Identification and validation of reference genes for RT-qPCR single-gene relative expression analysis in *Taenia solium* pre-adult stages

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SUMMARY

The larvae-to-adult development on the life cycle of zoonotic parasitic tapeworm *Taenia solium* involves striking –but clinically unappreciated– events with pivotal importance in cestode biology. Unlike the ones related to the intermediate host, the early-adult stages can be addressed *in vitro* offering a useful model to study evagination, strobilation and worm development. In the absence of a stage-specific transcriptome, postgenomic data exploration followed by single-gene relative expression analysis by RT-qPCR (reverse transcription-quantitative PCR) are useful strategies to gather information on the regulation of genes of interest during parasite development. However, this approach requires the validation of an endogenous reference gene (RG) to achieve accurate comparisons.

Therefore, we analyzed the expression stability of 17 candidate RGs on the context of the early-adult stages of *T. solium* classified as non-evaginated and evaginated larvae (cysts). The comprehensive tool RefFinder ranked malate dehydrogenase as the most stable gene within these conditions, and its suitability for relative quantification was validated by normalizing the expression of the transporter TGTP1 gene, known for being upregulated upon evagination. This study is the first attempt in finding reliable normalization standards for transcript exploration in genus Taenia.

INTRODUCTION

Genus *Taenia* records more than a hundred species of tapeworms that include parasites of wildlife and livestock. Among them, *Taenia solium* –together with *Taenia saginata* and *Taenia asiatica*–, is one of the three species of tapeworm that cause zoonotic infection and is responsible for teaniasis and cysticercosis; which are a type of helminthiasis belonging to the group of neglected tropical diseases currently prioritized by WHO[1].

The acquired disease may lead to life-threatening conditions, especially in endemic poor remote settings in developing countries of Africa, Asia, and Latin America, where the burden affects the health and livelihoods of rural farming communities. The latest report of WHO, identified *T. solium* as a leading cause of deaths from food-borne diseases, resulting in a devastating total loss of 2.8 million disability-adjusted life-years (DALYs)[2,3].

Like most helminths, *T. solium* has a complex life cycle that takes place in a usual intermediate host (pig) that harbors the larvae in its tissues (cysticercosis) and a unique definitive host (human) that carries the adult tapeworm in its intestines (taeniasis). The tapeworm carrier spreads, through faeces, thousands of microscopic parasite eggs

containing infective hexacanth embryo or oncospheres that will be accidentally ingested by free-roaming pigs or other humans. Active embryos are released from eggshells and cross the intestinal epithelium to finally be distributed by the bloodstream to skeletal muscle, heart and nervous system, where establish and develop into vesicle larvae (cysts) and may cause severe disease[4]. The viable cysts can remain in poorly cooked pig meat before being eaten by humans and undergo activation by gastric and bile acids. When placed in human intestine, the worm scolex evaginates from the larva and attaches by its hooks to the intestinal wall, from where starts growing to the adult tapeworm by producing continuous proglottids that will carry new eggs[4,5].

Due to the complexity of the host-parasite interactions through stages, the complete life cycle of *T. solium* is impossible to reproduce in the laboratory. Despite this, observations in experimental or naturally infected animal models produced valuable knowledge about the oncosphere-to-cyst development and the cyst-associated immunopathology in several tissues[6,7]. In counterpart, there is much less information related to cyst-to-adult development; due to the limited animal models available[5], and because this form of the disease generally has mild or non-specific symptoms[4,8]. Nonetheless, the study of the unappreciated stages involved in taeniasis is important for understanding factors of endemicity. Hence, a tapeworm carrier is the main risk factor for acquiring cysticercosis since it behaves as the spreader of the infective stage and thus, perpetuates the life cycle.

Unlike other parts of the cycle, the cyst-to-adult development involves several processes that have a pivotal importance in cestode biology, starting from cyst evagination and further strobilation of the tapeworm body[9]. Fortunately, the early-adult stage in *T. solium* can be addressed *in vitro* from cysts after chemical[10] or enzymatic treatment[11], offering a useful model to study evagination and worm development for this parasite.

With the publication of the *T. solium* genome[12] and with the first partial cyst transcriptomes obtained by RNA-seq[13,14]; a valuable amount of data is currently available to study stage-specific events involved in parasite's development[15]. Moreover, whole transcriptome analysis in other tapeworms (including families Taeniidae –genuses Taenia and Echinococcus–[16–20], Hymenolepididae[21] and Mesocestoididae[22]) lead to the identification of conserved pathways among cestodes that compliment previous datasets.

However, when single-gene expression analysis is required for comparisons among experimental conditions, the RT-qPCR is the appropriate method for gathering differences in the expression of specific mRNAs; based on its high sensitivity and specificity. Therefore, validation of the expression stability of candidate RG is strongly recommended before data normalization procedures that uses RT-qPCR to study any particular species or stages[23].

