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Original article

Stable internal reference genes for quantitative RT-PCR analyses in *Rhipicephalus microplus* during embryogenesis

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ABSTRACT

Studies on the transcriptional control of gene expression are crucial to understand changes in organism's physiological or cellular conditions. To obtain reliable data on mRNA amounts and the estimation of gene expression levels, it is crucial to normalize the target gene with one or more internal reference gene(s). However, the use of constitutive genes as reference genes is controversial, as their expression patterns are sometimes more complex than previously thought. In various arthropod vectors, including ticks, several constitutive genes have been identified by studying gene expression in different tissues and life stages. The cattle tick *Rhipicephalus microplus* is a major vector for several pathogens and is widely distributed in tropical and subtropical regions globally. Tick developmental physiology is an essential aspect of research, particularly embryogenesis, where many important developmental events occur, thus the identification of stable reference genes is essential for the interpretation of reliable gene expression data. This study aimed to identify and select *R. microplus* housekeeping genes and evaluate their stability during embryogenesis. Reference genes used as internal control in molecular assays were selected based on previous studies. These genes were screened by quantitative PCR (qPCR) and tested for gene expression stability during embryogenesis. Results demonstrated that the relative stability of reference genes varied at different time points during the embryogenesis. The GeNorm tool showed that *elongation factor 1α* (*Elf1a*) and *ribosomal protein L4* (*Rpl4*) were the most stable genes, while *H3 histone family 3A* (*Hist3A*) and *ribosomal protein S18* (*RpS18*) were the least stable. The NormFinder tool showed that *Rpl4* was the most stable gene, while the ranking of *Elf1a* was intermediate in all tested conditions. The BestKeeper tool showed that *Rpl4* and *cyclophilin A* (*CycA*) were the more and less stable genes, respectively. These data collectively demonstrate that *Rpl4, Elf1a*, and *GAPDH* are suitable internal controls for normalizing qPCR during *R. microplus* embryogenesis. These genes were consistently identified as the most stable in various analysis methods employed in this study. Thus, findings presented in this study offer valuable information for the study of gene expression during embryogenesis in *R. microplus*.

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1. Introduction

Studies on transcriptional control of gene expression play an essential role in the characterization of changes in physiological or cellular conditions of organisms. To ensure an accurate quantification of the mRNA amount and estimate the level of gene expression, it is essential to normalize the amount of the studied target gene with the amount of an internal reference gene (Huggett et al., 2005). A normalization is imperative to reduce experimental variability caused by various reasons, including the amount of mRNA, the quality of the mRNA, and the efficiency of the reaction (Busato et al., 2019).

Although numerous housekeeping or constitutive genes have been characterized, their utilization as reference genes is controversial, since the expression patterns of these genes are sometimes more complex than previously thought (Eisenberg and Levanon, 2013; Hounkpe et al., 2021; Wei and Ma, 2017; Zhu et al., 2008). In human, numerous genes with constant expression were validated as control for RT-PCR analyses, such as *actin γ 1*, ribosomal protein genes (*RpS18* and *RpS27*) and transmembrane nucleoporin C gene (*POM121*) (Caracausi et al., 2017). For arthropods, the situation is even more complex, several genes are routinely used as internal reference gene for qPCR data normalization (Lü et al., 2018), but the notion of relatively constant expression is not observed in all cases. Studies have shown that many genes considered constitutive are not stably expressed in all tissues during the development of an organism (Sagri et al., 2017; Singh et al., 2018; Uddin et al., 2011; Van Hiel et al., 2009). However, similar studies in different arthropod species, have led to the identification of various constitutive genes (Singh et al., 2018; Van Hiel et al., 2009). The expression of some genes has been studied by qPCR in different tissues, in different life stages, and different species to identify reference genes for data normalization in qPCR analyses. Since levels of expression in putative reference genes vary among the tissues in the various arthropod species studied, no single gene must be considered as a universal reference without first characterizing it (Koramutla et al., 2016; Singh et al., 2019). Various genes are commonly used as reference genes in molecular tests, including *ribosomal proteins, β-tubulin, elongation factor 1α, glyceraldehyde-3-phosphate dehydrogenase* and *β-actin* (Basu et al., 2019; Nolan et al., 2006; Pan et al., 2015; Shu et al., 2019). In ticks, information concerning constitutive genes is comparatively limited. In a few species, genes have been identified and characterized for use as endogenous constitutive control in gene expression studies, including *Ixodes ricinus* (Vechtova et al., 2020), *Ixodes scapularis* (Koči et al., 2013), *Amblyomma maculatum* (Browning et al., 2012), *Hyalomma anatolicum* (Salata et al., 2020), *Ornithodoros moubata* (Horigane et al., 2007), *Rhipicephalus microplus*, and *Rhipicephalus appendiculatus* (Nijhof et al., 2009).

The cattle tick or southern cattle tick, *R. microplus*, is widely distributed globally in the tropical and subtropical regions including Africa, the Americas, Australia, and Asia, and is the main vector for *Babesia bigemina, Babesia bovis* and *Anaplasma marginale* (Guglielmone, 1995). Moreover, it is estimated that losses in livestock production caused by tick infestation reach US$ 3.24 billion annually in Brazil (Grisi et al., 2014). It is essential to understand the physiology of parasites, and identify molecular targets (Ali et al., 2022; Bhowmick and Han, 2020; Xavier et al., 2021). Various studies have identified potential targets for tick control, including proteins involved in feeding processes (Chmelař et al., 2019; Francischetti et al., 2003; Xavier et al., 2019), blood digestion (Lu et al., 2019; Willadsen et al., 1995, 1989), reproduction (Leal et al., 2006; Seixas et al., 2018, 2008), development (Santos et al., 2013), and modulation of the host immune response (Sajiki et al., 2020; Tirloni et al., 2016).

