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Evaluation of the Suitability of Five Housekeeping Genes as Internal Control for Quantitative Real-Time RT-PCR Assays in Chickens Acutely Infected with IBV M41 Strain

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Abstract: Avian Infectious Bronchitis (IB) is an acute and highly contagious respiratory disease in chickens. To detect the virus in chickens, housekeeping genes were detected by quantitative real-time PCR for the normalization. The aim of study was to identify the most stable housekeeping gene (s) in IBV infection. Five housekeeping genes including *18S rRNA*, *β-actin*, Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Glucose-6-Phosphate Dehydrogenase (*G6PDH*) and Ubiquitin (*UB*) were investigated in nine tissues: trachea, thymus, liver, spleen, lungs, kidney, pancreas, proventriculus and bursa of Fabricius. The geNorm and Normfinder Software were used to calculate the stability of the candidate genes.

Key words: Avian infectious bronchitis virus, housekeeping genes, quantitative real-time reverse transcription-polymerase chain reaction, suitability, China

INTRODUCTION

Avian infectious bronchitis virus belongs to the Coronaviridae family and *Coronavirus* genus is an acute and highly contagious respiratory disease in chickens (Cavanagh, 2007). In various studies, Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (QR RT-PCR) was used for detection and rapid differentiation of avian infectious bronchitis virus isolates (Callison *et al*., 2005). SYBR Green, Taqman and LNA probe-based QR RT-PCR assays have been applied to detect and quantitate clinical samples (Benyeda *et al*., 2009; Chousalkara *et al*., 2009; Meir *et al*., 2010). In recent quantitative assays, broadly targeted multiprobe QR-PCR and high-resolution melt curve analyzing with a mathematical model leaded to more reliable, accurate and non-subjective systems for detection of IBV in poultry flocks (Hewson *et al*., 2009; Muradrasoli *et al*., 2009). To compensate the variations caused by sampling and processing, normalization is needed to control experimental error induced. Endogenous controls such as housekeeping genes which expressed at a constant level or at least be unaffected by the actual experimental conditions and variation were good candidates for normalization. In previous studies, β-actin was chosen in avian influenza virus and infectious bursal disease virus infection (Borm *et al*., 2007; Li *et al*., 2005) but the expression of housekeeping genes following IBV infection was not reported.

In this research, five housekeeping genes including *18S rRNA*, *β-actin*, *GAPDH*, *G6PDH* and *UB* were chosen as candidates genes. About 9 tissues were harvested at eight time points from IBV M41 strain challenged SPF chickens. After QR RT-PCR, data from different tissues and different time points were calculated by the geNorm program (Version 3.5) and Normfinder Software. Then, the genes with a moderate expression level were recommended in quantitative analysis after IBV infected.

MATERIALS AND METHODS

Specific Pathogen Free (SPF) chickens and virus inoculation: SPF embryos were purchased (SPF Experimental Animal Centre of Dahuanong Egg. Co., Ltd. Guangdong, P.R. China) and hatched, SPF chickens were housed into two groups of 24 chickens each named infected group and control group in Animal Disease Prevention and Food Safety Key Laboratory of Sichuan

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Province, Sichuan, P.R. China. *ad libitum* feed and water. The use of all laboratory animals and animal subjects in this study was approved by the scientific ethics committee of Sichuan University.

M41 strain was provided by China Institute of Veterinary Drug Control, Beijing, P.R. China, propagated in 10 days old SPF chicken embryos. The 50% chicken infection dose (EID_{50}) was determined by inoculation of serial 10 fold dilutions into SPF embryonated chicken eggs. At 1 day of age each bird in infected group was inoculated intranasally with 50 μL of the IBV with 1.5×10^3 EID_{50}/mL (Callison *et al*., 2005) and control groups 50 μL sterile purified water, respectively. All experimental chickens were examined twice daily for clinical signs and killed by cervical dislocation. About 9 tissues including trachea, thymus, lungs, liver, spleen, kidney, pancreas, proventriculus and bursa of Fabricius were harvested from three birds in each group on 1, 4, 7, 10, 14, 21, 28 and 35 days post inoculation (dpi) under aseptic conditions. Each of 2 trachea swab samples of the two groups was taken at 5 dpi for virus re-isolation.

Virus re-isolation: Each sample was inoculated into at least four SPF embryos and allantoic fluids were collected 36 h post inoculation for RT-PCR amplification then the remaining embryos were examined 1 week later for characteristic IBV lesions such as the dwarfing and stunting of embryos. Total 200 μL of allantoic fluid from each inoculated embryo was used for RT-PCR amplification following the oligonucleotide primers to amplify the whole *S1* gene sequence. The PCR products were analysed on a 1.0% agarose gel. The positive products were sequenced by Sangon Biological Engineering Technology and Services Co., Ltd. Shanghai, P.R. China.

