Korean J. Food Sci. An. Vol. 35, No. 4, pp. 459~465 (2015)
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DOI http://dx.doi.org/10.5851/kosfa.2015.35.4.459
ISSN 1225-8563 eISSN 2234-246X

ARTICLE

Comparison of Total RNA Isolation Methods for Analysis of Immune-Related microRNAs in Market Milks

Sangnam Oh, Mi Ri Park, Seok Jun Son, and Younghoon Kim*

BK21 Plus Graduate Program, Department of Animal Science and Institute of Agricultural Science & Technology, Chonbuk National University, Jeonju, 561-756, Korea

Abstract

Bovine milk provides essential nutrients, including immunologically important molecules, as the primary source of nutrition to newborns. Recent studies showed that RNAs from bovine milk contain immune-related microRNAs (miRNA) that regulate various immune systems. To evaluate the biological and immunological activity of miRNAs from milk products, isolation methods need to be established. Six methods for extracting total RNAs from bovine colostrums were adopted to evaluate the isolating efficiency and expression of miRNAs. Total RNA from milk was presented in formulation of small RNAs, rather than ribosomal RNAs. Column-combined phenol isolating methods showed high recovery of total RNAs, especially the commercial columns for biofluid samples, which demonstrated outstanding efficiency for recovering miRNAs. We also evaluated the quantity of five immune-related miRNAs (miR-93, miR-106a, miR-155, miR-181a, miR-451) in milk processed by temperature treatments including low temperature for long time (LTLT, 63°C for 30 min)-, high temperature for short time (HTST, 75°C for 15 s)-, and ultra heat treatment (UHT, 120-130°C for 0.5-4 s). All targeted miRNAs had significantly reduced levels in processed milks compared to colostrum and raw mature milk. Interestingly, the amount of immune-related miRNAs from HTST milk was more resistant than those of LTLT and UHT milks. Our present study examined defined methods of RNA isolation and quantification of immune-specific miRNAs from small volumes of milk for use in further analysis.

Keywords: immune-related miRNA, bovine milk, colostrum, market milks

Received March 11, 2015; Revised April 23, 2015; Accepted April 23, 2015

Introduction

Bovine milk is widely used as a dairy product, including in infant formula, and it is an important material in the food industry. Bovine milk contains the nutrients needed for growth and development of the calf and is a resource of lipids, proteins, amino acids, vitamins, and minerals. It contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes, and other bioactive peptides to support human health and immunity (Haug *et al*., 2007).

Recent papers have demonstrated the existence of microRNAs in human breast milk that can modulate the immune system (Chen *et al*., 2010; Hata *et al*., 2010; Kosaka *et al*., 2010). Immune-related microRNAs such as miR-181a, miR-155, and miR-223 were detected, but organ- and tissue-specific microRNAs have not been found. MicroRNAs (miRNAs) are small regulatory RNA molecules that generally down-regulate the expression of specific mRNA targets and play important roles in a wide range of physiologic and pathologic processes (Ambros, 2004; Kim *et al*., 2009; Kloosterman and Plasterk, 2006). Generally, microRNAs are initially transcribed as several-hundred nucleotide-long miRNA transcripts, termed primary miRNAs (pri-miRNAs). Subsequently, precursor miRNAs (pre-miRNA) and mature miRNAs were generated through nuclear-and cytoplasmic processing, respectively (Gregory *et al*., 2006; Lund and Dahlberg, 2006). It was recently reported that the exosomal vesicles (microvesicles) secreted by cells contained mRNA and miRNA that can be transferred to and function in neighboring cells (Sun *et al*., 2013; Zhang *et al*., 2010). A recent study provided evidence that the bovine colostrum-derived microvesicles can transfer immune-related miRNAs (miR-24, miR-93, miR-181a, and miR-451) and modulate immune responses such as cytokine production and macrophage proliferation (Sun *et al*., 2013). RNA isolation from exosomes was evaluated using seven different methods inclu-

*Corresponding author: Younghoon Kim, BK21 Plus Graduate Program, Department of Animal Science and Institute of Agricultural Science & Technology, Chonbuk National University, Jeonju, 561-756, Korea. Tel: +82-63-270-2606, Fax: +82-63-270-2612, E-mail: ykeys2584@jbnu.ac.kr

ding phenol-based methods, combined techniques, and commercial kits (Eldh *et al*., 2012). Although a previous study presented a unique comparison for extraction of exosomal RNA, exosomes extracted from mouse mast cell lines were separated using an ultracentrifuge which needed to take a long time as well as that study did not examine for extracting milk-derived miRNAs.

