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The Gene Expression of Virulence Factor Zinc-Metalloprotease GP63 in Mice Infected with Visceral Leishmaniasis

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Abstract: Visceral leishmaniasis is a zoonotic protozoal disease caused by Leishmania donovani in which the pathogen disseminates to visceral organs inside the macrophage that survive within phagolysosome & evade host defense mechanism result in potential fetal infection associated with hepatosplenomegaly, lymphadenopathy and progressive anemia.The present study was planned and conducted in order to evaluate the role of virulence factor zinc-metalloprotease GP63 in spleen of mice infected with visceral leishmaniasis by both real time PCR techniques. The result detected that the highest mean gene expression, in spleen tissue, recorded in passage four which is 7.21, while the lowest mean gene expression, in spleen tissue, recorded in passage four which is 4.81. (highly significant at $P \leq 0.01$).

KEYWORD: Visceral leishmaniasis, virulence factor, zinc-metalloprotease GP63

INTRODUCTION

Visceral leishmaniasis also called kala-azar or black fever/disease, reference to hyperpigmentation of the skin by stimulation of melanocytes during infection , another local name is given Described VL, such as Dumdum fever, Assam fever, and infantile splenomegaly, is the most acute form of leishmaniasis and generally affects the bone marrow, liver, spleen, or other lymphoid tissues. [1].

The major surface protein (MSP or gp63) is metalloproteinase (MP) which belongs to the metzincin class and is abundantly expressed on the surface of *Leishmania spp.* and other related trypanosomatide protozoans [2]. Gp63 is required for the resistance of promastigotes to complement-mediated lyse in the mammalian host, as the presence of an enzymatically active form of this proteinase significantly reduced the binding of terminal complement components to parasites [3]. Effect of gp63 occurs by activation of protein tyrosine phosphatases (PTPs) in macrophages, leading to minimize production of nitric oxide (NO) and attenuated innate inflammatory reactions, thus increasing the chances of survival of the parasite. [4].

Several functions have been proposed for GP63 and are likely to vary in the different life-cycle stages of *Leishmania*; GP63 is an endopeptidase that can hydrolyze a range of peptides, and common protein substrates include casein, gelatin, albumin, hemoglobin, and fibrinogen. [5]. Early studies in the search for a GP63 function have shown that it binds and breaks down the complement component C3, suggesting a role in the elimination of complementary-mediated lyses.[6].

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Materials and Methods

Experiment design

The study has included 20 Balb/c mice, males, (6~8) weeks-old divided into 4 majer passages, each mice injected intra-peritoneal with ($3x10^6$) promastigotes of *L. donovani*, were the first passage was after 30 days post infection, the second passage was after 30 days post infection, the third passage was after 30 days post infection, finally the fourth passage was after 30 days post infection.

Molecular study:Total RNA extraction

Total RNA was extracted from frozen tissue using (TRIzol ® reagent kit) and performed as follows following company instructions:

1- The spleen sample of 200 mg was homogenized with the addition of 750 µl TRIzol ® reagent.

2- In each tube 2- 200µl of chloroform was added and shaken vigorously for 15 seconds.

3- Incubate the mixture on ice for 5 minutes. Instead it centrifuged for 15 minutes at 12000 rpm, 4 ° C.

4- Supernatant was moved to a new Eppendorf pipeline, with the addition of 500µl isopropanol. Next, mixture is mixed 4-5 times by inverting the tube and incubated for 10 minutes at 4 ° C. Then centrifuged for 10 minutes at 12,000 rpm, 4C °.

5- Supernatant was discarded and 1ml 80 per cent of ethanol was applied and again combined with vortex. Then, centrifuge for 5 minutes at 12000 rpm, 4 ° C.

6- The supernatant was discarded, and the pellet of RNA was left to dry in air.

7- 100µl Nuclease-free water was applied to each sample to dissolve the RNA pellet, then the RNA sample extracted was held at -20.

