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Selection of reference genes for gene expression studies in porcine hepatic tissue using quantitative real-time polymerase chain reaction*

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Quantitative real-time polymerase chain reaction (RT-qPCR) has become an indispensable technique for accurate determination of gene expression in variety of samples. Accurate and reliable quantification, however, depends on a proper normalization strategy. Normalization with multiple uniformly expressed reference genes is becoming the standard, although the most suitable reference genes dependent on the used experimental factors as well as the tissue or cell type studied. In this study, the stability of various reference genes was investigated in porcine hepatic tissue. The study was conducted on Polish Large White, Polish Landrace, Pietrain, Pulawska and Duroc pigs slaghtered at different ages. Nine reference genes (*ACTB, B2M, GAPDH, HPRT1, RPL13A, SDHA, TBP, TOP2B* and *YWHAZ*) were investigated on 180 mRNA samples of porcine hepatic tissue. Based on *geNorm* and *NormFinder* analysis, three most stable (*HPRT1, TOP2B* and *TBP*) and three moderately (*GAPDH, ACTB* and *SDHA*) stable reference genes were identified. The study provides a

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new panel of reference genes for normalization of the expression of a gene of interest in porcine liver tissue. It is concluded 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 gene expression profiling studies of candidate genes for economic traits in pigs.

KEY WORDS: gene expression / *geNorm* / liver / *Normfinder* / pigs / reference genes / RT-qPCR

Selection of appropriate reference gene to normalize the quantitative PCR data is usually achieved *via* comparing expression profiles of studied genes to constitutively expressed genes known as reference or housekeeping genes (HKG) – Lee *et al.* [2007]. The normalization is commonly performed against total RNA taken, or against reference genes [Kadegowda *et al.* 2010]. Recently, concern about normalization to proper HKG has increased significantly and methods to select reference genes that are stably expressed under various experimental conditions and tissues of interest have received more attention [Vandesompele *et al.* 2002, Pfafl *et al.* 2004, Gabrielsson *et al.* 2005, Zhang *et al.* 2005]. The RT-qPCR became the method of choice for the quantification of mRNA and it enabled rapid and reliable quantification of mRNA transcription level [Bustin 2000, 2010] involving several optimizing steps.

In general, the expression stability varies greatly between genes, tissues and organisms. A summary of selected reference genes normalized for different porcine tissues, including liver, is presented in Table 1. In pigs, hepatic expression patterns of candidate genes with important functions in animal metabolism can help to identify potential molecular markers for meat quality traits. Porcine liver tissue plays major role in metabolism, energy storage and production of various cytokines and hormones.

Although several authors have normalized the RT-qPCR [Suzuki *et al.* 2000, Radonic *et al.* 2004], the use of a single reference gene still appears fully inadequate, and normalization by multiple controls is an upmost demand [Pfaffl *et al.* 2004]. Bemeur *et al.* [2004] and Lee *et al.* [2005] confirmed that the most commonly used reference genes cannot always be considered as reliable controls as they reveal different behaviour in different tissues [Ohl *et al.* 2005]. In light of this the nine porcine reference genes (Tab. 2) were selected to determine the most stable and appropriate among them for normalization of gene expression study.

Material and methods

Animals

The experimental material was consisted of 72 pigs of four breeds: Polish Large White, Polish Landrace, Duroc and Pietrain, slaughtered at the age of 60, 120 and 180 days. Each breed × age group was represented by 6 animals. Liver samples were collected and after slaughter immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation. Treatment and slaughter of animals were in accordance with the guiding principles for the care and use of experimental animals.

