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Reference Gene Selection for Normalization of PCR Analysis in Chicken Embryo Fibroblast Infected with H5N1 AIV*

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Abstract: Chicken embryo fibroblasts (CEFs) are among the most commonly used cells for the study of interactions between chicken hosts and H5N1 avian influenza virus (AIV). In this study, the expression of eleven housekeeping genes typically used for the normalization of quantitative real-time PCR (QPCR) analysis in mammals were compared in CEFs infected with H5N1 AIV to determine the most reliable reference genes in this system. CEFs cultured from 10-day-old SPF chicken embryos were infected with 100 $TCID_{50}$ of H5N1 AIV and harvested at 3, 12, 24 and 30 hours post-infection. The expression levels of the eleven reference genes in infected and uninfected CEFs were determined by real-time PCR. Based on expression stability and expression levels, our data suggest that the ribosomal protein L4 (RPL4) and tyrosine 3-monooxygenase tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) are the best reference genes to use in the study of host cell response to H5N1 AIV infection. However, for the study of replication levels of H5N1 AIV in CEFs, the β-actin gene (ACTB) and the ribosomal protein L4 (RPL4) gene are the best references.

Key words: Reference gene; Chicken embryo fibroblast; H5N1 avian influenza virus (AIV); Real-time PCR (RT-PCR)

Quantitative real-time PCR (QPCR) has become one of the most powerful quantification methods and a favorite tool in mRNA expression analysis and virus loading [12]. Because of its extreme sensitivity and accuracy, QPCR data analysis depends on a reliable reference gene to normalize for sample-to-sample and run-to-run variation, as variations arise from differences in nucleic acid integrity, the efficiency of the reverse transcription, and the amount of sample loaded [13,23]. However, a number of studies have suggested that the most stable reference genes may vary between cell types, tissues, and even different physiological and disease states [3, 4, 14, 19, 20, 22]. Similarly, the ideal stable reference genes can also vary between different cell types infected with different viruses [14, 23]. So, selection of a stable reference gene is critical for reliable performance of QPCR experiments.

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The highly pathogenic avian influenza caused by H5N1 has had devastating consequences for poultry production [1,5,24], and the virus has resulted in numerous infections in humans [25], making understanding of H5N1 viruses increasingly critical for public health. Quantitative analysis of H5N1 AIV and host mRNA levels is an important tool for the study of host-virus interaction. CEFs , the most commonly used cells in the study of host-avian virus interaction [9, 17], are the most popular cells used in the study of H5N1 AIV [15]. However, no determination of the ideal reference genes for QPCR in these cells has yet been carried out in the context of H5N1 AIV infection. In this study, the expression stabilities of 11 housekeeping genes commonly used in mammals were compared, in order to select a stable reference gene in normal CEFs and H5N1 AIV infected CEFs.

The eleven housekeeping genes examined were as follows: albumin (ALB), beta-2-microglobulin (B2M), ribosomal protein L4 (RPL4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L30 (RPL30), hypoxanthine phosphoribosyltransferase 1 (HPRT1), succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA), TATA box binding protein (TBP), tubulin, beta (TUBB), tyrosine 3-monooxygenase, tryptophan 5-monooxygenase activation protein,zeta polypeptide (YWHAZ) and the β-actin gene (ACTB).

MATERIAL AND METHODS

Virus propagation and the detection of $TCID_{50}$

H5N1 AIV virus isolate (CHSD003), identified and purified by the China Animal Health and Epidemiology Center, was propagated in SPF chicken embryos. The $TCID_{50}$ of the virus was determined in CEFs and calculated to be $10^{7.67}$/0.1 mL according to Reed-Muench [27].

Viral infection of CEFs

CEFs were cultured from 10-day-old SPF chicken embryos according to standard procedures [27]. Briefly, 1×10^7 cells were added per well in a 24-well culture plate. Monolayer cultures of CEFs were infected with 100 $TCID_{50}$ H5N1 AIV. Cells were harvested at 3h, 12h, 24h and 30h post-infection with RNAiso Reagent Trizol (TakaRa). Mock-infected CEFs were cultured and harvested in the same way. Five parallel samples were taken for each time point.

Extraction of RNA

Total RNA from each sample was extracted by RNAiso Reagent (TaKaRa) and prepared using an RNase-free DNase kit (TakaRa) according to the manufacturer's recommendations. RNA was quantified by Cary50Probe (Bio-Rad) to find the OD_{260}/OD_{280} value for each sample. The OD_{260}/OD_{280} values of all the RNA samples were between 1.8 and 2.0, and intact rRNA subunits of 28S, 18S and 5S were observed on gel electrophoresis, indicating that all the RNA samples used in this study were of good quality.

cDNA synthesis

cDNA was synthesized using the RT Reagents (BioBRK) with random primer according to the manufacturer's recommendations. cDNA synthesis was performed in a PCR instrument(Bio-rad) using 1μg of RNA, at 30℃ for 10min, 42℃ for 20min, 99℃ for 5min, and finished at 4℃. Then the cDNA were treated with RNaseH in order to ensure the cDNA was without RNA. Finally, cDNA was saved at -20℃ for further testing.

