ISSN 0015-5497, e-ISSN 1734-9168

Folia Biologica (Kraków), vol. 65 (2017), No 3
https://doi.org/10.3409/fb65_3.165

# The Reference Gene Selection to Study *PRNP* Gene Expression in Sheep

Agata PIESTRZYNSKA-KAJTOCH, Grzegorz SMOŁUCHA, Maria OCZKOWICZ, Anna KYCKO, Mirosław P. POLAK, Wojciech KOZACZYŃSKI, Anna KOZUBSKA-SOBOCIŃSKA, Jan F. ŻMUDZIŃSKI, and Barbara REJDUCH

*Accepted November 21, 2017* *Published online December 15, 2017* *Published December 21, 2017*

PIESTRZYNSKA-KAJTOCH A., SMOŁUCHA G., OCZKOWICZ M., KYCKO A., POLAK M.P., KOZACZYŃSKI W., KOZUBSKA-SOBOCIŃSKA A., ŻMUDZIŃSKI J.F., REJDUCH B. 2017. The reference gene selection to study *PRNP* gene expression in sheep. Folia Biologica (Kraków) **65**: 165-171.

The *PRNP* gene is connected to scrapie susceptibility in sheep (prion disease). Its polymorphism and expression may influence the occurrence of the disease. In order to study *PRNP* gene expression level in different ovine tissues, selection of reference genes is needed. Three housekeeping genes (*RPL27*, *RPS29*, *OAZ1*) were chosen for studying *PRNP* gene expression in ovine brain cortex, midbrain, cerebellum, brain stem, pituitary gland, spleen, liver, skeletal muscle and heart. The primers for gene sequencing were designed based on bovine reference sequences. *RPL27* was found to be the most stable reference gene (for brain tissues M=0.322, $SD_{Ct}$=0.486, Stability Value=0.0089; for all tissues M=0.489, $SD_{Ct}$=0.696; Stability Value=0.0093). Regardless of the housekeeping gene, the expression level of *PRNP* was higher in brain tissues than in other tissues analyzed. A normalization experiment indicated that all candidate reference genes could be used as endogenous controls for studying *PRNP* mRNA expression in different ovine tissues. However, *RPL27* seemed to be the most stable and appropriate for the experiment with many different tissue types and could be used as one of the reference genes for studying gene expression in ovine tissues. Our results confirmed that the *PRNP* gene is highly expressed in nervous tissue.

Key words: *PRNP* gene expression, *RPL27*, *OAZ1*, *RPS29,* real-time PCR normalization, sheep.

*Agata PIESTRZYNSKA-KAJTOCH, Grzegorz SMOŁUCHA, Maria OCZKOWICZ, Anna KOZUBSKA-SOBOCIŃSKA, Barbara REJDUCH, National Research Institute of Animal Production, Department of Animal Genomics and Molecular Biology, Krakowska 1, 32-020 Balice n. Kraków, Poland. E-mail: agata.kajtoch@izoo.krakow.pl*
*Anna KYCKO, Wojciech KOZACZYŃSKI, National Veterinary Research Institute, Department of Pathology, 57 Partyzantów Avenue, 24-100 Puławy, Poland.*
*Mirosław P. POLAK, Jan F. ŻMUDZIŃSKI, National Veterinary Research Institute, Department of Virology, 57 Partyzantów Avenue, 24-100 Puławy, Poland.*

Scrapie – a fatal, neurodegenerative prion disease of transmissible spongiform encephalopathies (TSE) – affects sheep and goats. The pathogenic prion protein ($PrP^{Sc}$) is thought to be the putative TSE agent. The *PRNP* gene encodes a physiological, cellular form of prion protein ($PrP^{C}$) and its polymorphism influences the scrapie susceptibility and incubation period. According to PRUSINER (1998), the conversion of normal $PrP^{C}$ into the pathological prion $PrP^{Sc}$ causes the prion diseases. $PrP^{Sc}$, the protease-resistant isoform of cellular $PrP^{C}$, accumulates in brain and other organs of affected animals leading to tissue degeneration (PRUSINER 1998; BAYLIS & GOLDMANN 2004; TRANULIS *et al*. 2011). Therefore, PRNP gene expression levels may influence scrapie pathogenesis. The disease has two forms: classical and atypical, which differ in e.g. $PrP^{Sc}$ glycosylation profile and genetic susceptibility. On the basis of *PRNP* genotype, scientists classified sheep into five classical scrapie risk groups (with increasing susceptibility). By contrast, atypical scrapie was diagnosed in animals with classical scrapie resistant genotypes and genotypes associated with classical scrapie susceptibility did not correlate with atypical scrapie occurrence (BAYLIS & GOLDMANN 2004; TRANULIS *et al.* 2011). Because of its role in the disease, researchers investigated the *PRNP*

gene and protein expression mainly in nervous tissue (BAYLIS & GOLDMANN 2004; GARCIA-CRESPO *et al.* 2005; 2006).