Table 1. An overview of recent studies of housekeeping genes utilized for normalization of expression levels measurement in different pig tissues

Investigated reference genes	Tissue	Tissue-specific best reference gene	Expression study on target gene*	Reference papers
ACTB, HPRT, GAPDH, cyclophilin	blood	ACTB, *GAPDH, cyclophilin*	*cytokines: IL-4, IFN-γ, IL-2; IL-10, IL-6, IL-1α;*	[Duvigneau *et al.* 2005]
ACTB, B2M, GAPDH, HMBS, HPRT1, RPL13A, SDHA, TBP, TOP2B,YWHAZ	backfat muscle, *longissimus dorsi* muscle	*ACTB, TBP, TOP2B*	*peroxisome proliferative activated receptor γ co-activator 1α (PPARGC1A)*	[Erkens *et al.* 2006]
ACTB, B2M, GAPDH, HMBS, HPRT1, RPL, 4SDHA, TPB, YWHAZ.	liver, kidney, thymus, adipose (subcutaneous), cortex cerebri, cerebellum, hippocampus, lymph nodules (jejunal), muscle *(longissimus dorsi*), heart (muscle), skin (dermis and epidermis) pancreas, bone marrow, bladder, lung, stomach (mucosal membranes), small intestine (mucosal membranes)	*ACTB, RPL4, TPB, HPRT1*	not analysed	[Nygard *et al.* 2007]
B2M, BACT, GAPDH, H2A, PGK1, SI8, UBC	oocytes and preimplantation embryos	*GAPDH, PGK1, S18, UBC*	not analysed	[Kuijk *et al.* 2007]
ACTB, *RPL32*, *POLR2A, AGPAT1, CANX*	prenatal skeletal muscles	*RPL32, AGPAT1, CANX*	*NME*, *IGSF1, HMGA*	[Muráni *et al.* 2007]
EEF1A1, GAPDH, HPRT1, TOP2B	diaphragm,	*EEF1A1/HPRT1*	not analysed	[Svobodová *et al.* 2008]
	heart	*GAPDH/HPRT1*		
	kidney	*EEF1A1/TOP2B*		
	liver	*GAPDH/TOP2B*		
	lungs,	*HPRT1/TOP2B*		
	longissimus dorsi muscle	*EEF1A1/HPRT1*		
	Spleen	*EEF1A1/HPRT1*		
ACTB	brain, thymus, heart, liver, spleen, lung, kidney, muscle, tongue, oral epithelium, respiratory tract, intestine (jejunum), genital tract, testes, ovaries	*ACTB*	*β-defensins 1,2, 3*	[Qi *et al.* 2009]
ACTB,*GAPDH*, *RPS23*, *RPS9*, *MTG1*, *ITGB4BP*, *MRPL39*, *RPS15A*, *UXT, TBK1*, *PCSK2*, *PTBP1*, *API5*, *VAPB*, *QTRT1*, *TRIM41*, *TMEM24*, *PPP2R5B*, *AP1S1*	mammary gland	*TBK1, PCSK2, PTBP1, API5, VAPB, QTRT1, TRIM41, TMEM24, PPP2R5B, AP1S1*	*CSN1S2*, *SCD*, *FABP3, LTF*	[Tramontana *et al.* 2008]
HPRT, cyclophilin	oviduct	*HPRT, cyclophilin*	*transforming growth factor beta 1 (TGFβ1)*	[Jiwakanon *et al.* 2009]
ACTB	*longissimus lumborum*	*ACTB*	*PKM2, CAST*	[Sieczkowska *et al.* 2010]
BANF1, DAK, PH3, GTF2H3, NSUN5, NUBP1, PRR3, SSU72, TIMM17b, VPS4A	subcutaneous back fat and bone marrow (mesenchymal stem cells)	*NSUN5, TIMM17B, VPS4A*	*osteogenic (COL1A1) and adipogenic (DBI) genes*	[Monaco *et al.* 2010]

Table 2. Primer sequences, amplicons length, annealing temperature and reference GeneBank accession numbers of investigated porcine reference gene

