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Determination of internal controls for quantitative real time RT-PCR analysis of the effect of *Edwardsiella tarda* infection on gene expression in turbot (*Scophthalmus maximus*)

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ABSTRACT

In recent years, quantitative real time reverse transcriptase-PCR (qRT-PCR) has been used frequently in the study of gene expression in turbot (*Scophthalmus maximus*) in relation to bacterial infection. However, no investigations on appropriate qRT-PCR reference genes have been documented. In this report, we determined the potential of eight housekeeping genes, i.e. β-actin (ACTB), ribosomal protein L17 (RPL17), α-tubulin (TUBA), elongation factor-1-α(EF1A), β-2-Microglobulin (B2M), RNA polymerase II subunit D (RPSD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S ribosomal RNA (18S rRNA), as internal standards for qRT-PCR analysis of gene expression in turbot as a function of bacterial infection. For this purpose, the expression of the eight housekeeping genes in seven turbot tissues was determined by qRT-PCR before and after bacterial challenge, and the data were analyzed with the geNorm and NormFinder algorisms. The results showed that the expression of all the examined genes exhibited tissue-dependent variations both before and after bacterial challenge. Before bacterial challenge, geNorm and NormFinder identified RPSD as the gene that showed least tissue specific expression. At 12 h post-bacterial infection, geNorm ranked ACTB/GAPDH, 18S rRNA/ACTB, ACTB/GAPDH, 18S rRNA/ACTB, RPL17/TUBA, RPSD/GAPDH, and RPSD/B2M, respectively, as the most stably expressed genes in liver, spleen, kidney, gill, heart, muscle, and brain. Comparable ranking orders were produced by NormFinder. Similar results were obtained at 24 h post-bacterial infection. Taken together, these results indicate that RPSD is the most stable gene across tissue types under normal physiological conditions and that, during bacterial infection, ACTB might be used as an internal standard for the normalization of gene expression in immune relevant organs; however, no single gene or single pair of genes in the examined set of housekeeping genes can serve as a universal reference across all tissue types under the condition of bacterial infection.

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1. Introduction

In the past few years, quantitative real time reverse transcription-PCR (qRT-PCR) has become a choice method for the detection and quantitation of mRNA in various organisms. Compared to traditional RNA quantitation techniques, qRT-PCR is highly sensitive and enables the detection of mRNA at very low copy numbers [1]. However, successful application of qRT-PCR depends on a number of technical parameters involved in PCR amplification and in data analysis, such as cDNA and RNA quality, primer specificity, PCR efficiency, and the selection of appropriate normalization factors, all which can affect the accuracy of qRT-PCR [2]. It is a general practice that in qRT-PCR, normalization is performed with a single internal control or reference gene, which in most cases is a housekeeping gene essential to the fundamental physiology of the organism and thus assumed to be expressed in a constitutive manner. The ideal control gene for real time PCR should exhibit constant expression in different tissues/cells or developmental stages and be unaffected by the experimental conditions under which the study is carried out [3–5]. However, accumulating evidences have indicated that the commonly used housekeeping genes, e.g., those encoding β-actin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal proteins, and ribosomal RNAs, vary at transcription level with tissue/cell type, development, or experimental design [6–10]. Therefore, the appropriateness of a reference gene should be validated before being applied to qRT-PCR analysis, as different reference gene or set of reference genes may be required under different situations.

Turbot (*Scophthalmus maximus*) is a species of flatfish belonging to the family of Scophthalmidae. It is an important economic species

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Table 1
The housekeeping genes used in this study.

Symbol	Name	Function	Accession number
ACTB	β-Actin	Cytoskeleton	EU686692
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis enzyme	DQ848904
RPL17	60S ribosomal protein L17	Ribosome Protein	DQ848879
TUBA	α-Tubulin	Cytoskeleton	DQ848853
EF1A	Elongation factor-1-α	Translation	AF467776
B2M	β-2-Microglobulin	Major histocompatibility complex	DQ848854
RPSD	RNA polymerase II subunit D	Transcription	DQ848899
18S rRNA	18S ribosomal RNA	Ribosome subunit	EF126038

Table 2
Primers and PCR amplification efficiencies.

Gene	Primer sequence (5′ → 3′)	Product size (bp)	PCR efficiency (%)	Correlation coefficient
ACTB	GTAGGTGATGAAGCCCAGAGCA CTGGGTCATCTTCTCCCTGT	204	98	0.996
GAPDH	TCCAATGTTTGTCATGGGAGTT CCAGAGGAGCCAGGCAGTT	101	95	0.995
RPL17	AICAGTGCGTCCCCTTCA CTCATCTTCGGAGCCTTGTTC	214	95	0.994
TUBA	CCCTCGTATCCAITTCCCTC GGTAGTTGATGCCCAICTTGA	287	100	0.993
EF1A	CAICGTCGCCTTCGTCC TGGCAICGCCCTCTTTG	113	105	0.997
B2M	CTCTGGCTGTTTTCGTCTGCT TCCTTTCCGTTCTCTCCCG	86	98	0.992
RPSD	CGGAGGAIGAGCAGGAAI TGCGGAIGGCAGTGATG	102	94	0.996
18S rRNA	GAATTGAIGGAAGGGCAIC GAIAAATCGCTCCAICAAITAA	166	90	0.995

cultured widely in Europe and Asia. In recent years, turbot industries worldwide have suffered heavily from disease outbreaks caused by bacterial infections, particularly those caused by *Edwardsiella tarda* and *Vibrios* [11,12]. Although qRT-PCR has been used in a large number of studies to investigate gene expression (such as the expression of immune genes) in turbot in relation to bacterial infection, no study on normalization strategy under infection conditions has been documented.

