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Evaluation of internal reference genes for quantitative expression analysis by real-time reverse transcription-PCR in somatic cells from goat milk

P. Modesto,*†[1] S. Peletto,* G. Pisoni,† P. Cremonesi,‡ B. Castiglioni,‡ S. Colussi,* M. Caramelli,* V. Bronzo,† P. Moroni,† and P. L. Acutis*

*Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, 10154 Turin, Italy
†Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare, Università degli Studi di Milano, 20133 Milan, Italy
‡Istituto di Biologia e Biotechnologia Agraria, Consiglio Nazionale delle Ricerche, 26900 Lodi, Italy

ABSTRACT

Reverse transcription (RT) quantitative real-time PCR (qPCR) is the most accurate and easy-to-perform technique to measure the expression level of a selected gene of interest by quantifying mRNA transcripts. The use of reference genes is commonly accepted as the most reliable approach to normalize RT-qPCR data and reduce possible errors generated in the quantification of gene expression. The optimal number and choice of reference genes are experimentally validated for specific tissues or cell types and experimental designs. To date, data on qPCR normalization in goats are scarce and the most suitable reference genes in this species have been identified for only a limited number of tissues. The aim of this study was to determine an optimal combination of stably expressed reference genes in caprine milk somatic cells (MSC) from healthy and infected mammary glands. For the purpose, we performed RT-qPCR for 10 commonly used reference genes from various functional classes and then determined their expression level in MSC from goats intramammary challenged with *Staphylococcus aureus* and in MSC from healthy controls, with a view to select genes whose stability would be unaffected under infection conditions. The geNorm and NormFinder algorithms were used for validating the reference genes. Furthermore, to demonstrate the importance of normalization of gene expression with appropriate reference genes, we tested the effect of using a combination of the least stable genes for expression analysis evaluation. On the basis of our evaluation, we recommend the use of a panel of reference genes that should include *G6PD*, *YWHAZ*, and *ACTB* for caprine MSC gene expression profiling. The expression of the 2 genes of interest, pentraxin-related protein (*PTX3*) and secreted phosphoprotein 1 (*SPP1*), was evaluated by RT-qPCR in all samples collected pre- and postinfection, and the recommended reference genes were used to normalize the data. Our study provides a validated panel of optimal reference genes for the identification of genes differentially expressed by qRT-PCR in caprine MSC. Moreover, we provided a set of intron-spanning primer sequences that could be suitable for gene expression experiments using SYBR Green chemistry on other caprine tissues and cells.

Key words: reference gene validation, quantitative reverse transcription-PCR, milk somatic cells, goat

INTRODUCTION

Fluorescence-based quantitative real-time PCR (**qPCR**) has become the standard for nucleic acid quantification. Because of its capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources, together with its combination of speed, sensitivity, and specificity, it is the most reliable and easy-to-perform technique to measure the expression level of a selected gene of interest (**GOI**) by quantifying mRNA transcripts (Bustin et al., 2009). Nevertheless, several variables associated with the different steps of reverse transcription (**RT**)-qPCR experimental procedures can lead to considerable intersample variation and possibly to erroneous results when comparing mRNA concentration across samples: the different amount and quality of starting material; RNA integrity; efficiency of cDNA synthesis and PCR amplification; and differences between tissues or cells in overall transcriptional activity (Vandesompele et al., 2002; Bionaz and Loor, 2007).

The reference genes or materials used for standardization are critical, and any assessment of the validity of an RT-qPCR experiment must also consider the appropriateness of the relative-quantification reference (Bustin et al., 2009). However, to date, no universal reference genes have been found.

Among the proposed strategies to control for technical and sample variation in RT-qPCR experiments, the use of reference genes is commonly accepted as the most

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[1]Corresponding author: paola.modesto@izsto.it

reliable approach to normalize RT-qPCR data and to reduce possible errors generated in the quantification of gene expression (Huggett et al., 2005). With this normalization strategy, reference genes are used as internal controls and submitted to the same experimental protocol as the GOI. The expression level of the target gene is then normalized according to the values of the internal controls.

Many studies have warned against the use of a single reference gene for gene expression normalization (Suzuki et al., 2000; Vandesompele et al., 2002; Everaert et al., 2011) and have established that genes traditionally thought to be stable for their ubiquitous expression and involvement in cell homeostasis (e.g., *GAPDH*, *ACTB*, *18S rRNA*) are not always the best reference genes, as they show different behaviors across various cell types and tissues and experimental conditions (Schmittgen and Zakrajsek, 2000; Selvey et al., 2001; Peletto et al., 2011). Nevertheless, because these genes are frequently used as a single endogenous control, many molecular analyses still contain qPCR data that are poorly normalized (Selvey et al., 2001; Bustin et al., 2009). Importantly, the use of a single unvalidated reference gene may give rise to biased study results, especially when study conditions are changed or experimental variability is increased (Schmittgen and Zakrajsek, 2000). The increase in reference gene variability becomes even more problematic if genes with relatively small expression differences are studied (Everaert et al., 2011). Vandesompele et al. (2002), for example, demonstrated that errors of up to 20-fold in expression data can be generated by the use of only a single reference gene. Moreover, the use of a single gene to normalize expression is no longer considered sufficient (Goidin et al., 2001; Dheda et al., 2004; Jemiolo and Trappe, 2004).

This implies that the choice for a given reference gene for gene expression normalization could bias relative mRNA expression results and alter study outcome (Everaert et al., 2011). It is clear, therefore, that an ideal reference gene should be stably expressed within the samples to be compared irrespective of experimental conditions or external factors; otherwise, the detection of small changes becomes unfeasible and unreliable (Peletto et al., 2011). Accordingly, the optimal number and choice of reference genes must be experimentally validated for particular tissues or cell types and specific experimental designs (Vandesompele et al., 2002; Andersen et al., 2004; Bustin et al., 2009).

To date, data on qPCR normalization in goats are scarce and no information is available on milk somatic cells (**MSC**) in this species. The most suitable reference genes in goats have been identified only for the preantral follicles, mammary gland, adipose tissue, muscle, and liver (Finot et al., 2011; Frota et al., 2011; Bonnet et al., 2013). Reverse transcription-qPCR studies to evaluate gene expression in other tissues and cells (e.g., chondrocytes, central nervous system cells, MSC, germ cells) are reported, but no experiments have been carried out in such contexts to identify suitable reference genes (Abdulmawjood et al., 2005; Pisoni et al., 2010; Vonk et al., 2010; Ren et al., 2011).