Although few analyses for expression stability of RG have been done in other cestodes[24,25], there is no report of a similar validation for the pre-adult stages of *T. solium*. Data normalization for RT-qPCR comparisons were performed previously, however, using canonical housekeeping gene targets such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[26] or Cu/Zn superoxide dismutase (TsCu/ZnSOD)[27].

In this study, a set of 17 transcripts were selected as candidate RG for T. solium pre-adult stages that were achieved *in vitro*. The expression stabilities of these genes were analyzed in evaginated and non-evaginated cysts using the RefFinder integrative tool that groups the statistical algorithms geNorm, NormFinder, BestKeeper and the delta-Ct method. Finally, to

validate the reliability of most stable gene, we quantified by RT-qPCR the relative mRNA expression of TGTP1 that was previously reported as differentially expressed after cyst evagination[26].

RESULTS

RT-qPCR amplification efficiency and product specificity

Primers of 17 candidate RG were used to amplify cDNA template by qPCR technology. The amplification efficiency for all the reactions performed with each primer sets reached values over 90% (Table 1). In addition, agarose gel electrophoresis revealed a single-band amplicon for each reaction, which was consistent with the expected product size (Fig. S1A). The graphs of the negative first derivative of the melting-curve were also plotted, all of which showed a single peak, indicating that the primers had strong specificity for target region and no non-specific amplification occurred (Fig. S1B).

Table 1. Primer sequences and RT-qPCR amplification features of 17 candidate RGs.

Gene	Wormbase accession no.	Primer sequence 5'– 3' (forward / reverse)	Product size (pb)	Tm (° C)	%E
αTUB	TsM_000816800	ACTCCGCGATAATGGGTCTT/ GGAGCTCTGGGAAGGGAATT	183	59.3	133.56
βACT-1	TsM_001199400	ATCGTAGCACCACCAGAACG/ CCCGATTCGTCGTACTCCTG	113	60	94.134
βACT-2	TsM_001001400	GTGCGAGACATCAAGGAGAAG/ GGAAGCGTTCATTGCCAACT	142	60.4	98.311
GAPDH1	TsM_000056400	TCCAAGAGATGAATGCCAATGC/ CAGAAGGAGCCGAGATGATGA	147	61.1	93.875
GAPDH2	TsM_001083000	CAAACTTGCCAAACCTGCCA/ GCGTCGAAAGTGGATGATGC	153	58.7	97.872
PGK1	TsM_000796500	GACACCGCTACTTGTTGTGC/ TCCGAAAGAGCTGATACGCC	125	59	105.076
MDH	TsM_000048200	TAAGGTGCTGGTTGTTGGCA/ CGACCTGATAGATGGCACGG	131	59.8	91.184
COX1	TsM_000113400	CCGTTAGGAGGTGGTGATCC/ ACCCATAAAAGCCAAAAGCA	154	60.1	101.694
RPB2	TsM_000754600	CACCACGAAGACACCACAAG/ TAGGAATGGCGTGAGGATTG	149	58.6	95.311
TBP	TsM_000071900	GCCAAAGTGCGAGATGAGAT/ CCCGATTTGTCTGAATCCAA	89	56.8	96.374
EIF4a	TsM_000855500	CTGCATTGGTACGCTTCAAC/ AGTGCTGTGACGACCTGATG	111	58.7	96.646
RPL13	TsM_000621100	TCCAATGCTATCGGGTGAAT/ CGCCTCGTGGATCGTATATT	74	56	103.092
EF1α	TsM_000233800	TTGCTGCTGGTACTGGTGAG/ CGCTATAATCAACCGCATCC	138	58.6	89.776
CYPA	TsM_000676800	TCGCAGTTCTTCATCACCAC/ TCTTGCCACTACTGCTACCG	127	58.1	91.710
UB3A	TsM_000891500	TACGCTTGTGCTGGTGGTAG/ GGGAAGAAGGAGATCCAAGG	87	58.5	93.421
UB2	TsM_001232600	TTGTCCCACTTATCCCAAGG/ ACGGCCTAAGCACACTGAAC	100	59.1	110.478
MAPK3	TsM_000447200	TCAAGCCGGACTACTTACCG/ CACGGTTGCTGTACATCTCG	140	58.6	101.564

In vitro parasite evagination and pre-adult stages nomination

Focusing on the early development of the pre-adult stages we considered two conditions: cysts directly extracted from naturally infected pigs and washed with PBS after its transport (non-evaginated) and *in vitro* evaginated cysts. For the latter group, we sought to identify culture conditions that achieve the complete evagination of parasites in order to set *bona fide* categories for further single-gene expression comparisons against non-evaginated. For this purpose, we first determined the best time point to take parasites after chemically inducing evagination with taurocholic acid (TA). We found that after 5 days in culture post TA-induction the parasites fully evaginated the scolex, neck and early strobila from the vesicle (Fig. 1A). This event was noticeable by either direct or macroscopic observation of the cultured plate and was confirmed by measures of the body lengths (Fig. 1B). Moreover, the percentage of evagination was the highest among the culture conditions tested (Fig. 1C). Based on these observations, we name the parasites obtained from this condition (5d, RPMI + TA 0.1%) as “evaginated” and served as contrast to the non-evaginated group.

