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Validation of reference genes for expression analysis in a teleost fish (*Catla catla* Hamilton) exposed to an endocrine-disrupting chemical, bisphenol-A

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Abstract
Analysis of gene regulation after bisphenol-A (BPA) challenge has become a major focus of toxicologic research, and gene-expression analysis through real-time quantitative RT-PCR (qPCR) requires appropriate normalization. Since activity of most genes tends to vary depending upon physiologic state and under different conditions, it is important to identify and validate stable reference genes for data normalization and analysis. The expression of five candidate reference genes was studied in two tissues (brain and liver) from *Catla catla* after exposure to graded concentrations of BPA (10, 100, and 1000 µg/l) for 14 days. Expression of each gene was plotted relative to control, and stability of candidate reference genes was determined using the algorithmic models NormFinder and Bestkeeper. Expression of two biomarker genes was studied, i.e., vitellogenin (*vtg*) in liver and aromatase (*cyp19b*) in brain. In liver, expression of *gapdh*, *eef1a,* and *actb* was strongly regulated by BPA treatment. However, in brain, *actb*, *18S,* and *of the predictedproduct size* were the most affected genes. Moreover, the magnitude of *vtg* expression in liver varied when normalized to different reference genes. In brain, *cyp19b* expression showed an inverted U-shaped curved when normalized to *gapdh*, *eef1a,* and *18S*, but an increasing trend was observed when normalized to *actb* and *tbp*. Our study shows that the abundance and expression of most genes were treatment and tissue dependent and pre-validation of internal control reference genes is very important for toxicologic studies that entail gene expression.

Keywords Bisphenol-A (BPA) · Reference genes · EDCs · Real-time quantitative RT-PCR · *Catla catla*

1 Introduction

Endocrine-disrupting chemicals (EDCs), either natural or synthetic compounds, are known to alter the endocrine system of organisms by blocking or mimicking the actions of endogenous steroids (Schug et al. 2011). The largest class of EDCs is the estrogenic endocrine disruptors, also known as environmental estrogens that mimic the action of 17-β estradiol with high estrogen receptor (ER) binding affinity. One important estrogenic EDC that has gained much attention is bisphenol-A, a monomer used in the synthesis of plastic and plastic products, including the epoxy resin that lines food and beverage containers. In addition to its use in the food plastic industry, it is also used in the manufacture of flame retardants, dental sealants and thermal paper (Vom Saal and Hughes 2005). Because of its numerous uses, it is the highest volume chemical produced worldwide. In 2013, 15 billion pounds BPA was produced and 8–15 billion pounds of BPA is produced annually (Vandenberg et al. 2013). Routes of BPA entry into the aquatic environment are through sewage treatment effluent, natural degradation of polycarbonate plastics, and through landfill leachates (Howdeshell et al. 2003). A number of adverse effects have been reported, including teratogenic effects, developmental abnormalities, increased cancer risk, and abnormal sexual development (Jobling et al. 2009; Fernandez and Russo 2010).

To completely understand the potential health implications of these estrogenic EDCs in aquatic organisms, a complete knowledge of their mode of action is required. Understanding molecular mechanism(s) of action of environmental estrogens at the level of gene expression using quantitative

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reverse transcriptase polymerase chain reaction (qPCR) is an active area of research. qPCR has the capacity to detect very minute amounts of nucleic acid in a wide variety of samples based on its simplicity, speed, sensitivity, and specificity. Real-time quantitative RT-PCR (qPCR) is used to estimate the abundance of specific gene transcripts, and is a more sensitive and accurate method for studying gene expression than traditional methods such as northern blotting and RT-PCR (McCurley and Callard 2008). Gene-expression analysis using qPCR requires the use of a reference or normalizer gene(s). Reference genes are used to normalize differences in RNA loading amounts, instrumental errors, variations in reverse transcription efficiency, and differences in RNA integrity among samples (Bustin et al. 2005).

Reference genes are often adapted from the literature and used without proper validation across a variety of experimental conditions assuming a constant level of expression. The housekeeping genes (HKGs) are thought to have consistent expression among cells, organs, and individual organisms, during different developmental stages, and with various experimental treatments. Many HKGs are used as reference genes in qPCR. Numerous investigators have reported that even without any chemical treatment, genes labeled as HKGs tend to vary depending upon tissue type and developmental stage (Fernandes et al. 2008; Liu et al. 2014a; Kumari et al. 2015). Unrecognized, unexpected changes in HKG expression could result in erroneous conclusions about real biologic effects (Dheda et al. 2005). If validation studies are performed, most use a single dose of EDCs for validation of reference genes. A large number of studies focus on validation of genes after estradiol (E2) exposure (Filby and Tyler 2007; McCurley and Callard 2008), and dose–response curves for BPA are different from those for endogenous E2 or synthetic ethinyl estradiol (EE2). To the best of our knowledge, there are presently only two studies that relate to validation of reference genes after BPA exposure in fish (Zhang and Hu 2007; Qin et al. 2013), and both these studies evaluated only a single concentration of BPA. Wan et al. (2011) also validated reference genes as part of their evaluation of gene expression in disk abalone after BPA exposure. A majority of published work related to BPA exposure in fish uses multiple concentrations, and it is necessary to validate genes for each of those concentrations. Moreover, in many studies, a single reference gene is used for various tissues, but tissue type must be considered when selecting reference genes for any experiment.

