Indian Journal of Animal Sciences **83** (9): 942–947, September 2013/Article

Evaluation of internal control genes for gene expression studies in skeletal muscle of riverine buffaloes

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Received: 27 January 2012; Accepted: 14 May 2013

ABSTRACT

Real-time PCR analysis is required to be normalized with internal control genes (ICG) for validating the whole-genome microarray data or a smaller set of genes. In riverine buffaloes very few Internal Control Genes (ICG) have been reported till date for transcriptional studies in meat quality. Present study utilized muscle tissues from fattening and non- fattening buffaloes. The treated animals (fattening) were fed with formulated diet and control animals (non-fattening) were fed with normal diet. A total of 10 previously known house keeping genes were evaluated to identify stably expressed ICGs in buffalo muscle tissue. Three softwares based on different algorithms: geNorm, Normfinder and BestKeeper were used to measure transcript stability. Results suggested that geometric average of UXT, RS18 and B2M would provide better normalization of gene expression data in buffalo muscle tissue.

Key words: Buffalo, Internal control genes, Muscle tissue, Normalization, q-PCR

The riverine buffalo (*Bubalus bubalis*) is established as an economically important species in many Asian and Mediterranean countries and its genetic improvement ranks high amongst agricultural research needs. India has about 105 million buffaloes (livestock census 2007), which is 60% of total population in the world. They contribute to 1.48 million metric tonnes of meat, amounting 24.54% of the total meat produced in the country (FAO 2008).

Marbling which describes fat storage in adipose tissue between the muscle fibers (Harper *et al*. 2004) plays a particularly important role in determining the juiciness and tenderness of beef. Several gene based studies related to carcass quality, tenderness and intramuscular fat deposition are limited to cattle and pig and no such work has been reported for buffalo meat quality traits.

Quantitative real time polymerase chain reaction (q-PCR) is the most efficient method to measure the gene expression and would be invaluable in measuring the transcriptional changes in buffalo muscle tissue. However, qPCR requires normalization of expression data as this technique is prone to analytical variations and confound the overall precision of the data (Huggett *et al.* 2005, Valasek *et al.* 2005). For accurate relative quantification of gene expression data across different samples, it is quite essential to take into account the variations that might occur in qPCR due to differing amount of starting material, pipetting errors, efficiencies of RNA extraction and reverse transcription (Vandesompele *et al.* 2002). Use of internal control genes (ICGs) with constant expression level between samples is now considered as effective method for normalization of qPCR data to account for the experimental variations (Vandesompele *et al.* 2002, Deindl *et al.* 2002, Radonic *et al.* 2004, Dheda *et al.* 2004, Tramontana *et al.* 2008, Kadegowda *et al.* 2009). Recently, the focus is on evaluating combination of multiple reference genes for normalization of expression data for different experimental set-up (Hembruff *et al.* 2005, Etschmann *et al.* 2006, Bionaz *et al.* 2007, Stamova *et al.* 2009, Setiawan *et al.* 2010, Li *et al.* 2011). Very limited studies have been reported on identification of suitable ICGs for normalization of qPCR data in muscle tissue of buffaloes. The present study was aimed to identify stably expressed ICG in buffalo muscle tissue from a battery of 10 housekeeping genes reported in cattle *viz*., glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)*, beta- actin (*ACTB*), ubiquitously expressed transcript (*UXT*), ribosomal protein S15a (*RPS15A*), beta 2-microglobulin (*B2M*), ribosomal protein S18 (*RS18*), ribosomal protein S9 (*RS9*), ribosomal protein S23 (*RPS23*), hypoxanthine-guanine phosphoribosyltransferase1 (*HPRT1*) and ubiquitin (*UBI*).

MATERIALS AND METHODS

Animals, feeding trial and tissue sampling: Buffalo calves

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(12) of about 1½ year were divided into 2 groups of 6 each on the basis of their body weight and age. After adaptation period of about 20 days these calves were put to 2 feedlot rations consisting of wheat straw *ad lib.* + 5–10 kg green and concentrate mixture as per their requirement. In group 2, the protein source in the concentrate mixture was replaced by limiting amino acids rich sources by combination of sunflower cake and cotton seed cake. The feeding period was continued for 14 weeks. The values for total DM intake and DM intake per 100 kg body weight were 6.89, 2.62 and 6.73, 2.61 in groups 1 and 2, respectively. The initial, final weight and weight gain per day was 177, 399, 0.526 and 176.4, 434 and 0.598 kg, respectively. At the end of the feeding period, muscular tissue samples were biopsied from individual animal. The tissue samples were transferred to RNA later and stored at –80 °C until RNA extraction.