The real-time quantitative PCR (qPCR) gene expression analysis is a very precise and sensitive method. It allows the detection of small differences in gene transcript levels between cell lines, tissues, samples, *etc.* However, the method requires data normalization against stably expressed endogenous reference genes to obtain accurate results. The expression level of endogenous controls often depends on many factors e.g. tissue, age, diet, living conditions, environment (BUSTIN *et al.* 2009; CHAPMAN & WALDENSTRÖM 2015). For gene expression experiments BUSTIN *et al.* (2009) and CHAPMAN and WALDENSTRÖM (2015) recommended: the selection of appropriate reference genes, careful organization of study groups, collect material, isolate and process RNA under appropriate conditions.

Many scientists have used *ACTB*, *GAPDH*, *HPRT1, 18srRNA* and *B2M* as endogenous controls for real-time PCR normalization (DE JONGE *et al.* 2007; LYAHYAI *et al.* 2009; LAMPO *et al.* 2009; O'CONNOR *et al*. 2013; MAHAKAPUGE *et al*. 2016). DE JONGE *et al.* (2007) showed large fluctuations in expression level among a set of diverse human samples and presented novel candidate housekeeping genes with enhanced stability. Thirteen genes encoding ribosomal proteins (e.g. *RPS13, RPL27, RPS29*) and two other genes, *OAZ1* and *SRP14,* were the most promising. He named them the "top 15 candidate housekeeping genes" (DE JONGE *et al.* 2007). The usefulness of *RPL27*, *RPS29* and *OAZ1* for farm animals in tissue gene expression studies was also investigated by OCZKOWICZ *et al.* (2010), ROPKA-MOLIK *et al.* (2012) and PEREIRA-FANTINI *et al.* (2016).

The aim of our experiment was to select and evaluate accurate endogenous controls for studying *PRNP* gene expression and reveal *PRNP* mRNA expression levels in different ovine tissues.

## Material and Methods

### Tissue collection and RNA extraction

Tissue fragments were collected directly after regular slaughter (intended for human consumption) of five adult healthy Polish Merino sheep (females, purebred, 101-125 months old) from the same flock. The tissues were immediately stored in liquid nitrogen. Total RNA was isolated from brain cortex, midbrain, cerebellum, brain stem (obex), pituitary gland, spleen, liver, skeletal muscle and heart (*apex cordis*) by using the SV Total RNA Isolation System (Promega). The RNA quantity and purity was evaluated by using a NanoDrop spectrophotometer (A260/280 and A260/230 ratios). The RNA quality was proved by gel electrophoresis (1% agarose). Next, concentration of RNA was normalized and reverse transcribed (1000 ng per sample; High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems). cDNA from the same tissue was poolled into one sample and a cDNA 5-point dilution series (1:10) was prepared separately for every tissue analyzed.

### Design of primers

The sequences of ovine *OAZ1* (ornithine decarboxylase antizyme 1), *RPL27* (ribosomal protein L27) and *RPS29* (ribosomal protein S29) were unknown prior to the experiment. By using primers designed on the basis of bovine reference sequences (*OAZ1*: NM_001127243.1; *RPL27*: NM_001034051.1; *RPS29*: AC_000167.1), the ovine gene fragments were sequenced (BigDye® Terminator v3.1 Cycle Sequencing Kit; Genetic Analyzer 3130xl; Applied Biosystems) and deposited in GenBank (NCBI) with Accession Numbers KU168744, KU168742 and KU168743, respectively. Sequences were analyzed in BioEdit Sequence Alignment Editor (TOM HALL) and BLAST. Next, TaqMan MGB probes and primers were designed for all genes studied (Table 1).

