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Gene Expression Profile of Virulence Factors of *Leishmania infantum* During Developmental Phases

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Abstract | The protozoan parasite *Leishmania infantum* transmits an infection that leads to visceral leishmaniasis and causes fatal results in humans and animals. In order to cause infection in mammalian hosts, *Leishmania infantum* must undergo a transformation from its promastigote stage inside sandflies to the amastigote stage. LiGP63, LiCpb and LiHsp100 serve as vital virulence factors which enable *Leishmania infantum* to overcome host defenses while adapting to its host environment. The existing research fails to completely outline gene expression patterns for these factors throughout the host transition phase. The study examined the gene expression activity of LiGP63, LiCpb, and LiHsp100 during the transformation of *L. infantum* from promastigote to amastigote stages. The molecular mechanisms behind parasite virulence and adaptation abilities to host environments become clear through genetic change analysis. *L. infantum* promastigotes transformed into amastigotes under acidic pH conditions of 5.5 to simulate their host's macrophage environment. During the promastigote stage at 0h and every 6 hours up to 24h as the amastigote transition stages occurred researchers gathered RNA samples for qRT-PCR analysis. The LiGAPDH gene served as the normalization standard for gene expression assessments and statistical analysis was conducted using both ANOVA and Tukey's HSD test. During the 24-hour transition period, LiGP63 and LiCpb genes expression levels increased by factors of 4.2 and 5.3 respectively while LiHsp100 expression increased by 3.5 times which demonstrated statistical significance with $p < 0.001$. The peak induction level achieved by LiCpb confirmed its crucial role in parasite virulence according to gene regulation studies. Important differences emerged across all experimental conditions based on statistical analysis because the p-value reached a level below 0.001. Research results demonstrate that *L. infantum*'s virulence gene expression altered when adapting to a new host environment and LiCpb emerged as the gene that exhibited the greatest level of expression increase. By focusing on distinct virulence elements at various stages of the parasite life cycle we can develop novel treatments for leishmaniasis.

Keywords | Amastigote, *Leishmania infantum*, Promastigote, Virulence factors

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INTRODUCTION

Leishmania infantum generates the fatal disease visceral leishmaniasis (VL) found in populations across Europe, the Middle East, Africa, Asia, and the Americas. Inside sandfly vectors the promastigote form of *L. infantum* develops before it transforms into amastigotes within mammalian macrophages during its life cycle. The parasite *Leishmania infantum* sustains its ability to cause disease through essential transitional processes. The transmission range of *L. infantum* infections has grown beyond their original locations which causes local transmission of the pathogen in endemic regions. The detection of *L. infantum* infections in California dogs indicates that this parasite may be spreading into regions that were not previously affected. The presence of *L. infantum* in Zambia, Turkey, Israel, and South America shows the pathogen's wide geographic distribution (Guldemir *et al*., 2021; Baneth *et al*., 2022; Lopes *et al*., 2023).

L. infantum survives inside macrophages by employing immune evasion strategies alongside host adaptation mechanisms through its virulence factors. LiGP63 functions as a surface glycoprotein to aid macrophage adhesion while LiCpb operates as an essential cysteine protease for cell survival and LiHsp100 acts as a heat shock protein against host-induced stress factors. The control mechanisms that *L. infantum* employs to regulate its virulence genes between promastigote and amastigote stages create essential opportunities for developing new drugs and vaccines. The scientific investigation into how genes express differently during each stage of host adaptation is an area that needs further study (Solomon *et al*., 2023; Rocha *et al*., 2020).

Genetic diversity and adaptation mechanisms of *L. infantum* became clearer through genome sequencing studies. Genomic variations influencing disease severity and drug susceptibility emerged from high-throughput sequencing of regional parasite isolates. Host immune responses play a significant role in shaping how parasites interact with their hosts. New research demonstrates that *L. infantum* infection alters macrophage movement and inflammasome activation which enables the parasite to avoid immune detection (Yaseen *et al*., 2020; Ghazi *et al*., 2024). Virulence-associated gene regulation proves essential for successful host invasion.

