Genome-wide microRNA profiling of bovine milk-derived exosomes infected with *Staphylococcus aureus*

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Abstract

Bovine milk is rich in exosomes, which contain abundant miRNAs and play important roles in the regulation of neonatal growth and development of adaptive immunity. Here, we analyzed miRNA expression profiles of bovine milk exosomes from three healthy and three mastitic cows, and then six miRNA libraries were constructed. Interestingly, we detected no scRNAs and few snRNAs in milk exosomes; this result indicated a potential preference for RNA packaging in milk exosomes. A total of 492 known and 980 novel exosomal miRNAs were detected, and the 10 most expressed miRNAs in the six samples accounted for 80–90% of total miRNA-associated reads. Expression analyses identified 18 miRNAs with significantly different expression between healthy and infected animals; the predicted target genes of differentially expressed miRNAs were significantly enriched in immune system process, response to stimulus, growth, etc. Moreover, target genes were significantly enriched in several Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways including inflammatory, immune, and cancer pathways. Our survey provided comprehensive information about milk exosomes and exosomal miRNAs involved in mastitis. Moreover, the differentially expressed miRNAs, especially miR-223 and miR-142-5p, could be considered as potential candidates for mastitis.

Keywords Bovine · Milk · Exosome · Mastitis · miRNA-sequencing

Introduction

Bovine mastitis is an inflammatory reaction of the mammary gland and is usually a consequence of microbial infection, caused by pathogens such as *staphylococci*, *streptococci* and *coliform* bacteria (Zadoks et al. 2011). Mastitis has long been considered a highly prevalent and costly disease with clinical or subclinical manifestations in the dairy industry (DeGraves and Fetrow 1993). To date, numerous studies have focused on bovine mastitis, including the nosogenesis, regulation mechanisms, and therapeutic approaches. For example, cytokines

and other inflammatory mediators showed lower expression in bovine milk infected by *Escherichia coli* than in milk infected by *Staphylococcus aureus* (Bannerman et al. 2004). Moreover, Toll-like receptor-4 has been suggested as an important contributor to robust mammary gland immune defense against *E. coli* mastitis (De Schepper et al. 2008). Nonetheless, few studies have focused on bovine mastitis milk, its vesicles, and gene expression.

Exosomes are a type of extracellular vesicle, such as microvesicles, apoptotic bodies, or ectosomes with a diameter of 50–150 nm, which are derived from MVBs fusing with the plasma membrane (Chivet et al. 2013; Kowal et al. 2016). Therefore, exosomal membrane molecules such as CD9, CD63, and CD81, as well as the proteins involved in exosomal biogenesis such as tumor susceptibility gene 101 (Tsg101), are considered to be exosomal markers (Kumar et al. 2015). Exosomes can be released from many cell types, including epithelial cells, endothelial cells, immunocytes, platelets, blood cells, and smooth muscle cells (Liao et al. 2014; Saunderson et al. 2014; Wieckowski and Whiteside 2006). Secreted exosomes can regulate the activity of recipient cells by delivering lipids, proteins, and nucleic acids, thus

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representing a novel intercellular communication pathway (Février and Raposo 2004). Furthermore, exosomes have been found to be involved in both immune stimulation and tolerance, depending on the cell origin (Raposo et al. 1996), and several studies have proposed the potential use of exosomes in immunotherapy (Aline et al. 2004; Pêche et al. 2003). Exosomes are present in body fluids including urine (Pisitkun et al. 2004), plasma (Wahlgren et al. 2012), saliva (Michael et al. 2010), malignant effusions (Andre et al. 2002), breast milk (Admyre et al. 2007), bronchoalveolar lavage fluid (Levänen et al. 2013), synovial fluid (Kolhe et al. 2017), and epididymal fluid (Gatti et al. 2005). The phenotype of exosomes derived from human breast milk varies with maternal sensitization and lifestyle, which might influence allergy development in the child (Torregrosa Paredes et al. 2014). Bovine milk exosomes exhibit cross-species tolerance with no adverse immune and inflammatory response, which can be considered as a carrier to enhance oral bioavailability and improve efficacy and safety of drugs (Munagala et al. 2016).