Moreover, analysis of the mRNA from MSC can be useful to investigate the transcriptional status of the mammary gland of an animal in relation to its genotype, nutritional, and pathologic status, and under the influence of hormonal factors (Boutinaud et al., 2002). So far, MSC have been used for gene expression analysis in cows (Murrieta et al., 2005; Lee et al., 2006; Tao and Mallard, 2007; Fonseca et al., 2009; Wickramasinghe et al., 2011, 2012), sheep (Bonnefont et al., 2011), and goats (Pisoni et al., 2010; Cremonesi et al., 2012).

The aim of this study was to determine an optimal combination of stably expressed reference genes in caprine MSC comparing healthy and infected mammary glands, to select genes whose stability was unaffected under inflammation conditions. In addition, the effects of using suboptimal combinations of reference genes for expression analysis were tested.

For the purpose, we performed RT-qPCR for 10 commonly used reference genes from various functional classes and then determined their expression level in MSC from goats intramammary challenged with *Staphylococcus aureus* and healthy controls, with a view to selecting genes whose stability was unaffected under infection conditions. Reference gene validation was performed using geNorm and NormFinder applets (Vandesompele et al., 2002; Andersen et al., 2004).

MATERIALS AND METHODS

Sample processing and experiments were carried out according to the *Minimum Information for Publication of Quantitative Real-Time PCR Experiments* (MIQE) guidelines (Bustin et al., 2009).

Sample Collection, Nucleic Acid Extraction, and cDNA Synthesis

Foremilk was collected from each left udder half of 10 healthy goats and from the same udder half at 24 and 30 h (hereafter 0, 24, and 30 h) after inoculation of 10^3 cfu of *Staphylococcus aureus*, as part of a larger experiment (Cremonesi et al., 2012). Goats were monitored before and after challenge for intramammary infections (particularly for *Staph. aureus*) by bacteriological analysis and SCC as previously described (Moroni et al., 2005). At the moment of challenge, no inflammation in the udders was present, as indicated

by the absence of symptoms and mastitis pathogens in foremilk samples tested for 11 consecutive days up to experimental challenge and by an SCC <250,000 cells/mL. To assess inflammation status, goats were monitored during experimental challenge by a general health check, udder examination, bacteriological analysis, and SCC (Cremonesi et al., 2012). *Staphylococcus aureus* in milk samples increased until a mean value of 6.1 $\log_{10}$ cfu/mL and the SSC reached a value of $4{,}925 \times 10^3$/mL (Cremonesi et al., 2012). All experimental procedures were performed according to Italian legislation, following approval by the ethics committee of University of Milan (Milan, Italy).

Milk collected aseptically from each mammary gland at each time point was transferred into 50-mL Falcon tubes and immediately centrifuged at 750 × *g* at 4°C for 10 min. After the fat layer and the supernatant were discarded, the cell pellet was suspended in 10 mL of PBS, pH 7.2 (Life Technologies, Foster City, CA) with added EDTA (Promega, Madison, WI). The entire solution (cells + PBS) was recovered and transferred into a new Falcon tube; PBS was added to a final volume of 50 mL to wash the pellet. After centrifugation at 450 × *g* for 10 min, the supernatant was discarded, the pellet was resuspended (according to its size) in 3 to 5 mL of Trizol (Life Technologies) and total RNA was extracted following the manufacturer's instructions. High-quality RNA was obtained through purification with RNeasy MinElute spin column (Qiagen, Carlsbad, CA) and eluted in RNase-free sterile water (Qiagen). Purity and concentration of total RNA were assessed using 2 independent techniques. Purity and concentration of RNA were evaluated by absorbance readings using a NanoDrop spectrophotometer (Agilent, Santa Clara, CA) measuring spectral absorption at 260 and 280 nm. The mean total RNA concentration was 1.96 μg/μL, whereas A_{260}/A_{280} and $A_{260}/_{230}$ ratios ranged from 1.92 to 2.05 and from 2.05 to 2.17, respectively. Therefore, all samples were pure and free from protein and organic pollutants derived from RNA extraction. Quality of RNA was determined with an RNA 6000 Nano LabChip Kit in the Agilent Bioanalyzer 2100 system (Agilent; Supplemental Figure S1; http://dx.doi.org/10.3168/jds.2012-6383). Quality was evaluated using the RNA integrity number (RIN); RIN values ranged from 8.1 to 9.6. The RNA was subsequently stored at −80°C.

To avoid any genomic DNA contamination during RT-qPCR, 500 ng of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions in a final volume of 20 μL. This kit uses a combined oligo(dT)/random hexamer primer strategy for reverse transcription (RT) and ensures complete digestion of genomic DNA by brief incubation of the sample at 42°C with a specific Wipeout buffer before retrotranscription. The cDNA was subsequently stored at −20°C.

Primer Design, RT-qPCR, and Selection of Candidate Reference Genes

Fourteen genes frequently used as references in RT-qPCR gene expression experiments were selected as candidate normalizers (Table 1); moreover, the expression of 2 genes of interest, pentraxin-related protein (*PTX3*) and secreted phosphoprotein 1 (*SPP1*), was evaluated by RT-qPCR in all samples collected pre- and postinfection. Primers for *SDHA*, *G6PD*, *TUBB*, and *18S rRNA* were as previously reported (Garcia-Crespo et al., 2005; Frota et al., 2011). Primer3 software, freely available online (http://bioinfo.ut.ee/primer3/), was used to design the other primers on conserved gene regions after alignment of the caprine sequences available in GenBank with bovine and ovine homologous genes (Table 2). SeqMan software (DNAStar, Madison, WI) was employed to perform all sequence alignments. Primers (Life Technologies) were not designed on exon-exon junctions, but they were selected to produce amplicons spanning 2 or more exons, taking into account the possibility to recognize nonspecific amplification of the genomic DNA. Preliminary PCR assays and sequencing using caprine pooled cDNA and genomic DNA were performed to determinate the exon-intron borders of the caprine genes and to test primer specificity. The exon-intron junctions were defined by aligning and comparing bovine and ovine GenBank sequences with genomic and mRNA sequences of the caprine genes (Supplemental Table S1; http://dx.doi.org/10.3168/jds.2012-6383).

