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Research Article

Identification of stably expressed reference genes in liver tissue of mouse fed with A1A1, A1A2 and A2A2 milk for normalization of transcriptional data

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Abstract

Milk protein is of two types- casein and whey. Beta casein, the second most abundant protein and crucial for casein micelle structure, has drawn a lot of attention in the last few years. The A1 and A2 variants that correspond to BCM7 and BCM9 peptides after proteolytic digestion have been the focus of debate due to their plausible relationship between disease risk and consumption of A1 or A2 type milk. In order to support animal trial studies with respect to feeding A1 and A2 milk for the long term and evaluate their effect on the expression of target genes in major metabolic organ, this study has aimed to identify a panel of stable reference genes (RGs) so that accurate normalization of expression data could be achieved. Therefore, 7 candidate RGs from different functional classes were assessed to identify the most stable RGs in liver tissue of mice after feeding them with A1A1, A2A2 and A1A2 milk-based diet for a trial period of 6 and 9 months. Four different statistical approaches (geNorm, NormFinder, Bestkeeper and RefFinder) were employed to find out the stable and most appropriate RGs. The ranking of RGs in 6-month and 9-month experimental trials revealed *SDHA* and *ACTB* as the most stable RGs in the 6-month trial, while *SDHA* and *HMBS* in the 9-month trial.

Key words: A1A2 milk, Expression stability, Liver tissue, q-PCR, Reference genes

Highlights

- Experimental mice were fed with milk-based diet (A1A1, A2A2 and A1A2).
- A panel of reference genes (RGs) were analyzed by statistical approaches.
- Liver tissue samples were taken for RNA extraction.
- *SDHA* and *ACTB* were found to be the most stable RGs in the 6-month trial, while *SDHA* and *HMBS* were the most stable RGs in the 9-month trial.

INTRODUCTION

Since thousands of years, cow milk has been used by humans across the world as a part of balanced nutrition. The two major class of proteins found in cow milk are; caseins (αS1-, αS2-, β-, k-casein) and whey (α-LA and β-LG) proteins in a proportion of 82% and 18%, respectively. In the last few years, A1 and A2 alleles of beta-casein (β-CN) have drawn worldwide attention. The milk from native cattlebreeds (*Bos indicus*) of India is generally referred to as A2 milk due to higher proportions of the A2 allele. The proteolytic digestion of A1 milk produces BCM-7, whereas digestion of A2 milk produces BCM-9. These differences are caused by point mutation (C8101A) in exon VII of the β-CN gene. The proline in A2 (codon; CCT) is replaced by histidine in A1 (codon; CAT/histidine) at position 67. Many reports indicated the association of A1 milk consumption with several diseases like type I diabetes, coronary heart disease, schizophrenia, infant death syndrome (Sun *et al*., 2003; Tailford *et al*., 2003; Woodford *et al*., 2008). The BCM-7 generated from A1 milk has opioid-like activity and has an affinity to bind with μ-opioid receptors present in our intestinal epithelium, gut system and brain. Infants with

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immature gastrointestinal tract (leaky gut) have more absorption of BCM-7. Further, animal and human trials have supported the linkage of type-1 diabetes with A1 milk consumption.

The BCM-7 derived from milk digestion could cross the blood-brain barrier leading to some neurological disorders and causing lymphocyte proliferation within crease in the level of inflammatory markers (Haq *et al*., 2014; Fiedorowicz *et al*., 2016; Trivedi *et al*., 2016).

The quantitative real time PCR (qRT-PCR) technique is considered to be quite efficient in evaluating the expression of target genes in a tissue or cell type. However, the accuracy of qRT-PCR results may be affected by several external factors such as quantity, quality of RNA, pipetting error, reverse transcription efficiency, PCR condition (Vandesompele *et al*., 2002; Dekkers *et al*., 2012; Xiang *et al*., 2018). Therefore, in order to evaluate the gene expression data accurately, it becomes necessary to normalize the target gene expression data with a panel of appropriate RG (Huggett *et al*., 2005; Palve *et al*., 2018; Verma *et al*., 2021). It is now an established fact that for each experimental condition involving various cell, tissue types or physiological, pathological, developmental stages, there is a need to identify a panel of stably expressed RGs (Kim *et al*., 2011). As a part of mice trial with respect to A1A1, A2A2, and A1A2 milk-based diet for a period of 6 and 9 months, this study was planned to identify a panel of stable reference genes in liver tissues so that accurate normalization of expression data could be achieved.

