DETECTION OF GENE EXPRESSION OF SERINE PALMITOYLTRANSFERASE (*SPT2*) IN MOUSE CELL LINE RAW264.7 INFECTED WITH *LEISHMANIA MEXICANA* AMASTIGOTES

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ABSTRACT

Leishmania **species are the causative agent of a tropical disease known as leishmaniasis. Previous studies on the old world species *Leishmania major,* showed that the amastigotes form which resides inside the macrophage of the vertebrate host, utilize host's sphingolipids for survival and proliferation. In this study, gene expression of serine palmitoyltransferase (SPT) subunit two (*MmLCB2*) of the mouse macrophage cell line (RAW264.7), which is the first enzyme in the *de novo* sphingolipid biosynthesis, was detected in both infected and non-infected macrophages. This was detected under condition where available sphingolipid was reduced, with the new world species *Leishmania mexicana.* Results of qPCR analysis showed that there was no difference in the expression of *MmLCB2* in infected and non-infected macrophages, under normal and serum-reduced media, suggesting that host sphingolipid did not up-regulated during infection. This can be concluded as a difference between the Old and New world *Leishmania* on the level of host-parasite interaction.**

Key words: ***Leishmania mexicana,*** **SPT, RAW264.7, myriocin.**

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التحري عن التعبير الجيني لإنزيم (*SPT2*) SERINE PALMITOYLTRANSFERASE في الخط الخلوي للفئران RAW264.7 المخمج بالأطوار عديمة السوط لطفيلي *Leishmania mexicana*

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الخلاصة

أن طفيلي اللشمانيا هو المسبب لداء اللشمانيات في المناطق الاستوائية. بينت دراسات سابقة إن الأطوار غير المسوطة للشمانيا العالم القديم *L. major* والتي تتواجد داخل المضيف الفقري، قد تستهلك من الدهون الاسفنجولية للمضيف للبقاء و الانقسام داخل خلايا المضيف. في هذه الدراسة تم تقييم التعبير الجيني للوحدة الثانية للجين المسؤول عن انزيم serine palmitoyltransferase (*MmLCB2*)، وهو الأنزيم الأول في عملية التخليق الحيوي للدهون، للخط الخلوي للبلاعم العملاقة للفئران (RAW264.7) في كل من الخلايا المخمجة و غير المخمجة بلشمانيا العالم الجديد *L. mexicana* في وسط زرعي بعد معاملته بمادة الميريوسين لتثبيط تخليق الدهون الاسفنجولية فيه. وقد أظهر تحليل النتائج بـ PCR الكمي (qPCR) إن التعبير الجيني للجين *MmLCB2* لم يختلف في كل من البلاعم العملاقة المخمجة و غير المخمجة، بوجود أو عدم وجود مادة الميريوسين مما يعني إن دهون المضيف لم يزداد إنتاجها أثناء الإصابة بالطفيلي. يمكن أن تعزى هذه النتائج الى إختلافات ما بين لشمانيا العالم القديم و لشمانيا العالم الجديد على مستوى علاقة الطفيلي بالمضيف.

