

Cloning, Sequencing and Evaluation of the β -tubulin gene from *Leishmania donovani* as a Reference Gene for RT- qPCR

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ABSTRACT

Leishmania is a group of trypanosomatid protozoan parasites that exist in two morphological forms: a promastigote form within their insect vector and an amastigote form in the mammalian host. To study gene expression in these two distinct lifecycle forms, real-time quantitative PCR (RT-qPCR) experiments were used to determine the copy number of control gene transcripts in the two lifecycle form populations. The goal of the current study was to clone the β -tubulin gene from *Leishmania donovani* and evaluate its role as a reference control for RT-qPCR gene expression studies. We cloned the β -tubulin gene from *Leishmania donovani* using primers designed based on the known sequences of the β -tubulin gene from the *L. major* database. Sequence analysis revealed a 1329bp ORF encoding a 443aa deduced protein with high homology to previously identified β -tubulin from other *Leishmania* species. The cloned *L. donovani* -tubulin gene, *LdBtub*, served as a reference control in RT-qPCR experiments with total RNA from *L. donovani* promastigotes and amastigotes. Results showed that *LdBtub* is constitutively expressed by both parasite developmental forms at a constant level and is therefore a useful reference control for real time gene expression studies within these organisms.

INTRODUCTION

Leishmania is a genus of parasitic trypanosomatid protozoa found in tropical and subtropical climates and is vectored by an infected female *Phlebotomus* or *Lutzomyia* sandfly. *Leishmania sp.* have two distinct life cycle stages: one in the human and the other in the sandfly vector (Courret, et al. 2002). The two major parasite developmental stages are: 1) extracellular flagellated promastigote form that resides and multiplies within the alimentary tract of the sandfly vector, and 2) obligate intracellular nonflagellated amastigote forms which reside and multiply within the phago-lysosomes of infected human macrophages (Handman, 2001). *L. donovani* is transmitted to humans when an infected sandfly takes a blood meal and injects flagellated promastigotes into the host's skin. The promastigotes are engulfed by the human macrophages and transform into aflagellated amastigotes in the phagolysosome compartment. A new

sandfly takes a blood meal on the infected host ingesting macrophages containing amastigotes which transform back into promastigotes in the sandfly midgut to complete the life cycle (Courret, et al. 2002).

Infection with *Leishmania donovani* results in visceral leishmaniasis. Symptoms of visceral infection include weight loss, fever, and enlargement of the spleen and liver (Saha, et al. 2007). There are approximately 500,000 new cases of visceral leishmaniasis each year (Desjeux 2004) many of which are fatal if left untreated (Bhattarai, et al. 2010). Moreover, drugs such as pentavalent antimony compounds that are used in the treatment of visceral leishmaniasis are nephrotoxic (Shakarian et al 2002).

Expression profiling at the mRNA transcript level can be a useful way to study the gene expression of this organism. Real-time quantitative PCR (RT-qPCR) is a technique used to precisely amplify and quantify nucleic acid molecules. The

starting quantity of the amplified target DNA molecule is inversely proportional to the threshold cycle (Ct), the cycle at which the fluorescent signal of the sample has increased above background fluorescence during the exponential phase. In the current study, RT- qPCR was used to determine the copy number of specific gene transcripts in promastigote and amastigote life cycle stages. In gene expression studies between the two life cycle stages, it is important to choose a reference gene for RT-qPCR studies that is constitutively expressed between the two life cycle stages. This will allow for the normalization of experimental results that examine differences in gene expression between the amastigote and promastigote life cycle forms.

In eukaryotic organisms β -tubulin is found in the cytoskeleton, flagella and ciliary axoneme structures. The cytoskeleton has several important functions including providing a framework and organization for cytoplasmic organelles and a scaffolding for cell membrane support and cell morphology. This protein also is a major component of flagella and cilia in eukaryotic cells allowing for cellular locomotion and movement of the extracellular environment, respectively. In addition, this protein is essential for cell division, and plays a critical role in the formation of mitotic spindles and therefore, is a vital component in cell division and cell cycle. Within the cytoplasm, the cytoskeleton coordinates intracellular transport (Vale, et al. 2003) and directs the secretion of vesicles by providing a network of tracks for vesicles to traffic upon (Huang, et al. 1984 & Wade, et al. 2007).

