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Validation of housekeeping genes as candidate internal references for quantitative expression studies in healthy and nervous necrosis virus-infected seven-band grouper (*Hyporthodus septemfasciatus*)

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Abstract

Background: In the present study, we evaluated four commonly used housekeeping genes, viz., actin- β , elongation factor-1 α (EF1 α), acidic ribosomal protein (ARP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal references for quantitative analysis of immune genes in nervous necrosis virus (NNV)-infected seven-band grouper, *Hyporthodus septemfasciatus*.

Methods: Expression profiles of the four genes were estimated in 12 tissues of healthy and infected seven-band grouper. Expression stability of the genes was calculated using the delta Ct method, BestKeeper, NormFinder, and geNorm algorithms. Consensus ranking was performed using RefFinder, and statistical analysis was done using GraphpadPrism 5.0.

Results: Tissue-specific variations were observed in the four tested housekeeping genes of healthy and NNV-infected seven-band grouper. Fold change calculation for interferon-1 and Mx expression using the four housekeeping genes as internal references presented varied profiles for each tissue. EF1 α and actin- β was the most stable expressed gene in tissues of healthy and NNV-infected seven-band grouper, respectively. Consensus ranking using RefFinder suggested EF1 α as the least variable and highly stable gene in the healthy and infected animals.

Conclusions: These results suggest that EF1 α can be a fairly better internal reference in comparison to other tested genes in this study during the NNV infection process. This forms the pilot study on the validation of reference genes in *Hyporthodus septemfasciatus*, in the context of NNV infection.

Keywords: Housekeeping gene, Expression stability, RT-qPCR, Nervous necrosis virus

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Introduction

The analysis and quantification of mRNA expression in different animal experimental settings are crucial in understanding the cause or outcome of a biotic or abiotic factor under study. The reverse transcriptase quantitative PCR (RT-qPCR) is a versatile and widely used technique to study the absolute or relative gene expression studies due to its accuracy, sensitivity, reproducibility, and wider dynamic range (Bustin et al. 2005; Huggett et al. 2005). The quality and accuracy of the data generated from a qPCR experiment relies on the normalization of the output with a constitutively expressed gene to avoid experimental errors caused by cDNA concentration, variations in RNA, reverse transcription efficiency, and PCR efficiency (Dheda et al. 2004). The ideal reference gene for qPCR should have a constant expression in different tissues/cells or developmental stages and should be unaffected by the experimental situations (Radonic et al. 2004). Housekeeping genes such as those encoding β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and elongation factor 1 alpha (EF1 α) are commonly used as internal references. Although housekeeping genes are generally involved in the maintenance of cellular homeostasis, they are assumed to be constitutively expressed; however, many studies demonstrated that the expression levels of these genes vary significantly with different factors (Ingerslev et al. 2006; McCurley and Callard 2008; Su et al. 2011; Paria et al. 2016). Therefore, it is crucial to evaluate suitable reference genes to choose the best performing candidate in all the experimental settings to prevent the misinterpretation of qPCR output and to get an accurate gene expression profile.

Grouper is an important warm and temperate water fish, distributed in the tropical, subtropical, and temperate zone waters (Meng et al. 1995) with great economic value for aquaculture. They belong to the family Epinephelinae, comprising of 159 marine species in 15 genera. The seven-band grouper, *Hyporthodus septemfasciatus*, is a high-valued marine finfish having an immense aquaculture potential in Southeast Asia. They inhabit the shallow water zones around 5–30 m in Korea, Japan, and China (Heemstra and Randall 1993). They are regarded as candidate species for aquaculture due to their limited resource and high economic value. The studies on the seven-band grouper have been increasing in recent years and has been focused mainly on the reproductive biology, early development, and disease pathology including the viral nervous necrosis (VNN) that causes heavy mortality in the larval and juvenile groupers during the summer season (Kim et al. 2012). The reports on gene expression profiling studies in seven-band grouper are limited; however, to understand the biology of the fishes and molecular mechanisms associated with infections, it is important to examine the functional genes involved in

these aspects. The identification of a suitable reference gene in seven-band grouper is crucial for accurate immune gene expression profiling. The objective of the current study was to validate housekeeping genes of seven-band grouper to identify a candidate reference gene as an internal control for expression profiling studies during NNV infection.

Materials and methods

Ethics statement

Juveniles of healthy seven-band grouper (7.8 g \pm 0.5 g) were maintained in the wet lab facility at 20–22 °C and daily fed with a commercial diet. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chonnam National University (CNU IACUC-YS-2018-3).

