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Journal Article

IDENTIFICATION OF STABLE REFERENCE GENES FOR NORMALIZATION OF GENE EXPRESSION IN AVIAN 25+IEL-NK CELLS FOLLOWING NEWCASTLE DISEASE VIRUS INFECTION

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SUMMARY

The use of multiple reference genes has long been advocated as a robust method for achieving accurate normalization in quantitative reverse transcription polymerase chain reaction (RT-qPCR). These internal reference genes are employed to normalize target gene expression and are expected to remain invariant regardless of treatment conditions, such as viral infection. However, several studies have reported instability in the expression of commonly used reference genes, including beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18s rRNA, under various experimental conditions. This study aimed to identify suitable reference genes for normalising genes associated with chicken intraepithelial lymphocyte natural killer (IEL-NK) cells following infection with different strains of Newcastle Disease Virus (NDV). Three commonly used reference genes were selected and two IEL-NK cell-related genes, B-NK and NK-lysin, for normalisation purposes. The mean M and V values for the combinations of reference genes (18S/ACTB; 18S/GAPDH; ACTB/GAPDH; and 18S/ACTB/GAPDH) were calculated using the Target Stability Value program in CFX Manager software version 3.1. Based on the recommended stability values, the combination of ACTB and GAPDH demonstrated the most stable expression among the reference gene combinations, while the inclusion of 18S resulted in unstable expression. Therefore, combining ACTB and GAPDH is recommended as suitable reference genes for normalising chicken IEL-NK cell-related genes following NDV infection.

Keywords: 18S, 25+IEL-NK cells, β-Actin, GAPDH, NDV, RT-qPCR

INTRODUCTION

With the ongoing global impact of Newcastle disease virus (NDV) on the poultry industry (Abdolmaleki et al., 2018; Hamisu et al., 2022), it is crucial to evaluate the transcriptional profiles of NDV-related target genes using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (Boo et al., 2020). qRT-PCR is a sensitive technique that combines accuracy and reproducibility for measuring target nucleic acid sequences across a broad dynamic range (Zhang et al., 2018). However, one of the primary challenges associated with qRT-PCR is the accurate and precise comparative determination of the template nucleic acid amplified between samples (Radonić et al., 2004). Variability in RNA quality and quantity, as well as differences in reverse transcription efficiencies among experimental samples, can significantly affect results (Huggett et al., 2005). Consequently, the reliability of this technique hinges on correcting inter-sample variability through normalisation with one or more housekeeping genes (Ferguson et al., 2010).

Housekeeping genes, unlike cellular genes, are expected to maintain stable expression levels under varying conditions; however, in some instances, they are influenced by experimental procedures (Watson et al., 2007). The selection of appropriate housekeeping genes is critical in RT-PCR, as any fluctuations in their expression across experimental groups can lead to assay insensitivity and misleading results (Thellin et al., 1999). Despite their presumed constitutive expression, not all housekeeping genes are stably expressed under all experimental conditions, and their expression can vary with tissue type, developmental stage, or stress, potentially introducing bias if not properly validated (Watanabe et al., 2021; Grigorova et al., 2024; Shen et al., 2024). Furthermore, traditional housekeeping genes may exhibit altered expression levels due to viral infections that manipulate key biological and molecular processes encoded by these genes (Boo et al., 2020). Each virus type can affect specific cellular transcription pathways differently, depending on the infected cell type and the viral strain involved. This variability underscores the challenge of identifying a universal reference gene applicable to all virus-infected cell types (Watson et al., 2007).

The three most commonly used reference genes for normalising qRT-PCR data are glyceraldehyde 3-

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phosphate dehydrogenase (GAPDH), β-actin (ACTB), and 18S rRNA. Previous research has highlighted the importance of reliable housekeeping genes for understanding the innate immune response in chickens (Boo et al., 2020). This study also identified GAPDH and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) as suitable reference genes for normalizing chicken IEL-NK cell expression following infection with very virulent infectious bursal disease virus (vvIBDV) (Boo et al., 2020). While some studies have found that 18S rRNA is stable in certain contexts, such as influenza A virus subtypes in chicken and duck lung cells (Kuchipudi et al., 2012), it has been shown to be unstable in chicken embryo fibroblasts after NDV infection (Yin et al., 2011).

