Evaluation of housekeeping genes for quantitative gene expression analysis in the equine kidney

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Housekeeping genes (HKGs) are used as internal controls for normalising and calculating the relative expression of target genes in RT-qPCR experiments. There is no unique universal HKG and HKGs vary among organisms and tissues, so this study aimed to determine the most stably expressed HKGs in the equine kidney. The evaluated HKGs included 18S ribosomal RNA (18S), 28S ribosomal RNA (28S), ribosomal protein L32 (RPL32), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex (SDHA), zeta polypeptide (YWHAZ), and hypoxanthine phosphoribosyltransferase 1 (HPRT1). The HKGs expression stability data were analysed with two software packages, geNorm and NormFinder. The lowest stability values for geNorm suggests that YWHAZ and HPRT1 would be most optimal (M=0.31 and 0.32, respectively). Further, these two genes had the best pairwise stability value using NormFinder (geNorm V=0.085). Therefore, these two genes were considered the most useful for RT-qPCR studies in equine kidney.

Key words: horse, housekeeping gene, kidney

RT-qPCR is a sensitive and accurate method for measuring gene expression in cells and tissues, but inaccurate pipetting, the quality of RNA, efficiency of cDNA synthesis by reverse transcriptase, and PCR amplification efficiencies can diminish RT-qPCR accuracy [15]. To reduce these factors and prevent misinterpretation of the results, reference genes are used as internal controls for normalising and calculating the relative expression of target genes [6, 8]. It is assumed that reference genes, commonly known as housekeeping genes (HKGs), are stably expressed in the tissues/cells, so choosing a suitable internal reference gene is an important way of ensuring accurate interpretation of the results [11]. These HKGs do vary across tissues and organisms, so it is recommended that a combination of HKGs is used in order to acquire a much more stable and reliable reference [3].

A number of studies on the appropriate internal controls for studying gene expression in equine tissues using RT-qPCR have been published [3, 4, 7, 9, 16, 21]. The aim of this study was to determine the most stably expressed HKGs in the equine kidney, which has not been studied yet, in order to use these for normalisation of gene expression in subsequent RT-qPCR experiments. The HKGs evaluated using RT-qPCR included 18S ribosomal RNA (18S), 28S ribosomal RNA (28S), ribosomal protein L32 (RPL32), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex (SDHA), zeta polypeptide (YWHAZ), and hypoxanthine phosphoribosyltransferase 1 (HPRT1).

Kidney samples were harvested post-mortem (Pathobiology post-mortem room, Massey University, Palmerston North, New Zealand) from nine adult horses (Thoroughbred (n=5) and Standardbred (n=4)). All horses were euthanized at the Pathobiology post-mortem room, Massey University, and procedures conformed to “The Code of Ethical Conduct for the Use of Animals for Teaching and Research” as approved under the New Zealand Animal Welfare Act 1999. Therefore, animal ethics approval was not required for this study. Samples were collected within half an hour of euthanasia, cut into small pieces, snap frozen in liquid nitrogen and stored at −80°C until processing. Adjacent samples from each horse were collected into 10% neutral buffered formalin and processed for histological examination. Haematoxylin and eosin (H & E) stained sections were...
examined to confirm the absence of significant lesions. Based on these evaluations, no lesions were detected.

Primers were designed according to primer sequences previously published [12, 13] or using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/nucleotide) to design primers. The best primer set was selected and the PCR amplicon sequence tested for secondary structures at 60°C using the mFold program (http://mfold.rutgers.edu/?q=mfold).

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA, U.S.A.) and included the optional on-column DNase digestion step (RNase-Free DNase Set, QIAGEN) during RNA isolation. RNA and DNA concentrations were measured using the Qubit® RNA HS and DNA HS Assay (Invitrogen, Life Technologies Corp., Carlsbad, CA, U.S.A.). The Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) was used to synthesise cDNA using Applied Biosystems® Veriti® Thermal Cycler (Applied Biosystems, Life Technologies Corp.).

Real-time PCR was performed using the StepOne Plus real-time PCR machine (Applied Biosystems, Life Technologies Corp.). Real-time PCR reactions (10 µl) contained 5 µl Fast SYBR Green real-time PCR Master Mix (Applied Biosystems, Life Technologies Corp.), the primer pairs, 10 ng of cDNA template and RNase-DNase free water. Negative controls of water and reaction mix without reverse transcriptase were included in every PCR run and all samples were run in duplicate. The real-time data were analysed using the StepOne plus software (Applied Biosystems, Life Technologies Corp.) and were exported into an Excel datasheet (Microsoft Excel 2010) for further analysis.

