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Selection of stably expressed internal control genes in circulating polymorphoneutrophils of periparturient Sahiwal (*Bos indicus*)cows

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ABSTRACT

Around parturition, animal undergoes a state of immune-suppression and become more susceptible to pathogens. This study was aimed to identify appropriate internal control genes (ICG) for transcriptional studies in polymorphoneutrophils (PMN)of Sahiwal (*Bos indicus*) cows during the periparturient period. The study involved 4 periparturient Sahiwal cows (–21 days to 0 day to +21 days relative to parturition) and 10 known internal control genes (ICG)from different functional classes. ForqPCR reaction following conditions were employed: 2 min at 50°C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60°C (annealing + extension).To measure the transcript stability of 10 ICG genes, threesoftwareprogrammes;geNorm, Normfinder and BestKeeper were used. All the genes showed acceptable expression stability as per the recommended threshold values. The geNorm analysis showed *RPS9*, *RPS15A* and *RPS23* genes to be most stably expressed while *HMBS* and *HPRT1* showed least stability.The expression stability for all the 10 ICG were within the acceptable range (M value <1.5).The pair wise variation analysis recommended the use of *RPS9*, *RPS15A*genes for normalization. The geNorm and Normfinder identified same set of most and least stable genes. BestKeeper analysis identified *ACTB*, *RPS9/ B2M*,*RPS23*, *RPS15A* genes showing consistent expression while *HMBS* and *HPRT1* showed relatively much higher variability. Our analysis identified *RPS9*, *RPS15A*and *ACTB*genesassuitable ICG to provide accurate normalization totranscriptional dataof PMN during the periparturient stages of Indian cows.

Key words: Indian cattle, PMN, Periparturient stage, Internal control genes, Normalization, q-PCR

During periparturinet period, dairy animals go through a series of physiological adaptations from late gestation through calving that could affect her overall health and subsequent productivity. Around parturition, they come into a temporary state of negative energy balance experience a state of immune-suppression and become more susceptible to pathogens. The negative energy balance that accompanies a reduction in feed intake around calving is thought to be a major contributor for higher incidence of infection. Although the reason for severe immune-suppression around the time of calving is not yet clear, it is hypothesized that metabolic challenges associated with the onset of lactation are factors capable of affecting immune function. Neutrophils from dairy animals provide an excellent model for studying periparturient disorders, as these cells are known to exhibit impaired immune function causing increase susceptibility of the animals to coliform bacteria (Kehrli*et al.* 1989, Cai *et al.*1994). Neutrophils provide the main immunologic defense against pathogens, but due to its depressed functional capacity during peripartum period; animals may become hypo-responsive to invading pathogens and thus more susceptible to infectious agents. However, little is known about the parturition induced changes in neutrophil gene expression and associated physiological changes in peripartum Indian zebu cattle. It is important to understand the mechanism that mediate changes in neutrophil function during the periparturient period in Indian zebu cattle and identify key genes that are directly involved in neutrophil mediated immune function. Such an effort will also provide basis to understand the basic neutrophil biology in Indian zebu(*Bos indicus*) cows. To undertake studies related to gene expression changes it is important to evaluate and identify battery of stably expressed ICG in Indian zebu cattle PMN cells during peripartum period. Real time quantitative PCR (*qPCR*) established as a preferred tool to measure gene expression changes is quite sensitive to variations that might occur

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due to differing amount of starting material, pipetting errors, efficiencies of RNA extraction and reverse transcription (Vandesompele *et al.* 2002, Huggett *et al.* 2005, and Valasek & Repa 2005) and require normalization to achieve accurate quantification. The use of internal control genes (ICG) or housekeeping genes (HKG) that have constant expression in response to experimental treatment or physiological state is an effective mean for normalization of expression data to account for the experimental variations (Deindl *et al.* 2002, Dheda *et al.* 2004, Radonic*et al.* 2004, Tramontana *et al*. 2008, and Kadegowda *et al.* 2009). To the best of our knowledge, no such report is available till date for Indian zebu cattle.The present study was conducted on animals of Sahiwal cattle with the aim to identify stably expressed ICG in PMN cells during peripartum period in order to initiate the transcriptional studies.

