Evaluation and Validation of the Six Housekeeping Genes for Normalizing Mrna Expression in the Ovarian Follicles and Several Tissues in Chicken

ABSTRACT

Expression of housekeeping genes is relatively constant in different tissues and cells by RT-qPCR analysis. Housekeeping genes (HGs) are usually utilized as the reference to evaluate and compare mRNA expression abundances of target genes in different cells or tissues sampled. However, the expression stabilities of different HGs in diverse samples may appear divergence. Currently, there is no exact reference data of HGs in hen ovarian follicular tissues during egg-laying period available yet. In this study, we detected the expression of 18SrRNA, ACTB, HOXC8, GAPDH, alpha-A, and alpha-D mRNA in the varied-size ovarian follicles (1-8 mm in diameter and F5), hearts, livers, spleens, lungs, and breast muscles of the laying hens by RT-qPCR, to analyze the results via Ct value, geNorm, Normfinder, and Bestkeeper. The data showed that the expression levels of 18SrRNA, alpha-A, and alpha-D transcripts were more significantly stable than the other three genes for normalizing mRNA expression in the hen ovarian follicles examination. Moreover, alpha-D, 18SrRNA, and alpha-A were also most suitable for the expression normalization in the tissues of the heart, liver, spleen, lung and breast muscle. In contrast, 18SrRNA has the most stable mRNA expression levels in all tissues sampled, so it can serve as an excellent inner control for the evaluation of the transcription levels in chickens. It is a remarkable fact that HOXC8 as a candidate reference should be avoided. Our study establishes a set of stably expressed candidate inner references in the hen ovarian follicles and several tissues, it firstly provided an exact data for validation of the inner references in normalizing transcription levels of a target gene in chickens.

INTRODUCTION

Egg production is one of the most important economic traits and reproduction trait for hens. However, the production performance is significantly different between high-yield and low-yield layers. The differences depend mainly upon the cyclic process of ovarian follicle development, follicle recruitment, and maturation. As known the process is complicated and under high coordination in hens, which was jointly regulated by the hypothalamus pituitary gonadal (HPG) axis endocrine hormone and many genes in the ovarian follicles in particular. Many studies determined that some genes such as FSHR, GDF9, STAR, and CYP11A1 were closely related to development and maturation of the follicles (Xu et al., 2018a). In addition to the follicles, the mRNA expression profiles of many genes in the other tissues and organs is also vital to the influence on the reproductive physiology in chicken, which are also involved in regulating the development of the ovarian follicles. The technique of RT-qPCR assay is a sensitive and powerful method to study the differences in gene expression (Liu et al., 2013). It can monitor gene expression by measuring mRNA levels and detect more
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subtle changes in gene expression (Nailis et al., 2006). However, there are some problems that restrict the use of RT-qPCR, such as extraction efficiency of RNA and the number of cells in different samples (Vandesompele et al., 2002). Mainly proper HGs are selected to normalize the experiments (Thellin et al., 2006; Lyahyai et al., 2009). HGs were usually used as internal controls in a variety of tissues and cells because of stabilisation and high expression at mRNA levels. Therefore, it was very important to normalize the data by selecting the suitable HGs according to the diverse samples, organ development process and experimental conditions. But some studies have demonstrated the expression level of some HGs will make a difference under certain circumstance (Yan et al., 2016; McGovern et al., 2018; Shen et al., 2019). Eight housekeeping genes were selected to detect the expression levels by geNorm, NormFinder and Bestkeeper software packages in ten types of tissues sourced from Boer goats. The results showed that in different tissue types, the most stable genes were different. 18SrRNA was the most stable in the heart and spleen; ACTB in the stomach, small intestine and ovary, GAPDH was in the muscle (Zhang et al., 2013). Five reference genes (Tuba1a, ACTB GAPDH, 18SrRNA, and Hist4h4) were validated in the stability of expression during different stages of mouse lung development. The results indicate that Tuba1a had the least variability in expression among the different stages of lung development (Mehta et al., 2015). Twelve commonly used HGs were evaluated in mouse oocytes and embryos cultured in vivo and in vitro by geNorm software. The results proved that the twelve genes showed different stabilities and ranking. The classical housekeeping gene ACTB showed the least stability (Mamo et al., 2007). Notably, there was no evidence to show that these HGs are suitable for all types of experiments. Accordingly, the best way is to identify the appropriate normalizing gene for each type of tissue, age or organism development process (Touchberry et al., 2006). Whereas, a limited number of studies related to the standardized HGs used in chicken ovarian follicles and related tissues during egg-laying periods.