One microliter of cDNA was used in a 25-μL PCR reaction using 2× QuantiFast SYBR Green PCR Master Mix (Qiagen) and 600 n*M* concentrations of each primer. The same batch of cDNA was used to amplify all candidate genes for each sample to control for variance due to different efficiency in cDNA synthesis. The PCR amplification was run on an Mx 3005P QPCR System (Stratagene-Agilent Technologies, Santa Clara CA). Amplification products were loaded and checked on 2% agarose gel, purified with a PCR Clean-Up System (Nucleospin Extract II, Macherey-Nagel, Düren, Germany), and sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, Foster City, CA) following producer's protocols. After purification with Illustra AutoSeq G50 Dye Terminator Removal Kit (GE Healthcare, Piscataway Township, NJ), the labeled amplicons were run on a 3130 Genetic Analyzer (Life Technologies). Finally, the sequences were checked for their specificity using NCBI Blastn

Table 1. Genes selected for the evaluation of internal reference genes for quantitative expression analysis by real-time PCR in somatic cells from goat milk

Symbol	Gene name	Biological function	Reference[1]	Species[1]
ACTB	β-Actin	Cytoskeletal structural protein	Hein et al. (2004)	Sheep
B2M	β-2-Microglobulin	Cytoskeletal protein involved in cell locomotion	Velculescu et al. (1999)	Human
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	Laud et al. (2001)	Sheep
HPRT1	Hypoxantine phosphoribosyltransferase 1	Metabolic salvage of purines in mammals	Caradec et al. (2010)	Human
PGK1	Phosphoglycerate kinase 1	Glycolytic enzyme, polymerase α cofactor protein	Falkenberg et al. (2011)	Human
RPL19	Ribosomal protein L19	Member of ribosome proteins	Rodriguez et al. (2012)	Human
SDHA	Succinate dehydrogenase complex	Electron transporter in the tricarboxylic acid cycle and respiratory chain	Swingler et al. (2009)	Human
TFRC	Transferrin receptor	Transferrin receptor	Majidzadeh-A et al. (2011)	Human
YWHAZ	Tyrosine 3-monooxygenase	Signal transduction by binding to phosphorylated serine residues on a variety of signaling molecules	Medina Villaamil et al. (2011)	Human
G6PD	Glucose-6-phosphate dehydrogenase	Cytosolic enzyme producing NADPH	Sanders et al. (2008)	Human
GYPC	Glycophorin C	Membrane glycoprotein	Brown et al. (2007)	Sheep
18S rRNA	18S Ribosomal RNA	Cytosolic small ribosomal subunit, translation	Grubor et al. (2004)	Sheep
UBQ	Ubiquitin	Polyubiquitin precursor	Frota et al. (2011)	Goat
TUBB	β-Tubulin	Major constituent of microtubules	Dupont-Versteegden et al. (2008)	Rat

[1]A reference to a study in which each gene was previously used as normalizer and the considered species are indicated.

software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison with sequences available on GenBank. For the primer pairs satisfying the requirements, the optimal concentration that generated the lowest cycle threshold (**Cq**) value and a sharp peak with no amplification of nonspecific products was determined. Preliminary RT-qPCR experiments, carried out to set up optimal reaction conditions, showed that all candidate reference genes were expressed in caprine MSC. Four out of 14 genes did not satisfy the requirements and were excluded from further analysis. Ten primer pairs generated melting curve profiles specific to cDNA and genomic DNA amplification. For each pair of primers, efficiency of RT-qPCR (E), median Cq value, and correlation coefficient (r) were determined using serial 1:10 dilutions of pooled cDNA on an Mx 3005P QPCR System (Table 2).

Retrotranscribed total RNA from the MSC isolated at each time point (0, 24, and 30 h) from the studied goats (n = 10) was amplified by RT-qPCR, using SYBR Green detection chemistry, run in triplicate in 96-well plates using the Mx 3005P QPCR system. Reactions were carried out in a total volume of 25 μL, containing 2 μL of cDNA, 1.5 μL of each 10 μ*M* primer (600 n*M* each), 12.5 μL of 2× QuantiFast SYBR Green PCR Master Mix, and 7.5 μL of RNase- and DNase-free sterile water. Nontemplate control was included in every run to exclude possible DNA contamination. Absence of genomic DNA contamination was verified by control reactions without reverse transcriptase. The cycle conditions were set as follows: initial template denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and elongation with measurement of fluorescence at 72°C for 30 s. The *TUBB* gene was quantified using an annealing and elongation temperature of 58°C for 45 s. The cycles were followed by a melting curve analysis of 95°C for 1 min, 55°C for 30 s, and a slow increase to 95°C with continuous fluorescent acquisition.

Data Analysis

Baseline and threshold values were automatically determined for all plates using MxPro QPCR software (Stratagene-Agilent Technologies). Raw Cq values were transformed into quantities (Q) by the comparative Ct method: $Q = E^{(\text{Min Cq} - \text{Sample Cq})}$, where min Cq is the lower Cq detected for each gene in a panel of samples. The RT-qPCR data were analyzed for reference gene expression stability using 2 different statistical algorithms: geNorm (version 3.5; Vandesompele et al., 2002) and NormFinder (version 0.953; Andersen et al., 2004) according to the developers' recommendations and using the default settings.

Table 2. Details of primers and amplicons for each of the 10 evaluated genes and of the 2 genes of interest quantified[1]