MATERIALS AND METHODS

Sampling and isolation of PMN cells

This study included 4 periparturient Sahiwal cows maintained at National Dairy Research Institute, Karnal. The 7 pre-parturition to peri-parturition stages (–21 days to 0 day to +21 days. were selected for isolation of polymorphonuclear leukocytes. All cows were fed a common lactation diet post-partum. The blood for neutrophil isolation was collected in EDTA vacutainers on –21, –14, –7, 0 (day of calving),+7, +14 and +21 days (relative to parturition). Neutrophils for RNA extraction were promptly isolated by centrifugation after lysis of erythrocytes. Approx. 25 ml.of blood was collected at each time point and brought to the laboratory at 4°C and PMN was isolated using theprotocol(Oh *et al.* 2008).The cells were transferred to a tube containing chilled Trizol reagent, homogenized, and stored at –80 °C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from isolated PMN collected at 7 time points during the peripartum period using ice-cold Trizol. RNeasy Mini Kit columns were used according to the manufacturer's protocolto remove traces of genomic DNA. RNA purity and concentration was evaluated by absorbance readings using the NanoDrop ND-1000 spectrophotometer. A260/A280 values range from 1.88 to 2.12, respectively. All the extracted RNAs were stored at –80°C and utilized within one month. 100 ng of total RNA was reverse transcribed using SuperScript IIIand cDNA was synthesized as described in our previous publication (Aggarwal *et al*.2013). The cDNA was subsequently diluted 1:4 (v:v) in nuclease free water and stored at –20°C. Sufficient cDNA was prepared in a single run to perform the *qPCR* experiments for all selected genes.

Selection of genes and primer design

A total of 10 candidate reference genes*viz*., glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)*, beta- actin (*ACTB*), ubiquitouslyexpressed transcript (*UXT*), ribosomal protein S15a (*RPS15A*), beta 2-microglobulin (*B2M*), alpha 2-microglobulin (*A2M*), ribosomal protein L–4 (*RL4*), ribosomal protein S18 (*RS18*), ribosomal protein S9 (*RS9*), ribosomal protein S22 (*RPS22*), ribosomal protein S23 (*RPS23*), hydroxymethylbilane synthase (*HMBS*), hypoxanthine-guanine phosphoribosyltransferase1 (*HPRT1*), GTP binding protein (*GTP*), eukaryotic translation elongation factor 1 alpha 1(*EEF1A1*) and ubiquitin C (*UBC*) from different functional categories were selected for the present study (Table 1). The primers were either designed on bovine GenBank sequences using Primer express 3 Software (Applied Biosystem) or taken from published literature. The primers employed in the present study generated minimum amplicon size ranging between 60–150 bp and had limited 3' G+C content. The speciûcity of primers wastested using a BLAST analysis against the genomic NCBI databaseand UCSC's Cow (*Bos taurus*), genome browser gateway. Primers information including sequences and product sizes are summarized in Table 1. Primers specificity was further confirmed in 20 µL PCR reaction using the similar protocol as described for *qPCR* except for the final dissociation protocol. Five µL of the