The present study was designed to identify appropriate reference gene(s) for robust molecular analysis of gene expression in fresh water cyprinid *Catla catla* after exposure to graded concentrations of BPA. Six genes were selected for their potential use as a reference: cytoskeletal actin beta (*actb*), the transcription factor *TATA-box binding protein (tbp)*, *elongation factor 1 alpha* (*eef1a*), *18S ribosomal RNA* (*18S*), *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), and *beta-2-microglobulin* (*b2m*). In the first part of this study, we cloned and sequenced *tbp* and the aromatase gene (*cyp19b)* for *C. catla*, as these sequences were not available for this particular species. We used several approaches to identify the most appropriate reference genes for liver and brain following BPA exposure in *C. catla*. To assess the importance of selecting an appropriately validated reference gene, we measured the expression of two genes of interest, *vitellogenin* in liver and *cyp19b* in brain, and compared the relative expression using the panel of putative reference genes. We determined that BPA influences the expression of many genes considered to be "housekeeping" genes. Our data show that using only a single reference gene for expression analysis of genes of interest can lead to inaccurate results related to BPA effects.

2 Materials and methods

2.1 Animals

Catla catla (mean length 18.8 ± 1.10 cm; mean weight 94.4 ± 5.97 g) were purchased from a commercial fish farm located in the suburbs of Lahore, Pakistan. Fish were acclimatized for 15 days in concrete tanks. During acclimatization, fish were fed with commercial carp pallet diet (*Oryza organics*) twice a day. Physico-chemical parameters, such as water temperature, dissolved oxygen, electrical conductivity, and hardness, were recorded every other day during acclimation and exposure after water renewal.

2.2 Experimental protocol and sample collection

After acclimatization, fish were divided into four groups (ten fish per group). One group served as control, while the other three groups were exposed to graded concentrations (10, 100, or 1000 µg/l) of BPA. Bisphenol-A was purchased from Sigma-Aldrich (St. Louis, MO, USA), and a stock solution of 2 mg/ml (in ethanol) was made. The control group was exposed to the maximal level of ethanol (0.5 ml/l) used for BPA dilution. The experiment was conducted in semi-static condition, following OECD guideline number 203 (OECD 1992). Seventy-five percent of the water was renewed every day and fresh toxicant was added (after water renewal) for 14 days. After the proper time, the liver and brain of fish were removed and snap frozen in liquid nitrogen, and stored at – 80 °C until RNA extraction.

2.3 RNA extraction and cDNA synthesis

Tissue samples were ground to a powder in liquid nitrogen and total RNA was extracted from a 100 mg sample

using Trizol reagent (Sigma) following manufacturer's instructions. Quantity of RNA was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and quality of RNA was checked on ethidium bromide stained agarose gel. RNA samples with 260/280 ratio of 1.8–2 and producing sharp 28S and 18S rRNA bands on agarose gel were used. Total RNA (1 µg) was reverse transcribed with oligo-dT primers using RevertAid MMLV cDNA synthesis kit (Thermo Fisher Scientific). Resultant cDNA was diluted 1:10 for use in qRT-PCR.

2.4 cDNA clone and sequence analysis

To amplify a partial gene sequence of *tbp* and *cyp19b* from *Catla catla*, primers were designed based on conserved sequences in teleost mRNA sequences from GenBank (http://www.ncbi.nlm.nih.gov). Fragments were generated by RT-PCR and the gel-purified product was cloned into a pMD 18-T Vector and sequenced. These sequences were used for qPCR primer design of *tbp* and *cyp19b*, and submitted to GenBank.

2.5 Primer design and real-time analysis

Primers for six reference genes were designed using the primer 3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Genebank accession number, product size, and annealing temperature are listed in Table 1. Validation of primer specificity was performed by the conventional PCR followed by gel electrophoresis to confirm amplification of a single band of the predicted product size. qPCR was performed using CFX 96 (Bio-Rad, Hercules, CA, USA) with Syber green fluorescent label. To ensure amplification of a single product, the melting curve analysis (60–95 °C) was performed at the end of each reaction. All gene-expression components of this study were carried according to Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) as much as possible (Taylor and Mrkusich 2013).

2.6 Data analysis and statistical analysis

Ct values (the number of PCR cycles required to reach the fluorescence threshold) were calculated by the instrument software (CFX Manager Software, Version 3.1) and were used for further analysis. Baseline and threshold values were automatically set by the software. The Ct values for each of the six reference genes were transformed into relative abundance using the $2^{-\Delta Ct}$ method, where $\Delta Ct = Ct\ treated - Ct\ control$. Microsoft excel-based tools were used to evaluate the stability of reference gene panel for this qPCR studies (Pfaffl et al. 2004; Andersen et al. 2004). The relative abundance of gene of interest was determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1 Gene accession numbers, primer sequences, and amplicon lengths of selected genes

Genes	Symbol	Accession no.	Primer Sequences (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	JX480499.1	ATCACAGCCACGCAGAAGACC CAGGAATGACTTTGCCCACAGC	126	60
18S ribosomal RNA	*18S*	GU967673.1	CGGTGAACCTTGGTGACTCT CTTGGATGTGGTAGCCGTTT	189	60
Elongation factor 1-alpha	*eef1a*	JX480501.1	CAATTTCTGGATGGCACGGTGAC GGCATCCAGGGCATCAAGAAGAG	127	60
β-Actin	*actb*	JQ991014.1	ACCCACACTGTGCCCATCTACG ATTTCCCTCTCGGCTGTGGTGG	146	60
Beta-2-microglobulin	*b2m*	AM690446.2	CTCCAGTCCCAAGATTCAGG TCAGTCTGCTTGCTCTCAGG	153	59
TATA-box binding protein	*tbp*	KX371090	AACAGCTTGTCCCTCCTGGA CAGGAGTGATGGGGGTCATA	213	60
Vitellogenin	*vtg*	EF190987.2	GTTGCTCTCCAGACCTTTGC GCAGAGCCTCCACCTTGTAG	180	60
Aromatase	*cyp19b*	KX371091	GGTTTCATCCAGTGGTGGAC GTAACGACTGGGAACGGTGT	185	60

