Indian Journal of Animal Sciences **88** (3): 319–00, March 2018/Article

Relative gene expression analysis of β-casein gene and its transcription regulatory genes in primary buffalo mammary epithelial cells of Surti and Jaffarabadi buffaloes

GAURAV PANDYA[1], UMED RAMANI[2], MAMTA JANMEDA[3], KULDEEP TYAGI[4],
BALKRUSHNA BRAHMKSHTRI[5] and VISHNU KHARADI[6]

Navsari Agricultural University, Navsari, Gujarat 396 040 India

Received: 11 July 2017; Accepted: 4 September 2017

ABSTRACT

The present study was designed to estimate the relative gene expression of major genes responsible for β-casein and its transcription regulation during early lactation cycle in Surti and Jaffarabadi buffaloes. Ten buffaloes of each breed maintained at Livestock Research Station, Navsari and Cattle Breeding Farm, Junagadh, respectively were selected for the study. The RNA was extracted from noninvasively isolated primary buffalo mammary epithelial cells (pBMEC) obtained using antibody mediated magnetic separation method from milk samples collected at day 15 and 60 postpartum (pp). Primers used for amplification of β-casein gene (*CSN2*) and its transcription regulatory genes were bovine specific. The mean relative expressions of C/EBPβ, RUNX2 and STAT5A genes were significantly higher in Jaffarabadi buffaloes as compared to Surti buffaloes at day 15 pp. However, between breed differences in the mean relative expressions of C/EBPβ, RUNX2 and STAT5A genes were not observed at 60 days pp. The relative expressions of CSN2 and YY1 genes did not differ significantly within or between breeds. The mean relative expression of KRT8 gene was almost comparable among all the groups under present study. Thus, only β-casein transcription regulatory genes C/EBPβ, RUNX2 and STAT5A amongst all studied genes showed significant between breed variability at day 15 pp, however this variability too got subsided as lactation stage advanced to day 60 pp.

Key words: Buffaloes, β-Casein gene, Gene expression, Jaffarabadi buffalo, Transcription regulatory genes, Surti buffalo

Milk is produced in the udder by mammary epithelial cells (MEC). Milk contains MEC, which are gradually exfoliated from the epithelium during lactation. Isolation of MEC from milk using immunomagnetic separation may be a useful non-invasive method to investigate transcriptional regulations in ruminants' udder. Isolation of viable MEC directly from fresh milk using antibody mediated magnetic bead separation method (Boutinaud *et al.* 2008) made repeatable gene expression studies possible throughout the lactation of the same animal.

Milk has a diverse composition of nutrients. Main constituents of bovine milk are lactose, protein and fat. Among those, fat appears to be the most affected by diet while the lactose and protein are mostly determined by genetic background (Bauman *et al.* 2006, Jenkins and McGuire 2006). Among the many nutrients provided in dairy milk and dairy products, milk protein is an important part of daily protein intake in the human diet. β-casein (encoded by *CSN2*), the second most abundant protein in bovine milk, is the most extensively studied milk protein. The proximal promoter of *CSN2* has lactogenic response elements that harbour multiple or a single binding site(s) for transcription factors, mainly including signal transducer and transcription activator 5 (STAT5), Runt-related transcription factor 2 (RUNX2), CAAT/enhancer binding protein β (C/EBPβ) and the repressive transcription factor Yin Yang 1 (YY–1) (Qian and Zhao 2014). Promoters of buffalo *CSN2* have also been isolated and characterized to drive mammary gland specific gene expression for the therapeutic purpose (Ganguli *et al.* 2015). Knowledge about the regulation of expression of the different milk protein genes during lactation can be a great tool for the genetic improvement of milk composition and milk yield. Most of the studies pertaining to milk protein synthesis and its transcriptional regulation in MEC have been carried out in mice, cattle, sheep or goats, but there are very few reports in buffalo. Considering these facts, the present study was designed to estimate the relative gene expression of major genes responsible for β-casein and its transcription

Present address: [1,3]Assistant Professor (gmpandya@nau.in, janmedamamta14@gmail.com), Department of AGB; [2] Assistant Professor (uvramani@nau.in), Department of ABT; [5]Professor (bpbkhatri@gmail.com), Department of ILFC, College of Veterinary Science and Animal Husbandry. [4]Assistant Research Scientist (naulrs@gmail.com), [6]Research Scientist (vbkharadi @nau.in), Livestock Research Station.

regulation, viz. *STAT5A, RUNX2, C/EBPβ* and *YY1* during lactation cycle in Surti and Jaffarabadi buffaloes.