MATERIALS AND METHODS

Animals and ethics: A total of 48 male C57BL/6 mice of six weeks of age were kept at small animal house facility of ICAR-National Dairy Research Institute (ICAR-NDRI), Karnal for conducting the animal trials in accordance with the Institutional Animal Ethics Committee (IAEC), ICAR-NDRI and Committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines.

Experimental procedure: The study was carried out in two phases, *viz*. 6 months and 9 months experimental trials. All the animals were acclimatized for two weeks. The mice were divided into four groups (Group I: control; Group II: fed with A1A1 diet; Group III: fed with A2A2 diet; Group IV: fed with A1A2 diet). The control group was fed with standard chow diet ad-libitum whilst the treated groups were fed with a milk-based diet prepared using spray dried milk samples from Karan Fries (cross-bred) cows with all the three genotypes (A1A1, A1A2 and A2A2) and provided with free access to water all the time. After accomplishment of trials i.e 6 months and 9 months, the mice of respective groups were treated with gaseous anaesthesia (diethyl ether) and euthanized by cervical dislocation. Liver tissue was carefully dissected from mice and collected in RNA later solution.

Total RNA extraction and cDNA synthesis: Total RNA was extracted from a total of 32 liver tissue samples; 16 samples each harvested from 6-month and 9-month trials (group I= 4, group II= 4, group III= 4 and group IV= 4). Total RNA was extracted using ice-cold Trizol reagent (Invitrogen, USA) followed by purification using RNaeasy mini kit (Qiagen, Germany) columns. Genomic DNA was removed by on column-digestion with DNAse I enzyme. First strand cDNA was synthesized using 200 ng RNA using Revertaid First strand cDNA synthesis kit (Fermentas, USA) as described in our previous studies (Kaur *et al*., 2018). Briefly, first strand cDNA was synthesized using 200 ng RNA, oligo-dT (18) primer, dNTP mix, random primers, RiboLock RNase inhibitor, M-MuLV reverse transcriptase using the program: 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. All cDNA samples were further diluted 1:4 in DNase/ RNase free water prior to real-time PCR. The quality of all the cDNA samples was confirmed by amplifying *GAPDH* gene using the similar protocol as described for qRT-PCR.

Selection of candidate (RGs): Primer and other details for all the 7 genes are given in Table 1. The primer specificity was further confirmed in a 20 μL PCR reaction using the same protocol described for qPCR except for

Table 1. Gene name, gene symbol, GenBank accession numbers, primer sequences, annealing temperature (Ta) and amplicon length for each evaluated RGs

Gene	Gene symbol	Accession number	Primer sequence F/R (5' to 3')	Ta	Product size (bp)
β-Actin	*ACTB*	ENSMUST00000100497.10	CTCTTTTCCAGCCTTCCTTC GGTCTTTACGGATGTCAACG	60°C	99
β2-Microglobulin	*B2M*	ENSMUST00000102476.4	CTGGTCTTTCTGGTGCTTGTC GTATGTTCGGCTTCCCATTC	60°C	109
β-Glucuronidase	*GUSB*	ENSMUST00000026613.13	ACCAGCCACTATCCCTACTCA GCCACAGACCACATCACAA	60°C	200
Hydroxymethylbilane synthase	*HMBS*	ENSMUST00000077353.14	GGATGTGCCTACCATACTACCTC GGTTTCCAGGGTCTTTCCA	60°C	115
Phosphoglycerate kinase 1	*PGK1*	ENSMUST00000081593.12	GAGGAAGAAGGGAAGGGAAA GCAGATTCACACCCACCAT	60°C	166
Peptidylprolyl isomerase A	*PPIA*	ENSMUST00000132846.1	CAGGGTGGTGACTTTACACG ATGGACAAGATGCCAGGAC	60°C	113
Succinate dehydrogenase	*SDHA*	ENSMUST00000022062.7	GCCAGGAACACTCCAAAAAC	60°C	139

the final dissociation protocol. Five microliters of the PCR product were evaluated on 2.5% agarose gel stained with ethidium bromide. The accuracy of primer pairs was also ensured by the presence of a unique peak during the dissociation step at the end of qPCR.