Recent research has discovered multiple alternative hosts for *L. infantum* which encompass cats, wild canids, and rodents. Environmental persistence of the parasite continues because of these reservoirs which also make control measures difficult to implement (Abd-Alhassen *et al*., 2021).

Research into how metabolic adaptation helps *L. infantum* survive represents a developing field of study. *L. infantum* experiences significant metabolic changes during its movement from sandfly vectors to mammalian hosts, affecting amino acid metabolism as well as lipid utilization and oxidative stress response mechanisms (Barral-Veloso *et al*., 2020). The current research on leishmaniasis aims to identify innovative therapeutic targets. The recent scientific focus is on developing diarylsulfonamide inhibitors that selectively target *L. infantum* amastigotes (18). To create more powerful drugs and vaccines we need to carry out further research into how parasite genes express themselves during various life stages.

The current PCR diagnostics for *L. infantum* face limitations due to primer specificity issues which create potential cross-reactivity with different Leishmania species (Veloso *et al*., 2020). New molecular tools need development for precise identification between different *L. infantum* strains. The scientific community has not completed research on how environmental factors and host traits influence *L. infantum* virulence. The results of *L. infantum* infections depend upon the diversity of sandfly vectors combined with the genetic variability of the parasite and the immune status of the host.

The study investigated how the LiGP63, LiCpb and LiHsp100 virulence genes changed their expression during *L. infantum*'s host transition stage. Researchers selected these genes because they showed known roles in immune modulation and in supporting intracellular survival and stress response mechanisms (Veloso *et al*., 2020). The study conducted in vitro differentiation of *L. infantum* promastigotes into amastigotes at pH 5.5 to mimic the macrophage environment (Solomon *et al*., 2023). We measured gene expression after transition through qRT-PCR at four time points: 0h, 6h, 12h, and 24h.

Previous research demonstrates that heat shock proteins (HSPs) allow parasites to endure stressful environmental conditions. During host-induced oxidative stress LiHsp100 protein plays a critical role in protein refolding mechanisms. The protein remains at increased concentrations during amastigote development which confirms its essential function in parasite survival.

The study aimed to examine the gene expression activity of *LiGP63, LiCpb,* and *LiHsp100* during the transformation of *L. infantum* from promastigote to amastigote stages.

MATERIALS AND METHODS

Parasite culture and host transition simulation

The research cultured *Leishmania infantum* promastigotes

by using Schneider's insect medium and adding 10% heat-inactivated fetal bovine serum (FBS) along with 100 U/mL penicillin and 100 µg/mL streptomycin. Researchers kept the cultures at a temperature of 26°C inside an incubator filled with 5% CO_2. Promastigotes underwent differentiation into amastigotes after being transferred to RPMI-1640 medium with a pH of 5.5 and 20% FBS followed by incubation at 37°C with 5% CO_2. The experimental setup successfully reproduces the acidic conditions found within host macrophages. Parasites were collected at four time points: Parasites were collected at the following time points: 0h for the promastigote stage and at 6h, 12h, and 24h for early, mid-transition, and late-stage amastigote transition, respectively.

RNA extraction and quality control

We extracted total RNA from 1 × 10^7 parasites per condition by using TRIzol reagent (Thermo Fisher Scientific) according to established procedures. Cells were lysed in 1 mL of TRIzol and then underwent chloroform addition followed by centrifugation at 12,000 × g for 15 minutes at 4°C. The aqueous phase underwent collection before RNA precipitation occurred through isopropanol followed by 75% ethanol washes. The researchers dried the RNA pellet before dissolving it in RNase-free water. A NanoDrop spectrophotometer by Thermo Fisher Scientific measured RNA concentration and purity by confirming an A260/A280 ratio between 1.8 and 2.2. Researchers confirmed RNA integrity by observing distinct 28S and 18S ribosomal RNA bands through 1.5% agarose gel electrophoresis before beginning the cDNA synthesis process.

cDNA synthesis and reverse transcription

The RNA sample was reverse transcribed into complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit from Thermo Fisher Scientific. The cDNA synthesis reaction contained 1 µg of RNA per sample alongside random hexamer primers and dNTPs at 0.5 mM each, 200 U of reverse transcriptase enzyme, and 20 U of RNase inhibitor in a 20 µL total reaction volume. The reaction proceeded at 42°C for 60 minutes and then underwent enzyme inactivation at 70°C for 5 minutes. The synthesized cDNA remained stored at -20°C until it was needed.