RNA extraction and cDNA synthesis: Total RNA was extracted from muscle tissue using ice-cold Trizol. To remove the traces of genomic DNA, RNeasy mini kit columns along with column digestion by RNAse free DNase enzyme were used. Total RNA concentration and purity was measured using a NanoDrop ND-1000 spectrophotometer. All the extracted RNA were stored at –80°C and utilized within 1 month.

A small aliquot of purified RNA was diluted to 100 µg / ml using nuclease free water prior to reverse transcription. cDNA was synthesized using 100 ng RNA, 1 µl dT_{12-18}, 1 µl 10 mmol/L dNTP mix, 1 µl random primers, and 7 µl DNase/ RNase free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 9 µl of master mix composed of 3 µl DNase/RNase free water, 4.5 µl 5x first-strand buffer, 1 µl 0.1 M DTT, 0.25 µl (50 U) of III RT, and 0.25 µl of RNase Inhibitor (10 U) was added. The reaction was performed in an applied biosystem using the program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:3 (v: v) with DNase/RNase free water.

Real time PCR (qPCR) assay and data analysis

The primers for each gene were designed using Primer express 3.0 software) Table 1 from *Bos taurus* sequences.

Quantitative PCR (qPCR) was performed using 4 µl diluted cDNA combined with 6 µl of a mixture composed of 5 µl 2×Light Cylcer 480 SYBR Green I master mix, 0.4 µl each of 10 µm forward and reverse primers, and 0.2 µl DNase/ RNase free water in a 96 well white plate. qPCR was conducted on the 8 cDNA samples with 10 primer-pairs. For each gene, samples were run in duplicate (technical replicates) along with 6 point relative standard curve plus the non-template control (NTC). The reactions were performed in a Light Cylcer using the amplification conditions: 5 min at 95°C (initial denaturation), 15 sec at

Table 1. Gene name, GenBank accession numbers, primer sequences, annealing temperature, amplicon length, PCR efficiency and regression coefficient for the studied genes

Gene Symbol	Accession number	Primers 5'-3'	T_a (forward, reverse)	Cellular localization	Biological function/component
ACTB	AY141970	GCGTGGCTACAGCTTCACC TTGATGTCACGGACGATTTC	60°C	Golgi membrane, plasma membrane	Cytoskeletal protein, immune response
B2M	NM_173893	AGTAAGCCGCAGTGGAGGT CGCAAAACACCCTGAAGACT	60°C	Cytoplasm	Cytoskeletal structural protein, nucleotide and ATP binding
GAPDH	BC102589	TGGAAAGGCCATCACCATCT CCCACTTGATGTTGGCAG	60°C	Plasma membrane	Glycolytic enzyme, Oxidoreductase in glycolysis and gluconeogenesis
HPRTI	BC103248	GAGAAGTCCGAGTTGAGTTT GGAAGGCTCGTAGTGCAAAT GAAGAGT	60°C		Purine synthesis in salvage pathway
RPS15A	BC108231	GAATGGTGCGCATGAATGTC GACTTTGGAGCACGGCCTAA	60°C	Cytoplasm	Protein synthesis/40S subribosome
RS18	DQ222453.1	CGGCTACCACATCCTATGAA TGGAGCTGGAATTACCGCGG	57°C	Cytoplasm	Component of the 40S ribosome
RPS23	BC102049	CCCAATGATGGTTGCTTGAA CGGACTCCAGGAATGTCACC	60°C	Cytoplasm	Protein synthesis/40S subribosome
RPS9	DT860044	CCTCGACCAAGAGCTGAAG CCTCCAGACCTCACGTTTGTTC	60°C	Cytoplasm	Protein synthesis/40S subribosome
UBI	BE668033	TCCCTACCTGCATCATGTGC GGAATTTGGGCCAGTGCTC	60°C	Cytoplasm	protein recycling
UXT	BQ676558	TGTGGCCCTTGGATATGGTT GGTTGTCGCTGAGCTCTGTG	60°C	Cytoplasm	Transcriptional activation, ATP binding, unfolded protein binding binding, microtubule