The monomer structure of β -tubulin is compact, but can be divided into three regions: the amino-terminal nucleotide-binding region, an intermediate taxol-binding region and the carboxy-terminal region which is believed to constitute the

binding surface for motor proteins (Wade, et al. 2007 & Marchler-Bauer, et al. 2009). Microtubules are dynamic structures that can rapidly assemble and disassemble according to the cells needs. The nucleotide binding region allows for the binding of a GTP molecule to β -tubulin. GTP binding on the amino terminal of the monomeric unit of β -tubulin is thought to be important for this rapid assembly and disassembly of the microtubule (Wade, et al. 2007 & Marchler-Bauer, et al. 2009). There is no clear function of the taxol binding domain however; it is the target site for the chemotherapeutic agent taxol, which when bound to β -tubulin disrupts the microtubule structure. Thus, taxol binding interferes with mitotic spindle formation and therefore blocks cell division in targets such as cancerous cells. The carboxyl-terminal region of β -tubulin is thought to bind the molecular motors dynein and kinesin. These two molecular motors are required to cleave ATP which in turn provides the energy required for the movement of vesicles on the microtubule tracks of the cytoskeleton within the cytoplasm of a eukaryotic cell (Wade, et al. 2007 & Marchler-Bauer, et al. 2009).

Because of its importance in a variety of cellular functions, we therefore cloned, sequenced and analyzed the relative amount of β -tubulin RNA to determine if there is a difference in expression levels of this gene between *L. donovani* promastigotes and amastigotes. This analysis was used to determine if it could serve as an appropriate reference control gene in expression studies using RT-qPCR (Dheda, et al. 2005). In the current study, we show that β -tubulin is constitutively expressed in both the amastigote and promastigote life cycle stages, by definition making it an appropriate reference control (Dheda, et al. 2005) for gene expression studies in these organisms.

MATERIALS AND METHODS

Reagents

All chemicals used, unless specified, were of analytical grade and purchased from Sigma-Aldrich Chemical Co. Enzymes used for molecular studies were obtained from New England Biolabs; DNA molecular mass standards were from Invitrogen, Inc. or from Roche.

Parasites and culture conditions

Parasites used in this study were *L. donovani* strain 1S-CL₂D from Sudan, World Health Organization (WHO) designation (MHOM/SD/62/1S-CL₂D). Promastigotes used for isolation of genomic DNA (gDNA) and total RNA were grown and maintained at 26°C in medium M199 (Invitrogen) supplemented according to Debrabant, et al. 2004. Axenic amastigote developmental forms of this *L. donovani* cell line were grown and routinely maintained at 37°C in RPMI1640 medium pH 5.5 containing (20% v/v) fetal calf serum as previously described (Debrabant, et al. 2004).

Isolation of gDNA

L. donovani parasite cultures were harvested at mid-log phase, which has been established to be when the cell culture has reached a density of ~2 X 10⁷ cells ml⁻¹ (Shakarian, et al. 1997), by centrifugation at 2100 x g for 15 min at 4°C (Shakarian, et al. 1997). The resulting cell pellets were washed twice in ice-cold phosphate buffered saline (PBS, 10 mM sodium phosphate, 145 mM NaCl, pH 7.4) by centrifugation as above and resuspended in the PBS buffer for isolation of gDNA. gDNA was prepared using the GNome DNA isolation kit (BIO 101) according to the manufacturer's instructions.

