EVALUATION OF MILK FAT AS AN ALTERNATIVE SOURCE OF RNA FROM BUFFALO MILK

Anju Sharma, Yashila Girdhar, Mandeep Kaur, Sonika Ahlawat, Ramesh Kumar Vijh and Reena Arora*

ABSTRACT

The study reports optimization of RNA extraction method from buffalo milk fat, to be used for gene expression analysis. Higher yields were obtained from buffalo milk fat as compared to skim milk, with manual method as well as commercial kits. Quality and integrity of the samples from both methods was comparable. Quantitative profiling of mammary gland specific genes from skim milk and fat derived RNA validated the efficiency of MFG as an alternate source of total RNA. This study confirmed that the RNA obtained from buffalo MFG was suitable for studies of mammary genes expression.

Keywords: buffaloes, *Bubalus bubalis*, fat, milk, qPCR, RNA

INTRODUCTION

Milk is a secretion of the mammary gland and a rich source of RNA (Lemay *et al*., 2013). Recent studies have begun to recognize milk somatic cells as a non-invasive source of DNA (Pokorska *et al*., 2016) as well as RNA (Boutinaud *et al*., 2002) for research on mammary tissue. Extraction of RNA from milk somatic cells is well established for humans (Munch *et al*., 2013), bovines (Boutinaud *et al.,* 2008), sheep (Mura *et al*., 2013) and goats (Maningat *et al*., 2007). Use of mammary epithelial cells (MEC) in milk as a source of RNA is considered a non invasive method for transcriptome studies of mammary gland (Boutinaud *et al*., 2015). Milk fat has been reported to be reliable and non-invasive source of MEC for gene expression studies in goat (Brenaut *et al*., 2012) and humans (Lemay *et al*., 2013). Choudhary *et al*. (2015) have suggested that milk fat globule (MFG) could serve as a source of RNA of mammary epithelial cells. Since MFG in the buffalo milk is larger than MFG of cow milk (Menard *et al*., 2010), greater amount of RNA is expected to be isolated. However, no specific commercial kits are available for extraction of RNA from MFG. The main aim of the study was to optimize and compare the RNA extraction protocol for isolation of RNA from buffalo milk fat by manual as well as commercial kits. Further, efficiency of the isolated RNA was also evaluated by real time gene expression analysis.

Milk samples from healthy buffaloes housed in cattle yard of ICAR-NDRI, Karnal were chosen. The samples were collected manually into sterile, RNase-free tubes, taking care to avoid any RNase contamination. The samples were immediately placed on ice and analyzed within 4 h. RNA was isolated from two different sources namely milk and fat using three different methods:

Animal Biotechnology Division, ICAR-National Bureau of Animal Genetic Resources, Haryana, India, *E-mail: rejagati@gmail.com

1) Trizol extraction method, 2) Commercial RNA isolation kit and 3) Combination of Trizol method and commercial kit. All the experiments were carried out in triplicate.

MATERIALS AND METHODS

Approximately 50 ml milk was used for RNA extraction. The milk sample was centrifuged at 2000 x g for 20 minutes at 4°C in order to pellet somatic cells and to separate the milk fat layer. The upper fat layer was removed by a sterile spatula and transferred to a new 50 ml centrifuge tube. The remaining milk was then discarded and the walls of the centrifuge tube were wiped carefully with sterile cotton ball to remove the remaining fat. The cell pellet obtained was washed twice with 10 ml of PBS (Phosphate Buffered Saline), pH 7.2, with 0.5 mM EDTA (Ethylene-D-amine-Tetra-Acetic Acid) by centrifugation at 2000 x g for 20 minutes at 4°C, to eliminate casein and fat globules.