Gene symbol	Gene name	Primer sequence (5' 3')	Amplicon length (bp)	Annealing temp. (°C)	GeneBank accession number
ACTB	*Beta-actin*	TCTGGCACCACACCTTCT TGATCTGGGTCATCTTCTCAC	114	60	GenBank:DQ178122
B2M	*Beta-microglobulin*	AAACGGAAAGCCAAATTACC ATCCACAGCGTTAGGAGTGA	178	60	GenBank:DQ178123
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	ACTCACTCTTCTACCTTTGATGCT TGTTGCTGTAGCCAAATTCA	100	57	GenBank:DQ178124
HPRT1	*Hypoxantine phosphoribosyltransferase I*	CCGAGGATTTGGAAAAGGT CTATTTCTGTTCAGTGCTTTGATGT	181	60	GenBank:DQ178126
RPL13A	*Ribosomal protein L13a*	AGTTAAAGTACCTGGCCTTCCT TGGCCTCTCTTGGTCTTG	136	59	GenBank:DQ178127
SDHA	*Succinate dehydrogenase complex, subunit A*	GAACCGAAGATGGCAAGA CAGGAGATCCAAGGCAAA	191	58	GenBank:DQ178128
TBP	*TATA box binding protein*	GATGGACGTTCGGTTTAGG AGCAGCACAGTACGAGCAA	124	59	GenBank:DQ178129
TOP2B	*Topoisomerase (DNA) II beta*	AACTGGATGATGCTAATGATGCT TGGAAAAACTCCGTATCTGTCTC	137	60	GenBank: AF222921.1
YWHAZ	*Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein*	ATGCAACCAACACATCCTATC GCATTATTAGCGTGCTGTCTT	178	60	GenBank:DQ178130

Analytical

Total RNA from 144 frozen liver tissue samples was isolated using Trizol reagent (INVITROGEN, USA) according to Chomczyński and Sacchi [1987]. The contamination of genomic DNA was removed by treating total RNA with RNase-free DNase (PROMEGA, USA) according to the ratio 1µl RQ1 RNase-free DNase (1U/µl). The quantity and quality of RNA preparations were measured spectrophotometrically (NANODROP, USA) at 260 nm. The purity of total RNA was determined by the A260/280 and A260/230 ratio and its integrity was checked by electrophoresis using 1% formaldehyde denaturing gel. The single strand (ss) cDNA was synthesized using M-MLV reverse transcriptase (PROMEGA, USA) in a 25µl reaction mixture according to the manufacturer's prescription. The reverse transcription reaction was performed for 1 h at 42°C in 60 µl of mixture containing 2 µg of total 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 RNase inhibitor, 0.5 µg of oligo (dT)15 primer, and 200 U of M-MLV reverse transcriptase (PROMEGA,USA). Nine porcine reference genes were selected belonging to various functional classes: *ACTB* – related to cell structure, *B2M* – related to cytoskeleton, *GAPDH* – related to carbohydrate metabolism, *SDHA* – related to energy metabolism, *HPRTI* – related to nucleotide metabolism, *RPL13A* – related to protein synthesis, *YWHAZ* – related to cell growth and death, *TBP* – related to transcription, *TOP2B* – related to transcription and replication of DNA. The sequence of primers, annealing

temperature, *GenBank* accession numbers of respective reference DNA sequence and estimated size of PCR products of selected reference genes are listed in Table 2.

The PCR amplification was performed in a LightCycler ® 480 real-time PCR system (ROCHE APPLIED SCIENCE) using 96-well optical plates with a SYBR green I master mix (ROCHE APPLIED SCIENCE). A PCR mix (20 µl) was prepared to reach the indicated final concentrations as follows: 8.6 µl of water, 0.2 µl of primers (forward and reverse; 10 µM), 1 µl (100 ng) of cDNA and 10 µl of SYBR green I master mix. Used was the following amplification programme: 5 min denaturation at 95°C, 40 cycles of four segment amplification with 10 s at 95°C (denaturation), 10 s at 58-60°C (annealing), and 10 s at 72°C (elongation). Annealing temperatures were optimized for individual genes and primers. The last step was melting, added to ensure that specific PCR product was obtained. The melting step consisted of 5 s at 95°C, 5 s at 64°C, and slow heating at a rate of 0.1°C per s up to 95°C, with continuous (5 times per 1°C) fluorescence measurement, finally followed by cooling down to 4°C. Efficiency during quantitative PCRs was estimated from five dilutions (1, 4, 16, 64 and 256) of cDNA specific to each tissue.

The variation of nine reference genes expressions was estimated based on a cycle threshold (Ct) using the LightCycler 480 software (ROCHE DIAGNOSTICS) following the manufacturer instructions. The Ct value of every single reaction and the mean efficiency of each amplicon were used to calculate their relative expression levels. For stability comparison of candidate reference genes two Visual Basic Applications (VBA) for Microsoft Excel – the *geNorm* version 3.4 [Vandesompele *et al.* 2002] and *NormFinder* [Andersen *et al.* 2004] – were used.