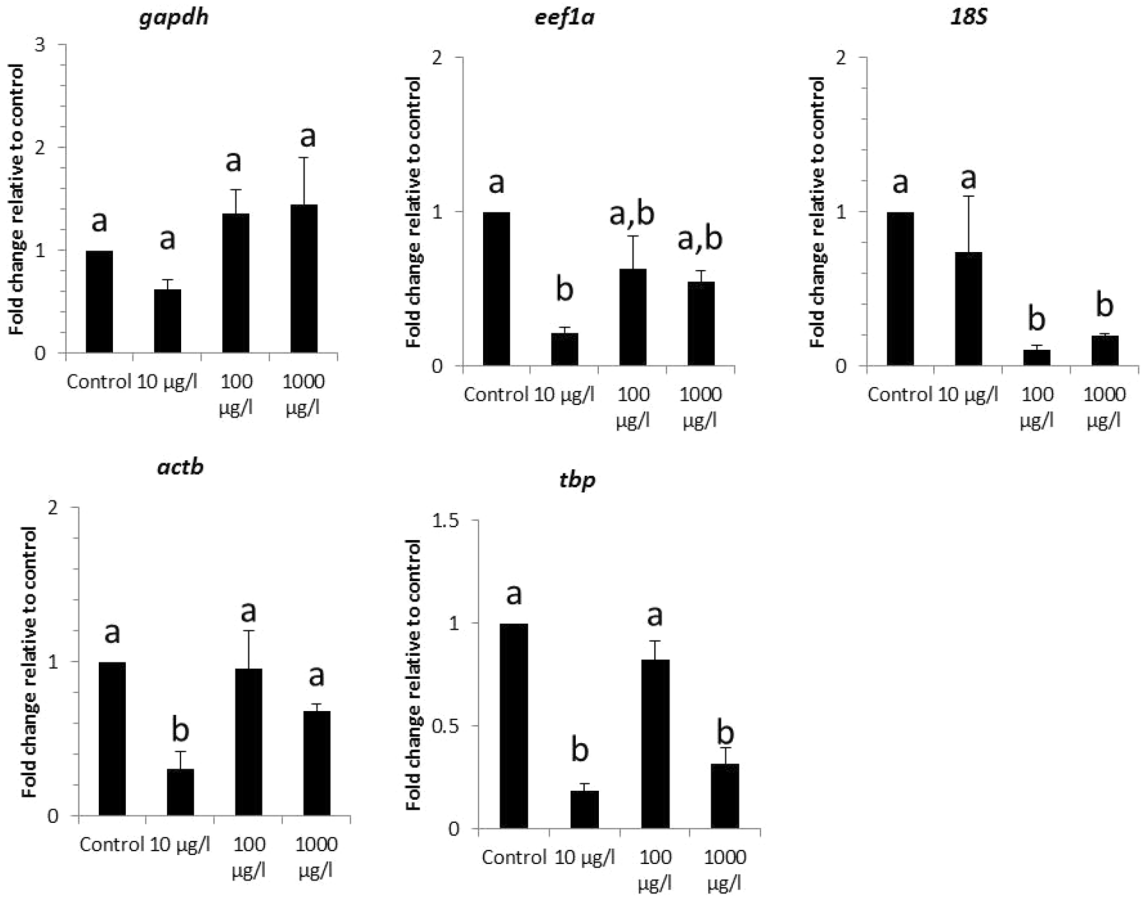

Fig. 1 Relative expression of five candidate reference genes following 14 days of exposure to graded concentrations of BPA in brain tissue of *Catla catla.* Different letters indicate significant differences among groups

3 Results

3.1 Abundance of candidate reference gene products

The six-selected genes code for proteins or RNAs that have different cellular functions and are regulated independently, thus reducing any error due to co-regulation of genes. *β-Actin* is a cytoskeleton protein, *TATA-binding protein* is a transcription factor, *18S ribosomal RNA* is a structural ribosomal RNA, *gapdh* is an important enzyme involved in glycolysis, and *b2m* is part of the major histocompatibility complex. *b2m* showed the highest variability after exposure to BPA in both tissues under study and was excluded from the final analysis. Each primer pair used in qPCR analysis (Table 1) amplified a single product of the expected size and displayed a single dissociation peak, ensuring high specificity. The number of PCR cycles required to reach the fluorescence threshold for each sample was defined as the Ct value. In brain and liver, *18S* showed the highest abundance (Ct ~ 12 and 15, respectively), followed by *eef1a*, *actb,* and *tbp*. *gapdh* is among the highest expressed genes in liver and is lowest expressed in brain (Table 2).