MicroRNAs (miRNAs) are stable small non-coding RNAs, typically 22 nucleotides in length, with high evolutionary sequence conservation. Numerous studies have found that miRNAs play crucial roles in the process of cellular proliferation and differentiation, tissue development, and immune response. Furthermore, only a few studies investigated the regulatory roles of miRNA in bovine immunity and infection. For example, a recent study revealed that miR-9, miR-125b, miR-146a, miR-233, and miR-155 were closely related with inflammation after stimulation of bovine monocytes with lipopolysaccharide and *S. aureus* enterotoxin B (Dilda et al. 2012). In another study, miRNAs were detected in exosomes and shown to be delivered to another cell as functional molecules (Valadi et al. 2007). Subsequently, numerous studies focused on the function of exosomal miRNAs. For example, miR-29c derived from urinary exosomes was discovered as a novel non-invasive marker for renal fibrosis (Lv et al. 2013). In addition, miR-21 was found to be enriched in serum exosomes and might serve as a potential biomarker for hepatocellular carcinoma (Wang et al. 2014). However, few studies focused on the function of bovine milk derived miRNAs.

The objective of this study was to investigate features of small RNAs, the expression pattern of miRNAs in bovine milk exosomes, and to comprehensively characterize differential expression of milk-derived exosomal miRNAs between normal and infected cows.

Materials and methods

Milk collection

Six 3- to 4-year-old Chinese female Holstein cattle were obtained from a dairy farm and divided into two groups

randomly. For the three healthy cows, the milk was inspected to have SCC < 100,000 cells/mL (Supplementary Table S1), and the udders were observed to show no mastitic symptoms, such as redness, swelling, or pain; bacterial isolation and identification from the milk showed the absence of pathogens. In the mastitic group, Holstein cows were infused with approximately 10^5 colony forming units (CFU) of *S. aureus* (ATCC29213, purchased from BNCC) suspended in 5 mL PBS. After 48 h, the milk SCC was > 1 million/mL (Supplementary Table S1), and the presence of mastitis was confirmed. Then, 400 mL of milk was aseptically collected under sterile conditions and stored at 4 °C until use.

Isolation of exosomes

Milk exosomes were isolated by differential centrifugation as described previously (Munagala et al. 2016). Briefly, milk was centrifuged at $10,000\times g$ in 50-mL centrifuge bottles (Thermofisher Scientific) at 4 °C for 30 min to remove fat globules, casein aggregates, and other debris. The whey was collected and transferred into 50-mL polycarbonate tubes and centrifuged at $100,000\times g$ at 4 °C for 60 min to remove large particles and microvesicles. The supernatant was carefully removed and filtered through 0.75 μm . This supernatant was centrifuged at $150,000\times g$ for 90 min at 4 °C. The exosome pellet was re-suspended in PBS and filtered through 0.22 μm and then collected and washed twice with PBS. Finally, exosomes were re-suspended in 200 μL PBS and stored at -80 °C until use.

NTA

Size distribution and particle concentration in bovine milk exosomes were analyzed by NanoSight and Zetasizer (Malvern Instruments Ltd., Malvern, UK). The particle Brownian movement was analyzed by NTA software (version 2.3, NanoSight). Ten microliters of exosomes was diluted in 1 mL with PBS, and size distribution and parameters were analyzed at 37 °C according to the manufacturer's instructions.

TEM

A drop of the re-suspended exosomes was placed on a sheet and then floated a carbon-coated copper grid on the drop for 10–30 s. The grid was removed, and excess liquid was drained by a piece of sterile filter paper. The same side of the grid was covered by a drop of 2% phosphotungstic acid, pH 7.0, for 5 s, and the excess liquid was removed. After several minutes, when the grid was dry, it was examined with Tecnai G2 F20 S-TWIN (FEI, Hillsboro, USA).