### cDNA synthesis and quantitative real-time PCR

Relative quantification (ΔΔCt method) and qPCR efficiency estimation (Standard Curve) were performed with standard protocols in the StepOnePlus Real-time PCR System (Applied Biosystems), in 15 µl reaction volume containing: 7.5 µl TaqMan Fast Advanced Master Mix, 900 nM of each primer, 250 nM of each TaqMan probe, 1 µl cDNA and nuclease-free water. The standard curve method was performed separately for all genes studied. After analysis, the most diluted samples ($5^{th}$ dilution) were rejected because of many outliers in replicate groups. In the comparative ΔΔCt method, the *PRNP* gene probe was multiplexed with a candidate endogenous control in three separate reactions for *OAZ1*, *RPL27* and *RPS29*. All reactions were carried out in triplicate. Skeletal muscle was used as a reference to calculate the relative *PRNP* gene expression (RQ) among tissues.

### Data analysis

The reaction efficiency was estimated in StepOne Software v.2.3 in standard curve dilution series analysis. Stability of the endogenous controls was estimated with geNorm (VANDESOMPELE *et al.* 2002) (on the basis of M-value calculation), Best-

Table 1

Primers and probes. A – primers used for reference gene sequencing; B – reference gene primers and TaqMan MGB probes for real-time PCR; C – *PRNP* primers and TaqMan MGB probes for real-time PCR

| Name | Sequence | Dye | Amplicon length (nt) | Reference sequence |
|---|---|---|---|---|
| A | | | | |
| OAZ1_1F | CTTCGCCAGAGAGAAGGAAG | | 560 | NM_001127243.1 |
| OAZ1_1R | CGGTCTCACAATCTCAAAGC | | | |
| OAZ1_2F | CTTCGCCAGAGAGAAGGAAG | | 810 | |
| OAZ1_2R | TGGAGTGAGCGTTTATTTGC | | | |
| RPL27_2F | CCTTTCTGCTGTAGTCCCAAG | | 418 | NM_001034051.1 |
| RPL27_2R | TTGTTCTTGCCCGTCTTGTA | | | |
| RPS29_2F | CGCTCTTGGTGAGAAACAGA | | 804 | AC_000167.1 |
| RPS29_2R | TTTGAGCGACTTGACCAAAG | | | |
| RPS29_3F | GTTGTTTGGAAGGGTTGCTT | | 1082 | |
| RPS29_3R | TTTGTCACAGAAATCGCACA | | | |
| B | | | | |
| OAZ1_F | GATCGAGCCGCCTTGCT | | 57 | KU168744 |
| OAZ1_R | CCGGTCTCACAATCTCAAAGC | | | |
| OAZ1_MGB | CGTACCTTCAGCTTTT | VIC | | |
| RPL27_F | TCATGCCCACAAGGTACTCTGT | | 70 | KU168742 |
| RPL27_R | CTCTGAAGACATCCTTGTTGACAAC | | | |
| RPL27_MGB | CCCTTGGACAAAAC | VIC | | |
| RPS29_F | CTCTTGCCGAGTCTGCTCAAA | | 62 | KU168743 |
| RPS29_R | GGCACATATTGAGGCCGTATTT | | | |
| RPS29_MGB | CGGCACGGTCTGAT | VIC | | |
| C | | | | |
| PRNP_F | TTTGTGGCCATGTGGAGTGA | | 58 | NM_001009481.1 |
| PRNP_R | CCTCCGCCAGGTTTTGGT | | | |
| PRNP_MGB | CCTCTGCAAGAAGC | FAM | | |

Keeper (Ct Standard Deviation - $SD_{Ct}$ and Correlation Coefficient – r) (PFAFFL *et al.* 2004) and NormFinder (Stability Value) (ANDERSEN *et al.* 2004). StepOne Software v.2.3, Excel (Analysis ToolPack) and Statistica v.11 (StatSoft) were used for gene expression data analysis, table preparation and statistics. To determine whether *PRNP* mRNA expression differed depending on various reference genes, Friedman's ANOVA and Wilcoxon signed-rank tests were performed.

## Results and Discussion

DE JONGE *et al.* (2007) placed *OAZ1*, *RPL27* and *RPS29* among the most stably expressed genes in human meta-analysis. We tested these candidate internal control genes along with the *PRNP* gene. The PCR efficiency (E) values were estimated for all genes and tissues separately and also for all brain tissues together on the basis of the Standard Curve method with a cDNA 4-point dilution series. Although all E values were close to 2, they differed between genes and tissues. The lowest PCR efficiency was obtained for *RPS29* in midbrain (1.880), and the highest for *PRNP* in heart and *OAZ1* in brain stem (2.013). The correlation coefficient values were between 0.985 and 1 (Table 2). Mean efficiencies were used for *PRNP* gene expression studies (ΔΔCt). The Ct Means ranged from 21.68 in brain cortex to 25.49 in heart for *OAZ1*, from 21.57 in brain cortex to 24.81 in heart for *RPL27* and from 20.01 in skeletal muscle to 23.25 in heart for *RPS29* (data not shown).