3.2 Relative abundance of selected candidate genes following BPA exposure

The expression levels of some of the candidate reference genes were altered in *Catla catla* after 14 days of exposure to BPA. The effect of BPA on the abundance of reference genes varied by tissue (Figs. 1, 2). In liver, *gapdh* was among the most unstably expressed genes. Abundance of *gapdh* significantly increased after the exposure to 10 or 100 µg/l of BPA. A trend toward an increase in abundance was also observed for *eef1a* and *actb*. *18S* abundance was relatively stable after exposure to low concentrations of BPA, but significantly increased up to fourfold after exposure to high BPA concentrations (1000 µg/l). The *TATA-box binding protein* was the most stable gene in the liver of *C. catla* after BPA exposure to all three concentrations. A different trend in expression of these genes was observed in brain. In the brain of *C. catla*, the *TATA-box binding protein* was significantly regulated

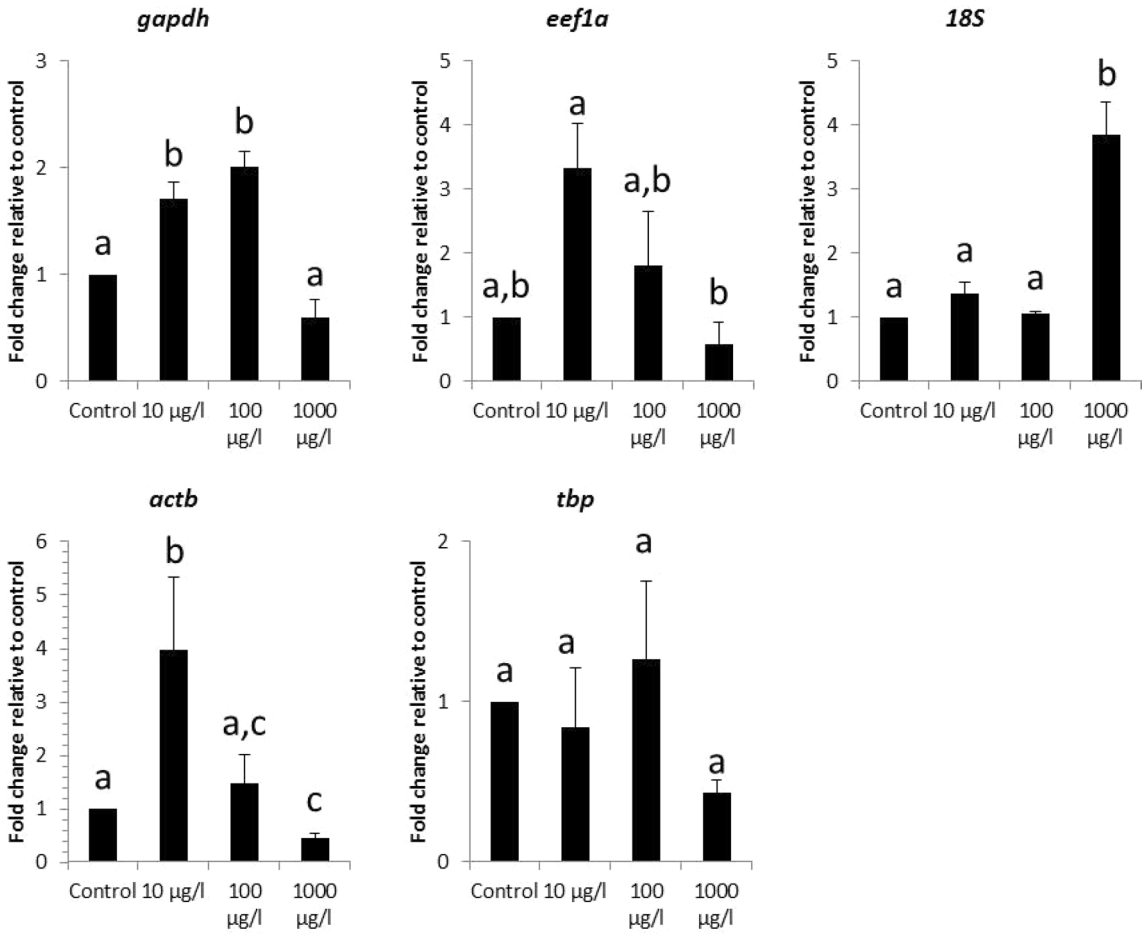

Fig. 2 Relative expression of five candidate reference genes following 14 days of exposure to graded concentrations of BPA in liver tissue of *Catla catla.* Different letters indicate significant differences among groups

Table 2 Abundance of candidate reference gene products (expressed as Ct) in *Catla catla* brain and liver tissues

Reference gene	Brain Ct	Liver Ct
gapdh	29.26 ± 0.28	21.21 ± 0.76
eef1a	21.19 ± 0.22	20.88 ± 1.03
18S	12.58 ± 0.49	15.13 ± 0.47
actb	20.59 ± 0.35	23.36 ± 0.98
tbp	27.70 ± 0.58	31.65 ± 0.32

Ct values are shown as mean ± SEM

after BPA treatment, whereas *gapdh* was among the most consistently abundant gene in brain.

The results of NormFinder showed that in liver, the most stable gene was *actb* followed by *tbp* and *gapdh* (Fig. 3a). In brain, *eef1a* was ranked as the most stable gene by NormFinder, with a stability value of 0.025, whereas *18S* was the least stable gene in brain, with stability value of 0.10 (Fig. 3b). In brain, *gapdh* was the most stable gene by BestKeeper, with an SD 0.5 followed by *actb*, *eef1a,* and *tbp*; *18S* was ranked as the least stable due to a high SD of 1.4 and a CV of 11. Results of BestKeeper ranked *18S* as the most stable gene in liver, with lowest SD, followed by *gapdh* and *tbp*. *eef1a* was ranked the most unstable gene in liver (Fig. 4a, b).