Commonly used HGs in hens were GAPDH, ACTB, 18SrRNA, 28SrRNA, and HSP70 (Lenart et al., 2017), but lacked strict and systemic experiment to validate, so as to evaluate which HGs may be the most suitable candidates for normalization in hens during egg-laying periods, especially in various sized follicles of hen ovary. Finally we found that 18SrRNA ribosomal RNA (18SrRNA), Beta-actin (ACTB), homeoboC8 (HOXC8), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chicken alpha-A-lglobingene (alpha-A) and chicken alpha-D-lglobingene (alpha-D) as candidate internal controls may be commonly used in chicken, but these genes changed a lot between various sample panels. 18SrRNA is a member of the ribosomal subunits, which is conservative and easy to use as general primer amplification in all eukaryotic cells. It’s interesting that the 18SrRNA gene does not transcribe into mRNA during the cell cycle, and is less affected by changes in the cell’s internal and external environment and functional state. The alpha-A and alpha-D belong to the a-globin gene family, they have been useful for studying the transcription regulation mechanisms in higher eukaryotes, and the chicken globin families have the characteristics of developmental regulator genes especially. Some finding indicated that there were certain coordinate expressions between alpha-A and alpha-D, but the mechanism remains unknown. GAPDH is a key enzyme in glycolysis and widely used as reference gene, nevertheless, its expression vary greatly in different development stages of different individuals (Kozera & Rapacz., 2013). ACTB is also one of the commonly used internal reference genes and is one of the main components of cytoskeletal actin. Several studies have reported that during the process of cell differentiation, the levels of GAPDH and ACTB varied greatly in different cell types (Lyahyai et al., 2009; Yan et al., 2016; McGovern et al., 2018; Shen et al., 2019). HOXC8 is a regulatory gene that controls embryonic development and cell differentiation, it was fairly conservative during evolution and expressed stably in the embryonic tissues of the chickens and quails. The reproduction of poultry was regulated by the HPG, and there are many studies on the mRNA expression of functional genes on chicken gonad axis now, but if the tissue expression stability of HG transcripts served as internal reference is not clear. Therefore, we currently investigated the variability of gene expression in the hearts, livers, spleens, lungs, breast tissues, ovarian follicles of 1-4.9 mm 5-6.9 mm 7-8 mm in diameter, and hierarchical follicle F5 in chicken during egg-laying periods in order to identify the most suitable internal controls for the normalization of targeting gene’s transcription in the sampled tissues and organs.

MATERIALS AND METHODS

Animals and sampling

Forty Hy-Line hens were obtained from the College of Animal Science and Technology of Jilin Agricultural
University, which were randomly selected from the Hy-Line chicken population, and raised in layer battery cages under the same rearing conditions, to include free access to water and feed in accordance with the nutrient requirements of Hy-Line hens (NY/T 33-2004, China). All of the hens were exposed to a 16L: 8D photoperiod, with lights on at 5:00 am and lights off at 9:00 pm. The relative humidity and temperature were adjusted according to the chicken’s behavior. All animal experimentations were carried out in accordance with the guidelines (Permission No. GR(J)19-039) approved by the Institutional Animal Care and Use Committee of Jilin Agricultural University (Changchun, China), which was issued on the basis of the Regulations for the Administration Affairs Concerning Experimental Animals of the State Council of the People’s Republic of China.

All layers (n=40) sampled for this experiment were randomly selected from the population and sacrificed at 21 weeks of age, which were divided into three groups for evaluation. The hearts, livers, spleens, lungs, breast muscular tissues, prehierarchical follicles (1-4.9 mm, 5-6.9 mm 7-8 mm in diameter), and hierarchical follicles F5 were collected from the hens and finally stored at -80°C. Various sized follicles were removed from the hen’s ovaries based on the method of Stepińska and Olszańska (Stepińska & Olszańska., 1996).

**RNA extraction and cDNA synthesis**

Total RNA was extracted using TRizol Reagent according to the manufacturer’s protocols (Gibco BRL, Grand Island, NY, USA). The RNA concentration was assessed by absorbance at 260 nm and the purity of RNA sample was monitored by inspection of the 260/280 nm ratio using a spectrophotometer of the type SmartSpec™ Plus (Bio-Rad, Hercules, and part USA). The samples were treated with DNase (Promega, Madison, WI, USA) to remove genomic DNA contamination. Then using a First Strand Synthesis Kit (QIAGEN, Crawley, UK) to reverse transcription (RT) of RNA, following the manufacturer’s protocol, and each sample was converted into cDNA in 30μl, stored at -20°C.