The geNorm algorithm calculates the gene expression stability measure (M) for internal control genes (VANDESOMPELE *et al.* 2002). The reference gene is stable if its M value is lower than 1.5.

Table 2

Real-time PCR efficiencies estimated by StepOne™ Software v2.3. E – efficiency; $R^2$ – correlation coefficient; SD – standard deviation

| Tissue | | *PRNP* | | | *OAZ1* | | | *RPL27* | | | *RPS29* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Slope | E | $R^2$ | Slope | E | $R^2$ | Slope | E | $R^2$ | Slope | E | $R^2$ |
| all brain tissues | | -3.511 | 1.927 | 0.990 | -3.417 | 1.962 | 0.985 | -3.427 | 1.958 | 0.995 | -3.601 | 1.895 | 0.993 |
| cerebellum | CB | -3.595 | 1.897 | 0.998 | -3.518 | 1.924 | 0.999 | -3.519 | 1.924 | 0.999 | -3.638 | 1.883 | 0.999 |
| brain cortex | BC | -3.619 | 1.889 | 0.998 | -3.449 | 1.950 | 0.999 | -3.390 | 1.972 | 1.000 | -3.597 | 1.897 | 0.999 |
| midbrain | MB | -3.479 | 1.938 | 0.997 | -3.410 | 1.965 | 0.999 | -3.415 | 1.963 | 0.999 | -3.646 | 1.880 | 0.999 |
| brain stem | BS | -3.439 | 1.953 | 0.999 | -3.290 | 2.013 | 0.999 | -3.411 | 1.964 | 0.997 | -3.568 | 1.907 | 0.999 |
| pituitary gland | PG | -3.591 | 1.899 | 0.997 | -3.428 | 1.958 | 0.999 | -3.406 | 1.966 | 0.999 | -3.564 | 1.908 | 1.000 |
| liver | LI | -3.464 | 1.944 | 0.997 | -3.420 | 1.961 | 0.999 | -3.395 | 1.970 | 0.997 | -3.581 | 1.902 | 0.999 |
| spleen | SP | -3.308 | 2.006 | 0.995 | -3.429 | 1.957 | 0.999 | -3.449 | 1.950 | 0.998 | -3.574 | 1.905 | 0.999 |
| skeletal muscle | MS | -3.513 | 1.926 | 0.998 | -3.412 | 1.964 | 1.000 | -3.459 | 1.946 | 0.999 | -3.629 | 1.886 | 0.999 |
| heart | HT | -3.291 | 2.013 | 0.998 | -3.359 | 1.985 | 1.000 | -3.351 | 1.988 | 0.998 | -3.560 | 1.909 | 0.999 |
| Mean | | | 1.941 | | | 1.964 | | | 1.960 | | | 1.897 | |
| SD | | | 0.039 | | | 0.023 | | | 0.017 | | | 0.021 | |

For all candidate endogenous controls in both groups: brain tissues and all tissues together (Table 3), the M value was lower than 1.5. Although the BestKeeper algorithm uses Ct Standard Deviation ($SD_{Ct}$) and Correlation Coefficient (r) for the gene expression stability calculation (PFAFFL *et al.* 2004), the results obtained for *OAZ1*, *RPL27* and *RPS29* were the same – all genes were stable (with $SD_{Ct}$ 1 and high r). NormFinder measures the stability value (combination of intra- and intergroup variation) – the lower the stability value, the greater the stability of the gene (ANDERSEN *et al.* 2004). Stability values in NormFinder were very low for all studied genes (Table 3). However, according to NormFinder, geNorm and BestKeeper, the most stable reference gene for all tissues including brain tissue was *RPL27* with the lowest M, the lowest $SD_{Ct}$ and the lowest Stability Value. As for the other two genes, on the basis of all algorithms *RPS29* stability was better than *OAZ1* in brain tissues (except for *OAZ1* r value for brain tissues in BestKeeper) and two algorithms (geNorm and NormFinder) showed that *OAZ1* stability was better than *RPS29* in all tissues (Table 3). *OAZ1* and *RPL27* have not been tested in sheep gene expression normalization studies before. However,

