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Evaluation of reference genes of *Mytilus galloprovincialis* and *Ruditapes philippinarum* infected with three bacteria strains for gene expression analysis

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Abstract – Quantitative real-time polymerase chain reaction (qPCR) is probably the most used method for gene expression quantification because of its high sensitivity and specificity. Nevertheless, this technology can undergo experimental errors and variations. Normalization of the results using a reference gene is therefore necessary to minimize these variations. As the study of immune genes in bivalve mollusks has increased in the last years, the establishment of adequate and stable reference genes for bivalves is strongly required. We analyzed the behavior of four putative reference genes: ribosomal RNA 18S, actin, elongation factor 1 – α and α-tubulin. The suitability of these four genes as internal control for qPCR was evaluated in mussel (*Mytilus galloprovincialis*) and clam (*Ruditapes philippinarum*) hemocytes after bacterial challenge. Four independent approaches (*BestKeeper, GeNorm, NormFinder* and *DeltaCt*) were used to assess the suitable genes for stable expression. For these particular circumstances, the most stable gene in hemocytes was elongation factor 1 – α for mussels and α-tubulin for clams.

Keywords: Shellfish immunity / Reference (housekeeping) genes / qPCR / *Mytilus galloprovincialis* / *Ruditapes philippinarum* / *Vibrio* spp / *Micrococcus lysodeikticus*

1 Introduction

Nowadays, quantitative real-time polymerase chain reaction (qPCR) is probably the most used method for gene expression quantification. This technique is a very popular tool because of its high sensitivity and specificity. Nevertheless, this technology is subject to considerable experimental errors and variations (sample-to-sample variation, differences in RNA integrity, DNAse treatment effectiveness and cDNA transcription efficiency as well as variations due to diverse experimental conditions, among others). Normalization of the results using a reference gene (also known as housekeeping gene) as an internal control is therefore necessary to minimize the systemic errors and inherent variations (Huggett et al. 2005). Our group has vast experience in gene expression analysis and we are conscious of all these warnings (Costa et al. 2009; Balseiro et al. 2013; Martins et al. 2014; Moreira et al. 2014).

Numerous bivalve mollusks species, such as the Mediterranean mussel (*Mytilus galloprovincialis*) and the Manila clam (*Ruditapes philippinarum*) have a great economic importance in the food industry around the world. Diseases caused mainly by *Perkinsus* (Waki et al. 2012) and *Vibrio* genera (Gestal et al. 2008) are associated with significant economic losses. The development and health status of these organisms can be especially affected by bacterial diseases (Allam et al. 2000; Gómez-León et al. 2005) which can alter the quality and price of the product. For this reason, the study of the immune defense against different pathogens is a field that is becoming increasingly important in the last years. The immune genes expressed in hemocytes, their main cellular defense against invading pathogens, have focused the attention of researchers especially in the last decade (Gueguen et al. 2003; Costa et al. 2008; Li et al. 2010; Romero et al. 2011; Moreira et al. 2012a). In some of these studies different putative reference genes such as 18S and 28S ribosomal RNA, the elongation factor 1 – α, the ubiquitin or the actin were used in qPCR experiments with no mention of previous housekeeping suitability evaluation.

It is accepted that reference genes are constitutively expressed genes due to their role in the maintenance of cell homeostasis and therefore are usually expressed at a constant level (Garcia-Vallejo et al. 2004). However, this assumption is not always correct and it is therefore necessary to establish reference genes whose expression, at mRNA level, do not change in the cell after experimental treatments to obtain reliable and repetitive results. For this reason, a specific search of the most suitable reference gene is necessary for each experimental condition. Indeed, several works have been reported in the last years regarding this topic not only in vertebrates such as mammals (Garcia-Vallejo et al. 2004) or fish (Mitter et al. 2009),

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but in mollusks (Martínez-Fernández et al. 2010) and also in bivalves (Araya et al. 2008; Cubero-Leon et al. 2012; Dheilly et al. 2011; Du et al. 2013; Mauriz et al. 2012; Siah et al. 2012); because different species in different circumstances are able to modify their transcriptome to overcome the situation.