INTRODUCTION

Leishmaniasis is a parasitic disease caused by infection with obligate intracellular protozoan of the genus *Leishmania*. Leishmaniasis typically presents as one of the three forms, either cutaneous, visceral or muco-cutaneous leishmaniasis and symptoms range between simple skin ulcer to severe visceral complications especially in the bone marrow, liver and spleen (1,2). About 12 million people worldwide suffer from leishmaniasis with an increase numbers in overseas travelers and/or troops (3). With absence of active vaccine, new treatments are needed to be found and many researches focus on the host-parasite relationship between the intra-cellular form of the parasite, amastigotes, and the hostile macrophages, where the parasite grow and proliferate (4). Sphingolipids are the key components of eukaryotic membranes, particularly, the plasma membrane and the lipid rafts are known to be a sub-domain in lipid-bi-layer rich in sphingomyelin, cholesterol and saturated lipids within the cell membrane (5). Serine Palmitoyltransferase (SPT) in suggested to be a key enzyme for the regulation of sphingolipids levels in cells, however, SPT is responsible for initial step of *de novo* biosynthesis in eukaryotic cells, and that sphingolipids are essential for the growth and development of animals (6). Genetic and biochemical studies have revealed that at least two different genes, *LCB1* and *LCB2*, are required for expression of SPT activity and that both the LCB1 and LCB2 proteins are subunits of the SPT enzyme (7, 8). Mammalian *LCB1* and *LCB2* encode 53- and 63-kDa proteins, respectively, and these subunits have mutual similarity (~ 20% identity) and this similarity is probably relevant to the formation of the hetero-dimer by the two subunits (7and8). To understand the role of *de* novo sphingolipid biosynthesis in *Leishmania*, previous study on the old world *L. major*, created a null mutant of SPT subunit two (*spt-2*) and this mutant cells were treated with myriocin (SPT potent inhibitor); the results showed that the latter sphingolipid products in the pathway, which is inositol phosphorylceramide (IPC) and ceramides synthesis, were inhibited and upon entry to stationary phase, the *spt-2* promastigotes failed to differentiate and died; this is to confirm that the sphingolipid synthesis is important for infectivity of the *Leishmania* (9). Another study (10) showed that during infection with *L. donovani*, the visceral species of leishmaniasis, stimulates the host macrophages to up-regulate the production of ceramide (which is the substrate of IPC synthase) indicating that the parasite may scavenge host sphingolipid for survival and proliferation inside the macrophage.

MATERIALS AND METHODS

1- Cell culture of *Leishmania mexicana* and RAW264.7 cell line.

Leishmania mexicana (MNYC/BZ/62/M379) was kindly provided by Dr Paul W. Denny (Department of Chemistry, Durham University, UK).

- Promastigotes was maintained at 26^0C in Schneider's *Drosophila* medium (Invitrogen) at pH=7.0, supplemented with 15% heated inactivated foetal bovine serum (HIFBS) purchased from (FBS, Biosera Ltd) (11).

- Amastigotes culture was prepared from promastigotes differentiation and maintained in Schneider's *Drosophila* medium supplemented with 20% HIFBS, pH=5.5 at 32^0C as described by a previous protocol (11).
- Continuous murine macrophages of RAW264.7 was maintained in dulbecco's modified eagle's medium (DMEM) (GIBCO®) with 10% HIFBS, 1% Pen/Strep, pH7 at 37^0C, 5% CO_2 (Another culture of RAW264.7 was maintained as above but with a condition where exogenous sphingolipids was reduced and 1% Nutridoma (Roche®) was added instead of FBS).

2- Macrophage Infection

Macrophage cultures were allowed to adhere in 24-well tissue culture plate (Nunc) and incubated for 24 hours in appropriate media (DMEM with10% HIFBS or 1% Nutridoma) with or without myriocin (50, 100 µM). Next day, *L. mexicana* amastigotes were applied in a ratio of 10:1 (parasite: macrophage) and the plates were then incubated for 48 hours in media of pH5.5, 32^0C,5%CO_2. Media were changed daily with myriocin where appropriate.

