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Research article

Detection the effect of the treatment of pentostam on LPG, CPA, and PPG virulence factors of *cutaneous Leishmania* by real time-PCR

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Abstract

The aim of this study was to evaluate the effectiveness of pentostam treatment through effect on some of the virulence factors of cutaneous Leishmania parasite by using the real-time PCR within five weeks, as well as the determination of parasite type by three virulence factors lipophosphoglycan, Cysteine protease and proteophosphoglycans by using nested PCR technique, since that these factors are already were diagnosed in Leishmania parasite. Fifty-five biopsies were taken from skin ulcers which clinically diagnosed as cutaneous leishmaniasis from patients attending Al-Diwaniyah education hospital in Al-Qadisiyah Province for the period between 1 \ 11 \ 2012-1 \ 5 \ 2013 ,whose age ranged from (10-80) years. The DNA was extracted, and then amplified by using primers selected on repetitive kDNA for identification of a Leishmania parasite; it is L. major species and the above three virulence factors, after that DNA electrophoresis was done for the amplified DNA. The results showed that Leishmania parasite was detected in 89.09% of samples. The L. major species was identified in the majority of Leishmania parasite positive samples 98%. RNA was extracted from positive samples collected before and after the treatment with pentostam to measure the amount of gene expression of virulence factors after treatment and to measure the effect of the drug on these factors. After amplification using four of primers, the first three of them were the target genes LPG, CPA, PPG whereas the fourth primer is called the initiator, which is GAPDH. These primers are designed using the NCBI gene bank database. Results showed that there was a high difference for each gene during the period of treatment in a level of the possibility of ($P \leq 0.05$) and by comparing the three genes it was found that there was a significant difference between cysteine protease and the other two factor lipophosphoglycan, proteophosphoglycan, whereas no significant difference between lipophosphoglycan, proteophosphoglycan. Hence, it has been clear that the treatment with pentostam has a high effect on parasite by its effect on the virulence factors (LPG, CPA, and PPG) and this explains that possibility of infection may happen again after having the treatment.

Keywords: Cutaneous Leishmania, Virulence factors, Pentostam, Real Time-PCR

Introduction

Leishmaniasis is a group of parasitic diseases caused by a number of protozoa species of Leishmania (1), It is an important tropical disease affecting 350 million people in 88 countries around the world with the risk of infection (2). Leishmaniasis is spread in tropical and subtropical regions of all inhabited continents except the Polar Regions and Australia. The disease is endemic in 88 countries (21 in the New World and 67 in the Old World) (3).The spread of the disease in poor sectors of society is exacerbated by

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overcrowding, lack of access to prevention services, diagnosis and treatment, lack of health consciousness (4). The World Health Organization classifies Leishmaniasis among neglected diseases that is associated with rural areas and poorness, but adapted to the urban environment (5). The prevalence of cutaneous leishmaniasis is noted in areas where it was not previously present as a result of urbanization, deforestation and the presence of domestic animals as hosts precursors (6), as well as natural disasters (7), economic difficulties (8), armed conflicts (9) and tourism all this makes the population vulnerable to migration to areas where there is a disease Cutaneous Leishmania (10). Pathogenic parasites of humans and animals have developed several factors of virility to ensure survival and replication within the host, The first role of these factors is to reduce the defenses of the host against it by innate and adaptive immunity, evidence suggests that the virulence determinants of the parasite are responsible for escape from host defenses, allowing these organisms to remain in an environment hostile immunologically to them in the host (11, 12). Leishmania parasites have many virulence factors, which can be defined as a set of parasitic components that help in survive within their mammalian parasites where they promote the survival of parasite and immune modification in the host of mammals ,These factors play an important role in the ability of these pathogenicity parasites (13). In Leishmania parasites, especially *Leishmania. major* , the virulence factor lipophosphoglycan play important rule in insurance of parasite transport throughout the host and staying inside the macrophage of the mammalian host without damage (13, 14, 15) whereas Cysteine protease helping the parasite in tissue invasion, survival in connective tissues and immune modification of the host (16), and finally proteophosphoglycans, participating in sand fly infection with parasite and aggravation of cutaneous and visceral Leishmania (17). Pentavalent antimonials, including meglumine antimoniate and sodium stibogluconate, have been used for more than half a century in the therapy of the parasitic disease Leishmaniasis (18). Sodium stibogluconate (Pentostam), this medicine is given by injection in muscle or veins but meglumine antimoniate (Glucantime) is given by injection in muscle only, the treatment phase consists of 10 injections to 20 syringes per day (19). The World Health Organization (20) recommends treating Leishmaniasis by antimoniate within the ulcer according to species and manifestations of clinical signs WHO recommends injecting 3 ml under the edges of the entire lesion until Bleaching surface area of the lesion for 5-7 days for 5-2 times (10).

