STABILITY OF REFERENCE GENES ESTIMATED BY REAL-TIME PCR IN PORCINE LIVER

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Abstract: It is essential for the method real-time PCR to control variables by validation of normalization of the data. The aim of this study was to develop a set of reference genes for relative quantification of mRNA expression in the porcine liver. The mRNA stability of expression was studied for five genes: GAPDH, HPRT1, PPIA, TOP2B, TBP1, where for each gene the Ct value characterized the level of expression in liver. With the help of geNorm range of stability of analysed genes was (from the most stable to the least): PPIA, TBP1, TOP2B, GAPDH, HPRT1.

Key Words: real-time PCR, pig, gene expression, reference gene

INTRODUCTION
Real-time PCR is an efficient method for studying quantification of gene expression in tissue. This method is sensitive, fast and has precise measurement of examined material in the sample (Gachon et al. 2004). However, number of variables should be controlled such as precise determination of the amount of starting material, differences in transcriptional activity and presence of inhibitors in different samples (Nygart et al. 2007). One of essential steps in gene expression analysis is validation of normalization of real-time PCR data (Svobodová et al. 2008). Normalization of the data is usually gained by comparison of expression profiles of studied genes with constitutively expressed genes known as reference genes (Lee et al. 2007). Since the reference gene is exposed to the same preparation steps as the gene of interest, this normalisation adjusts for any differences in the amount of starting material, RNA isolation and cDNA synthesis (Nygart et al. 2007).

Five reference genes was selected for validation of their stability in porcine liver: GAPDH, HPRT1, PPIA, TOP2B, TBP1. The most commonly used reference gene is GAPDH, which brings good results in many studies but in others it is not recommended for its variability of expression (Kozera and Rapacz 2013). HPRT1 gene is also commonly used reference gene, but doubted by some authors as stable reference gene (Erkens et al. 2006). TOP2B and TBP1 and their gene expression stability was previously analysed by Erkens et al. (2006). Studies has shown suitability of PPIA as reference gene in other species (Pérez et al. 2007) and this gene is also used as a reference gene in pigs.

In the present study the expression stability of five reference genes has been compared in porcine liver. This has enabled to estimate the suitability of these genes for normalisation of gene expression in porcine liver.

MATERIAL AND METHODS
Samples collection and isolation
Samples were collected from four male hybrid pigs from the right part of liver. Samples were immediately submerged in RNAlater (Qiagen, Hilden, Germany). Total RNA was extracted using
RNeasy Plus mini kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse transcribed at 42 °C using Quantitect reverse transcription kit (Qiagen, Hilden, Germany) with elimination of genomic DNA.

**Relative quantitative PCR with SybrGreen**

Standard curve was measured for each primer pair individually. Reaction for qPCR was prepared using Power Sybr® Green master mix (ThermoFisher scientific, Waltham, USA) in triplicate for each sample and for non-template negative control. Reaction consisted of 1 µl of cDNA, 10 pmol/µl of each primer, 10 µl of Sybr Green, 0.2 µl of AmpErase® Uracil N-glycosylase (UNG) (ThermoFisher scientific, Waltham, USA), 8 µl of RNase-free water in total volume of 20 µl. The qPCR was run on Rotor gene (Qiagen, Hilden, Germany) with cycling conditions 1 cycle of denaturation at 95 °C/10 min, followed by 40 cycles of 95 °C/10 min and 60 °C/1 min. This was followed by melting curve for verification of specificity of PCR products. Used primers are shown in table 1.

**Table 1 Details of primers used for analysis.**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Oligo sequence (5’ → 3’)</th>
<th>Amp. length</th>
<th>E (%)</th>
<th>Ref. seq.</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CAGCAATGCCTCCTGCACCA GATGCGGAAGTGTGCAGGA</td>
<td>70</td>
<td>80</td>
<td>AF141959</td>
<td>Svobodová 2011</td>
</tr>
<tr>
<td></td>
<td>AAGGACCCCTCGAAGTGTTG CACAAACATGATTCAGTCCTG</td>
<td>122</td>
<td>85</td>
<td>NM_001032376</td>
<td>Svobodová et al. 2008</td>
</tr>
<tr>
<td>HPRT1</td>
<td>CTGAGTGGTTGAGTGGCAAA CCACAGTCAGCAATGGTGATCT</td>
<td>130</td>
<td>79</td>
<td>NM_214353</td>
<td>Svobodová 2011</td>
</tr>
<tr>
<td>PPIA</td>
<td>CTAATGATGCTGGTGGCAAC CGACGATTCAGCTAGCCCG</td>
<td>100</td>
<td>89</td>
<td>AF222921</td>
<td>Svobodová et al. 2008</td>
</tr>
<tr>
<td>TOP2B</td>
<td>AACAGTTCAAGTTATGAGCCAG AAGATGTTCCTCAAACGCCTCG</td>
<td>153</td>
<td>99</td>
<td>DQ845178</td>
<td>Nygart et al. 2007</td>
</tr>
<tr>
<td>TBP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend: Amp. length – amplicon length, E – primer efficiency, Ref. seq. – reference sequence

