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Gene Expression of *Leishmania donovani* Exosome in BALB/c Mice by Real Time qPCR

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ABSTRACT

The aim of this study is to molecular detection of *Leishmania* donovani exosome by real time PCR gene expression. The results shows that the mean of gene expression in spleen tissue was (10.3300), in passage 1, while the mean of gene expression of was (24.6733), in passage 3. The control culture line, the mean of gene expression of was (8.0833), in passage 1, and (0.2300), in passages 3. This study explained that exosome gene expression was higher in spleen than in cultures media. The higher levels were in passage 3 and lowest levels in passage 1. The level of gene expression was declined in control culture line were the higher levels were in passage 1 and lowest levels in passage 3.

1. INTRODUCTION

The leishmaniasis is a vector-borne transmitted infection, the infection is prevalent in the tropical and subtropical areas in ~98 countries in Europe, America, Asia and Africa [1]. Nevertheless, more than 90% of recent infections occurred only in 13 countries. It is about between 0.9-1.7 million yearly of people are infected [2]. The exosomes are endocytic vesicles of approximately 40-100nm in size that are discharged from many types of eukaryotic cell including the parasitic protozoa *Leishmania* spp, and can assist intercellular communication [3]. Functionally, the *Leishmania* exosomes have been exist to be anti-inflammatory and its play a role in pathogenesis via modulating the expressed cytokines at the restricted site of infection [4]. Reports showed that the promastigote and amastigote forms of *L. donovani* can release exosomes that are discovered in host cells and selectively stimulate secretion of IL–8 from macrophages [5].

2. Materials and Methods

Primers: The primers for detection and quantifying of the target genes, and housekeeping gene (Actin), were designed by the NCBI-Gene Bank data base, these primers were made by (Bioneer company,Korea) as the following table:

Table (1): Primers sequence used in this study:

Primers	Sequence 5'-3'		Product size	Gene bank design code
Exosome	F	AACGCGTGATTTCGGTGAAG	139bp	XM_003859325.1

L. donovani	R	AGCAGCATAATCGCGGTTTG		
HKG-Actin	F	TGGCTCGATTTTGTCATCGC	92bp	MT786532.1
	R	TGTTGTGCACAATGCTTGGG		

Experimental design: The current study has incorporated 9 Balb/c mice (males), (~6-8) weeks old divided into 3 groups in each passages, each mice injected in intrapreitoneal cavity with $3x10^6$ promastigotes of *L. donovani*. All of the 3 groups were takes 1 month of infection. Specimens from spleen of each animals were cultured in semi solid and NNN media for growth and multiplication of parasite. The study also incorporated taking of 3 repeats of cultures at day 7 and day 12 following culturing of each passage and 3 repeats from the original culture line (without passing in animal).

Total RNA Extraction: The total RNA was extracted from frozen tissues using the TRIzol® reagent kit and performed according to company instructions:
A 200mg of spleen was homogenized with addition of 750µl TRIzol®reagent. In each tube 200µl of chloroform was then added and shaken vigorously for 15seconds. The mixture was incubated in ice for 5min then centrifuged for 15 min at 12000rpm 4°C. Then removed the supernatant to a new eppendorfs tubes, with adding of 500µl isopropanol. After that, the mixture was mixed 4-5times by inverting the tube and incubated for 10min at 4°C and then centrifuged at 12,000rpm. Supernatant was discarded and 1ml of 80% ethanol applied and mixed by vortex. Then, centrifuged for 5min at 12000rpm,4°C. The supernatant discarded, the precipitate of RNA was left to air drying. Finally, the sediment of RNA was resuspended in 100µl free nuclease water, then extracted RNA was saved in deep freeze.

Measurement the yield of extracted RNA: The RNA extracted was estimated using a Nanodrop spectrophotometer (THERMO,USA), two of quality controls on RNA were done. The first is to measure the volume of RNA (ng/µL), the second is to measure the purity of RNA through reading the spectrophotometer absorbance at 260nm and 280nm in similar Nanodrop operator as follows:
The suitable program (Nucleic acid→ RNA), selected after opening the machine. A dry wipe was applied, and the determined pedestals were cleaned several times. Afterward carefully pipette a 2µl of nuclease free water and positioned Nanodrop blanking on surface of the lower measuring pedestal. Then the pedestals are washed, and a 1µl RNA specimen is pipette for assessment."

