Selection of housekeeping genes for quantitative gene expression analysis in yellow-feathered broilers

Jie Zhang*, Yu-Yun Gao*, Yi-Qiang Huang, Qian Fan, Xin-Tao Lu and Chang-Kang Wang

College of Animal Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China

ABSTRACT
The aim of this study was designed to select housekeeping genes for quantitative gene expression analysis in yellow-feathered broilers. Twelve 3-week-old chickens were randomly selected from 60 yellow-feathered broilers. Then, 12 chickens were killed; the liver and jejunum samples were collected. The gene expression of housekeeping genes (β-actin, ACTB; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; hypoxanthine phosphoribosyl transferase 1, HPRT1; ribosomal protein L13, RPL13; TATA box binding protein, TBP; hydroxymethylbilane synthase, HMBS) were determined using quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, the expression stabilities of housekeeping genes were analysed using geNorm, Normfinder and BestKeeper programs. The result showed that RPL13 is the most proper gene in liver, GAPDH is the most proper gene in jejunum, and HMBS is the most proper gene in all tissues. In conclusion, this result provides the integrated reported evaluation of housekeeping genes for use in expression studies in yellow-feathered broilers. These findings further emphasise the need to accurately validate candidate housekeeping genes in the study before use in gene expression studies using RT-PCR.

Introduction
Real-time reverse transcriptase polymerase chain reaction (RT-PCR) is a sensitive technique for quantifying gene expression levels. RT-PCR has various applications, such as clinical diagnostics (Bustin and Dorudi 1998), gene expression and transcriptome analysis (Gachon et al. 2004). ‘Minimum Information for Publication of Quantitative Real-Time PCR Experiments’ (MIQE) was published to achieve more reliable and unequivocal interpretation from RT-PCR experiments (Bustin et al. 2009). It introduced precise normalisation, as an important requirement to ensure the accuracy of this method and obtain reliable gene expression measurement.

Because of differences in the quality and quantity of temple RNA and differences in efficiencies of reverse transcription between different samples (Huggett et al. 2005), the appropriate method for quantification is normalisation of the expression level of target gene to the expression level of housekeeping gene (Gutierrez et al. 2008; Udvardi et al. 2008; Løvdal and Lillo 2009). The housekeeping gene, such as ACTB, GAPDH and HPRT1 (Godornes et al. 2007; Wang et al. 2012; Marimoutou et al. 2015), is a stably expressed gene that is experimentally verified in given species and tissue under given experimental conditions (Løvdal and Lillo 2009). However, differences in the tissue and differences in experiment condition should have different choice criteria (Radonić et al. 2005; Watson et al. 2007; Boever et al. 2008; Cinar et al. 2012; Bages et al. 2015). There is a few reports about the stability of housekeeping genes in chickens (Bages et al. 2015; Nascimento et al. 2015). Therefore, this study was designed to evaluate the stability of housekeeping genes (ACTB, GAPDH, HMBS, HPRT1, RPL13 and TBP), aiming to provide the effective gist of housekeeping genes for use in expression studies in broilers.

Material and methods

Ethics statement
All animals used in this study were treated following the guidelines for experimental animals established by...
the Council of China. Animal experiments were approved by the Science Research Department of the Committee of Animal Care, Fujian Agriculture and Forestry University, Fuzhou, China (approval number: FAFU2016J15).

**Animal management**

A total of 60 three-week-old healthy yellow-feathered male broilers with an average initial body weight of 37.32 ± 0.22 g (mean ± standard deviation [SD]) were obtained from a commercial hatchery (Guangdong Wens Food Group Co. Ltd., China). A total of 12 chickens were randomly selected from 60 yellow-feathered broilers. Chickens were slaughtered by exsanguination and samples of liver and jejunum were collected within the following 15 min. Samples were immediately frozen in liquid nitrogen and then stored at −80°C until analysis.