MATERIALS AND METHODS

Animals and sample collection: Surti and Jaffarabadi buffaloes (10 each), maintained at Livestock Research Station, Navsari and Cattle Breeding Farm, Junagadh, respectively were selected for the study. Milk samples were collected in a sterile bucket to harvest pBMEC, repeatedly from the same buffaloes at 15th and 60th day pp. Buffaloes were categorized for data analysis and comparisons in to four groups, based on breed and stage of sample collection viz. S15 (Surti buffaloes 15th day pp), S60 (Surti buffaloes 60th day pp), J15 (Jaffarabadi buffaloes 15th day pp) and J60 (Jaffarabadi buffaloes 60th day pp). Fifteen hundred milliliter of the total milk was transferred in autoclaved glass bottles and was subjected for cell isolation.

Isolation of pBMECs and RNA isolation: Antibody mediated magnetic bead separation method was used for successful isolation of somatic cells and pBMEC from buffalo milk samples (Sigl *et al.* 2012) with minor modifications. Defattening of the milk and isolation of total somatic cells was carried out by centrifugation. Purification of pBMEC from total milk somatic cells, resuspended in 1% BSA-PBS, was performed applying an immunomagnetic bead based separation technique using 2.5 µl primary mouse monoclonal antibody against cytokeratin 8 antibody (clone C-43), which was specific to bovine epithelial cells. Dynabeads (25 µl) were used to collect antibody-bound cells by placing the sample vials into the DynaMag™ and were resuspended in 700 µl Qiazol®. The suspension was subjected to RNA isolation using miRNeasyMiniKit® as per the manufacturer's protocol.

cDNA synthesis and realtime PCR: An accurate amount (500 ng) of good quality RNA was taken and reverse transcription was carried out according to the manufacturer's instructions (QuantiTect® Reverse Transcription Kit) in a PCR thermocycler (AB_i). The genes responsible for the synthesis of β-casein and its transcription regulation were investigated for their differential expression along with marker gene for epithelial cells. The primers were commercially synthesized from a published reference (Sigl *et al.* 2014). *RPS9*, being reported as one of the most stable housekeeping gene, was used as reference gene (Yadav *et al.* 2012). Ten biological samples were run in technical triplicates for better precision of the data. Relative expression of β-casein, its transcription regulatory and keratin genes were quantified by Real Time PCR and analyzed using Applied Biosystems 7500 software v2.0.5.

Statistical analysis: Quantitative cycle (Cq) values were calculated by Applied Biosystems 7500 software v2.0.5. The ΔCq values were calculated as:

ΔCq = Cq target gene transcript – Cq reference gene transcript (Pfaffl 2001)

In order to avoid negative digits while allowing an estimation of a relative comparison between two time points, data were presented as Mean±SEm subtracted from the arbitrary values 10–ΔCq ($\log_2$) as suggested by Sigl *et al.* (2012). Thus, a high ΔCq value resembled high transcript abundance.

Additionally, the amount of target normalized to an endogenous reference gene and relative to a calibrator can be obtained by following expression.

Fold increase/decrease in target =$2^{-\Delta\Delta C}$q (Livak and Schmittgen 2001)

The data on various estimated values was subjected to statistical analysis using R (version 3.3.0) software. Descriptive statistics specifying Mean±SEm, were calculated for each group. One way ANOVA procedure was undertaken to compare means. Post Hoc multiple comparisons were made using Duncan's New Multiple Range Test (DMNRT).

RESULTS AND DISCUSSION

The mean relative expression '10-ΔCq ($\log_2$)' values of β-casein gene, its transcription regulatory genes and keratin gene among different groups are presented in Table 1.

