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Validation of Reference Genes for Real Time PCR Normalization in Milk Somatic Cells of Holstein Dairy Cattle

Research Article

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ABSTRACT

Real time-qPCR is the most reliable method for evaluation of mRNA expression levels. However, to obtain accurate results, selection of suitable reference genes is necessary for normalizing the real-time qPCR data. The aim of this research was to validate the expression stability of three potential reference genes (*ACTB*, *GAPDH* and *UXT*) in milk somatic cells of Holstein dairy cattle under different lactation stages. For this purpose two types of milk samples from eighteen healthy cows at three lactation stages (early, middle and late of lactation cycle) and four mastitic cows were included in this experiment. Total RNA was extracted from the milk somatic cells and then cDNA was synthesized. Real-time polymerase chain reaction (PCR) performed for *ACTB*, *GAPDH* and *UXT* genes as candidate reference genes. Then, the real-time PCR results were analyzed with BestKeeper program. The evaluation of selected genes by real-time PCR revealed that all genes were expressed in the healthy and mastitic dairy cows. In addition, the *UXT* and *GADPH* genes displayed the lowest and highest values of expression level, respectively. The *ACTB* gene was considered as the most suitable internal controls as it was stably expressed in milk somatic cells regardless of dairy cows conditions. Taken together, our results could help to select suitable reference gene for the normalization of expression levels in milk somatic cells of dairy cattle.

KEY WORDS BestKeeper, dairy heifers, milk somatic cell, reference gene.

INTRODUCTION

Quantitative real-time PCR (qPCR) technique is considered to be the most accurate and reliable method for gene expression analysis. It has the advantages of sensitivity, real time detection of reaction progress, speed of analysis and precise quantification of the material in the sample (Gachon *et al.* 2004). The qPCR is a multistage process and the accuracy of obtained results depends on several factors including the quality, stability and input of RNA, the efficiency of reverse transcription, primer performance, reference genes, PCR steps and method chosen for data analysis (Bustin, 2002; Bustin and Nolan, 2004; Pfaffl, 2001; Skern *et al.* 2005; Fleige and Pfaffl, 2006; Derveaux *et al.* 2010). Among them, the choice of suitable reference genes to normalize data is a great importance to obtain accurate results. A suitable reference gene should be expressed at a constant level among samples, and its expression is assumed to be unaffected by the experimental conditions (Bustin, 2002). The use of unsuitable reference genes may lead to errors in quantification and, then, the expression data may lead to misinterpretation. Reference gene validation was carried out in different organs of dairy and beef cattle, such as adipose tissue (Saremi *et al.* 2012), liver,

kidney, pituitary and thyroid (Lisowski *et al.* 2008), milk somatic cells (Varshney *et al.* 2012; Verbeke *et al.* 2015), mammary gland (Bionaz and Loor, 2007; Bougarn *et al.* 2011), oocyte (Macabelli *et al.* 2014; Mahdipour *et al.* 2015) and whole blood samples of cows (Devrim *et al.* 2012; Kishore *et al.* 2013; Kizaki *et al.* 2013).

Several statistical procedures or software packages have been reported to evaluate the stability expression in candidate reference genes, such as geNorm (Vandesompele *et al.* 2002), NormFinder (Andersen *et al.* 2004), BestKeeper (Pfaffl *et al.* 2004) and Stability index (Brunner *et al.* 2004), with the ranking of candidate reference genes depending upon the selected software. Up to now, based on our knowledge, there is no any report for validation of reference genes in milk somatic cells of Holstein dairy heifers. Therefore, the aim of this study was to evaluate the stability of β-actin, related to cell structure (*ACTB*), glyceraldehyde-3-phosphate dehydrogenese, related to carbohydrate metabolism (*GAPDH*) and ubiquitously-expressed transcript, related to activation of transcriptional activation (*UXT*) genes in milk somatic cells of Holstein dairy cows at first lactation under different lactation stages using BestKeeper program.

