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Assessment of internal controls for data normalization of gene expression after different bacterial stimulation by quantitative real-time PCR in golden pompano *Trachinotus blochii**

CHEN Xiaojuan[1, #], ZHANG Xiaoqi[1, #], SUN Yun[1, 2, **], TU Zhigang[1], CAO Zhenjie[1], WANG Shifeng[2], ZHOU Yongcan[1, 2, **]

[1] *State Key Laboratory of Marine Resource Utilization in South China Sea*, *Hainan University*, *Haikou 570228*, *China*

[2] *Hainan Academy of Ocean and Fisheries Sciences*, *Haikou 570228*, *China*

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Abstract *Trachinotus blochii* is one of the important commercial fish species. In this study, we aim to confirm the reliability reference genes in *T. blochii* during different bacterial challenge through quantitative real-time PCR (qRT-PCR). The expression of the seven selected genes in four immune organs (i.e., spleen, kidney, intestine, and gill) stimulated with *Vibrio harveyi*, *Edwardsiella tarda*, and *Streptococcus agalactiae* were determined by qRT-PCR. The PCR data was analyzed using the geNorm and NormFinder algorithms. The results showed the selection of the internal controls should be tissue specific when studying gene expression in response to bacterial stimulation. After 48 h of stimulation with *V. harveyi*, geNorm ranked EF1A/Actin, 18S rRNA/B2M, UBCE/B2M, and 18S rRNA/B2M, as the most stably expressed genes in spleen, kidney, intestine, and gill, respectively. After 48 h of stimulation with *E. tarda*, geNorm ranked 18S rRNA/EF1A, 18S rRNA/B2M, B2M/RPL13, and 18S rRNA/EF1A, as the most stably expressed genes in spleen, kidney, intestine, and gill, respectively. After 48 h of stimulation with *S. agalactiae*, 18S rRNA/EF1A, 18S rRNA/B2M, B2M/Actin, and 18S rRNA/B2M were ranked as the most stably expressed genes in spleen, kidney, intestine, and gill, respectively. Compared to the results analyzed by geNorm, reference genes received similar rankings when using NormFinder software. The results showed that the reference genes appeared to be not only tissue specific, but also specific to the infecting species of bacteria. If one gene is preferred when *T. blochii* were infected by bacteria, 18S rRNA, B2M, B2M, 18S rRNA may be used in spleen, kidney, intestine, and gill, respectively.

Keyword: *Trachinotus blochii*; housekeeping gene; expression stability; reference gene

1 INTRODUCTION

During the last several years, quantitative real-time PCR (qRT-PCR) has been in widespread use for quantitation and detection of mRNA in all kinds of organisms (Sun and Hu, 2015). In comparison with traditional methods of RNA quantitation, qRT-PCR is substantially more sensitive, accurate, and accessible (Heid et al., 1996; Haller et al., 2004; Ransbotyn and Reusch, 2006; Yoo et al., 2009). However, some studies have reported that the selected housekeeping genes were not necessarily appropriate for the given experimental conditions when using qRT-PCR (Liu et al., 2005; Qiu et al., 2013). Many studies have also shown that the expression of housekeeping genes was affected by some external environmental factors (Infante et al., 2008; Zhong et al., 2008; Dang and Sun, 2011; Zheng and Sun, 2011; Li et al., 2016), indicating that the housekeeping gene is not truly stable. Therefore, the selection and normalization of the most appropriate housekeeping genes under different environmental conditions are very important (Selvey et al., 2001; Huggett et al., 2005).

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** Corresponding authors: syshui207@126.com; zychnu@163.com

# CHEN Xiaojuan and ZHANG Xiaoqi contributed equally to this work and should be regarded as co-first authors.

The golden pompano *Trachinotus blochii* (Lacépède) was misapplied to *Trachinotus ovatus*, which is an Indo-Pacific species distributed from north to southern Japan and south to Australia (Liu et al., 2014). In China, *T. blochii* is one of the important commercial species. With the expansion of aquaculture industry, many bacterial diseases erupt, such as *Vibrio harveyi*, *Edwardsiella tarda*, and *Streptococcus agalactiae*, which cause substantial economic losses (Sun et al., 2009; Amal et al., 2012; Fransiska et al., 2019). At present, to our knowledge, there is no research related to the normalization of housekeeping genes of *T. blochii* during bacterial infection. In terms of qRT-PCR data analysis, geNorm, and NormFinder are typical algorithms (Vandesompele et al., 2002; Andersen et al., 2004). Specifically, the geNorm is used to assess the expression stability measure (M) value and to rank the optimum pair of reference genes (Vandesompele et al., 2002). However, in order to measure the systematic error, the NormFinder is applied to estimate the expression variation of each gene (Andersen et al., 2004).

In this study, we utilized qRT-PCR to analyze the accurate housekeeping gene of *T. blochii* following challenged by different bacterial diseases *V. harveyi*, *E. tarda*, or *S. agalactie*, using both geNorm and NormFinder to analyze the data. Seven common housekeeping genes were selected in the present study, including beta actin (Actin), 18S ribosomal RNA (18S rRNA), β-2-Microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin-conjugating enzyme E2 (UBCE), ribosomal protein L13 (RPL13), and elongation factor-1-α (EF1A). We identified and normalized the best internal genes after infecting juvenile *T. blochii* with each of the above pathogenic bacteria. These results will provide useful guidance for the selection of internal controls in future qRT-PCR studies in this species.