Embryogenesis, the process by which the embryo forms and develops, is one of the most important aspects of tick developmental physiology (Santos et al., 2013). During this period, many important developmental events occur, including oogenesis, early divisions, the formation of the embryonic axis and extraembryonic membranes, gastrulation, and organogenesis. These events are divided into 14 embryonic stages/phases and are influenced by the differential expression of many genes (Santos et al., 2013). In *R. microplus,* embryogenesis is typically completed in approximately 21 days under optimal environmental conditions (Moraes et al., 2007).

The characterization of stable reference genes is essential for obtaining reliable gene expression data. Therefore, it is essential to verify the stability of reference genes in a species- and stage-specific manner when conducting RT-qPCR experiments. Since this characterization has not been previously performed during embryogenesis in this important tick species, in this study, we analyzed several putative constitutive genes, including *β-actin* (*βactin*), *β-tubulin* (*βtub*), *cyclophilin A* (*CycA*), *elongation factor 1α* (*Elf1α*), *H3 histone family 3A* (*Hist3A*), *ribosomal protein l4* (*Rpl4*), *ribosomal protein S18* (*RpS18*), and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) as potential reference genes for *R. microplus* ticks.

2. Materials and methods

2.1. Ticks and tick eggs

The eggs of *R. microplus* ticks were collected from fully engorged females (Porto Alegre strain) that had fed on Hereford cattle (*Bos taurus*) and were maintained in the laboratory as described by Reck et al. (2009). To maintain ticks during oviposition, spontaneously detached engorged females ($n = 100$) were placed in a metal pan lined with double sided tape on the bottom surface to restrict ticks and then placed in a humidity chamber with conditions set at 80% relative humidity and 28 °C. Eggs were collected and appropriately dated every 24 h and placed in an environmental chamber (28 °C and 85% relative humidity). To determine the different developmental stages during embryogenesis, tick eggs were weighed in four 50 mg clutches for 1-, 3-, 5-, 7-, 9-, 12-, 15-, and 18-days post incubation and placed into a 1.5 mL tube. The eggs were then washed in DEPC-treated water, homogenized in 1 mL of TRIZOL reagent (Life Technologies, Carlsbad, CA, USA), and stored in -80 °C until total RNA extraction. The research was conducted in accordance with ethical and methodological guidance, in agreement with the International and National Directives and Norms and under approval by the Animal Experimentation Ethics Committee of Universidade Federal do Rio Grande do Sul (UFRGS) (project 27559).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using the TRIZOL reagent according to the manufacturer's instructions. Briefly, 200 μL of chloroform was added to the 1 mL of TRIZOL containing homogenized tick eggs, vortexed and incubated for 3 min at room temperature (RT). The RNA containing aqueous phase was obtained by centrifugation at 12,000 x g for 15 min at 4 °C and transferred to a new 1.5 mL tube. RNA was precipitated by mixing an equal volume of isopropanol and incubated at RT for 10 min. The total RNA pellet was obtained by centrifugation at 12,000 x g for 15 min at 4 °C and washed with 80% ethanol prepared with DEPC-treated water. Total RNA pellets were air dried and dissolved in DEPC-treated water. To remove excess DNA contaminants, total RNA was treated with DNase I (Life Technologies) and extracted again as described above to obtain DNA-free total RNA for quantification using a PicoDrop (PicoDrop Ltd., Hinxton, Cambridgeshire, UK). Total RNA (2 μg) was subsequently used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) following the manufacturer's instructions. The synthesized cDNA samples were stored in −20 °C until use.

2.3. Identification of reference genes

Candidate reference genes were selected based on previously published reference genes from *A. maculatum* (Browning et al., 2012),

I. scapularis (Koči et al., 2013), *R. microplus* and *R. appendiculatus* (Nijhof et al., 2009). Eight candidate genes were retrieved from NCBI GenBank Nucleotide database and GenBank BioProject ID PRJNA232001 consisting of *β-tubulin* (*βtub*) [accession number CK179480], *Cyclophilin A* (*CycA*) [accession number CV445080.1], *Elongation factor 1α* (*Elf1a*) [accession number EW679365], *β-actin* (*βactin*) [accession number AY255624.1], *H3 Histone family 3A* (*Hist3A*) [accession number CV442167.1], *Ribosomal protein l4* (*Rpl4*) [accession number CV447629.1], *Ribosomal protein S18* (*RpS18*) [accession number XM_037425301.1] and *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) [accession number CK180824].

