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Evaluation of housekeeping genes as references for quantitative real time RT-PCR analysis of gene expression in Japanese flounder (*Paralichthys olivaceus*)

Wen-jiang Zheng [a,b], Li Sun [a,*]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, PR China
[b] Graduate University of the Chinese Academy of Sciences, Beijing 100049, PR China

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ABSTRACT

Japanese flounder (*Paralichthys olivaceus*) is an important economic fish species cultured worldwide. In this report, we compared the potentials of ten housekeeping genes as quantitative real time RT-PCR (qRT-PCR) references for the study of gene expression in Japanese flounder under normal physiological conditions and during bacterial infection. For this purpose, the expression of the ten genes in eight flounder tissues (liver, spleen, kidney, heart, muscle, brain, gill, and intestine) was determined by qRT-PCR before and after bacterial infection. The expression levels of the housekeeping genes were then compared and evaluated with geNorm and NormFinder algorithms. The results showed that before bacterial infection, the tested genes exhibited tissue-specific expressions to various degrees, with β-actin and ubiquitin-conjugating enzyme being ranked as the most stable genes across tissue types. Following bacterial challenge, all the tested genes varied in expression levels in tissue-dependent manners and no cross-all-tissue type reference gene was identified among the examined panel of housekeeping genes; however, α-tubulin was recognized as the most stable gene in four (spleen, heart, muscle, and gill) of the eight examined tissues. These results indicate that for qRT-PCR analysis of gene expression in Japanese flounder as a function of bacterial infection, the choice of reference genes should be made according to tissue type.

1. Introduction

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) is a molecular technique whereby RNA molecules of very low copy numbers can be detected and quantified. For this reason, qRT-PCR has been used widely in the study of gene expressions in diverse organisms. Compared to other commonly used approaches for the quantitation of mRNA, e.g. northern blotting and ribonuclease protection assays, qRT-PCR has the advantage of being sensitive, reproducible, easy of use, and requiring very small amount of sample [1]. In qRT-PCR, the amplification products are detected by fluorescent signals generated by a fluorescent dye such as TaqMan, SYBR Green I, Molecular Beacons, and Scorpions, which bind DNA and emit fluorescence upon excitation. Real time PCR results can be analyzed via the standard curve method or, more frequently, the comparative C_t method, which is also known as the $2^{-\Delta\Delta CT}$ method [Applied Biosystems technical bulletin; [2]]. In this latter method, the C_t (the threshold cycle above which the increase in fluorescence is exponential) values of the target and control genes are normalized to an internal standard or reference. As a requirement, the reference gene should be universally expressed in all the tissues/cells in which the target gene is examined and unaffected at transcription level by the experimental conditions under which the target gene is studied [3–5]. Commonly used reference genes for qRT-PCR are mainly housekeeping genes, such as those encoding β-actin, glyceraldehyde-3-phosphate dehydrogenase, cyclophilin, certain ribosomal proteins, and ribosomal RNAs (18S or 28S rRNA). Although housekeeping genes are generally involved in the maintenance of basic cellular functions and hence are assumed to be constitutively expressed at constant levels, their expressions have in many cases been found to vary with tissue/cell type, developmental stage, and environmental stimuli [6–13]. Since the use of inappropriate reference genes can lead to erroneous normalization of the PCR data and consequently misinterpretation of the results [14], the selection of the most stable gene or set of genes under the experimental conditions is crucial to accurate profiling of gene expression.

Japanese flounder (*Paralichthys olivaceus*), a species of flatfish, is one of the most important fish species cultured in Asian countries including Japan, Korea, and China. Owing to its economic values, Japanese flounder has in recent years been studied extensively in various aspects, especially in those concerning immunology and

* Corresponding author. Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, PR China. Tel./fax: +86 532 82898829.
E-mail address: lsun@qdio.ac.cn (L. Sun).

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disease control. Like other aquaculture species, Japanese flounder is susceptible to a number of bacterial pathogens, notably *Edwardsiella tarda*, *Vibrios*, *Pseudomonas fluorescens*, and *Streptococcus iniae*, which are known to affect cultured flounder and cause heavy economic losses [15–17]. As a result, increasing numbers of qRT-PCR-involving investigations have been carried out to examine the effect of bacterial infection on gene expressions in flounder. In most of these studies, housekeeping genes, mainly β-actin, have been used as internal standards; however, the expressional stability of these housekeeping genes before and after bacterial encounter has not been investigated.