Western blot

For total protein, samples were incubated with RIPA (Beyotime, Shanghai, China) reagent for 10 min on a shaker, centrifuged at $10,000\times g$ for 5 min, and then the supernatant was collected. The protein content of the supernatant was measured with a BCA kit (Beyotime, Shanghai, China). Twenty micrograms of protein was loaded per well onto a 12% acrylamide gel. The protein was transferred onto a PVDF membrane and blocked with non-fat milk at room temperature for 2 h. Then, the membrane was incubated with either anti-calnexin (1:2000) (Abcam, USA), anti-Tsg-101 (1:300) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-CD81 (1:300) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in primary antibody diluent overnight at 4 °C. The membrane was washed 5×10 min with TBST. The secondary antibodies (1:5000) (Servicebio, Wuhan, China) diluted in TBST were incubated with the membrane at room temperature for 2 h. The membrane was washed 5×10 min and then incubated with ECL kit (Servicebio, Wuhan, China) following the manufacturer's recommendations.

RNA sequencing

Total RNA was extracted from bovine milk exosomes using the Trizol reagent (TAKARA, Japan) according to the manufacturer's protocol and dissolved in RNase free water. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), and the RIN was 7.0–8.0. A total amount of 2.5 ng RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra small RNA Sample Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations, and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System TruSeq PE Cluster Kit v4-cBot-HS (Illumina, NEB, USA) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina HiSeq 2500 platform and paired-end reads were generated. The raw FASTq files (raw reads) were first processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low-quality reads from raw data. Next, reads were trimmed and cleaned by removing the sequences smaller than 18 nt or longer than 30 nt. At the same time, Q20, Q30, GC-content, and sequence duplication level of the clean data were calculated.

cDNA synthesis and qRT-PCR

Fourteen differentially expressed miRNAs were reverse transcribed into cDNA by using mir-X™ miRNA First-Strand Synthesis Kit (Takara, Dalian, China) according to the manufacturer's protocol. Then, the SYBR™ Green II qRT-PCR kit (Takara, Dalian, China) was used to detect and quantify miRNA expression on a Bio-Rad CFX 96 (Bio-Rad, CA, USA) following manufacturer's recommendations. The cycle conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C 30 s. U6 small nuclear RNA was used as internal control to normalize the copy number of the miRNAs by the $2^{-\Delta\Delta C_t}$ method. After normalization, the relative miRNA levels of the infected group were expressed as fold change relative to those of the healthy group. The miRNA-specific primers were synthesized by Sangon Biotech Co. (Shanghai) (Supplementary Table 2). A miRNA was considered to be present only if its mean threshold cycle (C_t) was < 34 (Thomou et al. 2017).

Results

Isolation and characterization of bovine milk exosomes

Bovine milk exosomes were isolated by a serial centrifugations. Particle size ranged between 40 and 120 nm in diameter (Fig. 1a). Furthermore, exosomal size and morphology were confirmed to be around 100 nm and presented distinct membrane structure by TEM (Fig. 1b). Exosomal protein lysates were prepared and verified with western blot analysis for special exosomal membrane markers Tsg101 and CD81. The absence of the endoplasmic reticulum marker calnexin indicated that milk-derived vesicles were not contaminated with other multivesicular bodies (Fig. 1c). These results are consistent with previous exosome observations.

miRNA sequencing

Approximately 200 µL resuspended exosomes of each sample were used for RNA extraction and next-generation sequencing; six small RNA libraries were constructed and sequenced simultaneously. A total of 11.87 million high-quality reads were generated after removing low-quality reads and adaptor sequences (Table 1). Between the two groups (HH for Holstein healthy group and HM for Holstein mastitic group), nearly 96.72% of the total reads and 9.56% of unique reads were common (Fig. S1). After alignment with small RNA databases, Silva, GtRNAdb, Rfam, and Repbase databases, nearly 90.01% of the clean reads in the HH group and 83.26% in the HM group were identified as alignment reads (Fig. S1). Particularly, no small cytoplasmic RNA (scRNA)

Fig. 1 Isolation and characterization of bovine milk-derived exosomes. **a** The exosome suspension was analyzed by NanoSight. *X*-axis represented the size of particles, left *Y*-axis represented the concentration of particles (E6 particles/mL), and right *Y*-axis represented the cumulative percentage of particles. **b** A drop of exosomal suspension was imaged in TEM. **c** Exosomes isolated in two groups were analyzed for marker proteins with western blot

and few small nuclear RNA (snRNA) (389 reads) were detected in either group. The remaining reads were used to detect known miRNA, and novel miRNAs predicted based on the miRBase database.