Treatment DNase I: The extracted of RNA was treated with enzyme DNase I to eliminate trace amounts of genomic DNA from the eluted RNA using (DNase I enzyme kit) and done using the method defined by the company Promega,USA, instructions as follows:

Table (2): Components of DNase I treatment:

Solution	Volume
RNA 100ng/ul	10ul
DNase I enzyme	1ul
10X buffer	4ul
DEPC water	5ul
Total volume	20ul

After that, the mixture was incubated at 37°C for 30min. Then, for inhibition of DNase enzyme through adding 1µl stop reaction and incubated at 65°C for 10min.

Synthesizing of cDNA: DNase-I treated RNA samples were also used using the M-MLV Reverse Transcriptase kit and performed as follows:

Step 1

Table (3): Real Time Master Mix:

Master Mix	Volume
Total RNA 100ng/ul	8ul
Random Hexamer primer	1ul
DEPC water	1ul
Total	10ul

Then, RNA and priming were denatured at 65°C for 10min, immediately afterwards cooling on ice. Step 2

Table (4): Real Time Master Mix:

Master Mix	Volume
Step 1 RT master mix	10ul
M-MLV RTase (200u)	1ul
5X M-MLV RTase reaction buffer	4ul
100mM DTT	2ul
dNTP	2ul
RNase inhibitor	1ul
Total	20ul

The tubes were then placed in a vortex and spinning down briefly. In thermocycler the RNA was translated into cDNA under the following:

Table (5): Real Time PCR Cycle Condition

Step	Temperature	Time
cDNA synthesis (RT step)	42°C	1 hour
Heat inactivation	95°C	5 min

Quantitative Real-Time PCR (qPCR): The quantitative real-time PCR used in the quantification of gene expression analysis, which was normalized by the housekeeping gene (ACTIN) using the method of Real-Time PCR, as the following steps:"

1- qPCR Master Mixes Preparation: Through using GoTaq ®qPCR Master Mix Kit based on SYBER green dye target detection and ACTIN gene amplification in the Real-Time PCR system, qPCR master mix was prepared and contains the following:

Table (6): Components of qPCR Master Mix

Solutions	volume
cDNA template (100ng)	5µL
Forward primer(10pmol)	1 µL
Reverse primer (10pmol)	1 µL

qPCR Master Mix	12.5 µL
DEPC water	5.5 µL
Total	25 µL

The component of reaction solution placed in qPCR plate strip tubes and mixed for 3min by Exispin vortex centrifuge, then placed in Miniopticon Real-Time PCR.

2- Thermocycler Conditions: The qPCR plate and the following thermocycler protocol were loaded into the table below:

Table (7): Thermocycler Conditions of qPCR

qPCR step	Temperature °C	Time	Repeat cycle
Initial Denaturation	95	5min	1
Denaturation	95	20sec	45
Annealing \ Extension	60	30sec	

qRT-PCR Data analysis: The results of qRT-PCR data for the ACTIN and target genes were analyzed by the relative quantification of the gene expression (fold change), (the ΔC_T method Using the reference gene) as explained by [6], as following equation:
"Ratio (reference/target)= $2^{CT(reference) - CT(target)}$"

3. Results

The exosome gene expression of *L.donovani* in of Terms of Fold Change was conducted as follows:

3.1 Exosome Gene Expression of L. donovani Amastigote in Spleen Tissue

In this tissue 3 passages were collected. Three replicates of spleen tissue were separated from 3 animals to estimate the expression of exosome gene in each passage, the results recorded as shown in table (8), showed highly difference (p value=0.000), figures (1, A and B).

Table (8): Exosome gene fold change expression of *L. donovani* amastigote of spleen tissue in 3 passages.

Passages	CT (exosome)	CT (Actin)	ΔCT	Fold change (2^ΔCT)	Mean	SD
Passage 1	26.35	29.87	3.52	11.47	10.3300	1.7791
	26.91	30.40	3.49	11.24		
	26.80	29.85	3.05	8.28		
Passage 2	26.09	30.10	4.01	16.11	17.510	1.9728
	26.76	31.00	4.24	18.90		
	26.00	30.13	4.13	17.51		
Passage 3	26.77	31.32	4.55	23.43	24.6733	1.3875
	26.32	30.93	4.61	24.42		
	25.68	30.39	4.71	26.17		

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Total		17.5025	5.6874
value = 0.000 Highly significant			