**RNA isolation and reverse transcription**

The MIQE principle and operational method to determine gene expression were used in this paper (Bustin et al. 2009; Taylor and Mrkusich 2014; Gao et al. 2016). Total tissue RNA was extracted using RNAprep Pure Tissue Kit (DP431, Beijing Tiangen Biotechnology Co. Ltd., China) from liver and jejunum of chickens according to the manufacturer’s instructions. The RNA quantity and purity were determined at 260/280 nm by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). This ratio ranged from 1.8 to 2.0 for all samples. The 20 μL reverse transcription reaction mixture consisted of the following components: 1 μL of total RNA, 1 μg/μL, 0.5 μL of RNase inhibitor, 1 μL of oligo dT primer 500 μg/mL, 1 μL of random primers, 1 μL of PCR nucleotide mix 10 mM, 1 μL reverse transcriptase, 2 μL of MgCl₂ 25 mM, 4 μL of 5X reaction buffer, 8.5 μL of nuclease-free water. The reverse transcription was performed according to the Reverse Transcription System kit (A3500, Promega, Madison, WI). The reverse transcription products (cDNA) were stored at −20°C for RT-PCR.

**Housekeeping genes primer design**

Six housekeeping genes were selected based on their common use as endogenous control genes in gene expression studies. Primer pairs were designed for ACTB, GAPDH, HMBS and TBP based on the published sequences with the following respective Genbank accession numbers: NM_205518, NM_204305, XM_417846 and NM_205103, respectively. Primer pairs were designed for HPRT1 and RPL13 based on Primer-BLAST with the following respective Genbank accession numbers: NM_204848 and NM_204999, respectively. The designed primer sequences (Table 1) were validated on BLAST in order to ensure high efficiency. Primers were synthesised by Shanghai Sangon Biotechnology Co. Ltd., China.

**RT-PCR**

RT-PCR was performed in a 20 μL reaction mixture that included 10 μL 2X qPCR Master Mix Go Taq, 0.5 μL (10 μM) of each primer, 2 μL cDNA and 7 μL nuclease-free water as recommended in the manufacturer’s instructions provided with Go Taq™ qPCR Master Mix (A6001, Promega, Madison, Wisconsin, CA). Amplification was performed by one round of pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 58–60°C for 1 min. All reaction mixtures were incubated in an ABI 7500 RT-PCR System (Applied Biosystems, Foster City, CA). The PCR amplification efficiency of each primer pair was calculated from a standard curve using a fivefold dilution series (1/10³, 1/10², 1/10¹, 1/10⁰ and 1/10⁻⁴) of template cDNA. The efficiency values (Eff.) were obtained from the

| Name | Gene number | Strand | Sequence | Size (bp) | Annealing temperature (°C) |
|------|-------------|--------|----------|-----------|---------------------------|
| GAPDH | NM_204305 | Sense | ACTGTCAAGGCTGAGAACGG | 86 | 60 |
| ACTB | NM_205518 | Sense | ATCGCGAACCCTCATTGTC | 120 | 60 |
| HMBS | XM_417846 | Sense | GATGGATGCGATAGCCTGAA | 195 | 60 |
| RPL13 | NM_204999 | Sense | TGGATCCAGCGCAAGAAAC | 193 | 58 |
| HPRT1 | NM_204848 | Sense | TGGGAGTACCTCTCACAATC | 187 | 58 |
| TBP | NM_205103 | Sense | TGTGTCAGGCGACACTCTTG | 182 | 60 |
following equation (Pfaffl et al. 2004):

$$\text{Efficiency} \% = (10^{1-\frac{1}{\text{slope}}} - 1) \times 100\%$$

Data analysis

The RT-PCR results were analysed by ABI 7500 software (Applied Biosystems, Foster City, CA). Each sample was analysed in triplicate to obtain an average Ct value. To confirm results, three different programs, geNorm (Vandesompele et al. 2002), Normfinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004) were used. These programs calculate the gene stability value to rank the housekeeping genes.

Results

Integrity of total RNA

The results of agarose gel electrophoresis (Figure 1) showed that total RNA of liver and jejunum samples clearly exhibited the visible 28S/18S peaks. The 260/ 280 nm values (mean ± SD) of liver and jejunum samples were 1.8972 ± 0.09573 and 1.8852 ± 0.08327, respectively. Only those RNA samples with good integrity and high purity were used for RT-PCR.

