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Reference gene selection for quantitative real-time PCR normalization: Application in the caprine mammary gland

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ABSTRACT

In dairy animals, gene expression analysis has become increasing key to understand the biological processes occurring in mammary gland development that shape future milk potential. Selecting high-stability reference genes is crucial to interpret real-time qPCR data. This study investigated the expression stability of five top-ranked candidate reference genes in the goat mammary gland through three assays comparing different experimental conditions (physiological states, sample types and experimental treatments). The expression stability of genes including *β-actin*, *glyceraldehyde-3-phosphate dehydrogenase*, *18S rRNA*, *cyclophilin A* and *ribosomal protein large P0* was analyzed. Normalization for each experimental condition expression data revealed a different reference gene. Nevertheless, in our various assays, genes encoding for ribosomal proteins, *18S rRNA* and *RPLP0* presented the best expression stability. This result has been confirmed using a combined analysis of stability on the three assays. All genes showed the same distribution within and among the three assays and a different distribution between Ct variability and GeNorm normalization. In addition, the application on *Catenin B1* expression using an inappropriate reference gene confirmed erroneous variations in interpretation. To conclude, there is no single ideal reference gene for caprine mammary gland studies and we recommend using a panel of top-ranked reference genes, including RPLP0, at the beginning of each experiment to validate the most stable(s) gene(s).

1. Introduction

The shape of the lactation curve is determined by the number and secretory activity of mammary epithelial cells (Capuco et al., 2001, 2003). A thorough understanding of the mechanisms involved in mammary gland development is essential for increasing milk potential and lactation persistency and to achieve profitability of dairy farms. Many laboratories have investigated mammary gland function at a molecular level based on physiological models to understand the relations between mammary epithelial cell genetics and milk production (Capuco et al., 2001; Marti et al., 1997; Miller et al., 2005; Sorensen et al., 2008). The development of new powerful molecular biology tools allowing studies on RNA, such as real-time quantitative Polymerase Chain Reaction (RT-qPCR), has made it possible to elucidate gene regulation in the mammary gland.

RT-qPCR is widely used in all kinds of mRNA quantification studies due to its high sensitivity, good reproducibility and dynamic quantification range. In this technique, numerous factors (quality and integrity of RNA, unspecific PCR products, sampling methods, etc.) are sources of variability and can affect interpretation of RT-qPCR results. To control inter-sample differences due to these factors, normalization of RT-qPCR data is required and ensures an

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accurate measurement of gene expression. Actually, the most used method of normalization involves the analysis of target gene expression relative to a reference gene. A good reference gene is assumed to be expressed abundantly at a constant level in most tissues, at all stages of development, without being affected by the experimental treatments.

Historically, genes encoding for β-actin (*ACTB*), a cytoskeletal protein, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a glycolytic enzyme, have been widely used as reference genes. However, numerous studies have highlighted that the expression of these genes may be modulated during cellular and hormones processes (Revillion et al., 2000). It has become clear that data normalization using unstable reference genes can result in erroneous interpretation (Dheda et al., 2005).

Working on mammary gland development in ruminants is hampered by the lack of information on the expression stability of reference genes for RT-qPCR. There are a few published studies on the expression stability of reference genes in cows (De Ketelaere et al., 2006; Goossens et al., 2005; Lisowski et al., 2008; Robinson et al., 2007). Interestingly, Bionaz and Loor (2007) studied the expression stability of reference genes in the bovine mammary gland during the lactation cycle, and identified two genes encoding for ribosomal proteins, Ribosomal Protein S9 (RPS9) and Ribosomal Protein S15 (RPS15) as the most stable genes (Bionaz and Loor, 2007). However, very little is known on goats, and especially on the caprine mammary gland.

The objective of the present study was to evaluate the expression stability of five potential reference genes, i.e. β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, cyclophilin A (PPIA) and ribosomal protein large P0 (RPLP0), in goat mammary gland across different experimental conditions to identify a stable reference gene; and to illustrate the impact of choosing an appropriate reference gene by running data normalization on Catenin b1 expression (CNNTB1), a key gene in our research on mammary gland development, using candidate reference genes.

2. Materials and methods

All animal experimentation was conducted in accord with the relevant guidelines and licensing requirements of animal care (defined by the French Ministry for Agriculture) and approved by the French National Institute of Agricultural Research INRA).