3.3 Effects of using different reference genes on expression analysis of selected genes of interest

When normalized to each of the reference genes, *vtg* expression showed an inverted U-shaped curve. With the increases in BPA concentration, *vtg* abundance increased, but at the highest concentration *vtg* levels decreased. This may be due to the fact that at the highest concentration BPA becomes toxic to the liver. Histological analysis of liver exposed to 1000 µg/l BPA showed that BPA caused severe tissue damage at this concentration (Faheem et al. 2016). However, the fold increase in *vtg* abundance was over-estimated when

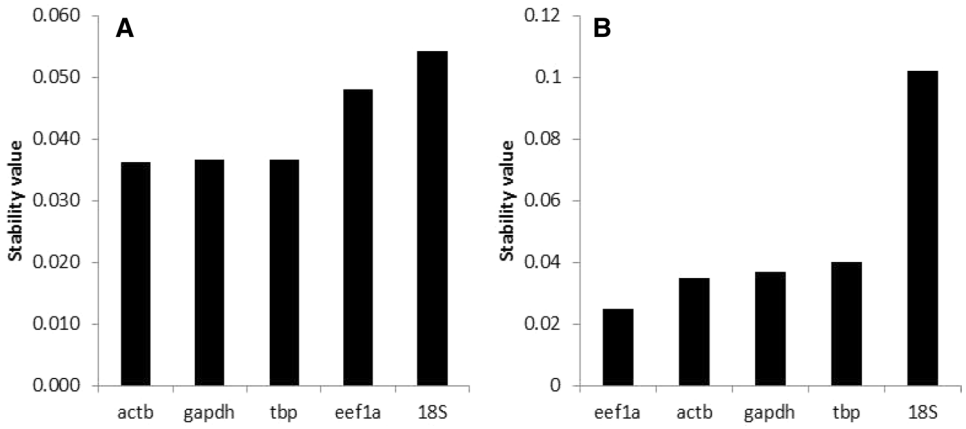

Fig. 3 NormFinder ranking of genes based on their stability values: **a** liver and **b** brain of *Catla catla*

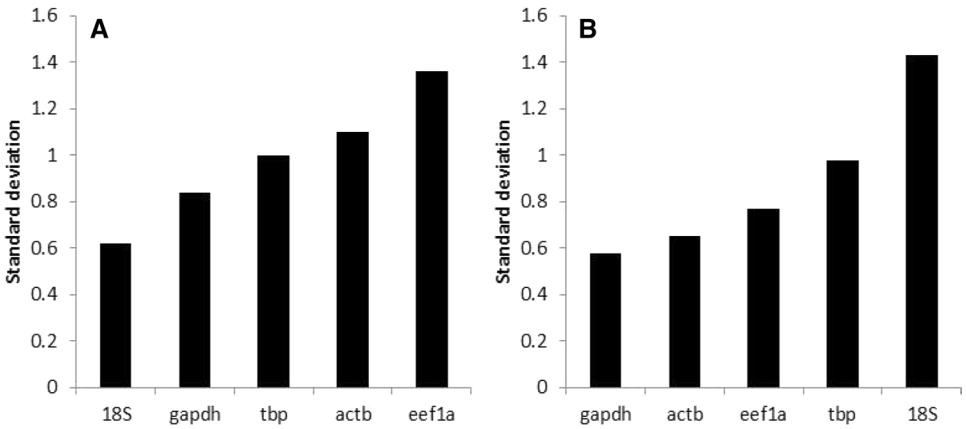

Fig. 4 BestKeeper output of reference genes ranked by their standard deviation: **a** liver and **b** brain of *Catla catla*

normalized to different reference genes. When normalized to the geometric mean of three reference genes with the lowest CV (> 4), the *vtg* abundance shows an inverted U-shaped (Fig. 5).

In this study, brain aromatase (*cyp19b*) expression increases or decreases to varying degrees when normalized to different reference genes. Interestingly, when the expression was normalized with *gapdh*, *18S,* and *eef1a*, *aromatase* mRNA level showed inverted U-shaped patterns after exposure to BPA, but when normalized to *actb* and *tbp*, *aromatase* mRNA expression increased in a concentration-dependent manner. Our results indicated that there is a need for validation of a set of reference genes for each tissue and for all concentrations in BPA toxicity experiments.

4 Discussion

Real-time PCR is extensively used for gene-expression analysis, but for accurate analysis of real-time data, an appropriate reference gene(s) for normalization is required. Validation of reference genes is extensively performed for mammalian and fish species, but less attention is given to the validation of genes prior to performing a dose-related toxicology or pharmacology study, as seen in the literature with BPA. Our study presents the first effort aimed at validating genes after graded BPA exposure in *Catla catla*. We choose *C. catla* as a model, because it is one of the most economically important food items in Pakistan and is an important aquaculture species.

Various methods have been proposed for identification of stable reference genes: e.g., calculating the biologic coefficient of variation (CV) of cycle threshold Ct (Tang et al. 2012), GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004). We choose two algorithmic tools, i.e., NormFinder and BestKeeper. NormFinder determines the stability of the candidate reference genes based on an estimate of the inter- and intragroup variations. It calculates the most stably expressed candidate genes and suggests two of the most stable genes as references genes (Andersen et al. 2004). BestKeeper is a Microsoft Excel-based tool that determines the "optimal" reference genes, employs pairwise correlation analysis of all pairs of candidate genes, and calculates the geometric mean of the "best"-suited ones. It calculates a BestKeeper index using the standard deviation of the Ct values between