**Selection of HGs and Primer design**

The six housekeeping reference genes were selected from different functions but commonly used HGs in hens, they are 18SrRNA, ACTB, HOXC8, GAPDH, alpha-A, and alpha-D respectively. RT-qPCR with TaqMan probes was employed to estimate the expression of the six genes. All primers that including probe primers were designed using Primer Premier 5.0 program by the GenBank sequences. The primer sequences are listed in Table 1.

**Reference standards preparation**

The target DNA fragments were obtained by PCR amplification. Furthermore, the PCR products were inserted into a pMD18-T vector (Takara, Tokyo, Japan) after extracted by SanPrep Column DNA Gel Extraction Kit (Sangon, Shanghai, China), and then transformed DH5a competent cells (Takara, Tokyo, Japan), the positive clones were selected from the DH5a competent cells. In addition, the plasmids which were extracted by SanPrep Column Plasmid Miniprep Kit (Sangon, Shanghai, China) were used as standard samples, and the concentrations of standard samples were used for calculating the copy numbers. The standard curve was prepared by a 1:10 dilution, each point of the standard curve was included in duplicate.

| Genes | Accession no. | Primer:forward/reverse/ Probe | Amplified fragment length (bp) | Amplification efficiency (%) | R2 |
|-------|---------------|-----------------------------|-----------------------------|-----------------------------|----|
| 18Sr RNA | AF173612.1 | TATTGGTGGAGCCGATTTGTCT CGGACATCTAAAGGGCATACA CTTGACATGCTACTAGTATGAGCAGACC | 169 | 112.5 | 0.995 |
| ACTB | NM_205518.1 | TGATATGTGCTGGCCTCTGGT TGTACCTCTTGGTCTGGGCTT CCTGACATACCAGGAGACATTGTC | 183 | 93.4 | 0.991 |
| HOXC8 | NM_204893.1 | CAATCTTATAGTGCTCTCAG GCTGCTCTGGTCTCAAT CAAAATCTGCTCTCCAGTCTGATTC | 248 | 103.9 | 0.990 |
| GAPDH | K01458.1 | TCTTCCACCTTTTGATCCGTT GTGCTCTTTGCTACTGCTTGT TGGTACATTGCAAGTCCAGAC | 146 | 108.2 | 0.997 |
| alpha-A | V00410.1 | CAAGGGAGCTTACCTCAAAAA TTGACGGAGGAGGATAGGA TTCAGCCATTGCTGCTCAC | 151 | 118.6 | 0.990 |
| alpha-D | V00411.1 | CCGTGGCGTATGAGTATAAGG TTCGTGCTGATACCTGTCTTG CTCCTATTACGTCCTGGTG | 153 | 117.1 | 0.994 |
**Quantitative real-time PCR (RT-qPCR)**

RT-qPCR was performed using a GeneAmp PCR System 9700 (ABI, Foster, USA). The reaction mixture contained 10 μl of TaqMan Fast qPCR Master Mix (2×) (Sangon, Shanghai, China), 0.5 μl of each of the forward, reverse and probe primers (10 μM), 2μl of diluted cDNA (1:10), and 6.5 μl of ddH2O in final volume of 20 μl. The following system protocol was: initial denaturation program (94°C for 3 m), melting program (94°C for 5 s), annealing program (55°C for 15 s), and extension program (72°C for 30 s). There were 40 cycles in amplification program; each run included a non-template control for assay. Triplicates of all reactions were run. Each assay also included three blanks. The data on the expression levels of the six HGs were obtained as Ct values by the GeneAmp PCR System 9700 and analyzed by SPSS18.0 software.

**Data analysis**

The geNorm (version 3.5), Normfinder (version 19) and Bestkeeper (version 1) software were used to calculate the stability of the candidate genes. geNorm measure M was used for a reference gene as the average pairwise variation V for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows the ranking of the tested genes according to their expression stability (Lin et al., 2013; De et al., 2017.).

Normfinder calculates the gene expression variation for all individual HGs based on delta-Ct values. It can count a stability value for each candidate gene, the best combination of two genes for a two-gene normalization factor, and a stability value for the best combination of two genes (Andersen et al., 2004).