Estimation of extracted total RNA yield

The total extracted RNA was evaluated and analyzed by Nanodrop spectrophotometer (THERMO. USA), two quality controls on extracted RNA were conducted. The first is to determine the volume of RNA (ng / µL), the second is the purity of the RNA by reading the spectrophotometer absorbance at 260 nm and 280 nm in the same Nanodrop system as follows:

1- The correct application (Nucleic acid, RNA) was chosen after opening the Nanodrop device.

2- A dry wipe was applied, and the measuring pedestals were washed several times. Then carefully piped 2µl of free nuclease water and placed Nanodrop blanking on the surface of the lower measuring pedestal.

3- The pedestals are then washed, and 1µl of the total RNA sample is piped for calculation**.**

2. 2. 10. DNase I Treatment

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

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 5087 - 5098
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Table (2.8) DNase I Treatment Components

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

After that, the mixture was incubated at 37 ° C for 30 minutes. Then, for inactivation of the DNase enzyme action 1µl stop reaction was added and incubated at 65C ° for 10 minutes.

cDNA synthesis

In the step of cDNA synthesis for the Pten and GAPDH genes, DNase-I treated RNA samples were also used using the M-MLV Reverse Transcriptase kit and performed as follows:

Step 1

Table (1) RT master mix of step 1

RT 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 10 minutes, immediately afterwards cooling on ice.

Step 2

Table (2) RT master mix of step 2

RT 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 put in a vortex and spinning down briefly. In thermocycler the RNA translated into cDNA under the following thermocycler conditions:

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Table (3) Real-Time PCR step cycle condition

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

Quantitative Real-Time PCR (qPCR)

The quantitative real-time PCR used in the quantification of GP63 gene expression analysis, which was normalized by the housekeeping gene (GAPDH) using the technique of Real-Time PCR, and this method involves the following steps:

1- The preparation of qPCR master mixes

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

Table (4) q PCR Master Mix Components

qPCR master mix Reaction solution	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 reaction solution component placed in qPCR plate strip tubes and mixed for 3 minutes by Exispin vortex centrifuge, then placed in Miniopticon Real-Time PCR.

2- qPCR The conditions of thermocyclers

After that, the qPCR plate and the following thermocycler protocol were loaded into the table below:

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 5087 - 5098
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Table (5) q PCR thermocycle condition

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extention Detection(scan)	60 °C	30 sec	

Data analysis of qRT-PCR

The results of the q RT-PCR data for the target and housekeeping genes were analyzed using the relative quantification of the gene expression (fold change) (the ΔCT method Using the reference gene) as described by (Livak and Schmittgen, 2001). as following equation:

Ratio (reference/target) = $2^{CT(reference) - CT(target)}$

RESULTTS

The first passage

At this passage, 5 mice were injected intra-peritoneal with promastigotes of *L. donovani*. The animals dissected after 30 days post infection. Three repeats of spleen were isolated from each animals to measure the gene expression of virulence factor zinc-metalloprotease GP63, the results recorded as shown in table1, the mean of higher value was 4. 14 while the lowest value was 0.544.

Table 6: Virulence factor zinc-metalloprotease GP63 gene fold change expression in the spleen tissue of the first passage.

The repeats	CT (GP63)	CT (GAPDH)	ΔCT	Fold change (2^ΔCT)	Mean of each animal	Mean of total animal
R1	37.14	32.49	-4.65	0.040	4.14	**2.847**
R2	30.66	33.29	2.63	6.190		
R3	30.66	33.29	2.63	6.190		
R4	32.18	33.32	1.14	2.204	1.717	
R5	35.28	32.59	-2.69	0.155		
R6	32.26	33.74	1.48	2.793		
R7	31.66	33.49	1.83	3.552	2.332	

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 5087 - 5098
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R8	33.36	32.90	-0.46	0.725		
R9	32.28	33.72	1.44	2.721		
R10	31.26	32.63	1.37	2.585		
R11	34.35	33.68	-0.67	0.627	1.235	
R12	33.74	32.73	-1.01	0.495		
R13	33.64	33	-0.64	0.642		
R14	34.34	33.82	-0.52	0.695	0.544	
R15	34.63	32.87	-1.76	0.295		

The second passage

At this passage, also 5 mice were injected intra-peritoneal with promastigotes of *L. donovani* isolated from the culture of the first passage. Three repeats of spleen from each one were isolated after 30 days post infection to measure the gene expression of virulence factor zinc-metalloprotease GP63 and the results recorded as shown in table 2, the higher value was 7.162 while the lowest value was 0.832.