Majority of RT-qPCR analyses use reference genes, which are genes that are stably expressed within various experimental conditions (Riedel et al., 2014). In RT-qPCR applications, relative fold changes of the target gene between experimental conditions are the most important, not absolute molecule numbers (Riedel et al., 2014). Although the ΔCT method is straightforward and most often used (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008), it does not allow for multiple reference genes. However, using more than one reference gene significantly strengthens data normalisation and accuracy in comparative gene expression analyses (Riedel et al., 2014). Some studies in both human and animal demonstrated that relying on a single reference gene can introduce systematic errors and lead to misleading or even contradictory results (Patrizi and Ho, 2021; Schmidt et al., 2022; Mirzaaghayi et al., 2022). Recent studies using high-throughput RNA-seq in chickens have revealed that no single housekeeping gene is consistently stable across all tissues, and commonly used genes like ACTB and HPRT1 may be unsuitable in certain contexts, so tissue-specific panels of stable reference genes are recommended for accurate normalisation (Mirzaaghayi et al., 2022). Therefore, employing a panel of validated housekeeping genes tailored to the experimental system greatly improves the reliability and reproducibility of gene expression studies in chickens and other animals (De Vries et al., 2007; Patrizi and Ho, 2021; Schmidt et al., 2022; Mirzaaghayi et al., 2022).

Statistical tools such as geNorm, NormFinder, and BestKeeper are widely used to assess the stability of candidate reference genes and recommend the optimal number and combination for normalisation (Kong et al., 2020; Tripathi et al., 2021). In this study, we investigated the stability of three common housekeeping genes, 18S rRNA, ACTB, and GAPDH, in chicken IEL-NK cells following infection with various NDV strains at different time points, using the method described by Vandesompele et al. (2002). The findings indicate that ACTB and GAPDH are stable reference genes suitable for normalizing gene expression related to IEL-NK cells after NDV infection. In contrast, 18S rRNA, combined with the other housekeeping genes, was unstable in avian IEL-NK cells following NDV infection.

MATERIALS AND METHODS

Acquisition and rearing of chickens

A total of 400 nine-day-old Specific Pathogen-Free (SPF) embryonated chicken eggs were obtained from the Veterinary Research Institute, Ipoh, Perak. These eggs were incubated, and more than 360 were successfully hatched under sterile conditions at the Laboratory of Vaccine and Biomolecules, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). Out of these, a total of 315 chicks were used for this experiment, where they were transferred to the Biosafety Level-2 (BSL-2) animal house facility at the Faculty of Veterinary Medicine, UPM, and were provided with feed and water *ad libitum*.

Viral strains

Two velogenic Newcastle Disease Virus (NDV) strains, genotype VII (IBS005/11) and genotype VIII (AF2400-1), along with a lentogenic strain La Sota (genotype II) were kindly provided by Prof. Dr. Abdul Rahman Omar from the Faculty of Veterinary Medicine, UPM, Malaysia.

Experimental design

A total of 315 SPF chickens were randomly divided into five groups (Groups 1 to 5), with 63 chickens in each group. Each group was further subdivided into three biological replicates, consisting of 21 chickens per replicate. At 24 days post-hatch, chickens in group 1 were inoculated with 0.1 ml of 106.5 embryo lethal dose (ELD50) NDV La Sota; while chickens in groups 2-5 were inoculated with 0.1 mL of 106.5 ELD50 NDV genotype VII, 0.1 mL of 106.5 ELD50 NDV genotype VIII, vaccinated with 0.1 ml 106.5 ELD50 NDV La Sota and challenged with 0.1 mL of 106.5 ELD50 NDV genotype VII, and vaccinated with 0.1 ml 106.5 ELD50 NDV La Sota and challenged with 0.1 mL of 106.5 ELD50 NDV genotype VIII, respectively.