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 425

1-Collection of the Samples and DNA Extraction

Blood samples were collected from infected patients from the Educational Maternity and Pediatric Hospital in Al-Diwaniyah city, for the first time before receiving treatment for diagnosed by conventional PCR and Nested-PCR. After receiving treatment (pentostam) for diagnosed by Real-Time PCR.1 cc of Normal saline was injected into the ulcers and the liquid was again withdrawn to get the parasites from the ulcer's edge and then placed in plastic tubes and kept in a frozen -20C^0 degree for diagnosed by PCR (21) .DNA Extraction from Infected patient's samples by using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) and done according to the protocol described by the manufacturer instructions.

2-PCR amplification

N- PCR were applied to detect the presence of *Leishmania major* . In the first round of the reaction, genus-specific primers CSB 1/2 were utilized to amplify the Leishmania spp.

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The products were then used as templates for the second round of PCR using the *L. major* - specific primers 13Z and LIR, which generate a product of 560 bp, nested PCR containing two primer sets: First step (CSB2XF:CGAGTAGCAGAAACTCCCGT TCA) (CSB1XR:ATTTTTCGCGATTTTCGCAG AACG),Second step(13Z:ACTGGGGGTTG GTGTAAAATAG) (LIR: TCGCAGAACGCCCCT) (22), The products were electrophoresed in a 1.5% agarose gel containing Atidium bromide dye and observed under UV light (23).

3-Quantitative Reverse Transcription Real-Time PCR (RT-qPCR):

RT-qPCR used for determine mRNA quantitative levels to detection gene expression of *Leishmania major* virulence factor gene which include (LPG1, CPA, and PPG1 gene) also used GAPDH gene as conservative gene for gene expression was done according to method described by(24) , Four primers were used in this study; the first 3 primer was Target genes(LPG,CPA,PPG) while fourth primer was GAPDH gene. These primers were designed by using NCBI- Gene Bank data base and Primer 3 design online, the primers used in quantification of gene expression using qRT-PCR techniques based SYBER Green DNA binding dye, and supported from (Bioneer, Korea) company.

Table (1): The Primers of virulence factor and GAPDH gene with their sequence (RT-PCR)

Primer	Sequences		Protect of Size
LPG	F	TGTTTCGCCTGCAATATCGC	150bp
	R	TTTGCCAGATGGACCTTTGC	
CPA	F	AACATCACAACGGCACTGTG	92bp
	R	AAACTCGCCTTTGTCATGGC	
PPG	F	AAGAACGACATCTGCTGCAC	102bp
	R	ACCGCTGCAAGTCACAATTG	
GAPDH	F	AGATGTGCTTGTGGTGAACG	132bp
	R	TGCAACTTGTCCGTGAACAG	

4-Real-Time PCR data analysis

The data results of q RT-PCR were analyzed by the relative quantification gene expression levels (fold change) Livak method that described by (25). The relative quantification method, quantities that are obtained from qRT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels.

5- Statistical analysis:

Statistical analysis were performed by using ANOVA (LSD) at $p \leq 0.05$ (26).

Results

Nested PCR technique

The results of DNA extraction for 55 samples showed that Leishmania parasite was detected in 49 samples of them (89.09%) and 6 samples were negative as is shown in the figure (1). While the results for positive samples showed that 47 samples were *L. major* (95.91%) as is shown in the Figure (2).