Table 7: Virulence factor zinc-metalloprotease GP63 gene fold change expression in the spleen tissue of the second passage.

The repeats	CT (GP63)	CT (GAPDH)	ΔCT	Fold change (2^ΔCT)	Mean of each animal	Mean of total animal
R1	34.39	33.43	-0.96	0.514		
R2	31.87	33.80	1.93	3.811	2.077	
R3	32.97	33.90	0.93	1.905		
R4	34.63	33.98	-0.65	0.637		
R5	32.77	33.19	0.42	1.338	0.832	
R6	35.20	34.26	-0.94	0.521		
R7	30.63	33.58	2.95	7.727		**3.735**
R8	30.80	34.03	3.23	9.383	7.162	
R9	31.07	33.20	2.13	4.377		
R10	32.09	33.88	1.79	3.458		
R11	30.52	34.17	3.65	12.553	6.912	
R12	31.37	33.61	2.24	4.724		

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R13	32.41	33.88	1.47	2.770	1.690	
R14	33.16	34.28	1.12	2.173		
R15	37.22	34.23	-2.99	0.126		

The third passage

At this passage, also 5 mice were injected intra-peritoneal with promastigotes of *L. donovani* preparation from the culture of the second passage. After dissected the animals, three repeats of spleen from each one were isolated after 30 days post infection to measure the gene expression of virulence factor zinc-metalloprotease GP63 and the results recorded as shown in table 3, the higher value was 6.909 while the lowest value was 2.895 .

Table 8: Virulence factor zinc-metalloprotease GP63 gene fold change expression in the spleen tissue of the third passage

The repeats	CT (GP63)	CT (GAPDH)	ΔCT	Fold change (2^ΔCT)	Mean of each animal	Mean of total animal
R1	30.44	33.03	2.59	6.021	6.660	**5.051**
R2	29.95	33.40	3.45	10.928		
R3	31.90	33.50	1.60	3.031		
R4	30.09	33.58	3.49	11.236	6.909	
R5	31.23	32.79	1.56	2.949		
R6	31.15	33.86	2.71	6.543		
R7	33.24	33.18	-0.06	0.959	4.739	
R8	30.20	33.63	3.43	10.778		
R9	31.49	32.80	1.31	2.479		
R10	32.22	33.48	1.26	2.395	2.895	
R11	31.79	33.77	1.98	3.945		
R12	31.98	33.21	1.23	2.346		
R13	34.23	33.48	-0.75	0.595	4.052	
R14	31.54	33.88	2.34	5.063		
R15	31.13	33.83	2.70	6.498		

The fourth passage

At this passage, also five mice were injected intra - peritoneal with promastigotes of *L. donovani* preparation from the culture of the third passage. After dissected the animals. Three repeats of spleen from each one were isolated after 30 days post infection to measure

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 5087 - 5098
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the gene expression of virulence factor zinc-metalloprotease GP63 and the results recorded as shown in table 4 the higher value was 14.912 while the lowest value was 6.181.

Table 9: Virulence factor zinc-metalloprotease GP63 gene fold change expression in the spleen tissue of the fourth passage

The repeats	CT (GP63)	CT (GAPDH)	ΔCT	Fold change (2^ΔCT)	Mean of each animal	Mean of total animal
R1	32.44	33.07	0.63	1.548	7.668	**9.119**
R2	29.95	33.87	3.92	15.137		
R3	31.90	34.56	2.66	6.320		
R4	29.09	33.90	4.81	28.051	14.912	
R5	30.23	33.17	2.94	7.674		
R6	31.15	34.32	3.17	9.011		
R7	31.24	34.07	2.83	7.103	6.181	
R8	30.20	33.48	3.28	9.682		
R9	33.49	34.30	0.81	1.758		
R10	30.22	33.21	2.99	7.945	6.911	
R11	32.79	34.26	1.47	2.762		
R12	29.98	33.31	3.33	10.026		
R13	32.23	33.58	1.35	2.549	9.924	
R14	31.54	34.40	2.86	7.239		
R15	29.13	33.45	4.32	19.985		