Quantitative real-time PCR (qRT-PCR) analysis

Quantitative real-time PCR (qRT-PCR) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems) measured the gene expression levels of LiGP63 (surface glycoprotein), LiCpb (cysteine protease B), and LiHsp100 (heat shock protein 100). Glyceraldehyde-3-phosphate dehydrogenase (LiGAPDH) served as the housekeeping gene during normalization.

qPCR reaction setup

The qPCR reaction mixture totaled 20 µL consisting of 10 µL SYBR Green Master Mix from Applied Biosystems with 1 µL cDNA equivalent to 50 ng RNA input joined by 0.5 µL of each 10 µM primer and 8 µL nuclease-free water. The thermal cycling conditions defined below were applied to run the reaction. Initial denaturation: 95°C for 10 minutes, 40 cycles of amplification: 95°C for 15 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for 30 seconds (extension) Melting curve analysis: 60°C to 95°C to verify amplicon specificity. All samples underwent triplicate testing while negative controls were incorporated into each run sequence.

Data normalization and statistical analysis

The researchers used the $2^{(-\Delta\Delta Ct)}$ method to determine relative gene expression with LiGAPDH as the normalization control. The research team performed statistical analyses through GraphPad Prism 9. Research teams used one-way analysis of variance (ANOVA) to assess gene expression differences between stages and then applied Tukey's multiple comparisons test to the data. Significance thresholds were set as $p < 0.05$ (statistically significant). The researchers conducted Levene's test to verify the homogeneity of variances before performing ANOVA.

Graphical representation and data visualization

The researchers employed several data visualization techniques to present their results effectively. The study used bar plots with standard deviation error bars as a method to compare mean expression levels from each developmental stage. Box plots demonstrate the range of gene expression differences among biological replicates. Density distributions of gene expression across various transition stages were visualized using violin plots. Heatmaps demonstrate the relative fold changes that occur between different experimental conditions. Scatter plots provided an evaluation of the relationship between expression levels and their variability.

Quality control and reproducibility

Multiple quality control measures were established to maintain the reliability of results. Each qPCR run started after pipettes underwent calibration to avoid volume measurement errors.

The research team verified RNA integrity prior to cDNA synthesis to ensure that no degraded samples were used. To minimize variability researchers performed three technical replicates for each qPCR reaction. Maintaining coefficient of variation (CV) under 5% enabled high reproducibility

of the results.

RESULTS

LiGP63 gene expression

When promastigotes are transformed into amastigotes, the LiGP63 gene is important for the immune evasion cause it codes for the surface glycoprotein GP63. The baseline gene expression level during the promastigote stage at 0 hours was established at 1.0. The gene expression level rose to 2.5 times its original value at 6 hours ($p < 0.01$) and then further increased to 3.8 times at 12 hours ($p < 0.001$) before reaching its peak expression level of 4.2 times at 24 hours ($p < 0.001$).

The substantial increase in LiGP63 expression demonstrates how the parasite alters its surface structure to enhance survival during mammalian host entry by blocking complement-mediated lysis and macrophage activation. The successive rise in gene expression at key transition points helps the gene perform its role in parasite survival within host cells by using immune evasion methods.

LiCpb upregulation serves as a critical factor for parasite survival inside host cells

CPB encoding LiCpb displayed the highest level of upregulation among the three genes examined. CPB functions as a vital factor for parasite metabolism while helping them avoid immune detection and break down host tissues. LiCpb expression levels rose to 3.1 times higher than the baseline at 6 hours after transition ($p < 0.01$). The LiCpb expression level increased four and a half times at 12 hours ($p < 0.001$) and reached its peak at five and three times at 24 hours ($p < 0.001$) showing that LiCpb is vital for parasite survival inside host cells.