2 MATERIAL AND METHOD

2.1 Experimental animal

Trachinotus blochii (45±2 g, n=60) were supplied by a commercial fish farm in Sanya, Hainan Province, China. The fish were maintained in oxygenated seawater at 26°C for two weeks to allow for acclimation to the new environment. Before initiating the experiments, fish were randomly selected for an inspection to confirm their health status (Wang et al., 2009). Tricaine methanesulfonate (Sigma, St. Louis, MO, USA) was used to anesthetize the fish prior to tissue collection (Schoettger and Julin, 1967; Hattingh, 1977).

2.2 Pathogen strains and culture conditions

The pathogens *V. harveyi*, *E. tarda*, and *S. agalactiae*, were previously isolated from diseased fish in Hainan Province. The isolated bacteria were cultured at 28°C in Luria-Bertani (LB) broth.

2.3 Bacteria challenge and tissue collection

The healthy *T. blochii* were randomly divided into four groups (A, B, C, and D), with 15 individuals per group. The pathogens *V. harveyi*, *E. tarda*, and *S. agalactiae* were cultured in LB medium to an OD_{600}≈0.8 and then re-suspended in phosphate-buffered saline (PBS). Since the fact that different bacteria have different lethal dose and disease time, we used different concentration of bacteria inoculum. Fish in the group A, B, and C was injected intraperitoneally with 100 μL *V. harveyi* (10^5 CFU/mL), *E. tarda* (10^6 CFU/mL) and *S. agalactie* (10^4 CFU/mL), respectively. While fish in the group D was injected intraperitoneally with 100 μL PBS as a control. After 48 h post challenge, five fish were randomly selected from each of the four groups, respectively. Tissues (spleen, kidney, intestine, and gill) were collected under sterile conditions after euthanasia. The tissues were then flash frozen in liquid nitrogen for preservation. The bacteria challenge experiment was repeated independently three times.

2.4 RNA extraction and cDNA library construction

Total RNA from the tissues samples (spleen, kidney, intestine, and gill; 30 mg per each) were extracted with the EZNA Total RNA Kit (Omega Bio-Tek, Doraville, GA, USA) and treated with RNase-free DNase I (Omega Bio-Tek, Doraville, GA, USA). The quality of the RNA was examined by determining 260/280 or 260/230 absorbance ratio using NanoDrop2000 (Thermo Scientific, USA) and gel electrophoresis. The final concentration of the extracted RNA was adjusted to 0.1 μg/μL with nuclease-free water. After that, cDNA was synthesized with 1 μg total RNA, random and Oligo (dT) primers, and the M-MLV reverse transcriptase (TaKaRa, Dalian, China) according to manufacturer's instructions.

Table 1 The housekeeping genes used in this study

Symbol	Name	Function	Accession number
Actin	Beta actin	Cytoskeletal protein	MK250485
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis enzyme	MK193811
18S rRNA	18S ribosomal RNA	Ribosome subunit	MK250487
B2M	β-2-Microglobulin	Majoyhistocum patibility complex	MK250486
EF1A	Elongation factor-1-α	Translation	MK210200
UBCE	Ubiquitin-conjugating enzyme E2	Protein degradation	MK210201
RPL13	Ribosomal protein L13	Ribosome protein	MK193810

Table 2 Primer pairs used in this study and the amplification efficiencies of PCR

Gene	Primer sequence (5′→3′)	Product size (bp)	PCR efficiency (%)	Correlation coefficient
Actin	CGTGCGTGACATCAAGGAGAA	178	98	0.998
	AAGGAAGGAAGGCTGGAAGAGG			
GAPDH	GAAGGGTGGTGCCAAGAGGG	137	96	0.998
	AGGCAGTTGGTTGTGCAGGAA			
18S rRNA	AGTTGGCATCGTTTATGGTCG	160	99	0.996
	GCATTCGTATTGTGCCGCTA			
B2M	AAGTCAGTCCACCCAAGGTTCA	139	95	0.993
	GGGATTTCCATTCCGTTCTTCATG			
EF1A	GTCCGTCAAGGAAATCCGTCG	174	102	0.999
	TTGAACTTGCAGGCAATGTGAG			
UBCE	CACGATGTCCAGCGAAGTACA	270	97	0.998
	GACCTCCACTCGTAGATGTTGTC			
RPL13	CCGTCTCATCGCTCCTCGTC	155	99	0.994
	CAATGGTGCGGGCTGTCTTT			

2.5 Determination of PCR efficiency

Seven housekeeping genes (Actin, 18S rRNA, B2M, GAPDH, UBCE, RPL13, and EF1A; Table 1) were selected to determine the most stably expressed genes of *T. blochii* following bacterial injection. The specific primer pairs used to amplify each candidate housekeeping gene are designed by the Primer Premier 5 software (Table 2) with the following parameters: 37–270 bp product, 50%–65% GC content, 58–62°C annealing temperature, and avoiding secondary structure. The specific of different PCR primer pairs was confirmed by the single band with appropriate size obtained after PCR amplification. There are three parallels for technical replicate comparison. The melting curve analysis was used to confirm the amplification specificity of each PCR. The amplification efficiency (E) and correlation coefficient (R^2) values were assessed according to the slopes of the standard curves obtained from the use of serial 10-fold dilutions of cDNA; while the calculated formula is: $E\,(\%)=(10^{-1/\text{slope}}-1)\times 100$ (Kubista et al., 2006). The acceptable E values range from 95% to 102%.