Real time quantitative PCR (q-PCR) of RGs transcripts: All the qPCR reactions were carried out in 96 well MicroAmp Optical Plate in Step One Plus instrument (Applied Biosystem, California). The final qPCR mixture comprised of 4 µL diluted cDNA combined with 6 µL of a master mix composed of 0.4 µL each of 10 µM forward and reverse primer, 3.2 µL nuclease free water and 2 µL of 5X EVA Green/ROX qPCR master mix (5X) (Solis Biodyne). All reactions were performed in duplicate along with non-template control. The reactions were performed using the following conditions: 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. A 6-point relative standard curve was constructed for each gene by running 5-fold serially diluted pooled cDNA samples in duplicates. The 10-point data (6 serial dilutions x 2 biological replicates) and equation $E = 10^{-1/slope}$ were used to calculate the PCR efficiency of each primer pair.

Evaluation of expression stability of RGs: The stability of the RGs was evaluated using three Excel-based visual basic macro tools: geNorm (Xiang *et al*., 2018), NormFinder (Andersen *et al*., 2004) and Bestkeeper (Pfaffl *et al*., 2004) along with RefFinder web tool (Xie *et al*., 2012). The input data for geNorm and NormFinder was generated using the relative quantities based on the comparative cycle threshold (Ct) method as described by Livak and Schmittgen (2001). Firstly the most stable RGs was identified by measuring the expression stability as 'M' value which is based on overall pairwise variations comparison among the RGs. The M value <1.5 indicates the stable expression of a gene. The genes with lowest M values were considered to have more expression stability and vice versa. In addition, pairwise variation analysis (V values) was also calculated using geNorm to select the optimal number of RGs to be used for normalization of expression data.

NormFinder algorithm ranks the RGs by calculating their expression stability taking into account possible variation across the different sample groups. Another statistical approach, BestKeeper uses the CT values instead of relative quantities as input data. BestKeeper calculates the descriptive statistics and crossing point standard deviations [(SD, ±CP) <1] for each RGs. Lastly, RefFinder analysis was performed that integrates the outcome of other tools like geNorm, NormFinder, BestKeeper to provide overall final ranking of RGs based on geometric mean.

RESULTS

Identification of RGs by geNorm: The M value that indicates the expression stability of individual RG was obtained in geNorm analysis both for 6-month and 9- month trials. The M values ranged from 0.33 (*SDHA* and *ACTB)* to 1.187 (*PPIA*). As per M values, *ACTB* were identified as the most stable RG in mice liver tissues harvested after 6-month trial. On the other hand, *B2M* and *PPIA* were identified as the least stable genes. The genes were ranked from most stable to least stable as *SDHA=ACTB>HMBS>PGK1>GUSB>B2M>PPIA* during 6 months of experiment trial (Fig. 1). However, in 9 months of experimental trial, the M values ranged from 0.348 (*SDHA* and *PGK1*) to 0.0805 (*ACTB*). According to M values, *SDHA* and *PGK1* were ranked as the most stable reference genes in liver tissues harvested after 9-month trial. On the other hand, *B2M* and *ACTB* were the least stable genes. Accordingly, genes were ranked from most stable to least stable as *SDHA=PGK1>HMBS>GUSB> PPIA>B2M>ACTB* during 9 months of experimental trial (Fig. 2).