95°C, 8 sec at 60°C, 20 sec at 72°C, 45 cycles (annealing + extension), 1 sec at 95°C, 1 min at 70°C, continuous at 95°C (melting curve) and 30 sec at 40°C (cooling). The qPCR expression data for each ICG in all samples were extracted in the form of crossing points. The data were acquired using the second derivative maximum method (Rasmussen *et al.* 2001) as computed by the light cycler software 3.5 and subjected for subsequent analysis. Three separate reference gene stability analysis software packages; geNorm (Vandesompele *et al.* 2002), Bestkeeper (Pfaffl *et al.* 2004) and NormFinder (Andersen *et al.* 2004) were used to analyse the RT qPCR data. The gene stability measures (M value) based on the average pair-wise variation of a particular ICG with all other control genes was calculated. Pair-wise variation (V) between the normalization factor (NF) obtained using n genes (best references) (NF_n) and the NF obtained using n+1 genes (addition of an extra less stable reference gene) (NF_{n+1}) was also calculated using geNorm. A cut-off value of 0.15 for the pair wise variation (V value) was considered acceptable**.** The standard deviations (SD) as well as the accumulated standard deviation (Acc. SD) that is a great indicator for optimal number of reference genes to be used were calculated by Normfinder. Best Keeper software was used to find out the pair wise correlations amongst the candidate ICGs.

RESULTS AND DISCUSSION

The average Ct values for the 10 ICGs were quite variable and showed a wide range of expression levels that ranged between 19.77 (GAPDH) and 31.39 (UXT). The ICGs were ranked according to their expression stability in the muscle tissue of animals fed with fattening and non- fattening diet using geNorm software. All the genes showed expression stability (M value) within the acceptable range (<1.5) and ranged between 0.346 (UXT/ RS18) and 0.932 (HPRT1, Table 2). The lower M value is indicative of higher expression stability of the genes (Vandesompele *et al.* 2002). UXT and RS18 genes with lowest M-value 0.346 were the most stably expressed genes. This was followed by B2M which was ranked as third most stable gene with M value of 0.496. Ranking of genes in terms of expression stability (from most stable to least stable) were: UXT/ RS18, B2M, ACTB, RPS23, GAPDH, RPS15A, RPS9, UBI and HPRT1.

Pair-wise variation (V) for studied genes was also calculated using geNorm software. The calculation of V is useful for deciding their optimal number to be used in an expression study (Vandesompele *et al.* 2002). A cutoff threshold of V = 0.15 is generally recommended. The analysis showed that V3/4 combination gave V value well within the acceptable limit (<0.15) (Fig. 1). In the present study, the set of only 3 genes (RS18, UXT and B2M) gave V value of 0.142. Taking into consideration the outcome of the geNorm analysis, we suggest RS18, UXT and B2M ICGs as the best choice to normalize the expression data in buffalo muscle tissue.

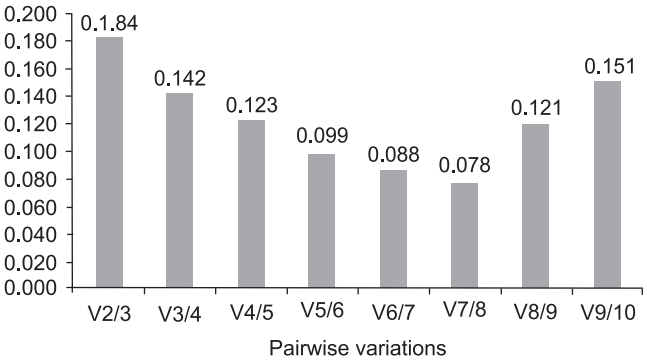

Fig. 1. Determination of optimal number of control genes for normalization by calculation of pair-wise variation (V) of normalization factor ratios for different number of control genes.

Normfinder tool was applied after converting the linear data in logarithmic scale. Interestingly, Normfinder analysis also identified same set of most stably expressed genes (UXT, RS18 and B2M). The bar plot showing variability for each gene is depicted in Fig. 2. As identified by geNorm, UXT was found to be the most stable gene by Normfinder as well. The standard deviations for each gene as well as the accumulated standard deviation (Acc.SD) are presented in Table 3.