Seven chickens from each group were sacrificed at 12, 36, and 60 hours post-challenge. The experiment was conducted with approval from the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, UPM, under reference number UPM/IACUC/AUP-R005/2019.

Isolation and characterization of CD3−/CD25+IEL-NK cell

The isolation of IEL-NK cells from duodenal samples was performed as previously described by Göbel (2000). Characterisation of CD3− cells was conducted using Magnetic Activated Cell Sorting (MACS) as described by Abdolmaleki et al. (2018). Briefly, 106 CD3− cells/mL were resuspended in 100 µL cold PBS-BSA-EDTA and stained with 10 µL of human anti-chicken CD25 conjugated with fluorescein isothiocyanate (FITC) (Bio-Rad Laboratories, USA). The cells were then passed through the MACS BS column, and positively selected

cells were collected. Viable cells were counted using ScepterTM 2.0 (MilliporeSigma, USA).

RNA extraction and conversion to cDNA

RNA from IEL-NK cells was extracted using the innuPREP RNA Mini Kit 2.0 (Analytik Jena, Germany) following the manufacturer's instructions. The purity and concentration of the RNA were evaluated using a spectrophotometer (Eppendorf, USA). The RNA was then converted into cDNA using the SensiFASTTM cDNA Synthesis Kit (Bioline, Meridian Life Science Company, USA).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed on a BioRad CFX96 Real-Time PCR System (BioRad Laboratories, Hercules, CA) using the SensiFAST Probe No-ROX Kit (Bioline). The primers used for the reference genes were listed in Table 1. The total reaction volume was 20 µL, containing 1x final concentration of 2x SensiFAST Probe No-ROX Mix, 400 nM each of 10 µM forward and reverse primers, 100 nM of 10 µM probe, 2 µL cDNA, and topped up to 20 µL with DEPC water. The thermal cycling conditions included: polymerase activation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 20 seconds. Non-template controls (NTC) were included in triplicate in each RT-qPCR run.

Table 1: Primer and probe sequences of the target and reference genes

Genes	Primer and Probe Sequences (5/-3/)	Annealing Temperature (°C)	References
GAPDH	Probe: (FAM)-CGCCATCACTATCTTCCAGG-(BHQ1) F: GAACGGGAAACTTGTGAT R: GACTCCACAACATACTCA	58	Abdolmaleki et al., 2018
18s	Probe: (FAM)-CCACAGTTA-ZEN-TCCAAGTAACGGGAGGG-(IBFQ) F: TCAGTTATGGTTCCTTTGGTCG R: CGTCGGCATGTATTAGCTCTAG	60	Rasoli et al 2014
Beta-Actin	Probe: (FAM)-ACCTTCAAC-ZEN-ACCCCAGCCATGTAT-(IBFQ) F: ACCCCAAAGCCAACAGAG R: CCAGAGTCCATCACAATACCAG	60	Abdolmaleki et al., 2018
B-NK	Probe: (FAM)-CCTACAACAGCACAGAGTCTGAT-(BHQ1) F: GGAAATGGGTGGACAACT R: ATTCTCCATCACGGAAAGGT	60	Abdolmaleki et al., 2018
NK-Lysin	Probe: (FAM)-CCGGTCCCATTGCGTC-(BHQ1) F: TGCGTGGGATGCAGATGAAG R: CAGAATCTGCATTTAATCCCCTTGC	60	Abdolmaleki et al., 2018

Data analyses

The stability of the combination of reference genes was evaluated using the Target Stability Value program in the CFX software version 3.1. The mean expression stability value (M value) and the mean coefficient of variation value (CV value) were generated. The interpretation of the results was based on the recommended stability values for samples in the software, where CV < 0.25 and M < 0.5 were recommended for homogeneous samples, and CV < 0.5 and M < 1 were recommended for heterogeneous samples.