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1  ATGCGTGAGA TCGTTCCTG CCAGGCCGGC CAGTGCGCCA ACCAGATCGG
51  CTCTAAGTTT TGGGAGGTCA TTGCCGACGA ACATGGTGTG GATCCGACGG
101 GTCCTTACCA GGGCGACTCG GATCTGCAGC TGGAGCGCAT CAACGTCTAC
151 TTCGATGAGT CGACGGGAGG CCGCTACGTG CCGCGCGCCG TGCTGATGGA
201 CCTCGAGCCC GGTACTAATG ACTCCGTTGG CGCCGGCCCG TACGGCCAGC
251 TGTTCGGCCC GGACAACTTC ATCTTTGGTC AGTCCGGCCG TGGCAACAAC
301 TGGGCCAAGG GCCACTACAC CGAGGGCGCG GAGCTGATCG ACTCCGTGTG
351 TGATGTGTGC CGCAAGGAGG CGGAGAGCTG CGACTGCTG CAGGGCTTCC
401 AGCTGTCTCA CTCCTCGGGC GSCGGCACGG GCTCCGGCAT GGGCACCGTG
451 CTCAITTCGA AGCTGCGCGA GGAGTACCCG GACCGGATCA TGATGACCTT
501 CTCCGTCATC CCCTCCCCCC GCGTCTCGGA TACCATTGTG GAGCCGTACA
551 ACACGACCCT CTCTGTGCAC CAGCTCGTGG AGAACTCCGA CGAGTCCATG
601 TGCAATGACA ACGAGGCGCT GTACGATAIT TGCTTCGCA CGCTGAAGCT
651 GACGACGCGC ACGTTCGGTG ACCTGAARCA CCTCGTCGCC GCTGIGATGT
701 CTGGCGTGAC CTGCTGCCGT GCTTCCCTG GCCAGCTGAA CTCTGACCTG
751 CGCAAGCTTG CCGTGAACTT CGTGCGTTC CCGCGCTGC ACTTTCAT
801 GATGGGCTTC GCGCGCTGA CGAGCCGCGG GTCCGACGAG TACCCGGCCG
851 TGTCCGTCGC GAGGCTGACG CAGCAGATGT TGCAGCCAA GAACATGATG
901 CAGCCGCGCG ACCCGCGCCA CCGCGCTAC CTCACCGCGT CCGCGCTGTT
951 CCGCGGCGCG ATGTGCAACA AGGAGGTGGA CGAGCAGATG CTGAACGTGC
1001 AGAACAGAAA CTCACGCTAC TTCAATTGAGT GGATCCGAA CAACATCAAG
1051 TCCTCCATCT GCGATATCCC GCCCARGGGT CTCAGATGT CCGTCCCTT
1101 CATCGGCAAC AACACCTGCA TCCAGGAGAT GTTCCGCGCC GTTGGTGAGC
1151 AGTTCACGGG TATGTTCCGC CGCAAGGCTT TCCTCCACTG GTACACCGGT
1201 GAGGGCATGG ACGAGATGGA GTTCAAGGAG GCCGAGTCCA ACATGAACGA
1251 CCTCGTCTCI GAGTACCAGC AGTACCAGGA CCCCACCGCT GAGGAGGAGG
1301 GCGAGTACGA GGAGGAGGAA GCCTACTAG
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Figure 1. *β-tubulin* gene sequence from *Leishmania donovani*. The 1329bp ORF encoding the *β-tubulin* is shown. The position of primers used to obtain the ORF, (BTUBULINFOR and BTUBULINREV are underlined. The two underlined and bolded sequences indicate the primers BTUBULINFOR₂ and BTUBULINREV₂ that were used as internal gene primers to amplify the a 272bp fragment. Numbers to the left of the sequence indicate the position of the nt within the gene sequence.

Oligonucleotide primers

PCR-Fwd and PCR-Rev (Figure 1) were designed to amplify the *L. donovani* homologue of *β-tubulin* ORF (LmjF33.0792) identified in the *Leishmania major* (Friedlin strain) genome database, GeneDB (Ivens, et al. 2005). These primers (BTUBULINFOR 5'–ATGCGTGAGATCGTTTCCTG–'3 and BTUBULINREV 3'–GATCATCCGAAGGAGGAGGA–'5) were synthesized by β cyanoethylphosphoramidite chemistry using an Expedite™ nucleic acid synthesis system (IDT). PCR amplifications with the *L. donovani* gDNA as template and the above primers were carried out using Sigma Ready Mix. Control reactions lacking gDNA or primers were carried out under identical reaction condition. After an initial “hot start” at 94°C for 2 min, the conditions used for amplification were: [95°C for 30 sec, 60°C for 1 min, 72°C for 1

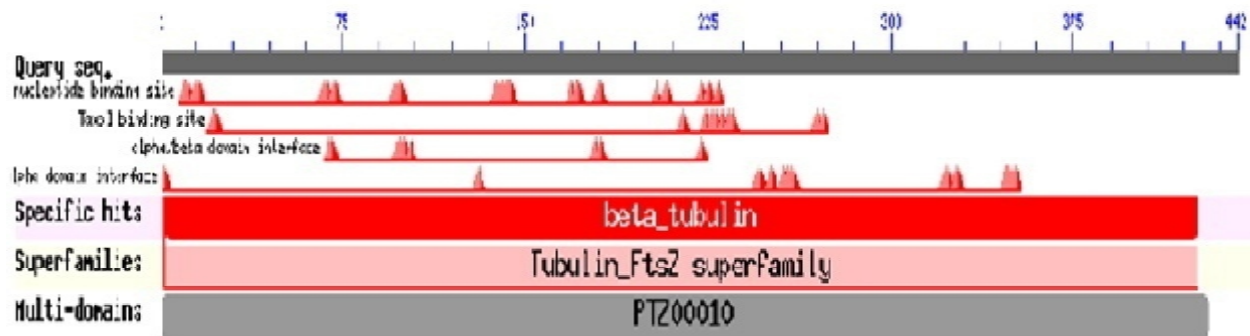


Figure 2. Conserved functional domains of β -tubulin from *L. donovani*. The deduced protein sequence of the β -tubulin from *L. donovani* was subjected to analysis with the Conserved Domain Database informatics. Several functional domains common to β -tubulin from other organisms were found including nucleotide binding sites, taxol binding sites, beta/alpha domains and alpha/beta domains. All conserved sites are indicated by triangles.