The relative transcript abundance of *CSN2* gene was steady among the four groups under study. Thus, the results of present study revealed that relative expression of *CSN2* was not affected by the stage of lactation (P>0.05) as well as breed of buffaloes, which was in agreement with the findings of the previous study in Surti and Mehsani buffaloes (Tyagi 2015). However, significant changes in

Table 1. Mean relative expression '10-ΔCq ($\log_2$)' values of β-casein gene, its transcription regulatory genes and keratin genes in different groups

Group	S15	S60	J15	J60	F values
N	10	10	10	10	
CSN2	4.04±0.07 (6.45)	3.94±0.09 (5.35)	4.08±0.07 (6.91)	4.07±0.06 (6.80)	0.41
C/EBPβ	3.39^{b}±0.06 (0.48)	3.55^{ab}±0.09 (1.71)	3.69^{a}±0.12 (2.91)	3.61^{ab}±0.07 (2.21)	4.03*
RUNX2	2.98^{b}±0.16 (–2.11)	3.26^{ab}±0.13 (–0.42)	3.42^{a}±0.16 (0.70)	3.40^{a}±0.11 (0.56)	5.20*
STAT5A	3.10^{b}±0.22 (–1.43)	3.39^{ab}±0.07 (0.48)	3.53^{a}±0.08 (1.55)	3.45^{ab}±0.08 (0.93)	4.01*
YY1	3.39±0.07 (0.48)	3.57±0.15 (1.88)	3.66±0.14 (2.64)	3.53±0.15 (1.55)	0.81
KRT8	3.37±0.06 (0.34)	3.45±0.10 (0.93)	3.50±0.11 (1.31)	3.52±0.06 (1.47)	1.72

*Significant at P≤0.05; Values in parenthesis are mean-ΔCq values. Means bearing different superscript between groups differed significantly.

relative expression of CSN2 gene at various stages of lactation have been reported (Sigl *et al.* 2012, 2014) in Holstein Friesian cows. Relative expression of *CSN2* from MECs during more advanced stage of lactation i.e. 70–80 days pp in Sahiwal cattle has also been studied (Janjanam *et al.* 2013). Mean relative expression of *C/EBPβ* differed significantly ($P \leq 0.05$) among breeds during early lactation. A significant increase in mRNA abundance of *C/EBPβ* from day 8 to day 57 pp has been reported (Sigl *et al.* 2014), which was in agreement with the findings of Surti buffaloes in the present study. The relative transcript abundance of *RUNX2* was significantly ($P \leq 0.05$) higher in Jaffarabadi buffaloes at both the stages of lactation as compared to those in Surti buffaloes. However, stage wise difference within breeds was not observed for mean relative expression of *RUNX2.* Thus, it can be said that relative expression of *RUNX2* was affected significantly by the breeds of buffaloes taken in the present study. Constant relative expression of *RUNX2* gene during first 155 days of lactation had also been reported in Holstein Friesian cows (Sigl *et al.* 2014). The mean relative expression of *STAT5A* gene was significantly ($P \leq 0.05$) higher in Jaffarabadi buffaloes as compared to Surti buffaloes during early lactation. Peculiarly, in the present study, increase of 7.87 and 5.11 folds in relative expression of *STAT5A* were observed in J15 and J60 groups as compared to S15 group, respectively. This may probably be attributed to the breed differences and gene environment interaction. However, stage-wise, no difference in relative expression of *STAT5A* was observed within breed. The similar finding has been reported in Holstein dairy cows up to 240 days of lactation (Bionaz and Loor 2011). No influence of lactation stage up to first 155 days of lactation in the transcript abundance of *STAT5A* was also reported (Sigl *et al.* 2014) in Holstein Friesian cows. The mean relative expression of *YY1* gene was at par in both the breeds at both the stages of lactation, which was in agreement with the previous report in Holstein Friesian cows upto first 155 days of lactation (Sigl *et al.* 2014).