2.6 qRT-PCR and data analysis

Quantitative real-time PCR was used to analyze the stabiblity of housekeeping genes using the SYBR® Premix DimerEraser (Perfect Real Time) (TaKaRa, Dalian, China). The reaction was amplified in a total volume of 20 μL mixture with three replications. The mixture contained 1 μL cDNA, 0.2 μL each of the forward and reverse primers (10 μmol/L), 10 μL SYBR Premix buffer, and 8.6 μL PCR grade water. The cycling program was set with the following profile: 94°C for 2 min, followed by 40 cycles at 94°C for 15 s, 59°C for 15 s, and 72°C for 30 s. geNorm is used to calculate the gene expression stability measure (M) 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 ranking of the tested genes

according to their expression stability (Vandesompele et al., 2002). NormFinder utilizes a mathematical model of gene expression that enables estimation of the overall variation of the candidate normalization genes and the variation between sample subgroups of the sample set (Andersen et al., 2004).

3 RESULT

3.1 Quality of qRT-PCR amplification

The E and R^2 values of the seven candidate reference genes ranged from 95% to 102%, and 0.993 to 0.999, respectively (Table 2).

3.2 The expression of housekeeping genes following stimulation with different bacteria

After stimulation for 48 h with various bacteria, there were variations in the transcription levels of all the genes in various tissues (Table 3). In terms of *V. harveyi* stimulation, the least changes were observed with the 18S rRNA and UBCE in spleen, while all other genes, particularly GAPDH, exhibited a maximum Ct variation of 5.1. In kidney, the RPL13 was the only gene that showed less change. In intestine, except for the 18S rRNA and EF1A, all other genes had Ct variations greater than 1, with the highest Ct variation (4.3) exhibited by the RPL13. In gill, the GAPDH and B2M exhibited high Ct variations, 3.1 and 2.8, respectively. In terms of *E. tarda* stimulation, the RPL13 showed a maximum Ct variation of 7.3 in spleen (Table 3). There were slightly difference of Ct variation observed with the EF1A in kidney (3.8), the GAPDH in intestine (3.5), the Actin in kidney (3.4), the 18S rRNA in kidney (2.9), the B2M in kidney (2.8), and UBCE in kidney (2.0). In terms of *S. agalactie* stimulation, the RPL13 showed a maximum Ct variation of 7.6 in spleen, following by the GAPDH, EF1A, B2M, 18S rRNA, Actin and UBCE with variations of 5.4 in intestine, 5.1 in intestine, 4.9 in spleen, 4.8 in spleen, 4.7 in intestine and 4.4 in kidney, respectively (Table 3).

Table 3 Ct values of the housekeeping genes expressed in the tissues of bacterial challenged golden pompano at 48 h after the challenge

Gene	Treatment	Spleen	Kidney	Intestine	Gill
Actin	PBS	21.7±0.3	21.5±0.5	16.6±0.3	21.7±0.6
	V. harveyi	24.7±0.7	24.6±0.6	17.8±0.6	23.6±0.4
	E. tarda	23.6±0.2	24.9±1.2	19.1±0.2	22.4±0.4
	S. agalactiae	18.2±1.0	24.0±0.5	21.3±0.1	24.0±0.2
18S rRNA	PBS	15.4±0.4	15.4±0.3	15.0±0.7	16.4±0.3
	V. harveyi	15.2±0.8	18.1±0.2	14.1±0.6	19.0±0.5
	E. tarda	15.8±0.8	18.3±0.3	17.3±0.1	17.8±0.8
	S. agalactiae	10.6±0.2	19.2±0.3	14.1±0.3	14.7±0.3
B2M	PBS	24.6±0.1	24.7±0.5	20.6±0.9	25.3±0.2
	V. harveyi	27.6±0.5	27.3±0.7	22.3±0.5	28.1±0.5
	E. tarda	22.2±0.6	27.5±1.0	22.6±0.4	26.1±0.5
	S. agalactiae	19.7±1.2	28.3±0.7	25.3±0.4	27.1±0.1
GAPDH	PBS	23.2±0.2	23.3±0.4	17.7±0.2	28.7±0.5
	V. harveyi	28.3±1.0	29.2±0.4	19.0±0.5	31.8±0.2
	E. tarda	24.3±1.3	25.9±0.7	21.2±0.6	30.3±0.3
	S. agalactiae	23.9±1.1	25.0±0.3	23.1±0.4	28.7±0.2
UBCE	PBS	30.0±0.5	31.6±0.1	30.6±0.3	33.4±0.5
	V. harveyi	31.0±0.7	34.5±0.3	32.4±0.6	32.7±0.6
	E. tarda	28.8±0.1	33.6±0.7	30.8±0.3	32.9±0.4
	S. agalactiae	26.3±0.3	27.2±0.2	34.2±0.2	33.3±1.2
RPL13	PBS	28.5±0.3	22.0±0.4	18.5±0.4	22.9±0.8
	V. harveyi	26.1±0.8	22.9±0.8	22.8±0.6	25.1±0.5
	E. tarda	21.2±1.3	23.5±0.5	20.6±0.6	23.7±0.7
	S. agalactiae	20.9±0.7	22.8±1.1	24.6±0.5	25.0±0.5
EF1A	PBS	22.5±0.2	22.0±0.8	16.7±0.1	21.9±0.9
	V. harveyi	25.3±1.0	23.1±0.2	17.4±0.2	22.5±0.2
	E. tarda	22.1±0.2	25.8±0.7	19.8±0.5	23.4±0.7
	S. agalactiae	17.6±1.2	26.2±0.3	21.8±0.3	24.3±0.7

3.3 The suitability of housekeeping genes following bacterial stimulation

3.3.1 geNorm

The geNorm analysis indicated that the M values of all the genes in the four examined tissues after three bacteria stimulated varied from 0.01 to 1.68 (Figs.1–3). Apart from the GAPDH in spleen after *S. agalactie* stimulated (M value=1.68, Fig.3), the M values under the other treatments were lower than the expression stability threshold (i.e., 1.5) proposed by geNorm. Following *V. harveyi* stimulation, in spleen, the M value of the GAPDH was the highest (1.32), which was followed in decreasing order by those of 18S rRNA, UBCE, RPL13, B2M, EF1A, and Actin (Fig.1), with the last two genes (EF1A/Actin) being recognized as the most stable genes. In kidney, intestine, and gill, the minimum M value was observed in 18S rRNA/B2M, UBCE/B2M, and 18S rRNA/B2M, respectively (Fig.1). Similarly, in response to *E. tarda* stimulation, 18S rRNA/EF1A, 18S rRNA/B2M, B2M/RPL13 and 18S rRNA/EF1A were indicated the minimum M value in spleen, kidney, intestine, and gill, respectively.