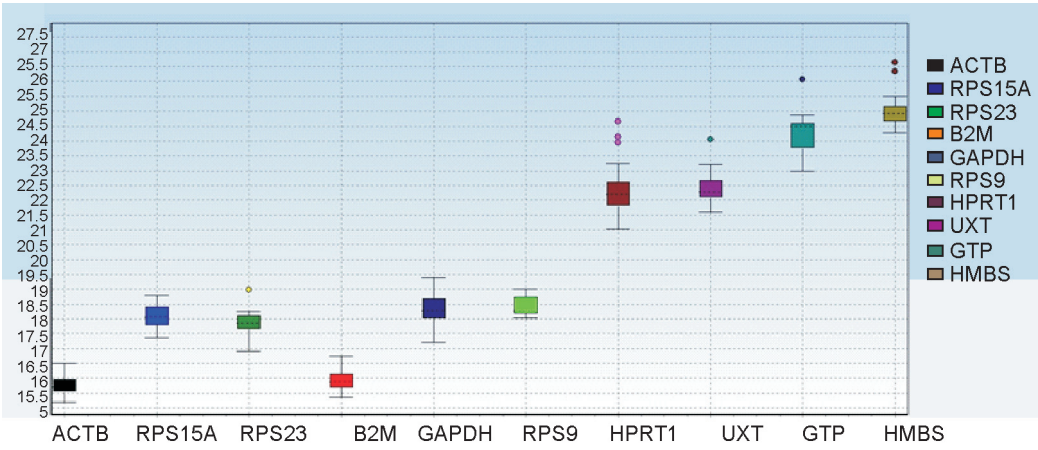

Fig. 1. Overall expression pattern of 10 genes evaluated in PMN of periparturient Sahiwal cows. The data is represented as q-PCR Ct values of each gene in the box and whisker diagram. The median is shown as a dashed line across the box. The boxes represent median, 1st and 3rd quartiles range while whiskers indicate maximum and minimum values.

x

Table 1. Gene name, accession numbers, cellular localization, biological functions, primer sequences, annealing temperature (T_a), amplicon length, PCR efficiency and regression coefficient for for set of candidate genes evaluated as ICGs in the present study

Gene Symbol & .Acc No	Cellular Localization	Biological Function/ Component	Primers 5'-3' (forward, reverse)	T_a	Amplicon Size (bp)	PCR Efficiency (%) *
ACTB -AY14 1970	Cytoplasm	Cytoskeletal structural protein, nucleotide and ATP binding	[1]GCGTGGCTACAGCTTCACC TTGATGTCACGGACGATTTC	60°C	56	1.93
B2M -NM_ 173893	Golgi membrane, Plasma membrane, early endosome membrane	Cytoskeletal protein, immune response, protein binding	CTGCTATGTGTATGGGTTC CGGAGTGAACTCAGCGTG	60°C	101	1.98
GAPDH- BC 102589	Plasma membrane	Glycolytic enzyme, Oxidoreductase in glycolysis and gluconeogenesis	[1]TGGAAAGGCCATCACCATCT CCCACTTGATGTTGGCAG	60°C	60	1.87
GTP- AK 074976	Cytoplasm and Nucleus	Biogenesis of the 60S ribosomal subunit	[1]CTTGGAATCCGAGGAGCCAC CTGGGATCACCAGAGCTGT	60°C	101	1.89
HMBS- BC112573.1	Cytoplasm	Heme synthesis, porphyrin metabolism, transferase activity	[2]CTTTGGAGAGGAATGAAGT GG AATGGTGAAGCCAGGAGGAA	60°C	80	1.86
HPRT1- BC103248	Cytoplasm	Purine synthesis in salvage pathway	[3]GAGAAGTCCGAGTTGAGTTTGG AAGGCTCGTAGTGCAAATGAAG	64°C 60°C	190 101	2.38 1.97
RPS15A- BC108231	Cytoplasm	Protein synthesis/40S subribosome	AGTAATGGTGCGCATGAATGTC ACTTTGGAGCACGGCCTAA			
RPS23- BC102049	Cytoplasm	Protein synthesis/40S subribosome	[1]CCCAATGATGGTTGCTTGAA CGGACTCCAGGAATGTCACC	60°C	101	1.90
RPS9- DT860044	Cytoplasm	Protein synthesis/40S subribosome	[1]CCTCGACCAAGAGCTGAAG CCTCCAGACCTCACGTTTGTTC	60°C	54	1.93
UXT- CR452243	Cytoplasm and Nucleus	Transcriptional activation, ATP binding, microtubule binding, unfolded protein binding	[1]TGTGGCCCTTGGATATGGTT GGTTGTCGCTGAGCTCTGTG	60°C	101	1.97

[1]Bionaz and Loor (2007), [2]Pérez et al. (2008),[3]Hernandez et al. (2009)

*qPCR efficiencies for each primer pair was calculated from 6 point standard curves using 5 fold dilution series of pooled cDNA from control and heat stressed samples

PCR product was run in a 2% 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*.