The aim of this work was the evaluation of four typically used reference genes in qPCR assays such as the 18S ribosomal RNA (18S rRNA) subunit, the actin, the elongation factor $1-\alpha$ (EF $1-\alpha$) and the α-tubulin to identify the most suitable reference gene in bivalve hemocytes after a bacterial challenge. For this purpose, BestKeeper, GeNorm, NormFinder and the DeltaCt methods were employed to study the stability of these genes. This investigation will provide useful information for researchers to obtain a more accurate quantification of specific mRNAs in the hemocytes of these two bivalve species after a bacterial infection.

2 Materials and methods

2.1 Animals and bacteria

Healthy adult *M. galloprovincialis* mussels (maximum 6 cm) and *R. philippinarum* clams (maximum 5 cm) were obtained from a commercial shellfish depuration facility (Vigo, Galicia, Spain) in summer. In the depuration facility bivalves are maintained starving for several days, filtering sterile seawater to eliminate possible toxins and pathogens. Both clams and mussels have passed the national quality controls for human consumption.

To challenge clams, the *Vibrio alginolyticus* TA15 strain was chosen because is a specific clam pathogen (Gómez-León et al. 2005). In the case of mussels, no specific pathogen is known to have a severe impact in its development, therefore we have chosen *Vibrio anguillarum* (gram negative) and *Micrococcus lysodeikticus* (gram positive) because they are widespread in the aquatic environment; these bacteria are present in the microbiota of mollusks and could induce metabolic changes in some species (Wu et al. 2013).

Bacteria were prepared from frozen aliquots. *V. alginolyticus* and *V. anguillarum* were seeded in lysogeny broth (LB) supplemented with 1% NaCl and let grow overnight at room temperature. *Micrococcus lysodeikticus* was grown in LB overnight at 37 °C. The next day the CFU number was determined and a bacterial suspension of 10^8 CFU ml^{-1} was prepared in phosphate-buffered saline (PBS).

2.2 Sampling

Mussels were notched in the shell next to the adductor muscle and injected with 100 μl of *V. anguillarum* ($n = 48$) or *Micrococcus lysodeikticus* ($n = 48$) at 10^8 CFU ml^{-1} in PBS 1×. One last group was injected with 100 μl of PBS 1× and used as control. Mussels were then returned to separate tanks and maintained at 15 °C until sampling at 3, 6 and 24 h post-infection.

Clams ($n = 64$) were notched in the shell next to the adductor muscles and intramuscularly injected with 100 μl of *V. alginolyticus*, strain TA15 (10^8 CFU ml^{-1} in PBS 1×). Controls ($n = 64$) were injected with 100 μl of PBS 1×. After stimulation, clams were returned to the tanks and maintained at 15 °C until sampling at 3, 8, 24, and 72 h after challenge.

In both cases, sixteen animals from each group were sampled at each sampling point and 1–2 ml of hemolymph sample was taken from the anterior adductor muscle with a 0.5 mm diameter sterile needle and syringe. The hemolymph from four animals of the same treatment was pooled and four biological replicates were taken at each sampling point. Samples were immediately centrifuged at 3000 × g during 15 min at 4 °C and the cells were resuspended in 250 μl of TRIzol® (Invitrogen) and stored at –80 °C until RNA isolation.

2.3 RNA isolation and cDNA synthesis

Total RNA isolation with TRIzol® (Invitrogen) was conducted following manufacturer's specifications in combination with the RNeasy mini kit (Qiagen) for RNA purification after DNaseI treatment. Next, concentration and purity of RNA were measured using a NanoDrop ND1000 spectrophotometer. RNA integrity was tested on an Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples that passed the Bioanalyzer quality test were used for further analysis.

Finally, the first strand cDNA synthesis for each pool was performed on 1 μg of total RNA using SuperScript^{TM}III Reverse Transcriptase (Invitrogen) following the manufacturer's protocol.