QR RT-PCR: Total RNA was extracted and measured by absorbance at 260 nm and the purity of the RNA sample was monitored by inspection of the 260/280 nm ratio before use for QR RT-PCR. The reverse transcription was performed by the PrimeScript™ RT Reagent kit (Takara Biotechnology (Dalian) Co., Ltd. Liaoning, P.R. China) following the manufacturer direction. A fixed amount of RNA (500 mg) from each sample was converted into cDNA in 20 μL. Similar amounts of total RNA of each sample were subjected to the cDNA synthesis protocol without the reverse transcriptase as negative samples. The primers were designed using the GenBank sequences for 18S rDNA (F: CTGAGAAACGGCT ACCACATCC; R: GCACCAGACTTGCCCTCCA), β-Actin (F: TGCTGCGCTCGTTGTTGAC; R: ACCTCTTTTGCT CTGGGCTTC), GAPDH (F: TGAGAAAGTCGGAGTC AACGG; R: GGGTCACGCTCCTGGAAGATA) and G6PDH (F: CGGGAACCAAATGCACTTCGT; R: CGCTGCCGTA GAGGTATGGGA) by Primer Premier 5.0 Software. The primer for Ubiquitin (UB) (F: GGGATGCA GATCTTCGT GAAA; R: CTTGCCAGCAAA GATCAA CCTT) was based on the sequences used by Li *et al*. (2005). The primers were tested by performing a BLAST search against the genomic NCBI database. QR RT-PCR was performed in iQ™5 Multicolor Real-Time PCR Dectection System (Bio-Rad Laboratories Inc., Hercules, USA). The following system protocol was run: the thermal cycling conditions included an initial denaturing step at 94 for 2 min, 40 cycles at 94 for 20 sec, 60 for 20 sec and 72 for 15 sec followed by an elongation step at 72 for 5 min. A single fiuorescence measure was taken after each annealing step.

Following amplification, the melting curves of PCR products were determined from 65-95 with a heating ramp of 0.5°C sec^{-1} and continuous fiuorescence measurement to determine the specificity of amplification. Each RNA sample was first controlled for genomic DNA contamination by running the no RT samples. For the real-time RT-PCR reaction, the system containing the following components was prepared: 10.0 μL iQTM SYBR Green Supermixe (Bio-Rad Laboratories inc., Hercules, USA), 0.5 μL forward primer (500 nM), 0.5 μL reverse primer (500 nM), 8.0 μL nuclease-free water and 1.0 μL template cDNA For each of the five genes, the reactions were run in triple as technique repeat.

Data analysis: The data on the expression levels of the five housekeeping genes from M41 strain infected groups were obtained as Cp values based of $E^{-\Delta CT}$ equation by the Bio-Rad iQ™5 Version 2.1 Software (Bio-Rad Laboratories Inc., Hercules, USA). Then the stability of the housekeeping genes was evaluated by the geNorm program (Version 3.5) and Normfinder Software. The geNorm calculated the gene expression stability value M for a reference gene as the average pairwise variation V of that gene with all other reference genes tested. Stepwise exclusion of the least stable gene (with highest M-value) allows ranking of the tested genes according to their expression stability and determines the most stable genes. Normalization factors based on the geometric mean of the expression levels of the n best reference genes were calculated by stepwise inclusion of an extra less stable reference gene. NormFinder is another reference tool for identifying the ideal normalization genes among various candidate genes. It ranked the various candidate reference genes according to their expression variation between

inter and intra groups. The gene which got lowest stablility value by this software was recommended the most stable.

RESULTS AND DISCUSSION

At 3 dpi >2/3 birds of IBV infected group were depressed with ruffled feathers and respiratory distress. At 4 dpi respiratory signs became obvious in all chickens and lasted until the 8 dpi. By the time of 11 dpi only slight respiratory signs were observed. No clinical sign was observed in the control group. No virus was recovered from any samples from control group. The IBV was isolated from all swab samples in challenged groups. The analysing on agarose gel and complete gene sequence of S1 identified IBV was inoculated positively.

