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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*

J. Equine Sci.
Vol. 27, No. 4
pp. 165–168, 2016

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*), *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

Received: June 13, 2016
Accepted: September 1, 2016
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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.rit.albany.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® 2.0 Fluorometer and 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 RNAase-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

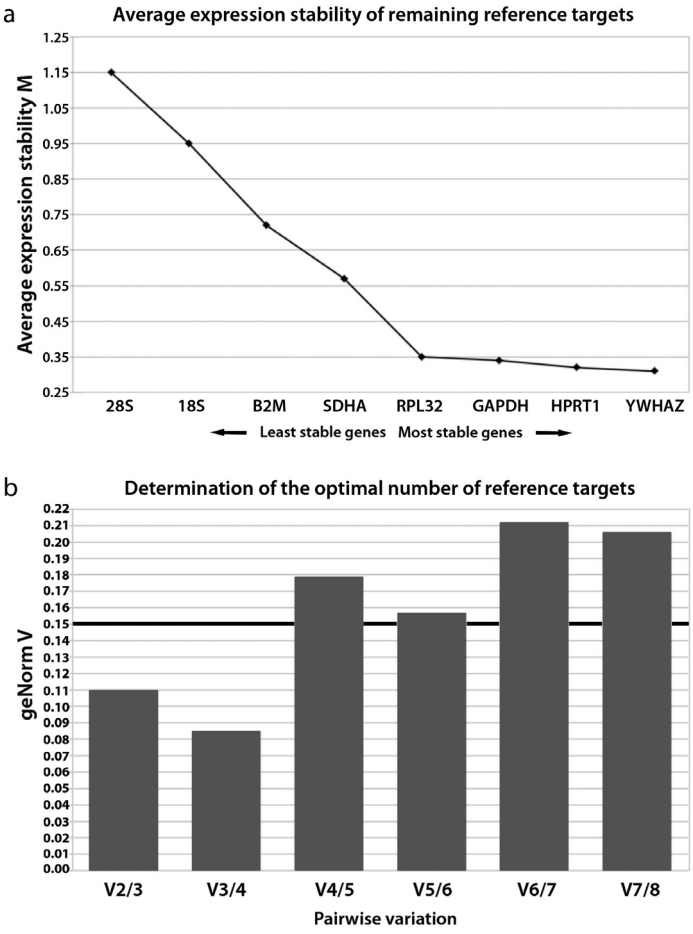

Fig. 1. Gene expression stability of the candidate housekeeping genes (HKGs) in equine renal tissue analysed by the geNorm program. a) Average expression stability values (M) of the HKGs in equine kidney measured during geNorm stepwise exclusion of the least stable reference genes. Lower M values correspond to the most stable and most suitable HKGs for normalisation. b) Evaluation of the optimum number of HKGs according to the geNorm software.

Table 1. Expression stability values of the candidate housekeeping genes calculated by the geNorm and Normfinder algorithms (ranking in parentheses)

Gene	*18S*	*28S*	*RPL32*	*B2M*	*GAPDH*	*SDHA*	*YWHAZ*	*HPRT1*
Stability Value	0.95	1.16	0.35	0.72	0.34	0.54	0.31	0.32
geNorm	(7)	(8)	(4)	(6)	(3)	(5)	(1)	(2)
Stability Value	0.9	1.12	0.1	0.86	0.14	0.49	0.24	0.16
Normfinder	(7)	(8)	(1)	(6)	(2)	(5)	(4)	(3)

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.

