Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2020 / v.22 / n.3 / 001-012

http://dx.doi.org/10.1590/1806-9061-2019-1256

Original Article

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

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■Keywords

Hen, Housekeeping gene, Ovarian follicle, Inner control.

Submitted: 25/December/2019
Approved: 07/July/2020

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

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 stabilization 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* (*GAP-DH*), *chicken alpha-Aglobingene* (*alpha-A*) and chicken *alpha-Dglobingene* (*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 to 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. Variously 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.

Table 1 – Primer information of housekeeping genes used in qRT-PCR.

Genes	Accession no.	Primer:forward/reverse/ Probe	Amplified fragment length (bp)	Amplification efficiency (%)	R^2
18Sr RNA	AF173612.1	TAGTTGGTGGAGCGATTTGTCT CGGACATCTAAGGGCATCACA CTGGCATGCTAACTAGTTACGCGACCC	169 27	112.5	0.995
ACTB	NM_205518.1	TGATATTGCTGCGCTCGTTG ATACCTCTTTTGCTCTGGGCTT CCTTGCACATACCGGAGCCATTGTC	183 25	93.4	0.991
HOXC8	NM_204893.1	CAATCGCTTTATGGTGCTCAG CGTCGCTTCCGTGTCAAAT CAAAATTCGTCTCCCAGTCTCATGTTCC	248 28	103.9	0.990
GAPDH	K01458.1	TTCTTCCACCTTTGATGCGG TGTGCTTGGCTCACTCCTTG TGGTCATTCAGTGCAATGCCAGCAC	146 25	108.2	0.997
alpha-A	V00410.1	CAAGGGCATCTTCACCAAAA TAAGGCAGGGAGGGATAGGA TTCCAGGGTCTCGGCGCCATACTCC	151 25	118.6	0.990
alpha-D	V00411.1	CCGTGCGGATAAGATAAGGC CCTGCTGGATGAGCTTCTTGT CCTCTTATAGCTCCCTGTACACCCGCC	153 27	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 ddH_2O 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 (R^2) 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

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

	Follicles (1-4.9 mm)		Follicles (5-6.9 mm)		Follicles (7-8 mm)		Follicles (F5)		All Follicles	
geNorm	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	*HOXC8*	2.382
	Gene	SV	Gene	SV	Gene	SV	Gene	SV	Gene	SV
Normfinder	*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	*HOXC8*	1.015

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

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 of *18SrRNA* had the smallest change range, while the Ct value of *HOXC8* had the largest change range. The mRNA expression stability of HGs was *18Sr RNA>alpha-D>alpha-A>ACTB>GAPDH>HOXC8* (Figure 1). The results indicated that the expression of *18SrRNA* mRNA was the most stable, while *HOXC8* was the most unstable gene.

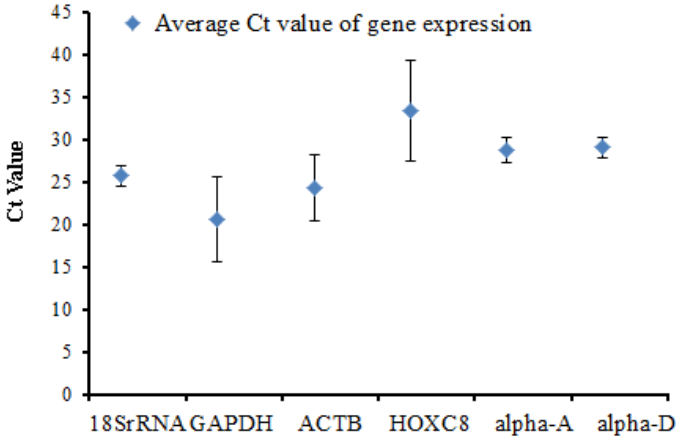

Figure 1 –Variation of Ct values of six housekeeping genes in all samples.

Note: The Ct value exhibited variance levels of the transcripts in different tissues. The 18SrRNA transcript has the smallest variation range, while HOXC8 transcript has the largest variation range, indicating that the 18SrRNA mRNA keeps the most stable expression levels in the tissues.