Primer design and sequencing of PCR products

All primers (shown in Table 1) of the eleven

Table 1. Information for primers of real-time PCR

Target gene	Access number(mRNA)	Amplicon size(bp)	Primer	Sequences(5'-3')
ALB	NM_205261	197	Forward	CCTGGACACCAAGGAAAT
			Reverse	TGTGGACGCCGATAGAAT
B2M	NM_001001750	194	Forward	CGTCCTCAACTGCTTCGTG
			Reverse	TCTCGTGCTCCACCTTGC
GAPDH	NM_204305	283	Forward	AGCACCCGCATCAAAGG
			Reverse	CATCATCCCAGCGTCCA
HRPT1	NM_204848	245	Forward	ACTGGCTGCTTCTTGTG
			Reverse	GGTTGGGTTGTGCTGTT
RPL30	NM_00100747	160	Forward	GAGTCACCTGGGTCAATAA
			Reverse	CCAACAACTGTCCTGCTTT
RPL4	NM_001007967	235	Forward	TTATGCCATCTGTTCTGCC
			Reverse	GCGATTCCTCATCTTACCCT
SDHA	XM_419054	187	Forward	CAGGGATGTAGTGTCTCGT
			Reverse	GGGAATAGGCTCCTTAGTG
TBP	NM_205103	470	Forward	CGTCAGGGAAATAGGCA
			Reverse	GACTGGCAGCAAGGAAG
TUBB	NM_205315	243	Forward	AAAACGAAGTTATCGGGTCTGA
			Reverse	ATGCGGCAACCAAATCG
YWHAZ	NM_00103134	358	Forward	TCCACCACGACAGACCA
			Reverse	CCAGCCTTCCAACTTCC
ACTB	NM_205518	139	Forward	CTGTGCCCATCTATGAAGGCTA
			Reverse	ATTTCTCTCTCGGCTGTGGTG

housekeeping genes were designed by the Primer 5.0 software package and checked by the oligo 6.0 software tool. The positive pMD-Recombined Plasmids inserted with the purified PCR products of housekeeping genes were sequenced (Unbiotech) to verify the validity of PCR amplification.

Real-time PCR of housekeeping genes

The annealing temperature and primer concentration were optimized for all 11 housekeeping genes. Real-time PCR was performed in an ABI 7300 Real-time PCR System (Applied Biosystems) in 96-well microtiter plates using a final volume of 20 μL. Reactions were performed in triplicate for each sample, and the mean value for each sample was calculated. Electrophoresis analysis of all the amplified products from real-time PCR showed single bands with the expected sizes, and no primer dimer was observed. The dissociation plots provided by the ABI 7300 also showed a single peak for each reaction.

Standard curve of real-time PCR

Standard curves were generated using copy number vs. threshold cycle (Ct). The linear correlation coefficients (R^2) of all eleven housekeeping genes were between 0.986 and 0.998. Based on the slopes of the standard curves, amplification efficiencies were between 95.66% and 109.95%, as derived from the formula $E = 10^{1/-slope} -1$. The Ct values of the standard curves of all the housekeeping genes had wide ranges.

Determination of stability and expression levels of housekeeping genes

The expression levels of eleven housekeeping genes were measured by calculating the Ct of each by real-time PCR, and the expression stabilities were evaluated by the GeNorm tool [21], which determined the most stable housekeeping genes from a set of tested genes in a given cDNA sample panel. Relative expression levels of each housekeeping gene were the average value of all the samples of each gene at all the

time points (3, 12, 24 and 30 hours post-infection) obtained by using the $2^{-\triangle\triangle Ct}$ calculation method.[6, 21].

RESULTS

The stability and expression levels of housekeeping genes in normal CEFs

The average expression stability *M* values of 11 housekeeping genes are shown in Table 2. The expression stability ranking from the most stable to the least stable was: GAPDH, HPRT1, RPL4, RPL30, ACTB, YWHAZ, B2M, ALB, TBP, SDHA and TUBB. The ranking of the relative expression levels (from high to low) was: ACTB, RPL4, GAPDH, YWHAZ, HRRT1, TBP, TUBB, RPL30, SDHA, ALB and B2M. Based on both the best expression stability and highest abundance gene transcripts in the normal CEFs, GAPDH and HRRT1 were the two best reference genes.

The stability and expression levels of housekeeping genes in the infected CEFs

The average expression stability *M* value of 11 housekeeping genes were shown in Table 2. The ranking of the expression stability (from the most stable to the least stable) was: ACTB, RPL4, YWHAZ, SDHA, GAPDH, B2M, TBP, ALB, HRRT1, RPL30 and TUBB. The ranking of the relative expression levels (from high to low) was: ACTB, RPL4, GAPDH, YHWAZ, HRRT1, TUBB, RPL30, TBP, SDHA, ALB and B2M. Based on both the best expression stability and high abundance gene transcripts, ACTB and RPL4 were the two best ideal reference genes in the infected CEFs.