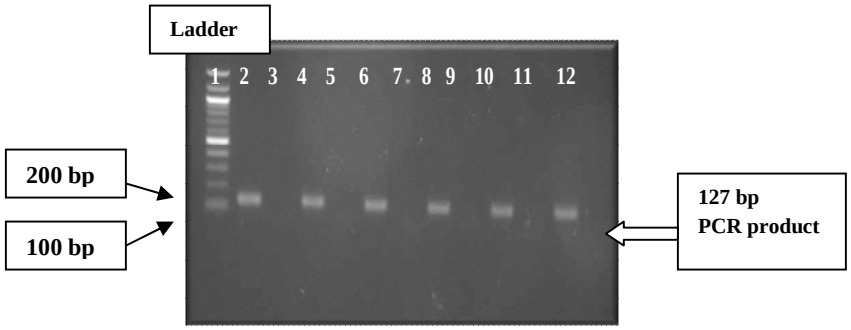
3- mRNA extraction and qPCR analysis

- Total RNA of macrophage cultures (serum-media and serum-reduced media) was extracted from culture of infected or non-infected macrophages using RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA samples were then treated with DNase (RQ1, Promega). cDNA was synthesized using ImProm-II Reverse Transcription System (Promega) according to the manufacturer's protocol.
- qPCR analysis: quantitative PCR was performed in a Rotor-Gene RG3000 (Corbett Research) using SYBR Green Jump-Start Tag Ready Mix (Sigma Aldrich) according to the manufacturer's procedure.
- *Mus musculus* SPT encoding subunit two (*MmLCB2*) was amplified using primer pair F: 5´AGGTGGATATCATGGAGAGA´3 and R: 5´GATCCAGTGTTCCTCGC ´3. Reference gene (*MmCasc3*) was amplified using primers previously chosen (12).
- qPCR was carried out in triplicate on 3 replicates with annealing temperature 52^0C for *MmLCB2* and 55^0C for *MmCasc3*.
- Gene expression analysis was analyzed using special software provided by Qiagen®, relative expression software tool (REST2009).

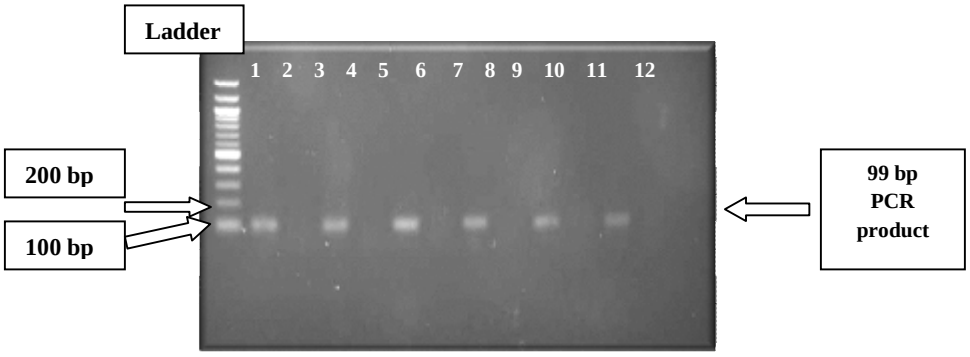
RESULTS AND DISCUSSION

Murine macrophage (RAW264.7) was used as a model for *ex vivo* infection with the new world *L. mexicana,* cultured in serum or reduced-serum DMEM, with or without myriocin, and the gene expression of *MmLCB2* was detected by qPCR. Specificity of RT–PCR products was documented with high resolution gel electrophoresis and resulted in a single product with the desired length in which cDNA has been made for all samples using ImProm-II Reverse Transcription System A3800 and the cDNA synthesized was then run on agarose gel 0.5%, each sample with its control, to verify the purity of cDNA with no contamination or primer dimer exist.

These results are shown in figures 1 and 2 displaying bands of cDNA at the exact molecular weight calculated, according to the DNA ladder of 1000bp, where *LCB2* of SPT is of 127 bp and *CASC3* is of 99 bp, no primer-dimers were generated during the applied 40 PCR amplification cycles.