Quantitative PCR (qPCR)

All *qPCR* reactions were performed *in a* 10 ?Lûnal volumecontaining6 ?L of a mixture composed of 5 ?L 2 X LightCylcer 480 SYBR Green I master mix (Roche, Germany), 50nM *of* each speciûc primer (0.4 ?L each of 10 ?M forward and reverse primers), 0.2 ?L DNase/RNase free water and 4 ?Lof diluted cDNA in a 96 well white plate (Roche, Germany).PCR ampliûcation on 21cDNA samples with 10 primer-pairswas run onLightCylcer 480 (Roche, Germany)using 96-well optical plates under the following conditions: 2 min at 50°C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60°C (annealing + extension). Each reaction was run induplicate (technical replicates) along with 6 point relative standard curveand a no-template control(NTC)was included using water instead of cDNA. To conûrmsingle gene-speciûc peaks dissociation protocol with incremental temperatures of 95 °C for 15 s plus 65°C for 15 s was used. The qPCR expression data for each ICG was extracted in the form of crossing points. The data was extracted using the 'second derivative maximum' method (Rasmussen *et al.* 2001) as computed by the LightCycler Software 3.5 (Roche Diagnostics) and subjected for subsequent analysis.

Data analysis

For assessment of reference genes expression stability,*qPCR* data was analyzed using three different statistical algorithms: geNorm version 3.5 (Vandesompele *et al.* 2002), NormFinder version 0.953 (Andersen *et al.* 2004) and BestKeeper version 1 (Pfafû *et al.* 2004) as per the developers' recommendations. For geNorm (http://medgen.ugent.be/~jvdesomp/geNorm/) and NormFinder (http://www.mdl.dk/), comparative Cycle threshold (Ct) values were converted to relative quantities whereas input data for BestKeeper (http://gene-quantification.com/bestkeepr.html) were the raw Ct values and the PCR efficiencies.

The expression stability of candidate reference genes was first analyzed using geNorm software that calculates the best ICG on the basis of most stable transcript levels (M value). Pair-wise variation (V), between the normalization factor (NF) obtained using n genes (best references; NF_n) and the NF obtained using n+1 (NF_{n+1}) genes (addition of an extra less stable reference gene) was also calculated using geNorm. A cut-off value of 0.15 for the pair wise variation (V value) was considered acceptable**.** Thestability of ICG was further analyzed using Normfinder software. Normfinder algorithm ranked the candidate ICGs by calculating their expression stability taking into account possible variation across the different sample groups. The standard deviation (SD) as well as the accumulated standard deviation (Acc.SD), a good indicator for optimal number of reference genes to be used was also calculated. Another statistical approach facilitated by Bestkeepersoftware was used to find out the pair wise correlations amongst the candidate ICGs (Pfaffl *et al.* 2004).The BestKeeper analysis included calculation of crossing point standard deviations [{SD,±CP.} <1] and results were displayed as standard deviation (S.D) and coefficient of variance (C.V). The programme has its assumption that the genes which are stably expressed should be highly correlated to each other.