The average expression stability of housekeeping genes in infected CEFs and normal CEFs

The average expression stability *M* values of 11 housekeeping genes (both in normal CEFs and CEFs infected with H5N1 AIV) were evaluated with the GeNorm tool and shown in Table 2. The ranking of the expression stability (from the most stable to the least stable) was: RPL4 and YWHAZ, ACTB, GAPDH, SDHA, B2M, TBP, HPRT1, ALB, RPL30 and TUBB. Based on both the expression stability and expression levels, RPL4 and YWHAZ were determined to be the two best reference genes for normalization of quantitative real-time PCR analysis of mRNA levels in host genes responses to H5N1 AIV.

Table 2. The average expression stability *M* value and relative expression levels of housekeeping genes in infected H5N1 AIV CEF and normal CEF

Genes	Average expression stability *M*			Relative expression levels	
	CEF infected with H5N1	Normal CEF	H5N1 AIV-Infected CEF and normal CEF	CEF infected with H5N1	Normal CEF
TUBB	2.049	1.265	1.806	982	902
RPL30	1.887	0.833	1.662	1395	9
HRRT1	1.784	0.646	1.521	854	1
ALB	1.706	1.053	1.577	1831	264
TBP	1.577	1.102	1.422	1748	10148
B2M	1.445	0.991	1.316	3579	551
GAPDH	1.237	0.646	1.08	395	848
SDHA	1.159	1.166	1.16	4	645
YHWAZ	1.031	0.921	0.856	1	1354
ACTB	0.935	0.887	0.976	179	1233
RPL4	0.935	0.737	0.856	6076	2307

DISCUSSION

The suitability of reference genes for QPCR depends on stability and expression levels. The release of the full chicken genome sequence has made it possible for a more extensive selection of reference genes in chicken, despite the fact that the functions of many genes in chicken are relatively uncharacterized compared to the mammal genome [10]. For years, ACTB and GAPDH have been widely used as reference genes in classical molecular methods for the analysis of mRNA expression, but they have sometimes been found to be unsuitable for normalization of gene expression by QPCR in mammalian cells [7, 16, 28]. However, studies on the selection of reference genes are rare in avian molecular biology. In this study, eleven housekeeping genes commonly used in mammal biology were selected in order to determine the ideal reference genes in the normal CEF and H5N1 infected CEF. Those genes were chosen because they are expressed extensively in all cells and have different functions, in order to avoid genes belonging to the same biological pathways that may be co-regulated. To the best of our knowledge, RPL4, YWHAZ, SDHA, HPRT1, ALB, RPL30, and TUBB were first investigated for their potential value as reference genes in chicken. Our result showed that SDHA, ALB, and B2M had low expression levels compared with other housekeeping genes both in the normal CEF and infected CEF, making them unsuitable reference genes for QPCR in chicken.

The expression stabilities of the eleven genes were determined at four time-points representing early, middle and late stages of infection. The gene expression stability showed certain differences between normal CEFs and infected CEFs. H5N1 AIV may have a different effect on transcription of these genes in the infected CEFs, making the ideal reference genes different in infected versus uninfected cells. As the $2^{-\triangle\triangle Ct}$ calculation method with respect to an internal control is the main method of relative quantitative analysis of real-time PCR in the study of host mRNA responses to virus infection [6], ideal reference genes should be expressed stably in both infected cells and mock-infected cells. Upon evaluation of the stability of 11 housekeeping genes with the GeNorm tool in normal and H5N1 AIV-infected CEFs, RPL4 and YWHAZ were determined to be the two best reference genes to use for the study of cellular responses to H5N1 AIV infection. This finding is useful in making quantitative analysis of host mRNA expression levels by real-time PCR more precise and reliable.

In this study, ACTB and RPL4 were the two most stably expressed housekeeping genes in the H5N1 AIV-infected CEFs. Thus, these genes can be used as ideal references in the quantitation of H5N1 replication by real-time PCR, because of that mock-infection cells are not concerned in this situation. ACTB was reported to be the best reference gene in CEFs infected with infectious bursal disease virus [11], but its transcription levels were not constant in NDV infected CEFs[18]. This indicates that the ideal reference genes in CEFs should be selected according to the virus being studied. GAPDH, as a commonly used reference gene in molecular methods, was not the most stably expressed housekeeping gene in the H5N1 AIV infected CEFs. This is consistent with the finding that GAPDH was an unsuitable reference gene in host cells infected with SARS corona virus, human herpesvirus-6, camelpox virus and cytomegalovirus [14]; however, it was found to be best reference gene in

cells infected with human immunodeficiency virus and herpes viruses [23]. Based on these data, it is important to select ideal reference genes for the precise normalization of gene expression levels and viral quantification in the study of the interaction between the cells and viruses.

CEF are not only used as model cells for the study of host responses to avian virus infection [15,26], but are also commonly used to study gene function in chicken [2]. Therefore, quantitative analysis of mRNA expression in CEFs is a useful tool for the study of gene functions in general. Our results showed that GAPDH and HPRT1 were the most stably and most highly expressed reference genes in normal CEFs, so we conclude that these genes are the most useful for the precise analysis of host gene mRNA expression levels by real-time PCR in normal CEFs.

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