In addition to M values, pairwise variation

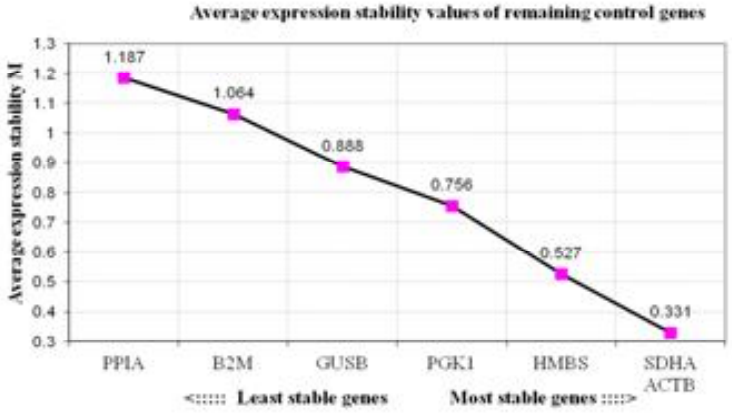

Fig. 1. Ranking of RGs based on average expression stability measures (M value) during 6 months trial

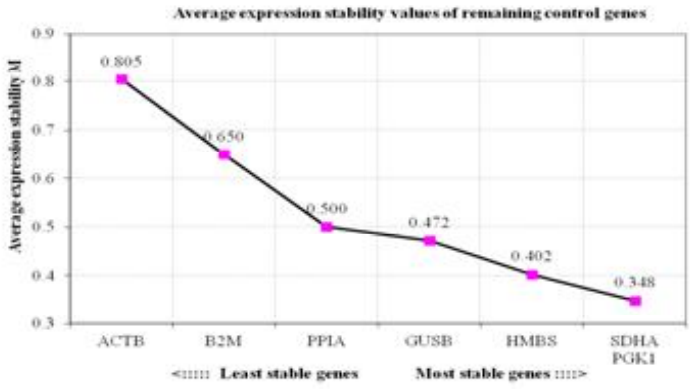

Fig. 2. Ranking of RGs based on average expression stability measures (M value) during 9 months trial

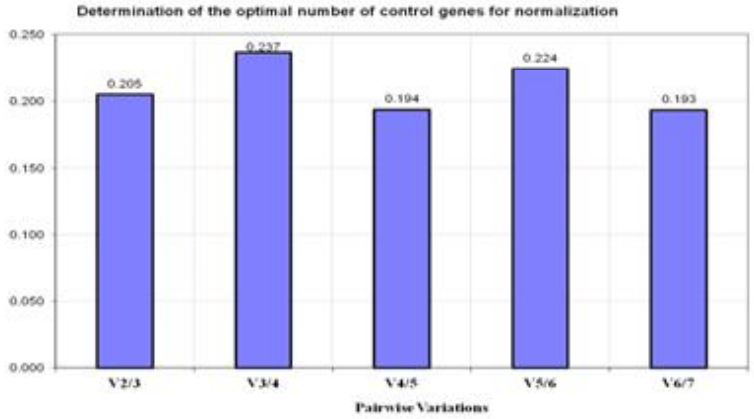

Fig. 3. The pairwise variation (V) analysis of candidate RGs for determining the optimal number of genes for normalization of different genes during 6 months trial

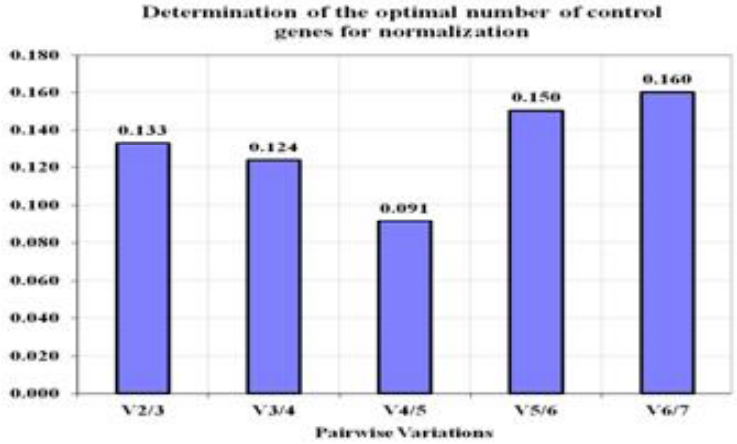

Fig. 4. The pairwise variation (V) analysis of candidate RGs for determining the optimal number of genes for normalization of different genes during 9 months trial