min] for 30 cycles, 72°C for 2 min, 4° hold. A 0.8% agarose gel in 1x TBE was run at 50 V for 1 hr to ensure that the amplification reactions were successful. Gels were stained with ethidium bromide and images were captured using Gel Logic 440 Imaging System (Kodak). The 1329-bp amplified-product was cloned into the *pCR®2.1-TOPO* vector (Invitrogen) and the resulting plasmid (*Ldon-PCR1329*) was subjected to nucleotide sequencing. Analyses of the sequence data obtained from the *Ldon-PCR1329* clone showed that it had high sequence identity with the *L. major* ORF (LmjF33.0792) (Ivens, et al. 2005). Based on this observation, the *L. donovani* ORF was designated as “*LdbTubulin*” to indicate its homology with this annotated β -tubulin.

Nucleotide sequencing and analyses

DNA was sequenced using the fluorescent di-deoxy chain terminator cycle sequencing method at the Genomics and Sequencing Center at the University of Rhode Island (Kingston, RI). Sequence data obtained from both strands were analyzed using the Genetic Computer Group (GCG) software package (Devereux, et al. 1984) running on an NIH Unix System and Sequencher 4.9 software (Gene Codes Corp., Ann Arbor, MI). Furthermore, such sequences were subjected to BLAST-N and BLAST-P analyses using the NCBI BLAST-link(<http://www.ncbi.nlm.nih.gov/BLAST/>

). Protein domain analysis was carried out using Conserved Domain Database (Marchler-Bauer, et al. 2009) and protein multiple sequence alignments were carried out using the ClustalW program (www.ebi.ac.uk/Tools/clustalw2/).

Nomenclature

The designations used in this report for genes, proteins and plasmids follow the nomenclature for *Trypanosoma* and *Leishmania* (Clayton, et al. 1998).

Isolation of RNA and Reverse Transcription-PCR control reactions

Total RNA was isolated from promastigote and axenic amastigote cultures of *L. donovani*, using TRIzol® according to the manufacturer’s instructions (Invitrogen). RNA samples were stored at -80 °C. Reverse transcription was carried out with RNase-free DNase I (Stratagene) treated total RNA from *L. donovani* promastigotes and axenic amastigotes using superscript II (Invitrogen, Carlsbad, CA) and oligo dT to generate cDNA according to manufacturer’s instructions. PCR amplification reactions contained the oligo primers as described above (BTUBULINFOR and BTUBULINREV), 2 μ l cDNA, dNTPs, Taq polymerase (Sigma) and the Taq 10x Reaction Buffer (Sigma) in a final volume of 50 μ l. The conditions for amplification were as described above. Control reactions lacking cDNA or primers were carried out under identical reaction