Milk-derived miRNAs in microvesicles were resistant to acidic conditions and RNase treatment, and could be detected in infant formulas purchased from markets (Izumi *et al*., 2012). However, little is known about the effects of processing, especially pasteurization, on milk miRNA levels. Although synthetic miRNAs were rapidly degraded, endogenous miRNAs were found to be resistant to harsh treatments such as acid and RNase, though those treatments are not representative of the treatments applied in commercial dairy processing.

Given that milk derived miRNAs could be transferred to human through dietary means, it is very important to quantify milk-derived miRNAs involving human health and immunity for evaluating the quality of milk products with standardized methods.

The aim of the current study was to determine a quick and easy method of RNA isolation from a small volume of milk to evaluate the immune-related miRNAs. To achieve this goal, we utilized bovine colostrum, which contains an abundance of immune-related miRNAs, in six different RNA isolation methods including phenol reagents, columns and a phenol-free column. In addition, in order to evaluate the changes in miRNAs with temperature treatment, we quantified the immune-related miRNAs from bovine colostrum, raw mature milk, market milks by using the most suitable isolation method and quantitative RT-PCR.

In the present study, we demonstrated the easy and quick isolation of total RNA from a small volume of bovine milk and quantified immune-related miRNAs for further analysis of dairy products.

Materials and Methods

Milk samples

Bovine colostrum and raw mature milk samples were collected from the 1st and 2nd d of lactating and mid-lactating healthy Holstein cows (Korea). Milk samples were frozen immediately after milking and were kept at -80°C until use. Two types of pasteurized (low temperature for long time, LTLT, 63°C for 30 min and high temperature for short time HTST, 75°C for 15 s) and sterilized milk, ultra heat treated milk (UHT, 120-130°C for 0.5-4 s) were obtained from a local market (Korea).

Milk preparation

The milk samples were centrifuged twice (12,000×g, 4°C, 10 min) to remove fat, cells, and large debris. The defatted supernatant was then centrifuged (20,000×g, 4°C, 30 min) to remove residual fat and casein.

Extraction of total RNA

Total RNA was extracted from bovine colostrums (n=3) using six different methods: three phenol methods using either Trizol® (Invitrogen, UK), Qiazol® (Qiagen, Germany), or RNazol® (MRC, USA), and three column methods using the miRNeasy® Mini kit (miRNeasy I), miRNeasy® Mini kit for serum (miRNeasy II) (both from Qiagen), and miRCURY™ RNA Isolation Kit (Exiqon, Denmark). All methods were used according to the manufacturer's protocol with some modifications and are summarized in Table 1. To extract total RNA from milk for the establishment of a simple and easy protocol, 0.2 mL of milk in its liquid state was used. The milk samples were homogenized with 4 volumes (0.8 mL) of Trizol®, Qiazol®, or RNazol® Lysis Reagent, mixed thoroughly by pipetting 20 times, and incubated for 5 min at room temperature. To normalize the miRNA expression data and generate a standard curve obtained by qPCR, synthetic *Caenorhabditis elegans* cel-miR-39 (miScript miRNA Mimic, Qiagen; 3.5 μL of 1.6×10^8 copies/μL), as a spike-in control, was added and thoroughly mixed. Next, chloroform (0.2 mL of volume, equal to the volume of milk) was added to the homogenate, which was mixed thoroughly by vortexing, incubated for 5 min at room temperature, and centrifuged (12,000×g, 4°C, 15 min).

The three phenol extraction methods were followed by alcohol precipitation and washing without using columns. The resulting aqueous phases were mixed with equal volumes of 100% isopropyl alcohol and centrifuged (12,000 ×g, 4°C, 15 min). RNA precipitants were washed with 75% ethanol and centrifuged (8,000×g, 4°C, 5 min), twice. For combined phenol and column methods, the resulting aqueous phases from Qiazol® homogenates were mixed with 1.5 volumes of 100% ethanol and passed through miRNeasy I or miRNeasy II columns. Total RNAs extracted from all methods were reconstituted with 50 μL of nuclease-free water. The quality, quantity, and integrity of the RNAs were assessed on an Agilent 2100 Bioanalyzer using an RNA 6000 Pico Kit (both from Agilent Technologies, USA), according to the manufacturer's protocol.

