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Vet J. Vol.189(3):278-83. 2011
doi: 10.1016/j.tvjl.2010.11.017.

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Abstract

Gene expression studies in blood cells, particularly lymphocytes, are useful for monitoring potential exposure to toxicants or environmental pollutants in humans and livestock species. Quantitative PCR is the method of choice for obtaining accurate quantification of mRNA transcripts although variations in the amount of starting material, enzymatic efficiency, and the presence of inhibitors can lead to evaluation errors. As a result, normalization of data is of crucial importance. The most common approach is the use of endogenous reference genes as an internal control, whose expression should ideally not vary among individuals and under different experimental conditions. The accurate selection of reference genes is therefore an important step in interpreting quantitative PCR studies.

Since no systematic investigation in bovine lymphocytes has been performed, the aim of the present study was to assess the expression stability of seven candidate reference genes in circulating lympho- cytes collected from 15 dairy cows. Following the characterization by flow cytometric analysis of the cell populations obtained from blood through a density gradient procedure, three popular softwares were used to evaluate the gene expression data. The results showed that two genes are sufficient for normalization of quantitative PCR studies in cattle lymphocytes and that YWAHZ, S24 and PPIA are the most stable genes.

Introduction

Quantitative PCR (q-PCR), also known as real-time PCR, has be- come the method of choice for quantifying mRNA transcripts due to its high sensitivity, reproducibility and large dynamic range. In addition, q-PCR is fast, easy to perform and provides simultaneous measurement of a limited number of genes in many different sam- ples (Bustin, 2002; Wong and Medrano, 2005). However, analysis of gene expression data requires accurate normalization in order to correct for inter-sample variation generated by several critical factors. These include the amount and quality of starting material, RNA integrity, yield in cDNA synthesis and differences between tissues or cells in overall transcriptional activity (Vandesompele et al., 2002).

A number of strategies have been proposed to control some of these variables, such as normalization against cell number, sample size (solid tissue) or RNA quantity. So far, the most reliable and common approach is the use of appropriate internal control genes (ICGs), since it takes into account the differences attributable to initial RNA quantity, RNA handling and variation in kinetics of the re- verse transcription reaction (Huggett et al., 2005). Ideally, the expression of an ICG should be stable within the samples to be com- pared regardless of tissue differences, developmental stage, physiological or pathological conditions, or experimental treatments.

Genes thought to retain this feature were initially selected among those ubiquitously expressed and involved in cell homeostasis, such as glyceraldehyde 3-phosphate dehydrogenase (GAP- DH) and b-actin (ACTB). Nevertheless, it is very difficult to find universal reference genes stably expressed across different cell types or tissues (Rubie et al., 2005; Lisowski et al., 2008; Kessler et al., 2009), because gene expression can be considerably affected by experimental conditions, biological processes and even different cell types (Bustin, 2000; Schmittgen and Zakrajsek, 2000; Radonic´ et al., 2004). This variation can seriously influence the interpreta- tion of data and result in false conclusions. Accordingly, a proper evaluation of several candidate ICGs should be performed before any gene expression study to select the most appropriate one in the system investigated and under individual experimental set- tings (Huggett et al., 2005). Moreover, Vandesompele et al. (2002) demonstrated that the common practice of single control normalization