Table 3

Gene expression stability calculations by geNorm, BestKeeper and NormFinder; M – stability measure (geNorm); $SD_{Ct}$ – Ct Standard Deviation (BestKeeper); r – Correlation Coefficient (BestKeeper); SV – Stability Value (NormFinder); bold font – the optimal value according to the calculation tools

| | geNorm - M | | |
|---|---|---|---|
| Tissue \ gene | *OAZ1* | *RPL27* | *RPS29* |
| brain tissues | 0.372 | **0.322** | 0.330 |
| all tissues | 0.575 | **0.489** | 0.635 |
| | BestKeeper - $SD_{Ct}$ / r | | |
| Tissue \ gene | *OAZ1* | *RPL27* | *RPS29* |
| brain tissues | 0.779 / **0.991** | **0.486** / 0.976 | 0.541 / 0.965 |
| all tissues | 0.820 / 0.953 | **0.696** / **0.972** | 0.762 / 0.918 |
| | NormFinder - SV | | |
| Tissue \ gene | *OAZ1* | *RPL27* | *RPS29* |
| brain tissues | 0.0128 | **0.0089** | 0.0095 |
| all tissues | 0.0192 | **0.0093** | 0.0257 |

they have been analyzed in other species. The *RPL27* gene was the second best in DE JONGE's human "top 15 candidate housekeeping genes" list (2007). ROPKA-MOLIK *et al.* (2012) confirmed that *OAZ1* is the best internal control for studying gene expression in porcine uterus and ovary and *RPL27* is the most stable in porcine oviduct. *OAZ1* and *RPL27* were the most suitable reference genes in porcine adipose tissue (PIÓRKOWSKA *et al.* 2011). *OAZ1*, *RPS29* and *RPL27* had the highest stability in porcine stomach (OCZKOWICZ *et al.* 2010). Conversely, *RPS29* had the lowest stability in a preterm lamb model with lung injury (PEREIRA-FANTINI *et al.* 2016) and was relatively unstable in epithelial and nonepithelial cells of mouse small intestine (WANG *et al.* 2010). For studying *PRNP* relative gene expression in ovine cerebrum, cerebellum, obex, spleen, terminal ileum and mesenteric lymph node, GARCIA-CRESPO *et al.* (2005) chose different housekeepeing genes e.g. *ACTB*, *YWHAZ*, *RPLI9*, *GAPDH*, *G6PDH* and *SDHA*. GOSSNER *et al*. (2009) used *YWHAZ*, *GAPDH*, *SDHA* and *HPRT1* for differential expression of *PRNP* and *SPRN* genes in scrapie infected sheep. *OAZ1*, *RPL27* and *RPS29* have not been as popular as *ACTB*, *GAPDH* or *18S RNA* for use as endogenous control genes. However, our findings showed that these three genes could be a good choice for gene expression normalization studies in sheep.

Furthermore, *PRNP* mRNA expression pattern in ovine tissues was investigated in relation to three candidate housekeeping genes in multiplexed reactions: *PRNP* + *OAZ1*, *PRNP* + *RPL27* and *PRNP* + *RPS29* (Fig. 1). As expected, the *PRNP* mRNA abundance was higher in nervous tissue and pituitary gland than in other tissues analyzed, regardless of the endogenous control. HAN *et al.* (2006) obtained similar results, but their experiment was conducted with SYBR Green instead of TaqMan probes. Research on *PRNP* gene expression did not reveal such differences between brain tissues and spleen (GARCIA-CRESPO *et al.* 2005). In fact, the mean *PRNP* mRNA expression in spleen was higher than in cerebrum and cerebellum. In our study, in analyzed tissues except brain tissue, the highest *PRNP* gene expression level was observed in heart. HAN *et al.* (2006) found that the *PRNP* gene expression level in heart was lower than in spleen, but higher than in liver. It is not clear, which part of the heart HAN *et al.* (2006) investigated.