MATERIALS AND METHODS

Eighteen healthy Holstein dairy cows at first lactation were classified according to their lactation stages (6 at 7-10, 6 at 140-150 and 6 at 290-295 days after parturition). The selection criteria was somatic cell count (SCC) less than 350000/mL milk for early lactation stage and SCC < 100000/mL for middle and late lactation stages. In addition of healthy cows, four dairy cows at first lactation with clinical mastitis were also included in this experiment. In healthy cows, one liter of milk sample representing all four quarters was collected in sterile tubes. The milk samples from cows with mastitis were collected from the quarter with clinical mastitis immediately after the onset of clinical signs and before drug treatment. Then, the milk sample was centrifuged for 20 min at 1500 g at 4 ˚C. The cell pellet was washed in phosphate-buffered saline (PBS) pH 7.4 twice and centrifuged for 20 min at 4 ˚C and 220 *g* according to Liebe (1996). The pellets were resuspended with 500 µL phosphate-buffered saline (PBS)- ethylenediaminetetraacetic acid (EDTA) and kept at -40 ˚C until RNA isolation.

Total RNA was extracted using Denazist kit according to the manufacturer's protocol. All samples were DNase I (Cinnagen) treated to eliminate DNA genomic contamination. RNA quality was assessed by electrophoresis on 1% agarose gel stained with ethidium bromide. All RNA samples were reverse transcribed using *AccuPower® RocketScript™ RT PreMix* kit (Bioneer) and random hexamer primers (Takapozist) according to the manufacture instructions. The final volume was adjusted to 50 µL with RNase free water. The amplified cDNA samples were then stored at -20 ˚C until use in real-time PCR. The primers sequences and their characteristics are shown in Table 1. Real-time PCR using CFX96 (BIORAD, USA) was performed on 3 candidate endogenous control genes for each of the 22 bovine milk cell samples using the HotTaq EvaGreen qPCR kit (Cinnagen). A PCR mix (10 µL) was prepared to give the end concentrations: 5 µL water, 1 µL each of the forward and reverse primers (10 pm), 1 µL of cDNA and 2 µL of *HotTaq EvaGreen* qPCR master mix. All reactions were performed in duplicate. The amplification conditions were based on reference papers (Table 1). In each reaction of real-time PCR, the cycle number at which the fluorescence rises appreciably above the background fluorescence is determined as crossing point (CP). Subsequently, a melting step was performed, consisting of 95 ˚C for 5 s. 65 ˚C for 5 s. and slow heating at a rate of 0.5 ˚C per 5 second up to 95 ˚C, with continuous fluorescence measurement, and finally followed by cooling down to 25 ˚C.

Expression stability of potential references genes are evaluated by BestKeeper program. To identify the most stable reference gene, this program use raw CP values and amplification efficiencies. The stability estimation of reference genes expression is based on the inspection of calculated variations (standard deviation (SD) and coefficient of variation (CV) values). According to the variability observed, reference genes can be ordered from the most stably expressed, exhibiting the lowest variation, to the least stable one, exhibiting the highest variation. Any studied gene with the SD higher than 1 can be considered inconsistent (Pfaffl *et al.* 2004). In addition, pearson correlations between each individual gene and the BestKeeper index (geometric mean between CP values of stable genes) were calculated as the BestKeeper correlation coefficient. Genes with the highest BestKeeper correlation coefficient were considered the most stably expressed (Bonefeld *et al.* 2008; Mehta *et al.* 2010). For each primer, the efficiency of qPCR and R^2 were estimated using a standard curve obtained from a pooled cDNA of all samples serially diluted 10-fold over 6 measuring points with two replications.

RESULTS AND DISCUSSION

The screening of three potential references genes by real-time PCR showed that all genes were expressed in the healthy (at different lactation stages) and mastitic Holstein dairy cows. Gene expression levels of the candidate reference gene (expressed in CP values) are displayed in Table 2. In this study, the expression levels of *UXT* and *GADPH* genes revealed lowest and highest values, respectively.