2.4 Primer design and efficiency

Specific PCR primers were designed from the sequences of the selected genes (Table 1) using Primer3 (Rozen and Skaletsky 2000) according to qPCR restrictions. Oligo Analyzer 1.0.2 (Kuopio University, Finland) was used to check for dimer and hairpin formation. Efficiency of each primer pair was then analyzed with seven serial five-fold dilutions of cDNA and calculated from the slope of the regression line of the quantification cycle versus the relative concentration of cDNA (Pfaffl 2001). A melting curve analysis was also performed to verify that only specific amplification occurred. If these conditions were not accomplished, new primer pairs were designed.

2.5 Quantitative real-time PCR

qPCR was performed in a 7300 Real Time PCR System (Applied Biosystems). One microliter of five-fold diluted cDNA template was mixed with 0.5 μl of each primer (10 μM) and 12.5 μl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 μl. The standard cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 30 s. All reactions were performed as technical triplicates.

Table 1. Putative reference genes analyzed and information about primer sequences and PCR products.

Primer name		Sequence	*Tm* (°C)	Product size (bp)	Amplification Efficiency	Accession number
Mussel actin	F	CGACTCTGGAGATGGTGTCA	59.8	153	1.96	AF157491.1
	R	GCGGTGGTTGTGAATGAGTA	59.6			
Clam actin	F	ACACCGTCCCAATCTACGAA	60.4	130	2.00	*
	R	GCGGTGGTTGTGAATGAGTA	59.6			
Mussel 18S	F	GTACAAAGGGCAGGGACGTA	59	106	2.00	L33452.1
	R	CTCCTTCGTGCTAGGGATTG	59			
Clam 18S	F	CCGAACATCTAAGGGCATCA	60.1	169	2.15	EF426293.1
	R	AGTTGGTGGAGCGATTTGTC	61.0			
Mussel EF-1α	F	GATATGCGCCAGTCTTGGAT	60.1	223	1.95	AB162021
	R	CTCATGTCTCGGACAGCAAA	60.0			
Clam EF-1α	F	GAAGACTTGCCCAAAGCAGT	59.5	128	2.05	*
	R	CGTACAGCAAAACGTCCAAG	59.4			
Mussel α-tubulin	F	CTTCGGTGGTGGTACTGGAT	59.8	173	1.90	HM537081.1
	R	AGTGCTCAAGGGTGGTATGG	60.0			
Clam α-tubulin	F	CTTCCACTCCTTCGGTGGT	60.1	120	2.00	*
	R	ACCTGTGGGGCTGGATAGA	60.5			

F: forward primer, R: reverse primer. (*) Moreira et al. (2012b).

2.6 Gene expression stability analysis

The expression stability of the four candidate reference genes selected in both bivalve species (n = 36 for *M. galloprovinvialis* and n = 30 for *R. philippinarum*) was evaluated using different approaches: BestKeeper (Pfaffl et al. 2004), GeNorm (Vandesompele et al. 2002), the comparative Ct method (Silver et al. 2006) and NormFinder (Andersen et al. 2004).

BestKeeper calculates the standard deviation (SD) of the crossing point (CP, also known as Ct or threshold cycle) of the whole dataset and determines the best reference genes employing the pair-wise correlation analysis of all pairs of candidate genes and calculates the geometric mean of the best suited ones. The genes with the lowest SD and the best correlation are then proposed as reference genes.

GeNorm is a pairwise variation method that relies on the principle that the expression ratio of two ideal reference genes is identical in all samples. If one or both of this pair of genes are not constantly expressed their expression stability decreases, while their variation in expression rises. The variation of a particular gene is the average of the variation with all the other studied genes.

The DeltaCt approach is a simple comparison of the relative expression of pairs of genes within each sample. The stability of the candidate genes is evaluated via the DeltaCt method and SD and it is ranked according to repeatability of the gene expression differences among the samples.