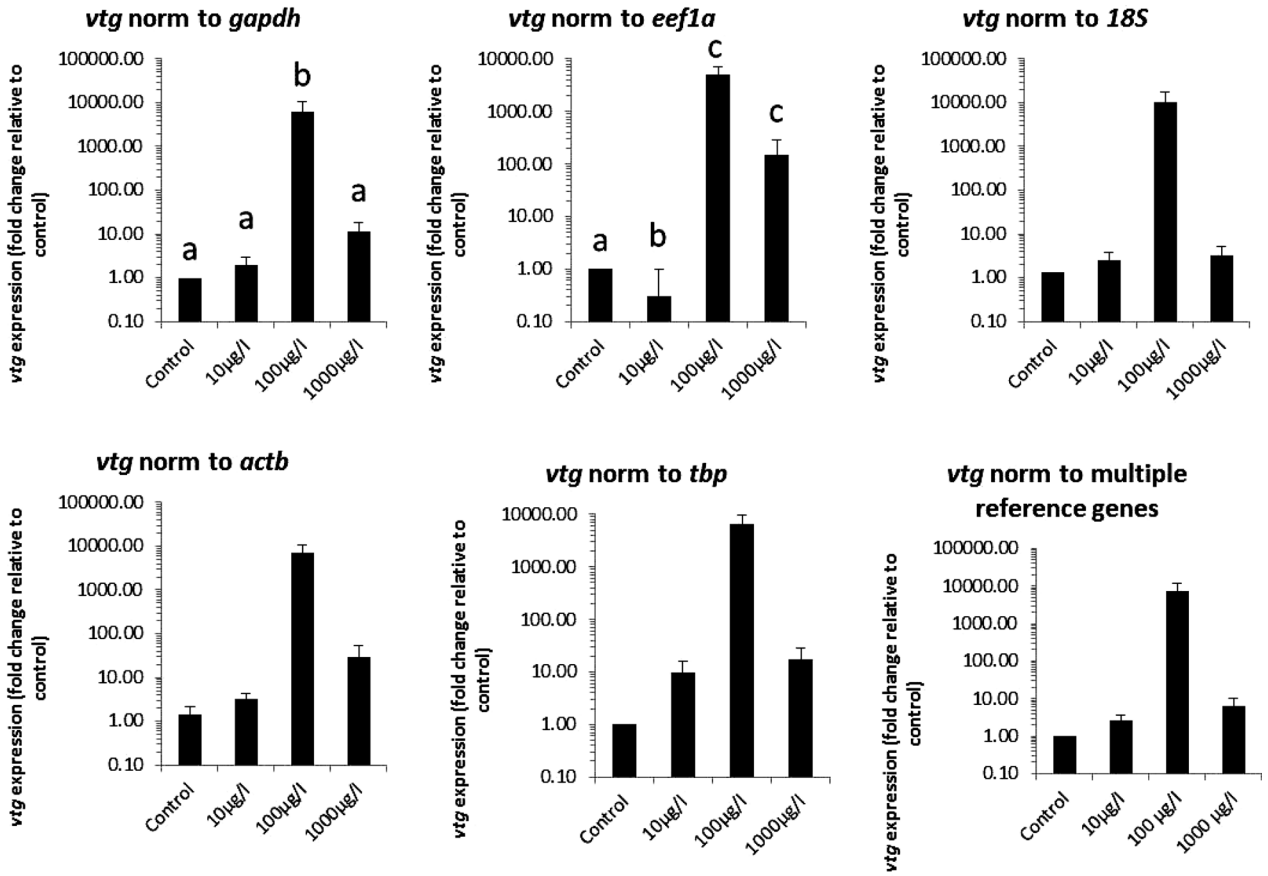

Fig. 5 Relative gene expression of the biomarker gene vitellogenin (*vtg*) normalized to different reference genes and mean of three selected genes (*gapdh*, *18S*, *tbp*), in liver of C. *catla* after 14 days of exposure to graded concentrations of BPA. Different letters indicate significant differences among groups

complete data sets, and genes with the lowest standard deviation (less than 1) are generally accepted for use as a reference gene for normalization purposes—a "normalizer" gene (Pfaffl et al. 2004).

Most of the studies on gene-expression analysis after BPA exposure used one reference gene, based on previously published studies without any further validation. Moreover, a single reference gene was used for all of the tissues studied. Rhee et al. (2011) used *18S* as a reference gene for 600 µg/l of BPA exposure for liver, intestine, gonads, and brain in *K. marmoratus*. *18S* rRNA was also used as an internal control in marine medaka (*Oryzias melastigma*) embryos exposed to 200 µg/l of BPA throughout their embryonic stages (Huang et al. 2012). Chan and Chan (2012) used *actb* as a reference gene in zebrafish larvae after exposure to 0.53–3.95 mg/l of tetrabromobisphenol-A. *actb* was used extensively as a reference gene in rare minnow exposed to various concentrations of BPA (Liu et al. 2012, 2014b; Guan et al. 2016), zebrafish (Ji et al. 2013), medaka (Lee et al. 2012), and sea bream (*Sparus aurata*) (Maradonna et al. 2014). *elongation factor alpha* (*eef1a*) was used as a reference gene in rainbow trout embryos (Birceanu et al. 2015).