Gene	Forward primer sequence (5′→3′)	Spanned exons	Amplicon length (bp)	Ta (°C)	Median Cq	E%	r	Reference/GenBank accession number
ACTB	CTTCCAGCCGTCCTTCCT	2nd	105	56	19.71	102.9	0.997	*Capra hircus* JX046106
	TGTTGGCATACAGGTCCTTTC	3rd						
GAPDH	GGGTCATCATCTCTGCACCT	7th	213	56	19.6	95.6	0.998	*Capra hircus* AJ431207
	ACAGTCTTCTGGGTGGCAGT	8th						
G6PD	TGACCTATGGCAACCGATACAA	10th	76	56	24.92	94.6	0.997	Garcia-Crespo et al. (2005)
	CCGCAAAAGACATCCAGGAT	11th						
PGK1	GGAAGGGAAGGGAAAAGATGC	4th	92	56	21.55	107.7	0.994	*Bos taurus* AC000187
	TCCCCTAGCTTGGAAAGTGA	5th						*Ovis aries* NC019484
18S rRNA	TTTGGTGACTCTAGATAACCTCGGGC	2nd	184	56	11.58	99.1	0.999	Frota et al. (2011)
	TCCTTGGATGTGGTAGCCGTTTCT	2nd						
RPL13A	CCCTGGAGGAGAAGAGAAAGG	7th	104	56	18.27	109.8	0.999	*Bos taurus* AC000175
	AATTTTCTTCTCGATGTTCTTTTCG	8th						*Ovis aries* NC019471
SDHA	CATCCACTACATGACGGAGCA	4th	84	56	26.61	99.9	0.985	Garcia-Crespo et al. (2005)
	ATCTTGCCATCTTCAGTTCTGCTA	5th						
YWHAZ	CTGAACTCCCCTGAGAAAGC	2nd	165	56	23.39	105.5	0.995	*Bos taurus* AC000171
	CTGCTTCAGCTTCGTCTCCT	3rd						*Ovis aries* NC019466
TUBB	TTCATTGGCAACAGCACAGCCA	4th	150	58	22.04	97.2	0.995	Frota et al. (2011)
	TCGTTCATGTTGCTCTCAGCCT	4th						
TFRC	TGGAAAAATCAGTTTTGCTGAA	6th	124	56	25.03	104.9	0.994	*Bos taurus* AC000158
	GTCCAAAAACTGGAAGATTTGC	7th						*Ovis aries* NC019458
PTX3	GATTCTGTTTTGTGCGCTCT	1st	177	56	23.57	93.6	0.998	*Bos taurus* AC000158
	CAGCATGGTGAAGAGCTTGT	2nd						*Ovis aries* NC019458
SPP1	TGAGAATTGCAGTGATTTGC	2nd	148	56	19.18	105.1	0.996	*Capra hircus* EU295699
	TGAGATGGGTCAGGCTTTAG	4th						

[1]All primers were used at a final concentration of 600 n*M*. Efficiency of quantitative reverse transcription-PCR (E), annealing temperature (Ta), median cycle threshold (Cq) value, and correlation coefficient (r) are also reported. Species and accession numbers of the sequences used for primer design are shown in the last column; references are indicated when primers were retrieved from a previous study.

The geNorm VBA applet for Microsoft Excel (now part of qbase+ software; Biogazelle, Zwijnaarde, Belgium) determines the 2 most stable reference genes or a combination of multiple stable genes for normalization from a set of tested genes in a given cDNA sample panel. geNorm calculates the gene expression stability measure (**M**) for a reference gene as the average pairwise variation (V) for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows for ranking of the tested genes according to their expression stability from the most stable (lowest M values) to the least stable (highest M values). Moreover, geNorm provides the optimal number of reference genes for normalization in a tested sample panel.

NormFinder is an algorithm for identifying the optimal normalization gene among a set of candidates. It ranks the set of candidate normalization genes according to their expression stability in a given sample set and given experimental design. The algorithm allows for estimating not only overall expression variation of the candidate normalization genes, but also the variation between sample subgroups of the sample set (e.g., normal and cancer samples). NormFinder calculates both intragroup variation, describing the stability of the gene expressions within each group, as well as the intergroup variation, which describes the stability of the gene expressions between the groups. For each candidate, the intergroup variation can be depicted as the difference between the 2 groups, and the intragroup variation can be depicted as a confidence interval for this difference (Andersen et al., 2004). The result is a pair of reference genes optimal for both groups. The resulting pair might have compensating expression, so that one gene, for example, is slightly overexpressed in one group, but the other gene is correspondingly underexpressed in the same group. Hence, the optimum pair may not include the optimum single gene, although it usually does.

Normalization of Relative Quantities of Transcripts of GOI in MSC

The 3 most stable genes identified by Normfinder and geNorm were used to normalize the data. This number was based on the geNorm outputs and fitted the recommendations proposed by Vandesompele et al. (2002). A further analysis was then carried out to evaluate the effect of the use of the 3 least stable genes on quantification.

Total RNA extracted from MSC isolated at 3 time points (0, 24, and 30 h) was used to analyze the expression level of the *PTX3* and *SPP1* genes. Primer design and RT-qPCR reactions were carried out using the same protocols described in the section "RT-qPCR Primer Design and Selection of Candidate Reference Genes."

The software REST2009 (version 2.0.13; Pfaffl et al., 2002) was used to analyze the gene expression data of the GOI using the 3 most stable reference genes and 2 least stable genes. This software applies a mathematical model that takes into account the different PCR efficiencies of the GOI and reference genes. It provides statistical information suitable for comparing expression in groups of treated and untreated samples in a robust manner. The integrated randomization and bootstrapping methods in this software test the statistical significance of differences in the calculated expression ratios and can be used even when the data include outliers. The expression ratio results of the investigated transcripts are tested for significance by a pair-wise fixed reallocation randomization test and plotted using standard error (SE) estimation via a complex Taylor algorithm. The analyses of experimental data were carried out by setting default values for randomization and bootstrapping.

The gene expression data of *PTX3* and *SPP1* evaluated by RT-qPCR were compared with the results obtained by microarray analysis of the same samples from a previous study (Cremonesi et al., 2012).

RESULTS AND DISCUSSION

Data Analysis

geNorm Analysis. Table 3 reports the data from healthy and infected goats analyzed at different time combinations (0 h plus 24 h; 0 h plus 30 h; 0 h plus 24 h plus 30 h). The M values were used to rank genes on the basis of their stability, wherein high M values indicate increased gene expression variability and the most stable genes should exhibit M values <1.5 (Vandesompele et al., 2002). However, recent studies suggest that adequate data analysis should be done using M values <1, in general, to compare minor differences in gene expression (Hellemans et al., 2007). Most of the genes reached an acceptable stable expression with low M values, below the limit of $M = 1$.