leads to relatively large errors and suggested the use of multiple ICGs especially if precise measurements are required.
To assess the suitability of different reference genes, a number of mathematical algorithms have been developed and are freely available. GeNorm ranks candidate genes by determining their expression stability as the average pairwise variation for each gene with all the others tested; then a normalization factor (NF) is cal- culated based on the geometric mean of the cDNA expression of a user-defined number of ICGs (Vandesompele et al., 2002). Best- Keeper also selects the optimal normalizer, but utilizes raw data instead of relative quantities expressed as copy number (Pfaffl et al., 2004). Finally, NormFinder uses a model-based approach able to choose the more appropriate gene, taking into account inter- and intra-group variations and avoiding artificial selection of co-regulated genes (Andersen et al., 2004).
Circulating lymphocytes are easily accessible cells for the development of minimally invasive assays to screen for toxicant expo- sure in humans and other species. Lymphocytes express several xenobiotic metabolizing enzymes (XMEs), especially cytochromes P450 (CYPs). CPYs are involved in the biotransformation of a wide variety of compounds, including environmental pollutants (Spencer et al., 1999; Dey et al., 2001; Nohara et al., 2006) which, in turn, may regulate the expression of CYPs or other XMEs in a way similar to that occurring in the liver, the major site of drug metabolism. Lymphocytes may therefore be considered a valuable surrogate to monitor hepatic enzymatic levels and to evaluate and characterize the exposure level of the whole organism (Furukawa et al., 2004). With the bovine species, the presence of toxic sub- stances in food products (milk, meat) is potentially dangerous for humans, so assessing exposure to chemical pollutants through minimally invasive procedures could be particularly useful.
Studies aimed at identifying the most suitable ICGs in the bovine species have been performed in muscle, liver, mammary gland, endometrium and blood cells (De Ketelaere et al., 2006; Bionaz and Loor, 2007; Janovick-Guretzky et al., 2007; Robinson et al., 2007; Perez et al., 2008; Kadegowda et al., 2009; Walker et al., 2009). However, no information is available specifically on peripheral lymphocytes. The aim of the present study was to develop a set of ICGs that can be used for normalizing q-PCR data from circulating lymphocytes. We designed seven PCR assays for commonly employed reference genes belonging to various functional classes and determined their expression stability in cells sampled from healthy cows. The validation of the ICGs was performed through the geNorm, NormFinder and BestKeeper applets
(Vandesompele et al., 2002; Andersen et al., 2004; Pfaffl et al.,2004).

Materials and methods

Animals, sample preparation and flow cytometry analysis

Fresh whole blood samples were collected by veniepuncture from 15 healthy, crossbreed dairy cows belonging to three different farms (five animals each). Peripheral blood mononuclear cells (PBMCs) were isolated by the method described by Dey et al. (2001) with slight modifications. In brief, 15 mL of EDTA blood were slowly layered over 15 mL of Histopaque 1077 (Sigma–Aldrich) and centrifuged at 400 g for 30 min at room temperature. The opaque interface containing PBMCs was transferred into a clean centrifuge tube, washed once with phosphate buffer saline (PBS) and re-centrifuged at 250 g for 10 min. To remove residual erythrocyte contamination, cells were incubated for 30 min with a lysing solution containing ammonium chloride (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in H_2O, pH
7.3). Finally, after repeated washing with PBS, the pellet was frozen at 80 C until
RNA extraction.
To assess the quality of lymphocyte separation, PBMCs from 9/15 cows were processed for flow cytometric analysis. Briefly, cell concentration was adjusted to
5000/lL and cell populations were characterized through an indirect labelling pro-

tocol using monoclonal antibodies against CD45 (clone CC1, Serotec) and CD14 (clone CAM36A, VMRD). A minimum of 10,000 events were acquired on a Coulter Epics XL cytometer and analyzed with Expo32 software. The analytical strategy is described in Fig. 1.

RNA extraction and cDNA synthesis

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the man- ufacturer's protocol. Contaminating genomic DNA was removed by treating each sample with DNase I (Qiagen). Purity, concentration and integrity of total RNA were assessed using two independent techniques. RNA purity was evaluated by absorbance readings using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A260/A230 and A260/A280 values range from 1.87 to 2.22 and from
2.08 to 2.16, respectively. RNA concentration and quality was determined with RNA 6000 pico LabChip Kit in the Agilent Bioanalyzer 2100 system. Quality was evaluated using the RNA Integrity Number (RIN) (Schroeder et al., 2006). All the samples had a RIN > 7.
Total RNA (1 lg) was reverse transcribed using SuperScript III First-Strand Syn-
thesis SuperMix for qRT-PCR (Invitrogen) according to the manufacturer's instruc- tions in a final volume of 20 lL. The cDNA was subsequently diluted in nuclease free water and stored at 20 C. Sufficient cDNA was prepared in a single run to
perform the q-PCR experiments for all selected genes.