Our data demonstrate that the expression levels of most of the putative reference genes evaluated are affected by BPA and that gene regulation is tissue dependent. In brain, *18S* was affected by BPA, and at higher concentrations (100 and 1000 µg/l), it was significantly down-regulated; whereas in liver cells, the expression was up-regulated significantly only at the higher concentration (1000 µg/l) and remained consistent at low-exposure concentrations (10 and 100 µg/l) of BPA. We suggest that *18S* can be used as an internal control in fish liver only at lower concentrations of BPA. Our data are in agreement with the findings of Filby and Tyler (2007), where *18S* was reported to be consistent after 10 ng/l of E2 exposure in liver. Rhee et al. (2011) used *18S* as a reference gene for BPA exposure in different organs of *K. marmoratus*. Expression of *gapdh* in liver was significantly up-regulated at 10 and 100 µg/l BPA. Zhang and Hu (2007) reported significantly up-regulated expression of *gapdh* in medaka liver after exposure to 100 µg/l BPA, and 200 ng/l EE2 and E2. Filby and Tyler (2007) observed down-regulation of liver

gapdh expression after exposure to EE2 for 21 days in fathead minnows. Contrary to these findings Qin et al. (2013) reported that GAPDH was among the most stable genes in juvenile rare minnow after 10 nM BPA exposure. Similarly, Zou and Ing (1998) reported estradiol down regulates *gapdh* mRNA level in liver. *gapdh* expression was also repressed in liver of rainbow trout (*Oncorhynchus mykiss*) and plaice after exposure to EE2 and other environmental estrogens (Hoyt et al. 2003; Brown et al. 2004; Hook et al. 2006).

In the present study, *actb* was strongly regulated by BPA exposure in both liver and brain. In liver, *actb* was up-regulated by 3.9-fold in groups exposed to 10 µg/l, but at higher exposures of BPA (100 or 1000 µg/l), there was a significant decrease in *actb* levels compared to control. Our data are in agreement Zhang and Hu (2007) who reported significant down-regulation of *actb* expression after exposure to 100 µg/l of BPA and 200 ng of EE2 and E2 in medaka. Array analysis of male sheepshead minnow (*Cyprinodon variegates*) liver showed that *actb* expression was down-regulated after exposure to the estrogenic chemicals methoxychlor, diethylstilbestrol, EE2, E2, and nonylphenol (Larkin et al. 2003). Similar findings were reported by Filby and Tyler (2007) in fathead minnow liver after 10 ng/l of EE2 exposure. This is in agreement with the observation that many estrogenic compounds (including E2) disrupt cytoskeletal compounds in vitro (Bocca et al. 2001). Contrary to these findings, *actb* expression increased after exposure to EE2 by liver in zebrafish (*Danio rerio*) and plaice (*Pleuronectes platessa*) (Brown et al. 2004; Hoffmann et al. 2006). Similar elevations in *actb* levels were observed in the pituitary of Atlantic salmon (*Salmo salar*) after nonylphenol and E2 exposure (Yadetie and Male 2002). This difference may be due to the difference in dose and exposure times employed. In disk abalone, *actb* expression was significantly down-regulated in females and up-regulated in males (Wan et al. 2011), further suggesting the need of gene validation in tissues and gender after estrogen exposure. Evidence from mammalian studies confirms estrogen regulation of *actb* and *gapdh* (Ing and Zhang 2004). In light of these studies, we suggest that the use of *gapdh* or *actb* alone as a reference gene may be inappropriate and might cause errors in data analysis. These genes should be used only after extensive validation.

Hepatic *elongation factor 1 alpha* expression showed an inverted U-shaped response after exposure to BPA, abundance of *eef1a* increased at 10 µg/l BPA exposure and a decrease was observed at higher concentrations. Filby and Tyler (2007) observed down-regulation of hepatic *eef1a* after EE2 exposure in fathead minnow, and similar down-regulation was observed in zebrafish embryos exposed to estrogens (Hoyt et al. 2003). Wan et al. (2011) observed significant increases in *eef1a* after BPA exposure in the hepatopancreas of disk abalone.

18S was relatively stable in liver under low concentrations (10 and 100 µg/l), but at higher concentrations, there was a significant up-regulation in expression of *18S*. In brain tissue, *18S* was strongly down-regulated by BPA exposure, confirming that gene regulation by estrogens is tissue specific. *18S* was considered to be a stable reference gene under EE2 exposure (Filby and Tyler 2007), and Zhang and Hu (2007) reported that mRNA expression of *18S* in liver was consistent after exposure to 100 µg/l of BPA. All of these studies employed only single BPA concentration, but in the present study of BPA concentration-dependent gene-expression experiments, *18S* was shown to exhibit variable patterns in liver and brain.

To assess the importance of selecting an appropriately validated reference gene for use as an internal control, in our final analysis, we measured the expression of two genes of interest, *vtg* in liver and *cyp 91b* in brain of *C. catla* exposed to graded concentrations of BPA, and compared the relative expression results obtained using the different reference genes. *vtg* and *cyp19b* were selected for this analysis because of their biological role in responsiveness to environmental estrogens. Vitellogenin protein and transcript level is commonly used as a biomarker of endocrine disruption in liver, and *cyp19b* catalyzes the rate-limiting step in estrogen biosynthesis in the brain.

In brain, aromatase abundance showed a variable pattern when normalized to different reference genes. Abundance of aromatase showed an inverted U-shaped curve when normalized to *gapdh*, *eef1a,* and *18S*. However, when normalized to *actb* and *tbp*, *cyp19b* abundance increased with increasing concentration (Fig. 6).

Interestingly, mRNA levels of *vtg* increased when exposed to 10 or 100 µg/l of BPA normalized to all selected reference genes, but the magnitude of expression was different with every reference gene. Moreover, *vtg* mRNA levels showed a decrease when exposed to 1000 µg/l of BPA. These data suggest that BPA at 1000 µg/l is toxic to liver cells, and thus, there is a decline in *vtg* expression. This speculation was confirmed by histologic analysis of liver at 1000 µg of BPA, which showed increased vacuolization, inflammation, and ruptured hepatocytes (Faheem et al. 2016).