RNA extraction By Trizol method

The cell pellet was suspended in 1ml Trizol while the fat layer was suspended in 1.5 ml trizol/ 500 mg milk fat. The contents of both tubes were then homogenized by vortexing and incubated on ice for 5 minutes. In case of fat, the tubes were centrifuged at 12000 rpm for 10 minutes at 4°C to remove lipids and the liquid phase obtained was transferred to new 2 ml tubes, while the cell pellet was processed as such to next step. Chloroform (250 µl per ml of trizol) was added to the tubes and mixed properly for 15 seconds and then incubated for 3 minutes on ice. The tubes were then centrifuged at 12000 rpm for 15 minutes at 4°C. The upper aqueous phase was transferred to fresh 2 ml tubes. Isopropanol was added to the tubes twice the volume of upper aqueous phase. Linear acrylamide (1 µl) was also added to each tube to enhance precipitation of RNA. The tubes were incubated for 20 minutes on ice and centrifuged to obtain the total RNA pellet. The RNA pellet was washed twice with 1 ml of 70% ethanol, centrifuged at 750 x g for 10 minutes at 4°C. The pellet was then dried at room temperature, dissolved in nuclease free water and DNase treatment was given to obtain DNA free RNA.

RNA isolation by commercial kit (Qiagen RNeasy)

Total RNA isolation was performed as per protocol recommended in kit.

RNA isolation using Trizol and filter columns

RNA isolation was proceeded as mentioned in trizol method till the isopropanol incubation step. After incubation, upto 700 µl of the sample was transferred to filter column (provided in commercial RNA isolation kit - Qiagen RNeasy), placed in a 2 ml collection tube and centrifuged for 15 seconds at 10000 rpm. The successive aliquots of the sample were centrifuged in the same column and the flow through was discarded after each centrifugation. On-column DNase treatment was given as per given protocol. Final elution of total RNA was done with 30 µl DEPC treated water. The column was kept at room temperature for 5 minutes and then centrifuged at 10000 rpm for 1 minute to elute the RNA. This step was repeated using elute to increase the RNA yield.

RNA samples obtained by all methods were immediately aliquoted and stored at -80°C. RNA concentration was measured with Nanodrop and purity was assessed using A_{260}/A_{280} as well as $A_{260/230}$ ratio. RNA integrity was assessed by electrophoretic analysis of 28S and 18S rRNA

subunits.

cDNA synthesis and qPCR

The total RNA extracted by the three procedures was used for synthesis of cDNA. The cDNA was synthesized using 100 ng of purified RNA, 1 µL 50 µM oligo (dT) 20, 1 µL 10 mM dNTP mix and nuclease free water to make the final volume 10 µl. The mixture was incubated at 65°C for 5 minutes and kept on ice for 2 minutes. A total of 10 µL of master mix composed of 2 µL 10X RT Buffer, 2 µL 0.1 M DTT, 4 µL 25 mM $MgCl_2$, 1 µL of SuperScript III RT (200 U/µl), and 1 µL of RNase OUT (40 U/µl) was added to cDNA mix. The reaction was performed in an Eppendorf Gradient cycler using the program: 50°C for 50 minutes and 85°C for 5 minutes. After chilling on ice for 5 minutes, RNase H (1 µl) was added to each tube and incubated for 20 minutes at 37°C followed by PCR amplification. Primer sequences published for some key genes like *RPS9, ACTB* and *CSN*2 (Cieslak *et al*., 2015) were used for validation of gene expression by qPCR. The qPCR reaction was performed in triplicate in a final volume of 10 µl containing 2 µl of cDNA, 8 µl of qPCR master mix (5 µl of SYBR Green Real-Time master mix, 0.3 µl of each primer, 2.4 µl of DNA/RNA-free water) on Roche Light cycler 480 system.

RESULTS AND DISCUSSION

In the present study, maximum yield of total RNA was obtained by Trizol method followed by purification using filter columns in case of both milk and fat. Least amount of mRNA was isolated using only commercial kits. However, much higher yields were obtained from buffalo milk fat as compared to skim milk, with manual method as well as commercial kits (Table 1). The quality of RNA obtained from all the three different procedures was similar. The $A_{260/280}$ ratio was observed to be between 1.9 and 2.05 in all cases. RNA has maximum optical absorption at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an $A_{260/A280}$ of 2.0, therefore, a value of 1.8 to 2.0 indicates that the RNA is pure (Gallagher, 1992, 1995). The $A_{260/230}$ ratio is also used to check contamination of certain residual chemicals such as phenol and guanidine used in the extraction process. A ratio of $A_{260/230}$ lying between 1.8 and 2.0 indicates that the RNA sample is free from these contaminants. In our experiment the ratio varied from 1.68 to 1.83 for milk and the readings were similar for fat derived RNA. A relatively lower $A_{260/230}$ ratio was obtained by Trizol extraction method. Our results indicate that Trizol method alone was not sufficient to remove the impurities, the use of filter columns however, improved the $A_{260/230}$ ratio. Hence the use of filter column after RNA extraction by Trizol method is recommended.