2.1. Animals and tissue preparation

2.1.1. Assay 1: lactating vs. prepubertal goats

Two experiments were conducted on 12 lactating and 24 prepubertal dairy Alpine goats from the experimental farm herd of the French National Institute for Agricultural Research at Le Rheu. All 12 dairy goats were milked twice daily for 2 wk postpartum. Mean stage of lactation was 25 ± 10 d at the beginning of the experiments. Samples of mammary tissue were collected by biopsy (Farr et al., 1996). The 24 prepubertal goats were slaughtered at 7 months of age for mammary gland sampling.

2.1.2. Assay 2: tissue sample vs. digested tissue (cells)

Mammary tissues of goats came from two different sample preparations: dissociation of mammary explants by single-cell suspension using enzymatic digestion (see the tissue dissociation section) or directly extracted mammary explants.

2.1.3. Assay 3: shammed vs. ovariectomized goats

24 alpine goats were assigned to one of two treatments: shammed (sham) or ovariectomized (ovx). Ovary resection concerned half the goats in the experiment, while the other half was only opened and stitched. Samples of mammary tissue were obtained from animals 1 month after surgery, at autopsy, under general anesthesia with subsequent euthanasia (Rompun i.v. 1 cc; Dolethal, i.v. 25 cc). The goat mammary glands were removed within 20 min from slaughter (Dessauge et al., 2009).

2.2. Sampling

Freshly-dissected tissue segments were cut into small pieces (1 g) and immediately frozen in liquid nitrogen for RNA extraction. Small pieces were then stored at −80 °C.

2.3. Tissue dissociation

Pieces of fresh tissue (5 g) were immediately washed after collection in vetedine solution, ethyl alcohol 70%, sterile water baths and sterile Hank's balanced Salt solution (HBSS). The tissues were then ground down minced with a scalpel and transferred to a sterile Erlenmeyer containing 20 ml Tissue Dissociation Solution (HBSS (antibiotic 1×, fungizone 1×, gentamicin 1×, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) complemented with 10 mg/ml collagenase and hyaluronidase. The tissues were gently dissociated on a rotary shaker at 37 °C for 2 h.

After discarding the fat layer, the dissociated tissue was filtered through a nylon filter (0.45 μm) and centrifuged at 450 × *g* for 5 min at 4 °C to pellet the cells. The supernatant was eliminated and the cell pellet was washed twice by resuspending the cells in 15 ml of HBSS followed by a repeat centrifugation step (450 × *g* for 5 min at 4 °C). The cells were then resuspended in 1 ml sterile phosphate buffer saline (PBS) to determine cell concentration and viability using a ViCell apparatus (Beckman Coulter, Roissy, France). Cells were finally pelleted and stored at −80 °C until analysis.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples (50 mg) using Trizol (Invitrogen Life Technologies, Germany) and RNeasy Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Tissue powder was ground in liquid nitrogen with a mortar and pestle and homogenized in 1 ml of Trizol reagent followed by aspiration of the mixture 10 times through a 21-gauge syringe needle. After 5 min incubation at room temperature, 200 μl of chloroform were added to each sample. The mixture was centrifuged at 12,000 × *g* for 15 min at 4 °C and the upper aqueous phase containing total RNA was recovered. The RNeasy Mini kit was used to precipitate and purify the RNA. A DNase digestion step was carried out to remove genomic DNA using an RNase-free DNase kit (Qiagen). The RNA was eluted from spin columns with 30 μl of sterile RNase-free water.

Total RNA concentration and the 260/280 nm and 260/230 nm absorbance ratios were measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France) by RNA profile and measurement of the RNA Integrity Number (RIN) using Agilent 2100 Expert Software, version B.02 (Agilent Technologies). Then, 1 μg of RNA was reverse-transcribed at 42 °C for 1 h using a ThermoScript RT-PCR System (Invitrogen, Germany) according to the manufacturer's protocol for oligo(dT) 15-primed cDNA synthesis. Prior to use in RT-qPCR, cDNA was diluted in diethyl pyrocarbonate (DEPC) water (1:50).

2.5. Quantitative PCR

Real-time PCR analysis was performed on an ABI Prism 7000 sequence detection system (Applied Biosystem, Courtaboeuf, France) using SYBR Green PCR master mix. Reactions were carried out in 96-well optical reaction plates with 200 nM of each specific primer and 2.5 μl of diluted cDNA. Each sample was run in technical triplicates with a non-templated control included.

The RT-qPCR program consisted of an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s and a combined primer annealing–extension step at 60 °C for 1 min in which fluorescence was measured. A melting curve was produced after completion