Evaluation of reference genes for gene expression studies in pig muscle tissue by real-time PCR

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ABSTRACT: The selection of reference genes is essential for gene expression studies when using a real-time quantitative polymerase chain reaction (PCR). Reference gene selection should be performed for each experiment because the gene expression level may be changed in different experimental conditions. In this study, the stability of mRNA expression was determined for seven genes: *HPRT1*, *RPS18*, *NACA*, *TBP*, *TAF4B*, *RPL32* and *OAZ1*. The stability of these reference genes was investigated in the skeletal muscle tissue of pig foetuses, piglets and adult pigs using real-time quantitative PCR and SYBR green chemistry. The expression of stability of the used reference genes was calculated using the geNorm application. Different gene expression profiles among the age categories of pigs were found out. *RPS18* has been identified as the gene with the most stable expression in the muscle tissue of all pig age categories. *HPRT1* and *RPL32* were found to have the highest stability in piglets and adult pigs, and in foetuses and adults pigs, respectively. The newly used reference gene, *TAF4B,* reached the highest expression stability in piglets.

Keywords: gene stability; reference genes; quantitative PCR; *Sus scrofa*; age categories of pigs; muscle tissue

To understand meat quality on a genetic basis and factors affecting meat quality, it is important to search for and identify the responsible genes. The real-time quantitative polymerase chain reaction represents an accurate, rapid, and sensitive method for the quantification of mRNA transcription and for the study of differential gene expression. Reference genes are commonly accepted and widely used for the normalization of data to compensate for differences among the samples and for the interpretation of the expression results. Ideally, reference genes should constitutively be expressed by all cell types and tissues, across developmental stages, and should not be affected by various experimental procedures (Thellin et al., 1999). Several studies have demonstrated that although reference genes are expressed by any cell, expression levels vary among different cell types, tissues and experimental conditions (Dheda et al., 2004; Radonić et al., 2004). Therefore, the suitability of reference genes has to be verified before each series of experiments (Bohle et al., 2007).

In our study, the expression stability of potential reference genes has been compared in the muscle tissue of different age categories of pigs (foetuses, piglets and adult pigs). It is unknown whether these genes are expressed in pigs of different ages in a stable manner. We tested seven genes of different functional classes for their potential to be used as reference genes. Two of the analysed genes are commonly used as reference genes in pigs: hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) and TATA box binding protein (*TBP*). Ribosomal protein S18 (*RPS18*), ribosomal protein L32 (*RPL32*),

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Table 1. Gene symbol, name and accession number of selected candidate reference genes

Gene symbol	Gene name	Accession number
*HPRT*1	hypoxanthine phosphoribosyltransferase 1	NM_001032376
*RPS*18	ribosomal protein S18	NM_213940
NACA	nascent-polypeptide-associated complex alpha polypeptide	AK237627
TBP	TATA box binding protein	DQ178129
TAF4B	TAF4B RNA polymerase II	EF133513
RPL32	ribosomal protein L32	NM_001001636
OAZ1	ornithine decarboxylase antizyme 1	NM_001122994

ornithine decarboxylase antizyme 1 (*OAZ1*), nascent-polypeptide-associated complex alpha polypeptide (*NACA*), and TAF4B RNA polymerase II (*TAF4B*) have not been analysed as reference genes in pigs so far.

MATERIAL AND METHODS

Selected genes, their accession numbers, and primer sequences are listed in Tables 1 and 2. For *HPRT1* and *TBP* genes, the primers described by Nygard et al. (2007) and Svobodová et al. (2008), respectively, were used. The other primers were designed on the basis of known pig sequences available at GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Porcine muscle tissues (nine samples from each age group of pigs) were collected from: (1) foetuses (hind limb; Czech Large White; 42 days of gestation), (2) piglets: (*musculus longissimus lumborum et thoracis*; commercial hybrid pigs; 1–18 days of age), (3) adult pigs (*musculus longissimus lumborum et thoracis*; commercial hybrid pigs; approximately 200 days of age). The tissue samples were immediately submerged in RNAlater Solution (Ambion) and stored at –20°C. Sample homogenization was carried out in FastPrep FP 120 (ThermoSavant) and the purification of total RNA was performed using the TRI Reagent (Sigma-Aldrich). RNA integrity was verified by 1.2% agarose gel electrophoresis. Contaminating DNA from each RNA sample was degraded with a DNA-free Kit (Ambion). For the verification of the absence of any DNA contamination, a control PCR was used. The primers (porcine *PLIN* gene) and PCR conditions to amplify this specific PCR product were applied according to Vykoukalová et al. (2009). Total RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The samples were standardised to contain approximately 1 µg of total RNA. Reverse transcription reaction (20 µl) was carried out with the Omniscript Reverse Transcriptase (Qiagen) and oligo $(dT)_{20}$ primers (Invitrogen).