The Bestkeeper software directly produces the pairwise correlation coefficient and Bestkeeper index in each gene. Bestkeeper index is generated by geometric mean (GM) of Ct values of all HGs. The stability of gene expression was determined mainly by a standard deviation (SD) and coefficient of variation (CV), the smaller values of SD and CV, the more stable the gene expression. Calculating the final ranking of HGs was performed according to the published method, thus for ACTB the geometric mean is 2.88[(4(2.8) 1/3)] (Chen et al., 2011; Vorachek et al., 2013).

**RESULTS**

**RNA Extraction and cDNA synthesis**

Total RNA was extracted from frozen tissues using TRIzol Reagent according to the manufacturer’s protocols (Gibco BRL, Grand Island, NY, USA). The distinct total RNA bands corresponding to 28S and 18S clearly were isolated from the prehierarchical follicles of 1-4.9 mm, 5-6.9 mm, 7-8 mm in diameter, hierarchical follicles F5, and the hearts, livers, spleens, lungs, breast muscles. A ratio of A260/280 was between1.8-2.0, which indicated that the quality of the RNA samples was appropriate. So, these RNA samples could be used in the coming tests.

**Identification and validation of the amplified fragment**

The samples were treated with DNase (Promega, Madison, WI, USA) to remove genomic DNA contamination. Reverse transcription (RT) of RNA was performed in 30μl reaction volumes using a First Strand Synthesis Kit (QIAGEN, Crawley, UK) based on the manufacturer’s protocol. Both RT-negative (containing template RNA but no reverse transcriptase enzyme) and RT water (containing reverse transcriptase but no template RNA) negative controls were used in every cDNA reaction. All samples were stored at -80°C until further use (Qin et al., 2015).

In order to ensure the accuracy of the target fragments, the fragments for 18SrRNA (169bp), ACTB (183bp), HOXC8 (248bp), GAPDH (146bp), alpha-A (151bp) and alpha-D (153bp) were cloned using specially designed primers then compared to the predicted sequences with the corresponding GenBank sequence (AF173612.1 for 18SrRNA, NM_205518.1 for ACTB, NM_204893.1 for HOXC8, K01458.1 for GAPDH, V00410.1 for alpha-A and V00411.1 for alpha-D) by BLAST software. To confirm the primer specificity, the PCR amplicon sizes were corresponding to the expected sequences of the candidate genes (Table 2).

**The efficiency of real-time PCR**

The standard curve indicated the linear relationship between Ct (cycle threshold) and template concentration with a good correlation, the range of correlation coefficient (R2) is 0.990-0.997 while the amplification efficiency was calculated according to the slope of the curves, results amplification efficiency was between 83.4%-118.6% (Table 1).

**Transcription levels of candidate internal controls**

Average cycle threshold (Ct) values can reflect the mRNA expression levels of HGs. Therefore, the variation range of Ct value of each candidate gene under certain conditions can be used as the basis
to evaluate the stability of gene transcript. We can obtain the information that the gene with the highest average abundance in chicken ovarian follicles (1-8 mm and F5) and tissues was HOXC8, the average Ct value was 33.58. In contrary, the gene with the lowest mRNA expression of grace was ACTB, the average Ct value was 20.87. Among the chicken follicles (1-8 mm and F5) and tissues collected in our study, the Ct value was 20.87. Among the chicken follicles (1-8 mm and F5), the average Ct value of HOXC8 had the lowest change range, while the Ct value of 18SrRNA had the smallest change range, indicating that the 18SrRNA transcript keeps the most stable expression levels in the tissues.