<i>L. donovani</i>	MREIVSCQAQCQGNQIGSKFWEVIADHGVDFTGSYQGDSDLQLERINVY	50
<i>L. infantum</i>	MREIVSCQAQCQGNQIGSKFWEVIADHGVDFTGSYQGDSDLQLERINVY	50
<i>L. braziliensis</i>	MREIVSCQAQCQGNQIGSKFWEVIADHGVDFTGSYQGDSDLQLERINVY	50
<i>L. tarentolae</i>	MREIVSCQAQCQGNQIGSKFWEVISEDHGVDFTGTYQGDSDLQLERINVY	50
<i>L. mexicana</i>	MREIVSCQAQCQGNQIGSKFWEVISEDHGVDFTGTYQGDSDLQLERINVY	50
<i>L. donovani</i>	FDSTGGRYVFRVAVLMDLEPGTMDSVRAGPYGQLFRPDMFIPGQSGAGNN	100
<i>L. infantum</i>	FDSTGGRYVFRVAVLMDLEPGTMDSVRAGPYGQLFRPDMFIPGQSGAGNN	100
<i>L. braziliensis</i>	FDSTGGRYVFRVAVLMDLEPGTMDSVRAGPYGQLFRPDMFIPGQSGAGNN	100
<i>L. tarentolae</i>	FDSTGGRYVFRVAVLMDLEPGTMDSVRAGPYGQLFRPDMFIPGQSGAGNN	100
<i>L. mexicana</i>	FDSTGGRYVFRVAVLMDLEPGTMDSVRAGPYGQLFRPDMFIPGQSGAGNN	100
<i>L. donovani</i>	WAKGHYTEGAELIDSVLDVCRKEAES CDC LQGPQLSHSLGGGTGSGMGT	L 150
<i>L. infantum</i>	WAKGHYTEGAELIDSVLDVCRKEAES CDC LQGPQLSHSLGGGTGSGMGT	L 150
<i>L. braziliensis</i>	WAKGHYTEGAELIDSVLDVCRKEAES CDC LQGPQLSHSLGGGTGSGMGT	L 150
<i>L. tarentolae</i>	WAKGHYTEGAELIDSVLDVCRKEAES CDC LQGPQLSHSLGGGTGSGMGT	L 150
<i>L. mexicana</i>	WAKGHYTEGAELIDSVLDVCRKEAES CDC LQGPQLSHSLGGGTGSGMGT	L 150
<i>L. donovani</i>	LI SKLRE EY PDR IMMTF SVI P SFRVS DTVVE PYNIT LSVHQLVENS DES M	200
<i>L. infantum</i>	LI SKLRE EY PDR IMMTF SVI P SFRVS DTVVE PYNIT LSVHQLVENS DES M	200
<i>L. braziliensis</i>	LI SKLRE EY PDR IMMTF SVI P SFRVS DTVVE PYNIT LSVHQLVENS DES M	200
<i>L. tarentolae</i>	LI SKLRE EY PDR IMMTF SVI P SFRVS DTVVE PYNIT LSVHQLVENS DES M	200
<i>L. mexicana</i>	LI SKLRE EY PDR IMMTF SVI P SFRVS DTVVE PYNIT LSVHQLVENS DES M	200
<i>L. donovani</i>	CI DNEAL YD ICFRT LKL TT PT PGDLNHLVA AVMS GVTCC LR FPGQLNSD L	250
<i>L. infantum</i>	CI DNEAL YD ICFRT LKL TT PT PGDLNHLVA AVMS GVTCC LR FPGQLNSD L	250
<i>L. braziliensis</i>	CI DNEAL YD ICFRT LKL TT PT PGDLNHLVA AVMS GVTCC LR FPGQLNSD L	250
<i>L. tarentolae</i>	CI DNEAL YD ICFRT LKL TT PT PGDLNHLVA AVMS GVTCC LR FPGQLNSD L	250
<i>L. mexicana</i>	CI DNEAL YD ICFRT LKL TT PT PGDLNHLVA AVMS GVTCC LR FPGQLNSD L	250
<i>L. donovani</i>	RKLA VNL VP FER LH FPM MG FA PLT SRGSQ QYRGL SV AEL TQ QMF DA KMM M	300
<i>L. infantum</i>	RKLA VNL VP FER LH FPM MG FA PLT SRGSQ QYRGL SV AEL TQ QMF DA KMM M	300
<i>L. braziliensis</i>	RKLA VNL VP FER LH FPM MG FA PLT SRGSQ QYRGL SV AEL TQ QMF DA KMM M	300
<i>L. tarentolae</i>	RKLA VNL VP FER LH FPM MG FA PLT SRGSQ QYRGL SV AEL TQ QMF DA KMM M	300
<i>L. mexicana</i>	RKLA VNL VP FER LH FPM MG FA PLT SRGSQ QYRGL SV AEL TQ QMF DA KMM M	300
<i>L. donovani</i>	QAAD PRHGR YLT AS ALF RGRMSTKEV DEQMLNVQNKNS YF IEWIPNNI K	350
<i>L. infantum</i>	QAAD PRHGR YLT AS ALF RGRMSTKEV DEQMLNVQNKNS YF IEWIPNNI K	350
<i>L. braziliensis</i>	QAAD PRHGR YLT AS ALF RGRMSTKEV DEQMLNVQNKNS YF IEWIPNNI K	350
<i>L. tarentolae</i>	QAAD PRHGR YLT AS ALF RGRMSTKEV DEQMLNVQNKNS YF IEWIPNNI K	350
<i>L. mexicana</i>	QAAD PRHGR YLT AS ALF RGRMSTKEV DEQMLNVQNKNS YF IEWIPNNI K	350
<i>L. donovani</i>	SS IC D I P EKGLKMS VTF I G N N T C I Q E M F R V G E Q P T G M F R R K A F L H W Y T G	400
<i>L. infantum</i>	SS IC D I P EKGLKMS VTF I G N N T C I Q E M F R V G E Q P T G M F R R K A F L H W Y T G	400
<i>L. braziliensis</i>	SS IC D I P EKGLKMS VTF I G N N T C I Q E M F R V G E Q P T G M F R R K A F L H W Y T G	400
<i>L. tarentolae</i>	SS IC D I P EKGLKMS VTF I G N N T C I Q E M F R V G E Q P T G M F R R K A F L H W Y T G	400
<i>L. mexicana</i>	SS IC D I P EKGLKMS VTF I G N N T C I Q E M F R V G E Q P T G M F R R K A F L H W Y T G	400
<i>L. donovani</i>	EGMDEME FTEAE SNMND LVSE YQQYQDATVEEAGEYEEEQEAY	44.2
<i>L. infantum</i>	EGMDEME FTEAE SNMND LVSE YQQYQDATVEEAGEYDEEQEAY	44.3
<i>L. braziliensis</i>	EGMDEME FTEAE SNMND LVSE YQQYQDATVEEAGEYDEEEAY	44.3
<i>L. tarentolae</i>	EGMDEME FTEAE SNMND LVSE YQQYQDATVEEAGEYDEEEPT	44.3
<i>L. mexicana</i>	EGMDEME FTEAE SNMND LVSE YQQYQDATVEEAGEYDEEQEAY	44.3