Expression profile and comprehensive stability analysis of candidate RGs

In order to determine the dispersion of Cq values of the selected candidate RGs under the conditions representing *T. solium* early-adult stages, initial qPCR runs were performed with cDNA generated from both evaginated and non-evaginated parasites. A small Cq value indicated higher gene expression abundance, and *viceversa*. The mean Cq values of all the RG were <35 cycles, suggesting that the abundances of the transcripts are not underrepresented. Based on a box-plot comparison built from the raw Cq values, the top 5 genes with lower variability (lower std. deviation) were RPL13, COX1, MDH, PGK1 and EF1a; while ETIF4AII, RPBII and UBE2 were the worst. Interestingly, the canonical reference genes GAPDH1, GAPDH2, aTUB, β-ACT1 and β-ACT2 showed a higher variability among the samples (Figure 2). However, set preferences on the distribution of the raw Cq values and their descriptive statistical measures might not be enough to assess the expression stability of candidate RGs, and other methods are required for more comprehensive and accurate evaluations.

In order to better explore the gene stability of the candidates, the web-based RefFinder tool that integrates the most common algorithms available to assess gene expression stability (geNorm, Normfinder, BestKeeper, and Delta-Ct) was employed to rank the most suitable RG. After the independent analysis with each program, a comprehensive ranking was built with all the outcomes and pointed at MDH (closely followed by RPL13) as the most stable gene from the initial panel. In contrast, ETIF4AIII and UBE2 were confirmed as the least consistent (Figure 3). The integrated ranking by RefFinder was concordant with the single-algorithm analysis in pointing MDH as the candidate RG with the highest expression stability.