Table 1. Flowchart showing the six different RNA isolation methods

Phenol-based RNA isolations	Step	Trizol®	Qiazol®	RNazol®
	1	Lysis and homogenization		
	2	Chloroform		RNase-free water
	3	RNA precipitation with isopropyl-OH		
	4	Wash twice		
	5	RNA reconstitution with RNase-free water		

Combined phenol and column methods	Step	Suitable Phenol reagent	
	1	Lysis and homogenization	
	2	Chloroform	
	3	RNA precipitation with EtOH	
		miRNeasy_I	miRNeasy_II
	4	Column load and RNA bind	
	5	Wash twice	
	6	RNA elution	

Phenol-free column method	Step	miRCURY™
	1	Lysis and homogenization
	2	Add Ethanol
	3	RNA bind
	4	Wash three times
	5	RNA elution

Total RNA from bovine colostrum was extracted using three phenol-based methods (Trizol®, Qiazol®, RNazol® lysis reagents), two phenol-combined column methods (miRNeasy I and II), and a phenol-free column kit (miRCURY™).

Quantification of miRNA by RT-qPCR

Quantitative RT-PCR was performed using the miScript System (Qiagen) according to the manufacturer's instructions with some modifications. In brief, to generate complementary DNA, 50 ng of total RNA was reverse-transcribed using the miScript II RT kit (Qiagen). The cDNA was diluted in 4 volumes of nuclease-free water and then subjected to qPCR on a StepOne Plus Real-Time PCR System (Applied Biosystems, USA) using the miScript SYBR green PCR kit. The miScript Primer assays (Qiagen, Germany) for the target miRNAs we used were as follows: MS00019789 for cel-miR39, MS00055027 for bta-miR-93, MS00050071 for bta-miR-106a, MS00044 835 for bta-miR-155, MS00050750 for bta-miR-188, MS00054166 for bta-miR-451. The real-time PCR protocol was used: PCR initial activation step of HotStarTaq DNA polymerase (95°C, 15 min); 40 cycles of denaturation (94°C, 15 s), annealing (55°C, 30 s), and extension (70°C, 30 s); and melting curve analysis.

To calculate the absolute expression levels of the target miRNAs, a series of synthetic miRNA oligonucleotides of known concentrations were also reverse-transcribed and amplified and used to generate a standard curve. The Ct value of cel-miR-39 was used to normalize the amount of immune-related miRNA in each sample and to compare the expression levels among milk samples.

Statistical analysis

The qRT-PCR assay was performed in triplicate and the entire experiment was repeated multiple times. The data shown are presented as the means±SEM of three or more independent experiments. The differences were considered statistically significant at $p<0.05$ and $p<0.001$ using Student's t-test.

Results and Discussion

Total RNA yield and size distribution

To establish an easy and quick method for isolating milk RNA, we used six different methods to extract total RNA from colostrum, which contains abundant microRNAs (Sun *et al*., 2013) (Table 1). We tested various ratios of volumes of milk, phenol reagents, and chloroform to optimize the extraction methods. The volume ratio used in the milk: phenol: chloroform was in 1:4:1, which produced higher ratios of A_{260}/A_{280} and more recovery of total RNA than did other ratios (data not shown). The quantities of total RNA extracted using the six different methods were assessed by a Bioanalyzer 2100 (Table 2). RNA integrity number (RIN), which is based on the ribo-

Table 2. Comparison of extraction efficiencies of six different isolating methods from bovine colostrums

Extraction method	Total RNA (ng)	Copies of spike in control	Extraction efficiency (%)
Trizol®	1041.25 ± 88.742	$2.899 \times 10^5 \pm 2.510 \times 10^4$	6.344 ± 0.549
Qiazol®	1152.75 ± 64.7	$2.090 \times 10^6 \pm 2.013 \times 10^5$	45.739 ± 4.405
RNazol®	288.75 ± 24.395	$6.419 \times 10^2 \pm 2.813 \times 10^0$	0.014 ± 0.050
miRCURY™	1266.25 ± 26.517	$2.196 \times 10^6 \pm 9.144 \times 10^4$	48.053 ± 2.001
miRNeasy I	1575.75 ± 143.189	$1.859 \times 10^6 \pm 2.579 \times 10^4$	40.674 ± 0.564
miRNeasy II	1603.25 ± 61.872 *	$3.622 \times 10^6 \pm 8.587 \times 10^4$	79.252 ± 1.879 **