To determine the optimal number of reference genes needed to calculate a normalization factor, geNorm measures the pairwise variation between 2 sequential normalization factors with an increasing number of reference genes. A cut-off value of 0.15 is usually considered acceptable; it indicates that the control gene combination ensures satisfactory stability and that an additional gene need not be included. In the panel of the candidate genes studied here, the use of 3 genes as references proved to be sufficient for accurate nor-

Table 3. Candidate reference genes for normalization of quantitative reverse transcription-PCR ranked according to their expression stability (calculated as the average M value after stepwise exclusion of the worst scoring genes) by the geNorm VBA applet[1]

	0 h plus 24 h		0 h plus 30 h		0 h plus 24 h plus 30 h	
Ranking order	Gene symbol	Average M value	Gene symbol	Average M value	Gene symbol	Average M value
1/2	*G6PD/ YWHAZ*	0.631	*ACTB/ YWHAZ*	0.416	*ACTB/ YWHAZ*	0.615
3	*ACTB*	0.760	*G6PD*	0.718	*G6PD*	0.782
4	*TUBB*	0.784	*TUBB*	0.820	*TUBB*	0.832
5	*SDHA*	0.817	*GAPDH*	0.870	*SDHA*	0.878
6	*TFRC*	0.843	*SDHA*	0.922	*18S rRNA*	0.941
7	*18S rRNA*	0.894	*18S rRNA*	0.973	*GAPDH*	1.010
8	*RPL13A*	0.948	*TFRC*	1.040	*TFRC*	1.076
9	*GAPDH*	1.023	*PGK1*	1.139	*RPL13A*	1.157
10	*PGK1*	1.166	*RPL13A*	1.224	*PGK1*	1.293

[1]Analyses were carried out in the samples collected at 0, 24, and 30 h. The data from healthy and infected goats were analyzed at different time point combinations.

malization in all group combinations, yielding V3/4 values (pairwise variation between 2 sequential normalization factors) below the proposed cut-off value of 0.15 (Vandesompele et al., 2002; Supplemental Figure S2; http://dx.doi.org/10.3168/jds.2012-6383). On this basis, the most stable candidate reference genes for normalization of RT-qPCR ranked according to their expression stability were *G6PD/ YWHAZ* (M = 0.631) and *ACTB* (M = 0.760) for the 0 h plus 24 h group, and *ACTB/ YWHAZ* (M = 0.416) and *G6PD* (M = 0.718) for 0 h plus 30 h group. When all samples were analyzed together (0 h plus 24 h plus 30 h group), *ACTB/ YWHAZ* (M = 0.615) and *G6PD* (M = 0.782) represented the optimal reference gene combination.

On average, *ACTB*, *G6PD*, *YWHAZ*, and *TUBB* were found to be stable genes in all 3 combinations of samples; *RPL13A* and *18S rRNA* exhibited similar behavior, being the least stable genes and ranking together in the bottom half of the list in all 3 analyses; *PGK1* ranked at the bottom of the list in the 3 groups.

NormFinder Analysis. NormFinder ranks a set of candidate genes according to their expression stability measure (**ρ**) based on the similarity of their expression profiles, wherein lower values are assigned to the most stable genes. The results of the NormFinder analysis applied to our data are shown in Table 4. In the group including 0 h and 24 h samples, the most stable genes were *G6PD* and *YWHAZ*, with ρ of 0.147 and 0.260, respectively; *ACTB* (ρ = 0.261) constituted an additional stable gene. In the 0 h plus 30 h group, *G6PD* (ρ = 0.187) and *YWHAZ* (ρ = 0.200) occupied the highest positions, and *SDHA* (ρ = 0.204) constituted an additional stable gene. The combinations *SDHA/ YWHAZ* (ρ = 0.115) and *G6PD/ YWHAZ* (ρ = 0.115) were the best combinations in the 0 h plus 24 h and 0 h plus 30 h groups, respectively. In the whole panel of noninfected and infected samples (0 h plus 24 h plus 30 h), the most stable gene was *G6PD* (ρ = 0.170), and the best combination of 2 genes was *G6PD/YWHAZ*, with a stability of 0.144; *RPL13A* showed a low stability value in all 3 combinations of samples. Taking into account the analysis carried out using combined data from all 3 groups of samples that shows the intragroup variation of the reference genes, the ranking appeared to be consistent, if not identical, with that determined using geNorm.

NormFinder software allows estimation of not only the overall expression variation of the candidate normalization genes, but also the variation between subgroups of the sample set (i.e., pre-and postinfection samples); Figure 1 shows the intergroup variation of the 10 candidate reference genes. The best candidate genes were those with an intergroup variance as close to zero as possible (*G6PD* and *YWHAZ*). The genes with the maximal intergroup variation were *PGK1*, *TFRC*, and *RPL13A*.

Stability of Reference Genes in Caprine MSC

We examined the expression of 10 genes in caprine MSC by means of 2 commonly accepted software algorithms (geNorm and NormFinder). Both are frequently used and NormFinder is freely available, but they have a different working rationale. NormFinder selects, out of a set of potential reference genes, one single best-performing reference gene that shows the minimum variation within the group analyzed. NormFinder might be less stable than geNorm against outliners and sampling errors (Żyżyńska-Granica and Koziak, 2012). In contrast, because geNorm focuses on pairwise comparisons of reference gene expression in experimental samples, it is less robust toward expression co-regulation of the candidate genes (Andersen et al., 2004). On the other hand, assessment of reference genes based on pairwise

Table 4. Candidate reference genes for normalization of quantitative reverse transcription-PCR listed according to their expression stability (stability value ρ) within all 3 groups of samples calculated by the NormFinder VBA applet[1]

	0 h plus 24 h		0 h plus 30 h		0 h plus 24 h plus 30 h	
Ranking order	Gene	ρ	Gene	ρ	Gene	ρ
1	*G6PD*	0.147	*G6PD*	0.187	*G6PD*	0.170
2	*YWHAZ*	0.260	*YWHAZ*	0.200	*YWHAZ*	0.241
3	*ACTB*	0.261	*SDHA*	0.204	*ACTB*	0.260
4	*SDHA*	0.296	*GAPDH*	0.238	*SDHA*	0.304
5	*18S rRNA*	0.310	*ACTB*	0.246	*18S rRNA*	0.326
6	*TFRC*	0.360	*18S rRNA*	0.317	*GAPDH*	0.375
7	*GAPDH*	0.405	*PGK1*	0.371	*TUBB*	0.381
8	*TUBB*	0.436	*TUBB*	0.412	*RPL13A*	0.492
9	*RPL13A*	0.509	*RPL13A*	0.441	*TFRC*	0.598
10	*PGK1*	0.713	*TFRC*	0.514	*PGK1*	0.62
Best combination	*SDHA/YWHAZ*	0.115	*G6PD/YWHAZ*	0.115	*G6PD/YWHAZ*	0.144

[1]Analyses were carried out in the samples collected at 0, 24, and 30 h. The data from healthy and infected goats were analyzed at different time point combinations.

comparisons takes into account a potential dilution effect on gene expression, as reported in a previous study with bovine mammary tissue, in which mRNA of stably expressed genes decreased during lactation because of a dilution effect brought about by large increases in the expression of highly abundant genes (Bionaz and Loor, 2007). Therefore, the use of more than one type of algorithm for the validation of reference genes is advisable because comparison of reference gene rankings obtained from more than one program will give more reliable results.