Results and discussion

A set of stable reference genes for expression analysis of porcine hepatic tissues was determined by RT-qPCR. Selected reference genes (*ACTB, B2M, GAPDH, SDHA, HPRTI, RPL13A, YWHAZ, TBP* and *TOP2B*) were amplified on single strand (ss) cDNA synthesized from RNA derived from porcine liver. The RT-qPCR assays produced a single peak in the melting curve for examined samples. Reference genes with different functions in cell were chosen in order to avoid those belonging to the same biological pathways that may be co-regulated (Tab. 2). The porcine sequences of the genes were obtained by FASTA search with the human cDNA sequence for each gene against a porcine EST database [Gorodkin *et al.* 2007].

The standard curves were generated using relative concentration *vs*. the threshold cycle (Ct) with the LightCycler 480 software (ROCHE DIAGNOSTICS). Standard PCR efficiency curves were prepared with five-fold serial dilutions of the cDNA. A negative control was also included to determine possible amplification from contamination of genomic DNA. Only primers with single peaks and good negative controls were used. The RT-qPCR reaction performed on serial cDNA dilution showed high efficiency ranging – 92 to 98% (Tab. 3). The different levels of mRNA transcripts

of reference genes were confirmed by various ranges of Ct values (the fractional PCR cycle at which the fluorescent signal significantly rises above the background level and cross the threshold value).

Table 3. Mean values of cycle threshold (*Ct*) for individual genes transcripts and PCR efficiency

Liver	B2M	ACTB	GAPDH	YWHAZ	SHDA	HPRT	TBP	RPL13A	TOP2B
Mean Ct	16.5	19.1	20.0	24.3	22.8	23.3	26.1	27.0	28.0
Range of Ct	12.2-19.7	16.3-22.8	17.8-23.7	19.1-28.2	17.3-25.5	21.9-25.1	23.5-28.2	23.5-32.1	26.2-32.5
E (%)	98	96	97	96	92	92	93	94	96

The obtained Ct and E values were used in the *geNorm* programme to estimate hepatic expression stability (M value) in the investigated reference genes. The M values were used to rank the porcine reference genes on the basis of their stability using *geNorm* calculations [Vandesompele *et al.* 2002, 2004]. Stability of expression based on Ct value demonstrated that hepatic expression levels of porcine reference genes ranged from most stable to the least stable as: *TBP, TOP2B, HPRT1, GAPDH, ACTB, SDHA, RPL13A, YWHAZ, B2M* (Fig. 1).

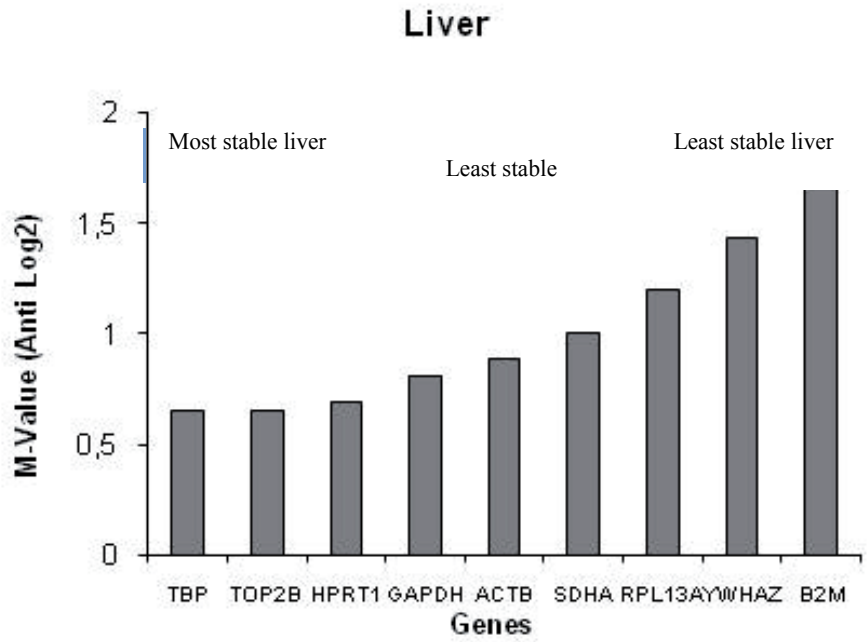

Fig. 1. The mRNA expression stability indices of porcine reference genes according to *geNorm* calculation in liver.