Table 1 Primers characteristics of 3 potential reference genes in real time PCR

Gene	Primer	Sequence (5'-3')	Length bp	Accession	Reference
ACTB	β-actin.f38 β-actin.r428	CCTTTTACAACGAGCTGCGTGTG ACGTAGCAGAGCTTCTCCTTGATG	391	AH00130	(Lee *et al.* 2006)
GADPH	GADPH.463f GADPH582r	GGCGTGAACCACGAGAAGTATAA CCCTCCACGATGCCAAAGT	120	AF022183	(Leutenegger *et al.* 2000)
UXT	UXT.323F UXT.423R	TGTGGCCCTTGGATATGGTT GGTTGTCGCTGAGCTCTGTG	101	BQ676558	(Bionaz and Loor, 2007)

Gene expression variation was calculated for all three candidate reference genes based on CP-values and displayed as the standard deviation (SD) and coefficient of variation (CV). BestKeeper highlighted *ACTB* as the reference gene with the least overall variation from the three candidate genes with an SD of 0.96 (Table 2), which represents an acceptable 1.95 fold change in expression. The variation in expression of *UXT* and *GADPH* genes was greater than two-fold (SD greater than 1.0). Among the examined three candidate reference genes, *UXT* lies in middle with respect to its stability (Figure 1). Subsequently, pair-wise correlation between genes and also correlation between each gene and the BestKeeper index were calculated (Table 3). Correlations between the three genes ranged from -0.05 for *UXT*/*ACTB* to 0.44 for *GADPH*/*ACTB*. The highest correlation between candidate genes and the BestKeeper index was obtained for *GADPH* gene (0.88) that was as the least stable expressed reference gene. However, the *ACTB* gene also displayed significant correlation with the BestKeeper index (P<0.01).

A number of authors have studied expression profiles of housekeeping genes in milk somatic cells of cattle, goat and yak using qRT-PCR, but based on our knowledge; this is the first report that the validation of housekeeping gene was investigated in Holstein dairy cows at first lactation in three lactation stages. The three candidate housekeeping genes that were evaluated in this experiment were selected from three studies investigating gene expression in milk somatic cells of Holstein dairy cattle (Table 1). PCR amplification products were obtained for three housekeeping genes in all samples but *UXT* was displayed consistently high CP values (greater than 35) in both sample groups and suggesting it is not expressed in sufficient quantity to be used as an effective housekeeping gene in milk somatic cells (Table 2). Very low level of amplification was also observed for *UXT* gene in milk somatic cell of Sahiwal dairy cattle through lactation (Varshney *et al.* 2012). In similar study, *GAPDH* showed the highest expression, whereas the expression of *UXT* was the lowest in mammary epithelial cells of buffalo during all stages of lactation (Yadav *et al.* 2012).

In the present study, *ACTB* was the most stable reference genes in Holstein dairy heifers at different conditions, making it as a suitable reference gene for normalization of real-time PCR data in milk somatic cells.

