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Origial article

Evaluation of reference genes for studies of gene expression in the bovine liver, kidney, pituitary, and thyroid

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Abstract. Expression patterns of candidate genes with important functions in animal metabolism can help to identify potential molecular markers for cattle production traits. Reverse transcription followed by polymerase chain reaction is a method for rapid and accurate mRNA quantification. However, for exact comparison of mRNA quantity in various samples or tissues, it is important to choose appropriate reference genes. In cattle, little information is available on the expression stability of housekeeping genes (HKGs). The aim of the present study is to develop a set of reference genes that can be used for normalization of concentrations of mRNAs of genes expressed in the bovine liver, kidney, pituitary and thyroid. The study was performed on 6-, 9-, and 12-month-old bulls of dairy and meat cattle breeds. Six HKGs were investigated: *ACTB*, *GAPDH*, *HPRTI*, *SDHA*, *TBP*, and *YWHAZ*. The most stably expressed potential reference HKGs differed among tissues/organs examined: *ACTB*, *TBP*, *YWHAZ*, *GAPDH*, *HPRTI*, and *SDHA* in the liver; *GAPDH* and *YWHAZ* in the kidney; *GAPDH* and *SDHA* in the pituitary; and *TBP* and *HPRTI* in the thyroid. The results showed that the use of a single gene for normalization may lead to relatively large errors, so it is important to use multiple control genes based on a survey of potential reference genes applied to representative samples from specific experimental conditions.

Keywords: cattle, housekeeping genes, normalization, reference genes, real-time PCR.

Introduction

Quantitative real-time polymerase chain reaction (PCR) has been recognized as the most accurate and sensitive method for quantifying mRNA transcripts (Bustin 2002). However, data processing can affect interpretation of real-time PCR results. Reference procedures and various options for data processing are available in several publications (Livak and Schmittgen 2001; Pfaffl 2001; Pfaffl et al. 2002). Real-time PCR is a complex procedure, with 3 main steps needed to be optimized: the standard curve of serial cDNA dilutions, PCR efficiency, and a normalization factor based on reference genes. Moreover, the quality and integrity of nucleic acids, tissue degradation, sampling method, unspecific PCR products, DNA dyes or PCR inhibitors, are critical factors in real-time PCR (Pfaffl 2001; Bustin 2002). The accurate quantification of reference genes enables normalization of differences in the amount of cDNAs of interest in individual samples. These differences could be generated by different quality or amount of a starting material, or differences in RNA preparation and yield of cDNA synthesis. RNA is sensitive to inadequate sample handling and storage (Holland et al. 2003), and the stability of mRNAs may differ between genes. It has been shown experimentally that transcripts of some genes differ in sensitivity to RNA degradation, but even de-

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graded RNA samples may give correct amplification curves in real-time PCR assays (Perez-Novo et al. 2005). In most real-time PCR analyses, only one reference gene is used (e.g. *GAPDH*, *ACTB* or 18S rRNA). However, in several publications (Hamalainen et al. 2001; Deindl et al. 2002; Glare et al. 2002) authors claimed that these genes are unsuitable references for real-time PCR analysis. All genes tested so far are regulated to some extent, and therefore of limited value as quantitative references (Blanquicett et al. 2002). In order to circumvent these problems, numerous other genes should be investigated to choose potential reference genes that are the least regulated under individual experimental conditions (Radonic et al. 2004).

The expression patterns of tissue-specific genes, which have important functions in metabolism, could aid to identify potential molecular markers for cattle production traits, like meat or milk yield and quality. However, in cattle, information on the stability of expression of housekeeping genes (HKGs) is scarce. Although some studies (Rhoads et al. 2003; De Ketelaere et al. 2006; Janovick-Guretzky et al. 2007) have been performed in the bovine liver, leukocytes and mammary gland under different physiological and dietary experimental conditions, the studies were limited to dairy cows during pregnancy or lactation periods.