The M values of *TBP, TOP2B* and *HPRT1 (most stable)* lower than 0.7 in porcine liver tissue, showed highest expression stability. However, the M values ranging from *0.7 to* 1.0 (moderately stable) were observed for *GAPDH, ACTB* and *SDHA,* respectively. The normalization factor (NF) of these most and moderately stable reference genes was estimated on the basis of geometric mean of the Ct values. The stability estimations are shown in Table 4. The ranking of relative hepatic expression levels (from high to low) was revealed as follows: *B2M, YWHAZ, RPL13A, SDHA, ACTB, GAPDH, HPRT1, TOP2B* and *TBP* (Fig. 1). Furthermore, *TOP2B* and *TBP* showed the lowest level of mRNA transcript (Fig. 3) despite the most stable porcine reference genes (Fig. 1). Conclusively, the results of *GeNorm* indicate that normalization with the use of a set of three most stably expressed porcine reference genes (*TBP, TOP2B, HPRT1*) would provide reliable results for mRNA expression analysis in porcine liver tissue.

Table 4. Stability of the reference genes according to *geNorm* and *NormFinder* depending the way of the samples were grouped. The stability values were obtained with *geNorm* – M values for a reference gene as the average pairwise variation of the reference gene and *NormFinder* – V that combines intra-and intergroup variation in the expression of each gene

GeNorm M-value		NormFinder – age grouped		NormFinder – breed grouped	
gene symbol	M	gene symbol	V	gene symbol	V
TBP	0.66	TBP	0.10	TBP	0.14
TOP2B	0.66	HPRT1	0.15	HPRT1	0.17
HPRT1	0.69	ACTB	0.16	ACTB	0.19
GAPDH	0.80	TOP2B	0.17	TOP2B	0.20
ACTB	0.89	GAPDH	0.17	GAPDH	0.20
SDHA	1.00	SDHA	0.24	SDHA	0.27
RPL13A	1.20	RPL13A	0.29	RPL13A	0.33
YWHAZ	1.43	YWHAZ	0.38	YWHAZ	0.44
B2M	1.67	B2M	0.49	B2M	0.57

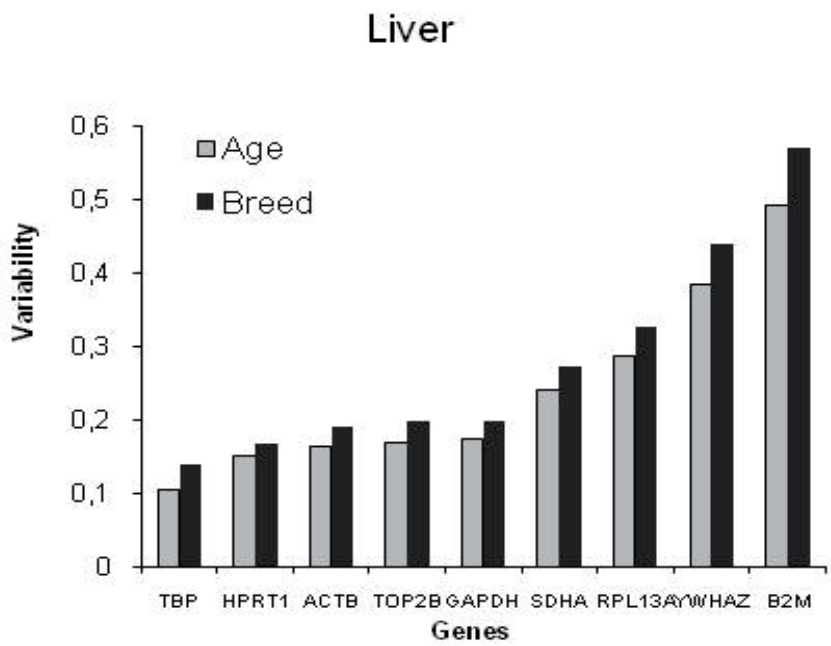

Fig. 2. The mRNA expression stability indices of porcine reference genes according to *Normfinder* calculation in liver with reference to age and breed