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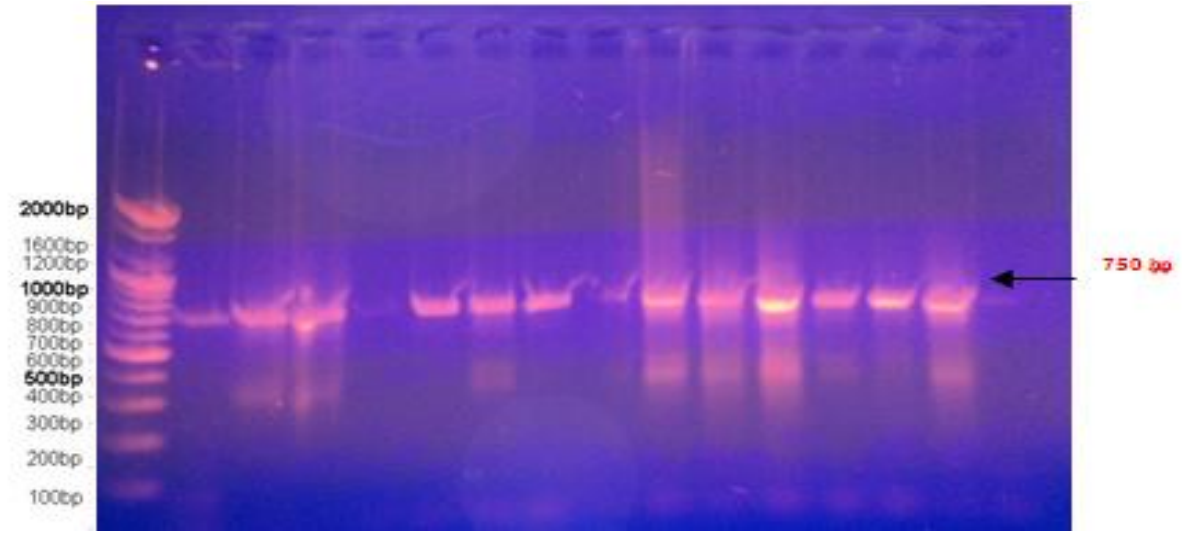

Figure (1): Agarose gel electrophoresis image that show PCR product analysis of Leishmania parasite gene Phospholipase . Where, Lane (M) DNA marker (2000-100bp), Lane (1,2,3, ,5,6,7,8,9,10,11,12,13,14) positive at Nested PCR product size 750bp

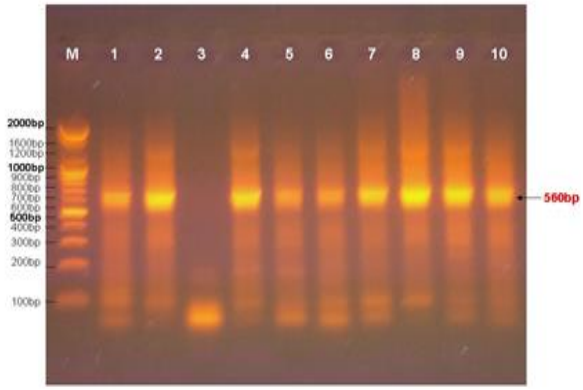

Figure (2): Agarose gel electrophoresis image that show PCR product analysis of L. *major* parasite gene Phospholipase. Where, Lane (M) DNA marker (2000-100bp), Lane (1, 2, 4, 5, 6, 7, 8, 9, 10) positive at Nested PCR product size 560bp

Relative measure of genotypes determined by Q Time -Real PCR

The relative measure of genotypes identified in the study was calculated as virulence factors of genes CAP, LPG, PPG In positive samples of *L. major* by method $^{2-\Delta\Delta CT}$(Livak) using genetic marker (GAPDH) which is a conservative gene that transit from generation to generation without change, which use to normalized the above gene productivity levels in qReal Time-PCR. Results showed amplification of specific primers of the gene that mean to the gene expression of the virulence factor CPA gene in the first week of treatment was 0.5 fold and began to decline gradually as it reached (0.51, 0.14 ,0.08, 0.03, 0.006) fold respectively for the subsequent four weeks of treatment with pentostam in comparison with pre-treatment group 1 the samples are untreated as shown for the number of folds in the reproduction level mRNA of CAP gene as is shown in the figure (3) and there was a significant difference in the relative measure of the gene during the five weeks of treatment at a probability level ($P \leq 0.05$).