The aim of the present study was to develop a set of reference genes that can be used for normalizing real-time PCR data from 4 bovine tissues/organs of interest among breeds distinctly different in meat and milk productivity and quality, and among animals differing in age. We designed six PCR assays of potential reference genes belonging to various functional classes (*ACTB*, *GAPDH*, *SDHA*, *YWHAZ*, *HPRTI*, *TBP*) for determination of their expression stability, and normalization purposes in the bovine liver, kidney, pituitary and thyroid. The expression stability of potential reference genes was calculated by the geNorm applet (Vandesompele et al. 2002).

Materials and methods

Animals and tissue collection

Experimental groups consisted of young bulls of 4 breeds of cattle differing in production and utility type: Black-and-White (i.e. Polish Holstein-Friesian, a dairy breed); Polish Red (a dual-purpose breed: combined milk and meat); Limousine (a beef breed of high-meat low-fat production, late-maturing); Hereford (a beef breed, high-meat high-fat production, early-maturing). Bulls were slaughtered at the age of 6, 9 or 12 months (5 animals in each age and breed group, in total 60 animals). Samples were carefully collected from the liver, kidney, anterior pituitary, and thyroid, next flash-frozen in liquid nitrogen, and stored at –80ºC until RNA isolation.

All procedures involving animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals, and were approved by the Local Ethics Commission (permission No. 3/2005).

RNA extraction and reverse transcription

Total RNA from 60 frozen tissue samples was isolated using an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The quantity and quality of RNA preparations were estimated using Nanodrop (USA) and Bioanalyzer (Agilent, USA) equipment.

The reverse transcription (RT) reaction was performed for 1 h at 42°C in 40 µL containing 2 µg of RNA, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.3 mM dNTP mix, 25 U of RNasin, 0.5 µg of oligo $(dT)_{15}$ primer, and 200 U of M-MLV reverse transcriptase (Promega, USA).

Primers and real-time PCR quantification

We selected 6 genes belonging to various functional classes, frequently used as references in real-time PCR gene expression experiments: β-actin (*ACTB*, related to cell structure); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, related to carbohydrate metabolism); succinate dehydrogenase, subunit A (*SDHA*, related to energy metabolism); hypoxanthine phosphoribosyl- transferase I (*HPRTI*, related to nucleotide metabolism); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*, related to cell growth and death); and TATA-box-binding protein (*TBP*, related to transcription).

Primers for *YWHAZ* were based on the paper by Goossens et al. (2005). Other primers were designed using Primer 5 software and bovine GenBank sequences. With the exception of *YWHAZ*, primers were selected to produce amplicons spanning 2 exons. The sequence of primers (including annealing temperature), GenBank accession numbers, and estimated size of PCR products are listed in Table 1.

Table 1. Housekeeping genes evaluated in this study

Gene name	Accession number	Oligo sequence (5'→3')	Product size (bp)	Spanned exons	Annealing temp. (°C)
Beta actin	NC_007326	GAGCGGGAAATCGTCCGTGAC GTGTTGGCGTAGAGGTCCTTGC	278	3–4	60
Glyceraldehyde-3-phosphate dehydrogenase	NC_007303	ACCACTTTGGCATCGTGGAG GGGCCATCCACAGTCTTCTG	75	6–7	60
Hypoxanthine phosphoribosyl-transferase I	NW_001501830	TGCTGAGGATTTGGAGAAGG CAACAGGTCGGCAAAGAACT	154	2–3	58
Succinate dehydrogenase complex, subunit A	NC_007318	GCAGAACCTGATGCTTTGTG CGTAGGAGAGCGTGTGCTT	185	13–14	60
TATA-box-binding protein	NW_001503202	ACAACAGCCTCCCACCCTATGC GTGGAGTCAGTCCTGTGCCGTAA	111	3–4	60
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide	NW_001493253	GCATCCCACAGACTATTTCC GCAAAGACAATGACAGACCA	120	3' UTR	60