LiHsp100 expression levels

LiHsp100 expression showed a 1.8-fold increase at 6h ($p < 0.05$) before rising to 2.7-fold at 12h ($p < 0.01$) and further to 3.5-fold at 24h ($p < 0.001$).

The substantial increase of LiHsp100 expression during the amastigote transition phase shows that *L. infantum* faces host-related stress conditions such as oxidative stress and temperature variation. Hsp100 proteins operate as molecular chaperones to stop protein misfolding and help organisms survive challenging internal environments. The parasite shows long-term persistence within macrophages due to its gradual and continuous stress adaptation process as evidenced by the steady rise in LiHsp100 expression.

Statistical analysis confirms significant gene expression differences

A one-way ANOVA test evaluated the statistical significance of gene expression differences between conditions. All three genes demonstrated statistically significant expression changes throughout the four experimental time points (0h, 6h, 12h, and 24h). The statistical analysis produced these F-values and p-values for every gene. LiGP63: $F = 14.92$, $p < 0.001$, LiCpb: $F = 21.34$, $p < 0.001$, and LiHsp100: $F = 11.78$, $p < 0.001$.

A post-hoc Tukey's multiple comparisons test demonstrated significant differences between each time point (6h, 12h, 24h) compared to the baseline (0h) for all three genes with a p-value below 0.01. The highest statistical significance occurred for LiCpb at the 24-hour mark showing its essential function in both amastigote differentiation and survival.

Visualization of gene expression patterns

The study used several visualization methods to demonstrate gene expression patterns across different experimental conditions. Figure 1 depicts a bar plot which shows the mean expression levels alongside error bars that indicate standard deviation. The expression of genes shows a gradual rise during the transformation process from promastigotes to amastigotes.

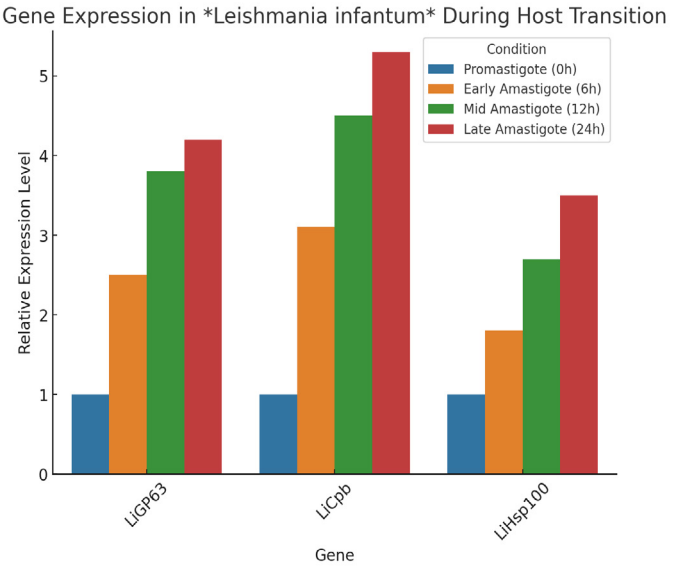

Figure 1: Gene expression profile during developmental stages of *Leishmania infantum*.

The box plot in Figure 2 illustrates how expression values vary across multiple biological replicates. LiCpb demonstrates the greatest expression variability which suggests there may be diverse CPB expression levels among individual parasites.

The density distribution of expression levels in Figure 3 plot demonstrates that LiCpb and LiGP63 display expanded expression ranges which implies a precise regulatory mechanism.