Selection of genes and primer design

We selected seven genes frequently used as references in q-PCR experiments: ACTB, GAPDH, succinate dehydrogenase complex, subunit A (SDHA), tyrosine 3- monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), hypoxanthine phosphoribosyl-transferase I (HPRT1), ribosomal protein S24 (S24), and peptidylprolyl isomerase A (cyclophilin, PPIA). To reduce possible co-regulation of genes, the genes were selected from different functional classes.
Primers for GAPDH, SDHA, HPRT1, YWHAZ, S24 and PPIA were based on previous publications (Goossens et al., 2005; Bevilacqua et al., 2006; Robinson et al., 2007).
The primers for ACTB were designed on bovine GenBank sequences using Primer 3 Software v. 0.4.0 (Rozen and Skaletsky, 2000). The specificity of primers was tested using a BLAST analysis against the genomic NCBI database. Primers informa- tion including sequences and product sizes are summarized in Table 1.

Quantitative PCR

All PCR reactions were performed in a 20 lL final volume containing 2 POWER SYBR Green PCR Master Mix buffer (Applied Biosystems), 300 nM of each specific primer (900 nM for SDHA) and 5 lL of diluted cDNA. PCR amplification was run on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using
96-well optical plates under the following conditions: 10 min at 95 C for polymer- ase activation, and 40 cycles of 15 s at 95 C and 60 s at 60 C. A melting curve was produced to confirm single gene-specific peaks and to detect primer/dimer formation by heating samples from 60 to 95 C. PCR efficiencies were calculated using a relative standard curve derived from a pooled cDNA mixture (a 10-fold dilution series with four measuring points). Each reaction was run in triplicate and a no-tem- plate control was included using water instead of cDNA.

Analysis of ICG expression stability

q-PCR data were analyzed for reference genes expression stability using three different statistical algorithms: geNorm version 3.5 (Vandesompele et al., 2002), NormFinder version 0.953 (Andersen

et al., 2004), and BestKeeper version 1 (Pfaffl et al., 2004) according to the developers' recommendations. For geNorm and Norm- Finder, raw quantification cycle (Cq) values were converted to relative quantities using the comparative Cq method, whereas input data for BestKeeper were the Cq values and the PCR efficiencies.

Results

Flow cytometry analysis indicated high lymphocyte purity of the separated PBMC with a percentage of lymphocytes ranging from 90.2% to 98% (mean ± SD = 94.3% ± 2.7). A screening of tran- scription profiles of the selected reference genes by q-PCR showed
that all were expressed across all samples. Gene-specific amplification was confirmed by a single peak in melt-curve analysis, except for HPRT1, which was therefore excluded from the study.
The highest expression was obtained with ACTB and S24 with Cq averages of 22.66 and 23.84, respectively, whereas the lowest ex- pressed gene was SDHA with a mean Cq value of 29.45. For all investigated genes, the relative standard curves gave correlation coefficients greater than 0.99 and efficiencies higher than 90%.
To determine the optimal choice and number of ICGs, expression values of the candidate genes were processed in the applications geNorm, NormFinder and BestKeeper.

GeNorm analysis

The expression stability measures (M) of the ICGs as calculated by the geNorm applet are reported in Table 2. High M values indicate increased gene expression variability, whereas the most stable genes should exhibit M values <1.5 (Vandesompele et al., 2002). Initially, YWHAZ was shown to be the most stably expressed gene with an average M value of 0.351, followed by PPIA and S24. However, when we performed the stepwise exclusion of the worst-scoring reference genes, the recalculation of the new M values indicated that S24 and PPIA offered the most suitable gene combination (Fig. 2A).
All candidate genes performed sound displaying values <0.55, although the commonly employed reference genes, ACTB and GAPDH, were the least stable. To determine the optimal number of references genes needed for the NF calculation, geNorm measures the pairwise variation between two sequential NF with increasing number of ICGs. Since an arbitrary cut-off value of
0.15 indicates acceptable stability of the control gene combination (the inclusion of an additional reference is not required), the use of two ICGs (i.e. S24 and PPIA) was determined to be sufficient for accurate normalization (Fig. 2B).