Integrity of RNA was also checked by agarose gel electrophoresis. Distinct bands of 28S and 18S rRNA subunits revealed undegraded RNA for all the methods used (Figure 1), therefore, quality and integrity of the samples from all methods used in the study was comparable. Similar results have been reported for goat MFG (Brenaut *et al*., 2012), lower quantity as well as quality of RNA was obtained from bovine MFG (Canovas *et al*., 2014).

In order to confirm that the total RNA extracted from buffalo milk fat represented the mammary epithelial cells, gene expression analysis of some marker genes was done. The quantitative-PCR (qPCR) was performed on cDNA derived from

Table 1. Summary of results from RNA extractions.

Methods used	Milk			Fat		
	RNA yield (ng/µl)	$A_{260/280}$	$A_{260/230}$	RNA yield (ng/µl)	$A_{260/280}$	$A_{260/230}$
Trizol method	10.26	2.01	1.68	130.70	1.81	1.56
Commercial kit method	6.80	2.05	1.82	7.70	2.06	1.79
Trizol + spin column method	29.63	1.99	1.83	371.20	2.08	1.82

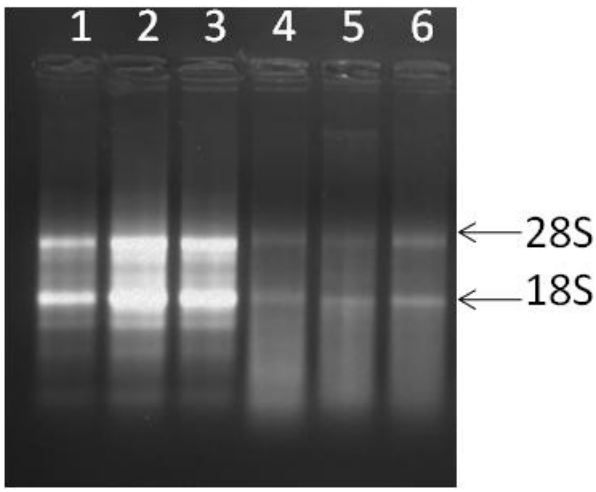

Figure 1. Agarose gel electrophoresis of total RNA extracted from buffalo milk fat (1, 2, 3) and skim milk (4, 5, 6).

both milk and fat RNA, using the specific primers for *RPS9, ACTB* and *CSN*2 genes. The average Ct value ranged from 23.02 to 26.69 for all the genes analyzed. Previous studies have also validated the expression of some major milk genes by qPCR, from RNA derived from human milk fat (Lemay *et al*., 2013) and goat milk fat (Brenaut *et al*., 2012). Hence, quantitative profiling of mammary gland specific genes from skim milk and fat derived RNA validated the efficiency of MFG as an alternate source of RNA. This study confirmed that the RNA obtained from buffalo MFG was suitable for studies of mammary genes expression. Hence, buffalo milk fat can be successfully used as an alternative source of total RNA for gene expression analysis.

ACKNOWLEDGEMENTS

This work was financially supported by ICAR Consortium Research Platform on Genomics-Buffalo genomics. We are grateful to

Director, ICAR-NBAGR for providing laboratory facilities. We are grateful to Director, ICAR-NDRI for permitting the collection of milk samples.

REFERENCES

Boutinaud, M., L. Herve and V. Lollivier. 2015. Mammary epithelial cells isolated from milk are a valuable, non-invasive source of mammary transcripts. *Frontiers in Genetics*., **6**: 323.