(V score) was estimated by geNorm in order to calculate the optimal number of RGs to be required for normalization. In 6-month trial, the pairwise V score was 0.237 in V3/V4, 0.194 in V4/V5, 0.193 in V6/7 combinations. These values were slightly on higher side than the recommended threshold criteria of ≤0.15. The panel of 4 (V4/V5) or 5 (V5/V6) most stable RGs with a V score of 0.194 and 0.193, respectively should be utilized for normalization of expression data of liver tissues harvested from 6-month trial. In 9-month trial, pair of only two most stable genes (V2/V3 combination) gave V value below the threshold of 0.15. Therefore, as per V value, the combination of two RGs could be suggested to normalize the qPCR data in liver tissue of mice during 9-month trial.

Identification of RGs by NormFinder: NormFinder that is also excel-based statistical approach works intra and inter group variation. In the present analysis, NormFinder identified *SDHA* (0.155), *HMBS* (0.299) and *ACTB* (0.318) as most stable genes whereas *B2M* (0.642) and *GUSB* (0.685) were least stably expressed genes in 6-month trial (Table 2). However, during the 9-month trial, NormFinder identified *PGK1* (0.078), *GUSB* (0.191) and *SDHA* (0.238) as the most stable genes, whereas *B2M* (0.329) and *ACTB* (0.458) as least stable (Table 3).

Table 2. Identification of RGs by NormFinder during the 6-month trial

Gene name	*SDHA*	*HMBS*	*ACTB*	*PPIA*	*PGK1*	*B2M*	*GUSB*
Stability value	0.155	0.299	0.318	0.47	0.62	0.642	0.685
Best gene						*SDHA*	
Stability value						0.155	
Best combination of two genes						*SDHA* and *HMBS*	
Stability value for best combination of two genes						0.172	

Table 3. Identification of RGs by NormFinder in 9-month trial

Gene name	*PGK1*	*GUSB*	*SDHA*	*HMBS*	*PPIA*	*B2M*	*ACTB*
Stability value	0.078	0.191	0.238	0.25	0.257	0.329	0.458
Best gene					*PGK1*		
Stability value					0.078		
Best combination of two genes					*PGK1* and *GUSB*		
Stability value for best combination of two genes					0.102		

Table 4. Standard deviation (SD) and coefficient of variance (CV) analysis by using BestKeeper in 6-month trial

Column data	***PPIA***	***SDHA***	***ACTB***	***PGK1***	***HMBS***	***GUSB***	***B2M***	**Best**	**Keeper**
n	13	13	13	13	13	13	13	13	13
geo Mean [CP]	22.41	27.48	24.09	27.78	27.98	26.31	21.88	—	25.30
ar Mean [CP]	22.50	27.58	24.23	27.96	28.06	26.45	21.92	—	25.40
min [CP]	18.08	23.90	20.28	22.89	24.56	22.13	19.57	0.00	21.76
max [CP]	24.31	29.80	26.77	30.58	30.00	29.89	23.96	7.00	27.37
std dev [± CP]	1.55	2.16	2.38	2.77	1.87	2.35	1.21	—	2.01
CV [% CP]	6.90	7.83	9.82	9.92	6.66	8.89	5.53	—	7.93

n: number of samples; geo Mean [CP]: geometric mean of cycling point; ar Mean [CP]: arithmetic mean of CP; min [CP] and max [CP]: extreme values of CP; std dev [±CP]: standard deviation of the CP; CV [%CP]: coefficient of variation expressed as a percentage on the CP values