There are several different mathematical methods for determining the relative expression stability of genes including geNorm [20], Normfinder [2], Bestkeeper [18] and the comparative Delta Ct method [19]. In this study the HKGs expression stability data were analysed with two software packages; geNorm (qbase+ 3.0, Biogazelle, Zwijnaarde, Belgium) [10, 20], and NormFinder (MOMA, Aarhus University Hospital, Denmark http://moma.dk/normfinder-software) [2]. Both methods generate a measure of HKGs stability, which can be used to rank the HKGs.

The geNorm program calculates the gene expression stability value M, which corresponds to the average pairwise variation (V) of a particular gene with all other control genes, to determine the benefit of adding extra reference genes for the normalisation process [20]. The most stable HKG has the lowest M value, while the least stable has the highest M value. Although the geNorm program indicates genes with M values below the threshold of 1.5 as the most stable HKGs, it is suggested that M values lower than 1.0 consider as the most stable HKGs [20]. To obtain reliable results from real-time PCR data, two or more reference genes should be used for data normalisation. The optimal number of reference genes can be determined by calculating the pairwise variation (V), and this was calculated for all the samples analysed. The proposed cut-off value for V is 0.15 [20], below which the inclusion of an additional control gene is not required (Fig. 1).

NormFinder is a Microsoft Excel-based Visual Basic application that estimates the stability values of a single HKG according to the similarity of their expression profiles by using a model-based approach [2]. The results of the NormFinder analysis were similar to those of geNorm, where NormFinder ranked RPL32, GAPDH, HPRT1 and YWHAZ, and geNorm ranked YWHAZ, HPRT1, GAPDH and RPL32 as the top four most stably expressed genes, respectively. Both methods ranked 18S and 28S as the least stable genes (Table 1). The difference in rankings may be explained by the different algorithms used and the different methodologies employed; where NormFinder identifies the single best gene with the most stable expression in the tissue, geNorm mostly detects the two (or more) optimal genes with the least variation in their expression ratio compared to other genes, but the results can be skewed by coregulation of genes in similar functional classes. NormFinder also takes the inter- and intra-group variations into account and is not significantly affected by coregulation of HKGs [3]. Most HKGs that were chosen in this study, apart from 18S and 28S, belong to different functional classes thus avoiding coregulation as a problem in the analysis.

While the results from the two programs were similar, because the geNorm ranking calculates V values for the proposed groups of HKGs. This is useful for deciding the optimal number of HKGs to be used in gene expression studies [20]. Therefore, it was decided that since YWHAZ and HPRT1 genes had good stability in both geNorm and NormFinder they would be appropriate to use as HKGs in further gene expression studies on kidney tissue from horses.

Studies have been reported where the most stable HKGs in kidneys of other species were determined. The most stable renal HKGs reported in the cat were RPL30 (ribosomal protein L30), HMBS (hydroxymethylbilane synthase), YWHAZ and B2M [16], in dogs RPS19 (ribosomal protein S19), RPS5 (ribosomal protein S5), B2M, and HPRT [5] and RPL13A (ribosomal protein L13a) and RPL32 [17], and in cattle GAPDH and YWHAZ [14]. This suggests that reference genes tend to be species independent. However, YWHAZ and HPRT appear to be fairly stably expressed in the kidney of many species.

Two other studies have examined the stability of HKGs in equine renal tissue [1, 21]. However, in these studies, pan-tissue HKG which were not specific to the kidney were examined. The proposed HKGs for normalisation in these
studies were *Ubiquitin B (UBB)* [1], and *18S* [21]. *UBB* was not one of the HKGs that were used in the current study and *18S* ranked as the one of the least stable HKGs by both geNorm and NormFinder programs for equine kidney. The previous studies examined different numbers of HKGs and validated them across a large number of tissues, and then chose the most stable HKG to be used in all of those tissues. The main focus of the current study was to determine the most stable HKGs in the kidney. Therefore, any comparison of different gene expression between individuals should consider that the expression of HKGs could vary according to the target tissue. Another limitation of previous studies is that only one HKG was suggested. Using a single HKG is often not suitable and not accurate for normalisation in gene expression studies.

In summary, the current study is the first to specifically examine the stability of HKGs in the equine kidney and suggests that a combination of *YWHAZ* and *HPRT1* genes
can be used as HKGs for RT-qPCR studies.

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