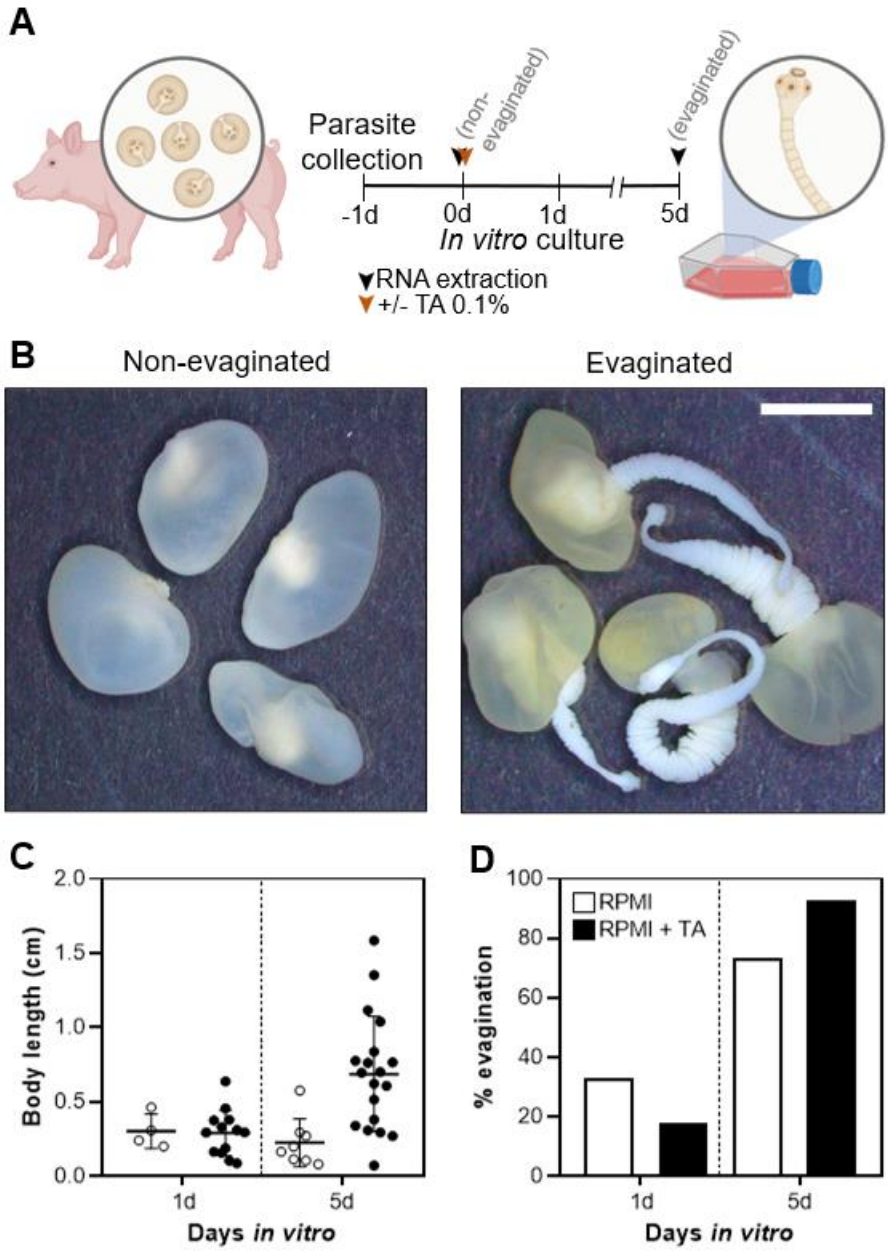

Figure 1. Taurocholic acid induces complete *in vitro* evagination of *T. solium* cyst after 5 days. (**A**) Study design and analysis workflow. (**B**) Representative macroscopic images of directly extracted non-evaginated (left) and evaginated parasites cultured for 5d after 0.1% TA induction (right) that were considered for single-gene analysis comparisons. Scale bar is 5 mm. (**C**) Quantification of body length (distance from the base on the vesicle until the distal scolex) in cm and (**D**) percentage of evagination for all the culture conditions tested.

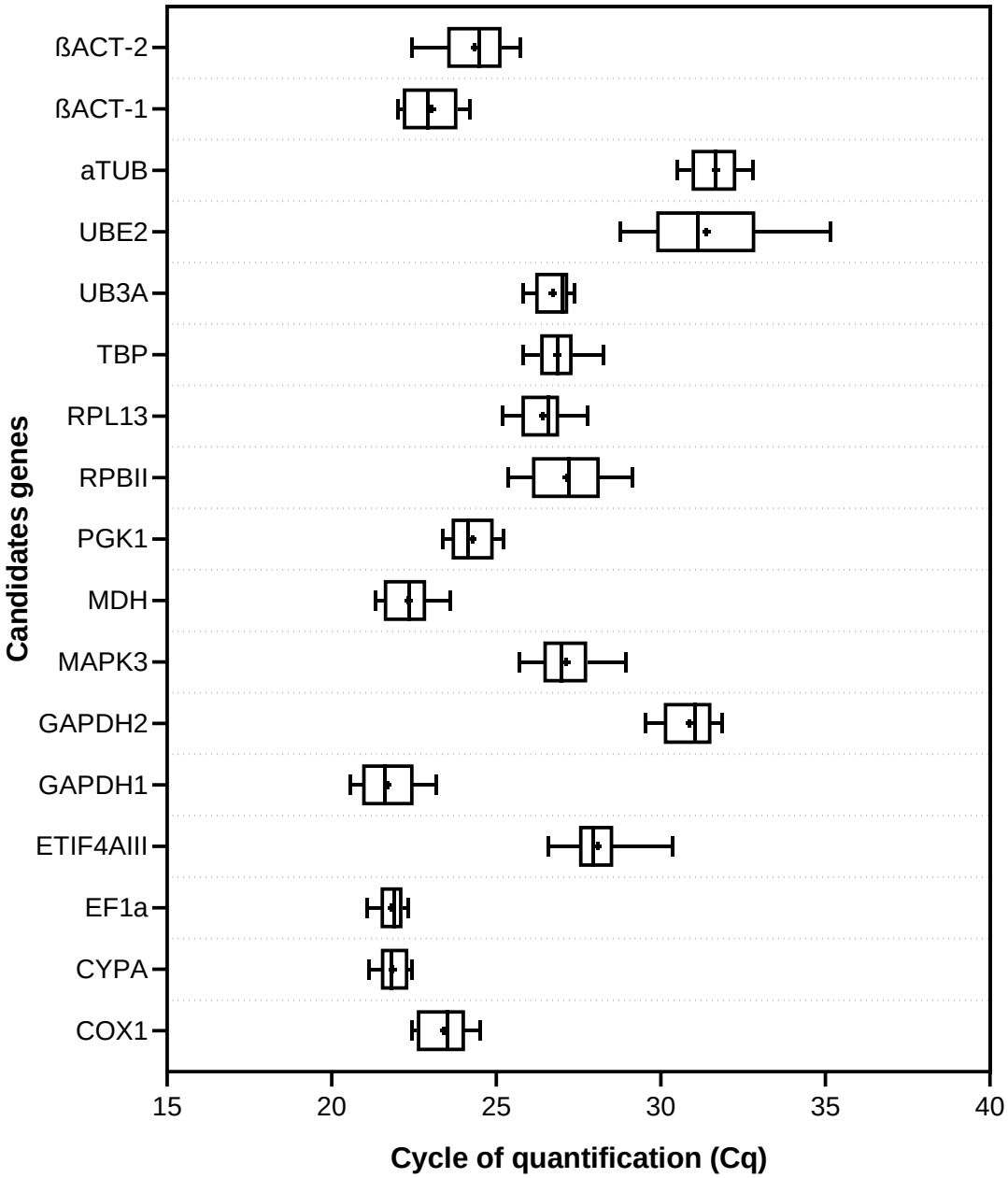

Figure 2. Transcript abundance and raw Cq distribution of RG. A box-plot comparison was built based on the Cq values obtained for every candidate reference gene. Cq mean values (cros), the median (line across the box) and interquartile ranges for the 25% and 75% percentiles are represented for each candidate reference gene in all samples. Whiskers represent the maximum and minimum values.