QR RT-PCR Efficiency (E) values for the virus and five genes were as follows: *18S rRNA*, 115.7%; *GAPDH*, 90.1%; *β-Actin*, 112.8%; *G6PDH*, 106.8% and *UB*, 93.8%. The correlation coefficients were 0.994, 0.997, 1.000, 0.999, 0.999 and 0.995, respectively. The high correlation coefficient and large dynamic range ensured the amplification efficiency closely comparatively and reproducibility. The expression stability of candidate genes was sorted by geNorm from least to most stable in Fig. 1: *18S rRNA*, *G6PDH*, *β-Actin*, *GAPDH* and *UB*. The M-values compared to all other tested genes were 0.86273, 0.76009, 0.66207 and 0.64152, respectively. *GAPDH* and *UB* got the same value and could be considered as the most stability genes in this experimental set-up.

The pairwise variation $V_{n/n+1}$ between two sequential normalization factors Nf_n and Nf_{n+1} were shown in Fig. 2. So, *GAPDH* and *UB* were considered realism candidate genes. The stablility values of the five cadidate genes calculated by NormFinder were *18S rRNA*, 0.597; *GAPDH*, 0.238; *β-Actin*, 0.410; *G6PDH*, 0.451 and *UB*, 0.376. GAPDH which got the stablility value of 0.238 was also identified as the best gene by Normfinder. As suggested by the two housekeeping genes selecting software, *GAPDH* with *UB* should be identified and validated as internal control in IBV M41 strain acutely infected chickens. Real-Time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. Normalization strategies are necessary to correct for experimental error during the procedures of extraction and processing of the RNA. Reference genes which proved suitable for some experimental conditions are not necessarily similarly appropriate for others. β-actin is mainly used as an internal control for normalization of cytokine expression

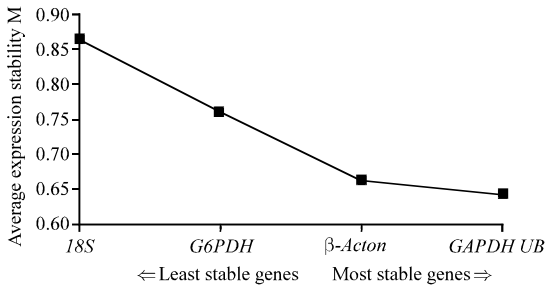

Fig. 1: Average expression stability values of the five reference genes. The data was calculated by geNorm Software and listed from least to most stable

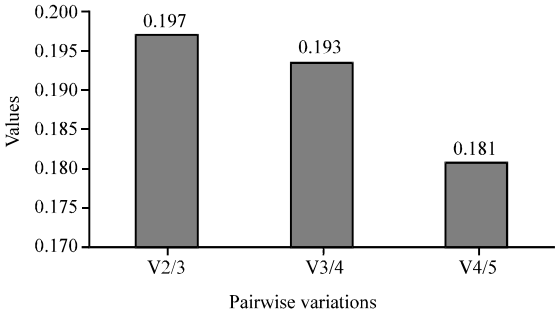

Fig. 2: The optimal number of control genes for normalization was selected by pairwise variation analysis between the normalization factors NFn and NFn+1. According to geNorm, the inclusion of two or three reference genes has no significant effect

in several experimental designs (Sundaresan *et al*., 2007). GAPDH is a multifunctional enzyme involved in cellular metabolism. But it is reported that GAPDH mRNA levels can be influenced by experimental conditions such as nutritional manipulations and advancing age. Therefore, a proper validation of the suitability of a given reference gene is required for each experimental setting.

The pairwise variation $V_{n/n+1}$ between two sequential normalization factors Nf_n and Nf_{n+1} showed $V_{2/3}$ (0.197) and $V_{3/4}$ (0.193) has no obvious difference indicating the added 3rd or 4th reference genes didn't play important roles. The $geNorm_{TM}$ Housekeeping Gene Selection kit handbook suggestted that the proposed 0.15 value must not be taken as a too strict cut-off; variations in different tissues may lead to the value of $V_{n/n+1}$ >0.15 inevitably. β-actin which got the higher M value (0.66207) of the stepwise exclusion by geNorm program was eliminated. Though the principle of geNorm is that the expression ratio of two ideal internal control genes is identical in all tested samples; Normfinder program focuses on the two genes

with the least intra and inter-group expression variation. Taken together, GAPDH with UB were recommended by the two programs in SPF chickens infected by M41 strain as several serotype IBVs circulated all over the world more experiments should be carried on to establish the stability of internal controls in IBV infection.

CONCLUSION

As the result, 18S rRNA and G6PDH which got the M-value of 0.86273 and 0.76009 showed least stable in this experimental set-up; GAPDH and UB combination were suggested by geNorm. The Normfinder program indicated GAPDH as best candidate genes. So, GAPDH and UB together could be recommended to normalize the expression in IBV M41 strain infected chickens.

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