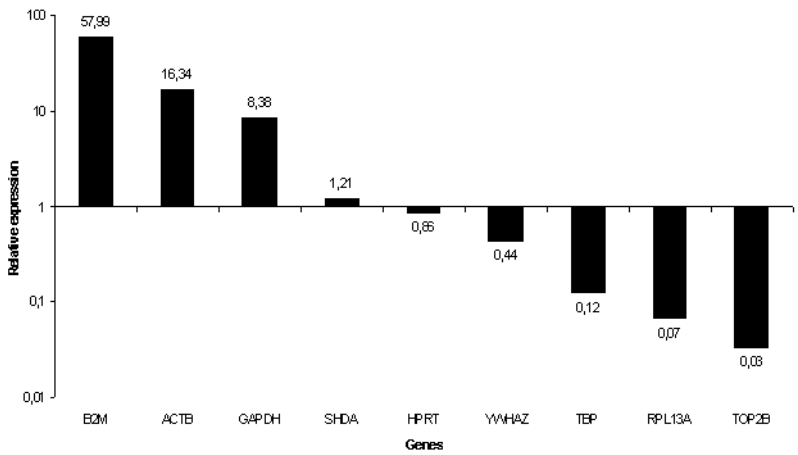

Fig. 3. The relative expression levels of porcine reference genes in the liver.

The hepatic expression levels of candidate genes with crucial function for important traits in animal breeding would be eventually implemented into gene assisted- selection programme (GAS). Because such genes would influence metabolic pathways in liver and muscles towards improvement of porcine meat quality. In recent years, several studies aiming at tissue specific evaluation and selection of best stable reference genes in pigs have been reported. However, fewer studies have normalized the porcine hepatic gene expression (Tab. 1). Identified were *ACTB, RPL4, TPB* and *HPRT1* [Nygard *et al.* 2007], *GAPDH* and *TOP2B* [Svobodová *et al.* 2008] and *ACTB* [Qi *et al.* 2009] as most stable reference genes recommended for the porcine hepatic gene expression profiling (Tab. 1). Thus, the present results are in accordance with those reported earlier. The results based on NormFinder analysis [Andersen *et al.* 2004] identified *TBP, HPRT1, ACTB, TOP2B, GAPDH* and *SDHA* as most stable porcine reference genes for hepatic tissue (Fig. 2). The *NormFinder* programme uses a model based approach for computing gene stability value for either the most stable reference gene or the best combination of two genes [Andersen *et al.* 2004], which allows to estimate the variations between time points. In accordance to *geNorm*, the *NormFinder* also showed differences in ranking of reference genes in porcine hepatic tissue. For instance, the top three porcine reference genes are the same in both *geNorm* and *NormFinder* except the *TOP2B* replaced by *ACTH* in the latter (Tab. 4). The rankings obtained with geNorm occurred not similar to those by NormFinder and the latter were not similar to the most stable and moderately stable reference genes (Tab. 4). However, both programmes indicated as the least stable porcine reference genes – *RPL13A, YWHAZ* and *B2M*. Finally, it may be concluded that *TBP, TOP2B* and *HPRT1* are the most suitable porcine reference genes for hepatic tissue samples. Moreover, this study revealed a newly developed set of porcine reference genes for normalization of mRNA expression data from porcine hepatic tissue. The most stable porcine reference genes expressed in hepatic tissues providing a basis for possible investigation of potential candidate genes such as insulin-like growth factor 1 *(IGF-1),* insulin-like growth factor 2 *(IGF-2*), insulin-like growth factor 1 receptor (*IGF1R),* growth hormone receptor *(GHR)* as well as other genes important for postnatal growth and development process such as *myogenesis,* and ultimately towards improved quality of pork.

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