Table 2. Ranking of ICG according to their expression stability

Ranking order	Gene name	M-value
1	UXT	0.346
2	RS18	0.346
3	B2M	0.496
4	ACTB	0.559
5	RPS23	0.612
6	GAPDH	0.645
7	RPS15A	0.669
8	RPS9	0.69
9	UBI	0.788
10	HPRT1	0.932

Table 3. Standard deviation (SD) and accumulated standard deviation (Acc SD) for each gene

Gene name	SD	Acc. SD
UXT	0.1729	0.1729
RS18	0.3736	0.2059
B2M	0.392	0.1895
GAPDH	0.5552	0.1987
RPS23	0.5748	0.1961
ACTB	0.6138	0.1928
RPS15A	0.6142	0.1871
RPS9	0.8196	0.1931
UBI	0.91	0.1992
HPRT1	1.5018	0.2339

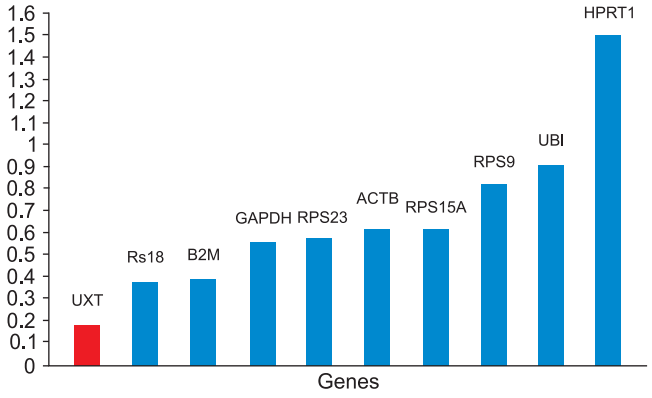

Fig. 2. Bar plot showing gene variability in 10 candidate ICGs by *Normfinder*. The optimum reference gene is highlighted in red (UXT).

The gene expression variation based on Ct values for the analyzed ICGs was calculated using Bestkeeper algorithm. The analysis included calculation of crossing point standard deviations [{stddev (±CP)} <1] and results were displayed as standard deviation (SD) and coefficient of variance (CV). The *RPS9* gene with lowest crossing point SD value (0.69) revealed minimum variations (Table 4). This was followed by GADPH and B2M genes with SD value of 0.98, 1.01 respectively. These values correspond to an acceptable range of fold change expression (< 2.0). On the other hand, the HPRT1 gene found to be least stable as its variation in expression was greater than two fold (S D > 1.0; or fold change > 2.0).

Additionally, the inter-gene relation for 10 ICGs pairs was estimated. The highly correlated ICGs were combined into the Bestkeeper index and the correlation between each candidate ICGs and Bestkeeper was estimated (Table 5). The relationship between ICGs and Bestkeeper was described in terms of Pearson correlation coefficient (r), coefficient of determination (r^2) and the *p*-value (Table 5) The best correlation between Bestkeeper index and ICGs was observed for *UXT* (r = 0.981). The statistically significant correlation shown by UXT with the Bestkeeper index appeared to be consistent with their good performance as assessed by geNorm and Normfinder.

The data presented here describe the evaluation of 10 housekeeping genes as ICGs for gene comparison studies in buffalo skeletal muscle. Our analysis strongly indicated UXT, RS18 and B2M to be most appropriate endogenous control genes for data normalization in q-PCR experiments involving buffalo skeletal muscle samples.

Studies conducted in cattle (Perez *et al.* 2008, Clark *et al.* 2011) and pig (Feng *et al.* 2010) examined ICGs in skeletal muscle. Our study is the first report to evaluate the stability of candidate reference genes in buffalo skeletal muscle during fattening and non- fattening dietary experimentation.

Unlike the present study, Feng *et al.* (2010) identified HPRT to be most stably expressed ICGs in skeletal muscles of porcine. Similarly Perez *et al.* 2008 did not find B2M to be stably expressed in bovine muscle tissue. Instead they identified EEF1A2 (Eukaryotic translation elongation factor 1 alpha 2) and SF3A1 (Splicing factor 3 sub unit 1) to be stably expressed in their experiments. These studies clearly indicated that test suited ICGs for each species and experimental condition need to be evaluated due to species specific tissue difference in the physico-chemical events, skeletal muscle development and its response to difference dietary interventions. This argument was strengthened by the earlier studies of Feng *et al.* (2010) where different set of ICGs were identified for skeletal muscle of Yorshire, Meishan and chinese pig breeds. Similarly, Lisowski *et al.* (2008) suggested different set of ICGs to be used separately for bovine tissues (liver, kidney pituitary and thyroid) and recommended that no single gene could be used for expression data normalization in all the tissues. The present study thus provide panel of ICGs that can be used in gene