References

1. Ahn, K., Bae, J.H., Nam, K.H., Lee, C.E., Park, K.D., Lee, H.K., Cho, B.W., and Kim, H.S. 2011. Identification of reference genes for normalization of gene expression in thoroughbred and Jeju native horse (Jeju pony) tissues. *Genes Genomics* **33**: 245–250. [CrossRef]
2. Andersen, C.L., Jensen, J.L., and 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. [Medline] [CrossRef]
3. Beekman, L., Tohver, T., Dardari, R., and Léguillette, R. 2011. Evaluation of suitable reference genes for gene expression studies in bronchoalveolar lavage cells from horses with inflammatory airway disease. *BMC Mol. Biol.* **12**: 5. [Medline] [CrossRef]
4. Bogaert, L., Van Poucke, M., De Baere, C., Peelman, L., Gasthuys, F., and Martens, A. 2006. Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. *BMC Biotechnol.* **6**: 24. [Medline] [CrossRef]
5. Brinkhof, B., Spee, B., Rothuizen, J., and Penning, L.C. 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. *Anal. Biochem.* **356**: 36–43. [Medline] [CrossRef]
6. Brito, A.B., Lima, J.S., Brito, D.C., Santana, L.N., Costa, N.N., Miranda, M.S., Ohashi, O.M., Santos, R.R., and Domingues, S.F.S. 2013. Validation of reference genes for ovarian tissue from capuchin monkeys (Cebus apella). *Zygote* **21**: 167–171. [Medline] [CrossRef]
7. Cappelli, K., Felicetti, M., Capomaccio, S., Spinsanti, G., Silvestrelli, M., and Supplizi, A.V. 2008. Exercise induced stress in horses: selection of the most stable reference genes for quantitative RT-PCR normalization. *BMC Mol. Biol.* **9**: 49. [Medline] [CrossRef]
8. Feng, H., Huang, X., Zhang, Q., Wei, G., Wang, X., and Kang, Z. 2012. Selection of suitable inner reference genes for relative quantification expression of microRNA in wheat. *Plant Physiol. Biochem.* **51**: 116–122. [Medline] [CrossRef]
9. Figueiredo, M.D., Salter, C.E., Andrietti, A.L.P., Vandenplas, M.L., Hurley, D.J., and Moore, J.N. 2009. Validation of a reliable set of primer pairs for measuring gene expression by real-time quantitative RT-PCR in equine leukocytes. *Vet. Immunol. Immunopathol.* **131**: 65–72. [Medline] [CrossRef]
10. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* **8**: R19. [Medline] [CrossRef]
11. Huggett, J., Dheda, K., Bustin, S., and Zumla, A. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* **6**: 279–284. [Medline] [CrossRef]
12. Kayis, S.A., Atli, M.O., Kurar, E., Bozkaya, F., Semacan, A., Aslan, S., and Guzeloglu, A. 2011. Rating of putative housekeeping genes for quantitative gene expression analysis in cyclic and early pregnant equine endometrium. *Anim. Reprod. Sci.* **125**: 124–132. [Medline] [CrossRef]
13. Klein, C., Rutllant, J., and Troedsson, M.H. 2011. Expression stability of putative reference genes in equine endometrial, testicular, and conceptus tissues. *BMC Res. Notes* **4**: 120. [Medline] [CrossRef]
14. Lisowski, P., Pierzchała, M., Gościk, J., Pareek, C.S., and Zwierzchowski, L. 2008. Evaluation of reference genes for studies of gene expression in the bovine liver, kidney, pituitary, and thyroid. *J. Appl. Genet.* **49**: 367–372. [Medline] [CrossRef]
15. Paris, D.B., Kuijk, E.W., Roelen, B.A.J., and Stout, T.A.E. 2011. Establishing reference genes for use in real-time quantitative PCR analysis of early equine embryos. *Reprod. Fertil. Dev.* **23**: 353–363. [Medline] [CrossRef]
16. Penning, L.C., Vrieling, H.E., Brinkhof, B., Riemers, F.M., Rothuizen, J., Rutteman, G.R., and Hazewinkel, H.A.W. 2007. A validation of 10 feline reference genes for gene expression measurements in snap-frozen tissues. *Vet. Immunol. Immunopathol.* **120**: 212–222. [Medline] [CrossRef]
17. Peters, I.R., Peeters, D., Helps, C.R., and Day, M.J. 2007. Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. *Vet. Immunol. Immunopathol.* **117**: 55–66. [Medline] [CrossRef]
18. Pfaffl, M.W., Tichopad, A., Prgomet, C., and 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**: 509–515. [Medline] [CrossRef]
19. Silver, N., Best, S., Jiang, J., and Thein, S.L. 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol. Biol.* **7**: 33. [Medline] [CrossRef]
20. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**: H0034. [Medline] [CrossRef]
21. Zhang, Y.W.W., Davis, E.G., and Bai, J. 2009. Determination of internal control for gene expression studies in equine tissues and cell culture using quantitative RT-PCR. *Vet. Immunol. Immunopathol.* **130**: 114–119. [Medline] [CrossRef]