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Validation of housekeeping genes for gene expression analysis of bone broilers samples using real-time PCR

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Gene expression studies using real-time PCR for mRNA quantification has been widely used. This methodology has high accuracy, sensitivity and reproducibility. However, to ensure a reliable relative quantification, a stable housekeeping gene is required. These genes should not change over the treatments and they are often required to correct variations from sample to sample, such as the amount and integrity of the starting material, the efficiency of the RNA extraction, enzymatic efficiency and the differences between tissues or cells. Several genes are commonly used (GAPDH, 18S ribosomal RNA and actin), but their variations have already been reported depending on the experiments. Regarding this, it is suggested that for each experiment, many genes should be tested and the more stable ones chosen. Besides that, no housekeeping gene was defined for chicken bone tissues to date. Hence, the aim of this study was to evaluate and validate stable housekeeping genes in samples of affected and non-affected chickens with femoral head necrosis. Bone samples of 20 commercial broilers with 45 days of age (10 affected and 10 non-affected) were collected, submitted to total RNA isolation using Trizol (Invitrogen), followed by cDNA synthesis (SuperScript III - Invitrogen). Primers were designed in exon-exon junction for 6 genes previously described as housekeeping genes: *HBMS-2, RPL30*, *RPL4, HPRT-1, RPLP1* and *GAPDH*. The qPCRs were performed in ABI 7500 SDS (Applied Biosystems) real time PCR equipment using SYBR Green as dye and with samples in duplicate. The Ct (cycle threshold) values were obtained and submitted to specific software for analysis of housekeeping gene stability: Genorm, Normfinder, BestKeeper, and also for DeltaCT analysis. After, the genes were ranked according to their stability using the link: http://www.leonxie.com/referencegene.php. For all software used, the *HBMS* gene was the most stable, followed by the *RPL-30*, which varied around 2.3 Cts. In contrast, *RPLP1* and *GAPDH* genes were the most unstable, varying 4.09 and 4.11 Cts, respectively. It is interesting to note that the *GAPDH,* one of most commonly used housekeeping gene in mRNA expression analysis, was the most variable gene in this study, reinforcing the need to evaluate several reference genes before the relative quantification analysis. Besides that, according to Genorm software, the combination of *HBMS* and *RPL-30* was indicated when two genes are used together in the relative quantification. Thus, the *HBMS* and the *RPL-30* are effective housekeeping genes for relative quantification on chicken femur samples.

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