Table 10 : Comparison of mean virulence factor zinc-metalloprotease GP63gene fold change expression among passages in the spleen tissue

passages	***n***	**Mean fold change**	**SD**	***P***
passage 1	15	2.86	4.81	0.006 HS
passage 2	15	3.73	3.61	
passage 3	15	5.06	3.57	

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 5087 - 5098
Received 05 March 2021; Accepted 01 April 2021.

passage 4	15	9.12	7.21	

n: number of cases; SD: standard deviation; HS: highly significant at $P \leq 0.01$

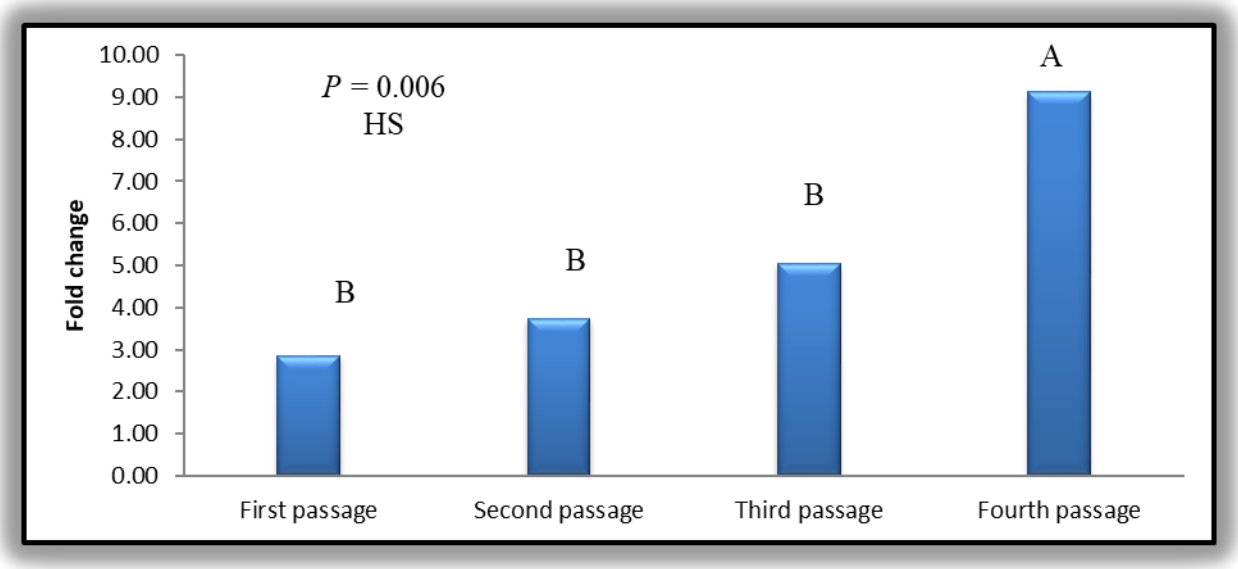

Figure 1: Bar chart showing comparison of mean virulence factor zinc-metalloprotease GP63gene fold change expression among passages.

DISCUSSION

The use of virulence factor zinc-metalloprotease GP63 gene fold change expression may provide a good tool to evaluate the virulence of *Leishmania* parasite, on the other hand, mice infected with *L. donovani* do not get sick; therefore, the sole dependence of clinical features to assess the virulence of *Leishmania* is not successful [7].