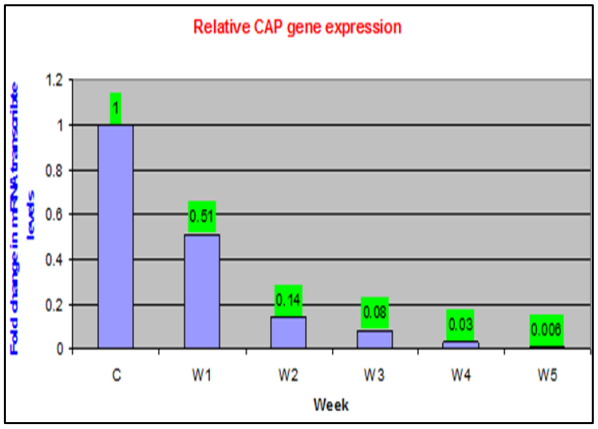

Figure (3): Relative CAP gene expression

Results showed amplification of specific primers Of the gene that mean to the gene expression of the virulence factor LPG gene

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in the first week of treatment was 0.27 fold and began to decline gradually as it reached (0.15, 0.14, 0.05, 0.01) fold respectively for the subsequent four weeks of treatment with Pentostam in comparison with pre-treatment group 1 the samples are untreated as shown for the number of folds in the reproduction level mRNA of LPG gene as is shown in the Figure (4) and there was a significant difference in the relative measure of the gene during the five weeks of treatment at a probability level (P≤ 0.05).

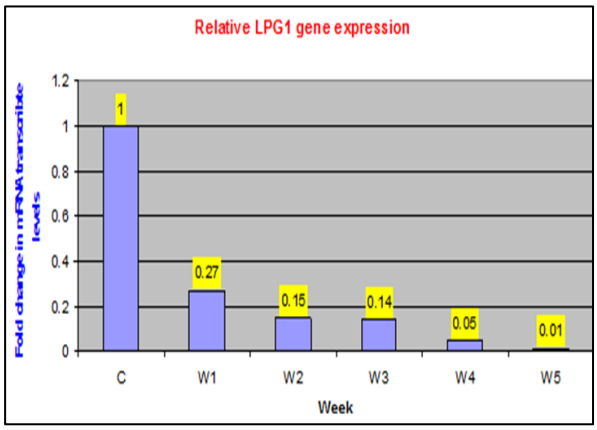

Figure (4): Relative LPG1 gene expression

Results showed amplification of specific primers of the gene that mean to the gene expression of the virulence factor PPG gene in the first week of treatment was0.06 fold and began to decline gradually as it reached (0.06 ,0.007 ,0.0026,0.0025,0.0003) fold respectively for the subsequent four weeks of treatment with pentostam in comparison with pre-treatment group 1 the samples are untreated as shown for the number of folds in the reproduction level mRNA of PPG gene as is shown in the Figure (5) and there was a significant difference in the relative measure of the gene during the five weeks of treatment at a probability level (P≤ 0.05).

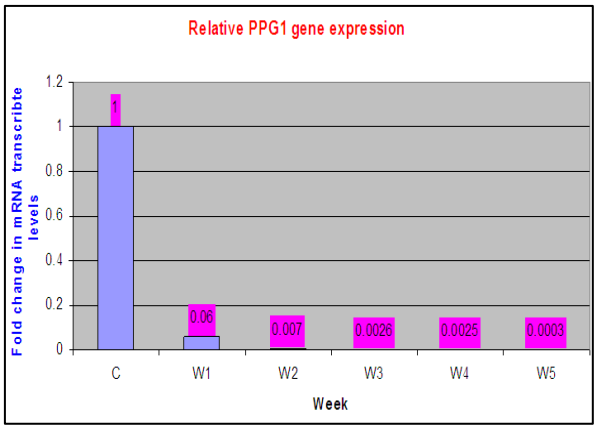

Figure (5) Relative PPG1 gene expression

The comparison among the three genes during the five-week treatment period showed a significant decrease in the rate of heterogeneous folds after the end of the treatment. The highest was the LPG virulence factor, which was mean in the fifth week of 0.01, 0.006 for the CPA and 0.0003 for PPG as is shown in the Table (2).