Analysis of housekeeping genes’ specificity

The melt curves (Figure 2) of ACTB, GAPDH, HMBS, HPRT1, RPL13 and TBP exhibited only one specific peak without primer dimer and nonspecific amplification, and the melt temperatures (mean ± SD) were 82.0 ± 0.2 °C, 81.8 ± 0.2 °C, 84.5 ± 0.2 °C, 80.9 ± 0.2 °C, 86.6 ± 0.2 °C and 81.1 ± 0.2 °C, respectively. The amplification efficiency of ACTB, GAPDH, HMBS, HPRT1, RPL13 and TBP were 105.23%, 102.87%, 107.62%, 96.78%, 95.12% and 100.56%, respectively. Therefore, the result of RT-PCR was accurate and reliable.

Determination of the stability of housekeeping genes by geNorm

geNorm program relies on the identical expression ratio of two ideal reference genes in all samples, independent of the treatment, condition or tissue. In this principle, the housekeeping gene expression stability measure (M value) is calculated as the average pairwise variation between an individual gene and all other housekeeping genes included in this analysis (Vandesompele et al. 2002). Low M values characterise genes with the most stable expression, that is, geNorm program has a cut-off limit of variability suggesting that any gene with an M < 1.5 should be considered reliable as a stable housekeeping gene. In this study, each data was transformed to relative quantities using the formula: $2^{-\Delta Ct}$, in which $\Delta Ct = \text{the corresponding Ct value} – \text{minimum Ct value}$. From the transformed data, geNorm produced a graph based on M values. From these results (Table 2), the rank in liver showed that the most stably expressed gene was GAPDH, and followed by RPL13, HMBS, HPRT1, ACTB and TBP. The rank in jejunum showed that the most stably expressed gene was GAPDH, and followed by RPL13, TBP, HMBS, ACTB and HPRT1. Meanwhile, the rank in all tissues showed that the most stably expressed gene was TBP, and followed by HMBS, ACTB, HPRT1, RPL13 and GAPDH.

Determination of the stability of housekeeping genes by Normfinder

Normfinder program is a RT-PCR data normalisation tool that ranks the potential housekeeping genes expression stability values for all samples. Normfinder programme calculates a stability value that is inversely correlated with the stability of gene expression (Andersen et al. 2004). Therefore, the lowest stability value will be top ranked. In our study, all raw Ct values were transformed to relative quantities using the $2^{-\Delta Ct}$ method, and each data was calculated and ranked using Normfinder (Table 2). The rank in liver showed that the most stably expressed gene was RPL13, followed by GAPDH, HMBS, HPRT1, ACTB and TBP. The rank in jejunum showed that the most stably expressed gene was GAPDH, followed by RPL13, TBP, HMBS, ACTB and HPRT1. Meanwhile, the rank in all tissues showed that the most stably expressed gene was ACTB, followed by HMBS, TBP, HPRT1, GAPDH and RPL13.

Determination of the stability of housekeeping genes by BestKeeper

BestKeeper program determines the variability in expression level of a set of housekeeping genes by analysing Ct values and classifying variability by the
coefficient of variance (CV) and the SD. BestKeeper creates BestKeeper Index which is the geometric mean of Ct values of all candidate reference genes grouped together. To define the most stable housekeeping gene, BestKeeper index is compared to each housekeeping gene resulting in a value for the Pearson correlation coefficient. And BestKeeper employs a pairwise correlational analysis which selects the optimal housekeeping genes (Pfaffl et al. 2004). It is recommended that any gene with a SD < 1 should be considered as a stable housekeeping gene and, in this study, all studied housekeeping genes with SD < 1

Figure 2. Melt curves generated for housekeeping genes. The melt curves of six housekeeping genes showed one specific peak.
could be considered as the stable housekeeping genes. From BestKeeper Index (Table 2), HPRT1 was shown to be the most stable gene in liver, followed by RPL13, HMBS, GAPDH, TBP and ACTB. ACTB was shown to be the most stable gene in jejunum, followed by GAPDH, HPRT1, TBP, RPL13 and HMBS. Meanwhile, HPRT1 was shown to be the most stable gene in all tissues, followed by HMBS, TBP, ACTB, RPL13 and GAPDH.