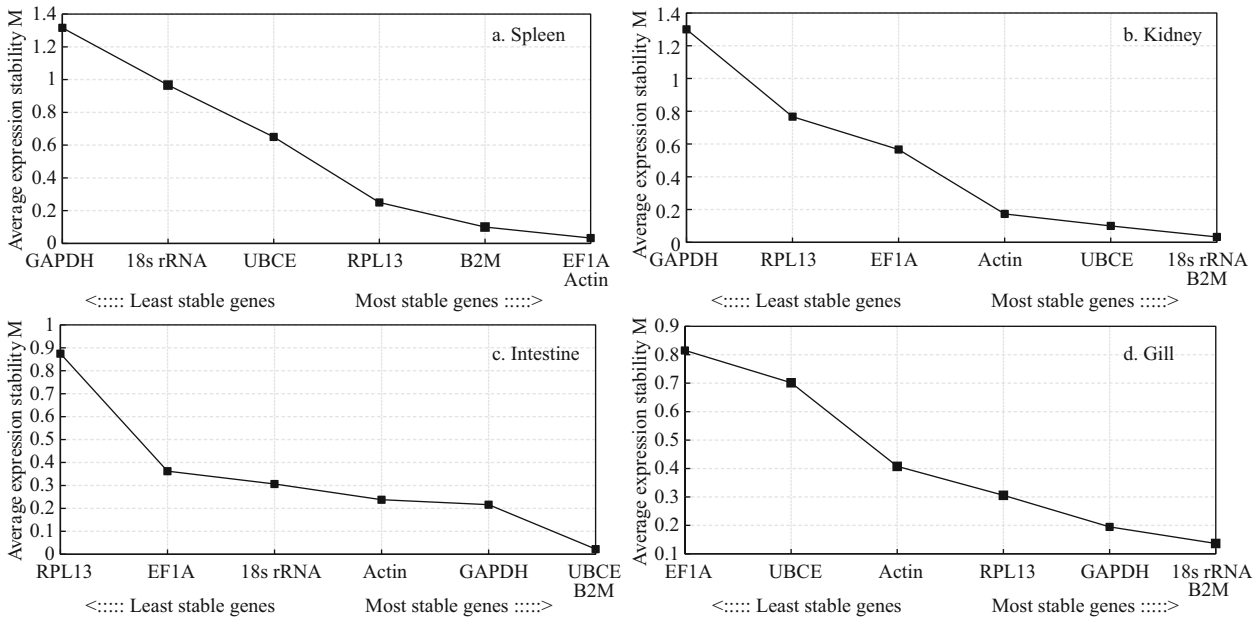

Fig.1 Expression stability of the housekeeping genes confirmed by geNorm at 48 h post-infection by *V. harveyi* in *T. blochii* tissues

The expression of the housekeeping genes after challenged by PBS or *V. harvayi* at 48 h post-infection in *T. blochii* tissues was determined by qRT-PCR, the expression stability (*M*) of each of the genes was calculated by geNorm. The *M* value was lower, the expression was more stable.

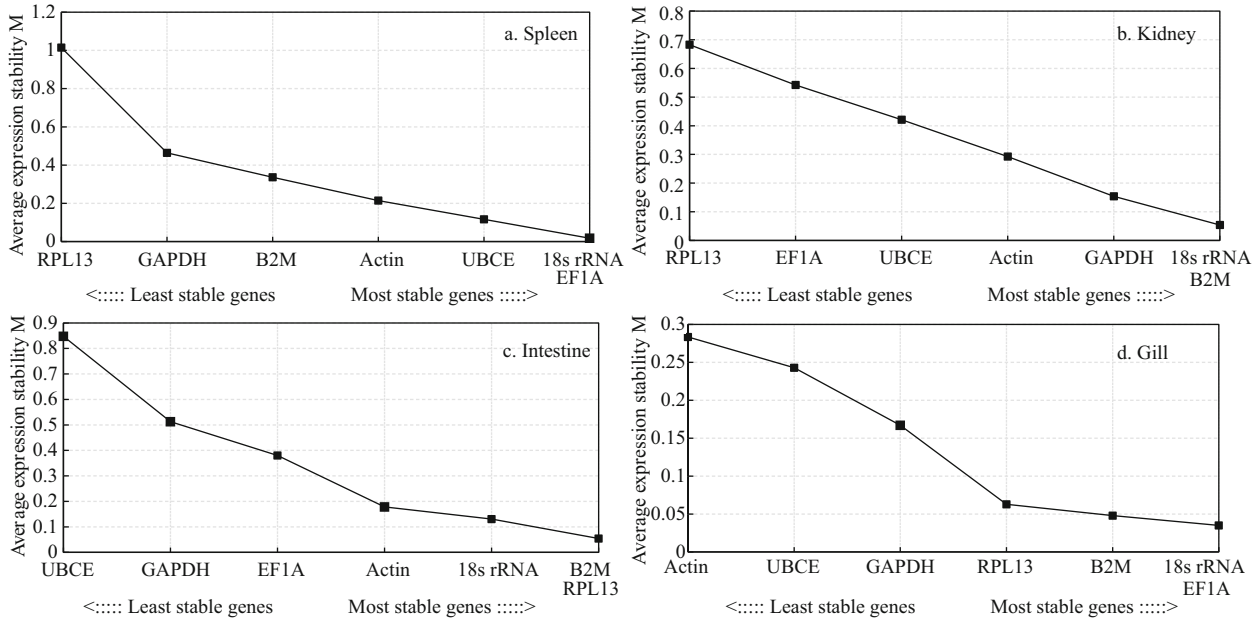

Fig.2 Expression stability of the housekeeping genes confirmed by geNorm at 48 h post-infection by *E. tarda* in *T. blochii* tissues