Currently, real time PCR are available in four different chemistries, i.e., TaqMan® (Applied Biosystems), Molecular Beacons, Scorpions® and SYBR® Green (Molecular Probes), all which detect PCR products through the generation of a fluorescent signal [13]. Of the different fluorescent dyes in use, SYBR Green is a cyanine dye that binds preferentially to double-stranded DNA, and the resulting complex emits green light upon excitation. Owing to its inexpensiveness and high sensitivity, SYBR Green-based PCR product detection has been used very often. In this study, we compared the expression stability of available turbot housekeeping genes during bacterial infection and evaluated their potential as internal controls in the normalization of SYBR Green-based qRT-PCR.

2. Materials and methods

2.1. Sample preparation

E. tarda TX1, a fish pathogen that has been described previously [14], was cultured in Luria-Bertani broth (LB) medium at 28 °C to mid-logarithmic phase (OD_{600} ~0.8) as described previously [14]. The cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to 1×10^7 cells/ml. Turbot (~11 g) were purchased from a commercial fish farm in Shandong Province, China and divided randomly into two groups (three or four fish/group). Fish in each group were administered via intraperitoneal (i.p.) injection with 100 μl of *E. tarda* suspension or PBS (control). At 12 h and 24 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 [14].

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 random and Oligo (dT) primers (Promega, USA) and the Superscript II reverse

Table 3
C_t values of the housekeeping genes expressed in the tissues of PBS- and *E. tarda*-challenged turbot at 12 h after the challenge.

Gene	Challenging agent	Liver	Spleen	Kidney	Heart	Muscle	Brain	Gill
ACTB	PBS	19.7 ± 0.1	23.2 ± 0.1	20.9 ± 0.1	20.8 ± 0.1	25.1 ± 0.3	20.8 ± 0.1	18.7 ± 0.1
	E. tarda	22.6 ± 0.1	24.6 ± 0.3	23.5 ± 0.2	17.6 ± 0.2	19.9 ± 0.2	18.7 ± 0.2	19.4 ± 0.1
GAPDH	PBS	26.6 ± 0.1	27.8 ± 0.2	24.7 ± 0.2	27.2 ± 0.2	29.5 ± 0.9	22.9 ± 0.1	22.9 ± 0.1
	E. tarda	29.4 ± 0.1	28.6 ± 0.1	27.2 ± 0.3	23.2 ± 0.1	26.2 ± 0.1	21.4 ± 0.1	23.5 ± 0.4
RPL17	PBS	18.7 ± 0.2	23.6 ± 0.2	21.4 ± 0.5	21.5 ± 0.1	24.7 ± 0.4	20.8 ± 0.2	20.8 ± 0.2
	E. tarda	21.9 ± 0.1	26.7 ± 0.1	23.7 ± 0.2	19.0 ± 0.2	21.1 ± 0.1	20.3 ± 0.2	19.8 ± 0.1
TUBA	PBS	23.3 ± 0.1	26.7 ± 0.2	23.5 ± 0.2	23.7 ± 0.1	25.2 ± 0.7	21.1 ± 0.3	23.8 ± 0.1
	E. tarda	25.5 ± 0.1	26.9 ± 0.2	24.1 ± 0.3	21.2 ± 0.1	25.1 ± 0.1	20.4 ± 0.1	22.8 ± 0.1
EF1A	PBS	24.5 ± 0.2	30.5 ± 0.3	30.6 ± 0.6	23.4 ± 0.1	23.1 ± 0.5	24.9 ± 0.1	26.2 ± 0.2
	E. tarda	27.6 ± 0.1	30.4 ± 0.5	31.5 ± 0.7	21.6 ± 0.1	21.3 ± 0.2	23.1 ± 0.3	27.2 ± 0.1
B2M	PBS	20.6 ± 0.1	23.2 ± 0.1	22.5 ± 0.1	22.1 ± 0.1	23.7 ± 0.4	23.1 ± 0.1	19.8 ± 0.1
	E. tarda	23.0 ± 0.1	23.8 ± 0.1	24.5 ± 0.6	19.9 ± 0.1	22.9 ± 0.1	22.4 ± 0.7	19.9 ± 0.1
RPSD	PBS	24.3 ± 0.1	28.4 ± 0.1	26.6 ± 0.4	26.5 ± 0.1	30.1 ± 0.5	26.1 ± 0.1	25.2 ± 0.1
	E. tarda	27.3 ± 0.1	29.5 ± 0.1	28.0 ± 0.2	24.2 ± 0.1	27.1 ± 0.1	25.4 ± 0.1	24.7 ± 0.2
18S rRNA	PBS	17.7 ± 0.1	20.4 ± 0.1	20.0 ± 0.1	18.6 ± 0.2	23.9 ± 0.1	17.3 ± 0.2	14.1 ± 0.1
	E. tarda	18.5 ± 0.1	21.7 ± 0.1	20.9 ± 0.3	16.6 ± 0.1	17.6 ± 0.1	16.5 ± 0.2	17.7 ± 0.1

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