Sample preparation

To study the expression of the housekeeping genes in normal seven-band grouper, tissues including brain, gill, eye, heart, spleen, liver, gut, head kidney, trunk kidney, blood, muscle, and skin were collected and stored immediately at –80 °C. Tissues of three animals were pooled, and five such replicates were used to study the gene expression. For NNV challenge study, fish were injected intramuscularly with 100 μ L of 10^{3.5} TCID₅₀ NNV in L-15. Fish injected with sterile L-15 were used as control. At 0, 12, 24, 48, and 72 h of viral challenge, fish were sacrificed and the aforementioned tissues were collected and pooled in the same manner as described above.

RNA extraction and cDNA synthesis

Total RNA from the tissues were extracted using Tri reagent (MRC, USA) according to manufacturer's instruction. One microgram of the DNase treated total RNA was reverse-transcribed by ReverTraAce qPCR RT Kit (Toyobo, Japan) primed with random hexamers according to manufacturer's instruction. The prepared first-strand cDNA was diluted in nuclease-free water to get 100 ng/ μ L and stored at –20 °C until use.

Primer designing for reference genes and PCR efficiency

A total of four reference genes were selected for gene expression analysis viz., actin- β , elongation factor-1 α (EF1 α), acidic ribosomal protein (ARP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Specific primers for each of the genes were designed based on the nucleotide sequences from the *H. septemfasciatus* brain transcriptome data (Kim et al. 2017) using Primer-BLAST suite (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). The optimal annealing temperature of each primer was about 60 °C and the amplicons were within the range of 100–150 bp. PCR specificity was confirmed with a single melt peak in dissociation curve analysis. PCR efficiency was calculated

Table 1 Sequence, efficiency and correlation coefficient of the primers used in the present study

Gene	Primer sequence (5'-3')	Efficiency E (%)	Correlation coefficient (R^2)
Actin- β	AGCAGGAGTACGATGAGTCC GTATGCAGAGCCAGTCGTTTG	102	0.995
Glyceraldehyde-3-phosphoate dehydrogenase (GAPDH)	TGCCACGCAAAACATCATTC TGAATGCCATGCCTGTCCAGC	104	0.994
Acidic ribosomal protein (ARP)	GCCACGTGGAAGTCCAACATA ACAGACGGATGGTCTGCATC	101	0.999
Elongation factor 1- α (EF1 α)	CGAGAAGTTCGAGAAGGAAGC GATGAGCTGCTTACACCAAG	100	0.992

based on the slope of a standard curve generated using ten-fold serial dilutions (10 , 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}) of liver cDNA.

Quantitative real-time PCR

All reactions were carried out on in an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using the AccuPower 2XGreenStar qPCR Master Mix (Bioneer, Korea) following the manufacturer's instructions. The reactions were performed in triplicates with $10 \mu\text{M}$ of each primer and 100 ng of cDNA per reaction. The thermal profile consisted of 95°C for 10 min , followed by 40 cycles of 95°C for 10 s and 60°C for 10 s . A dissociation curve analysis of the amplification was performed from 60 to 95°C at either $0.1^\circ\text{C}/\text{s}$ melting rate with a smooth curve setting averaging 1 point to confirm that only the specific PCR product was amplified and detected.

Fold change expression of immune genes

To further evaluate the expression stability of housekeeping genes, a comparative fold change expression analysis of immune genes, IFN-1 and Mx, at 48 h post-infection was undertaken. Fold change was separately calculated for both immune genes with actin- β , EF1 α , ARP, and GAPDH as internal controls using the comparative CT method ($2^{-\Delta\Delta\text{CT}}$ method) (Livak and Schmittgen 2001).

Data analysis

Gene expression stability was evaluated using delta Ct method (Silver et al. 2006) and four commonly used programs viz., geNorm V3.5 (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper V1 (Pfaffl et al. 2004). The comprehensive ranking of the expression stability was evaluated using the RefFinder program (Xie et al. 2012). Statistical analysis for the differences in the expression level of actin- β , EF1 α , ARP, and GAPDH was done by linear regression and two-way analysis of variance (ANOVA) using GraphPad Prism 5.0 (La Jolla, CA,

USA). The difference was considered to be significant at $p < 0.05$.