RESULTS

In this study, three reference genes were utilized to normalize two target genes associated with avian 25+IEL-NK cells, specifically B-NK and NK-lysin (Hamisu, et al., 2022), at various time points following infection with different Newcastle Disease Virus (NDV) strains. The stability values for combinations of two or more reference genes were assessed using the Target Stability Value tool in CFX Manager software, version 3.1 (Bio-Rad, U.S.A.). The results were evaluated based on the recommended Stability Values for Samples provided by the software.

As shown in Tables 2, 3, and 4, the mean coefficient of variation (CV) and mean M value for combinations of 18S and β-actin; 18S and GAPDH; and 18S, β-actin, and GAPDH exceeded the recommended CV of <0.25 and M value of <0.5 for homogeneous samples. However, the mean CV and mean M value for combinations of β-actin and GAPDH were lower than the recommended stability values (Table 5).

Therefore, the combination of β-actin and GAPDH serves as stable reference genes suitable for normalizing gene expression related to chicken 25+IEL-NK cells following NDV infection. Furthermore, the results indicated that the inclusion of 18S in each combination

Table 2. Stability values for combination of *18s* rRNA and *β-actin* used for target gene normalization at different time points. The results were obtained from three biological replicates, where each biological replicate consisted of 25+IEL-NK cells harvested from 7 chickens.

Stability parameters	Reference genes combination at different time points					
	18s	*β-actin*	*18s*	*β-actin*	*18s*	*β-actin*
	12 hrs	12 hrs	36 hrs	36 hrs	60 hrs	60 hrs
CV	0.83	1.43	1.03	2.12	0.83	1.46
M Value	5.47	5.47	9.82	9.82	5.83	5.83
Mean CV	1.13		1.57		1.15	
Mean M Value	5.47		9.82		5.83	

Table 3. Stability values for combination of *18s* rRNA and GAPDH used for target gene normalization at different time points. The results were obtained from three biological replicates, where each biological replicate consisted of 25+IEL-NK cells harvested from 7 chickens.

Stability parameters	Reference genes combination at different time points					
	18s	GAPDH	*18s*	GAPDH	*18s*	GAPDH
	12 hrs	12 hrs	36 hrs	36 hrs	60 hrs	60 hrs
CV	0.84	1.26	0.96	2.19	0.76	1.31
M Value	5.68	5.68	9.96	9.96	6.30	6.30
Mean CV	1.05		1.58		1.04	
Mean M Value	5.68		9.96		6.30	

Table 4. Stability values for combination of *18s* rRNA, *β-actin* and GAPDH used for target gene normalization at different time points. The results were obtained from three biological replicates, where each biological replicate consisted of 25+IEL-NK cells harvested from 7 chickens.

Stability parameters	Reference genes combination at different time points								
	18S	*β-actin*	GAPDH	*18S*	*β-actin*	GAPDH	*18S*	*β-actin*	GAPDH
	12hrs	12hrs	12hrs	36 hrs	36 hrs	36 hrs	60hrs	60 hrs	60 hrs
CV	0.91	1.20	1.06	1.14	1.78	2.00	0.85	1.23	1.15
M Value	5.58	2.89	3.00	9.89	5.09	5.16	6.07	3.05	3.28
Mean CV	1.06			1.64			1.08		
Mean M Value	3.82			6.71			4.13		

Table 5. Stability values for combination of *β-actin* and GAPDH used for target gene normalization at different time points. The results were obtained from three biological replicates, where each biological replicate consisted of 25+IEL-NK cells harvested from 7 chickens.