To investigate the influence of mammary gland inflammation on the stability of the candidate reference genes, the analyses were performed in pre- and

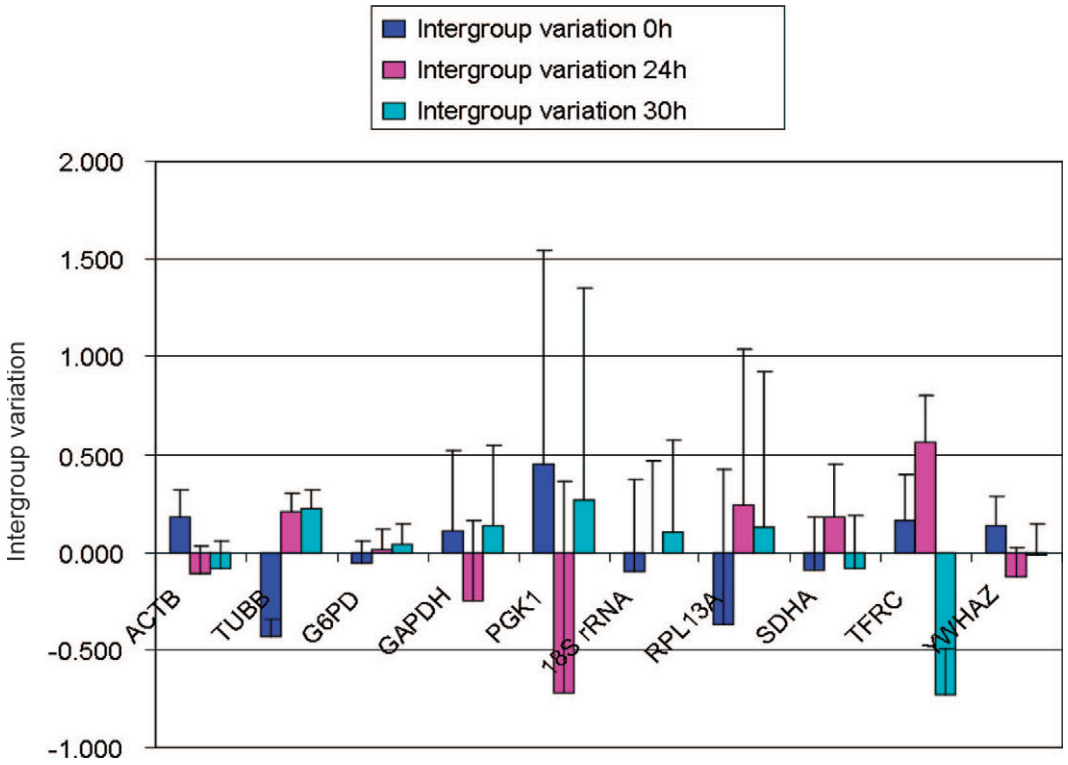

Figure 1. Intergroup variation of the 10 candidate reference genes between the samples collected at 0, 24, and 30 h, according to analysis with NormFinder software (Andersen et al., 2004). The error bars show the confidence intervals on the intergroup variances. The candidate genes picked by NormFinder are those with an intergroup variation as close to zero as possible and, at the same time, having the smallest errors bars. Color version available in the online PDF.

postinfection samples with the aim to uncover genes that were not affected by inflammation. To monitor gene expression stability, 2 different time points (24 and 30 h) after challenge infection were analyzed, and the stability of candidate reference genes was evaluated in different time point combinations. In all groups of samples, the results obtained with geNorm and Normfinder were consistent although not identical, similar to previous reports (Piehler et al., 2010; Peletto et al., 2011; Spalenza et al., 2011). However, the evaluation of all 3 time points together was likely the most useful analysis to determine the optimal set of genes for application in gene expression studies on MSC collected from goats during mammary gland inflammation and from control animals. We combined all data sets for the analysis with geNorm and ran the intra- and intergroup variation estimation function in NormFinder. This software provides, in general, a more precise and robust measure of gene expression stability than geNorm, especially when sample subgroups exist. It relies on a model-based approach, which entails application of a mathematical model to describe the expression values measured by RT-qPCR, to separate analysis of the sample subgroups, to estimate both the intra- and interexpression variation, and to calculate the candidate gene stability value. Nevertheless, the results obtained by both algorithms were consistent (Tables 3 and 4), with *G6PD*, *YWHAZ*, and *ACTB* being the best combination of reference genes for gene expression studies in caprine MSC in pre- and postinfection goats (M = 0.615–0.782; ρ = 0.170–0.260). In contrast, *RPL13A*, *TFRC*, and *PGK1* were the least stable genes in both the geNorm and the NormFinder analysis (M = 1.076–1.293; ρ = 0.492–0.620), suggesting that inflammatory status influenced the expression stability of these genes, as was particularly evident for the genes ranking at the bottom of the list.

Standardization of reporting procedures and, indeed, reporting of a minimum amount of relevant technical information of molecular strategies is of paramount importance to guarantee the reliability of gene expression studies in human and animal science (Bustin and Penning, 2012). Few studies to date have attempted to validate suitable reference genes in goats; this makes comparison between them difficult because of the differences in the number and type of genes evaluated in different tissues and physiological conditions. In a previous study on caprine mammary tissue, the most stable reference genes were the 2 ribosomal protein genes—ribosomal protein large, P0 (*RPL0*) and *18S rRNA*—whereas *GAPDH* and *ACTB* were the least stable genes (Finot et al., 2011). In another study, carried out on goat preantral follicles, *18S rRNA* was one of the least stable genes, as ranked by geNorm, whereas *ACTB* and ubiquitin were the most stable genes, and *PGK1* and *TUBB* held a middle position in the ranking list (Frota et al., 2011). More recently, Bonnet et al. (2013) analyzed 8 candidate genes (none in common with our study) using the geNorm procedure to determine the most stable reference genes in bovine and caprine adipose tissue, muscle, liver, and mammary gland. The authors proposed 29 sets of reference genes that differed depending on the tissue and species. Because the only 3 studies that have evaluated reference genes in caprine tissue (Finot et al., 2011; Frota et al., 2011; Bonnet et al., 2013) did not test the *G6PD* and *YWHAZ* genes, no comparisons with our study are possible.