Total RNA isolated using six different methods was analyzed using the RNA Pico kit with an Agilent 2100 Bioanalyzer. To compare the efficiency of RNA isolation, 5.6×10^8 copies of cel-miR-39, as a spike in control, were added to each homogenate from the six different methods and then amplified and quantified by RT-qPCR. From the analysis, the total RNA amounts and isolation efficiencies were obtained. Mean values are shown with standard deviations. Statistical analysis was conducted beween miRNeasy I and miRNeasy II in total RNA recovery and extraction efficiency (*p<0.05, n=5 and **p<0.001, n=5)

somal RNA, was not valid for milk RNA from microvesicles such as exosomes. Most of the methods tested were shown to be clear of protein contamination, as the A_{260}/A_{280} ratios were over 1.9 except for the RNazol® method. Since Qiazol® was shown to provide the highest yield among the three phenol-based extraction methods with good lysing ability, we applied the liquid phase from this method, including the RNA pool, to two column kits (miRNeasy_I and miRNeasy_II) followed by Qiazol® extraction. The two column kits resulted in outstanding yields of total RNA from milk, with greater than 1.5 mg per 200 microliters of bovine colostrum being recovered by RNA binding to the column matrix throughout the ethanol washing steps. Bioanalyzer electropherograms from all methods were shown to isolate total RNA with distribution patterns of small RNA, while the cellular apparent ratio of 28S rRNA to 18S rRNA was approximately 2:1 (Fig. 1).

To evaluate the extraction efficiencies of the different methods, focused on miRNA, we added synthetic *C. elegans* miR-39-1 miRNA mimic as a spike-in control with an exact number of copies (5.6×10^8 copies per sample) to

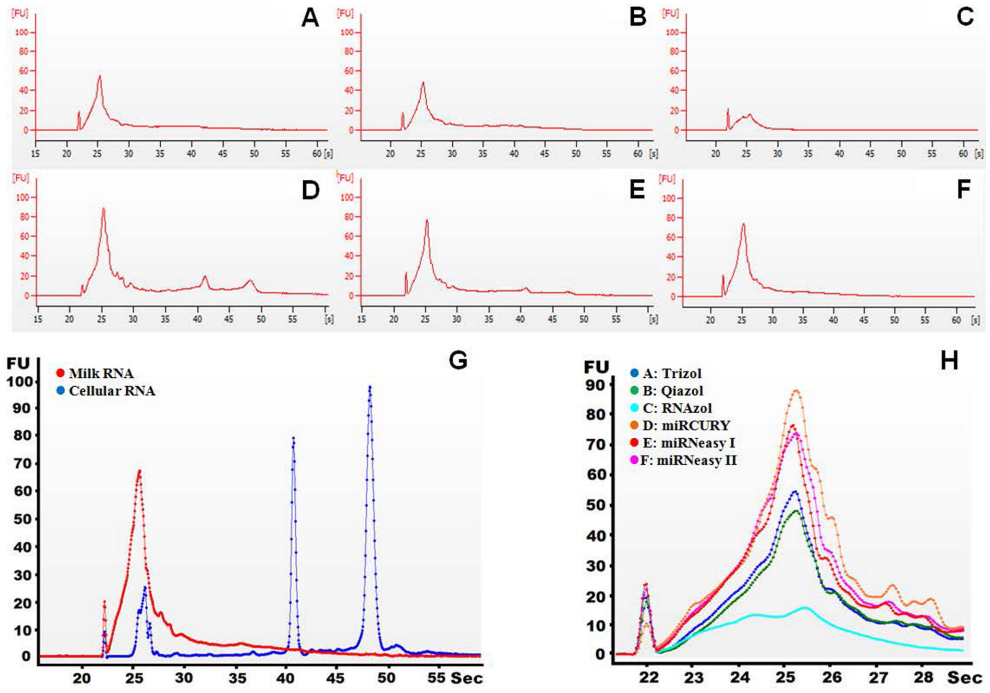

Fig. 1. RNA analysis of total RNA from bovine colostrum. Total RNA isolated with six different methods, (A) Trizol®, (B) Qiazol®, (C) RNAzol®, (D) miRCURY™, (E) miRNeasy_I, (F) miRNeasy_II, and (H) merged electropherograms was analyzed using RNA Pico 6000 kit in an Agilent 2100 Bioanalyzer. The electropherograms show the size distribution dependent on times (s) and fluorescence unit (FU) of total RNA in colostrum. (G) shows apparent different distribution of total RNA from bovine milk and mammalian primary cells (mouse mesenchymal stem cells), respectively.