Characteristics of known and novel miRNAs

In total, miDeep2 generated 1472 miRNAs in the high-throughput sequencing data from both groups; from these, 492 miRNAs were identified as known bovine miRNAs. The majority of total and known miRNAs were 18–24 nucleotides in length (Fig. 2a; Supplementary Table 3), consistent with the characteristics of miRNA. Among the known miRNAs in the six libraries, ten highly expressed miRNAs accounted for approximately 80–90% of the total reads (Fig. 2b). In addition, 980 unique reads were considered as novel miRNAs (Supplementary Table 4). The novel miRNAs were named as unconservative miRNAs, followed by chromosome number and the serial number generated when the sequence was detected. On average, the total read counts of novel miRNAs were much lower than of known miRNAs. A total of 613 unconservative miRNAs had read counts < 10, and

only 66 unique sequences had a total read count > 100 (Supplementary Table 4). Furthermore, removing the 613 unique sequences, 179 sequences belonged to 40 miRNA families, and 115 sequences had significant homology with mir-2284.

Differential expression of miRNAs

Differential expression (DE) analysis of the two groups was performed using the DESeq R package (1.10.1), and miRNAs with an adjusted $p < 0.05$ were assigned as differentially expressed. Finally, a total of 18 differentially expressed miRNAs were detected, of which 12 miRNAs were upregulated and six were downregulated. Four of these DE miRNAs were novel (Table 2; Supplementary Table 5). Among these miRNAs, bta-miR-142-5p and bta-miR-223 were also identified to be significantly upregulated in a previous study (Sun et al. 2015). The other differentially expressed miRNAs showed different expression patterns in our studies. Differences in miRNA expression between the two groups are illustrated by Volcano Plot (Fig. 3).

Table 1 Total number of sequence reads from six small RNA sequencing libraries with number of alignments

	HH1	HH2	HH3	HM1	HM2	HM3
Total raw reads	21,050,342	23,613,507	20,946,610	28,659,439	20,224,894	28,260,353
Clean reads	17,897,078	22,034,379	16,171,810	26,511,440	16,088,610	20,025,618
Q30 (%)	98.27	98.78	98.79	98.8	98.4	97.08
Number of alignments	161,14,983	20,570,860	13,810,757	24,904,303	14,142,023	13,097,810
Mapped reads	10,204,349	16,070,343	9,262,399	18,930,800	9,440,008	8,406,855

Fig. 2 The length and expression pattern of miRNAs in all libraries. **a** The distribution by length (nt) of all and known miRNA from bovine milk exosomes. **b** Top ten miRNAs with the highest expression levels in

six bovine milk exosomal miRNA libraries. And 15 miRNAs that are present in the top 10 miRNAs

Target gene prediction and enriched GO functional categories and KEGG pathways

Two software, miRanda and TargetFinder, were employed to predict target genes. In total, 18,028 target genes were identified for all miRNAs, and 1112 target genes might be regulated by the 18 DE miRNAs. Gene ontology (GO) enrichment analysis of all miRNAs and differentially expressed genes was performed by the Goseq R packages based on Wallenius non-central hyper-geometric distribution. The results revealed enrichment of 22 biological processes, such as immune

system process, response to stimulus, and growth (Fig. 4). KOBAS software was used to statistically test the enrichment of differential expression genes in KEGG pathways. Interestingly, the target genes of the 18 DE miRNAs were enriched in 11 pathways (Supplementary Table 6).

Verification of miRNA expression by RT-PCR

Fourteen known differentially expressed miRNAs were verified by real time PCR. Among them, three miRNAs (miR-423-5p, miR-1468, let-7b) were downregulated, while nine