Figure 3. Clustal W Alignment of the *L. donovani* the β -tubulin. The Clustal W alignment informatics was used to compare the *L. donovani* β -tubulin aa sequence to four other *Leishmania* species. The β -tubulin deduced protein from *L. donovani* shows a 99% identity with that from *L. infantum* and *L. braziliensis* and a 98% identity with the *L. tarentole* and *L. mexicana* β -tubulin proteins.

condition. PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining.

Generation of a standard curve using qPCR

A standard curve was generated using six serial dilutions of plasmid template (*Ldon*-PCR1329, at an initial concentration of 3 ug/ul) with a known copy number ranging from 3.3×10^7 - 3.3×10^2 copies per ul. To generate a standard curve

for experimental data analysis, a reaction volume of 25 ul was prepared for each qPCR reaction using a final concentration of 1X SYBR Green Supermix (BioRad, Hercules, CA), 0.5 uM of each forward and reverse β -tubulin primer (BTUBULINFOR2, 5'-ATCATGATGACCTTCTCCGT and BTUBULINREV2, 5'-GAAGCCCATCATGAAGAAGT) and 1 ul of the serially diluted *Ldon*-PCR1329 plasmid template. This primer set (BTUBULINFOR2 and BTUBULINREV2) amplified a 272bp product within the 1329 bp *L. donovani* β -tubulin gene (see Figure 1 for position of

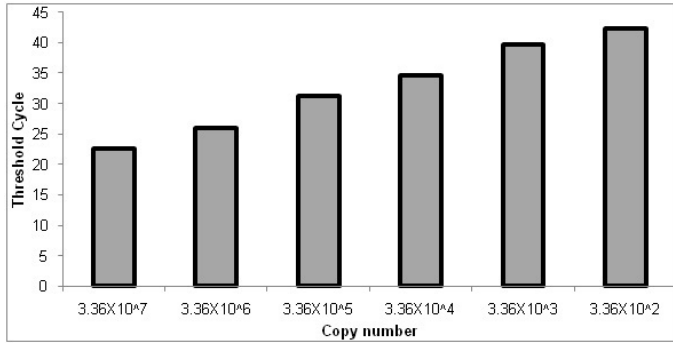


Figure 4 Standard curve generated from serial dilutions of the cloned *Leishmania donovani* β -tubulin gene. This standard curve was generated from a 10-fold dilution series of the cloned β -tubulin gene. The dilutions range from 3.36×10^7 copies/ul to 3.36×10^2 copies/ul. The points obtained show evenly spaced threshold cycle values ~ 3 - 32 cycles apart which produced a linear standard curve. This indicates that the conditions chosen for the amplification reactions were optimal. Thus the standard curve was used to extrapolate the starting quantities of the β -tubulin transcripts in test samples.

primer set within the β -tubulin gene sequence). Amplification reaction conditions were 95°C for 5 min; 95°C for 10 sec and 50°C for 30 sec (45 cycles); 95°C for 1 min and 55°C for 1min; 55°C for 10sec with an increase of 0.5°C per cycle (81 cycles).

RT- qPCR

To determine the copy number of β -tubulin mRNA transcripts in total RNA from *L. donovani* amastigotes and promastigotes RT-qPCR experiments were carried out. For these studies 25 ul reactions using a final concentration of 1X SYBR Green Supermix (BioRad, Hercules, CA), $0.5 \mu\text{M}$ of each forward and reverse primer (BTUBULINFOR2 and BTUBULINREV2), 1 ul of reverse transcriptase and 100ng of total RNA from either *L. donovani* amastigotes or promastigotes were prepared. Reactions were performed in 96-well plates in triplicate with optimized cycling parameters of 50°C for 10 min to synthesize cDNA followed by amplification reactions as described using an iQ5 Imaging Module and iCycler (BioRad).