2.4. Quantitative (q) RT-PCR expression analysis

Primers for RT-qPCR were designed using primer3 plus (Untergasser et al., 2012) and were tested using 18 day post-oviposited *R. microplus* eggs. A quantitative real time PCR approach (RT-qPCR) with SYBR® Green (Life Technologies) detection was used to analyze the transcription profile and stability of eight commonly used reference genes listed above. In four biological replicates, 20 µL reaction volumes in technical triplicate consisted of ten-fold diluted cDNA that was originally synthesized from 2 µg total RNA, 300 nM each of forward and reverse primers, and 2X SYBR® Green Real Time PCR Master Mix (Life Technologies). The RT-qPCR cycling conditions were as following: stage one at 50 °C for 2 min, stage two at 95 °C for 10 min, and stage three containing two steps with 40 cycles of 95 °C for 15 s and 60 °C for 1 min using the StepOnePlus™ Real-Time PCR system (Life Technologies). Data were collected at every cycle of the second step in stage three. In addition, RT-qPCR assay was included to show similar transcription patterns. cDNA from tick eggs (in the eight time points listed above) was used as a template in the reaction, and the transcription pattern was evaluated targeting three *R. microplus* genes: *R. microplus secreted protein 20* [accession number GBBR01000092] (homologous to *AV422* from *Amblyomma americanum)* (Mulenga et al., 2007, 2013; Tirloni et al., 2014a), *R. microplus glycine-rich protein* [accession number KY271084] (homologous to *PA107* from *A. americanum*) (Leal et al., 2006; Mulenga et al., 2007) and *R. microplus serpin – 15 (RmS15* (accession number KC990114) (Tirloni et al., 2014b). The results were normalized with the inclusion of *Elf1a* and *Rpl4* as reference genes in the analysis.

To determine the purity and specificity of PCR amplicons, a dissociation curve analysis was performed subsequently after amplification from 60 to 95 °C to ensure the absence of primer-dimer formation and amplicon specificity. Additionally, samples were resolved on 2% agarose gels to confirm primer specificity. The Ct values and PCR efficiency of each reaction were determined using the LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009 The samples used to calculate PCR efficiency were derived from cDNA synthesized at various time points during *R. microplus* embryogenesis. These eggs were collected, and we conducted a 10-fold dilution series with a minimum of five data points. Controls without templates were included to ensure primer quality.

2.5. Determination of reference gene expression stability

The expression stability of the eight selected candidate genes was evaluated using GeNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004) for all experimental samples. Raw Ct (threshold cycle) sample values were used for Bestkeeper analyses. For GeNorm and NormFinder analyses, Ct values were transformed to linear scale expression quantities using the formula ΔΔCt, where the sample with highest Ct value (minimum expression) between the eight time points during embryogenesis was used as calibrator with a value equal to "1″.

Using the highest Ct values as the calibrator, ΔCt values were obtained using the formula: Ct_{sample} - $Ct_{calibrator}$, where Ct_{sample} is the Ct value of each sample obtained and $Ct_{calibrator}$ is the lowest value from the average Ct. Average ΔCt values were determined for each biological replicate for each time point during embryogenesis. The ΔΔCt values were calculated using the formula: $\Delta Ct_{sample} - \Delta Ct_{calibrator}$, where ΔCt_{sample} is the ΔCt value of each sample obtained and $\Delta Ct_{calibrator}$ is the lowest value from the average ΔCt. To calculate the fold determination, the formula $2^{-\Delta\Delta Ct}$ was used as described in Livak and Schmittgen (2001).

3. Results

3.1. Identification and selection of housekeeping genes

Data mining of NCBI GenBank nucleotide database and *R. microplus* transcriptome GenBank BioProject ID PRJNA232001 using the BLASTX resulted in the identification of eight putative reference genes*: βactin, Elf1a, Hist3A, Rpl4, GAPDH, CycA, βtub* and *RpS18*. Screening by PCR using specific primers (Table 1) yielded a single specific amplicon of the expected size for each analyzed gene (Fig. 1).

3.2. Evaluation of candidate reference genes stability

The stability of eight commonly used reference genes in *R. microplus* eggs at eight time points during embryogenesis (days 1, 3, 5, 7, 9, 12, 15, and 18) was determined by RT-qPCR. Primer specificity was tested by melting curve analysis and visualized by electrophoresis on a 2% agarose gel (data not shown). To confirm that secondary structures such as primer dimers, self-complement and hairpins were avoided in primer sets, a dissociation curve analysis was used from temperatures of 60–95 °C. Only one peak was observed for all primer sets in the dissociation analysis, which confirmed that the primers were gene-specific (data not shown). The average PCR efficiency for all primer pairs was >90% (Table 1).

3.3. Stability of candidate reference genes during different embryogenesis time points

Raw Ct values for each candidate reference gene for all replicates were compiled for the eight time points during embryogenesis (Fig. 2 and Supplementary Table 1). The highest Ct values (ranging from 20 to 24, approximately), indicating a lower transcriptional level, were obtained for *GAPDH* in all embryogenesis time points evaluated in this work. In contrast, *RpS18* presented highest transcription level (Ct values from 14 to 20, approximately) from the fifth day of embryogenesis. Interestingly, from t day 1 until day 3 of embryogenesis, the highest transcription was observed only for *Hist3A* (Ct values from 16 to 19, approximately).