### Table 2 – The expression stability of housekeeping genes in hen ovarian follicles by geNorm and Normfinder.

| geNorm        | Follicles (1-4.9 mm) | Follicles (5-6.9 mm) | Follicles (7-8 mm) | Follicles (F5) | All Follicles |
|---------------|----------------------|----------------------|-------------------|----------------|--------------|
| Gene          | MV       | Gene     | MV       | Gene     | MV       | Gene     | MV       | Gene     | MV       |
| 18SrRNA ACTB  | 0.431    | 18SrRNA  | 0.209    | 18SrRNA  | 0.506    | alpha-A  | 0.581    | alpha-A  | 0.634    |
| GAPDH         | 0.431    | ACTB alpha-A | 0.209 | ACTB GAPDH | 0.506    | alpha-D  | 0.581    | alpha-D  | 0.634    |
| alpha-A       | 0.771    | alpha-D  | 0.438    | HOXC8    | 0.610    | 18SrRNA  | 1.247    | 18SrRNA  | 1.334    |
| alpha-D       | 0.917    | GAPDH    | 0.572    | alpha-D  | 0.701    | ACTB HOXC8| 2.079    | ACTB     | 1.727    |
| HOXC8         | 1.188    | HOXC8    | 0.952    | alpha-A  | 1.525    | GAPDH    | 2.278    | GAPDH    | 2.076    |
|               | 1.545    |          | 1.585    |          | 1.796    |          | 3.066    |          | 2.382    |
| Normfinder    |          |          |          |          |          |          |          |          |          |
| Gene          | SV       | Gene     | SV       | Gene     | SV       | Gene     | SV       | Gene     | SV       |
| GAPDH         | 0.023    | 18SrRNA  | 0.048    | 18SrRNA  | 0.117    | 18SrRNA  | 0.311    | 18SrRNA  | 0.194    |
| 18SrRNA       | 0.300    | ACTB     | 0.048    | ACTB     | 0.371    | ACTB alpha-D | 0.650 | ACTB     | 0.410    |
| alpha-A       | 0.389    | GAPDH    | 0.302    | GAPDH    | 0.474    | HOXC8    | 0.846    | GAPDH    | 0.705    |
| ACTB          | 0.571    | alpha-A  | 0.449    | HOXC8    | 0.752    | alpha-A  | 1.045    | alpha-A  | 0.740    |
| alpha-D       | 0.583    | alpha-D  | 0.659    | alpha-D  | 0.964    | GAPDH    | 1.082    | alpha-D  | 0.763    |
| HOXC8         | 0.957    | HOXC8    | 1.305    | alpha-A  | 1.039    |          | 2.021    |          | 1.015    |

Note: MV: M value; SV: Stability Value; N = 40.

### Ranking of the candidate internal controls

To establish the rankings of stability, the ranking of HGs for the tissues and follicles were evaluated respectively by geNorm, Normfinder, and Bestkeeper (Table 2 to Table 5). The results calculated by geNorm as described earlier, M value expresses the stability of the HGs. The smaller the M value, the higher the stability. An M value below the threshold of 1.5 was recommended by geNorm to identify sets of HGs with stable expression (Selvarajah et al., 2017). Therefore, the genes with M value higher than 1.5 would not be selected in the research. As shown in Table 2, 18SrRNA and ACTB had the same and lowest M value in the prehierarchical follicles of 1-4.9 mm, 5-6.9 mm, 7-8 mm in diameter, which implied these two genes were the most stable genes in the prehierarchical follicles. But in hierarchical follicles F5, the first three stable genes were alpha-A, alpha-D, and 18SrRNA. When the prehierarchical follicles and hierarchical were put together to analyze the stability of the HGs, we found alpha-A and alpha-D had the same value 0.634, which were more stable than 18SrRNA. Of course, these findings were only from geNorm tools, in order to avoid the imprecise results, Normfinder backed up from another approach, whose analysis confirmed the gene which has the lowest stability value is the most stable gene. The results proved that 18SrRNA was the best gene in every group of follicles, except in the prehierarchical follicles of 1-4.9 mm in diameter (Table 2). Stability value for the best combination of two genes is 18SrRNA and ACTB is 0.228 in all follicles. The results of Bestkeeper as shown in Table 3, the SD and CV values of 18SrRNA are the lowest, so the expression of 18SrRNA is the most stable in chicken follicles (1-8 mm and F5) during the lying period, the second is alpha-D, in contrast, HOXC8 is the most unstable.

Furthermore, all tissues were pooled together to analyze the stability of genes, the findings showed alpha-A and alpha-D have the same M value 0.714, which is the lowest individual M value and the most stable genes in our research model, 18SrRNA came...
Table 3 – The expression stability and related parameters of reference genes in hen ovarian follicles analyzed by Bestkeeper.

| Parameters       | 18SrRNA | GAPDH | ACTB | HOXC8 | alpha-A | alpha-D |
|------------------|---------|-------|------|-------|---------|---------|
| GM[Cp]           | 24.72   | 20.52 | 23.59| 32.39 | 28.92   | 29.44   |
| Min[Cp]          | 23.73   | 15.48 | 21.30| 29.39 | 27.07   | 27.83   |
| Max[Cp]          | 26.88   | 25.99 | 28.14| 37.47 | 33.77   | 33.18   |
| SD[±Cp]          | 0.76    | 2.09  | 1.76 | 2.71  | 1.06    | 0.85    |
| CV[%Cp]          | 2.07    | 8.34  | 7.43 | 10.12 | 3.67    | 2.88    |

Note: Measured in N = 40 hens samples. Tissues include heart, liver, spleens, and lungs and breast muscles.