The results of *PRNP* gene expression analysis varied depending on endogenous control used (Friedman's ANOVA: Chi^2 ANOVA = 6.250 (N=9, df=2), p=0.04394). When normalized with *OAZ1*, the highest *PRNP* mRNA expression was observed in brain stem (followed by midbrain, brain cortex and cerebellum) and the lowest in

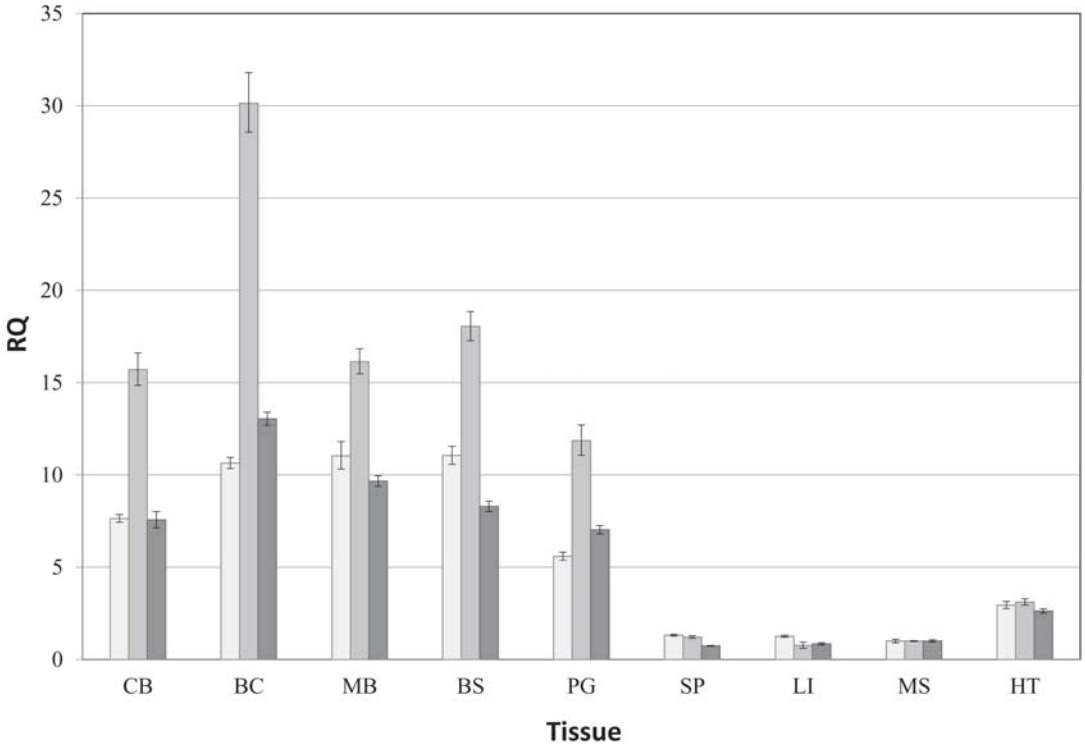


Fig. 1. *PRNP* relative gene expression in different ovine tissues, normalized with *OAZ1* (light grey), *RPS29* (grey) and *RPL27* (dark grey); CB – cerebellum, BC – brain cortex, MB – midbrain, BS – brain stem (*obex*), PG – pituitary gland, SP – spleen, LI – liver, MS – skeletal muscle, HT – heart (*apex cordis*); RQ value calculated in StepOne Software v2.3; the error bars show min and max RQ values.