Table (2): comparison among the three genes during the five-week treatment

Factors	First week	Five week
CPA	0.515812	0.006295 aE
LPG	0.272108	0.01362 bC
PPG	0.069292	0.000373 bE

Different large letters indicate significant differences between the first week and the subsequent four weeks at a probability level of 0.05 using the LSD test.
Similar large letters indicate no significant differences between the first week and the subsequent four weeks at a probability level of 0.05 using the LSD test.
Different small letters indicate significant differences between the three genes in the fifth week of treatment at a potential level of 0.05 using the LSD test.
Similar small letters indicate no significant differences between the three genes in the fifth week of treatment at a potential level of 0.05 using the LSD test.

Discussion

In the current study, the method of Nested-PCR was used as a basic method in diagnosing the Leishmania parasite and then identifying it and recording the infection for several reasons: The traditional methods for diagnosis of cutaneous leishmaniasis are not so sensitive and high quality that 100% can be relied upon. For example, a Leishmania

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test for skin is very sensitive but lacks specificity (27). The laboratory diagnosis of Cutaneous Leishmania is usually required either watch the amastigotes stage or to double the isolation of Leishmania parasites from the lesion (28), The methods most commonly used in most Cutaneous leishmaniasis are microscopic examination of abrasion from lesion, biopsy impression smears and histopathology, The most traditional methods of diagnosis are the cultivation of the lesion in the appropriate culture and this is only available in reference laboratories or related to Cutaneous leishmaniasis and sensitivity is more positive in only 70% of acute cases when performed optimally(29). The need for more sensitive methods for the diagnosis of Cutaneous leishmaniasis has led to the development of DNA-based diagnostic techniques and Kinetoplastids which containing approximately 10,000 small ring DNAs, known as the KDNA that range from 600 to 800 bp in species belonging to the genus of leishmanial, The abundance of numbers and other properties of these molecules have made it a target for a number of PCR-based techniques (30). Traditional techniques commonly used in the diagnosis of leishmaniasis are attributed to the fact that they do not differentiate between the types of Leishmaniasis and their sensitivity is less than Molecular techniques (31) (32) (33). Nested-PCR technique provides an alternative, quick, sensitive and specific method for traditional techniques. In this way, the diagnosis of leishmaniasis is diagnosed and the species identified at the same time (34). Based on this, we used the Nested-PCR technique to diagnose cutaneous Leishmaniasis for patients attending Al-Diwaniyah education hospital, PCR results for this study showed that. 89.09% of the samples were positive while 10.90 % only they were negative , The percentage of *L. major* was in positive samples 98% While only two were negative and thought to be *L. tropica*, Where There are only two species in Iraq, causing Cutaneous leishmaniasis (35) (36). This is inconsistent with what both of (37) (38) have found in Al-Qadisiyah Province that all positive samples were *L. major* ,whereas (39) found in Kufa, after samples were tested by PCR, 90% of the samples were positive and a *L .major* percentage of which (51%) while the proportion of *L. tropica* (49%). The Results of current study agree with results of (40) and (41) in Iran which confirmed that the Nested-PCR technique is a sensitive and accurate method for detection and differentiation between the types of Leishmania parasite. The results of the current study confirmed that the diagnosis in the PCR-Nested method is more accurate and faster than other tests and distinction this disease from skin diseases similar to cutaneous Leishmaniasis diffuse in the region. The pentostam is the best treatment for sufferers of Cutaneous and Visceral leishmaniasis (42) (43). When measuring the gene expression of these factors before and after treatment, There was a significant difference for each gene during the treatment period at the probability level ($P \leq 0.05$) and when comparing the three genes with each other during the fifth week of treatment, There was a significant difference between the CPA gene and the other two genes LPG and PPG. There was no significant difference in the calculation of the relative measure of the LPG and PPG genotypes with each other. The difference between the presence of these factors before and after treatment was observed. In all cases, the virulence factors after the treatment period is very small compared to the presence in the first weeks, as well as the work of the immune system, which contributes to increasing resistance to the body and suppress the effectiveness of the virulence factors this explains why there is no second relapse of cutaneous Leishmaniasis. As for the explanation of this apparent decrease in the amount of these factors, the method of action of the Sodium stibogluconate (Pentostam) is not clearly understood but exposure the parasite in stage of amastigotes to mg\ml500 Pentostam leads

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to a reduction of about 50% of the DNA and protein of the RNA ,This can be attributed to a decrease in the production of ATP (adenosine triphosphate) and GTP (guanosine triphosphate) of the parasite, thus contributing to the lower synthesis of these molecules.

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