NormFinder analysis

This model-based approach ranks genes according to the similarity of their expression profiles and generates a stability measure (q) that assigns the lower values to the most stable genes. Norm-Finder identified YWHAZ as the least variable gene with a stability value of 0.068 followed by GAPDH (0.105) and S24 (0.113) (Table
2). Like the geNorm results, ACTB performed poorly compared to all the other candidate reference genes, displaying the highest stability value (0.16).

BestKeeper analysis

Initially, BestKeeper calculates the variations (SD and CV) for each investigated gene in the samples providing overall stability in gene expression. The software's developers suggest excluding genes with a SD > 1.0, yet all candidate ICGs under study showed minor fluctuations in expression levels with S24 and YWAHZ exhibiting the lowest SD and CV (Table 3). Further data

processing deter- mined the most stable genes based on the coefficient of correlation (r) to the BestKeeper Index, which is the geometric mean of Cq vaues of the candidate ICGs. The best correlations were obtained for YWHAZ, GAPDH, and PPIA with P = 0.001 (Table 2). Although ACTB was ranked as the fourth stable gene, it had the highest SD and thus was considered the worst reference gene.

The consistency and reliability of the BestKeeper Index was further assessed through the analysis of sample integrity among the investigated genes. The intrinsic variance (InVar) of all samples displayed low Cq variation, as well as a fold expression regulation
<0.5 (data not shown).

Discussion

The selection of ICGs is critical for the interpretation of expression data generated by q-PCR. It is difficult to identify universal reference genes and validation of the chosen reference genes is necessary for each experimental setting (Rubie et al., 2005; Lisowski et al., 2008; Kessler et al., 2009). Consequently, numerous studies have been con- ducted to identify reliable ICGs in specific tissues in various species (Nygard et al., 2007; Cappelli et al., 2008; Schlotter et al., 2009). For the bovine species, analyses of expression stability of candidate ref- erence genes have been performed in various types of samples, including muscular tissue (Perez et al., 2008), liver (Janovick- Guretzky et al., 2007), endometrium (Walker et al., 2009), pre- implantation embryos (Goossens et al., 2005), and the lactating mammary gland (Bionaz and Loor, 2007; Kadegowda et al., 2009).

Blood cells are attractive because of their accessibility and use- fulness in monitoring several physiological and pathological condi- tions. An earlier report has determined the most stable genes for q- PCR data analysis in bovine polymorphonuclear (PMN) leukocytes (De Ketelaere et al., 2006). The application of the geNorm software to gene expression data from 16 dairy cows selected SDHA, YWHAZ and 18S ribosomal RNA (18S rRNA) as the most suitable reference genes for these cells. A subsequent study using blood samples from heifers, either uninfected or experimentally infected with the cattle tick Boophilus microplus, determined that ACTB and GAPDH were the most stable genes compared to 18S rRNA and acidic ribosomal protein large (RPLP0) (Robinson et al., 2007).

Although these authors claimed that the experiment was con- ducted on RNA from PBMCs, the method used to isolate cells (lysis with ammonium chloride) has been reported to separate all the leukocyte populations, including granulocytes, whereas the density gradient procedure assures a selective loss of PMN cells (Pelegrí et al., 1995). Our study is the first dealing with the valida- tion of ICGs for bovine peripheral lymphocytes. Indeed, the flow cytometry analysis demonstrated that the cells obtained through Histopaque isolation were almost exclusively composed of lymphocytes.

Seven reference genes were evaluated using three different and commonly accepted programs. Since the geNorm and BestKeeper algorithms seem to be influenced by the co-regulation of genes, due to the use of pairwise comparisons, we selected the candidate references on the basis of the difference in their physiological functions, namely, cytoskeleton (ACTB), carbohydrate metabolism (GAPDH), signalling pathways (YWHAZ), metabolic salvaging of nucleotides (HPRT1), energy metabolism (SDHA), protein synthesis (S24) and protein folding (PPIA).