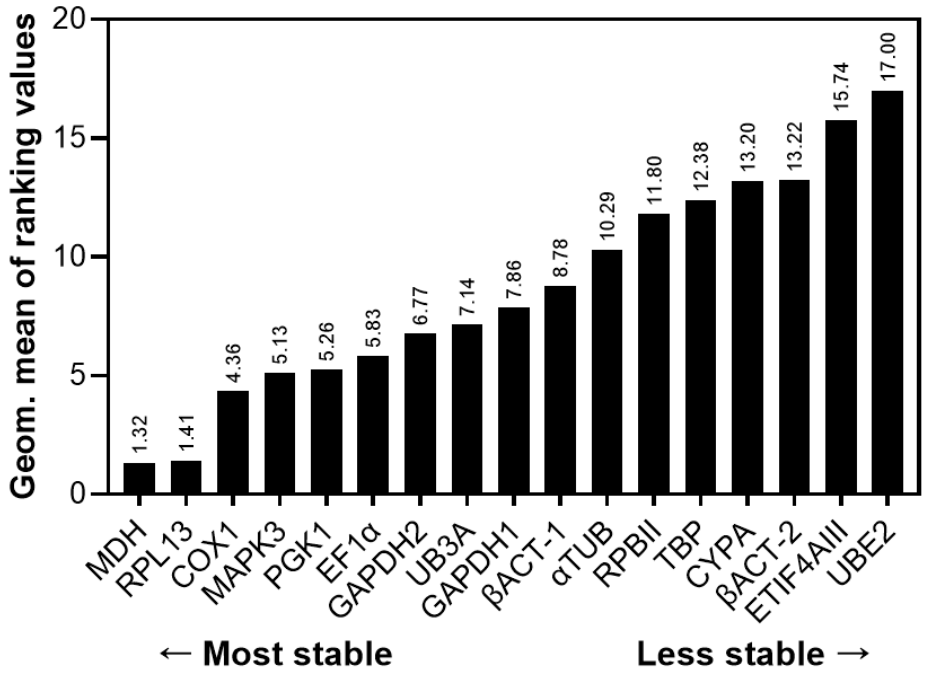

Figure 3. Refinder comprehensive ranking of gene stability scores. The output scores for gene stability analysis obtained by the algorithms geNorm, Normfinder, BestKeeper and the comparative Delta-Ct method were integrated into a comprehensive score calculated by the geometric mean of the previous four. Lower values indicate less variability and therefore more gene stability.

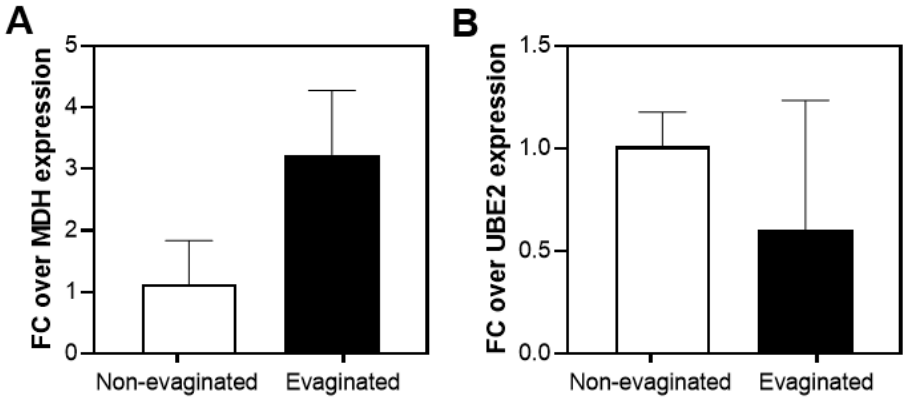

Figure 4. Relative quantification of the glucose transporter TGTP1 normalized with the best and worst candidates. The expression of the TGTP1 gene, known for being upregulated on evagination, was normalized with (**A**) the top-ranked MDH gene and (**B**) the worst-ranked UBE2 using the $2^{-\Delta\Delta Cq}$ method.

DISCUSSION

Like in other neglected infectious diseases, the research on parasite biology of *T. solium* is a mostly unexplored field that holds crucial information to better understand the interactions with the hosts, the developmental changes through life cycles, and therefore the associated disease endemicity. In the absence of stage-specific transcriptomes, single-gene

expression analysis is a powerful approach to explore the roles of genes of interest in the context of well-defined biological conditions. Thus, the achievement of controlled in vitro evagination of cysts is a valuable model to study events on the early-adult development.