To evaluate the reference genes to be used in transcription analyzes at different times during embryogenesis, a timed experiment was performed. Using RT-qPCR, the expression profiles of all putative constitutive genes were analyzed at different times (Fig. 3). Eight candidate reference genes used in this study showed different relative stabilities during the time points evaluated. In the NormFinder analysis (Fig. 3A), from day 1 to 5, it is possible to observe that *Rpl4* exhibited the best stability level, while *RpS18* had the lowest relative values. The same pattern was not observed in results from day 7 to 9, as *CycA* and *βtub* presented the best and lowest relative stability, respectively, and from 12 to 18 days, *Elf1a* was the most stable gene and *βactin* with the smallest relative stability. In contrast, GeNorm tool (Fig. 3B) identified *Rpl4* and *Elf1a* as adequate candidates for reference genes at different embryogenesis points (from 1 to 5 and from 7 to 9 days), while *Hist3A* and *RpS18* switched between the least stable genes in all conditions tested. These results were also observed at the last time point, with *Rpl4* remaining in the first position along with *GAPDH*. According to the GeNorm results, the pairwise analysis (Fig. 3C) suggests that the use of more than one reference gene, at least two, are needed to normalize the RT-qPCR results.

Table 1
Primers used in candidate reference *Rhipicephalus microplus* genes during embryogenesis.

Symbol	Gene name	Function	Accession number	Forward primer	Reverse primer	Amplicon length (bp)	Primer efficiency (%)
Btub	β-tubulin	Component of microtubules	CK179480	TCAAGCGTATCTCCGAGC	GCCTCTGTGAACTCCATTTC	100	90
CycA	Cyclophilin A	Facilitate protein folding	CV445080	ATGCTGGCCCCAACACTAAT	CATGCCTTCAACAACCGAGC	104	95
Elf1a	Elongation factor 1α	Component of the eukaryotic translational apparatus	EW679365	AGCACGCTCTACTGGCCTAC	TTCTGGATTTCCTCGAAACG	112	90
Bactin	β-actin	Cytoskeletal structural protein	AY255624	CCCATCTACGAAGGTTACGCC	CGCACGATTTCACGCTCAG	140	93
Hist3A	H3 Histone family 3A	Involved in structure of chromatin	CV442167	AAGCAGACCGCCCGTAAGT	GTAACGACGGATCTCCCTGAG	153	90
Rpl4	Ribosomal protein L4	Structural component of the large 60S ribosomal subunit	CV447629	AGGTTCCCCTGGTGGTGAG	GTTCCTCATCTTTCCCTTGCC	149	93
RpS18	Ribosomal protein S18	Structural component of the 40S ribosomal subunit	XM037425301	TCTCTCGTGATTCCTGACAAGT	CTTGATGGCGGTCAGGGCGAA	99	90
GAPDH	Glyceraldehyde- 3-phosphate dehydrogenase	Oxireductase in glycolysis and gluconeogenesis	CK180824	AGTCCACCGGC GTCTTCCTCA	GTGTGGTTCACA CCCATCACAA	124	91

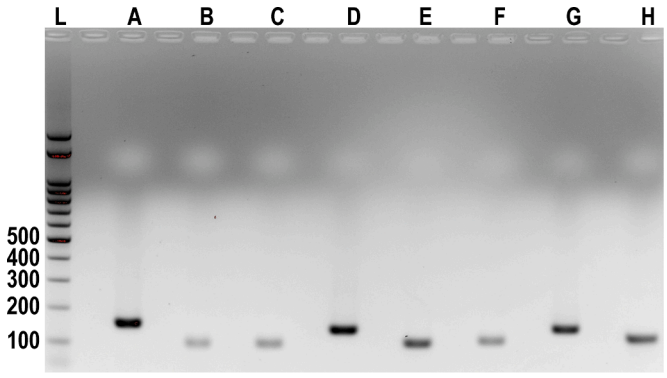


Fig. 1. Validation of amplicons from eight candidate reference genes from *Rhipicephalus microplus* by RT-qPCR. Eight candidate reference gene targets were amplified by qPCR and resolved on a 2% agarose gel. The letters above represent the reference gene target as the following *L* = 100 bp ladder, *A* = *H3 Histone family 3A* (*Hist3A*) (152 bp), *B* = *Ribosomal protein S18* (*RpS18*) (99 bp), C = *β-tubulin* (*βtub*) (100 bp), *D* = *β-actin* (*βactin*) (139 bp), E = *Cyclophilin A* (*CycA*) (104 bp), *F* = *Elongation factor 1α* (*Elf1a*) (112 bp), *G* = *Ribosomal protein l4* (*Rpl4*) (152 bp), and *H* = *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (123 bp).

3.4. Comparative evaluation of candidate reference genes

The results from the GeNorm analysis indicated that *Elf1a* and *Rpl4* had the most stable transcription throughout all analyzed points, while *Hist3A* and *RpS18* presented the lowest stability, respectively (Fig. 4A). Similar findings were obtained using the NormFinder tool, which identified *Rpl4* as the most stable gene, although *Elf1a* showed intermediate stability in all tested conditions, and *Hist3A* followed by *RpS18* had the lowest stability levels (Fig. 4B). According to BestKeeper, *Rpl4* and *CycA* were the most and least stable genes, respectively (Fig. 4C). The pairwise variation analysis (Fig. 4D) revealed that the use of two or four reference genes in the same assay yields reliable data, whereas the inclusion of five or more constitutive genes does not improve data reliability.

3.5. Similar transcription pattern analysis

A comparative analysis of RT-qPCR using *Elf1a* and *Rpl4* as internal reference controls showed that *R. microplus secreted protein 20* had a similar transcription pattern throughout embryogenesis (Fig. 5A and B). Although no differences were observed in the early stages, some variations in relative transcriptional levels of *R. microplus glycine-rich protein* (Fig. 5C and D) and *RmS15* (Fig. 5E and F) were noted at the later stages. When *Rpl4* was used as the normalizer, a decrease in relative expression of *R. microplus glycine-rich protein* was observed on day 18 compared to *Elf1a* (Fig. 5C and D). Interestingly, when putative *Elf1a* was used, fewer transcripts were present on day 15 of the *RmS15* target assay, but this was not observed in the *Rpl4* assay (Fig. 5E and F).