Table 2 Differential expression of miRNAs in bovine milk-derived exosomes between HH and HM. (* $p < 0.05$, ** $p < 0.01$)

miRNA name	<i>p</i> value	log2FC	Regulated	Significance level
bta-let-7b	2.20E-02	-1.54	Down	*
bta-miR-103	3.12E-02	1.69	Up	*
bta-miR-142-3p	1.22E-02	3.27	Up	*
bta-miR-142-5p	3.07E-02	4.68	Up	*
bta-miR-1468	4.92E-02	-1.92	Down	*
bta-miR-146a	1.18E-02	3.15	Up	*
bta-miR-146b	1.70E-06	3.97	Up	**
bta-miR-147	3.97E-02	3.13	Up	*
bta-miR-221	1.32E-02	2.97	Up	*
bta-miR-223	1.70E-04	5.16	Up	**
bta-miR-2284w	3.97E-04	5.02	Up	**
bta-miR-2285b	4.82E-02	2.22	Up	*
bta-miR-23a	1.78E-02	1.96	Up	*
bta-miR-423-5p	2.44E-02	-1.41	Down	*
unconservative_25_329551 ^a	3.52E-02	-2.19	Down	*
unconservative_25_333271 ^a	3.72E-02	-2.58	Down	*
unconservative_3_387931 ^a	1.49E-02	2.88	Up	*
unconservative_8_520328 ^a	3.45E-02	-2.46	Down	*

^a Novel miRNAs were named as unconservative miRNAs, which were named as “unconservative_ the number of chromosome_ the serial-number generated when the sequence was detected”

Fig. 3 Volcano plot for differential expression of miRNAs (red points represent miRNAs significantly upregulated, and green points represent miRNAs significantly downregulated) (color figure online)

(miR-142-5p, miR-142-3p, miR-103, miR-147, miR-23a, miR-223, miR-146a, miR-146b, and miR-221) were upregulated, in agreement with the high-throughput sequencing results (Fig. S2). However, miR-2284w expression levels were below the threshold cycle (C_t) 34, and the expression pattern of miR-2285b detected in qRT-PCR was different from miRNA-seq.

Discussion

Effective ways to cure mastitis include stimulation of the immune responses related to mastitis at the molecular level and effective prophylactic treatments in the early stage. So far, several pathways and cytokines have been identified as inflammatory responses and tissue injuries, such as JAK-STAT, NF- κ B, TNF- α , iNOS, and COX-2 (Rossol et al. 2011). A genome-wide association study revealed TRAPPC9 and ARHGAP39 as indicators for bovine mastitis susceptibility (Wang et al. 2015); miRNA profiling of bovine mammary epithelial cells (Jin et al. 2014; Li et al. 2015) and mammary gland (Kirsty et al. 2013) identified miRNAs and mRNAs related to mastitis. Recent studies have hypothesized that exosomal miRNA may be a potential biomarker for disease (Lv et al. 2013). The present study confirmed the presence of exosomes derived from bovine milk, identified the characteristics of exosomal small RNAs, and measured the expression of miRNA in exosomes.

In this study, we performed miRNA sequencing of milk exosomes to provide insight on differential expression of miRNAs and the molecular mechanisms of mastitis. We identified 11.87 million high-quality reads. Interestingly, no scRNAs and few snRNAs were identified based on alignments with small RNA databases. It has been proposed that exosomal cargo can change in response to different cellular conditions (Skog et al. 2008). The mechanisms for selective packaging and release of exosomal RNAs are not well known; however, it has been reported that RNA profiles from exosomes are different from those of host cells. RNA packaging may occur by specific interactions with RNA binding proteins; these interactions are necessary for membrane invagination into the interior of an MVB (Janas et al. 2015). Previous studies have reported that exosomes and parental cells have different types of RNA, with the apparent lack of ribosomal RNA in exosomes (Hessvik et al. 2012; Mittelbrunn et al. 2011).