However, the relative quantification analysis lacks a suitable RG in genus *Taenia* and the normalization of the expression data is performed using canonical RG that are not validated either for the organism and the stage.

In our analysis, we aimed on the identification of genes that are stably expressed in evaginated and non-evaginated experimental conditions. Therefore, we selected 17 candidates based on their previously reported stability in the close-related parasitic cestode *Echinococcus* sp. (aTUB, RPL13, TBP, CYPA, ETIF4A-III, MAPK3, and EF1a) and for being widely used for relative quantification normalization (COX1, βACT-1, βACT-1, GAPDH1, GAPDH2). Moreover, other genes were considered based on its role in housekeeping cellular processes e.g metabolism (PGK1, MDH) and transcription (RPB2, UB3A, UB2).

The analysis of gene expression stability for candidate RGs by RefFinder brings together different algorithms that takes a dataset of Cq values obtained in experimental conditions as an input to build an ordinal ranking on the consulted candidates. Therefore, the cytosolic NAD+ supplier enzyme MDH was chosen as the most stable RG from the panel. Although there is no report of its stability on other platelminthes, MDH has been previously reported and a good RG on the crop pathogen nematode *Meloidogyne hapla* through its several developmental stages, and under temperature and inorganic compound experimental treatments.

Finally, we performed a relative quantification analysis of the Wnt4 gene using MDH as RG. The upregulation of TGTP1 upon evagination has been previously reported using GAPDH1 as normalizer. The results of our validation are consistent with the upregulated behavior of TGTP1 in the context of scolex evagination.

Taken together, the results of this work validated that MDH could be used as suitable reference genes for normalizing single-gene relative expression qRT-PCR data in the context of *T. solium* cyst evagination. This study provides a basis for future studies of gene function in *T. solium* early-adult development.

MATERIALS & METHODS

Selection of candidate RGs and primer design

Primers were designed using Primer3[28] and BLAST® software based on the 17 mRNA sequences of *T. solium* candidate RGs retrieved from GeneDB databases. The basic parameters used for primer design were as follows: primer length between 18-21 bp, GC content between 45-55%, melting temperature between 55-60 °C, and amplicon length between 60-200 bp. Every primer was 100% complementary to the expected primer binding sites, except for the COX1 primers, which had less (97%) complementarity to the used mRNA sequence. The details of each selected candidate gene and the biological features of each primer set are shown in Table 1 and Table S1.

Parasites collection and *in vitro* evagination

Taenia solium cysts were collected from muscular tissue of a naturally infected pig, as determined by a positive tongue examination, from a farm at Huancayo, Junin, Peru. Only undamaged and complete cysts were selected and placed in phosphate buffered-saline (PBS; 0.1 M, pH 7.4) supplemented with gentamicin (20 µg/mL) and benzylpenicillin (10 µg/mL) for transport.

The evagination of a group of cysts was induced with taurocholic acid 0.1% (Sigma, St. Louis, MO) diluted in RPMI 1640 medium supplemented 2 g/L of $NaHCO_3$ (Merck®, Burlington, MA) and 10% fetal bovine serum (Gibco®, Thermofisher Scientific, Waltham, MA). The parasites were cultured in 5% CO_2 with 2 mL of described media, in 12-well plates (Corning, Corning, NY) for up to 5 days at 37°C. The scolex evagination was monitored by direct observation of the plates at 1 and 5 days to set the best conditions for complete evagination (5d, RPMI + TA 0.1%). After culture, all parasites were washed with PBS to remove residuals of either transport or culture media, from previous steps. The percentage of evagination (understood as the proportion of parasites with appreciable scolex) and the measures of the length of the growing body (distance from the distal extreme of the scolex until the base on the vesicle) was assessed for every culture condition and parasite, respectively.

RNA extraction and DNAse treatment

Evaginated and non-evaginated parasites were embedded independently in 1 mL of TRIzol (Invitrogen, Carlsbad, CA) and admixed with a tissue homogenizer tip (Omni International, Kennesaw, GA) on ice. A first RNA isolation was performed following the manufacturer's protocol, considering an extra washing step with 75% ethanol. After the solubilization, whole RNA was treated with 2 U RNAse-free DNAse I (Invitrogen, Carlsbad, CA) for 30 min at 37° C. The DNAse was inactivated with TRIzol, and the RNA was re-purified. RNA yield was measured by spectrophotometry with NanoDrop® (Thermofisher Scientific, Waltham, MA) and approximately 100 ng of RNA were loaded in a 1.5% agarose gel electrophoresis to test its integrity.

Two-step RT-qPCR reaction

The cDNA was generated from 100 ng of RNA of every parasite by reverse transcription with the High-Capacity Reverse Transcription Kit (Thermofisher Scientific, Waltham, MA) in a final volume of 20 µL. The reaction mix was incubated 15 min at 25° C, 2 h at 37° C and 5 min at 85°C and stored at 4° C until required.