Results

Quantitative real time PCR efficiency and intra- and inter-assay variability

The four housekeeping genes, viz., actin- β , EF1 α , ARP, and GAPDH were amplified by qPCR from 12 different tissues of the healthy and NNV-infected animals. The amplified products ranged from 100 to 150 bp . The PCR efficiency was calculated using the equation: PCR efficiency (E%) = $(10^{-1/\text{slope}} - 1) \times 100$. All reactions displayed efficiency between 100% and 105% (Table 1). Intra-assay variation was $< 1.19\%$ and inter-assay variation was $< 0.98\%$ in this study, indicating high reproducibility of the assay.

Expression level and stability of housekeeping genes in healthy tissues

Expression levels were evaluated in 12 different tissues for all four housekeeping genes. The mean expression levels for all the studied genes were in the range of 14 – 18 Ct values (Fig. 1, Additional file 1). However, GAPDH showed significant variation in its expression across the normal tissues with Ct value of 14.7 in the eye and 25.6 in the liver. Compared with all the tissues, the gut showed expression for all the housekeeping genes. The highest variation in expression of the house keeping genes was in the muscle. Heart displayed significant variation in its expression for all the four genes: skin and spleen for actin- β and GAPDH, respectively; liver for EF1 α and GAPDH, respectively; and head kidney and trunk kidney for EF1 α and actin- β , respectively (Fig. 2).

For expression stability analysis, the stability ranking for the four genes obtained from the delta Ct method, Bestkeeper, geNorm, and NormFinder programs were compared. ARP was found to be the most stable expressed gene by the delta Ct, norm finder, and the geNorm, whereas, EF1 α by the best-keeper and geNorm (Fig. 3). The overall ranking for stability of candidate reference genes determined by

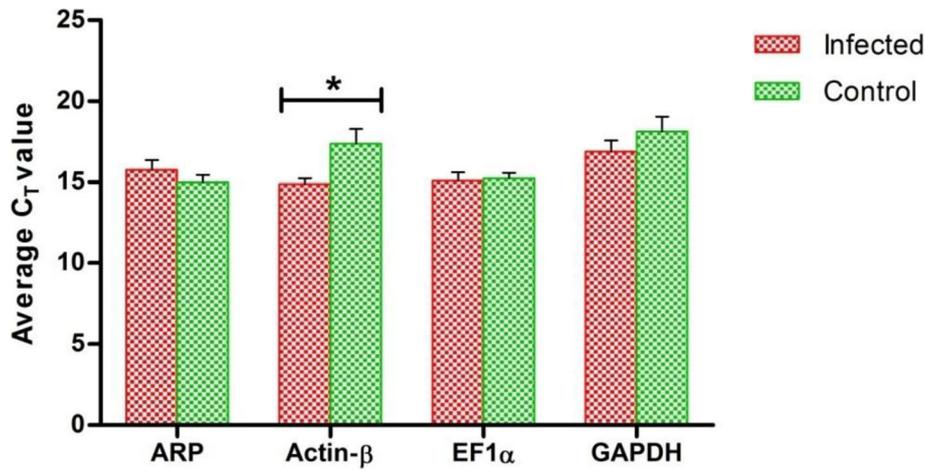


Fig. 1 Graphical representation of absolute Ct values for each gene analyzed in healthy and infected seven-band grouper. Whiskers represent the maximum and minimum values. Data represented as mean ± SEM, asterisk shows significant difference at $p < 0.05$