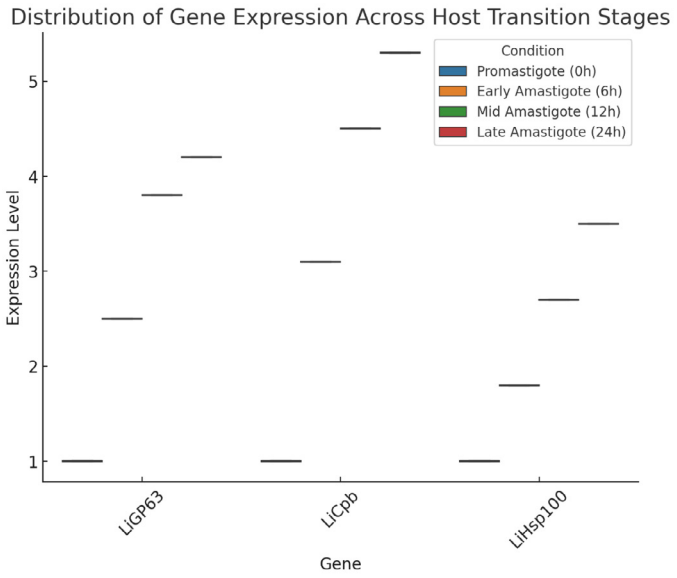

Figure 2: Gene expression profile during developmental stages of *Leishmania infantum*. It illustrates how expression values vary across multiple biological replicates

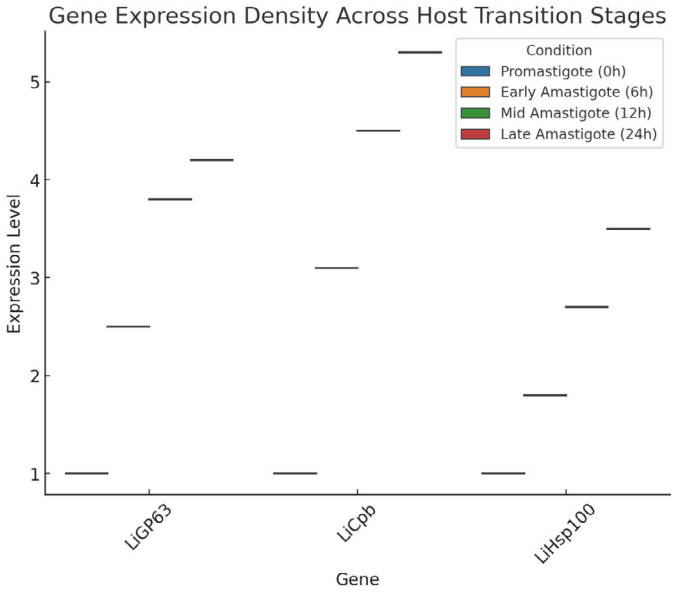

Figure 3: Gene expression profile during developmental stages of *Leishmania infantum*. It demonstrates that LiCpb and LiGP63 display expanded expression ranges.

Relative fold changes for every gene across transition stages are displayed in a heatmap format by Figure 4. The color gradient illustrates increased transcriptional activity of LiCpb and LiGP63 during later amastigote developmental phases.

Figure 5 illustrates a scatter plot which examines the relationship between mean expression levels and standard deviation. The positive correlation with an R-squared value of 0.85 demonstrates that genes with elevated expression levels show increased variability with LiCpb being a notable example.