Finally, NormFinder estimates the intra- and the intergroup expression variation and sums them to calculate the candidate gene stability value. This method is known to show less sensitivity toward coregulation of the candidate genes and is useful to contrast with the pairwise comparison approaches, in which sensitivity to coregulation could be a handicap.

3 Results

After the bacterial challenge or the intramuscular PBS injection mussels and clams showed a different pattern of mortality. Mussels suffered no mortality during the experiment. Regarding clams, the cumulative mortality rate in the challenged animals at the end of the experiment, 72 h, was 44%. The controls showed an end point mortality of 10%.

To find the appropriate samples to perform the analyses, after the Bioanalyzer run, we could not use the RNA integrity number (RIN) to discriminate between suitable and degraded samples. In the particular case of many protostomes, including mussels and clams, heating the RNA sample splits 28S rRNA in two fragments that migrate closely with 18S rRNA. This result should not be misinterpreted as degradation. We used the electrophoretic profile of the samples to assess the integrity of RNA as described by Winnebeck et al. (2010). According to this, two of the clam samples were removed from the analysis.

Considering the CP values for each gene and species, it is important to note that both in mussel and clam 18S seemed to be, by far, the most stable gene with a maximum CP oscillation of 1.62 for *M. galloprovincialis* and 1.54 for *R. philippinarum*. On the contrary, the other three genes suffered oscillations within samples, sometimes more than 10 CPs as in the case of actin (Figs. 1A and 1B). It could be also inferred from Figure 1A that, in *M. galloprovincialis*, EF 1 – α and α-tubulin had a similar modulation after a bacterial challenge, whereas actin showed a different modulation pattern and 18S was barely modulated. In Figure 1B, corresponding to *R. philippinarum*, only 18S showed an independent pattern. This representation of the CP values could motivate to think that 18S is the most stable gene, but the analyses conducted using the four software showed that EF 1 – α and α-tubulin were the most stable genes for mussel and clam, respectively (Figs. 1C and 1D). The different methods showed, in general,