Boutinaud, M., M.H. Ben Chedly, E. Delamaire and J. Guinard-Flament. 2008. Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. *J. Dairy Sci*., **91**: 988-998.

Boutinaud, M., H. Rulquin, D.H. Keisler, J. Djiane and H. Jammes. 2002. Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. *J. Anim. Sci*., **80**: 1258-1269.

Brenaut, P., R. Bangera, C. Bevilacqua, E. Rebours, C. Cebo and P. Martin. 2012. Validation of RNA isolated from milk fat globules to profile mammary epithelial cell expression during lactation and transcriptional response to a bacterial infection. *J. Dairy Sci*., **95**: 6130-6144.

Canovas, A., G. Rincon, C. Bevilacqua, A. Islas-Trejo, P. Brenaut, R.C. Hovey, M. Boutinaud, C. Morgenthaler, M.K. VanKlompenberg, P. Martin and J.F. Medrano. 2014. Comparison of five different RNA sources to examine the lactating bovine mammary gland transcriptome using RNA-sequencing. *Scientific Reports*, **4**: 5297.

Cieslak, J., M. Mackowski, G. Czyzak-Runowska, J. Wojtowski, K. Puppel, B. Kuczynska and P. Pawlak. 2015. Screening for the most suitable reference genes for gene expression studies in Equine Milk Somatic Cells. *PLoSONE.,* **10**(10), e0139688. http://doi.org/10.1371/journal.pone.0139688

Choudhary, R.K., H. Kaur, S. Choudhary and R. Verma. 2015. Distribution and analysis of milk fat globule and crescent in Murrah buffalo and crossbred cow. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, **87**(1): 167-172.

Gallagher, S.R. 1992. Quantitation of DNA and RNA with absorption and fluorescence spectroscopy, p. A3-5-A3-8. *In* Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (eds.) *Short Protocols in Molecular Biology*. Greene Publishing Associates and John Wiley and Sons, New York, USA.

Gallagher, S.R. 1995. Quantitation of DNA and RNA with absorption and fluorescence spectroscopy, p. A.3D.1-A.3D.8. *In* Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (eds.) *Current Protocols in Molecular Biology.* Vol. 3. John Wiley and Sons, New York, USA.

Lemay, D.G., R.C. Hovey, S.R. Hartono, K. Hinde, J.T. Smilowitz, F. Ventimiglia, K.A. Schmidt, J.W. Lee, A. Islas-Trejo, P.I. Silva, I. Korf, J.F. Medrano, P.A. Barry and J.B. German. 2013. Sequencing the transcriptome of milk production: milk trumps mammary tissue. *BMC Genomics*, **14**: 872.

Maningat, P.D., P. Sen, A.L. Sunehag, D.L. Hadsell and M.W. Haymond. 2007. Regulation of gene expression in human mammary epithelium: Effect of breast pumping. *J.*

Endocrinology., **195**: 503-511.

Menard, O., S. Ahmad, F. Rousseau, V. Briard-Bion, F. Gaucheron and C. Lopez. 2010. Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. *Food Chem.,* **120**: 544-551.

Munch, E.M., R.A. Harris, M. Mohammad, A.L. Benham, S.M. Pejerrey, L. Showalter, M. Hu, C.D. Shope, P.D. Maningat, P.H. Gunaratne, M. Haymond and K. Aagaard. 2013. Transcriptome profiling of microRNA by Next-Gen deep sequencing reveals known and novel miRNA species in the lipid fraction of human breast milk. *PLoS One*., **8**: e50564.

Mura, M.C., C. Daga, S. Bodano, M. Paludo, S. Luridiana, M. Pazzola, M.L. Dettori, G.M. Vacca and V. Carcangiu. 2013. Development of a RNA extraction method from milk for gene expression study in the mammary gland of sheep. *Mol. Biol. Rep*., **40**: 2169-2173.

Pokorska, J., D. Kulaj, M. Dusza, J. Zychlinska-Buczek and J. Makulska. 2016. New Rapid Method of DNA Isolation from Milk Somatic Cells. *Anim. Biotech*., **27**(2): 113-117.