In a recent study on transcriptomic analysis of MSC in sheep (Bonnefont et al., 2011), the stability of 7 reference genes was assessed, and the most stable genes selected by geNorm analysis for the normalization of RT-qPCR were *RPL19*, *HPRT*, *SDHA*, and *GAPDH*.

Normalization of Relative Quantities of Transcripts of GOI in MSC

Changes in the expression of *PTX3* and of *SPP1* were quantified by RT-qPCR, and samples collected at 0, 24, and 30 h postinfection were compared using *G6PD*, *YWHAZ*, and *ACTB* as the reference genes. The data were analyzed by REST2009 software, and the results for *PTX3* and *SPP1* are reported in Table 5 and Figure 2. Previous analysis (Cremonesi et al., 2012) of the same samples using microarrays showed a significant change in the expression of these 2 genes, with *PTX3* being upregulated in samples at both 24 and 30 h (log fold-changes of 5.66 and 5.35, respectively; $P < 0.01$) and *SPP1* being downregulated in samples at 24 h (fold-change of −2.47; $P < 0.01$), but the change in its expression level in samples at 30 h was not considered significant (log fold-change <1.5; $P < 0.01$). The REST2009 software determines whether a significant difference exists between samples and controls, while taking into account issues of reaction efficiency and reference gene normalization by using randomization techniques. Analysis of RT-qPCR data by using REST2009 confirmed the significant ($P < 0.05$) differential expression levels of *PTX3* and *SPP1* in samples at both 24 and 30 h.

Comparison between samples collected at 24 h postinfection and control samples (before infection) showed that *PTX3* was upregulated by a mean factor of 62.36 and *SPP1* was downregulated by a mean factor of 0.28. These results are consistent with the data from the microarray analysis showing *PTX3* differentially

Table 5. Differential expression levels between the healthy and infected animals at 24 and 30 h postinfection, using the 3 best and 3 worst reference genes as resulted by geNorm and NormFinder analyses

	Best reference genes						Worst reference genes					
Period	Gene	Type[1]	Reaction efficiency	Expression ratio (infected/healthy)	$P(H1)^2$	Result	Gene	Type	Reaction efficiency	Expression ratio (infected/healthy)	$P(H1)^2$	Result
24 h vs. 0 h												
	G6PD	REF	0.95	0.87			*RPL13A*	REF	1.0	0.31		
	ACTB	REF	1.0	0.91			*PGK1*	REF	1.0	2.06		
	YWHAZ	REF	1.0	1.26			*TFRC*	REF	1.0	1.56		
	PTX3	TRG	0.94	62.36	0.000	↑	*PTX3*	TRG	0.93	116.93	0.000	↑
	SPP1	TRG	1.0	0.28	0.015	↓	*SPP1*	TRG	1.0	0.54	0.361	
30 h vs. 0 h												
	G6PD	REF	0.95	0.82			*RPL13A*	REF	1.0	0.16		
	ACTB	REF	1.0	0.85			*PGK1*	REF	1.0	3.74		
	YWHAZ	REF	1.0	1.44			*TFRC*	REF	1.0	1.72		
	PTX3	TRG	0.94	100.85	0.000	↑	*PTX3*	TRG	0.93	271.28	0.000	↑
	SPP1	TRG	1.0	0.38	0.011	↓	*SPP1*	TRG	1.0	1.08	0.887	

[1]REF = reference; TRG = target.

[2]$P(H1)$ = probability of the alternative hypothesis that the difference between the samples and control groups is due only to chance. $P(H1) < 0.05$ was considered statistically significant.

expressed by a factor of 50.53 and *SPP1* differentially expressed by a factor of 0.18 (Supplemental Figure S3; http://dx.doi.org/10.3168/jds.2012-6383).

Comparison between the samples collected at 30 h postinfection and control samples revealed that *PTX3* was upregulated by a mean factor of 100.85 and *SPP1* was downregulated by a mean factor of 0.38. According to the microarray results, *PTX3* expression was differentially expressed by a factor of 40.67, whereas *SPP1* was not (Supplemental Figure S3; http://dx.doi.org/10.3168/jds.2012-6383). Although the results are consistent, discrepancies between RT-qPCR and microarray analysis have been reported (Lutzow et al., 2008; Swanson et al., 2009; Bonnefont et al., 2011). Amplification-based technology has a wider dynamic range (Allanach et al., 2008) and often results in a greater differential expression factor compared with hybridization-based analysis (Tao and Mallard, 2007; Swanson et al., 2009; Mitterhuemer et al., 2010). The reason may reside in the technical differences between the 2 methods (hybridization vs. amplification) or the different sensitivities of the 2 technologies (Lutzow et al., 2008). Importantly, normalization of microarray data is also carried out differently from normalization of RT-qPCR data (Gyorffy et al., 2009; Git et al., 2010): the former requires global normalization, whereas the latter generally utilizes the expression of one or more reference genes against which all other gene expression is calibrated. Other studies have emphasized that microarray should be applied as a discovery tool rather than for quantitative analysis and that the differentially expressed genes identified by microarray analysis be validated by the more sensitive RT-qPCR method (Rajeevan et al., 2001; Swanson et al., 2009).

To highlight the importance of selecting and using validated reference genes, we carried out the normalization of gene expression RT-qPCR data by REST2009 using 3 less-stable genes: *RPL13A*, *TFRC*, and *PGK1*. The results for samples at 24 and 30 h are shown in Table 5 and Figure 2. The *PTX3* gene was upregulated in samples at both 24 and 30 h; however, it was differentially expressed by a factor of 116.93 and 271.28, respectively, which is 2 to 3 times higher than the values obtained previously. Moreover, according to this analysis, *SPP1* was not differentially expressed in samples at either 24 or 30 h, demonstrating that the choice of the reference gene in data normalization affects the reliability of results and can lead to misinterpretation.