RESULTS AND DISCUSION

RNA quality and expression levels of the candidate ICG

The RNA extracted from all the PMN samples were of high quality as indicated by mean A260/280 ratio of 2.05±0.028. Gene-specific amplification was confirmed by a single peak in melt-curve analysis and a single band with the expected size in agarose gel electrophoresis(data not shown). High amplification efficiencies ranging from 0.88 to 1.06 were observed for all the analyzed genes. The characteristics of each of the 10 genes based on their Ct values are shown as box whisker plot in Fig. 1. The average Ct values for the 10 ICG were quite variable (15.77 for *ACTB* to25.15 for *HMBS*) and showed a wide range of expression levels. Based on broad range of expression, the ICG were grouped into three classes. Class 1 comprised of 2 genes*;* (*ACTB*, *B2M*) with mean Ct values of 15.23 while class 2 included 4 genes*; RPS23*, *GAPDH*, *RPS15A* and *RPS9* that clustered around mean Ct value of 18.16. Class 3 also included 4 genes; *HPRT1*, *UXT*, *GTP*, and *HMBS* and showed lower expression with mean Ct values of 23.54. Most of the 10 genes evaluated showed consistent expression level in PMN at different time point of peripartum Sahiwal cows (Suppl. Fig. 1). *ACTB* and *B2M* were the most abundant while *HMBS* was the least abundant transcripts in PMN of Sahiwal cows. The consistency of expression for 10 internal control genes has also been demonstrated by the heat map (Suppl. Fig. 2).

Fig.1 Overall expression pattern of 10 genes evaluated in PMN of periparturient Sahiwal cows. The data is represented as q-PCR Ct values of each gene in the box and whisker diagram. The median is shown as a dashed line across the box. The boxes represent median, 1st and 3rd quartiles range while whiskers indicate maximum and minimum values.

Expression stability evaluation by geNorm

The expression stability measures (M) of the 10 candidate ICGs was determined using geNorm applet.The M value ranged from 0.236 (*RPS9*, *RPS15A* to 0.636 (*HPRT1,* Suppl. Table 1). All candidate genes performed well displaying M values below the default limit of 1.5, below which the candidate ICG can be referred as stable expressed. The genes were ranked from the most stable (lowest M value) to the least stablegenes(highest M value): RPS9,RPS15A>RPS23>*ACTB*>*B2M*>*UXT*>*GAPDH*> *GTP*>*HMBS*>*HPRT1*. The lower M value is indicative of higher expression stability of the gene (Vandesompele *et al.* 2002). The ranking of each of the 10 ICGs according to their M value is shown in Fig.2. This plot was generated by a step wise exclusion of the least stable genes (highest M value) until only two candidate genes is left. The stability in terms of M value refers to constancy of the expression ratio between two non-co regulated genes among all samples tested. The more stable is the expression ratio among two genes, more likely the genes are appropriate internal controls, *i.e*. two ideal internal control genes should have an identical expression ratio in all samples regardless of experimental conditions, cell, and/or tissue type (Vandesompele *et al.* 2002).The presentgeNorm analysis showed that *RPS9*, *RPS15A*genes were the most stably expressed while *HPRT1* was the least stable gene amongst the 10 candidate ICG evaluated in PMN of periparturient Sahiwal cows. Similar approach has been used in a number of other studies to find out the suitable ICG (Brunner*et al.* 2004,Garcia-Crespo*et al.* 2005, Zhang*et al.* 2005, Bionaz and Loor 2007, and Stamova*et al.* 2009).Additionally, pair wise variation termed as 'V value' was also calculated to determine the optimal number of genes necessary to calculate a normalization factor. In this analysis, we started with the two most stably expressed genes and then sequentially included the less stably expressed genes. The contribution of each gene to the variance of normalization factor ratio was calculated to illustrate the effect of adding or removing a particular gene from the final set of reference genes. A large variation meant that the added gene had a significant effect and should probably be included for

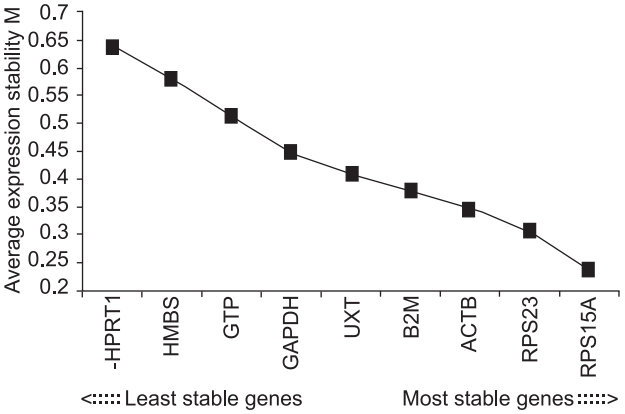

Fig. 2. Ranking of genes based on average expression stability values (M). The M values of each reference genes was plotted from least stable (left) to most stable (right).

calculation of the normalization factor.As per the recommendations of Vandesompele *et al.*(2002), the least number of genes showing Vvalue below the cut off limit of 0.15 was selected as optimal number of genes for normalization.