The aim of this study was to evaluate the appropriateness of the commonly used housekeeping genes as qRT-PCR references in the profiling of gene expression in Japanese flounder tissues as a function of bacterial infection. In our study, two normalization algorithms were used, i.e. geNorm [18] and NormFinder [19]. GeNorm determines the expression stability measure (M) for each of a set of potential reference genes as the average pairwise variation (V) for that gene with all other examined reference genes. The reference genes were ranked according to their expression stability by stepwise exclusion of the gene with the highest M value (i.e. least stable). NormFinder identifies the optimal reference gene from a set of candidate genes by ranking the genes according to their expression stability in a given group of samples and under the given experimental conditions. It provides for each of the examined gene a stability value that represents the calculated expression variation of that gene under the specified experimental condition, thus allowing evaluation of the suitability of that gene as a normalization factor.

2. Materials and methods

2.1. Sample preparation

E. tarda TX1, a fish pathogen that has been described previously [20], was cultured in Luria-Bertani broth (LB) medium at 28 °C to mid-logarithmic phase (OD_{600} ~ 0.8) as described previously [20]. The cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to 5×10^6 cells/ml. Japanese flounder (*P. olivaceus*) (~9 g) were purchased from a commercial fish farm in Shandong Province, China and divided randomly into two groups (3 fish/group). Fish in each group were administered via intraperitoneal (i.p.) injection with 100 μl of *E. tarda* suspension or PBS (control). At 48 h post-infection, the fish were sacrificed, and liver, spleen, kidney, heart, brain, gill, and muscle were collected under aseptic conditions. To verify *E. tarda* infection, liver, spleen, and kidney of TX1- and PBS-infected fish were homogenized in PBS, and the homogenates were plated on LB agar plates. After incubation at 28 °C for 48 h, colonies appeared on all the plates containing homogenates from TX1-infected fish but not on the plates containing homogenates from the fish injected with PBS. The genetic nature of the colonies was verified by PCR analysis using primers specific to the *eseB, orf26*, and *luxS* genes of TX1 as described previously [20].

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from 30 mg tissue samples with the EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA) and treated with RNase-free DNase I (TaKaRa, Dalian, China). The quality of the RNA was examined by determining 260/280 absorbance ratio using a Gene QuantPro spectrophotometer (Amersham Pharmacia Biotec, Arlington Heights, IL) and by gel electrophoresis. The purified RNA was adjusted to 0.3 μg/μl with nuclease-free water. One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

2.3. Primer design and PCR efficiency

Ten flounder housekeeping genes, i.e. β-actin (ACTB), eukaryotic translation initiation factor 5A (eIF5A), ribosomal protein L17 (RPL17), α-tubulin (TUBA), elongation factor-1-α (EF1A), β-2-Microglobulin (B2M), ubiquitin-conjugating enzyme (UBCE), ornithine decarboxylase (ODC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S ribosomal RNA (18S rRNA) (Table 1), were examined for the potential to serve as qRT-PCR references in the study of gene expression in Japanese flounder before and after bacterial infection. Oligonucleotide primers specific to each of the genes were designed with the Primer Premier 5.0 software (Table 2). PCR efficiency (E) and correlation coefficient (R^2) were determined based on the slops of the standard curves generated using serial 10-fold dilutions of sample cDNA. The efficiency was calculated as follows: $E\ (\%) = (10^{-1/\text{slope}} - 1) \times 100$ [21]. The acceptable E value was defined as between 90 and 110%.

2.4. Quantitative real time RT-PCR (qRT-PCR) and data analysis

qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China). The reaction was performed in triplicate in a total volume of 20 μl containing 10 μl SYBR Premix buffer, 2 μl cDNA, 0.2 μl each of the primers, and 7.6 μl PCR-grade water. The PCR program was 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. All reactions were run in duplicate. Negative control without cDNA was included in each

Table 1
The housekeeping genes used in this study.

Symbol	Name	Function	Accession number	Reference
18S rRNA	18S ribosomal RNA	Ribosomal subunit	EF126037	[26]
RPL17	Ribosomal protein L17	Structural component of the large 60S ribosomal subunit	AF220552	[26]
ODC	Ornithine decarboxylase	Polyamine synthesis.	AY214169	[27]
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	AB029337	[28]
eIF5A	Eukaryotic translation initiation factor 5A	Protein synthesis	FJ390056	[29]
ACTB	β-Actin	Cytoskeletal protein	EU090804	[30]
UBCE	Ubiquitin-conjugating enzyme	Protein degradation	AU050388	[26]
TUBA	α-Tubulin	Cytoskeletal protein	AU050297	[26]
B2M	β-2-Microglobulin	Small subunit of the MHC1	AF433657	[26]
EF1A	Elongation factor-1-a	Protein synthesis	AB240552	[26]