toxic effects against amastigotes but could induce the expression of the inducible nitric oxide synthase (iNOS) gene and release of NO (nitric oxide) from both infected and noninfected macrophages. So the macrophages can remove the amastigotes (23). There is no beneficial effect of combination a four week course of treatment with 5% imiquimod cream and a standard course of treatment with meglumine antimoniate in patients with cutaneous leishmaniasis in endemic area of *L. tropica* (18). Also in our study the combination of glucantime and imiquimode had the least reduce of *Leishmania* DNA load.

Recent researches showed acquired mutations in *L. tropica* leading to decreased responsiveness to antimonial drugs such as glucantime (24). There are several mechanisms for resistance of

Leishmania treatment with pentavalent antimonial group, but all of them result from decreasing the concentration of drug in the cell. This could be either by decreasing drug uptake by integral membrane proteins or by increasing efflux and or sequestration of the active metabolites (8).

Dendritic cells (CD1a positive cells) and macrophages (CD68 positive cells) play important role in *Leishmania* detecting and eliminating. *Leishmania* promastigotes (the infectious form of *Leishmania*) are taken up by skin macrophages. Promastigotes transform into amastigotes within macrophages and replicate. Then macrophages are lysed and amastigotes are released. Free macrophages infect skin dendritic cells. These activated dendritic cells migrate to lymph nodes through dermis and activate T cells to become TH1 cells. Cytokines released from Th1 cells induce macrophages to up -regulate and eliminate intracellular organisms (25).

In terms of IHC findings, we have shown that in all cases with reduced Leishmania DNA load, the number of CD1a positive cells (dendritic cells) had decreased in epidermis and increased in dermis. The other finding was decrease of CD68 positive macrophages in these cases. It means that in successfully treated patients dendritic cells migrate from epidermis to dermis in order to activate immune system. Macrophages are the main reservoir of the parasite, thus reducing the number of them demonstrated efficacy of drugs in reducing parasites.

Beta actin, a human protein, is present in almost all tissues. Although its expression can change in some diseases or due to some drugs but it is probably the best housekeeping gene as internal control in real time PCR; since beta actin levels do not change under numerous treatments in a multiple samples(26).

Conclusion

This study showed that these primers and probes as appropriate candidates can be used in identification of *L. tropica* species and parasite load in endemic areas. Further studies using different clinical samples to optimize the identification of *Leishmania* genus and species, and also parasite load are essential.

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