The results obtained with each software program were quite comparable, albeit not identical, as already reported in studies per- formed in various tissues and species (Anstaett et al., 2010; Mehta et al., 2010; Piehler et al., 2010). YWHAZ and GAPDH were consid- ered the most stably expressed genes according to both BestKeeper and NormFinder, whereas geNorm selected S24 and PPIA as the best reference gene combination. However, S24 and PPIA ranked as the third stable gene in NormFinder and BestKeeper, respec- tively, and the geNorm analysis suggested YWHAZ as a single con- trol gene, although the use of two ICGs is highly recommended.

Our findings were surprising with regard to GAPDH, which can be regulated under a large number of physiological states and is of- ten not considered a good reference gene (Olsvik et al., 2005;

Brattelid et al., 2010). Nevertheless, Shen et al. (2010) and Robinson et al. (2007) have proposed reference gene combinations including GAPDH for normalization. Proper evaluation of this gene stability in the cell type or tissue of interest is therefore mandatory before reporting q-PCR results.
Interestingly, the widely employed reference gene ACTB, along with SDHA, had the lowest expression stability with the three soft- ware programs in contrast to reports on leukocytes isolated follow- ing lysis with ammonium chloride where ACTB has appeared to be the most suitable control gene (Robinson et al., 2007). Taking into account the stability values assigned to each gene by the three algorithms, none of the candidate ICGs exhibited a high degree of variation, suggesting that the expression of the commonly used reference genes in bovine circulating lymphocytes is modulated to a lesser extent by physiological or environmental conditions.

Conclusions

YWAHZ, S24 and PPIA are good ICGs for q-PCR studies of peripheral blood lymphocytes from dairy cows and the geometric mean of two reference genes is an accurate NF. However, the expression stability of ICGs may change in specific experimental conditions and thus their variability should be carefully evaluated in every experimental setting.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This work has been partly supported by the grants of the Regione Piemonte ‘Progetto Ricerca Sanitaria Finalizzata 2008: Ricerca di biomarcatori di esposizione a diossine e policlorobifenili diossino-simili in bovine da latte appartenenti ad allevamenti ad alto rischio’ (P. Sacchi) and ‘Ricerca di biomarcatori di esposizione a diossine e policlorobifenili diossino-simili in specie animali produttrici di alimenti provenienti da allevamenti ad alto rischio di contaminazione’ (C. Nebbia).

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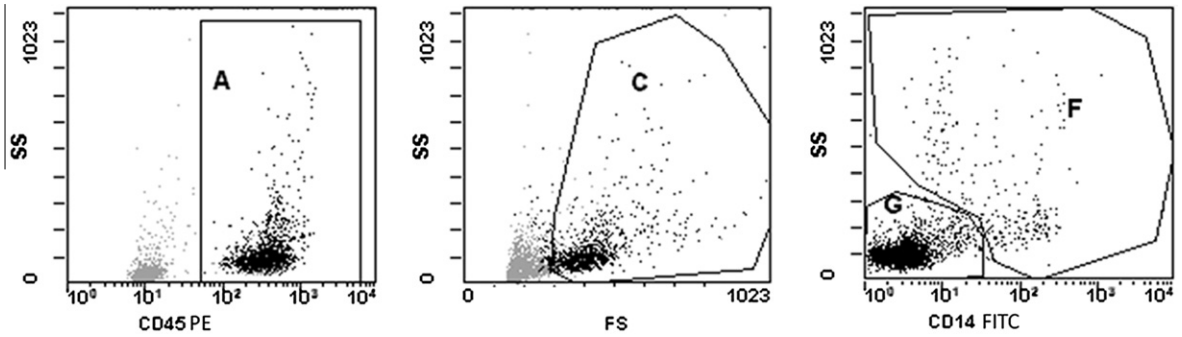

Fig. 1. Representative acquisition dot plots to analyze subpopulations of bovine peripheral blood mononuclear cells. CD45 positivity (region A) was used to define the gate of analysis excluding debris (region C); backgating on C, two different regions were depicted on the basis of side scatter properties and CD14-exposure: F (granulocytes and monocytes) and G (lymphocytes). SS, side scatter; FS, forward scatter; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

Table 1

Candidate reference genes used for quantitative PCR analysis.