The qPCR reactions were done with the PowerUp® SYBR Green Master Mix (Thermofisher Scientific, Waltham, MA). For every 10-µL reaction, forward and reverse primers were added at a final concentration of 500 nM each, and 1 µL of cDNA was used as the template. The cycling conditions were set in the PikoReal™ Real-Time PCR System (Thermofisher Scientific, Waltham, MA) as follows: 2 min at 50°C, 2 min at 95°C; and 40 cycles of 15 s at 95°C and 1 min at 60°C. Every sample was run by triplicates and a melting curve step was done at the end of every experiment ranging from 60°C to 95°C with increments of 0.2°C. Additionally, the size and specificity of amplification products were analyzed trough a 1.5% agarose gel electrophoresis. RG datasets with Cq values >35 were excluded for having low abundance.

Amplification efficiency and analytical performance

To determine the PCR amplification efficiency for every candidate RG, 5-point calibration curves of cDNA were prepared using five 2-fold, 5-fold or 10-fold serial dilutions. The linear regression, slopes and *y*-axis intercepts for every standard curve were calculated with the Graphpad Prism® software version 6.01 (Graphpad®, San Diego, CA). Every dilution was test by triplicate and amplification efficiency was determined based on the slope of the log-linear portion of the calibration curve ($10^{-1/slope} - 1$), as described in MIQE guidelines[29].

Data analysis for gene expression stability

The gene expression stability of the candidate RGs was compared and ranked with different normalization algorithms integrated on the web-based tool RefFinder (available at: https://www.heartcure.com.au/reffinder/). This program integrates the analysis performed by the extensively-used computational algorithms geNorm[31], Normfinder[32], BestKeeper[33], and the comparative Delta-Ct method[30]. The selection of the most stable RG was made based on the recommended comprehensive ranking calculated by the geometric mean of the stability scores obtained from each method.

Validation of RG in relative quantification

In order to validate the reliability of the selected RG, the relative expression of the transporter TGTP1 was normalized by the most and the less stable genes that were obtained by the normalization algorithms. Indeed, TGTP1 RT-PCR target has been reported previously as differentially expressed upon evagination in *T. solium* cysts[26]. Five biological replicates per condition and three technical replicates per sample were set up for this analysis. Fold-change for TGTP1 at experimental conditions was analyzed using the $2^{-\Delta\Delta Cq}$ method[34]. The sequence and other technical details for TGTP1 primer set is shown in Table S2.

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AUTHOR CONTRIBUTIONS

RGL and JBLT conceptualized and designed this work. JBLT, SDA, DC and VV performed experiments, RGL and JBLT performed all data analysis. RGL wrote the manuscript with input from all authors and CGG finished the critical revision of the article.

DECLARATION OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTAL INFORMATION

Table S1. Descriptions of candidate reference genes

Gene symbol	Gene name	Function	Wormbase parasite ID
αTUB	Alpha tubulin	Structural constituent of cytoskeleton	TsM_000816800
βACT-1	Beta actin-1	Structural constituent of cytoskeleton	TsM_001199400
βACT-2	Beta actin-2	Structural constituent of cytoskeleton	TsM_001001400
GAPDH1	Glyceraldehyde 3-phosphate dehydrogenase 1	Glycolytic enzyme	TsM_000056400
GAPDH2	Glyceraldehyde 3-phosphate dehydrogenase 2	Glycolytic enzyme	TsM_001083000
PGK1	Phosphoglycerate kinase 1	Glycolytic enzyme	TsM_000796500
MDH	Cytosolic malate dehydrogenase	NAD+ supplier in glycolysis	TsM_000048200
COX1	Cytochrome c oxidase subunit 1	Oxidative phosphorilation enzyme	TsM_000113400
RPB2	DNA directed RNA polymerase II subunit RPB2	Transcription	TsM_000754600
TBP	TATA box binding protein	Transcription	TsM_000071900
EIF4a	Eukaryotic translation initiation factor 4A III	Translation and protein synthesis	TsM_000855500
RPL13	Ribosomal protein L13	Translation and protein synthesis	TsM_000621100
EF1α	Elongation factor 1 alpha	Translation and protein synthesis	TsM_000233800
CYPA	Cyclophilin A	Protein folding, trafficking and regulation	TsM_000676800
UB3A	Ubiquitin protein ligase	Protein quality control	TsM_000891500
UB2	Ubiquitin conjugating enzyme E2	Protein quality control	TsM_001232600
MAPK3	Mitogen activated protein kinase 3	Protein kinase in cell signaling	TsM_000447200