RT-qPCR data analysis

Analyses of the qPCR amplifications for both the standard curve and the experimental RNA from *L. donovani* promastigotes and amastigotes were carried out using the iQ5 Imaging Module software that runs the iCycler real-time thermocycler (BioRad). In particular, melt curve analysis of the qPCR reactions was carried out. With melt curve analysis the presence of a single peak at a single temperature for all of the reactions demonstrates that the same single and specific product was amplified in each of the reactions. The melt curve analysis therefore validates the parameters and assay conditions used in the qPCR assays and indicates that they are specific. Similarly, r^2 values for the reactions were analyzed by the iQ5 Imaging Module software that runs the iCycler real-time thermocycler (BioRad). An r^2 value of 1.0 indicates a 100% efficient reaction. Therefore, the closer to 1.0 the r^2 value, the more robust or efficient the reaction was.

RESULTS

Analysis of *L. donovani* β -tubulin deduced protein

Sequence analysis of the cloned *L. donovani* β -tubulin gene revealed the deduced protein of the full length 1329 bp gene is 443 aa and has a predicted molecular mass of 49.7 kDa. The deduced protein contains highly conserved sites common among known members of the β -tubulin family. For example, the Conserved Domain Database (Marchler-Bauer, et al. 2009) showed several functional domains common to β -tubulin proteins from other organisms. These conserved sites include 30 nucleotide binding sites, 11 taxol bind sites, 19 beta/alpha domain interfaces and 9 alpha/beta domain interfaces (Figure 2). The C-terminus of the deduced protein contains neither a predicted GPI anchor signature sequence nor a hydrophobic trans-membrane domain, which is consistent with β -tubulin being a structural, non-membrane-bound protein in these parasites. The putative β -tubulin protein

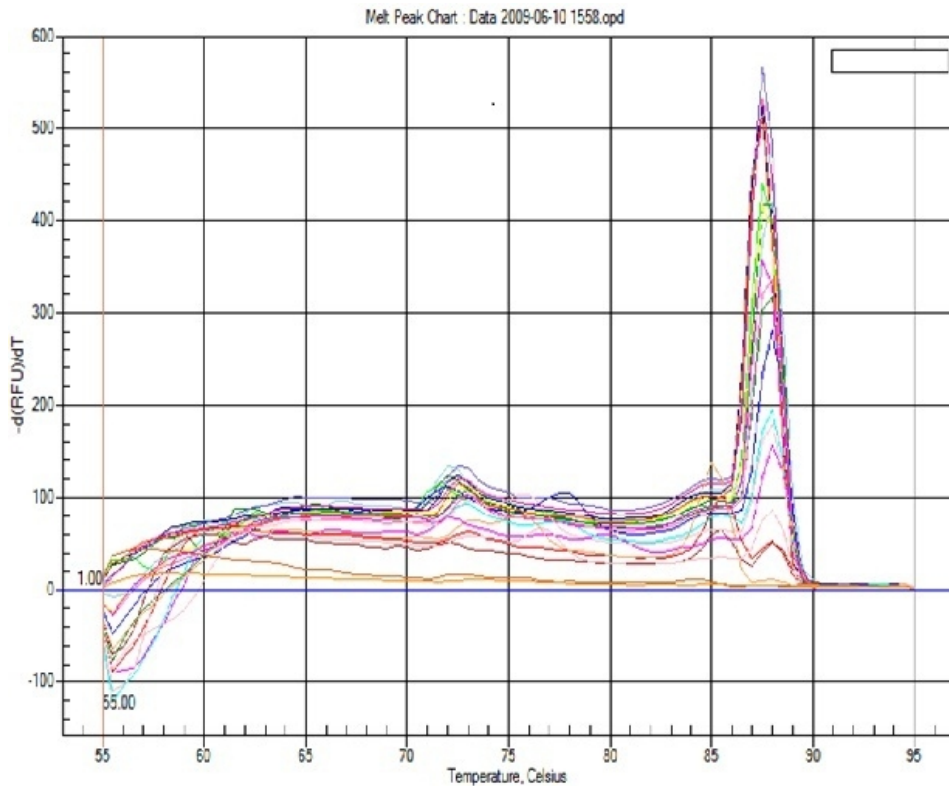


Figure 5: Melt curve analysis. Each line in the graph represents an individual reaction each with a different dilution of β -tubulin template. Melt curve analysis demonstrated that each serial dilution reaction product generated for the standard curve melted at the same temperature, 87°C. The presence of a single peak indicated that only one product was generated in these assays, the 272bp target amplicon.

from *L. donovani* shows a 99% identity with that from *L. infantum* (Jackson, et al. 2006) and *L. braziliensis* (Jackson, et al. 2006) and a 98% identity with the *L. tarentole* (Yakovich, et al. 2006) and *L. mexicana* (Fong and Lee, 1988) β -tubulin proteins. The clustal W alignment for the *L. donovani* β -tubulin deduced aa sequence with those known from other species of *Leishmania* is shown (Figure 3).