The PCR amplification was performed in a 7500 ABI PRISM apparatus (Applied Biosystems, USA) using 96-well optical plates with a SYBR GREEN PCR Master Mix technique (Applied Biosystems, USA). A PCR mix (25 μL) was prepared to give the indicated end concentrations: 11.1 μL of water, 0.2 μL of primers (forward and reverse; 10 μM), 1 μL (100 ng) of cDNA, and 12.5 μL of SYBR GREEN PCR Master Mix. The following amplification program was used: 10 min of denaturation at 95°C, 40 cycles of 4-segment amplification with 15 s at 95°C (for denaturation), 30 s at 58–60°C (for annealing), and 40 s at 72°C for elongation. Annealing temperatures were optimized to individual genes and primers. A dissociation step was added to ensure that the desired amplicon was detected. The dissociation eliminated a non-specific fluorescence signal and ensured accurate quantification of the desired product. Subsequently, a melting step was performed, consisting of 2 s at 95°C, 5 s at 58°C, and slow heating at a rate of 0.1°C per second up to 95°C, with continuous fluorescence measurement, and finally followed by cooling down to 35°C. Standard curves were made for calculating the amplification efficiency during real-time PCR. Five dilutions of each cDNA (1, 5, 25, 125, 625) were primed separately for each tissue/organ and each reference gene.

Data analysis

Gene expression variation was calculated for individual reference genes based on cycle threshold (*Ct*) values and real-time PCR efficiencies (*E*). Real-time *E* was calculated from the given slopes in the 7500 Real Time PCR System software according to the equation: ($E = 10^{-(1/b)} - 1$; where b = regression coefficient). *Ct* and *E* values were entered into the geNorm applet, which then ranks the genes basing on *M* values, where the gene with the most stable expression has the lowest value (Vandesompele et al. 2002). This measure is based on the principle that the expression ratio of 2 ideal control genes is identical in all samples, regardless of the experimental conditions.

Results

Six reference genes were amplified in 4 examined tissues/organs, and all real-time PCR assays produced a single peak in the melting curve. Single-band products of appropriate size were obtained with agarose gel electrophoresis (not shown). Reaction efficiencies, measured using serial dilution of cDNA, were between 92% and 98%. Mean *Ct* values, range of *Ct* values, and reaction efficiencies for the genes in each tissue are shown in Table 2.

The obtained *Ct* and *E* values were used in the geNorm applet to calculate expression stability (*M* value) of the investigated HKGs for each tissue. The *M* values were used to rank the genes on the basis of their stability, from the least to the greatest (Figure 1). The sets of most stably expressed genes differed between the tissues/organs examined. Using the proposed cut-off value of $M = 1$, two HKGs were proposed for accurate normalization of gene expression in quantitative real-time PCR experiments, with the exception of the liver, in which all 6 HKGs appeared suitable. According to the results obtained with the geNorm

Table 2. Cycle threshold (*Ct*) and reaction efficiency (*E*, %) values for individual genes in examined tissues

	ACTB	*GAPDH*	*HPRTI*	*SDHA*	*TBP*	*YWHAZ*
KIDNEY						
Mean *Ct*	21.8	23.3	25.6	29.4	33.2	23.8
Range of *Ct*	18.3–24.7	22.1–25.5	23.3–28.3	27.0-32.4	30.7–36.9	22.7–26.1
E (%)	98	96	92	92	93	96
LIVER						
Mean *Ct*	19.1	22.0	23.5	25.5	29.0	23.8
Range of *Ct*	16.1–20.4	20.9–32.6	21.3–25.7	23.2-27.2	27.4–30.3	21.7–24.6
E (%)	97	98	95	93	92	93
PITUITARY						
Mean *Ct*	25.4	25.1	29.4	33.1	33.7	28.6
Range of *Ct*	21.7–29.9	21.9–29.3	25.3–37.7	29.5–36.1	30.5–36.7	23.6–39.2
E (%)	95	94	92	93	96	94
THYROID						
Mean *Ct*	24.1	23.5	27.8	30.6	32.0	23.9
Range of *Ct*	22.1–33.4	18.4–26.9	25.0–31.8	26.6–37.8	30.5–37.2	22.3–25.6
E (%)	94	94	93	94	93	94