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	gene					
	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; CP: Crossing Point..

second (Table 4). Moreover, geNorm sorted the stability of genes and the first three were *18SrRNA*, *alpha-D* and *alpha-A* in spleens, lungs and breast muscles (Table 4). In contrast, the results of the hearts and livers were different from other tissues, it showed the genes from the least to the most stable: *HOXC8*, *GAPDH*, *18SrRNA*, *ACTB*, *alpha-D,* and *alpha-A* in hearts, *HOXC8*, *ACTB*, *18SrRNA*, *alpha-A*, *alpha-D* and *GAPDH* in the livers. According to Normfinder analysis results, the stability value of *18SrRNA* is the lowest in the hearts, livers, lungs, breast muscles, and all tissues, which demonstrated *18SrRNA* is the best gene (Table 4). Only in spleens, *alpha-D* is the best gene, *18SrRNA* is the second stable. Normfinder also confirmed that the best combinations of two genes are *18SrRNA* in all tissues, and stability value for the best combination of two genes is 0.472. The most stable gene is *alpha-D* in the tissues, the second is *18SrRNA*, the last one is *HOXC8,* and these results are from Bestkeeper (Table 5).

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

	Hearts		Livers		Spleens		Lungs		Breast muscles		All tissues	
	Gene	MV	Gene	MV	Gene	MV	Gene	MV	Gene	MV	Gene	*MV*
geNorm	*alpha-A*	0.335	*GAPDH*	0.383	*18SrRNA*	0.837	*alpha-A*	0.187	*18SrRNA*	0.177	*alpha-A*	0.714
	alpha-D	0.335	*alpha-D*	0.383	*alpha-D*	0.837	*alpha-D*	0.187	*alpha-D*	0.177	*alpha-D*	0.714
	ACTB	0.647	*alpha-A*	0.660	*alpha-A*	0.725	*18SrRNA*	0.656	*alpha-A*	0.562	*18SrRNA*	1.052
	18S rRNA	0.709	*18SrRNA*	0.929	*HOXC8*	1.596	*HOXC8*	0.898	*HOXC8*	1.023	*ACTB*	1.615
	GAPDH	1.136	*ACTB*	1.632	*GAPDH*	1.373	*GAPDH*	1.320	*ACTB*	1.277	*HOXC8*	2.045
	HOXC8	1.802	*HOXC8*	2.056	*ACTB*	1.965	*ACTB*	1.403	*GAPDH*	1.769	*GAPDH*	2.291
	Gene	SV	Gene	SV	Gene	SV	Gene	SV	Gene	SV	Gene	SV
Normfinder	*18SrRNA*	0.137	*18SrRNA*	0.216	*alpha-D*	0.154	*18SrRNA*	0.182	*18SrRNA*	0.041	*18SrRNA*	0.494
	ACTB	0.137	*alpha-A*	0.281	*18SrRNA*	0.381	*HOXC8*	0.183	*alpha-D*	0.041	*alpha-D*	0.748
	GAPDH	0.481	*GAPDH*	0.684	*alpha-A*	0.694	*alpha-D*	0.549	*alpha-A*	0.347	*alpha-A*	0.811
	alpha-D	0.520	*alpha-D*	0.758	*GAPDH*	0.824	*alpha-A*	0.661	*HOXC8*	0.568	*ACTB*	0.947
	alpha-A	0.657	*ACTB*	0.851	*ACTB*	0.883	*GAPDH*	0.682	*ACTB*	0.916	*GAPDH*	1.032
	HOXC8	1.414	*HOXC8*	1.289	*HOXC8*	0.908	*ACTB*	0.685	*GAPDH*	1.234	*HOXC8*	1.054

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

It can be noted that *HOXC8* has the highest M value in all follicles (geNorm), and it also was the most unstable gene in all follicles and tissues from the Normfinder and Bestkeeper results, thus it should be avoided in normalizing mRNA levels in follicles of hens.

The optimum number of HGs

Determination of the optimal number of HGs for normalization is based on the pairwise variation (V*n*/V*n+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

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

Parameters	gene					
	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 V2/3 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 V2/3 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 (V3/4=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 V3/4(0.129) was lower than 0.15 (Figure 3), which indicate that the use of three HGs was sufficient.