The expression of the housekeeping genes after challenged by PBS or *E. tarda* at 48 h post-infection in *T. blochii* tissues was determined by qRT-PCR, the expression stability (*M*) of each of the genes was calculated by geNorm. The *M* value was lower, the expression was more stable.

Therefore, these genes were determined to be the optimal housekeeping genes in these tissues (Fig.2). Following *S. agalactie* stimulation, 18S rRNA/EF1A, 18S rRNA/B2M, B2M/Actin, and 18S rRNA/B2M were observed to have the minimum *M* values in spleen, kidney, intestine, and gill, respectively. Thus, these genes were the best target housekeeping genes in these tissues for analysis (Fig.3).

The pairwise variation (*V*) between two sequential normalization factors containing a growing number of genes was assessed to determine the optimal number of genes required for data normalization. For expressions in four tissues following *V. harveyi* stimulation, all of the V2/3 values exhibited less than 0.15 (Fig.4) that indicating the third reference gene was not necessary included in each case. Similarly, in

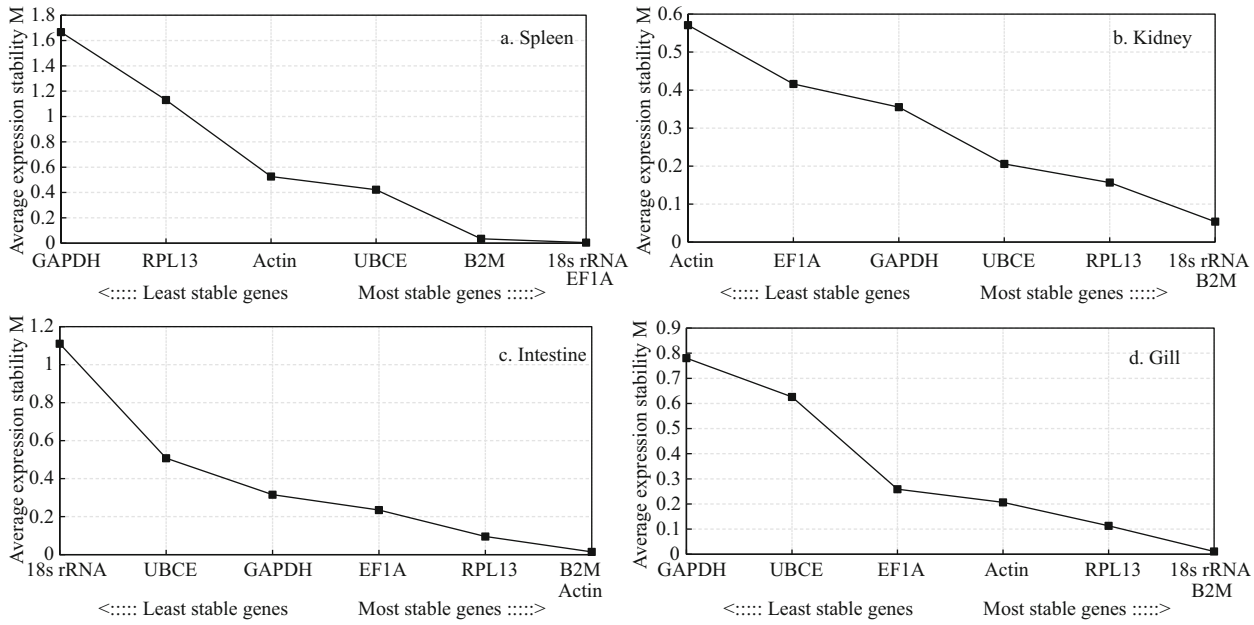

Fig.3 Expression stability of the housekeeping genes confirmed by geNorm at 48 h post-infection by *S. agalactiae* in *T. blochii* tissues

The expression of the housekeeping genes after challenged by PBS or *S. agalactiae* at 48 h post-infection in *T. blochii* tissues was determined by qRT-PCR, the expression stability (*M*) of each of the genes was calculated by geNorm. The *M* value was lower, the expression was more stable.