Stability parameters	Reference genes combination at different time points					
	β-actin	GAPDH	*β-actin*	GAPDH	*β-actin*	GAPDH
	12 hrs	12 hrs	36 hrs	36 hrs	60 hrs	60 hrs
CV	0.10	0.11	0.12	0.12	0.09	0.09
M Value	0.31	0.31	0.36	0.36	0.26	0.26
Mean CV	0.11		0.12		0.09	
Mean M Value	0.31		0.36		0.26	

raised the stability values beyond the recommended thresholds. Thus, the instability can be attributed to the presence of 18S, indicating that it is not a stable reference gene for normalizing avian IEL-NK cell-related genes after NDV infection.

DISCUSSION

The selection of appropriate reference genes is crucial for accurate gene expression analysis, particularly in lymphocytes across different species, including chickens. For instance, in mouse lymphocytes, ubiquitin C (Ubc) has been identified as the most stable reference gene,

while beta-actin (Actb) is unsuitable due to its high variability (Albershardt et al., 2012). In human T cells, such as Jurkat cells, genes like ABCA5, GAPDH, HPRT1, PLA2G4A, and RPL13A have been identified as stably expressed across different gravity conditions (Thiel et al., 2017). This suggests that these genes could serve as reliable reference genes for studies involving immune cells under altered gravity conditions.

Similarly, in chicken intraepithelial lymphocytes, GAPDH and YWHAZ were found to be the most stable reference genes in chickens infected with infectious bursal disease virus (Boo et al., 2020), but not in rabbit tissues (Deindl et al., 2002), and nucleated human blood cells (Bustin et al., 1999). However, ACTB is generally considered less stable in these cells (Boo et al., 2020). In fact, our study also found that the combination of β-actin and GAPDH is a stable reference gene combination for normalising avian IEL-NK cell genes following infection with NDV. This finding aligns with previous studies indicating β-actin is stable in lymphoid cells (Anstaett et al., 2010), although it is unsuitable as a normaliser for the expression of interstitial cells in the heart valves in sheep (Yperman et al., 2004).

The 18S rRNA gene is often considered highly stable across various cell types and conditions. In studies involving human, pig, chicken, and duck cells, 18S rRNA was found to be the most stable reference gene (Kuchipudi et al., 2012). However, its stability can vary depending on the specific cell type and experimental conditions. For example, in lymphocytes from patients with ALS, 18S rRNA was found to be highly variable (Usarek et al., 2017). In our study, the inclusion of 18S rRNA resulted in unstable combinations, making it unreliable as a normalizer for IEL-NK cell genes following treatment with NDV strains.

While GAPDH is commonly used as a reference gene, its stability can vary. In human lymphocytes, it is stable under certain conditions (Thiel et al., 2017), but in chicken IEL-NK cells, it is stable when combined with YWHAZ (Boo et al., 2020). In some studies, GAPDH has shown variable expression in lymphocytes (Usarek et al., 2017). Our findings are consistent with Van Tol et al. (2008), who reported stable expression of GAPDH in bovine oocytes (Van Tol et al., 2008). However, GAPDH was found to be unstable in zebrafish embryos and in fibroblast cell lineages of mice under different treatment conditions with antidepressants (Lin et al., 2009; Sugden et al., 2010).

Some studies on lymphocytes in humans and mice did not agree with our findings. For example, Bas and co-workers (2004) reported that ACTB and GAPDH have variable gene expression (Bas et al., 2004). Similarly, 18S rRNA and GAPDH in macrophages showed non-stable expression (Stephens et al., 2011). Additionally, Dheda's team found that GAPDH, ACTB, and some other reference genes were less stable in peripheral blood mononuclear cells (Dheda et al., 2004). In contrast, Kaszubowska and colleagues (2015) showed that GAPDH was a stable reference gene for human NK-92 cell lines following stimulation with IL-2 or TNF for 2, 24, or 72 hours (Kaszubowska et al., 2015).

CONCLUSION

This study highlights the importance of selecting stable reference genes for gene expression analysis. The ACTB/GAPDH combination is recommended for normalizing chicken IEL-NK cell genes following NDV infection. It is crucial to screen additional reference genes using various stability programs to ensure reliable normalisation in studies involving chicken IEL-NK cells and NDV infection.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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