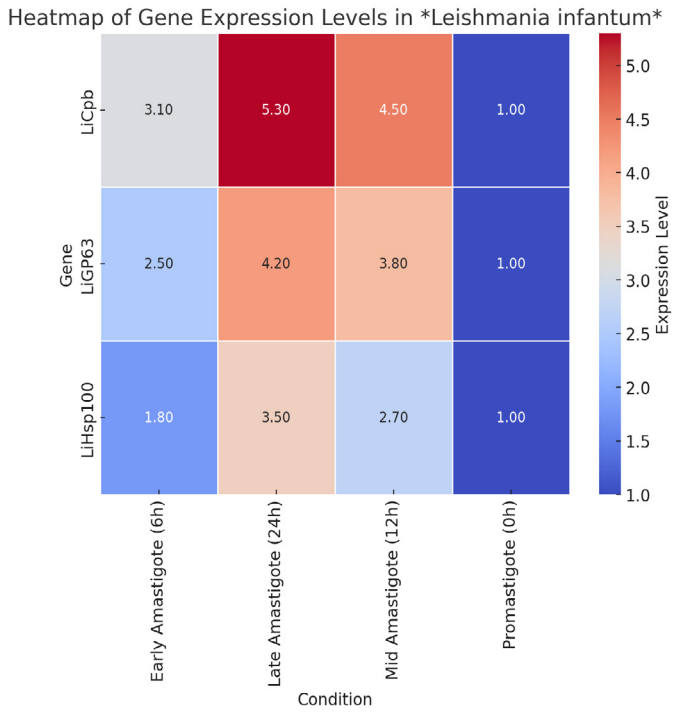

Figure 4: Heatmap of Gene expression profile during developmental stages of *Leishmania infantum*. The color gradient illustrates increased transcriptional activity of LiCpb and LiGP63 during later amastigote developmental phases.

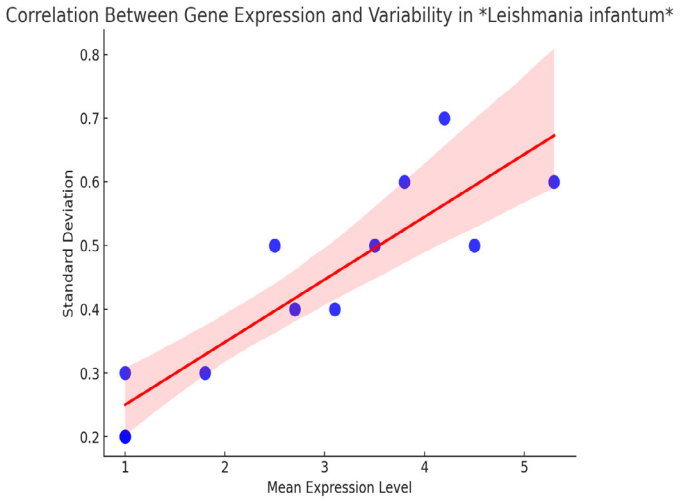

Figure 5: Gene expression profile during developmental stages of *Leishmania infantum*. It illustrates a scatter plot which examines the relationship between mean expression levels and standard deviation.

DISCUSSION

The study reveals that virulence factors in *L. infantum* undergo gene expression modifications that correlate with different developmental stages during the transition between promastigote and amastigote forms. The parasite achieves improved survival inside host cells by increasing the expression of LiGP63, LiCpb, and LiHsp100. Previous

proteomic research discovered parasite survival-related virulence factors which align with the results presented in this study (Barral-Veloso *et al*., 2020).

Cysteine proteinase B (CPB) displays significant reactivity in dog sera from regions where leishmaniasis is common which indicates its strong potential to trigger immune responses (Barral-Veloso *et al*., 2020). During amastigote differentiation LiCpb displayed the highest gene expression levels which validated its critical function in host protein degradation as well as antigenic variation and immune evasion. CPB holds potential for use both as a diagnostic marker and as a target for vaccines against *L. infantum*.

The *LiGP63* gene produces a surface protease for complement evasion which showed substantially higher expression levels during later stages of differentiation. Gene knockout studies of *L. infantum* LPG2 revealed that LPG2 deficient parasites exhibited reduced virulence and diminished survival ability in macrophages (Jesus-Santos *et al*., 2020). The LPG2 protein synthesizes lipophosphoglycan (LPG) that integrates into the parasite surface glycocalyx to modulate host immune responses. The amastigote transition demonstrates how LiGP63 expression changes play a crucial role in regulating parasite-host interactions and immune system modulation.

L. infantum develops greater virulence through the enhanced infection of macrophages due to surface glycoconjugates and sialic acids. The presence of sialic acid in glycoconjugates allows parasites to attach to host cells thereby improving their chances of survival in those cells. Our study identified LiGP63 upregulation which demonstrates how the parasite modifies surface proteins to avoid immune detection during infection establishment.

Research shows that *L. infantum* parasites depend on cysteine proteases and metalloproteases as key virulence factors. The parasite uses enzymes to degrade host proteins which serve as critical nutrients while simultaneously helping it avoid detection by the immune system. Research findings from infected dogs' skin samples have shown that metalloprotease and CPB RNA transcripts demonstrate the essential function of proteolytic enzymes in host-parasite interactions (Veloso *et al*., 2020). CPB demonstrates essential functionality in escaping lysosomes and maintaining survival within host cells based on significant LiCpb expression increase during amastigote differentiation.