Table 4. Descriptive statistical analysis for 10 ICG based on cycle threshold (Ct)

	GAPDH	ACTB	RPS9	RPS23	RPS15A	RS18	B2M	HPRT1	UXT	UBI
N	10	10	10	10	10	10	10	10	10	10
geo Mean [CP]	19.73	24.89	25.03	27.18	22.95	24.92	25.02	28.49	31.36	27.21
ar Mean [CP]	19.77	24.93	25.05	27.21	22.98	24.95	25.05	28.59	31.39	27.28
min [CP]	17.27	22.85	23.10	25.50	20.84	23.51	22.84	26.50	29.73	24.96
max [CP]	21.61	27.10	26.40	29.59	24.94	27.38	27.21	33.17	33.83	31.70
std dev [± CP]	0.98	1.16	0.69	1.05	1.03	1.16	1.01	2.16	1.06	1.67
CV [% CP]	4.96	4.67	2.75	3.86	4.48	4.65	4.05	7.57	3.37	6.12
min [x-fold]	–5.49	–4.14	-3.81	–3.21	–4.31	–2.66	–4.55	–3.97	–3.11	–4.76
max [x-fold]	3.68	4.61	2.58	5.33	3.96	5.51	4.55	25.58	5.53	22.45
std dev [± x-fold]	1.97	2.24	1.61	2.07	2.04	2.24	2.02	4.48	2.08	3.18

N, number of samples; GM [Ct], geometric mean of Ct; AM[Ct], arithmetic mean of Ct; min [Ct]and max [Ct], extreme values of Ct; SD [± Ct], standard deviation of the Ct;CV [% Ct], coefficient of variation expressed as a percentage on the Ct values; min [x-fold] and max [x-fold], extreme values of expression levels expressed as absolute x-fold over or under coefficient; SD [± x-fold], standard deviation of the absolute regulation coefficients.

Table 5. Repeated pair-wise correlation between genes and with *BestKeeper* index (BI)

vs.	GAPDH	ACTB	RPS9	RPS23	RPS15A	RS18	B2M	HPRT1	UXT	UBI
ACTB	0.865	-	-	-	-	-	-	-	-	-
p-value	0.005561	-	-	-	-	-	-	-	-	-
RPS9	0.93	0.779	-	-	-	-	-	-	-	-
p-value	0.001	0.022803	-	-	-	-	-	-	-	-
RPS23	0.849	0.802	0.794	-	-	-	-	-	-	-
p-value	0.007625	0.016614	0.0186	-	-	-	-	-	-	-
RPS15A	0.954	0.811	0.935	0.834	-	-	-	-	-	-
p-value	0.001	0.014496	0.001	0.00997	-	-	-	-	-	-
RS18	0.833	0.871	0.768	0.898	0.788	-	-	-	-	-
p-value	0.010209	0.004823	0.025948	0.002477	0.020068	-	-	-	-	-
B2M	0.896	0.962	0.877	0.854	0.851	0.909	-	-	-	-
p-value	0.002604	0.001	0.004237	0.006959	0.007367	0.001761	-	-	-	-
HPRT1	0.785	0.864	0.734	0.856	0.783	0.982	0.896	-	-	-
p-value	0.021116	0.005686	0.038029	0.006651	0.021663	0.001	0.002604	-	-	-
UXT	0.9	0.868	0.872	0.929	0.9	0.972	0.926	0.962	-	-
p-value	0.002311	0.005206	0.00477	0.001	0.002311	0.001	0.001	0.001	-	-
UBI	0.885	0.843	0.835	0.912	0.897	0.945	0.908	0.95	0.981	-
p-value	0.003504	0.008559	0.009852	0.001587	0.002502	0.001	0.00183	0.001	0.001	-

expression studies involving buffalo skeletal muscle. However, similar to the present study, Perez *et al.* 2008; Clark *et al*. 2011 reported 18S rRNA as reliable ICG for normalization of real time PCR data in porcine and bovine skeletal muscle respectively.

In conclusion, this study identified UXT, RS18 and B2M as the most stably expressed control genes in buffalo skeletal muscle. These genes will certainly allow accurate and reliable normalization of q-PCR studies in this important dairy/meat species of India.

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