4. Discussion/conclusion

Specific genes, called housekeeping genes, are needed to maintain basal physiological processes. Therefore, it is expected that these genes have stable and constitutive transcription in different organs and conditions (Eisenberg and Levanon, 2013; Koonin, 2002). However, the selection of genes that are constantly expressed in all tissues still remains a challenge (Kozera and Rapacz, 2013). An appropriate endogenous reference gene must have a constant transcription level, even in different physiological conditions, since a gene with variable expression will interfere in the reliability of the results, and data analysis will be based on the presence of validated reference genes (Fu et al., 2013). Microarray and RT-qPCR are methodologies that allow the joint analysis of the transcription of several genes, but the normalization of the results is necessary (Heid et al., 1996; Schena et al., 1995; Vandesompele et al., 2002). These standardizations adjust the alterations induced by differences in sample quantification, reaction efficiency or other technical factors. qPCR is extensively used in different biology fields; however, it is difficult to identify only one or few to normalize the data in all tissues or species (Kozera and Rapacz, 2013). Several genes originally characterized as housekeeping showed alterations in expression levels (Radonić et al., 2004). Therefore, it is essential to validate the reference genes before using them as constitutive genes in a newly studied species.

Several housekeeping genes are commonly used as reference genes in different species (Ruiz-Villalba et al., 2017; Sagri et al., 2017). As a comparative result, *GAPDH* and *βactin* were suitable candidates in midgut of *Rhodnius prolixus* after *Trypanosoma cruzi* infection, however the same results were not observed in this tissue after *Trypanosoma*

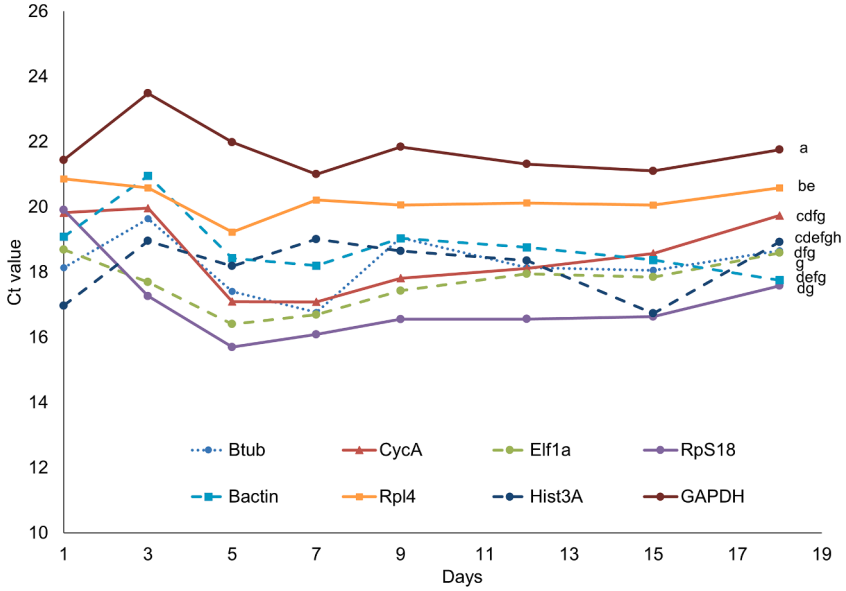


Fig. 2. Ct values of candidate reference genes from *Rhipicephalus microplus* during embryogenesis. RT-qPCR using cDNA from eggs (from 1 to 18 days) was used as target. The transcription of *β-actin* (*βactin*), *β-tubulin* (*βtub*), *cyclophilin A* (*CycA*), *elongation factor 1α* (*Elf1a*), *H3 histone family 3A* (*Hist3A*), *ribosomal protein l4* (*Rpl4*), *ribosomal protein S18* (*RpS18*) and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were evaluated.