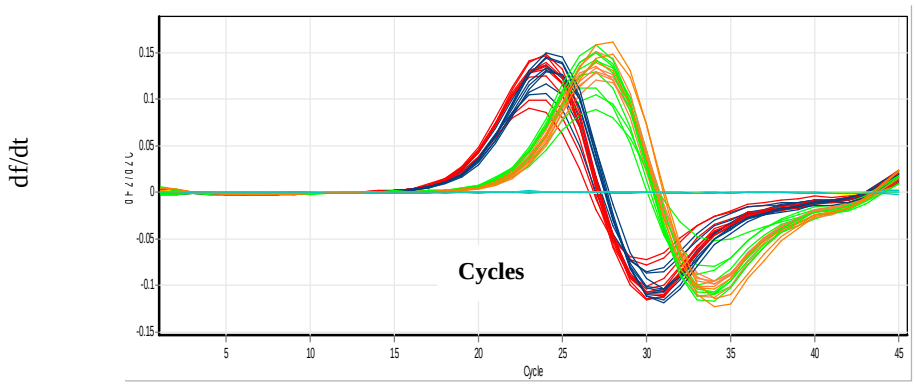


Figure(1):PCR product of 127 bp *SPT-LCB2*, cDNA synthesized of RNA extracted from six biological samples of RAW264.7 (3 of infected and 3 of non-infected), DNA ladder of 100 bp. (1, 3 and 5: triplicates of biological samples of infected macrophages, 7, 9 and 11: triplicate of biological samples of non-infected macrophages; 2, 4, 6, 8, 10 and 12: the negative control of each sample).

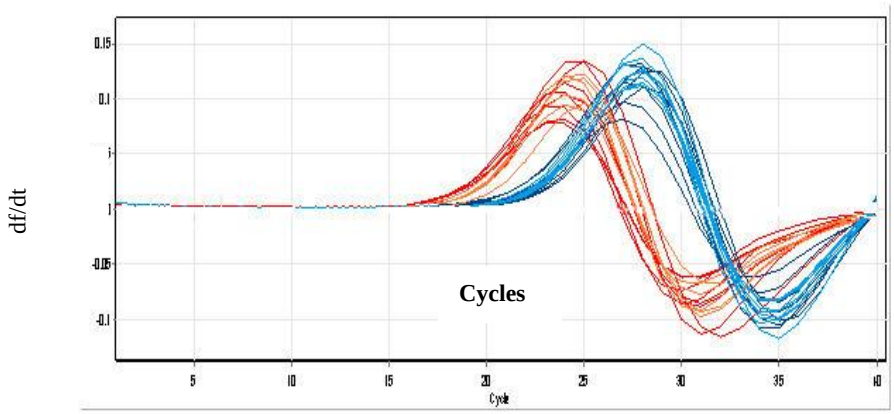


Figure(2):PCR product of 99 bp *CASC3*, cDNA synthesized of RNA extracted from six biological samples of RAW264.7 (3 of infected and 3 of non-infected), DNA ladder of 100 bp. (1, 3 and 5: triplicates of biological samples of infected macrophages, 7, 9 and 11: triplicate of biological samples of non-infected macrophages; 2, 4, 6, 8, 10 and 12: the negative control of each sample).

Rotor gene amplification for both genes at the two different conditions are shown below in figures 3 and 4, in which cycles were run correctly according to the plot of delta fluorescence/delta temperature (df/dt) with the number of cycles, as given by qPCR.



Figure(3):qPCR report for *SPT- LCB2* and *CASC3* of cDNA of RAW264.7 cultured in FBS-DMEM, (Rotor-Gene™ version 6.1.93).



Figure(4): qPCR report for *SPT- LCB2* and *CASC3* of cDNA of RAW264.7 cultured in FBS-reduced DMEM, (Rotor-Gene™ version 6.1.93).

The software used to determine the significant difference between both genes was relative expression software tool 2009 (REST 2009), QIAGEN, which is usually used to determine whether there is a significant difference between samples and controls, taking into account issues of reaction efficiency and reference gene normalization because the normalization and efficiency calculations involve ratios and multiple sources of error, it would be extremely difficult to devise a traditional statistical test, and so randomization techniques are used instead, this method is also designed to allow entry of the Rotor-Gene's comparative quantization outputs: take-off point and amplification (13).

The two most commonly used methods to analyze data from real-time, quantitative PCR experiments are absolute quantification and relative quantification; absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve.

Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control (14). Results have shown that *MmLCB2* expression has no significant difference at transcript level of infected and non-

infected macrophages, cultured in FBS-DMEM or FBS-reduced DMEM, in relative to the reference gene (*Casc3*) used in this experiment (figures 5 and6); as it can be seen in these figures as graphs outcome in REST2009 for the relative expression of SPT in infected and non-infected macrophages (test and control) in both media of DMEM FBS or FBS-reduced; in each group, the relative gene expression of SPT is not different in both treated and non-treated samples with the SPT inhibitor, myriocin.