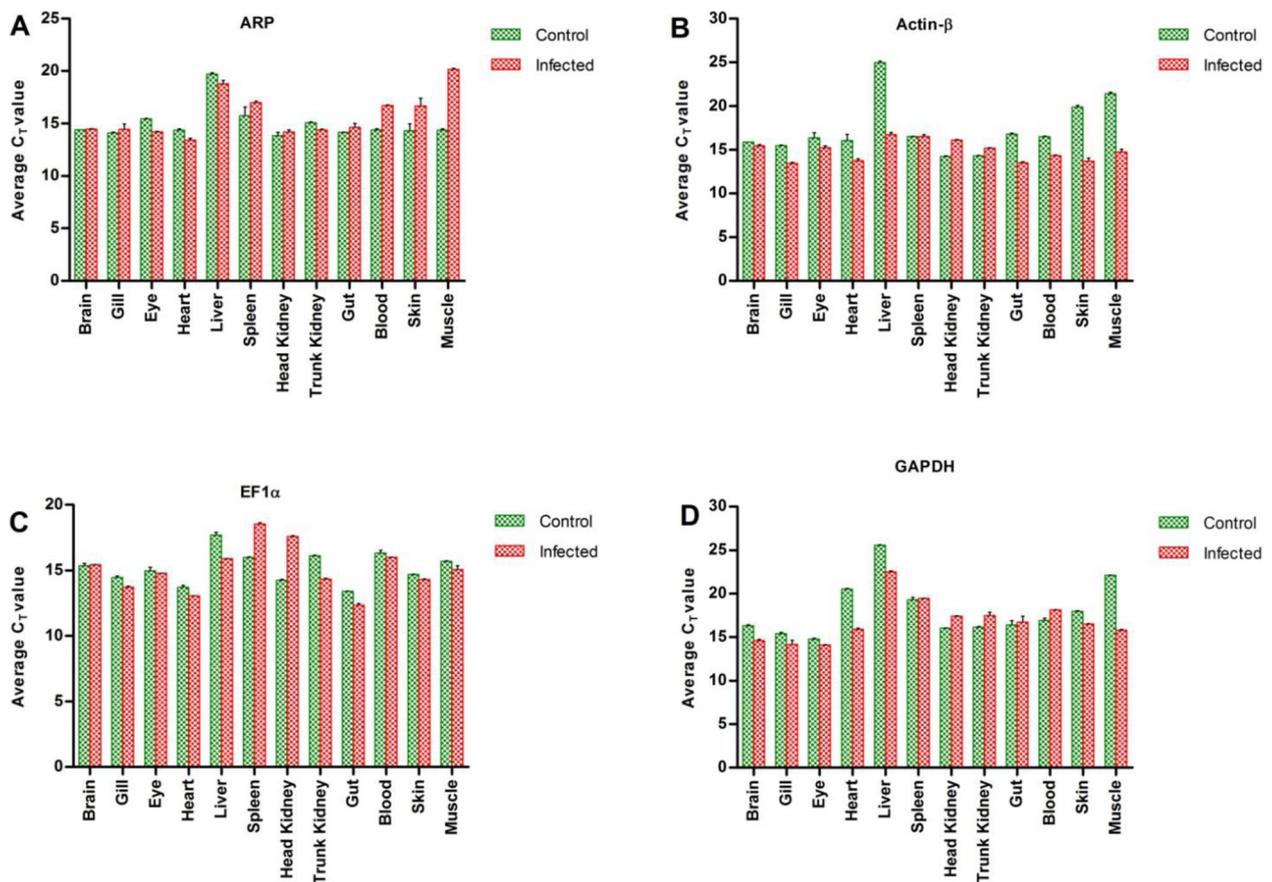


Fig. 2 Expression levels of candidate reference genes in different tissues of healthy and infected seven-band grouper. Data represented as mean ± SEM of ten replications. **a-d** indicates the expression levels of ARP, actin-β, EF1α and GAPDH respectively