Before using only one gene as a reference control, it should be confirmed that no significant regulation occurs under experimental conditions (Pfaffl et al. 2004). Vandesompele et al. (2002), reported that the conventional normalization of gene-expression analysis based on one gene leads to erroneous results by up to 3–6.4 fold, and recommended the use of the geometric mean of at least three reference genes as an accurate way to estimate mRNA transcript abundance. To avoid such errors, we used the mean of the three most stable genes described by NormFinder and BestKeeper. We choose the combination of *gapdh*, *eef1a,* and *actb* in

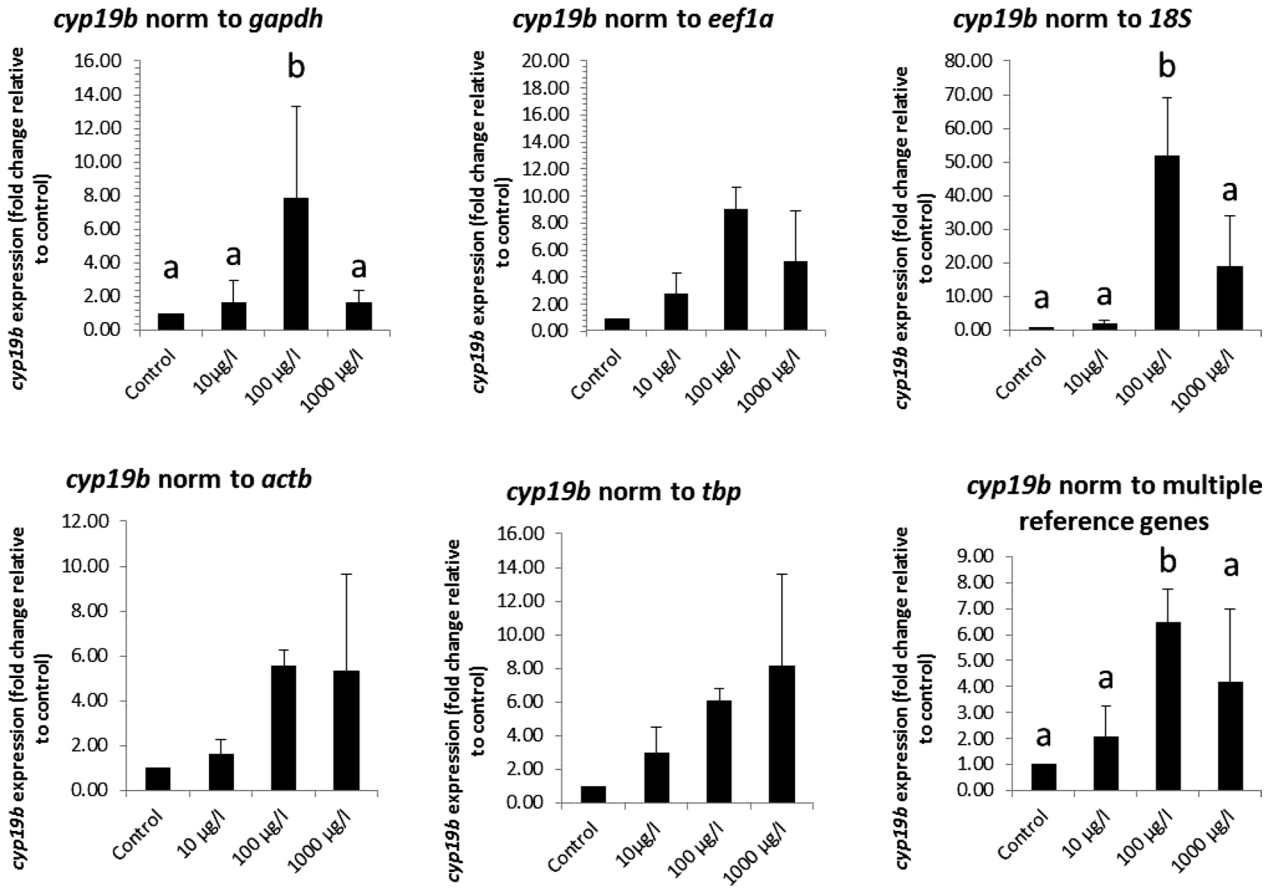

Fig. 6 Relative gene expression of the biomarker gene, *cypl9b* normalized to different reference genes and means of three selected genes (*gapdh*, *eefla*, and *actb*), in brain of *C.* catla after 14 days of exposure to graded concentrations of BPA. Different letters indicate significant differences among groups

brain and combination of *gapdh*, *18S,* and *tbp* as reference control in liver.

5 Conclusions

Based upon our results, most of the genes previously used as reference genes in fish are regulated by BPA exposure. We do not recommend using just a single gene for every tissue. We especially do not recommend using *gapdh* or *actb* as a single gene for expression analysis following BPA exposure. It is critical to validate a set of reference genes for each tissue, and selecting genes having Ct with the lowest SD and coefficient of variation. Our data show that using only a single reference gene for expression analysis of our genes of interest (*vtg* and *cyp19b*) can lead to erroneous results related to the effects of BPA. Using the geometric mean of Ct values for stable reference genes will support robust analysis of gene-expression levels.

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Author contributions MF carried on the experiments and prepared the manuscript under supervision of NJ and KPL. MF and SK analyzed data. All authors critically revised the manuscript and approved the final manuscript.

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