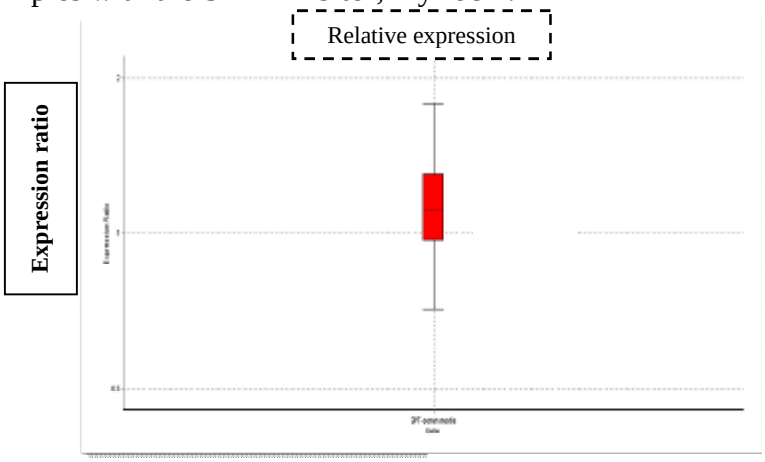


Figure (5): Relative expression of *MmLCB2* in test and control cells of RAW264.7 cultured in FBS-DMEM, whisker box as shown in REST2009.

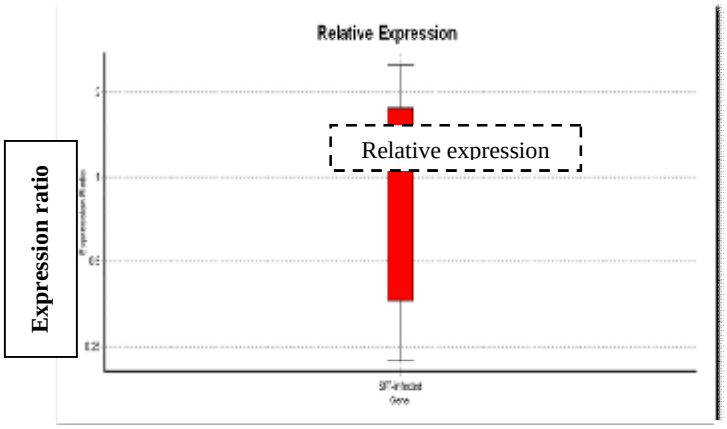


Figure (6): Relative expression of *MmLCB2* in test and control cells of RAW264.7 cells cultured in FBS- reduced DMEM, whisker box as shown in REST2009.

REST 2009 is a new software tool to estimate up and down regulation for gene expression studies. The software addresses issues surrounding the measurement of uncertainty in expression ratios by using randomization and bootstrapping techniques. Graphical output of the data via whisker-box plots provides a visual representation of variation for each gene that highlights potential issues such as a distribution skew; this

Software is a standalone tool for analysis of gene expression data from quantitative, real-time PCR experiments (15). Proteomic analysis by two-dimensional gel electrophoresis visualized by silver staining, of axenically differentiated *L. mexicana* of the same isolate we have used in this study, found about 2000 protein species in each developmental stages: procyclic promastigotes, metacyclic promastigotes and amastigotes; in which 47 spots were stage-specific while further 1000 spots changed in intensity during differentiation; the majority of the spots were expressed during the infective stages of parasite differentiation (16). However, one of the proteins that had been identified from the macrophage isolated amastigotes, was serine palmitoyltransferase like-protein, such result strongly support our findings that host SPT did not up-regulated during infection; in which *L. mexicana* amastigotes have the ability to invade and proliferate withing macrophages when host sphingolipids biosynthesis is inhibited Further knowledge of more differences between new and old world leishmaniasis are required when considering lipid biosynthesis as a target of new therapies.

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