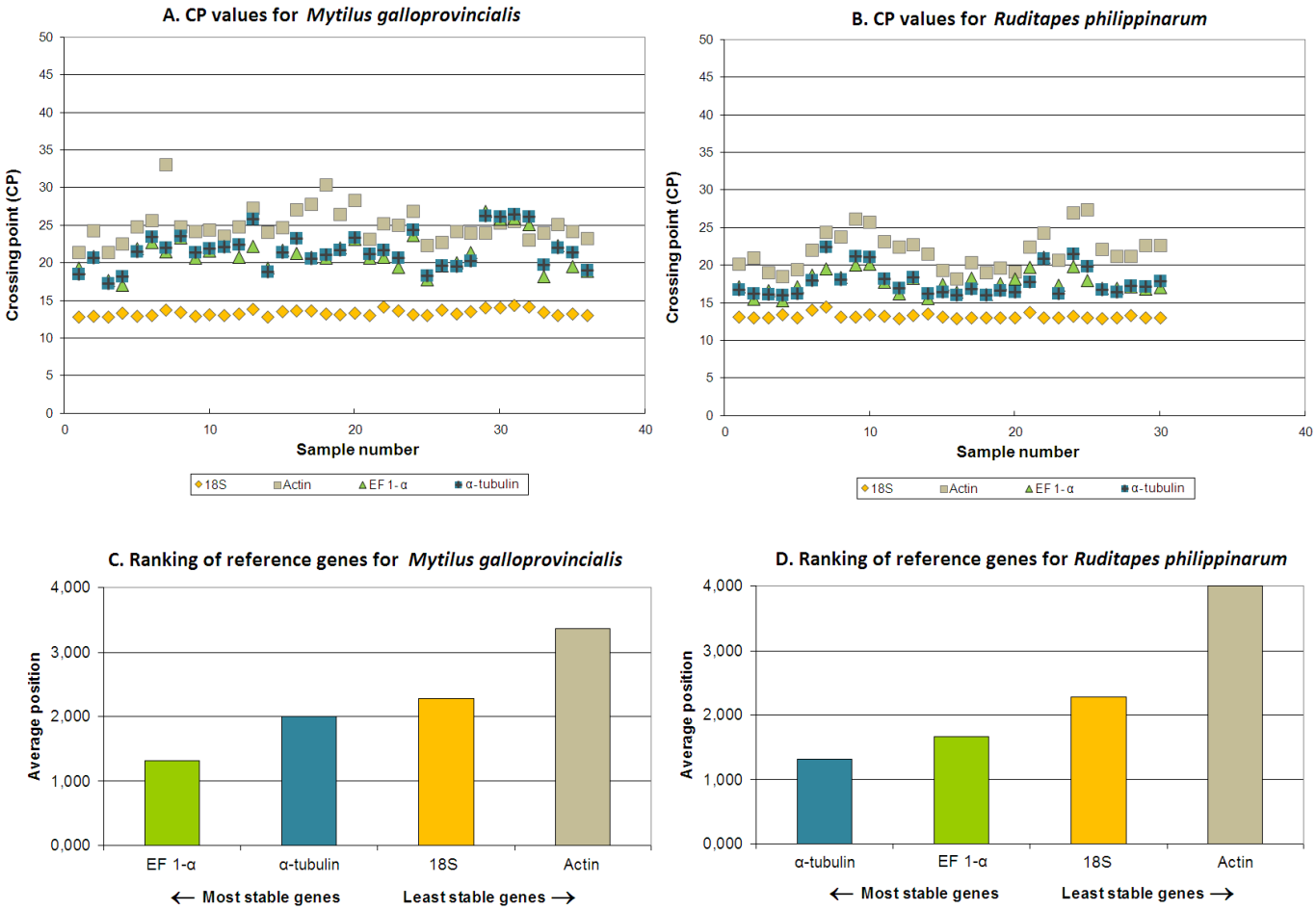

Fig. 1. Graphical representation of the crossing points (CP) values (A and B) obtained for the four candidate reference genes (sample number includes control and challenged groups), and average position in the comparison of the four methods to find the most stable reference gene (C and D), in both species *M. galloprovincialis* and *R. philippinarum*).

the same result to find the best candidate, except BestKeeper (Table 2). It highlights not only the importance of analyzing several candidate genes, but also the importance of using several mathematical methods to find a good group of reference genes.

Taking into account the standard deviation (SD) data (Table 2) in both species, only 18S could be used as reference gene following BestKeeper criterion: "any studied gene with the SD higher than 1 can be considered inconsistent". On the other hand, the correlation analysis between the expression pattern of all the samples of the four candidate genes (Table 2) showed that for *M. galloprovincialis* EF 1 – α and α-tubulin, were the most suitable to be reference genes, meanwhile for *R. philippinarum* α-tubulin and actin had the best correlation coefficients. None of these two conditions alone is used by any software but it is possible to guess that BestKeeper gives more weigh to the CP stability meanwhile GeNorm and DeltaCt are more focused on the pairwise comparisons. The most diverse method, NormFinder, showed similar results to GeNorm and DeltaCt and it was important to finally find the most stable genes.

The comparison of all the mathematical approaches provided by BestKeeper, GeNorm, NormFinder and DeltaCt and the average of the rank position for each candidate gene (Fig. 1C and D) showed EF 1 – α and α-tubulin as the optimal reference genes for Mediterranean mussel and Manila clam, respectively, in hemocytes and after a bacterial challenge using *Vibrio* and *Micrococcus*. In both species, these two genes were the most stable according to the four analyses together and 18S and actin were the least reliable to be used as housekeeping genes.

4 Discussion

Some studies have been recently published to find the best reference genes for qPCR analysis in bivalves (Araya et al. 2008; Dheilly et al. 2011; Cubero-Leon et al. 2012; Mauriz et al. 2012; Siah et al. 2012; Du et al. 2013) and showed that for each species, tissue, challenge and even reproductive state the optimal reference gene may change. Although it is known that the selection of an inappropriate reference gene could alter the results, no routine analysis to find the most stable gene are usually made (Bustin et al. 2009).