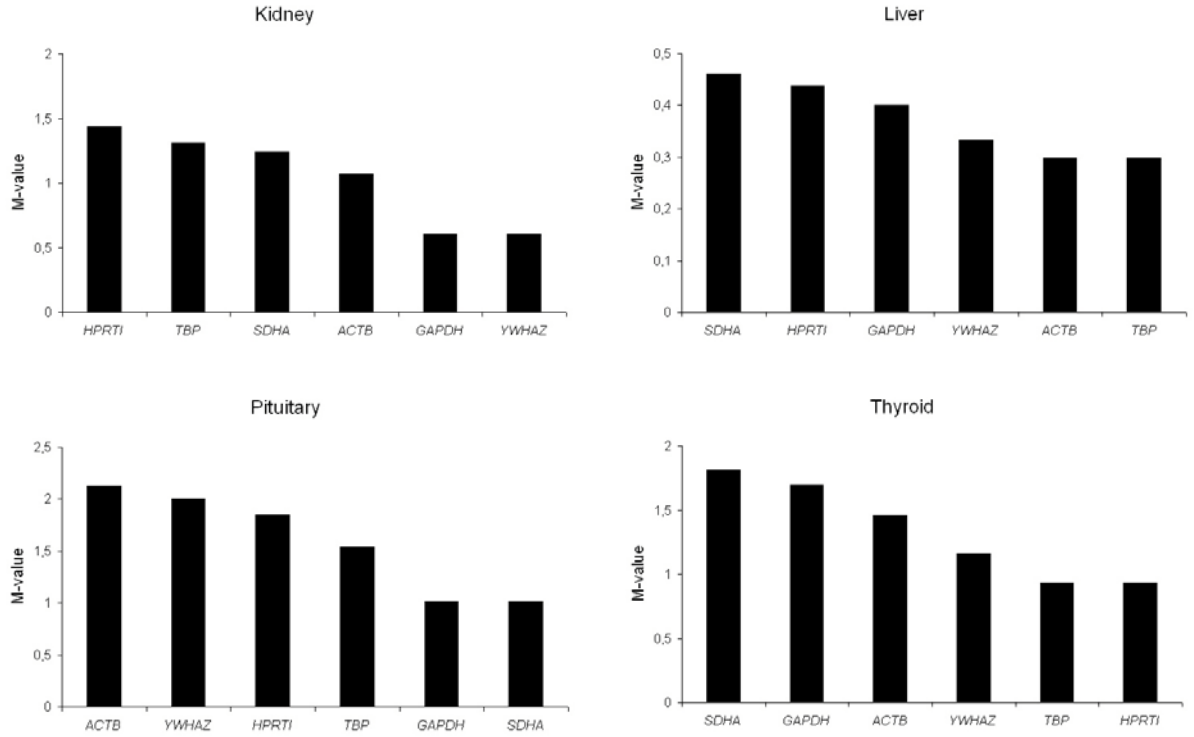

Figure 1. Expression stability (*M*) of housekeeping genes in various bovine tissues analysed with the geNorm software. Genes with the lowest *M* values have the most stable expression.

applet, the most stably expressed genes are (ordered according to their expression stability): *GAPDH*, *YWHAZ* in the kidney; *ACTB*, *TBP*, *YWHAZ*, *GAPDH*, *HPRTI*, and *SDHA* in the liver; *GAPDH* and *SDHA* in the pituitary; *TBP* and *HPRTI* in the thyroid.

Discussion

Numerous studies have shown that HKGs used as references are regulated and vary under specific experimental conditions. This is a fundamental problem for normalization of relative mRNA expression in quantitative real-time PCR experiments, as well as in microarray experiments. Therefore several possible reference genes need to be tested and should pass the same steps of analysis as the gene to be quantified (Pfaffl et al. 2002).