**Data Analysis**

Mean of Ct (cycle of threshold) values were measured using Rotor gene gene software and were converted into input data for geNorm application. The geNorm algorithm is based on the principle of identical expression ratio of the 2 most stable reference genes in all samples. The obtained M value is the average pairwise variation of a particular gene with all other control genes (Vandesompele et al. 2002).

**RESULTS AND DISCUSSION**

Five commonly used reference genes was selected (table 1). Efficiency of the qPCR amplification of analysed genes was between 79–99%. Average Ct values of GAPDH, HPRT1, PPIA, TOP2B and TBP1 genes at each sample are shown in table 2.

**Table 2 Average Ct values of analysed samples for genes GAPDH, PPIA, HPRT1, TOP2B and TBP.**

<table>
<thead>
<tr>
<th>sample</th>
<th>GAPDH</th>
<th>PPIA</th>
<th>HPRT1</th>
<th>TOP2B</th>
<th>TBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.53</td>
<td>14.25</td>
<td>15.38</td>
<td>21.43</td>
<td>21.68</td>
</tr>
<tr>
<td>2</td>
<td>18.74</td>
<td>14.68</td>
<td>15.62</td>
<td>22.47</td>
<td>22.65</td>
</tr>
<tr>
<td>3</td>
<td>17.20</td>
<td>14.03</td>
<td>16.04</td>
<td>21.52</td>
<td>21.57</td>
</tr>
<tr>
<td>4</td>
<td>18.67</td>
<td>14.85</td>
<td>16.87</td>
<td>22.46</td>
<td>22.50</td>
</tr>
</tbody>
</table>

M values were obtained using the geNorm algorithm. The range of expression stability in analysed genes was (from the most stable to the least stable): PPIA, TBP1, TOP2B, GAPDH and HPRT with M values: 0.387, 0.389, 0.399, 0.640 and 0.737 (Figure 1).
Figure 1 Reference gene mRNA expression stability in porcine liver according to geNorm (M values)

The least stable gene in our study was \textit{HPRT1}. This result is in disagreement with Nygart et al. (2007), who suggested that the gene \textit{HPRT1} is good reference gene for low abundant transcript expression studies, but is in agreement with Erksen et al. (2006), who studied stability of gene expression in porcine back fat and \textit{longissimus dorsi} muscle and found this gene unstable in his study. The second least stable gene is \textit{GAPDH} which result is in agreement with Svobodová et al. (2008), who studied stability of the reference genes in several porcine tissues, and Erksen et al. (2006). Our data suggest that the most stable reference genes for liver are \textit{PPIA}, \textit{TBP1} and \textit{TOP2B}. These results are in agreement with findings in study of Erksen et al. (2006) who indicated for normalisation to use \textit{TOP2B} and \textit{TBP1}. Also Gu et al. (2011) recommends \textit{TOP2B} as one of the most stable genes in several porcine tissue samples. Park et al. (2014) studied stability of reference genes in four pig breeds and several tissue samples and suggested as appropriate reference genes in three pig breeds genes \textit{PPIA} and \textit{TBP} and in fourth breed genes \textit{PPIA} and \textit{TOP2B}, which is in agreement with our findings.

According to Vandesompele et al. (2002) expression results are considerably more reliable when they are normalized using the geometric mean of multiple reference genes. Recommended number of reference genes for normalisations is three, because as GeNorm analysis indicates adding third reference gene to the normalisation factor had large impact on reducing variability (Erksen et al. 2006). Also expression stability of reference genes varies among different tissues and different breeds so it is important to test reference genes before analysing gene of interest.

CONCLUSION

Our study provides recommendation for the choice of endogenous control genes in mRNA expression studies in porcine liver tissue. We have investigated expression stability of five genes (\textit{GAPDH, HPRT1, PPIA, TOP2B, TBP1}) in the porcine liver. Three of the investigated genes (\textit{PPIA, TOP2B, TBP1}) were found to be the most stable in studied tissue, therefore we recommend using this genes as endogenous control in real-time reverse transcription PCR analysis for gene expression in porcine liver. Genes \textit{GAPDH} and \textit{HPRT1} were found out to be the least stable in our study.
ACKNOWLEDGEMENTS
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