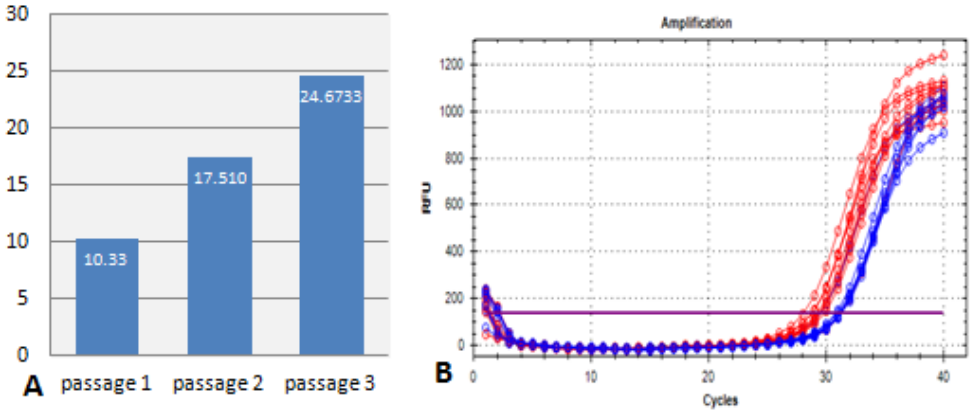

Figure (1): A-Levels of *L. donovani* amastigote exosome gene expression of spleen tissue in 3 passages. B-Real time PCR amplification plots of exosome gene in *L. donovani* amastigote (spleen tissue). Where, the red qPCR plots (exosome gene), and the blue qPCR plots (Actin gene).

3.2 Exosome Gene Expression of L. donovani Promastigote

Culture at day 7: Samples of spleen tissue from each animals were isolated for cultured in media and 3 repeats of promastigotes taken from the culture at day 7 of culturing, to measure the gene expression of exosome, table (9), these result showed highly difference (p value=0.003), figures (2, A and B).

Table (9): Exosome gene fold change expression of *L. donovani* promastigote at day 7 culture in 3 passages.

Passages	CT (exosome)	CT (Actin)	ΔCT	Fold change (2^ΔCT)	Mean	SD
Passage 1	26.67	29.64	2.97	7.84	8.7567	0.8225
	27.34	30.51	3.17	9.00		
	27.11	30.35	3.24	9.43		
Passage 2	26.78	29.89	3.11	8.63	10.6200	1.8545
	26.53	29.98	3.45	10.93		
	27.56	31.18	3.62	12.30		
Passage 3	24.93	28.89	3.96	15.56	16.1100	1.9249
	25.59	29.78	4.19	18.25		
	25.87	29.73	3.86	14.52		
Total					11.8289	3.5938

value = 0.003 Highly significance

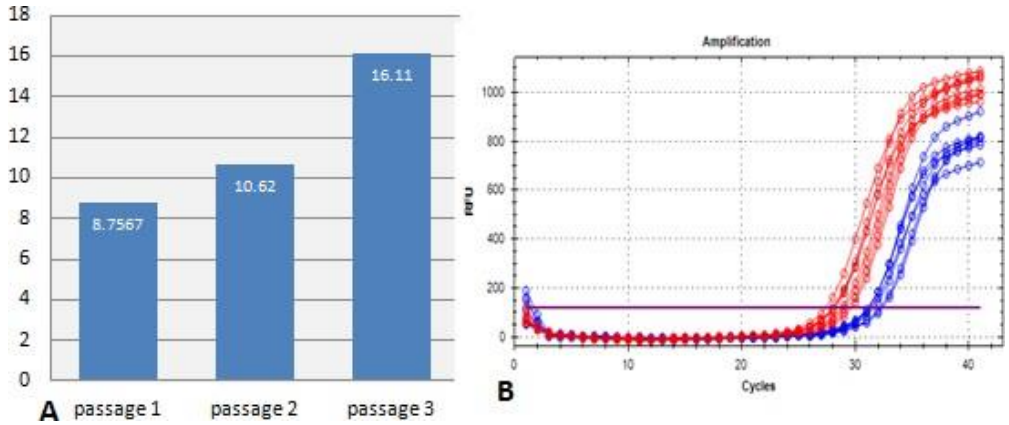

Figure (2): A- Levels of *L. donovani* promastigote exosome gene expression at day 7 culture in 3 passages. B-Real time PCR amplification plots of exosome gene in *L. donovani* promastigote (culture day 7). Where, the red qPCR plots (exosome gene), and the blue qPCR plots (Actin gene).

Culture at day 12: Samples of spleen tissue from each animals were isolated for cultured in media and 3 repeats of promastigotes taken from the culture at day 12 of culturing, tables (10), showed a difference (p value=0.031), figures (3 A and B).

Table (10): Exosome gene fold change expression of *L. donovani* promastigote at day 12 culture in 3 passages.