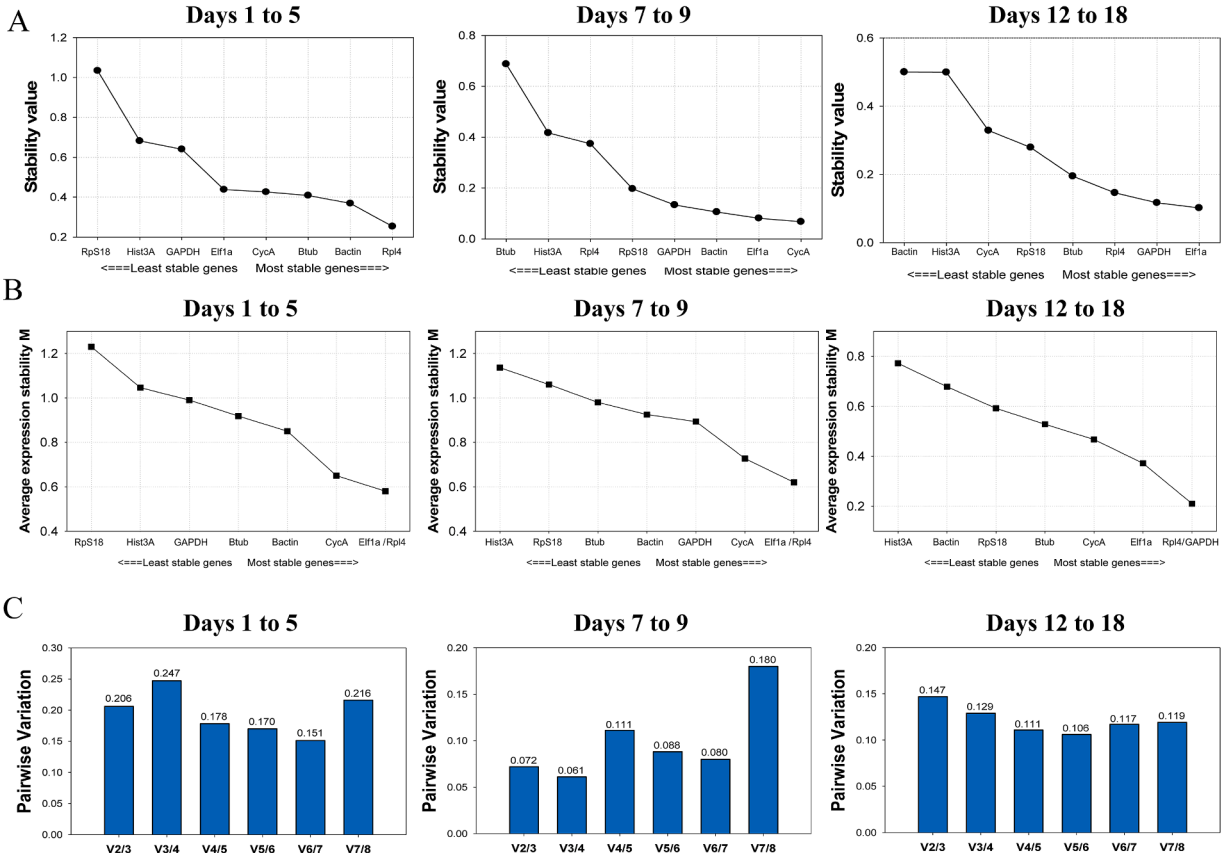
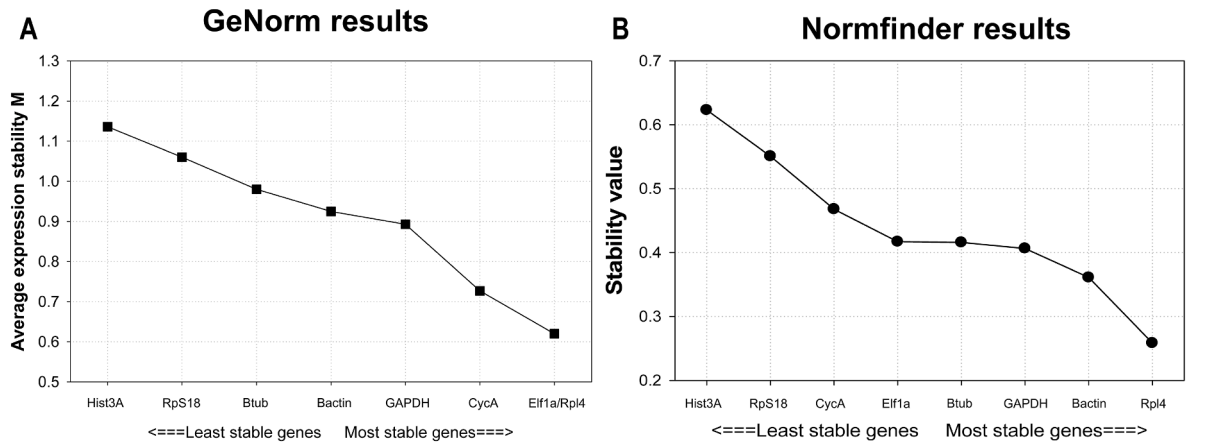


Fig. 3. Transcription profiles of eight putative constitutive genes in different days of embryogenesis of *Rhipicephalus microplus*. *A*= NormFinder analysis; *B*=GeNorm analysis; C= Determination of optimal number of control genes for normalization by GeNorm tool.

rangeli infection and blood feeding condition, since *Tubulin* and *βactin* presented the best combination followed by *GAPDH* and *Tubulin*, respectively (Paim et al., 2012). *βactin* and *GAPDH*, along with *ribosomal protein 49* were also reliable to normalize qPCR results in different life stages of Calliphoridae species (Cardoso et al., 2014).