Several studies of reference genes directed towards specific tissues have been carried out for use in some livestock animal species, e.g. pigs and sheep (Garcia-Crespo et al. 2005; Erkens et al. 2006; Svobodová et al. 2008). In cattle, studies of expression stability of HKGs were performed in the polymorphonuclear leukocytes, where *SDHA*,

YWHAZ, and 18S rRNA were selected as being the most stable genes for accurate normalization of real-time results (De Ketelaere et al. 2006). In the developing bovine embryo the *YWHAZ*, *GAPDH*, and *SDHA* genes were found to be the most stably expressed and were recommended as references to study embryo gene expression (Goossens et al. 2005). Among the 9 genes analysed in the cow's mammary gland during lactation, UXT, RPS9, and RPS15 had the most stable expression ratios across cows and time (Bionaz and Loor 2007). In cattle subject to parasitic challenge with ticks, *ACTB* and *GAPDH* appeared the most suitable reference genes in blood (Robinson et al. 2007).

The present study provides the first detailed analysis of the stability and expression levels of HKGs in various bovine tissues/organs. Six reference genes were examined in 4 different bovine tissues. The expression stability (*M* values) of the investigated genes varied among examined tissues/organs, and therefore no single set of HKGs can be used for normalization in all the tissues examined. Furthermore, the level of expression (mean *Ct* values) of each gene varied, as *ACTB*, *GAPDH* and *YWHAZ* tended to be the most highly expressed and *HPRTI*, *SDHA* and *TBP* the least expressed in all the tissues tested (Table 2). *GAPDH* and *ACTB*, commonly used as reference genes, were the least stably expressed in the thyroid and pituitary, respectively. *ACTB* is 1 of 6 different isoforms of the cytoskeletal actin and 1 of 2 non-muscle cytoskeletal actins (Garrels and Gibson 1976), which are involved in cell motility, structure and integrity. Therefore, any changes in cell structure and integrity due to the animals' intensive growth may have also resulted in a change in *ACTB* expression, rendering it an inappropriate reference gene (Deindl et al. 2002; Glare et al. 2002; Radonic et al. 2004; Erkens et al. 2006). In the present study, *GAPDH* appeared the second most highly expressed gene in the tissues examined, which might be due to its important role in carbohydrate metabolism. *GAPDH* is quite unstable and not suitable as a reference gene in the thyroid, but in agreement with results of Garcia-Crespo et al. (2005), it showed the lowest variation among the genes in the kidney, liver and pituitary. The least expressed genes, *SDHA* and *TBP*, presented the worst stability score in the thyroid and kidney, respectively. However, an analysis of the expression stability of these genes showed that *SDHA* could be used for normalization in the liver and pituitary, and *TBP* in the liver and thyroid. According to Nygard et al. (2007) *TBP* is the most relevant for low-abundance transcripts, whereas *ACTB* is suitable for highly abundant transcripts.

In summary, the gene expression results obtained in cattle might be more reliable if they are normalized by using the geometric means of multiple reference genes, as also recommended for cattle by Bionaz and Loor (2007), and for other species by Vandesompele et al. (2002). Our results demonstrated that 2 or more reference genes should be used as controls, to validate gene expression data from the investigated tissues/organs of bulls belonging to breeds differing in meat and milk performance potential and in various periods of individual development of intensive body and muscle growth.

Profiling of gene expression experiments are carried out in many laboratories. Search for genes differentially expressed among various breeds, genotypes, animals differing in milk or meat production capacity, has a potential to provide new tools for marker-assisted selection in cattle, e.g. by discovering the so-called expression quantitative trait loci – eQTLs (Hocquette et al. 2007). Studying differential gene expression with real-time PCR or microarray techniques requires using stably expressed reference genes. Results of the present study may provide new information on the HKGs to be used as references in gene expression experiments carried out with bovine tissues.

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