Note: MV: M value; SV: Stability Value; N = 40.

Table 4 – The mRNA expression stability of housekeeping genes in hen tissues by geNorm and Normfinder.

| Gene         | GeNorm MV | GeNorm LD | Normfinder MV | Normfinder SD | Normfinder CV |
|--------------|-----------|-----------|---------------|---------------|---------------|
| 18SrRNA      | 0.335     | 0.383     | 0.709         | 0.216         | 0.137         |
| GAPDH        | 0.647     | 0.660     | 1.136         | 1.632         | 1.802         |
| ACTB         | 0.647     | 0.660     | 1.136         | 1.632         | 1.802         |
| alpha-A      | 1.414     | 1.289     | 1.414         | 1.289         | 1.414         |
| alpha-D      | 1.414     | 1.289     | 1.414         | 1.289         | 1.414         |

The optimum number of HGs

Determination of the optimal number of HGs for normalization is based on the pairwise variation (Vn/ Vn+1) value for geNorm. A large variation means that the added gene has a significant effect and should preferably be included for calculation of a reliable normalization factor. Based on the Genome Biology data, 0.15 as a cut-off value is proposed, below which the inclusion of an additional reference gene is not preferred.

Table 5 – The expression stability and related parameters of housekeeping genes in hen tissues analyzed by Bestkeeper in hen tissues.

| Parameters       | 18S rRNA | GAPDH | ACTB | HOXC8 | alpha-A | alpha-D |
|------------------|---------|-------|------|-------|---------|---------|
| GM[Cp]           | 25.63   | 20.85 | 25.09| 34.26 | 29.28   | 29.47   |
| Min[Cp]          | 23.38   | 16.89 | 20.66| 29.14 | 27.90   | 28.35   |
| Max[Cp]          | 27.38   | 26.01 | 28.39| 41.43 | 31.00   | 31.28   |
| SD[±Cp]          | 0.98    | 2.08  | 2.15 | 2.87  | 0.87    | 0.85    |
| CV[%Cp]          | 3.82    | 8.33  | 8.53 | 9.89  | 2.98    | 2.87    |

Note: Measured in N = 40 hens samples. Tissues include heart, liver, spleens, and lungs and breast muscles.
required. If pairwise variation \( V_{n/(n+1)} < 0.15 \), the optimum number of HGs is \( n \).

The optimum numbers of reference genes for chicken follicles are shown in Figure 2, the value of \( V_{2/3} \) is 0.108, 0.144 and 0.150 in follicle (1-4.9 mm, 5-6.9 mm and 7-8 mm) respectively, the value was lower than 0.15, so the two genes are enough to simultaneously and accurately normalize gene expression in follicle (1-4.9 mm, 5-6.9 mm and 7-8 mm). But the values of \( V_{2/3} \) in follicles V3/4, V4/5 and V5/6 in all follicles (1-8 mm, F5) were 0.151, 0.102, 0.280, 0.247, so the value \( (V_{3/4} = 0.102) \) was lower than 0.15, which suggested that the optimal number of HGs were three. \( 18SrRNA \), \( \alpha-D \), and \( \alpha-A \) were the best combination of genes in follicles (1-8 mm, F5) of laying hens. In all hen tissues, the results have shown that only the value of \( V_{3/4} (0.129) \) was lower than 0.15 (Figure 3), which indicate that the use of three HGs was sufficient.

**Figure 2** – Determination of the optimal number of housekeeping genes for normalization of mRNA expression in hen ovarian follicles.

Note: Determination of the optimal number of housekeeping genes for normalization of mRNA expression are based on the pairwise variation \((V_n/(n+1))\) value, if pairwise variation \( V_n/(n+1) < 0.15 \), the optimum number of housekeeping genes is \( n \). Data on the figure show that the value of \( V_{2/3} \) in follicles (1-4.9 mm, 5-6.9 mm and 7-8 mm) was lower than 0.15, so the two genes were enough to simultaneously and accurately normalize gene expression. The value of \( V_{3/4} \) was lower than 0.15 in all follicles, which suggested that the optimal number of housekeeping genes are three, \( 18SrRNA \), \( \alpha-D \), and \( \alpha-A \). F, Follicles, measured in \( N = 40 \) hens samples.

**Figure 3** – Determination of the optimal number of housekeeping genes for normalization of mRNA expression in five tissues of hens.