In ticks, selection of endogenous targets has been described in different tissues and conditions. In *I. scapularis* the constitutive expression of six genes were validated in salivary glands and synganglion



C

CP data of housekeeping Genes by BEST KEEPER

	CycA	RpS18	Btub	Hist3A	Bactin	Elf1a	GAPDH	Rpl4
n	96	96	96	96	96	96	96	96
geo Mean [CP]	18.49	17	18.19	18.2	18.8	17.61	21.72	20.21
AR Mean [CP]	18.53	17.06	18.23	18.22	18.82	17.64	21.74	20.21
min [CP]	16.32	13.38	13.93	15.72	17.59	15.16	20.64	18.8
max [CP]	20.45	20.34	19.99	20	21.54	19.04	24.37	21.28
std dev [+/- CP]	1.03	0.98	0.94	0.82	0.73	0.71	0.64	0.44
CV [% CP]	5.56	5.76	5.13	4.49	3.88	4.05	2.95	2.15
min [x-fold]	-4.5	-12.32	-19.13	-5.57	-2.31	-5.48	-2.12	-2.65
max [x-fold]	3.89	10.1	3.49	3.49	6.68	2.69	6.27	2.11
std dev [+/- x-fold]	2.04	1.98	1.91	1.76	1.66	1.64	1.56	1.35

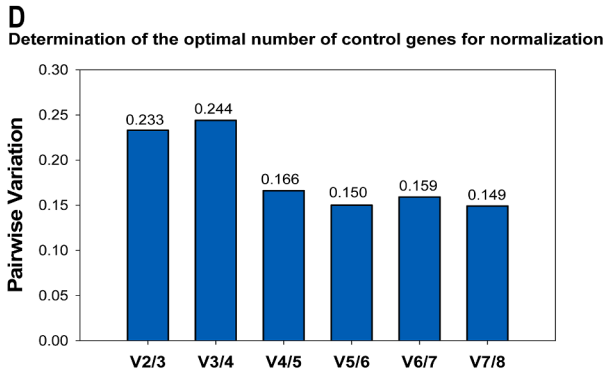


Fig. 4. Statistical analysis of endogenous genes stability using GeNorm, Normfinder and BestKeeper tools. *A*= GeNorm; *B*= NormFinder; C= BestKeeper; *D*= Determination of optimal number of control genes for normalization by GeNorm tool.

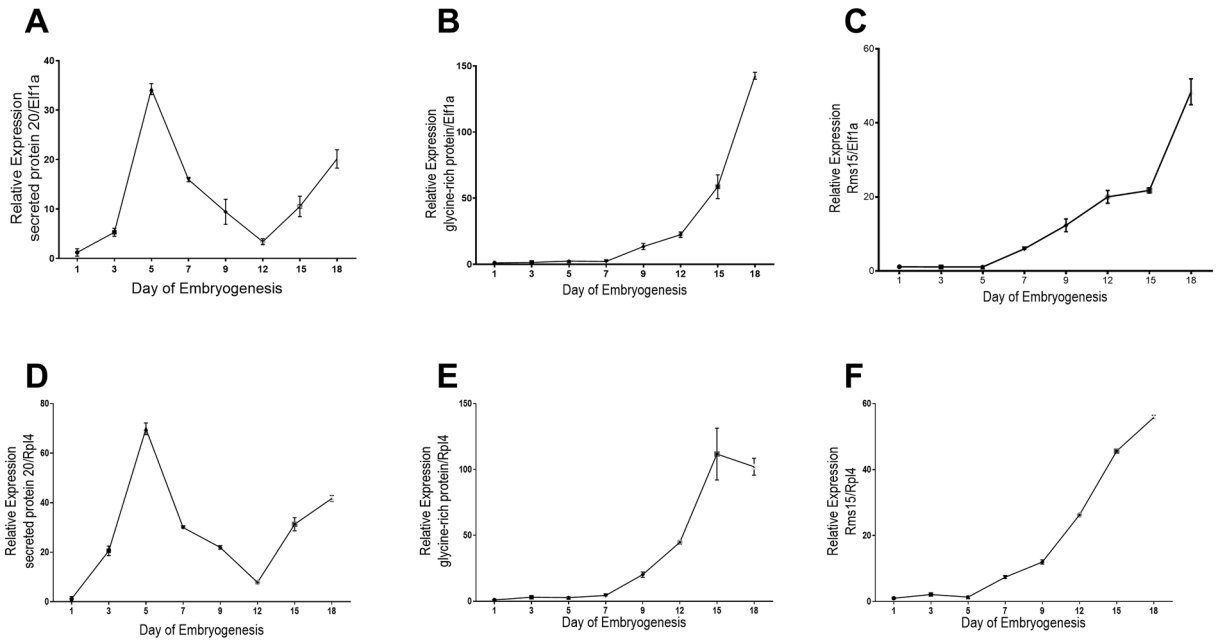


Fig. 5. Comparative analysis of RT-qPCR using *Elf1a* and *Rpl4* as internal reference control. *Elf1a* and *Rpl4* were used as internal control in a quantitative transcription assay evaluating the relative expression of *Rhipicephalus microplus secreted protein 20* using (A and B). *Rhipicephalus microplus glycine-rich protein* (C and D) and *RmS15* (F and G).

(Koči et al., 2013). In *H. anatolicum*, the transcription of four reference genes was evaluated in tick cell lines (Salata et al., 2020). In *R. microplus* and *R. appendiculatus*, nine constitutive genes were evaluated for their stability in eggs, larvae, nymphs, and adults under unfed and fed conditions (Nijhof et al., 2009). However, despite being studied for many years, there is a lack of reports about the validation of constitutive genes during embryogenesis of ticks, even though it is used extensively as a model in chelicerates (Laumann et al., 2010; Santos et al., 2013;