Symbol	Full gene name	Primers	Sequence 5′ ‘? 3′	Amplicon size (bp)	Origin
ACTB	b-actin	Forward	CCCAGATCATGTTCGAGACC	95	Primer 3 (v. 0.4.0)
		Reverse	GAGGCATACAGGGACAGCAC		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward	GGCGTGAACCACGAGAAGTATAA	118	Robinson et al. (2007)
		Reverse	CCCTCCACGATGCCAAAGT		
SDHA	Succinate dehydrogenase complex, subunit A	Forward	GCAGAACCTGATGCTTTGTG	185	Goossens et al. (2005)
		Reverse	CGTAGGAGAGCGTGTGCTT		
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Forward	GCATCCCACAGACTATTTCC	120	Goossens et al. (2005)
		Reverse	GCAAAGACAATGACAGACCA		
HPRT1	Hypoxanthine phosphoribosyl-transferase I	Forward	TGCTGAGGATTTGGAGAAGG	154	Goossens et al. (2005)
		Reverse	CAACAGGTCGGCAAAGAACT		
S24	Ribosomal protein S24	Forward	TTTGCCAGCACCAACGTTG	66	Bevilacqua et al. (2006)
		Reverse	AAGGAACGCAAGAACAGAATGAA		
PPIA	Peptidylprolyl isomerase A (cyclophilin A)	Forward	TGACTTCACACGCCATAATGGT	62	Bevilacqua et al. (2006)
		Reverse	CATCATCAAATTTCTCGCCATAGA		

Table 2

Stability ranking of candidate reference genes derived from geNorm, NormFinder and BestKeeper analyses.

geNorm[a]		NormFinder		BestKeeper	
Gene symbol	Stability value (M)	Gene symbol	Stability value (q)	Gene symbol	Stability value (r)
YWHAZ	0.351	YWHAZ	0.068	YWHAZ	0.920
PPIA	0.375	GAPDH	0.105	GAPDH	0.910
S24	0.379	S24	0.113	PPIA	0.899
GAPDH	0.407	PPIA	0.115	ACTB	0.897
SDHA	0.457	SDHA	0.147	S24	0.874
ACTB	0.526	ACTB	0.160	SDHA	0.828

YWHAZ, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; PPIA, Peptidylprolyl isomerase A (cyclophilin A); S24, Ribosomal protein S24; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SDHA, Succinate dehydrogenase complex, subunit A; ACTB, b-actin.

[a] Ranking of the candidate reference genes before the stepwise exclusion analysis.

Table 3

BestKeeper software statistics for candidate reference genes based on quantification cycle (Cq) values.

	ACTB	GAPDH	SDHA	YWHAZ	S24	PPIA
GM (Cq)	22.66	27.62	29.45	26.96	23.84	27.54
AM (Cq)	22.67	27.63	29.46	26.97	23.84	27.54
Min (Cq)	21.68	26.76	28.39	26.24	23.06	26.77
Max (Cq)	23.99	28.83	30.60	27.85	24.43	28.59
SD (±Cq)	0.67	0.45	0.46	0.36	0.33	0.46
CV (%Cq)	2.94	1.63	1.55	1.32	1.40	1.66

Descriptive statistics were calculated for all 15 samples. GM (Cq), geometric mean of Cq; AM (Cq), arithmetic mean of Cq; Min (Cq) and Max (Cq), extreme values of Cq; SD (±Cq), standard deviation of Cq; CV (%Cq), coefficient of variation expressed as a percentage of the Cq level. ACTB, b-actin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SDHA, Succinate dehydrogenase complex, subunit A; YWHAZ, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; S24, Ribosomal protein S24; PPIA, Peptidylprolyl isomerase A (cyclo- philin A).