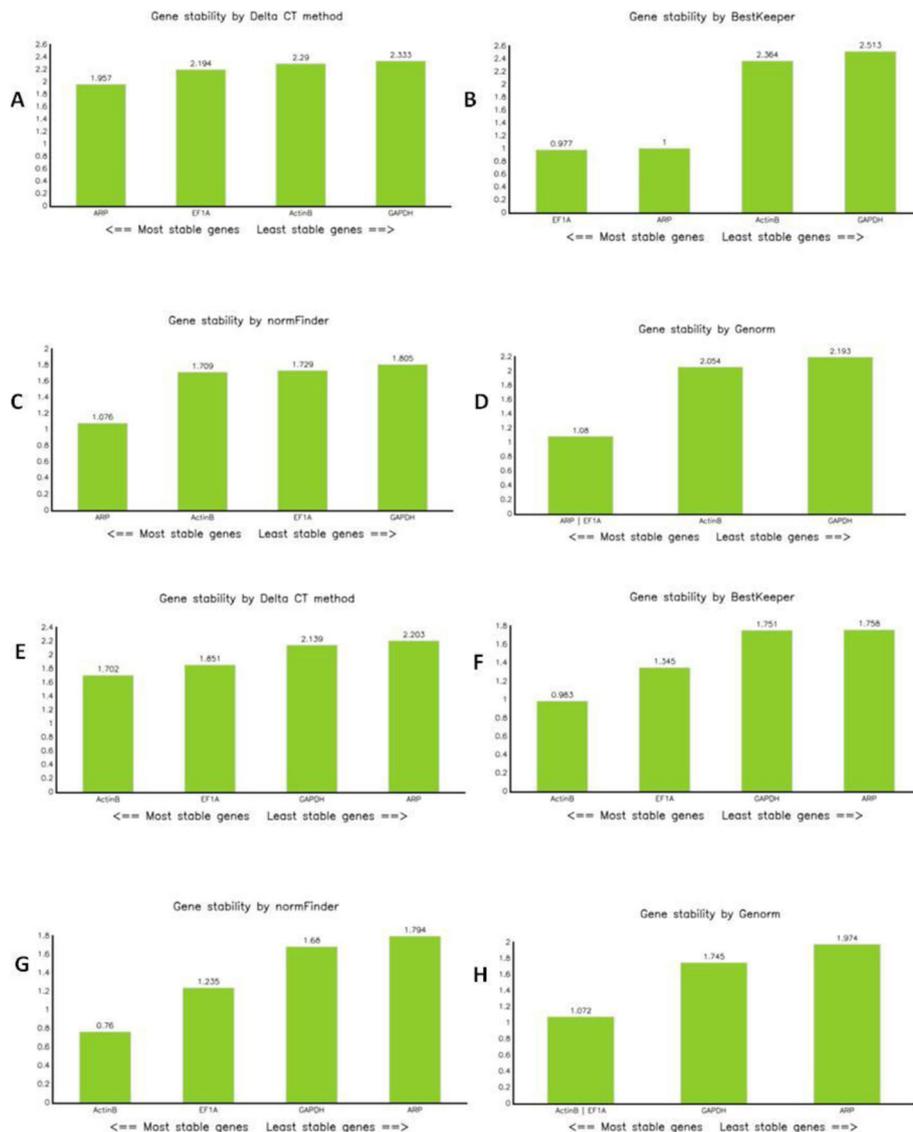


Fig. 3 Determination of the expression stability in healthy (a–d) and NNV-infected (e–h) seven-band grouper tissues evaluated using different programs (viz., Delta Ct, Bestkeeper, NormFinder, and geNorm)

RefFinder was in the order: ARP > EF1α > actin-β > GAPDH.

Expression level and stability of housekeeping genes in NNV-infected tissues

Variation in the expression profile of selected housekeeping genes was analyzed in all the 12 tissues of fish challenged with the NNV. As shown in Fig. 1, the Ct values of the four genes ranged between 14.89 and 16.88. The most highly expressed gene was EF1α in the gut followed by ARP, actin-β, and EF1α in the heart. The most variable expression was recorded for GAPDH with the expression level of 14.08 in the eye and 22.52 in the liver. Significant variation in the expression of all the four genes was observed in the

spleen while the expression of GAPDH, actin-β, and ARP was variable in the liver. EF1α and GAPDH showed significant expression in the gut, actin-β showed significant expression in the eye, and EF1α showed significant expression in the gill, which was followed by significant expression of ARP in the blood, skin, and muscle.

Actin-β was found to be the most stable expressed gene in the NNV-infected tissues as predicted by all the four methods (Fig. 3). The decreasing order of stability was further EF1α followed by GAPDH and ARP. Comprehensive stability ranking according to the refFinder suggested that actin-β is the most suitable gene with a geomean value of 1.0, and the overall stability order was actin-β > EF1α > GAPDH > ARP.

Time-dependent expression of housekeeping genes post-NNV challenge

Following the NNV challenge, the variation in the expression of housekeeping genes was evident. As represented in Fig. 4a, the expression of EF1 α was found to be more consistent compared with other genes. Statistical analysis showed a significant difference in the expression among all genes and also with time for ARP, actin- β , and GAPDH. Expression of EF1 was not statistically different across the time points showing a stable expression. Further analysis by linear regression (Fig. 4b) also represented a stable expression of EF1 α followed by actin- β , whereas there was a significant difference in the expression of ARP and GAPDH.

Fold change expression of immune genes

To evaluate the performance of housekeeping genes in the fold change evaluation of immune genes, expression of IFN-1 and Mx in the brain, eye, spleen, kidney, and blood was analyzed (Fig. 5). Significant variation in the fold change expression and calculation thereof was observed in the tissues using different housekeeping genes. Fold change varied from 0.5–1.5 fold for IFN-1 in the blood and spleen between the four housekeeping genes. In the case of Mx expression, pronounced variation in the fold change was evident in the blood and kidney.