Wagner, 1893). The genes *β-actin* and *GAPDH* showed a reduced degree of variation in *A. maculatum* (Browning et al., 2012). When observing the results from GeNorm, NormFinder and BestKeeper analyses, both genes were among the most stable positions when transcription in the salivary glands, midguts and different stages was evaluated (Browning et al., 2012). In *H. anatolicum*, four genes (*Elf1a, βactin, GAPDH* and *ribosomal protein L13A*) were validated for use in qPCR assays (Salata et al., 2020). In *O. moubata*, the transcription of *βactin* was stable considering unfed and fed females and has been suggested as a candidate to be used as an internal control (Horigane et al., 2007). In contrast, in this work, the *βactin* showed a stable transcription pattern at different embryogenesis points. Interestingly, in the last points of embryogenesis (from 12 to 18 days), *β-actin* presented the highest degree of variability by NormFinder and GeNorm and does not seem to be a good candidate as an internal control to evaluate the transcription of genes during tick embryogenesis. Meanwhile, despite having the lowest transcription in embryo, *GAPDH* remained among the most stable genes to all the tools applied, making it a suggested good candidate as a reference gene by GeNorm at the later stages of embryogenesis.

In salivary glands and synganglion of *I. scapularis*, a relatively stable transcription of some ribosomal proteins, like *L13A* and *ribosomal protein S4* was observed, making them useful for qPCR normalization. These can be used either as a set or individually (Koči et al., 2013). In addition, in *R. microplus* and *R. appendiculatus, Elf1a, βactin, Hist3A, Rpl4, Tubulin, CycA, GAPDH, glutathione S-transferase* (GST), and *TATA box-binding protein* (*TBP*) transcription were measured in all life stages of ticks. According to Ct values, *βactin, GAPDH, TBP* and *GST* showedlow transcriptional level in *R. microplus*. GeNorm analysis indicated that *Elf1a* and *Rpl4* are the best candidates to be used as internal control genes, while NormFinder also selected *Elf1a*, followed by *GAPDH* (Nijhof et al., 2009). In this work, *Elf1a, Rpl4* and *GAPDH* also exhibited stable transcription levels during the evaluated embryogenesis periods.

Discussions regarding the optimal number of reference genes have been reported (Yang et al., 2015). The use of a single internal control is not sufficient for performing quantitative analyses, and using incorrect endogenous genes could lead to errors in results analysis (Fu et al., 2013). Some reports suggest that six or seven genes are recommended to normalize the qPCR results (Nijhof et al., 2009). For example, in *Plutella xylostella*, the lowest pairwise variation was obtained when four genes were used, interestingly the inclusion of the fifth gene resulted in high variations compromising the normalization process (Fu et al., 2013). Meanwhile in our study, the lowest pairwise values were observed for V7/8 (0.149), but the use of four constitutive genes was sufficient to reach reliable results.

Different algorithms have been used to identify the most stable genes (Shakeel et al., 2018). GeNorm use the average expression stability (M value) to promote accurate analysis of the transcription of housekeeping genes, additionally, a pairwise analysis is performed to estimate the optimal number of reference genes to applied in an assay (Vandesompele et al., 2002). Meanwhile, BestKeeper can be in both housekeeping expression analysis and other target gene analyzes (Pfaffl et al., 2004). NormFinder, is an approach that takes into account differences of each sample, followed by individual transcription of the candidate genes, allowing the identification of gene variation between subgroups (Andersen et al., 2004). In a study on *Nilaparvata lugens*, these algorithms showed that a *ribosomal protein* (*RPS11*) was an adequate endogenous control in different tested conditions, such as developmental stages and tissues (Yuan et al., 2014). Similarly, in this study, *Rpl4* could be indicated as the best reference gene, presenting the lowest variability in *R. microplus* embryo tissue, according to GeNorm, NormFinder, and BestKeeper. However, differences were observed when different embryogenesis time points were evaluated. NormFinder demonstrated that *Rpl4* was the most stable gene from day 1 to 5, *CycA* was chosen from day 7 to 9, and *Elf1a* could be used from day 12 until 18. On the other hand, in the GeNorm analysis, *Rpl4* remained the appropriate reference gene throughout the evaluated period, along with *Elf1a* (1 to 9 days) and *GAPDH* at the last time point.

Since the use of constitutive genes, it is a requirement for accurate quantitative analysis (Shakeel et al., 2018), more than one internal control may be used to normalize RT-qPCR data. We have analyzed for the first time the transcriptional pattern of eight candidate constitutive genes throughout *R. microplus* embryogenesis. Variations in relative stability during *R. microplus* embryogenesis were observed and different candidates for internal control in molecular assays might be applied. Overall, this study highlights the importance of selecting appropriate reference genes for accurate RT-qPCR analysis at different stages of tick embryogenesis. In addition to the eight candidates evaluated in this work, there are others constitutive genes that can be tested for different purposes. Although no universal constitutive gene was found, the analysis of the eight candidate genes showed that *Rpl4, Elf1a*, and *GAPDH* are suitable internal controls for normalization of RT-qPCR data during *R. microplus* embryogenesis. It is recommended to use more than one reference gene to ensure accurate and reliable results. This study provides valuable information for future molecular studies on *R. microplus* and other tick species.

CRediT authorship contribution statement

Tae Kwon Kim: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Jéssica Waldman:** Formal analysis, Writing – original draft, Writing – review & editing. **Freddy Ibanez-Carrasco:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Lucas Tirloni:** Formal analysis, Writing – original draft, Writing – review & editing. **Camila Waltero:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Christiano Calixo:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Gloria R. Braz:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Albert Mulenga:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Itabajara da Silva Vaz Junior:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Carlos Logullo:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2023.102251.

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