skeletal muscle; with *RPL27* – the highest *PRNP* mRNA expression was noticed in brain cortex (followed by midbrain, brain stem and cerebellum), and the lowest in spleen; with *RPS29* – the highest *PRNP* mRNA expression was observed in brain cortex (followed by brain stem, midbrain and cerebellum) and the lowest in liver. With *RPS29* as the endogenous control, the *PRNP* expression pattern in tissues varied significantly from the pattern obtained with *OAZ1* (Wilcoxon signed-rank test: Z=1.9604, P=0.0499) and *RPL27* (Wilcoxon signed-rank test: Z=2.3805, P=0.0173). There was no such difference between *PRNP* + *OAZ1* and *PRNP* + *RPL27* (Wilcoxon signed-rank test: Z=0.7001, P=0.4838) expression patterns. Thus, this result confirmed the findings of BestKeeper and NormFinder that apart from *RPL27*, *OAZ1* is a better reference gene than *RPS29* for studying *PRNP* gene expression in all tissues. Taking into account normalization with only *RPL27* and *OAZ1*, the highest *PRNP* gene expression in nervous tissue was observed in brain cortex and brain stem followed by midbrain and cerebellum. However, the differences in the *PRNP* expression level between brain tissues were smaller than 2-fold. Studies conducted in natural scrapie-infected ewes and healthy controls showed higher *PRNP* expression in cerebellum and obex than in cerebrum in both groups (GARCIA-CRESPO *et al.* 2006). Research on sheep with different genotypes showed the highest *PRNP* expression level in obex followed by ileum, lymph node, spleen, cerebellum and cerebrum (GARCIA-CRESPO *et al.* 2005). HAN *et al.* (2006) showed that the highest mRNA abundance was observed in obex and neocortex followed by cerebellum, spinal cord, hippocampi, conarium and thalamus. Some discrepancies between our results and the mentioned studies could be explained by differences in chosen housekeeping genes, sheep age and breed, number of samples and, in some cases, also in method. GOSSNER *et al*. (2009) showed that *PRNP* gene expression might be influenced by *PRNP* genotype. They studied VRQ/VRQ, ARR/VRQ and ARR/ARR sheep and found that in heterozygotes the *PRNP* levels were higher than in homozygotes. However, in previous studies GARCIA-CRESPO *et al.* (2005) did not confirm the hypothesis that *PRNP* mRNA levels vary between genotypes. Animals used in our study had ALRR/ALRR, ALRR/AFRQ and ALRR/ALRQ genotypes. Because of the small number of animals, we couldn't check statistically whether these genotypes influenced *PRNP* gene expression level in our study. Quite interesting results were obtained in our experiment for pituitary gland, where *PRNP* mRNA abundance was almost as high as in cerebellum. To our knowledge, *PRNP* mRNA expression has not been reported to date in ovine pituitary gland. In cattle, *PRNP* expression in pituitary gland was lower than in nervous tissues, spleen, liver and had the same level as in muscle (TICHOPAD *et al.* 2003). Further research is needed to reveal the role of *PRNP* in the pituitary gland.

## Conclusions

Our results confirmed that the *PRNP* gene, which plays an important role in scrapie pathogenesis, is highly expressed in nervous tissue and showed that the *PRNP* mRNA level was also high in pituitary gland. According to geNorm, BestKeeper and NormFinder analysis, the *RPL27* housekeeping gene was the most stable among the three tested candidate endogenous controls (*RPL27*, *OAZ1*, *RPS29*) for studying *PRNP* mRNA abundance in ovine tissues. Therefore, *RPL27* can be considered as one of the reference genes for qPCR normalization studies in ovine tissues.

## Acknowledgments

The study was financed by the Ministry of Science and Higher Education grant project no. NN311406339.

## References

ANDERSEN C.L., JENSEN J.L., ØRNTOFT T.F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. **64**: 5245-5250. https://doi.org/10.1158/0008-5472.Can-04-0496

BAYLIS M., GOLDMANN W. 2004. The genetics of scrapie in sheep and goats. Curr. Mol. Med. **4**: 385-396.

BUSTIN S.A., BENES V., GARSON J.A., HELLEMANS J., HUGGETT J., KUBISTA M., MUELLER R., NOLAN T., PFAFFL M.W., SHIPLEY G.L., VANDESOMPELE J., WITTWER C.T. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time pcr experiments. Clin. Chem. **55**: 611-622; https://doi.org/10.1373/Clinchem.2008.112797

CHAPMAN J.R., WALDENSTRÖM J. 2015. With reference to reference genes: A systematic review of endogenous controls in gene expression studies. Plos One **10**: E0141853. https://doi.org/ 10.1371/Journal.Pone.0141853

DE JONGE H.J.M., FEHRMANN R.S.N., DE BONT E.S.J.M., HOFSTRA R.M.W., GERBENS F., KAMPS W.A., DE VRIES E.G., VAN DER ZEE A.G., TE MEERMAN G.J., TER ELST A. 2007. Evidence based selection of housekeeping genes. Plos One **2**: E898. https://doi.org/10.1371/Journal.Pone.0000898

GARCIA-CRESPO D., JUSTE R.A., HURTADO A. 2005. Selection of ovine housekeeping genes for normalisation by real-time RT-PCR; analysis of prp gene expression and genetic susceptibility to scrapie. BMC Vet. Res. **1**: 3. https://doi.org/10.1186/1746-6148-1-3

GARCIA-CRESPO D., JUSTE R.A., HURTADO A. 2006. Differential gene expression in central nervous system tissues of sheep with natural scrapie. Brain Res. **1073-1074**: 88-92. https://doi.org/10.1016/J.Brainres.2005.12.068