The real-time PCR study was performed on the 7500 Real-Time PCR System (Applied Biosystems),

Table 2. Primer pairs used for PCR

Gene symbol	Forward primer sequence (5'→ 3')	Reverse primer sequence (5'→ 3')	Product size (bp)
*HPRT*1	AAGGACCCCTCGAAGTGTTG	CACAAACATGATTCAAGTCCCTG	122
RPS18	GGGTGTAGGACGGAGATATGCT	ATTACACGTTCCACCTCATCCTC	102
NACA	AAGAGGAGAGTGAAGAGGAAGAGGT	GCTCGGACTGCCTTTGCTC	106
TBP	AACAGTTCAGTAGTTATGAGCCAGA	AGATGTTCTCAAACGCTTCG	153
TAF4B	GCAGCCAAGAGTCGTTCCA	CCTCACTTCCAGACCCAGATTC	178
RPL32	AGCGGAACTGGCGGAAAC	TGTGAGCAATCTCAGCACAGTATG	210
OAZ1	AGTTCCAGGGTCTCCATCAA	TTAATGTGTTTGGCATCTGTGA	243

and each PCR reaction mixture (final volume 20 µl) contained 8.4 µl nuclease-free water (TopBio), 0.2 µl Uracil N-glycosylase (UNG) (Applied Biosystems), 10 pmol forward and reverse primer, 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems), and 1 µl of cDNA sample or water as a negative control. The real-time PCR program started with 2 min of UNG incubation step at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. Subsequently, the melting curve was constructed for the verification of PCR product specificity by increasing the temperature from 60 to 95°C in sequential steps of 0.2°C for 15 s. 10-fold dilution series of cDNA was included for each gene and tissue to determine PCR efficiency by constructing a relative standard curve. Each sample was run in triplicate and all experiments contained a negative control. All PCR amplicons were further verified by electrophoresis on 3% agarose gels, and by sequencing using ABI PRISM 3100 – Avant Genetic Analyser (Applied Biosystems). Gene stability was evaluated using the geNorm software program (Vandesompele et al., 2002).

RESULTS AND DISCUSSION

In this study, the expression levels of mRNA of a number of potential reference genes were measured. The expression data of the seven selected genes (*HPRT1*, *RPS18*, *NACA*, *TBP*, *TAF4B*, *RPL32* and *OAZ1*) were determined in a set of 9 samples taken from the porcine muscle tissue of foetuses, piglets and adult pigs.

Dilution curves of PCR amplification of the analysed genes in all samples showed efficiency between 87.1% and 108.3%, linear correlation coefficient (R^2) from 0.993 to 1.000, and average Cq values from 13.46 to 29.5 cycles. The expression stability and *M* values of the investigated genes are shown in Table 3. The *M* values for the analysed genes were lower than 1.5. It indicates the high expression stability of all seven reference genes in muscle tissues in all studied age categories of pigs.

Our results showed that *RPL32* and *RPS18* were the most stable genes (the lowest *M* value) in muscle tissue across the age categories of pigs. These genes encode components of the 60S ribosomal subunit (*RPL32*) and 40S ribosomal subunit (*RPS18*). *RPL32* and *RPS18* have not been evaluated as reference genes in pig tissues so far, nevertheless they are commonly used as suitable reference genes for the normalisation of real-time quantitative PCR data. *RPL32* gene was selected as an ideal reference gene in the majority of canine tissues including muscle. *RPS18* was the gene with the most stable expression in canine lungs and spleen, and it is also a suitable reference gene in muscle tissue (Peters et al., 2007).

HPRT1 and *TBP* are often used as reference genes. Our analysis showed that *HPRT1* reached the highest expression stability in muscle tissue of both piglets and adult pigs. This result is in agreement with Nygard et al. (2007) and Svobodová et al. (2008). In agreement with Erkens et al. (2006) and Nygard et al. (2007), the *TBP* is also a suitable reference gene for the expression analysis in porcine tissues (including muscle).

TAF4B and *NACA* were not analysed as reference genes in other studies. *TAF4B* was a gene product with the most stable expression in piglets, but its expression was least stable in foetuses and adult pigs. Nevertheless, the respective *M* values were 0.95 and 0.60, and this indicates a good stability of the *TAF4B* gene. This gene regulates the expression of genes controlling the cell proliferation (Mengus et al., 2005) and plays a role in both male and female fertility (Falender et al., 2005). The *NACA* gene

Table 3. Gene expression stability of the reference genes according to geNorm program

	Ranking of genes by expression stability values (*M*)					
	least stable genes					most stable genes
Foetuses	*TAF4B* 0.826	*HPRT1* 0.625	*TBP* 0.574	*NACA* 0.457	*OAZ1* 0.391	*RPS18*/*RPL32* 0.377
Piglets	*NACA* 0.879	*RPL32* 0.782	*OAZ1* 0.699	*TBP* 0.560	*RPS18* 0.443	*HPRT1*/*TAF4B* 0.356
Adult pigs	*TAF4B* 0.596	*NACA* 0.489	*TBP* 0.380	*OAZ1* 0.344	*RPL32* 0.335	*HPRT1*/*RPS18* 0.281

was selected as a suitable control gene for real-time quantitative PCR using microarray data in human breast cancer tissue (Popovici et al., 2009). The highest expression stability was reached in foetuses, in contrast with piglets and adult pigs.

GeNorm calculates the normalisation factor assessing the optimal number of reference genes for generating the *M* factor by calculating the pairwise variation *V*. It proposes 0.15 as a cut-off value, below which the inclusion of an additional reference gene is not required. *V* value did not decrease when more than four reference genes in foetuses and piglets, and more than five reference genes in adult pigs, were included. Our results suggest that the number of reference genes for optimal normalisation is either two or three in foetuses and piglets, or three possibly four in adult pigs.

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