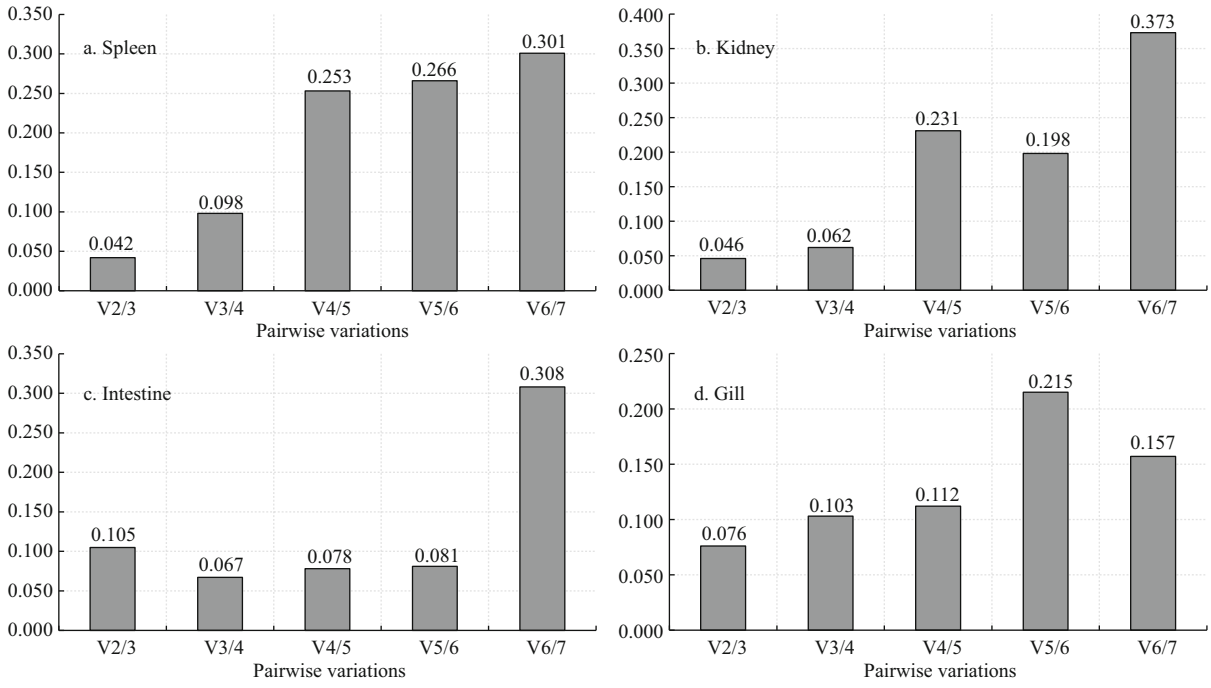

Fig.4 Determination of the optimal number of reference genes by NormFinder for normalization after *V. harveyi* infection

The mRNA levels of the housekeeping genes in various tissues of *T. blochii* after challenged by PBS or *V. harveyi* were determined by RT-qPCR. The pairwise variation between normalization factors was determined by NormFinder.

response to *E. tarda* and *S. agalactie* stimulation, the results of measurements on 48 h samples indicated that the V2/3 values were all lower than 0.15 of all the genes in the four tissues tested (Figs.5 & 6). Therefore, the target pairs V2/3 are considered the most reliable and effective housekeeping genes in these tissues.

3.3.2 NormFinder

The ranking orders produced by NormFinder for the expressions in spleen, kidney, intestine, and gill were mostly similar to those obtained by geNorm. NormFinder ranked the candidate reference genes by scores. Following stimulation with *V. harveyi*, the

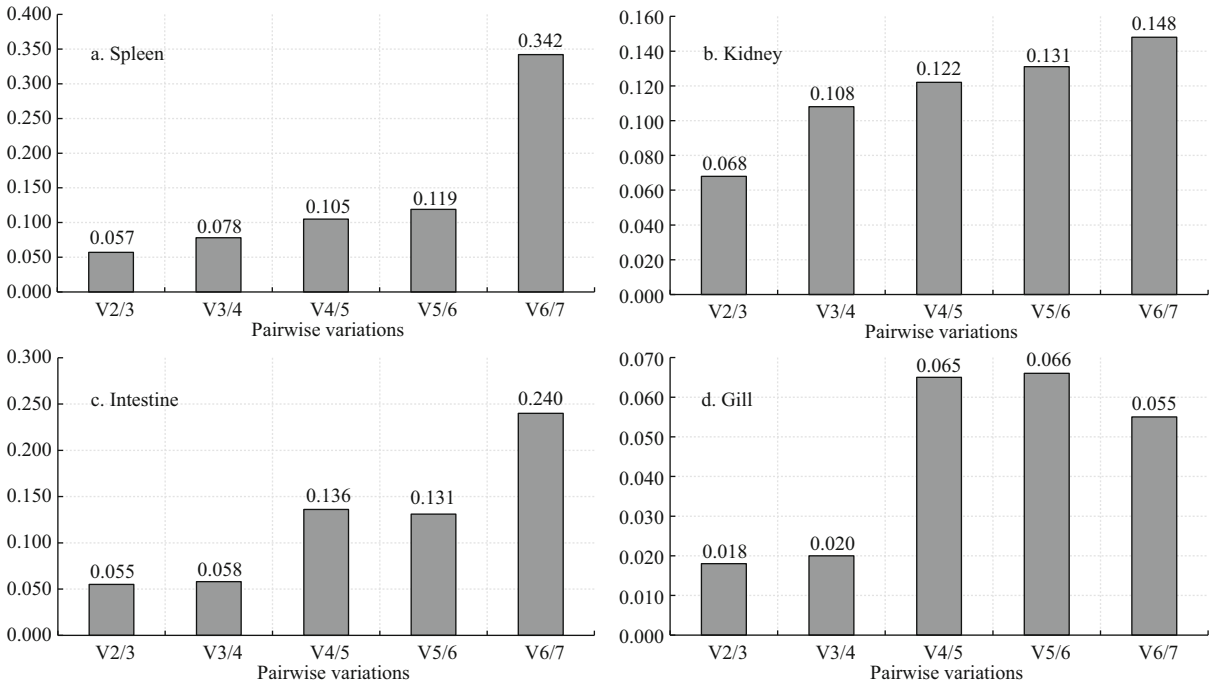

Fig.5 Determination of the optimal number of reference genes by NormFinder for normalization after *E. tarda* infection

The mRNA levels of the housekeeping genes in various tissues of *T. blochii* after challenged by PBS or *E. tarda* were determined by RT-qPCR. The pairwise variation between normalization factors was determined by NormFinder.