Actins are the main structural protein of cytoplasm and play important role in cell secretion, motility, cytoplasm flow and cytoskeleton maintenance (Hunter and Garrels, 1977). Verbeke *et al.* (2015) identified ubiquitin C (UBC), ribosomal protein S15a (*RPS15A*) and *ACTB* as the most stable genes based on their expression in bovine milk somatic cells. The expression stability of ribosomal protein L4 (*RPL4*), elongation factor 1 alpha (*EEF1A1*), *GAPDH* and *ACTB* genes were also reported in mammary epithelial cells across different lactation stages of Indian cows (Jatav *et al.* 2016). Nine candidate reference genes including *UXT*, *GAPDH* and *ACTB* were assessed in milk somatic cells of Sahiwal dairy cattle and results revealed that PPP1R11 (Protein phosphatase 1, regulatory (inhibitor) subunit 11), *ACTB*, *UBC* and *GAPDH* were stably expressed genes among all candidate reference genes (Varsheny *et al.* 2012). Jarczak *et al.* (2014) evaluated six potential reference genes and found that peptidylprolyl isomerase A (*PPIA*) and ribosomal phosphoprotein P0 (*RPLP0*) are the most suitable internal controls as they were stably expressed in goat milk somatic cells regardless of disease status. In addition, the expression stability of ten commonly used reference genes such as *ACTB* and *GAPDH* were examined in milk somatic cells from goats in mammary gland challenged with *Staphylococcus aureus* and in milk somatic cells from healthy controls. The Glucose 6-phosphate dehydrogenase (*G6PD*), Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), and *ACTB* gens were recommended as reference genes to normalize the qPCR data (Modesto *et al.* 2013). Bai *et al.* (2014) investigated the transcriptional stability of 10 candidate reference genes in milk somatic cells of lactating yak, including the genes of our study**.** Four genes, ribosomal protein S9 (RPS9), *PPP1R11*, *UXT*, and mitochondrial ribosomal protein L39 (*MRPL39*), were identified as being the most stable genes in milk somatic cells of lactating yak.

Expression evaluation of nine candidate reference genes including *ACTB*, *GAPDH* and *UXT* was investigated in bovine mammary gland during the lactation cycle. *UXT*, *RPS9* and *RPS15* displayed the most expression stability across cow and time (Bionaz and Loor, 2007). Bonnet *et al.* (2013) studied eight candidate reference genes including *UXT* in different bovine and / or caprine tissues.

Table 2 Descriptive statistics of the 3 potential reference genes

Gene*	*UXT*	*GADPH*	*ACTB*
N	22	22	22
GM (CP)	37.04	28.88	32.26
AM (CP)	37.12	28.95	32.29
Min (CP)	32.04	24.03	29.71
Max (CP)	40.76	32.59	33.87
SD (±CP)	1.85	2.00	0.96
CV (% CP)	4.98	6.9	2.99
Min (x-fold)	-32.01	28.85	-5.87
Max (x-fold)	13.45	13.08	3.05
SD (±x-fold)	3.6	4.00	1.95
Ranking	2	3	1
r^2	0.97	0.95	0.98
E (%)	92.3	94.7	97.23

GM: the geometric mean and AM: arithmetic mean.
Min: minimal value and Max: maximal value.
SD: standard deviation and CV: coefficient of variance.
Min (x-fold) and Max (x-fold): the extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient.
SD [± x-fold]: standard deviation of the absolute regulation coefficient.
r2: coefficient of correlation estimated by qPCR.
E: polymerase chain reaction (PCR) efficiency.