GOSSNER A.G., BENNET N., HUNTER N., HOPKINS J. 2009. Differential expression of *Prnp* and *Sprn* in scrapie infected sheep also reveals *Prnp* genotype specific differences. Biochem. Biophys. Res. Commun. **378**: 862-866. https://doi.org/10.1016/J.Bbrc.2008.12.002

HAN C.X., LIU H.X., ZHAO D.M. 2006. The quantification of prion gene expression in sheep using real-time RT-PCR. Virus Genes **33**: 359-64. https://doi.org/10.1007/S11262-006-0076-6

LAMPO E., VAN POUCKE M., VANDESOMPELE J., ERKENS T., VAN ZEVEREN A., PEELMAN L. J. 2009. Positive correlation between relative mRNA expression of PRNP and SPRN in cerebral and cerebellar cortex of sheep. Mol. Cell. Probe **23**: 60-64.

LYAHYAI J., SERRANO C., RANERA B., BADIOLA J. J., ZARAGOZA P., MARTIN-BURRIEL I. 2009. Effect of scrapie on the stability of housekeeping genes. Anim. Biotechnol. **21**: 1, 1-13, https://doi.org/10.1080/10495390903323851

MAHAKAPUGE T.A., SCHEERLINCK J.P., ROJAS C.A., EVERY A.L., HAGEN J. 2016. Assessment of reference genes for reliable analysis of gene transcription by RT-qPCR in ovine leukocytes. Vet. Immunol. Immunopathol. **171**: 1-6. https://doi.org/10.1016/j.vetimm.2015.10.010

O'CONNOR T., WILMUT I., TAYLOR J. 2013. Quantitative evaluation of reference genes for real-time PCR during *in vitro* maturation of ovine oocytes. Reprod. Dom. Anim. **48**: 477-483. https://doi.org/10.1111/rda.12112

OCZKOWICZ M., RÓŻYCKI M., PIÓRKOWSKA K., PIESTRZYNSKA-KAJTOCH A., REJDUCH B. 2010. A new set of endogenous reference genes for gene expression studies of porcine stomach. J. Anim. Feed Sci. **19**: 570-576. https://doi.org/10.22358/Jafs/66323/2010

PEREIRA-FANTINI P.M., RAJAPAKSA A.E., OAKLEY R., TINGAY D.G. 2016. Selection of reference genes for gene expression studies related to lung injury in a preterm lamb Model. Sci. Rep. **6**: 26476. https://doi.org/10.1038/Srep26476

PFAFFL M.W., TICHOPAD A., PRGOMET C., NEUVIANS T.P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett. **26**: 509. https://doi.org/10.1023/B:Bile.0000019559.84305.47

PIÓRKOWSKA K., OCZKOWICZ M., RÓŻYCKI M., ROPKA-MOLIK K., PIESTRZYŃSKA-KAJTOCH A. 2011. Novel porcine housekeeping genes for real-time RT-PCR experiments normalization in adipose tissue: Assessment of leptin mrna quantity in different pig breeds. Meat Sci. **87**: 191-195. https://doi.org/10.1016/J.Meatsci.2010.10.008

PRUSINER S.B. 1998. Prions. Proc. Natl. Acad. Sci. USA **95**: 13363-13383.

ROPKA-MOLIK K., OCZKOWICZ M., MUCHA A., PIÓRKOWSKA K., PIESTRZYNSKA-KAJTOCH A. 2012. Variability of mRNA abundance of leukemia inhibitory factor gene (LIF) in porcine ovary, oviduct and uterus tissues. Mol. Biol. Rep. **39**: 7965. https://doi.org/10.1007/S11033-012-1642-8

TICHOPAD A., PFAFFL M.W., DIDIER A. 2003. Tissue-specific expression pattern of bovine prion gene: quantification using real-time RT-PCR. Mol Cell. Probes **17**: 5-10.

TRANULIS M.A., BENESTAD S.L., BARON T., KRETZSCHMAR H. 2011. Atypical prion diseases in humans and animals. Top. Curr. Chem. **305**: 23-50. https://doi.org/10.1007/128_2011_161

VANDESOMPELE J., DE PRETER K., PATTYN F., POPPE B., VAN ROY N., DE PAEPE A., SPELEMAN F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3**: 34.1-34.11

WANG F., WANG J., LIU D., SU Y. 2010. Normalizing genes for real-time polymerase chain reaction in epithelial and nonepithelial cells of mouse small intestine. Anal. Biochem. **399**: 211-217. https://doi.org/10.1016/J.Ab.2009.12.029