Passages	CT (exosome)	CT (Actin)	ΔCT	Fold change (2^ΔCT)	Mean	SD
Passage 1	29.18	30.11	0.93	1.91	2.0033	0.3204
	29.65	30.89	1.24	2.36		
	29.13	29.93	0.80	1.74		
Passage 2	29.21	31.39	2.18	4.53	3.2067	1.2524
	28.18	29.21	1.03	2.04		
	28.78	30.39	1.61	3.05		
Passage 3	27.63	30.18	2.55	5.86	5.3533	1.5054
	28.03	29.90	1.87	3.66		
	27.27	29.98	2.71	6.54		
Total					3.5211	1.7732
value = 0.031 Significance						

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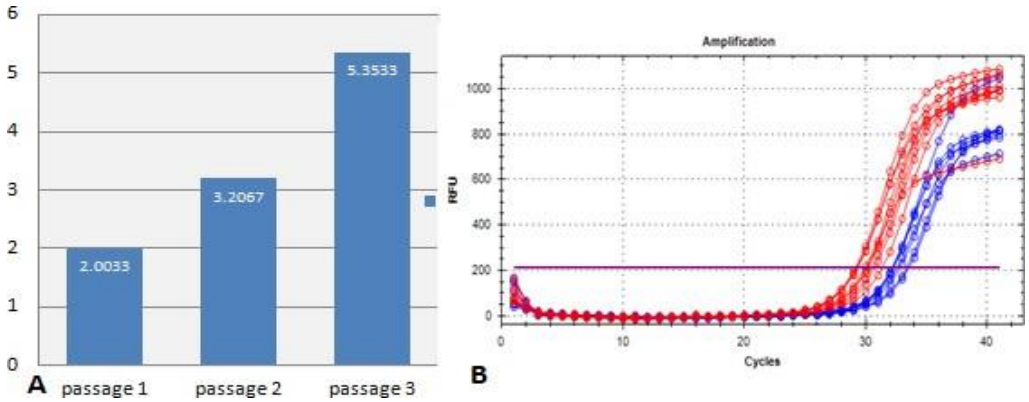

Figure (3): A-Levels of *L. donovani* promastigote exosome gene expression at day 12 culture in 3 passages. B-Real time PCR amplification plots of exosome gene in *L. donovani* promastigote (culture day 12). Where, the red qPCR plots (exosome gene), and the blue qPCR plots (Actin gene).

Control culture line: Control samples of original culture line was taken and made a serial subcultures corresponding with the culture of passage experiment and 3 repeats were taken for measuring exosome, table (11), highly difference (p value=0.000), figures (4, A and B).

Table (11): Exosome gene fold change expression of *L. donovani* promastigote at control culture line in 3 Passages.

Passages	CT (exosome)	CT (Actin)	ΔCT	Fold change (2^ΔCT)	Mean	SD
Passage 1	27.21	30.10	2.89	7.41	8.0833	1.1232
	28.85	31.75	2.90	7.46		
	28.20	31.43	3.23	9.38		
Passage 2	29.27	30.24	0.97	1.96	2.1133	0.5561
	30.13	31.58	1.45	2.73		
	31.21	31.93	0.72	1.65		
Passage 3	34.06	30.90	-3.16	0.11	0.2300	0.1039
	33.00	31.23	-1.77	0.29		
	33.74	31.97	-1.77	0.29		
Total					3.4756	3.6060
value= 0.000 Highly significance						

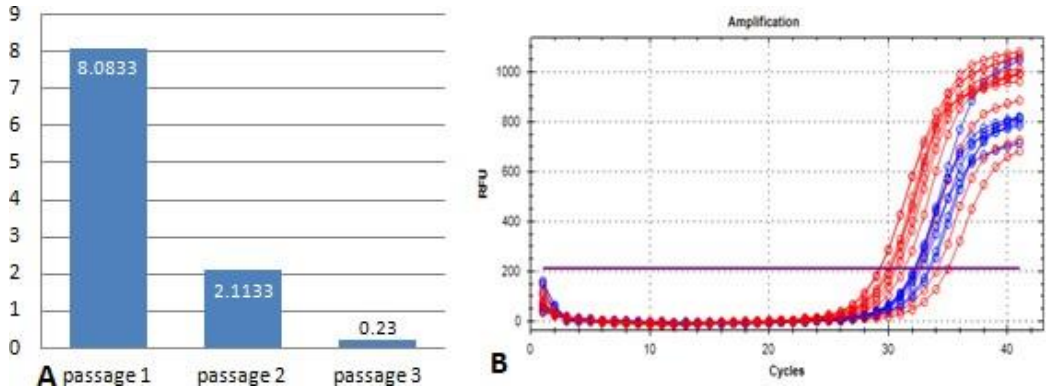

Figure (4): A-Levels of *L. donovani* promastigote exosome gene expression at culture line control in 3 passages. B-Real time PCR amplification plots of exosome gene in *L. donovani* promastigote (culture line control). Where, the red qPCR plots (exosome gene), and the blue qPCR plots (Actin gene).