Identification of a candidate reference gene for infection studies in *H. septemfasciatus*

Consensus ranking of each gene was calculated from the geometric mean of the reffinder (Fig. 3) stability values of healthy tissues and infected tissues. Thus, EF1 α (1.624) was found to be the most suitable internal reference control for infection-related gene expression studies in *H. septemfasciatus* followed by actin- β (1.646), ARP (2.181), and GAPDH (3.464).

Discussion

In the present study, we analyzed the stability of four seven-band grouper housekeeping genes as internal standards for quantitative immune gene expression studies in relation to NNV-infection. The expression of housekeeping genes varies with various factors such as development, stress, and infection, and a thorough validation of housekeeping genes for different factorial conditions is desirable (Bustin 2000). Studies on reference gene validation have been undertaken in various fish species for different experimental conditions (Olsvik et al. 2005; Fernandes et al. 2008; Tang et al. 2007; Zheng and Sun 2011; Purohit et al., 2016; Paria et al. 2016; Wang et al. 2017). In our study, EF1 α was found to be the most stable gene in all the healthy tissues, whereas GAPDH was ranked as the least stable gene by all the four methods. The concurrent finding has also been observed for the gene expression profile of *Epinephelus akaara*, wherein GAPDH and actin- β were the least stable genes (Wang et al. 2017). Paria et al. (2016) reported that EF1 α and actin- β as the most stably expressed transcripts in the normal tissues of Asian seabass. EF1 α was found to be the most consistently expressing gene in Atlantic salmon tissues (Olsvik et al. 2005; Ingerslev et al. 2006; Jorgensen et al. 2006) and showed least tissue specific expressions in Atlantic halibut (Øvergård et al. 2010). These observations were contradictory in case of zebrafish and Japanese founder, where actin- β was the most stable expressed gene (Casadei et al. 2011; Zheng and Sun 2011). These differences in the expression patterns of housekeeping genes can be as a result of physiological differences pertaining to the different fish species.

Actin- β , GAPDH, and B2M are commonly used internal controls for grouper gene expression studies using the RT-qPCR method (Tang et al. 2008; Huang et al. 2009; Luo et al. 2010; Liu et al. 2012). However, the evidence based on the previous studies cannot be generalized because the expression stability of these genes in grouper species per se is still unclear. From our observations,

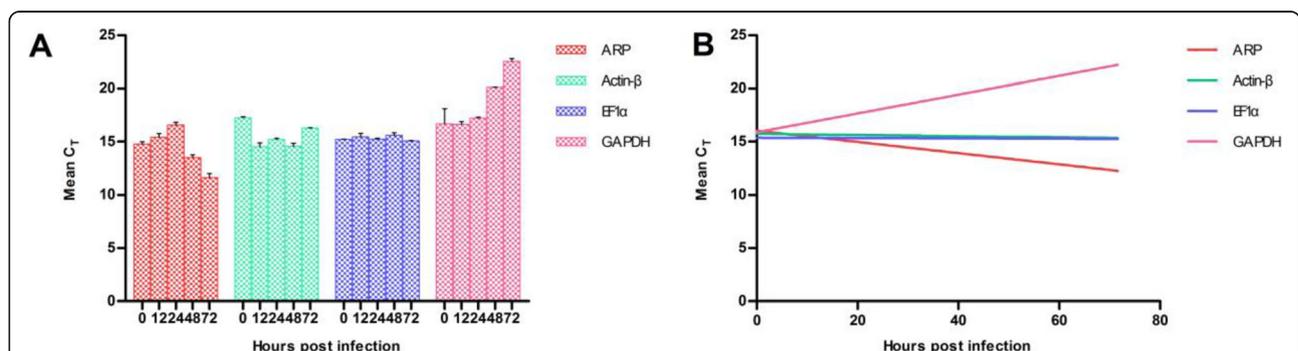
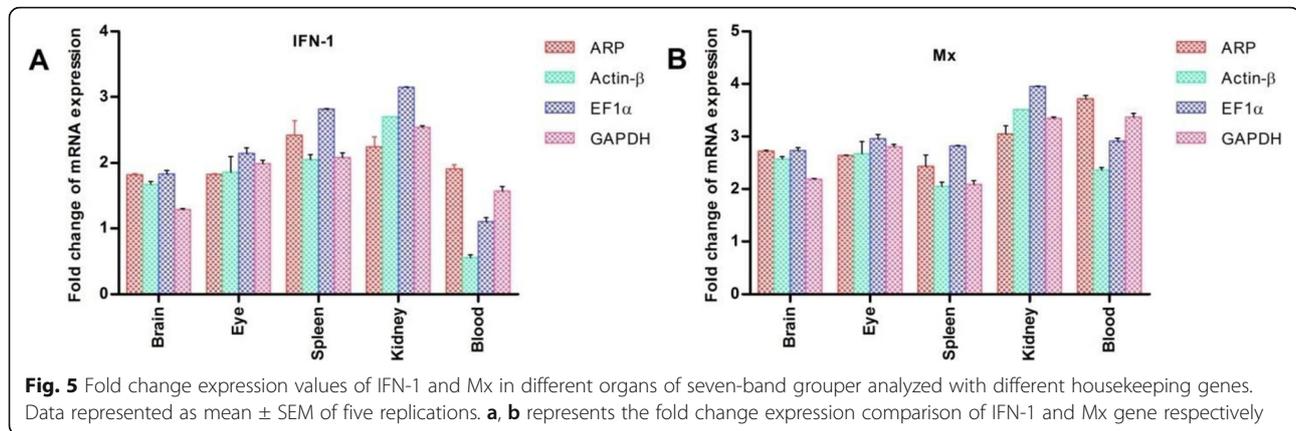