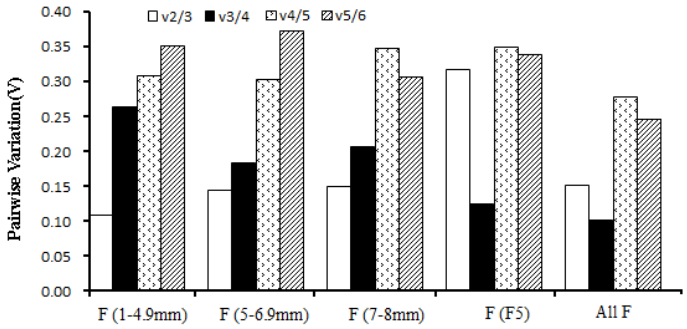

Figure 2 – Determination of the optimal number of housekeeping genes for normalization of mRNA expression in hen ovarian follicles.

Note: Determination of the optimal number of housekeeping genes for normalization of mRNA expression are based on the pairwise variation (Vn/Vn+1) value, if pairwise variation Vn/(n+1)<0.15, the optimum number of housekeeping genes is n. Data on the figure show that the value of V2/3 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 V3/4 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.

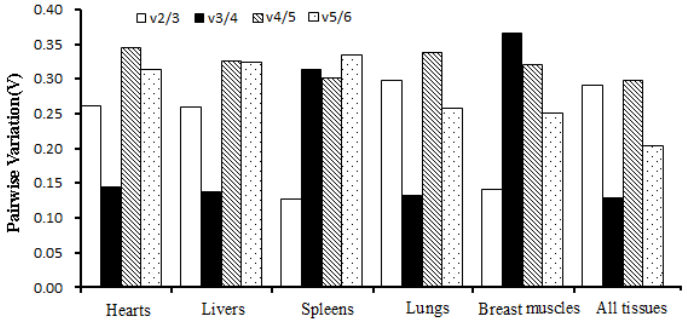

Figure 3 – Determination of the optimal number of housekeeping genes for normalization of mRNA expression in five tissues of hens.

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 V3/4 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-A* (2.29) < *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

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	*HOXC8*	*GAPDH*	*ACTB*	*GAPDH*
6	*GAPDH*	*HOXC8*	*HOXC8*	*HOXC8*

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

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 the internal reference gene. In addition, *GAPDH* was a key enzyme in the glycolysis pathway, which was strengthened in tumorigenesis, therefore, *GAPDH* should not be used as internal references in the study of glucose metabolism because changes were involved. It is clear that the *ACTB* and *GAPDH* were not suitable HGs for the laying hens.

18SrRNA exists in all eukaryotic cells and has the advantages of high conservatism and easy amplification with universal primers. It was one of the most abundant conserved genes in cells. *18SrRNA* was not transcribed into mRNA during the cell cycle, and it was subject to small changes in the internal and external environment and functional state of the cells, so it was the best reference gene in the cell culture system. Researches have shown that *18SrRNA* was used as the housekeeping gene to study the expression of *CDK5* gene in egg ovary of Hy-Line hens (Zhang *et al*., 2012), during a research on inhibitory effect of *SLIT2* on granulosa cell proliferation in the prehierarchical follicles of the chicken ovary, *18SrRNA* was used as 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, *18SrRN*A was highly expressed in the hypothalamus (Yuan *et al*., 2017). This

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 1*8SrRNA* 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 1*8SrRNA* 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 method is used in our study. Therefore *HOXC8* gene should be avoided.

In this study, we screened out six HGs (*18SrRNA*, *GAPDH*, *ACTB*, *HOXC8*, *alpha-A,* and *alpha-D*) to examine the least predictable in terms of stability, Ct value, geNorm, Normfinder and Bestkeeper were used to select optimum gene for follicles and tissues of laying hens. Our results provide suitable HGs for high-precision normalization in RT-qPCR analysis, thereby making it more convenient to analyze gene expression under these experimental conditions. As far as we know, no accurate results about the validated HGs have been used for the normalization of expression data in the follicles and tissues of laying hens. Our results provide appropriate HGs for the high-precision normalization of RT-qPCR analysis, so as to facilitate the analysis of gene expression in follicles and tissues at different 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.

ACKNOWLEDGMENTS AND FUNDING

Authors are thankfully acknowledging the financial support of this work including National Natural Science Funds (No. 31902145, No. 31672407, No. 31272431), Project of Education Development Plan of Jilin Province (No. JJKH20180646KJ), Project of Science and Technology Development Plan of Jilin Province (No. 20170101019JC), China Agriculture Research System (No. CARS-41).

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