4. Discussions

In the first passage of exosome gene expression in spleen of *L. donovani*, primary generation of the parasite create a positive level of virulence that has been measured by gene expression fold change, while the next passage exposed higher expression of exosome than 1st passage which indicates that the second generation of parasites were more virulent than the first one, the exosome expression level continue to increase through the 3rd passage (table, 8), as a result, it can be concluded that with additional generations, the parasite develop more virulent strains, this result agreed with the a study conducted by [7], [8], were mentioned that the infection of experiment animals is frequently required for increase the parasite virulence. [9], exposed that *Leishmania* GP63 was the only *Leishmania* protein enriched in exosomes released by macrophages upon infection. At the 7th day of culture, there was an increase in level of gene expression of exosome, that it was lowest expression in passage one, and the highest level in passage three (table, 9). The higher gene expression of exosome at day 7 culture may due to of high quantities of promastigote present in the infective form metacyclic step, this results agreed with [7], were they exposed that exosome is a vital for promastigote, associated to metacyclic promastigotes and increased its virulence and infectivity. Promastigotes expresses exosome vesicles (carrying Gp63), it's important for the survival of the parasite inside the gut milieu of sand flies [10]. [11] showed that in culture media, the parasites excrete a high infective potential, at early stationary stage through days 4-7. At the day 12th of culture, the passage one has the lowest, and the highest level was in passage three. In addition, the recent work revealed that the gene expression is higher in day 7 and tend to decreased in day 12 in all passages, (1, 2 and 3), as shown previously (table, 10). The low gene expression gene levels at day 12 of culture might be result from the effect of contamination, [12] noticed that biphasic culture media is sensitive to bacterial contamination and couldn't support the long term *in vitro* growth of *Leishmania* promastigotes. [13], suggested that mutants virulence genes can effect gene expression and that *Leishmania* parasite mutants display significantly lower virulence in the culture through sub cultures suggesting a critical role for this protein in *Leishmania* pathogenesis. At the culture control, the gene expression of exosome was noticed to be decreased dynamically among passages (in difference to culture days (7 and 12), and tissues), there was a decline in exosome gene expression level of *L. donovani* in passages in which the first passage has the highest expression (active parasite growth and proliferation, recently isolated from infected tissue), then it was declined in the second passage (less active promastigote than first passage), and the lowest level was noticed in passage three (less active than second passage and with low virulence, continuous culturing without activation in lab animal), these outcomes resemble to results shown by [7], that the virulence and infectivity of parasite decreases with continuous culturing. In a related study conducted by [14], on *L. donovani* to detect and measuring the level of the virulence factor and showed that the gene expression declines in control culture.

The suitable temperature and PH have a major role which controls the increasing or decreasing the virulence and infectivity of parasite, in this study the levels of the gene expression was decreased at control culture as a result of acidification of the growth medium at pH~5.4 [15], [20- 35]. The current study showed that, with time, there may be changes in culture media like as decrease in oxygen and nutrient supply; so, the high rate of parasitic activity is related with early days due to the availability of sufficient nutrient and then may lead to change in PH of culture media which cause changed to the oval shaped promastigote cell bodies that considered less virulent to the host macrophage. It is previously known that the *in vitro* maintenance of *Leishmania* rapidly leads to a reduce of virulence [16]. [17], made daily estimations of parasite number, virulence and viability promastigote in Schwarz differential medium (SDM), NNN and Schneider medium enabled a quick parasite increase that peaked at 7th day followed by a sharp decrease, the decrease in numbers of parasite was concomitant with significant decrease in viability and proliferative parasites. In control culture lines, the virulence of the parasite and the virulence factors gene expression will be decline with time during sub cultures this results agreed with [18], [19], they revealed that in *L. donovani*, the expression of virulence factors was decreased along with the time of the subculture. Also with long time the number of parasite and the virulence of the parasite will be decrease.

5. Conclusions

This study showed that exosome gene expression was higher in spleen tissue than in cultures, and the exosome gene expression at day 7 have higher levels than at day 12 in all passages. The gene expression was at higher levels in passage three and lowest levels in passage one (in spleen and cultures day 7 and 12). The level of exosome gene expression was declined with progress days in control line culture.

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