Standard curve generated with the cloned L. donovani β -tubulin gene

A standard curve using six serial dilutions of the cloned *L. donovani* β -tubulin gene was generated by RT-qPCR (Figure 4). Primers were chosen to amplify a 272 bp portion of the β -tubulin gene (see Figure 1 for sequence and position of primers). Analysis of the standard curve amplifications resulted in a graph with an r^2 value of 0.984 as generated by the iQ5 Imaging Module and iCycler software (BioRad). This high r^2 value indicates that the conditions chosen for the amplification

reactions were robust, as an r^2 value directly correlates to the efficiency of the amplification reaction. In other words, the closer to 1.0 that the r^2 value is, the more efficient and therefore optimal the reaction conditions were. Moreover, melt curve analysis of the qPCR reactions for the standard curve showed a single distinct peak at 87°C (Figure 5). The presence of a single peak at the same temperature for all of the standard curve dilution reactions demonstrated that one specific product was amplified in each of the reactions and therefore validated the parameters used for these assays. Thus, the standard curve generated under these conditions (Figure 4) was subsequently used to extrapolate the starting quantities of the β -tubulin transcripts in test samples of the total RNA isolated from amastigote and promastigote life cycle forms.

RT-qPCR with total RNA from amastigotes and promastigotes

To determine if β -tubulin could serve as a reference gene in RT-qPCR

studies, we quantified β -tubulin transcripts in equivalent amounts of total RNA isolated from *L. donovani* amastigotes and promastigotes. The total RNA of the amastigote and promastigote revealed threshold cycle (C_t) values of 30.01 and 33.58, respectively. Analysis of the assay by iQ5 Imaging Module software showed an amplification efficiency of 93.3% with an r^2 value of 0.816. The copy number of transcripts per μ l in the *L. donovani* amastigotes and promastigotes for β -tubulin was calculated to be 2113 and 2364 copies, respectively. The fold difference was calculated to be 0.056.

DISCUSSION

The β -tubulin gene was chosen as a potential reference gene for RT-qPCR studies because of its importance in cellular division, intracellular transport and secretion, regulation of cellular morphology, and motility of flagella- all processes undertaken by these parasites. RT-qPCR is a technique used in gene comparison studies to identify genes that may code for proteins that are a part of an organism's pathogenicity. The accuracy and success of RT-qPCR assays are reliant on a reference gene that is constitutively expressed throughout the organism's life cycle. *Leishmania donovani* has two distinct stages: the amastigotes and the promastigotes. Each stage has distinct morphological differences and is found in the human host and insect vector, respectively. A reference gene that is expressed constitutively by each of these parasite stages should provide a baseline for future gene expression studies that compare RNA levels of specific gene transcripts found in these distinct life cycle stages. Hence for the first time the β -tubulin gene was cloned and sequenced from the human pathogen *Leishmania donovani* [clone 1S₂D]. The deduced aa sequence of the *L. donovani* β -tubulin contained all of the conserved hallmarks of β -tubulin from other organisms such as nucleotide binding sites,

taxol binding sites, alpha/beta domains and beta/alpha domains.

If a gene such a β -tubulin is used as a reference gene for RT-qPCR studies it must be maintained at constant levels between experimental groups such as the *L. donovani* promastigote and amastigote life cycle forms. The use of conventional reference genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and -actin, is inappropriate in these studies due to their variability in expression among cell population (Dehda, 2005). Results of the RT-qPCR assays to determine the presence and copy number β -tubulin transcripts with total RNA from *L. donovani* amastigotes and promastigotes determined that the β -tubulin gene is constitutively expressed in both of these life cycle stages. Our high amplification efficiency and r^2 values from the standard curve support that our experimental optimization of β -tubulin as a promising choice for a reference gene in future reactions. Moreover, a fold difference of 0.056 β -tubulin transcripts indicates that there is a near identical level of amplification with RNA from amastigotes and promastigotes. Taken together, these results show that β -tubulin is constitutively expressed between both lifecycle stages and that it is a satisfactory reference gene for future gene expression studies.

As Leishmaniasis infections become more prevalent, additional research on the organism is needed. Treatments for this infection are toxic and expensive, and though effective, there is a chance of the disease reoccurring. Finding new, more effective drug targets is essential in successfully treating this disease. With the identification of a reference gene that is constitutively expressed in both lifecycle stages of *L. donovani*, comparative gene expression studies can be carried out using RT-qPCR to identify possible gene targets that may be essential in the pathogenicity of this organism and therefore lead to the development of new treatment options for this important human pathogen.

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