Our geNorm result showed that a combination of only two most stable genes (RPS9 and RPS15A), gave V value of 0.107 which is well within the acceptable limit (Fig. 3). The lower the pairwise variation better is the combination of genes. From this perspective, the V value was also calculated by adding the third and fourth less stable genes i.e. V3/V4 and V4/V5 combinations. This further reduces the V value to 0.086 and 0.074, respectively. However, as the aim in such studies is to achieve an overall variation of less than 0.15 with minimum genes possible, we suggest that as per geNorm outcome, *RPS9* and *RPS15A* gene combinations could be appropriate to normalize the target genes expression in PMN of periparturient Sahiwal cows.

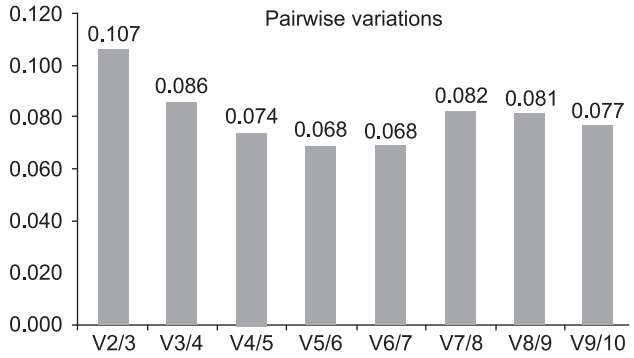

Fig. 3. Pair-wise variation (V) of normalization factor ratios to determination of optimal number of genes for normalization.

Normfinder expression stability analysis

The result of the Normfinder analysis is shown in Fig.4. Similar to geNorm, Normfinder also identified *RPS9*as most stableand *HPRT1* as least stablyexpressed genes (Fig. 4). In contrast to geNorm, Normfinder measure the expression stability of each gene independently from each other. However, more often than not the results from geNorm and Normfinder are comparable as both use relative quantities as raw input data. In the present study as well, there was

Table 2. Standard deviation (S.D.) and accumulated standard deviation (Acc.S.D.) estimated by Normfinder

Gene Name	S.D.	Acc. S.D.
RPS9	*0.2536*	0.2536
ACTB	*0.2862*	*0.1912*
RPS15A	0.2917	*0.1603*
UXT	0.3359	0.1467
B2M	0.3359	0.1352
RPS23	0.3397	0.1261
GAPDH	0.5042	0.1299
GTP	0.5705	0.1342
HMBS	0.7141	0.1432
HPRT1	0.7551	0.1494

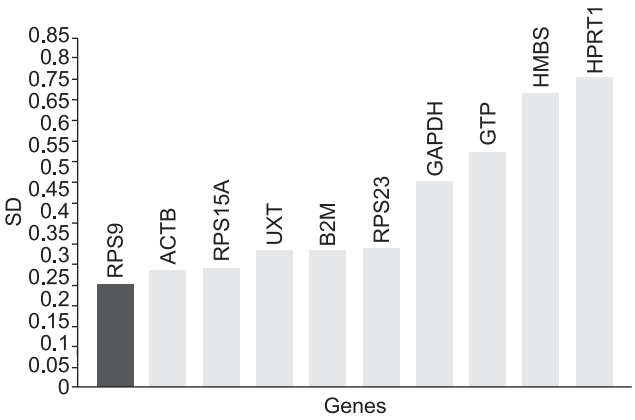

Fig. 4. Bar plot showing gene variability in 10 candidate ICG by Normfinder. The optimum reference gene is highlighted in red (RPS9).

good agreement between geNorm and Normfinder outcome, albeit slight variation was observed in the ranking of other genes.The analysis revealed *ACTB* to be the second most stable gene followed by *RPS15A* and *UXT*. In geNorm analysis, *ACTB* gene was ranked at third position. But similar to geNorm, Normfinder also found *GAPDH*, *GTP*, *HMBS* and *HPRT1* as most variable genes and were ranked last amongst the 10 candidate genes. The bar plot showing variability for each gene is depicted in Fig.4. The standard (SD) and accumulated standard deviation (Acc SD) of each gene are presented in Table 2.