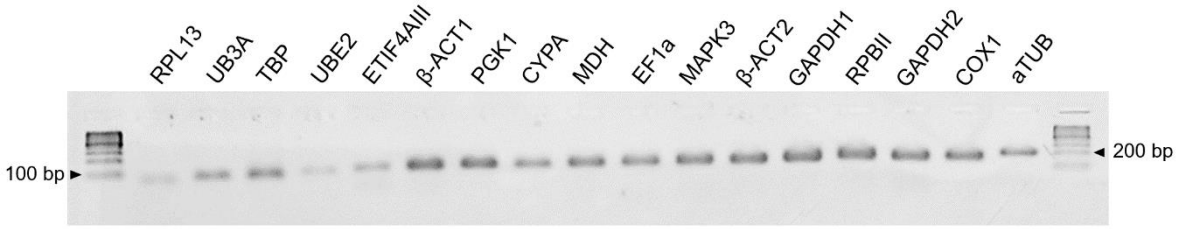

Figure S1. Specificity of the RT-qPCR amplification. The product of amplification obtained for each RG was loaded on in a 4% agarose electrophoresis gel. 20 ng of GeneRuler 100 bp ladder was added on the first and the last wells of the gel.

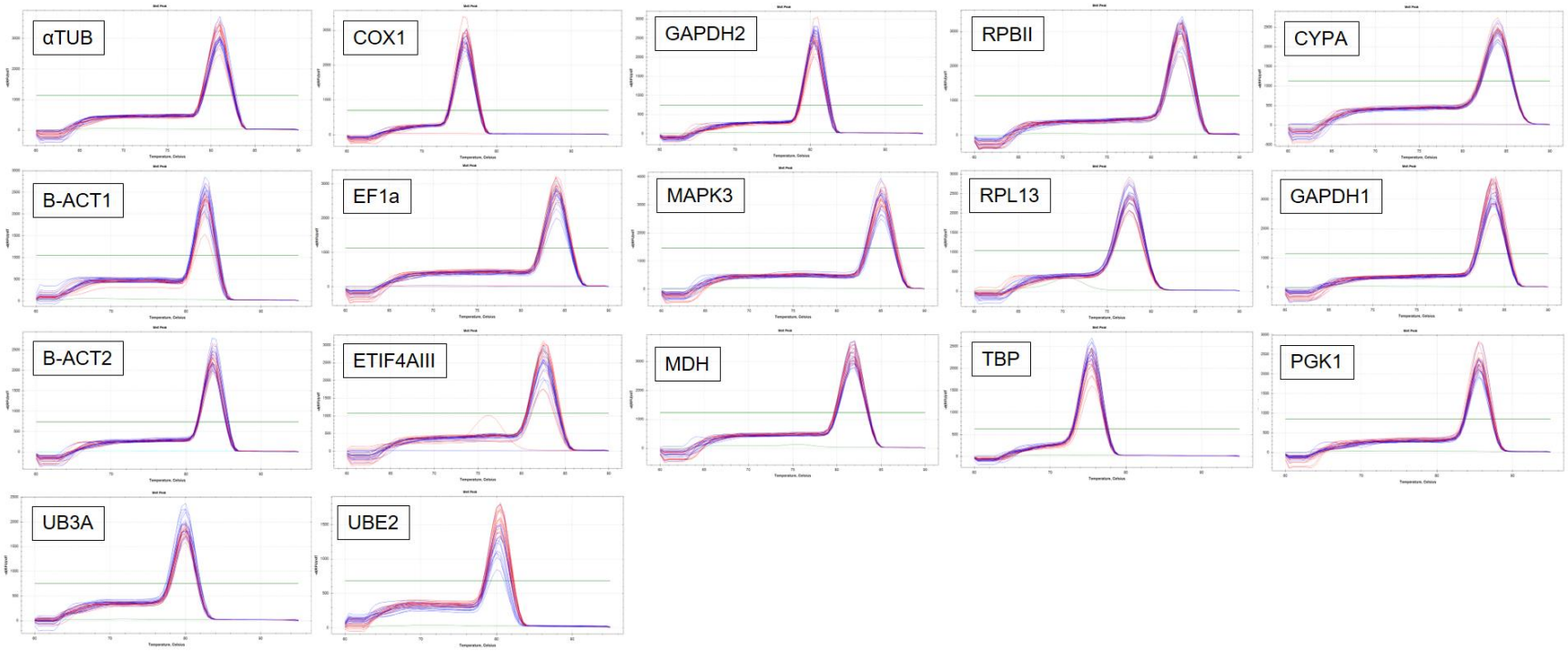

Figure S2. Melting-curve from RT-qPCR products of 17 RGs. The melting curves obtained for each candidate RG showed a single peak for each reaction.

Table S2. Descriptions of TGTP1 target gene

Gene symbol	Gene name	Function	Wormbase parasite ID	Primer sequence 5'– 3' (forward / reverse)	Product size (pb)	Tm (° C)
TGTP1	Taenia Glucose Transporter Protein 1	Glucose transporter	TsM_000722000	ACTCCGCGATAATGGGTCTT/ GGAGCTCTGGGAAGGGAATT	183	59.3