**Discussion**

RT-PCR has become a powerful tool for sensitive gene expression measurement, especially when samples quantities are limited or a transcript is expressed at a relatively low amount of RNA (Wong and Medrano 2005; Nolan 2006). However, the sensitivity of gene expression also means that experimental conditions of variation should be considered, such as differences in sample, differences in reverse transcriptase reaction efficiency and the amount of cDNA template used in each RT-PCR (Walker et al. 2008). Therefore, selecting appropriate housekeeping genes is an essential step for analysing gene expression using RT-PCR, and this essential step can improve the fidelity of RT-PCR result (Vandesompele et al. 2002; Guo et al. 2014). However, many researches make use of these housekeeping genes without proper validation of their stability of expression levels. This means that the proper housekeeping gene should be identified for a specific species under study or in a new experimental set up. In this study, we used RT-PCR technique for ACTB, GAPDH, HMBS, HPRT1, RPL13 and TBP as the housekeeping gene, and tested the stability of candidate housekeeping genes in liver and jejunum of broilers.

It is well known that choice of one, or preferably more, suitable reference genes are a key factor for any gene expression analysis applying RT-PCR (Engdahl et al. 2016; Huang et al. 2016; Xu et al. 2016). GAPDH or ACTB is commonly used housekeeping genes for gene expression studies in many researches (Niu et al. 2011; Guo et al. 2013; Buldak et al. 2014; Bronkhorst et al. 2016). ACTB was the most appropriate housekeeping gene for cell-free DNA and mRNA quantification (Bronkhorst et al. 2016), as well as GAPDH was shown to be a stable gene under heat shock in *Ditylum brightwellii* (Guo et al. 2013). However, other studies suggested that the conventional housekeeping genes were not always highly stably expressed in other animal species or in a complex range of experimental treatments (Schmittgen and Zakrajsek 2000; Selvey et al. 2001; Zhu et al. 2001; Glare et al. 2002; Bas et al. 2004). For example, Bas et al. (2004) demonstrated that ACTB was not suitable for housekeeping gene using RT-PCR in human T lymphocytes. In addition, GAPDH was described as the least stable gene in salmons infected by virus (Jorgensen et al. 2006). In our study, we also found that the most commonly used GAPDH should not be considered as reliable housekeeping gene in all tissues rank (Table 2).

The systematic validation of housekeeping genes demonstrated that none of them performed consistently well for all sample types and that the stability of genes varied according to tissue analysis (Table 2). All in all, this study employed geNorm, Normfinder and BestKeeper programs to validate the housekeeping gene stability. However, three programs did not rank the candidate housekeeping genes in the same ranks. This is due to differences in algorithms. geNorm calculates the stable value of gene expression, and
estimates the number of housekeeping gene (Jain et al. 2006; Jian et al. 2008). Normfinder focuses on the coefficient of variation of a gene across all samples (Andersen et al. 2004). BestKeeper depends on pairing correlation analysis about Ct values, and analyses original data directly (Kumar et al. 2011). According to the ranking provided by geNorm, GAPDH and RPL13 were the top two genes in liver and jejunum, and TBP and HMBS were the top two genes in all tissues. According to the ranking provided by Normfinder, GAPDH and RPL13 were also the top two genes in liver and jejunum, and ACTB and HMBS were the top two genes in all tissues. According to the ranking provided by BestKeeper, HPRT1 and RPL13 were the top two genes in liver, ACTB and GAPDH were the top two genes in all tissues. From the above, we conclude that RPL13 is the most proper gene in liver, GAPDH is the most proper gene in jejunum, and HMBS is the most proper gene in all tissues. Therefore, choice of housekeeping genes should apply some kinds of programs and analyse result at the same time. Only in this way, can we select the appropriate housekeeping gene for RT-PCR.

In conclusion, this study is aimed to validate a set of commonly used housekeeping genes in chicken tissues for the normalisation of gene expression analysis using RT-PCR. Our experimental purpose was to define the most stable expression of housekeeping gene, rather than determining a ‘best’ method for all experimental conditions. Therefore, the selected housekeeping genes may improve the reliability of gene expression studies in broilers. At the same time, these findings further emphasise the necessity to accurately validate candidate housekeeping genes in the study before use in gene expression studies using RT-PCR.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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