BestKeeper expression stability analysis

The Bestkeeper algorithm was used to calculate gene expression variation based on Ct values. Each of the 10 candidate reference showed consistent expression levels (Table 3). The *ACTB*gene revealed minimum variation with lowest crossing point S.D. value of 0.26. This was followed by *RPS9/B2M*,*RPS23*, *RPS15A*genes with S.D. value of 0.27/0.27, 0.30, and 0.31, respectively. All these genes were stably expressed as the values were well within the acceptable range of SD <±1.0 Ct. On the other hand,*HMBS* and *HPRT1* genes were again found to be the least stable.

The slightly different outcome with BestKeeper in comparison to geNorm and Normfinder could be explained due to the fact that this algorithm uses Ct values input data while the other two softwares uses raw relative quantities. Further, in BestKeeper we took into account the standard deviations of Ct values as BestKeeper Index. The highly correlated ICG were combined into BestKeeper index and the correlation between each gene and BestKeeper was estimated (Table 4). The relationship between ICG and BestKeeper index was described in terms of Pearson correlation coefficient (r), coefficient of determination (r^2) and the *p*-value (Table 4). The best correlation between BestKeeperand ICG was observed for *UXT* (r = 0.812) followed by *HPRT1*, *RPS9RPS23* and *RPS15A* genes. The *HPRT1* gene was ranked as most unstable gene by different algorithm, but correlation analysis revealed second best value for *HPRT1* (r=0.779).

Overall inference and final ranking of ICG

Apart from minor variations, the analysis using different algorithm indicated *RPS9, RPS15A, RPS23*and *ACTB*to be stable genes. Further, the mean Ct value for these genes (Ct <19) indicated their higher expression in PMN of periparturient Shaiwal cows. Traditionally, *GAPDH* and *ACTB, RS18*genes have commonly been employed as housekeeping genes in many studies (Bustin, 2000, Vandesompele *et al.* 2002,Musters*et al.* 2004, Huggett *et al.* 2005, Thorn *et al.* 2006, 2007, and Deindl*et al.* 2008). However, because of the sensitivity and dynamic range of real-time pCR, the expression of so called housekeeping genes was found to be unstable and varied with the treatment, type of tissue/cells (Suzuki *et al*. 2000, Vandesompele *et al.* 2002,Bionaz and Loor, 2007,Janovick Guretzky *et al.* 2007, Stamova*et al.* 2009, and Tong *et al.* 2009). Zhang *et al.* (2005) identified panel of suitable reference genes in human neutrophils, however their data suggested the use of *GNB2L1*, *HPRT1*, *RPL32*, *ACTB*, and *B2M* genes for normalization. Interestingly, in contrast to our findings, *HPRT1* was reported to be reasonably stable in human neutrophils (Zhang *et al.* 2005). Similarly De Ketelaere *et al.* (2006) reported the SDHA, YWHAZ, and 18S rRNA as being the most stable genes in bovine PMN indicating the necessity to identify appropriate ICG for each species and experimental types.