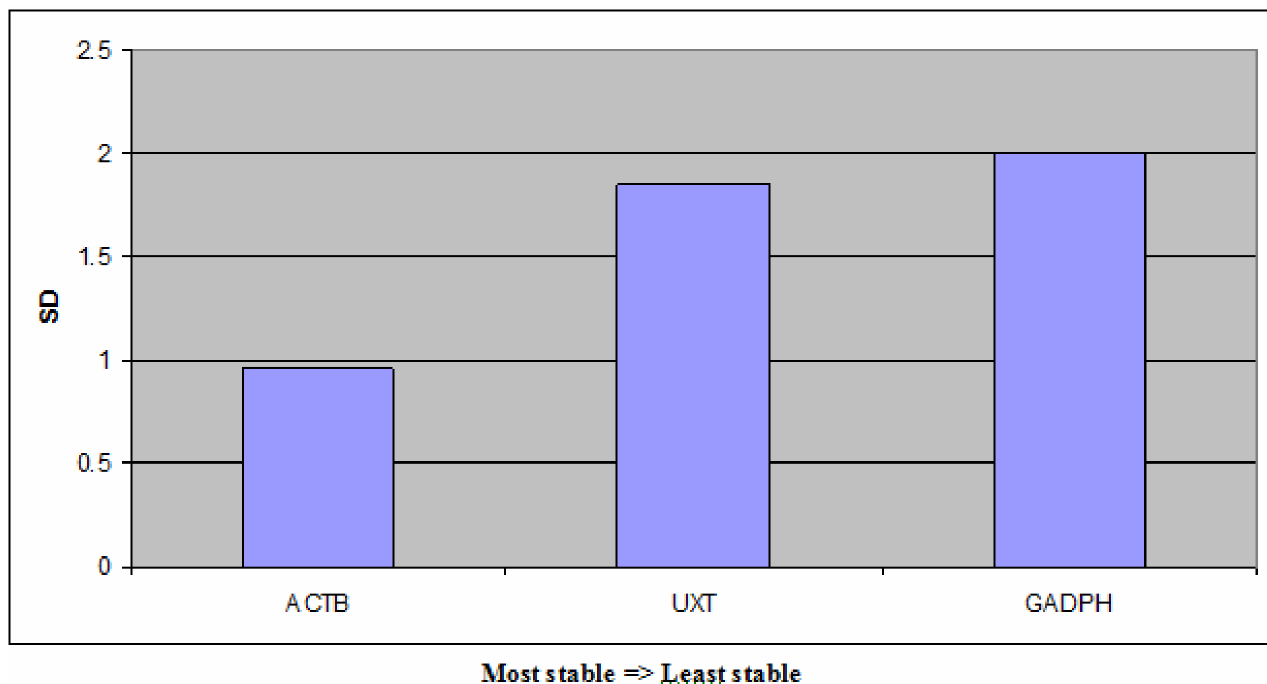

Figure 1 Expression stability of the 3 candidate reference genes in milk somatic cells of dairy heifers

Table 3 Repeated pair-wise correlation analysis of three candidate reference genes and correlation analysis reference genes versus BestKeeper index

Gene	*UXT*	*GADPH*	*ACTB*
GADPH	0.30	-	-
P-value	0.17	-	-
ACTB	-0.05	0.44	-
P-value	0.83	0.05	-
BestKeeper *vs.*	UXT	GADPH	ACTB
r^1	0.57	0.88	0.81
P-value	0.001	0.001	0.001

r: coefficient of correlation.

In bovine, *UXT*, initiation factor 3 subunit K (*EIF3K*) and *TBP* were the most stable genes in mammary gland while in caprine, genes with the highest stability in the mammary gland were *UXT*, *PPIA* and *MRPL39*. The most stable genes in bovine and caprine mammary gland were *UXT*, *EIF3K* and ceroid- lipofuscinosis neuronal 3 (*CLN3*). Finot *et al*. (2011) reported that among six potential reference gene such as *ACTB* and *GAPDH*, the genes encoding for ribosomal proteins, 18S rRNA and *RPLP0* presented the best expression stability in caprine mammary gland. Evaluation of appropriate housekeeping genes in the bovine mammary gland tissue samples and epithelial cell revealed that *UXT* and *GAPDH* were the most stable reference genes (Jedrzejczak and Szatkowska, 2014).

The results of our study showed unstable expression with respect to the one of the most commonly used reference gene, i.e. *GAPDH*. Historically, *GAPDH* gene has been used quite frequently as single endogenous control gene in the most of studies on bovine gene expression. In the other hand, the expression of this reference gene has been shown to highly unstable in bovine mammary gland (Kadegowda *et al*. 2009). Therefore, to correct interpretation of qPCR results, evaluation of *GAPDH* as reference gene in any tissue of interest is mandatory.

CONCLUSION

In conclusion, this study identified that *ACTB* gene can be used as reference genes in genes expression studies on bovine milk somatic cells as it demonstrated stable expression under different experimental conditions.

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