The glycoprotein (GP63) is a zinc-dependent metalloprotease that is found in high amount on the promastigotes surface, connected via a (Glycosyl-phosphatidylinositol) GPI-anchor [8]. Breakdown of the GPI anchor by phospholipase C leads to constant shedding of GP63 to the extracellular compartment. Added to that, GP63 is also directly secreted by the parasite through the pocket of the flagella. It has been observed that intracellular amount of GP63 found that can be produced upon certain triggers in the extracellular compartment [9]. The encoding genes for GP63 found as a multigene chain in the *Leishmania* genome. Various GP63 genes possess subtle variations in sequence as well as pattern expression; however the exact variation among these GP63 genes are not fully understood [10].

In the first passage, first generation of parasite leads to creation level of virulence that has been measured by expression of the virulence factor zinc-metalloprotease GP63 fold change, while in the second passage appear more expression of the virulence factor zinc-metalloprotease GP63 which means that the second generation parasites were more virulent, The elevation of virulence factor continuous increasing in the next passages. Therefore it be can concluded that with further generations, visceral *Leishmania* develop more virulence strains. This agree with the results of the study by Alcolea, and Alonso, [11] where

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mentioned that infectivity and virulence decreases with culture passages and infection of laboratory animals is frequently required for increase the virulence.

On the other hand, WHO [12] have provided the following explanation for the perpetuation, that is the continuation in nature, of these parasite populations in that it depends on the existence Two hosts: insects that feed on blood and mammals. They also suggested that the production of tissue parasites, including blood, liver, spleen, bone marrow, skin, relies on their ability to weaken the hosts through mechanisms that bare either specific to the parasites or common to other forms of parasites. It has been shown by Baneth *et al.,[*13] that the comprehensive Research into the events that occur in mammalian hosts that are linked to persistence and infectivity can provide for valuable information about parasite dissemination mechanisms and pathogenesis, and this may provide ability to discover better therapeutic approaches. It appears that the dependence on assessment of virulence by the factor zinc-metalloprotease GP63 will reduce the time and number of animals needed in comparison, with dependence on histological assessment alone.

Beside that, Aslan *et al*.,[14] have shown few in spleen tissue models that were proposed to study visceral leishmaniasis, and that dogs and hamsters are the most reliable experimental animals, because these animals shown similar clinicopathological features as visceral leishmaniasis in human. Those authors have shown that the experimental models of visceral leishmaniasis in mice The premature regulation of the liver parasite load and the delayed burden of the spleen parasite were highlighted.Therefore, histological evaluation of histological sections is essential to identify virulence of the parasite.

In line with our previous observation that new generations of *Leishmania* produced by inoculating new animals by strains taken and cultured from previous animals causes the appearance of more virulent strains, this might be related to that, due to their numerous mechanisms of immune subversion and evasion and modulation of the macrophage, *Leishmania* parasites are able to successfully infect mammalian macrophages. this suggestions correspond with as shown by previous studies Abu-Dayyeh *et al.*, [15] and Contreras *et al*., [16] where it have been described that upon infection of the macrophage, numerous signaling proteins for example Interleukin-1 receptor-associated kinase 1 (IRAK-1), Janus kinase 2 (JAK2) and mitogen-activated protein kinase (MAP Kinases), transcription factors sign transducer and activator of transcription 1 (STAT-1), Activator protein 1 (AP-1) and nuclear factor kappa beta (NF-κB), The mammalian/mechanistic target of rapamycin (mTOR) translational protein is also altered.

Previous and recent studies have shown that *Leishmania* infection affects protein kinase C PKC sensitivity to its natural substrates, for example di-acyl-glycerol, and has established GP63 as the main virulence factor responsible for the abrogate ROS production of reactive oxygen species [17].

However, a study by Isnard *et al,*. [18] has shown that *Leishmania* GP63 is capable of reaching the nucleus, degrading multiple Activator Protein 1 AP-1 subunits. In addition, it has recently been shown that GP63 reaches the perinuclear portion by gluing specific nucleo-pore proteins and further explaining its interaction with various transcription factors.

CONCLUSION

The gene expression of virulence factor zinc-metalloprotease GP63 in spleen tissue of passage four has the highest, followed by passages three, then by passages two and finally by passages one. Therefore it be can concluded that with further generations, visceral *Leishmania* develop more virulence strains.

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