Identification of RGs by BestKeeper: BestKeeper algorithm was used to find the optimal RGs by analyzing the pairwise correlation between the genes. It allows a comparative analysis across potential reference genes by estimating correlations of the expression levels between all possible candidates. Gene expression variation was analyzed based on C_T-values and results were displayed as standard deviation (SD) and coefficient of variance (CV). All the candidate genes showed SD within limit (<1) and thus could be termed as stage RGs for data normalization in 6 (Table 4) and 9 months of trial (Table 5). Ranking of genes with least SD analysis of HKG vs. BestKeeper during the 6 months and 9 months of experimental trials are presented in Table 6 and 7, respectively. Ranking of genes with least coefficient of correlation [r] analysis of HKG vs. BestKeeper during 6 months and 9 months of experimental trial is presented in Table 8 and 9, respectively. Ranking of genes with least regression analysis of HKG vs. BestKeeper during 6 months and 9 months of experimental trial presented in Table 10 ans Table 11, respectively.

Identification of RGs by RefFinder: Additionally, RefFinder algorithm was used to evaluate the overall ranking of RGs in 6 months

Table 5. Standard deviation (SD) and coefficient of variance (CV) analysis by using Bestkeeper in 6-month trial

Column data	*PPIA*	*SDHA*	*ACTB*	*PGK1*	*HMBS*	*GUSB*	*B2M*	Best	Keeper
n	17	17	17	17	17	17	17	17	17
geo Mean [CP]	17.87	24.24	22.22	23.64	26.72	26.45	18.17	—	22.50
ar Mean [CP]	17.88	24.25	22.25	23.64	26.72	26.45	18.20	—	22.51
min [CP]	16.37	23.58	19.95	23.00	25.86	25.69	14.83	0	21.88
max [CP]	18.61	25.34	23.49	24.25	27.55	27.79	19.27	7	23.18
std dev [± CP]	0.41	0.29	0.92	0.33	0.38	0.43	0.50	—	0.37
CV [% CP]	2.31	1.18	4.14	1.38	1.41	1.62	2.73	—	1.64

Table 6. Ranking of genes with least SD analysis of HKG vs. BestKeeper in 6-month trial

Pearson correlation coefficient (r)	*PPIA*	*SDHA*	*ACTB*	*PGK1*	*HMBS*	*GUSB*	*B2M*
vs.	HKG 1	HKG 2	HKG 3	HKG 4	HKG 5	HKG 6	HKG 7
HKG 2	0.85	-	-	-	-	-	-
p-value	0.00	-	-	-	-	-	-
HKG 3	0.85	1.00	-	-	-	-	-
p-value	0.00	0.00	-	-	-	-	-
HKG 4	0.75	0.98	0.98	-	-	-	-
p-value	0.00	0.00	0.00	-	-	-	-
HKG 5	0.84	0.99	0.98	0.97	-	-	-
p-value	0.00	0.00	0.00	0.00	-	-	-
HKG 6	0.75	0.93	0.93	0.94	0.92	-	-
p-value	0.00	0.00	0.00	0.00	0.00	-	-
HKG 7	0.88	0.97	0.97	0.92	0.95	0.85	-
p-value	0.00	0.00	0.00	0.00	0.00	0.00	-

Table 7. Ranking of genes with least SD analysis of HKG vs. BestKeeperin 9-month trial

Pearson correlation coefficient (r)	*PPIA*	*SDHA*	*ACTB*	*PGK1*	*HMBS*	*GUSB*	*B2M*
vs.	HKG 1	HKG 2	HKG 3	HKG 4	HKG 5	HKG 6	HKG 7
HKG 2	0.52	-	-	-	-	-	-
p-value	0.03	-	-	-	-	-	-
HKG 3	0.18	0.03	-	-	-	-	-
p-value	0.49	0.92	-	-	-	-	-
HKG 4	0.51	0.63	0.63	-	-	-	-
p-value	0.04	0.01	0.01	-	-	-	-
HKG 5	0.25	0.38	0.36	0.66	-	-	-
p-value	0.33	0.13	0.15	0.00	-	-	-
HKG 6	0.59	0.31	0.36	0.66	0.32	-	-
p-value	0.01	0.23	0.15	0.00	0.21	-	-
HKG 7	0.22	0.36	-0.06	0.23	-0.24	0.54	-
p-value	0.39	0.16	0.81	0.38	0.36	0.02	-