Fig. 4 Time-dependent expression profile of housekeeping genes following NNV infection in the tissues of seven-band grouper. **a** Mean Ct value of housekeeping gene transcription in grouper tissues following NNV infection (n = 5). **b** Linear regression curve of housekeeping gene expression (p < 0.05, n = 5)



actin- β was the most stable expressed gene in NNV-infected tissues. In half-smooth tongue sole, actin- β was found to be the stable expressing gene in all the 12 tested tissues following LPS or *Vibrio anguillarum* challenge (Li et al. 2010). In NNV-infected Atlantic halibut, RPL7 and EF1 α were found to be the candidate reference genes (Øvergård et al. 2010), whereas, in European seabass, ribosomal protein L13a was identified as the stably expressing gene following NNV challenge (Mitter et al. 2009). GAPDH was not a suitable choice for the infection-related gene expression studies in seven-band grouper owing to its high degree of variability in the infected tissues. This shortcoming of GAPDH as an internal control has been reported in other fish species, including infectious salmon anemia virus infection in Atlantic salmon and *Edwardsiella tarda* infection in Japanese flounder (Jorgensen et al. 2006; Zheng and Sun 2011). The instability in the expression of GAPDH might be due to its diverse range of functions in glycolysis, DNA replication and repair, protein phosphotransferase/kinase reactions, membrane transport and fusion, translational regulation and phosphotransferase activity, and nuclear RNA export, resulting in its transcript abundance sensitive to cell homeostasis which could alter during a viral or bacterial infection.

Using the four methods, stability in the expression of housekeeping genes was highly variable in healthy and NNV-infected tissues. Based on the integration of the stability results in healthy and NNV-infected tissues, only EF1 α satisfied the stability threshold for the infection-related gene expression studies out of the four reference genes studied. The results were in concordance with the findings of Paria et al. (2016) highlighting EF1 α as a candidate internal reference in normalizing RT-qPCR data in infection-related gene expression studies.

Conclusion

In conclusion, the expression and stability of the four housekeeping genes tested in this study have tissue-dependent variation in healthy as well as NNV-infected

fish seven-band grouper tissues with significant variation in the fold change calculations for immune genes. Considering the consensus ranking of overall stability, EF1 α was found to be the most suitable reference gene in infection studies of grouper. The outcome of this study may potentiate future research works on gene expression studies in response to NNV infection in *H. septemfasciatus*.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s41240-019-0142-3>.

Additional file 1: Table S1. Mean overall Ct value of all the organs analyzed from five replications (pool of 3 animal in each replication) in control and infected group.

Abbreviations

ARP: Acidic ribosomal protein; Ct: Threshold cycle; EF1 α : Elongation factor-1 α ; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IFN-1: Interferon-1; Mx: Interferon-induced GTP-binding protein; NNV: Nervous necrosis virus

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Authors' contributions

RK and OMJ designed this study and wrote the manuscript. RK, SSNQ, JOK, and JOK performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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Availability of data and materials

All datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chonnam National University (CNU/IACUC-YS-2018-3).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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