Because very little information is available on qPCR normalization in goats, the main aim of this study was to increase the number of validated reference genes for gene expression studies in caprine MSC. A distinctive strength of our study is the effort put into primer design, with the aim to validate only oligonucleotide se-

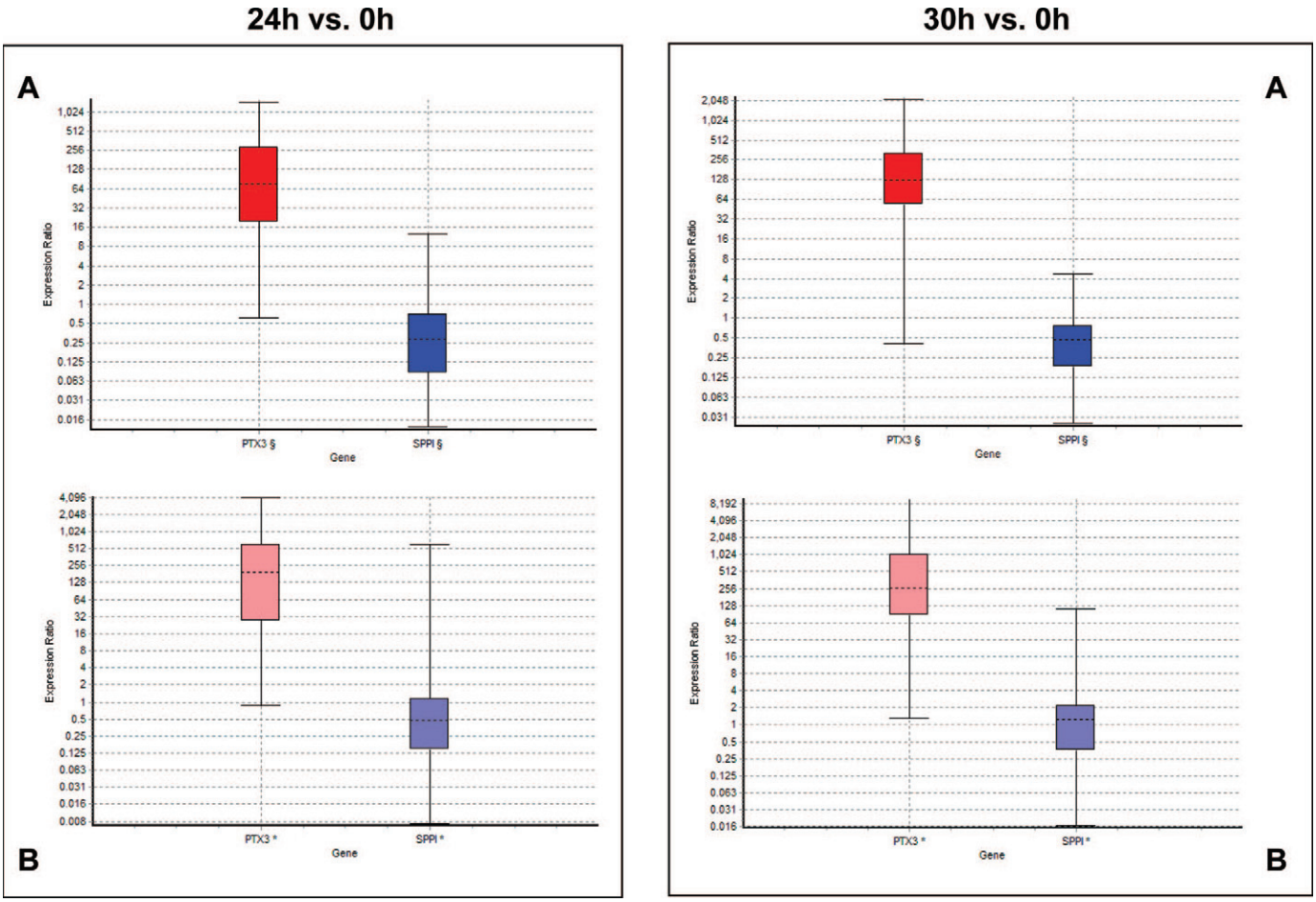

Figure 2. Expression ratios of pentraxin-related protein (*PTX3*) and secreted phosphoprotein 1 (*SPP1*) between samples collected at 24 h versus 0 h (left panel) and 30 h versus 0 h (right panel). Boxes represent the interquartile range, or the middle 50% of observations; the dotted line represents the median gene expression; and whiskers represent the minimum and maximum observations. Boxplot after analysis by REST2009 (Pfaffl et al., 2002), using (A) 3 stable reference genes; (B) 3 not stable reference genes. Color version available in the online PDF.

quences spanning at least one intron. Previous works on reference gene validation in goats did not consider this aspect, probably because of the lack of caprine genomic DNA sequences available in public databases. Indeed, we were able to retrieve intron-spanning primers from previous publications for only 2 genes (*SDHA*, *G6PD*) from among those included in our study. Also, at the time of writing, in RTPrimerDB (http://medgen.ugent.be/rtprimerdb/), a reference database for qRT-PCR primers (Pattyn et al., 2003, 2006; Lefever et al., 2009), 8,603 real-time PCR primer sets for 27 organisms were available, but no assays were deposited under *Capra hircus*. Our strategy of primer design and reaction optimization, although it entailed more effort, ensured specific amplification of mRNA transcripts. This approach is highly recommended in combination with DNase I treatment to avoid or recognize co-amplification of contaminating genomic DNA (Vandesompele et al., 2002).

CONCLUSIONS

The aim of this study was to develop a set of reference genes for normalizing RT-qPCR data from caprine MSC by following MIQE guidelines. Many publications do not provide sufficient experimental details to permit the reader to critically evaluate the quality of the results presented or to repeat the experiments. The MIQE guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. Our results demonstrate the importance of the normalization of gene expression with appropriate reference genes, which should be stably expressed under the conditions of the experiment. This stability must be validated experimentally for each species, tissue, or sample because the choice of the reference gene in data normalization affects the reliability of the

results and can lead to misinterpretation. On the basis of our evaluation, we recommend the use of a panel of reference genes, which should include *G6PD*, *YWHAZ*, and *ACTB* for caprine MSC gene expression profiling. Importantly, change of any experimental condition (e.g., purification of epithelial cells from the somatic cells) can alter the expression ratio between genes and it is essential to validate reference genes in each single study.

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