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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

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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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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