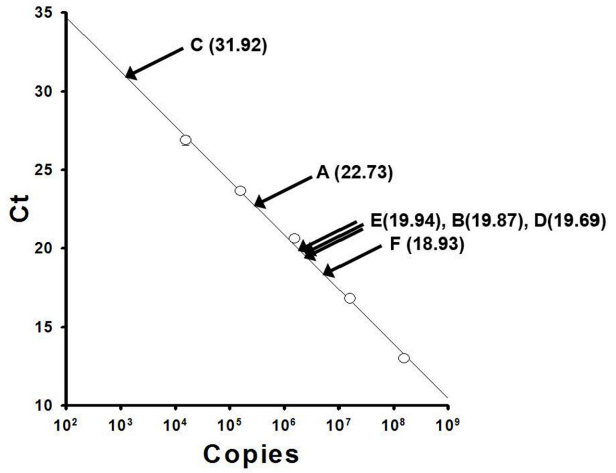

Fig. 2. Comparison of extraction efficiency of spike in control. Standard curve of cel-miR-39-1 was generated by 10 times serially diluted cDNA from reverse transcription reaction. Mean C(t) values of cel-miR-39-1 from six different isolating methods were plotted on the standard curve and used to calculate the numbers of isolated mimic copies and extraction efficiencies. (A) Trizol®, (B) Qiazol®, (C) RNAzol®, (D) miRCURY™, (E) miRNeasy I, and (F) miRN easy_II.

the Qiazol® homogenizing step to enable us to generate a standard curve and to allow us to assess the recovery of cel-miR-39-1 after RNA purification. The standard curve was generated by serially diluting the cDNA from a reverse transcription reaction (Fig. 2). The mean C(t) values of cel-miR-39-1 from six different isolation methods were plotted on the standard curve and used to calculate the numbers of isolated mimic copies and extraction efficiencies. As shown in Table 2, the miR Neasy_II kit (the kit for serum) resulted in the highest extraction efficiency of nearly 80 percent while total RNA recovery was similar to those of the two other columns. Trizol® and RNazol® showed very low extraction efficiency for cel-miR-39-1, meaning that most of the endogenous milk miRNAs were lost through the purification steps. The high extraction efficiency of the synthetic miRNA mimic suggests that the endogenous miRNAs from milk would be enriched as much as possible for further analysis.

In this study, we evaluated six different RNA isolation methods, based on phenol reagents and columns, to determine their total RNA recovery and extraction efficiency. Since milk RNAs are exosomal sources carrying small RNA, there was a need to standardize a quick and easy method using a small volume of milk (0.2 mL) to isolate RNA from exosomes to apply to further analysis and evaluation of dairy products. Qiazol® was shown to be the best homogenizing phenol reagent for milk exosomes, and the miRNeasy II method resulted in the best recovery and extraction efficiency among the six different isolation methods tested, based on the amount of cel-miR-39-1 recovered.

Relative amount of immune-related microRNAs in pasteurized and sterilized milk samples

To further evaluate the changes in miRNAs under temperature treatment, we used the miRNeasy_II method to quantify the amounts of immune-related miRNAs from bovine colostrum and raw mature milk as well as from LTLT, HTST, and UHT milks that were produced from the same milk industrial cooperation. We compared the amounts of bovine miR-93, miR-106a, miR-155, miR-181a, and miR-451, which are known to be abundant in bovine colostrum, using real time PCR analysis (Kosaka *et al*., 2010; Pedersen and David, 2008; Sun *et al*., 2013). Only a limited amount of information has been identified experimentally regarding the roles of miRNA in milk, but immune-related miRNA are relatively well characterized. Given that milk has a role in infant development, we selected five miRNAs, miR-93, miR-106a, miR-155, miR-181a, and miR-45 (Bidarimath *et al*., 2014; Pedersen and David, 2008; Rosenberger *et al*., 2012; Sharma *et al*., 2009; Vigorito *et al*., 2013) for quantification.

As shown in Fig. 3A, the amounts of total RNA from colostrum were approximately 1.96-fold and 3.5-fold higher than those of raw mature milk, pasteurized, and sterilized milk products, respectively. As expected, total RNA in raw mature milk was destroyed through all heat treated forms. However, HTST and UHT methods did not result in the same degree of reduction in RNA quantity as was seen with LTLT. Purity of colostrum showed the highest ratio and those were gradually reduced in the raw mature milk to pasteurized milk samples.