The present study successfully demonstrated the utility of three software tools to test multiple reference genes and identify panel of genes whose expression stability was maximum in PMN samples of periparturient Sahiwal cows. From the present data set, *RPS9, RPS15A, RPS23* and *ACTB* genes outscoredother candidate reference genesin terms of

Table 3. Descriptive statistics for 10 candidates ICG based on cycle threshold (Ct)

	ACTB	RPS15A	RPS23	B2M	GAPDH	RPS9	HPRT1	UXT	GTP	HMBS
n	21	21	21	21	21	21	21	21	21	21
geo Mean [Ct]	15.77	18.07	17.84	15.93	18.31	18.41	22.35	22.37	24.24	25.14
ar Mean [Ct]	15.77	18.08	17.84	15.93	18.32	18.41	22.37	22.38	24.25	25.15
min [Ct]	15.19	17.38	16.90	15.35	17.22	18.02	21.03	21.59	23.00	24.28
max [Ct]	16.49	18.80	18.98	16.74	19.39	19.00	24.64	24.06	26.07	27.54
std dev [± Ct]	0.26	0.31	0.30	0.27	0.40	0.27	0.67	0.38	0.52	0.52
CV [% Ct]	1.63	1.72	1.69	1.72	2.20	1.46	3.00	1.70	2.15	2.08
min [x-fold]	-1.49	-1.62	-1.91	-1.50	-2.13	-1.31	-2.49	-1.72	-2.37	-1.82
max [x-fold]	1.65	1.66	2.21	1.75	2.11	1.51	4.90	3.22	3.55	5.27
std dev [± x-fold]	1.19	1.24	1.23	1.21	1.32	1.21	1.59	1.30	1.43	1.44

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 4. Repeated pair-wise correlation analysis amongst 10 genes and with BestKeeper Index (BI). Pearson coorelation coefficient (r), and the probability value (p) are shown.

		ACTB	RPS15A	RPS23	B2M	GAPDH	RPS9	HPRT1	UXT	GTP	HMBS
RPS15A	r	0.245	-	-	-	-	-	-	-	-	-
	p-value	0.285	-	-	-	-	-	-	-	-	-
RPS23	r	0.412	0.667	-	-	-	-	-	-	-	-
	p-value	0.064	0.001	-	-	-	-	-	-	-	-
B2M	r	0.149	0.321	0.434	-	-	-	-	-	-	-
	p-value	0.517	0.155	0.049	-	-	-	-	-	-	-
GAPDH	r	0.719	0.046	0.401	0.094	-	-	-	-	-	-
	p-value	0.001	0.844	0.071	0.686	-	-	-	-	-	-
RPS9	r	0.520	0.779	0.576	0.157	0.168	-	-	-	-	-
	p-value	0.016	0.001	0.006	0.499	0.468	-	-	-	-	-
HPRT1	r	0.336	0.330	0.380	0.609	0.282	0.249	-	-	-	-
	p-value	0.135	0.145	0.089	0.003	0.216	0.277	-	-	-	-
UXT	r	0.376	0.605	0.591	0.319	0.318	0.696	0.570	-	-	-
	p-value	0.093	0.004	0.005	0.158	0.161	0.001	0.007	-	-	-
GTP	r	-0.015	0.546	0.193	0.266	-0.232	0.406	0.534	0.481	-	-
	p-value	0.945	0.010	0.401	0.245	0.311	0.067	0.013	0.027	-	-
HMBS	r	0.416	0.068	-0.054	0.066	0.207	0.211	0.312	0.310	0.472	-
	p-value	0.061	0.767	0.813	0.775	0.369	0.359	0.169	0.172	0.031	-
BI-10 genes	r	0.626	0.667	0.674	0.546	0.47	0.677	0.779	0.812	0.601	0.511
p-value	p-value	0.002	0.001	0.001	0.010	0.032	0.001	0.001	0.001	0.004	0.018

expression stability. From these findings, we conclude that although any of the top four genes might be sufficient as reference gene in PMN of peripartum Sahiwal cows, still more than one gene has always been preferred for producing more accurate data. Based on the stability analysis carried out in the present study, we suggest the inclusion of *RPS9, RPS15A*and *ACTB*genes as reference genes for normalization of PMN transcriptional data.To our knowledge as this is the first ever report on testing the stability of prospective ICG in PMN of Indian native cattle, the present analysis was restricted to some of the well known house- keeping genes reported in other studies.The ICG identified in the present study would be helpful for accurate normalization of PMN expression data during peripartum period- an important stage affecting the performance and overall well-being of dairy animals.

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