Note: The optimal number of housekeeping genes required for reliable normalization of mRNA expression was statistically predicted by geNorm with \( V \) value (cut-off=0.15), representing pairwise variation. Data on the graph show that the value of \( V_{3/4} \) in all tissues was lower than 0.15, so three housekeeping genes were sufficient. \( \alpha-A \), \( \alpha-D \) and \( 18SrRNA \) were proved to be the optimal genes combination in all tissues, measured in \( N = 40 \) hens samples.

Alpha-A, \( \alpha-D \), and \( 18SrRNA \) were proved to be the optimal gene combination in all tissues in the laying hens, using the three best reference genes was a valid normalization strategy in most cases, and results in much more accurate and reliable normalization compared to the use of only one single reference gene.

**Final Ranking of Candidate Reference Genes**

Considering the ranking results from geNorm, Normfinder and Bestkeeper, we used the mean of standard deviation values derived from comparisons among HGs. As showed in Table 6, the most stable housekeeping gene in follicles (1-8 mm and F5) was \( 18SrRNA \) (1.44), the overall ranking with geometric mean was \( 18SrRNA \) (1.44) < \( \alpha-D \) (2.71) < \( ACTB \) (2.88) < \( GAPDH \) (4.22) < \( HOXC8 \). Additionally, the most stable housekeeping gene in tissues (heart, liver, spleen, lung and breast muscle) was \( \alpha-D \) (1.59), the overall ranking with geometric mean was \( \alpha-D \) (1.59) < \( \alpha-A \) (1.82) < \( 18SrRNA \) (2.08) < \( ACTB \) (4.31) < \( GAPDH \) (4.93) < \( HOXC8 \) (5.65) (Table 7).

**DISCUSSION**

The expressive disciplinarian of genes can be conducive to research the hereditary effects of significant economic traits. Nevertheless, HGs are inevitable for accurate data normalization and thus authentic results in studies of gene expression (Zhu et al., 2015). The process of correcting and normalizing target genes by RT-PCR was affected by many factors, it was therefore of great concern that different experiments must use the right HGs. Many studies revealed that different HGs are used in a different organ, tissue and stage. However, when researching the gene expression of laying hens, how to appropriate HGs to calibrate and standardize the target gene is unclear.

\( Ct \) values can reflect the expression levels of HGs. In addition, geNorm, NormFinder and Bestkeeper can select the most suitable HGs simply and directly. geNorm can analyze the suitable number of HGs and find the optimal combination. Normfinder can not only compare differences in the expression of HGs, but also calculate variations between sample groups. Bestkeeper software can be used to compare the expression levels of 10 HGs and 10 target genes in 100 samples, and finally obtain relatively stable internal reference genes. It should be noted that the outputs of the most stable housekeeping gene may be different depending on
the different kinds of algorithms, so we used the brief procedure that according to the geometric mean to obtain the final ranking, as is done in the present study, the gene with smaller geometric mean being the most stable one. In this study, the results from three software programs are not the same in the follicles and tissues, the reason may be that geNorm only considers the overall expression level variation of housekeeping reference genes, however other methods include both expression level variation and overall expression level.

Some reliable HGs have been identified in bird tissues that include ACTB and GAPDH (Olias et al., 2014; Bagés et al., 2015; Borowska et al., 2016; Katarzyńska et al., 2017; Hassanpour et al., 2018). Previous studies showed ACTB was the best stable gene in 12-days cultured goat preantral follicles, while 18SrRNA was the least stable gene (Frota et al., 2011). Moreover, in a lipopolysaccharide inflammation model in chickens, ACTB appeared to be the most stable single gene in this model, but GAPDH should be avoided (De et al., 2008). Conversely, ACTB and GAPDH were the two most stable HGs in bovine oocytes (Van et al., 2008). Furthermore, GAPDH was more strongly expressed and suitable in the brain tissue, heart muscle, liver, and kidney of a chicken embryo, while ACTB was more strongly expressed in the gizzard and almost absent from cardiac muscle cells (Lin & Redies., 2012). In order to select the most suitable HGs for normalizing the follicles and other tissues in hens. In this study, we ultimately investigated the comprehensive ranking of ACTB and GAPDH which was the 4th in follicles (1-8mm and F5) and the 5th in the tissues (heart, liver, spleens, lung, and breast muscles) respectively. As we know, ACTB was one of the cytoskeleton actin proteins. If the cytoskeleton system was abnormal or the target gene is involved in cytoskeleton formation, ACTB should not be used as internal references in this model, but GAPDH should be avoided (De et al., 2008). Conversely, ACTB and GAPDH were the two most stable HGs for normalizing (Xu et al., 2018b). These results are consistent with the results of this study, our results showed that 18SrRNA was the best suitable housekeeping gene in the follicles, especially follicles of 5 to 8 mm in diameter. Conversely, another published study indicated ACTB was the best in the ovary of Jining Bairi chicken at different stages of sexual development, however, 18SrRNA was highly expressed in the hypothalamus (Yuan et al., 2017). This