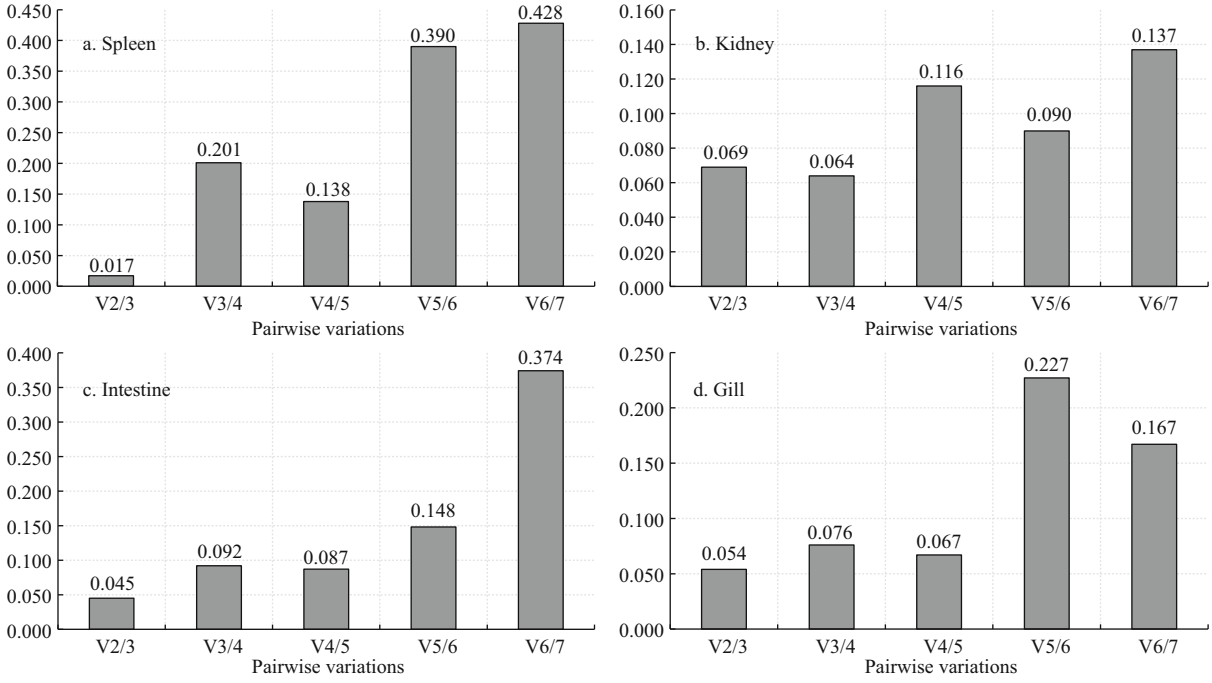

Fig.6 Determination of the optimal number of reference genes by NormFinder for normalization after *S. agalactiae* infection

The mRNA levels of the housekeeping genes in various tissues of *T. blochii* after challenged by PBS or *S. agalactiae* were determined by RT-qPCR. The pairwise variation between normalization factors was determined by NormFinder.

18S rRNA/Actin (0.002) was identified as the most stable genes in spleen by NormFinder analysis, followed in order of decreasing stability by UBCE (0.041), B2M (0.232), EF1A (0.872), RPL13 (1.110), and GAPDH (1.574) (Table 4). The most stable genes in kidney, intestine, and gill after *V. harveyi* stimulated, ranked by NormFinder were 18S rRNA/B2M, UBCE/B2M, and 18S rRNA respectively. Following *E. tarda*

Table 4 Ranking of the candidate reference genes generated by NormFinder for analyzing expression at 48-h *V. harveyi* infection

Ranking order	Spleen	Kidney	Intestine	Gill
1	18S rRNA	18S rRNA	UBCE	18S rRNA
	(0.002)	(0.010)	(0.007)	(0.037)
2	Actin	B2M	B2M	Actin
	(0.002)	(0.010)	(0.007)	(0.087)
3	UBCE	UBCE	GAPDH	PRL13
	(0.041)	(0.042)	(0.208)	(0.087)
4	B2M	Actin	Actin	B2M
	(0.232)	(0.054)	(0.276)	(0.249)
5	EF1A	EF1A	18S rRNA	GAPDH
	(0.872)	(0.670)	(0.456)	(0.405)
6	RPL13	RPL13	EF1A	UBCE
	(1.110)	(0.898)	(0.607)	(0.836)
7	GAPDH	GAPDH	RPL13	EF1A
	(1.547)	(1.895)	(1.373)	(0.857)

The stability values were denoted by numbers and shown in brackets.

Table 5 Ranking of the candidate reference genes generated by NormFinder for analyzing expression at 48-h *E. tarda* infection

Ranking order	Spleen	Kidney	Intestine	Gill
1	18S rRNA	18S rRNA	B2M	18S rRNA
	(0.019)	(0.018)	(0.019)	(0.125)
2	B2M	B2M	RPL13	PRL13
	(0.019)	(0.018)	(0.019)	(0.133)
3	GAPDH	GAPDH	18S rRNA	B2M
	(0.128)	(0.061)	(0.042)	(0.173)
4	UBCE	Actin	Actin	EF1A
	(0.592)	(0.246)	(0.083)	(0.177)
5	EF1A	UBCE	EF1A	Actin
	(1.167)	(0.420)	(0.614)	(0.236)
6	Actin	EF1A	GAPDH	GAPDH
	(1.177)	(0.546)	(0.815)	(0.240)
7	RPL13	RPL13	UBCE	UBCE
	(2.613)	(0.768)	(0.989)	(0.337)

The stability values were denoted by numbers and shown in brackets.

stimulation, the most stable genes in spleen, kidney, intestine, and gill were 18S rRNA/B2M, 18S rRNA/B2M, B2M/RPL13, and 18S rRNA respectively (Table 5). In response to stimulation with *S. agalactie*, the most stable genes in spleen, kidney, intestine, and gill were 18S rRNA, B2M, B2M/Actin, and 18S rRNA/B2M, respectively (Table 6).