To compare the amount of immune-related miRNAs in colostrum, raw milk, and pasteurized milk products, cel-miR-39-1 was added to the homogenization step for normalization to evaluate the relative RNA quantity at each level. We hypothetically set the 2^(-Ct) of UHT as 1 because the Ct of UHT was lowest in all samples (Fig. 3B). The quantity of immune-related miRNAs of colostrum was significantly higher than in raw mature milk; 18.7-fold higher for miR93, 7.7-fold higher for miR106a, 5.4-fold higher for miR155, 5.4-fold higher for miR188a, and 6.3-fold higher for miR451. All of the miRNAs of raw mature milk were dramatically decreased throughout the pasteurizing steps. Surprisingly, all levels of immune-related

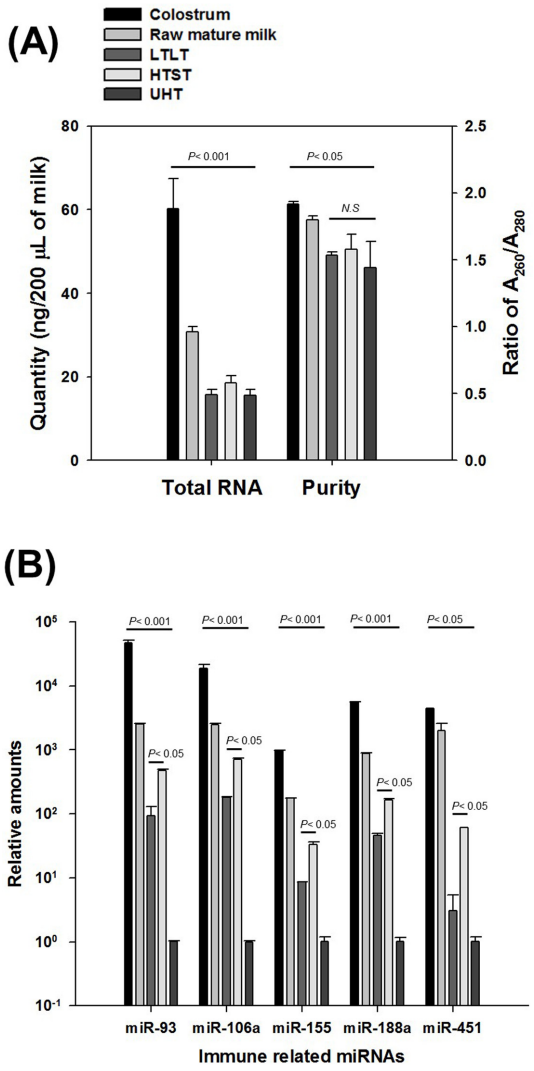

Fig. 3. Relative amounts of immune-related miRNAs in bovine colostrum, raw mature milk, and pasteurized milk products (LTLT, HTST, and UHT). (A) Total RNA isolated using miRNeasy_II method was quantified by NanoQ spectrometry. (B) Complementary DNA was subjected to qPCR on a StepOne Plus Real-Time PCR System using miScript SYBR green PCR kit. To normalize the amount of each miRNA, cel-miR-39-1 was added to each milk sample and C(t) value was obtained. To compare the relative amount of each miRNA, 2^(-Ct) of UHT was set as 1, hypothetically.

miRNAs of HTST milk were higher than those of LTLT milk; 5.0-fold higher for miR93, 3.9-fold higher for miR106a, 3.8-fold higher for miR155, 3.5-fold higher for miR188a, and 20-fold higher for miR451. This result implies that milk miRNA enclosed by exosomes was more resistant to the 75°C for 15 s of the HTST conditions than to 63°C for 30 min of the LTLT conditions. Although it has been known that LTLT pasteurization minimizes the destruction of nutrients within milk, it appears to result in increased destruction of the immune-related miRNA-containing exosome under 30 min at 63°C.

Our present study demonstrated that immune-related miRNAs in market milks as well as colostrum and raw milk could be evaluated by a simple and quick method of RNA isolation and quantitative RT-qPCR. Given that little experimental study in the field of dairy food has been performed on milk miRNAs and their functions, our present study provides some sound prospective molecular work as well as basic technology that can be used in further studies.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2014R1A1A2008481) and the High Value-Added Food Technology Development Program of the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (iPET), the Ministry for Food, Agriculture, Forestry, and Fisheries of the Republic of Korea (111137-03-3-SB010).

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