### Table 6 – Six housekeeping genes ranked by different methods in hen ovarian follicles.

| Ranking | geNorm   | Normfinder | Bestkeeper | Overall ranking |
|---------|----------|------------|------------|-----------------|
| 1       | alpha-A  | 18S rRNA   | 18S rRNA   | 18S rRNA        |
| 2       | alpha-D  | ACTB       | alpha-D    | alpha-A         |
| 3       | 18S rRNA | GAPDH      | alpha-A    | alpha-D         |
| 4       | ACTB     | alpha-A    | ACTB       | ACTB            |
| 5       | GAPDH    | alpha-D    | GAPDH      | GAPDH           |
| 6       | HOXC8    | HOXC8      | HOXC8      | HOXC8           |

Note: Measured in N = 40 hens samples.

### Table 7 – Six housekeeping genes ranked in hen tissues by different methods.

| Ranking | geNorm   | Normfinder | Bestkeeper | tissues |
|---------|----------|------------|------------|---------|
| 1       | alpha-A  | 18S rRNA   | alpha-D    | alpha-D |
| 2       | alpha-D  | alpha-D    | alpha-A    | alpha-A |
| 3       | 18S rRNA | alpha-A    | 18S rRNA   | 18S rRNA |
| 4       | ACTB     | ACTB       | GAPDH      | ACTB    |
| 5       | GAPDH    | HOXC8      | HOXC8      | HOXC8   |
| 6       | HOXC8    |            |            |         |

Note: Measured in N = 40 hens samples. Tissues include heart, liver, spleens, and lungs and breast muscles.
result was inconsistent with our experimental results, the main reason may be the differences between different chicken species. 18SrRNA and ACTB have been used as normalization genes in gene expression of the chicken reproductive axis at present. However, one challenge is associated with 18SrRNA use: the random primers of 18SrRNA must be used for cDNA synthesis rather than oligo-(dT). In order to avoid this problem, we referenced the methods of coapplication reverse transcription (Co-RT) to improve the sensitivity and accuracy of reverse transcription (Zhu & Altmann., 2005; Kuchipudi et al. 2012). Furthermore, it has been previously demonstrated that 18SrRNA was the most stable housekeeping gene in poultry lungs, this was the same with Normfinder results of our study.

It’s worth noting that alpha-D was the best housekeeping gene in all the tissues by calculating the mean of standard deviation values of three methods, alpha-D and alpha-A belonged to the chicken globin gene family, which is characterized as developmentally regulated genes. These two genes were found to be expressed in 10-day old chick embryo erythrocytes and alpha-D only in the late embryo of a pigeon but not in the adult stage, however, alpha-D has been expressed in adult chickens just as our results (Gavrilov & Razin., 2008a). In addition, the promoter of the two genes was located within the CpG Island in chicken, CpG Island is one of the important markers of HGs, it regulates the stability of gene mRNA expression at the transcriptional level (Gavrilov & Razin., 2008b). Although there are no reports on the two genes as internal reference genes, the results of this experiment prove that they have good expression stability in the follicles and tissues of hens, which can be considered as internal reference in the future.

Housekeeping gene expression is not always constant, on one hand, it may use a domestic gene as a housekeeping gene blindly making small differences in gene expression which are difficult to identify, on the other hand, it may make mistakes or even contrary conclusions. HOXC8 gene was the most unstable in all follicles and tissues in the hens regardless of which development stages. In conclusion, our data show that the expression levels of 18SrRNA was the most stable in all chicken samples (1-8 mm in diameter and F5 follicles and tissues), 18SrRNA, alpha-A, and alpha-D were the most stable genes for normalizing the gene expression in chicken follicle (1-8 mm in diameter and F5) synthetically. Moreover, alpha-D, 18SrRNA, and alpha-A were the best in chicken tissues (heart, liver, spleen, lung and breast muscle) (Table 6, 7). It should be pointed out that 18SrRNA was the most stable mRNA expression levels in all chicken tissues sampled, it can serve as an excellent inner control for evaluation of the transcription levels in chickens. On the contrary, HOXC8 should be avoided in normalizing the gene expression in chicken.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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