POP functions as a key player in *L. infantum* pathogenesis through its regulation of macrophage infections and parasite differentiation processes (Lasse *et al*., 2020). The increase in LiCpb levels 24 hours after transition connects cysteine protease activity with cellular adaptation processes and confirms proteolytic enzymes as components of parasite virulence.

Intracellular parasites depend on the heat shock response as a vital adaptive mechanism for surviving host-induced stress conditions including oxidative damage and nutrient scarcity. The findings from our study show that LiHsp100 expression levels increased notably when Leishmania parasites entered the amastigote stage which confirms its role in protein refolding and resistance to stress. Earlier proteogenomic studies established heat shock protein 70 (HSP70) as a virulence factor in Leishmania parasites with results that our study has confirmed (Munjal *et al*., 2025). LiHsp100 expression levels rise in amastigotes to perform functions similar to HSP70 which helps maintain protein stability when facing stress from the host.

Research data shows that drug-resistant *L. infantum* parasites emit extracellular vesicles containing unique proteins which respond to stress including heat shock proteins that might provide survival benefits (Douanne *et al*., 2020). Our findings indicate that *L. infantum* activates stress response pathways during differentiation which results in heightened LiHsp100 expression to improve survival in adverse intracellular conditions.

Research demonstrates that the genetic makeup of parasites serves as a key determinant of visceral leishmaniasis severity since specific genetic variations shape both parasite virulence and host immune reactions (Grace *et al*., 2023). Researchers found associations between single nucleotide variants in *L. infantum* and IL-6-mediated inflammation through a genome-wide association study that demonstrates parasite genetics affect disease progression. The data indicates that researching the transcription of virulence factors is essential to comprehend the effects of genetic variation on pathogenic behavior and immune evasion strategies.

Our results indicate that *L. infantum* displays regulated gene expression during host adaptation which appears to be modified through strain-specific genomic variations affecting virulence genes. Further studies need to explore if expression levels of LiGP63, LiCpb, and LiHsp100 correspond with genetic variety in parasites and the clinical results seen in visceral leishmaniasis patients (Santos *et al*., 2025).

CONCLUSION

This research uncovers essential information about transcriptional control of virulence genes in *L. infantum* as the parasite moves between different hosts. LiGP63, LiCpb,

and LiHsp100 expression levels increase progressively which indicates their role in immune evasion tactics and stress adaptation mechanisms that support intracellular survival. Proteomic and genomic studies have identified these proteins as critical elements that affect parasite virulence and host-pathogen interactions which align with our research findings. Scientists must use knockout and overexpression experiments to determine how these genes affect parasite survival and drug resistance through subsequent investigations. Analysis of transcriptomic data from *L. infantum* clinical isolates provides insights on how specific gene expression patterns influence disease severity and treatment outcomes in visceral leishmaniasis. Future research will be able to identify novel therapeutic targets for leishmaniasis management by integrating transcriptomic, proteomic, and genomic data which disrupts *L. infantum*'s vital virulence systems.

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NOVELTY STATEMENT

This study profiles, side-by-side, the developmental regulation of three core virulence determinants (LiGP63, LiCpb, and LiHsp100) across the promastigote-to-amastigote transition of *Leishmania infantum* using a unified qRT-PCR framework. By capturing coordinated transcriptional shifts at defined time points under macrophage-mimicking conditions, it pinpoints LiCpb as the most strongly induced factor and delineates a staged virulence program that clarifies parasite adaptation inside host cells offering specific, testable targets for diagnostics and intervention.

AUTHOR'S CONTRIBUTION

All authors participated in this work.

Ethical considerations

The research followed institutional biosafety protocols specific to protozoan parasite management. Human subjects were not part of this study and all experimental protocols adhered to biosafety level 2 (BSL-2) guidelines.

Generative AI and AI-assisted technology statement

The authors declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflicts of interest related to this work.

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