Table 8. Ranking of genes with least coeff. of corr. [r] analysis of HKG vs. BestKeeperin 6- month trial

Best Keeper vs.	*PPIA*	*SDHA*	*ACTB*	*PGK1*	*HMBS*	*GUSB*	*B2M*
coeff. of corr. [r]	0.87	1.00	0.99	0.97	0.99	0.94	0.97
p-value	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 9. Ranking of genes with least coeff. of corr. [r] analysis of HKG vs. BestKeeper in 9- month trial

BestKeeper vs.	*PPIA*	*SDHA*	*ACTB*	*PGK1*	*HMBS*	*GUSB*	*B2M*
coeff. of corr. [r]	0.66	0.59	0.6	0.86	0.41	0.83	0.58
p-value	0	0.01	0.01	0	0.1	0	0.02

Table 10. Ranking of genes with least regression analysis of HKG vs. BestKeeper in 6-month trial

Regression analysis: HKG vs. Best Keeper	*PPIA*	*SDHA*	*ACTB*	*PGK1*	*HMBS*	*GUSB*	*B2M*
	TG 1	TG 2	TG 3	TG 4	TG 5	TG 6	TG 7
	vs.	vs.	vs.	vs.	vs.	vs.	vs.
	BK	BK	BK	BK	BK	BK	BK
coeff. of corr. [r]	0.87	1.00	0.99	0.97	0.99	0.94	0.97
coeff. of det. [r^2]	0.76	1.00	0.99	0.95	0.97	0.89	0.93
intercept [CP]	2.33	0.47	-5.24	-6.45	4.56	-2.86	6.56
slope [CP]	0.79	1.07	1.16	1.35	0.93	1.15	0.60
SE [CP]	±1.073	±0.162	±0.307	±0.753	±0.362	±0.967	±0.389
p-value	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Power [x-fold]	1.73	2.10	2.24	2.56	1.90	2.23	1.52

Table 11. Ranking of genes with least regression analysis of HKG vs. BestKeeper in 9-month trial

Regression analysis: HKG vs. Best Keeper	*PPIA*	*SDHA*	*ACTB*	*PGK1*	*HMBS*	*GUSB*	*B2M*
	TG 1	TG 2	TG 3	TG 4	TG 5	TG 6	TG 7
	vs.	vs.	vs.	vs.	vs.	vs.	vs.
	BK	BK	BK	BK	BK	BK	BK
coeff. of corr. [r]	0.66	0.59	0.6	0.86	0.41	0.83	0.58
coeff. of det. [r^2]	0.44	0.35	0.36	0.74	0.17	0.69	0.33
intercept [CP]	-1.91	11.43	-14.37	6.33	16.63	1.88	-10.25
slope [CP]	0.88	0.57	1.63	0.77	0.45	1.09	1.26
SE [CP]	±0.445	±0.349	±0.969	±0.204	±0.445	±0.326	±0.796
p-value	0	0.01	0.01	0	0.1	0	0.02
Power [x-fold]	1.84	1.48	3.09	1.7	1.36	2.13	2.4

Table 12. Overall ranking of RGs in 6-month and 9-month trials

Ranking	6 Month	9 Month
1	*SDHA* (1.41)	*SDHA* (1.19)
2	*ACTB* (2.45)	*HMBS* (1.86)
3	*HMBS* (2.71)	*PGK1* (2.45)
4	*B2M* (3.50)	*ACTB* (3.72)
5	*GUSB* (4.47)	*PPIA* (5)
6	*PPIA* (5.12)	*GUSB* (6)
7	*PGK1* (5.63)	*B2M* (7)

and 9 months experimental trial (Xie F *et al*., 2012). In this data set, *SDHA* (1.41) and *ACTB* (2.45) was most stable RGs followed by *HMBS* (2.71), *B2M* (3.50) in 6-month experimental trial, while *SDHA* (1.19) and *HMBS* (1.86) most stable RGs followed by *PGK1* (2.45), *ACTB* (3.72) in 9-month experimental trial. However, *PPIA* (5.12), *PGK1* (5.63) was unstable RGs in 6-month experimental trials and *GUSB* (6), *B2M* (7) was unstable RGs in 9-month experimental trial in Table (12).