In this work, the behavior of four putative reference genes (18S, actin, EF 1 – α, and α-tubulin) was analyzed.

Table 2. Descriptive statistics of the expression results of the selected genes for *Mytilus galloprovincialis* and *Ruditapes philippinarum*. The best reference genes, according to the different methods, are highlighted in grey. CP: crossing point; *n*: number of samples; min: minimum; max: maximum, *SD*: standard deviation; *CV*: coefficient of variation, *r*: coefficient of correlation.

	M. galloprovincialis				*R. philippinarum*			
	18S	Actin	EF 1 – α	α-tubulin	18S	Actin	EF 1 – α	α-tubulin
n	36	36	36	36	30	30	30	30
Mean [CP]	13.35	24.96	21.29	21.70	13.21	21.89	17.71	17.72
min [CP]	12.78	21.41	17.06	17.24	12.90	18.15	15.32	16.02
max [CP]	14.40	33.03	26.90	26.43	14.44	27.33	21.10	22.37
SD [±CP]	0.37	1.63	1.75	1.87	0.24	2.03	1.18	1.49
CV [% CP]	2.74	6.53	8.22	8.60	1.82	9.28	6.64	8.40
min [x-fold]	–1.49	–10.19	–15.45	–16.02	–1.26	–12.18	–5.36	–3.06
max [x-fold]	2.07	244.15	45.55	22.68	2.59	47.84	11.85	26.67
r	0.639	0.619	0.910	0.944	0.430	0.916	0.871	0.976
p-value	0.001	0.001	0.001	0.001	0.018	0.001	0.001	0.001
BestKeeper SD [±x-fold]	1.29	3.10	3.36	3.64	1.20	4.74	2.46	3.13
GeNorm stability value	1.42	1.65	1.09	1.09	1.73	2.20	0.91	0.91
DeltaCt average SD [Ct]	1.88	1.89	1.47	1.38	2.21	2.66	1.92	1.99
NormFinder stability value	1.63	1.66	0.67	0.14	1.46	2.34	1.08	1.30

The sequences for the selected genes were obtained from the GenBank public database or a *R. philippinarum* hemocytes transcriptome (Moreira et al. 2012b). The experiment was performed in parallel in two bivalve species *M. galloprovincialis* and *R. philippinarum* after a bacterial challenge (*V. anguillarum* and *Micrococcus lysodeikticus* for *M. galloprovincialis* and *V. alginolyticus* for *R. philippinarum*). Control and challenged hemocytes were taken into account to perform the stability studies through four different approaches: BestKeeper, GeNorm, NormFinder and DeltaCt.

Elongation Factor 1 – α and α-tubulin seems to be the optimal reference genes for gene expression analysis through qPCR in *M. galloprovincialis* and *R. philippinarum* hemocytes, respectively, after bacterial challenge. However, the different methods used showed different results. This apparent discrepancy shows up that the different calculation methods to evaluate the gene stability may not result in the same optimal housekeeping gene, even in species and tissues subjected to similar experimental approaches. Moreover, different tissues or cell types may present different suitable reference genes within the same organism. Therefore, one thing is clear: when using qPCR for expression analysis studies, besides primer design restrictions for qPCR, primer efficiency and RNA quality evaluation, a search for the optimal internal control for each experimental condition is absolutely required.

5 Conclusion

Among the four potential optimal reference genes analyzed in hemocytes from two bivalve species after stimulation with bacterial pathogens, the elongation factor 1 – α (*M. galloprovincialis*) and α-tubulin (*R. philippinarum*) were found to be the optimal for being used in the normalization of the results in qPCR experiments performed under these conditions. Nevertheless, each experimental approach requires the evaluation of its own batch of housekeeping genes in order to obtain the reliable and acute results. Therefore, this work is a useful tool for those investigators interested in the study of genetic modulation analysis in hemocytes after bacterial challenge both in Mediterranean mussel and Manila clam.

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