The relative expression of *KRT8* gene was almost similar among all the four groups. It was constantly expressed irrespective of the breed and stage of lactation. The epithelial keratins had earlier been also useful markers for epithelial cells (Taylor-Papadimitriou *et al.* 1989). Transcript abundance of *KRT8* gene was also constant at different lactation stages in Holstein Friesian cows (Sigl *et al.* 2012, 2014) and Surti and Mehsani buffaloes (Tyagi 2015) at various stages of lactation.

Overall, the relative expression of the *CSN2* gene in the present study showed no change in the expression pattern among Surti and Jaffarabadi buffaloes at various stages of lactation. Relative expression of *C/EBPβ*, *RUNX2* and *STAT5A* was lower in Surti buffaloes as compared to Jaffarabadi buffaloes at day 15 pp. The relative expression of *YY1* was constant in all the four groups under study. The relative expression of *KRT8* was also constant in all the four groups under study, suggesting the role of *KRT8* as epithelial cell marker gene. Extension of this study with proteomics analysis can throw more light on the transcription regulation of *CSN2* gene at various stages of lactation in Indian buffaloes.

ACKNOWLEDGEMENT

We acknowledge the Dean, Veterinary College, NAU, Navsari for providing financial support and necessary laboratory facilities to carry out this research work. We also thank Research Scientist, Cattle Breeding Farm, Junagadh Agricultural University, Junagadh for providing milk samples and data to carry out this study.

REFERENCES

Bauman D E, Mather I H, Wall R J and Lock A L. 2006. Major advances associated with the biosynthesis of milk. *Journal of Dairy Science* **89**: 1235–43.

Bionaz M and Loor J J. 2011. Gene networks driving bovine mammary protein synthesis during the lactation cycle. *Bioinformatics and Biology Insights* **5**: 83–98.

Boutinaud M, Ben-Chedly M H, Delamaire E and Guinard-Flament J. 2008. Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. *Journal of Dairy Science* **91**: 988–98.

Ganguli N, Ganguli N, Usmani A and Majumdar S S. 2015. Isolation and functional characterization of buffalo (*Bubalus bubalis*) β-casein promoter for driving mammary epithelial cell-specific gene expression. *Journal of Biotechnology* **198**: 53–59.

Janjanam J, Jamwal M, Singh S, Kumar S, Panigrahi A K, Hariprasad G, Jena M K, Anand V, Kumar S, Kaushik J K, Dang A K, Mukesh M, Mishra B P, Srinivasan A, Reddy V S and Mohanty A K. 2013. Proteome analysis of functionally differentiated bovine (*Bos indicus*) mammary epithelial cells isolated from milk. *Proteomics* **13**: 3189–3204.

Jenkins T C and McGuire M A. 2006. Major advances in nutrition: impact on milk composition. *Journal of Dairy Science* **89**: 1302–10.

Qian X and Zhao F. 2014. Current major advances in the regulation of milk protein gene expression. *Critical Reviews™ in Eukaryotic Gene Expression* **24**: 357–78.

Sigl T, Meyer H H D and Wiedemann S. 2012. Gene expression of six major milk proteins in primary bovine mammary epithelial cells isolated from milk during the first twenty weeks of lactation. *Czech Journal of Animal Science* **57**: 469–80.

Sigl T, Meyer H H D and Wiedemann S. 2014. Gene expression analysis of protein synthesis pathways in bovine mammary epithelial cells purified from milk during lactation and short-term restricted feeding. *Journal of Animal Physiology and Animal Nutrition* **98**: 84–95.

Taylor-Papadimitriou J, Stampfer M, Bartek J, Lewis A, Boshell M, Lane E B and Leigh I M. 1989. Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to *in vivo* phenotypes and influence of medium. *Journal of Cell Science* **94**: 403–13.

Tyagi K K. 2015. Relative gene expression analysis of milk protein genes in primary milk epithelial cells of Surti and Mehsani buffaloes. Thesis submitted to Navsari Agricultural University, Navsari.

Yadav P, Singh D D, Mukesh M, Kataria R S, Yadav A, Mohanty A K and Mishra B P. 2012. Identification of suitable housekeeping genes for expression analysis in mammary epithelial cells of buffalo (*Bubalus bubalis*) during lactation cycle. *Livestock Science* **147**(1–3): 72–76.