DISCUSSION

In the present study, expression stability of seven candidate RGs *PPIA*, *ACTB*, *PGK1*, *SDHA*, *HMBS*, *GUSB*) and *B2M* was evaluated to identify the most appropriate panel of RGs in mice liver tissues fed with A1 and A2 milk. Four statistical tools (geNorm, NormFinder, BestKeeper and RefFinder) were utilized to evaluate the expression stability of these candidate RGs. Therefore, selection of suitable RGs become very necessary for reliability of qPCR data. To the best of our knowledge, till now, no studies have reported the panel of RG for normalization of target gene expression data in liver tissue of mice fed with different variants of A1 and A2 milk.

Numerous qPCR based studies have been conducted in mice to evaluate the effect of different experimental conditions such as growth, nutritional supplements, feeding, obesity, disease, drug treatment, physiological and development stages on gene expression pattern of target genes. The accurate interpretation of such studies was based on correct panel of RGs identified for each experimental condition. For example, *HMBS* and *B2M* was identified as most stable RGs in liver tissue of mice using geNorm, NormFinder and BestKeeper (Gong *et al*., 2016). Similarly, *ACTB* and *YWHAZ* were identified as most stable RGs in liver of C57BL/6 mouse during standard or high polyphenol feeding (Day *et al*., 2018). Another notable mice based study has identified *RPLP0* or *HPRT1* as most stable genes in a concanavalin A induced hepatitis model (Shi *et al*., 2010).

Apart from these, *PPIA* and *TBP* genes were identified as stable RGs in regenerating liver tissue of partially hepatectomy mouse (Tatsumi *et al*., 2008). In addition, Wang and Xu (2010) reported panel of the internal control genes in eight types of cells involved in liver regeneration in mice. In another study, Hildyard *et al*. (2019) reported *RPL13a*, *CSNK2A2*, *AP3D1* and *ACTB* as stable RGs in healthy and dystrophic muscles in mouse. Several other studies have been conducted to identify panel of RGs for normalization of qPCR in mouse with various disease such as disease type-I diabetes or obesity (Almeida-Oliveira *et al*., 2017), Colitis (Eissa *et al*., 2016), stroke (Li *et al*., 2015), muscular atrophy (Nakao *et al*., 2015), epilepsy (Pernot *et al*., 2010) and liver regeneration (Tatsumi *et al*., 2008). In past, our group has also published many studies related to identification of appropriate RGs in tissues of buffaloes (Kaur *et al*., 2018), heat stressed MECs of riverine buffaloes (Kapila *et al*., 2013)

and heat stressed PBMCs of native cows and riverine buffaloes (Kishore *et al*., 2013).

In the present investigation, *SDHA*, *PGK1* and *ACTB* genes were identified as most appropriate panel of RGs in liver tissue of mice fed with milk-based diet (A1A1, A2A2 and A1A2) for a period of 6 months and 9 months. This information will be quite useful to assess the impact of feeding mice with milk based diet derived from cows with diverse genetic background on liver tissues.

In conclusion, the four statistical tools were consistent in identifying the set of RGs as the most stable. Amongst the seven candidate RGs, *SDHA* was identified as the most stably expressed RG in the liver tissue of mice fed with the milk based diet and thus could be utilized as an effective normalizer for qPCR based expression data of target genes.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: MM, MS, RSK and AKM: Conceived and designed the study; VC, AK, GB, VS, SKN, SD and PS: Performed the feeding trials and collected the liver samples; VC, AK, PAS and PV: Performed qRT-PCR experiments; VC, MT and MM: Conducted bioinformatics and data analysis; MS, MM, VC and MT: Drafted manuscript.

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