Table 6 Ranking of the candidate reference genes generated by NormFinder for analyzing expression at 48-h *S. agalactiae* infection

Ranking order	Spleen	Kidney	Intestine	Gill
1	18S rRNA	B2M	B2M	18S rRNA
	(0.007)	(0.022)	(0.005)	(0.004)
2	B2M	18S rRNA	Actin	B2M
	(0.071)	(0.090)	(0.005)	(0.004)
3	EF1A	Actin	UBCE	RPL13
	(0.168)	(0.301)	(0.259)	(0.215)
4	GAPDH	EF1A	EF1A	Actin
	(0.475)	(0.483)	(0.464)	(0.400)
5	UBCE	UBCE	GAPDH	EF1A
	(0.504)	(0.590)	(0.614)	(0.492)
6	RPL13	GAPDH	RPL13	UBCE
	(0.670)	(0.851)	(1.059)	(0.757)
7	Actin	RPL13	18S rRNA	GAPDH
	(1.448)	(1.366)	(1.758)	(0.786)

The stability values were denoted by numbers and shown in brackets.

3.4 Data analysis summary

To normalize the most suitable housekeeping genes following challenged with different bacteria, the geNorm and NormFinder algorithms were both applied. In summary, the results showed that the 18S rRNA was the best reference genes in spleen and gill following different bacterial stimulation; while the B2M was the best reference genes in kidney and intestine (Table 7).

4 DISCUSSION

In other previous studies, NormFinder, bestKeeper and geNorm (Vandesompele et al., 2002; Andersen et al., 2004; Pfaffl et al., 2004) have been used in conjunction with qRT-PCR to determine the optimal genes to use as internal references when examining gene expression in cells and tissues of various taxon. A normalization factor generated by geNorm based on multiple internal controls can be used in the future qRT-PCR analysis, whereas NormFinder can be applied to select the optimal reference gene referring to the expression stability of the candidate genes (Andersen et al., 2004).

In this study, we selected seven commonly used reference genes, which have also been used in many other species as internal controls for qRT-PCR, including Actin, 18S rRNA, B2M, GAPDH, UBCE, RPL13, and EF1A (Zhong et al., 2008; Bower and

Table 7 Expression stability of the housekeeping genes in *T. blochii* tissues as determined by geNorm and NormFinder

		Spleen	Kidney	Intestine	Gill
V. harveyi	geNorm	EF1A/Actin	18S rRNA/B2M	UBCE/B2M	18S rRNA/B2M
	NormFinder	18S rRNA/Actin	18S rRNA/B2M	UBCE/B2M	18S rRNA
E. tarda	geNorm	18S rRNA/EF1A	18S rRNA/B2M	B2M/RPL13	18S rRNA/EF1A
	NormFinder	18S rRNA/B2M	18S rRNA/B2M	B2M/RPL13	18S rRNA
S. agalactiae	geNorm	18S rRNA/EF1A	18S rRNA/B2M	B2M/Actin	18S rRNA/B2M
	NormFinder	18S rRNA	B2M	B2M/Actin	18S rRNA/B2M

Johnston, 2009; Dang and Sun, 2011; Sun and Hu 2015; Li et al., 2016). To normalize the most stable reference genes following challenged with different bacteria in *T. blochii*, geNorm and NormFinder were utilized to analyze the data. In some previous studies, housekeeping genes were observed to follow a tissue-specific expression trend in red drum (*Sciaenops ocellatus*) and zebrafish (*Danio rerio*) (McCurley and Callard, 2008; Sun and Hu, 2015). The other studies have reported that the most stable housekeeping gene varies with experimental conditions. For example, in different tissues or at different growth stages, the suitable internal reference genes do not remain the same (Filby and Tyler, 2007; Infante et al., 2008; Fernandes et al., 2008; Zhong et al., 2008; Bower and Johnston, 2009; Li et al., 2010; Øvergård et al., 2010; Løvoll et al., 2011; Dang and Sun, 2011; Zheng and Sun, 2011). For instance, in red drum during bacterial infection, RPS35, ACTB, EF1A, ND1, TUBB, EF1A, ACTB, and ACTB were analyzed and determined to be the ideal internal references in brain, gill, heart, intestine, kidney, liver, muscle, and spleen, respectively (Sun and Hu, 2015). geNorm identified the most suitable internal reference genes from different genes. The most suitable internal reference genes were also analyzed by NormFinder.

5 CONCLUSION

To improve the accuracy of evaluation in this study, we normalized the optimal reference genes of *T. blochii* at 48 hpi with different bacteria using geNorm and NormFinder. When studying *T. blochii* gene expression after being stimulated by different bacteria, the most stable internal controls should be selected for each tissue being tested. Following bacterial challenge, the most stable genes identified by geNorm and NormFinder were 18S rRNA, B2M, B2M, and 18S rRNA, for reference in spleen, kidney, intestine, and gill of *T. blochii*, respectively.

6 DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the article.

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