Only a few miRNAs were highly expressed, ranking in the top ten within each of the six samples and accounting for approximately 80–90% of all known bovine miRNAs. This finding was consistent with those of studies on human breast milk exosomes (Zhou et al. 2012); bovine mammary epithelial cells infected with *Streptococcus uberis* (Lawless et al. 2013), *S. aureus*, and *E. coli* (Jin et al. 2014); and bovine milk exosomes (Sun et al. 2015). Among these miRNAs, bta-miR-148a and bta-30a-3p were also identified by Sun. miR-148a was reported to be abundant in human breast milk exosomes, porcine breast milk exosomes (Gu et al. 2012), and in human and murine plasma cells (Porstner et al. 2015). miR-148a was also highly expressed in bovine milk and was predicted to be a potential biomarker for identifying the quality of raw milk or milk-related commercial products (Chen et al. 2010). miR-30a-5p might ameliorate inflammatory response and oxidative stress by targeting Neurod 1 (Fu et al. 2017). Moreover, the other four miRNAs (miR-200a, miR-21-5p, let-7g, and let-7c) identified in our study have been reported to play roles in immunity. Using miRDeep 2 for novel miRNA prediction, we identified 980 novel miRNAs in bovine milk exosomes. Only 367 novel miRNAs had read counts > 10, which is consistent with previous reports (Sun et al. 2015). A previous study identified seven members of the bta-miR-2284/2285 family among the eight more novel candidate miRNAs (Guduricfuchs et al. 2012). We also detected 115 unique sequences showing significant homology with mir-2284. It is possible that these highly expressed miRNAs in bovine milk exosomes may be ingested and play post-transcriptional regulation in calves.

A total of 492 known miRNAs were identified, and 14 known differentially expressed miRNAs were found in the two groups. Among these DE miRNAs, miR-223 ($\log_2FC = 5.16$) and miR-142-5p ($\log_2FC = 4.68$) were highly regulated post-infection, which is consistent with previous reports on

Fig. 4 Gene ontology functional annotation of the target genes of differentially expressed and all miRNAs in HH and HM

bovine milk exosomes (Sun et al. 2015) and bovine mammary gland infected with *S. aureus* or *S. uberis* (Li et al. 2015; Naeem et al. 2012). In bovine colostrum milk, a higher level of miR-223 was reported to be involved in immune response and immune system development (Fazi et al. 2005). Interestingly, miR-223 was also identified to inhibit classic pro-inflammatory pathways and was therefore considered as a novel regulator of diet-induced adipose tissue inflammatory response (Chen et al. 2004). Furthermore, a novel miR-223–CEBP- β –LMO2–miR-142 regulatory pathway has been proposed to have pivotal functions in hematopoiesis (Sun et al. 2010). Thus, a deeper analysis of the biological regulation of miR-223 and miR-142-5p in mammary epithelial cells is needed, as these miRNAs may play crucial roles in bovine mastitis. Further, miR-146a/b was also significantly upregulated in milk exosomes post-infection. Similar upregulation of miR-146a and miR-146b was detected in bovine mammary gland after injection with *S. aureus* (Wang et al. 2016) and in human monocytes reacting to lipopolysaccharides (Taganov et al. 2006). Furthermore, miR-146b was proposed to be involved in luminal alveolar progenitor cell maintenance by regulating STAT3 (Elsarraj et al. 2013). Regarding the down-regulation of miRNAs, miR-1468 participates in the regulation of cell cycle progression by targeting RRM1 (Jiang et al. 2017). Let-7b (Teng et al. 2013) and miR-423 (Wang et al. 2017) have been shown to regulate the activation of NF- κ B

signaling by targeting TLR4 and TNIP2, respectively. Our results provide important information about miRNAs differentially expressed in bovine milk exosomes and specific miRNAs that could be potential candidate for early diagnostic detection of mastitis. Furthermore, differentially expressed miRNAs packaged by exosomes post-infection might be delivered to recipient cells, such as macrophages, for cell-to-cell communication, and may be functional in the new location.

It was notable that predicted gene targets of DE miRNAs were enriched in GO terms, such as cellular process, developmental process, metabolic process, immune system process, and growth. This result indicated the involvement of miRNAs in numerous developmental and physiological processes, including disease and immune development. The results of the KEGG pathway analysis indicated that target genes were associated with cancer, inflammatory, and immune pathway. This result further illuminated the function of bovine milk-derived exosomes in growth and productive capacities as well as defense capabilities in infants.

Conclusion

Using deep sequencing, we characterized the miRNA profiling of bovine milk-derived exosomes in healthy and *S. aureus*-infected mastitic groups. We found that no scRNA

and few snRNA existed in bovine milk exosomes. In addition, 492 known and 980 novel miRNAs were detected, and ten highly expressed miRNAs accounted for 80–90% of the total reads. Differential expression analysis showed that 14 known miRNAs and four novel miRNAs had significant differential expression. Among these miRNAs, miR-223 and miR-142-5p were considered as potential targets for early detection of mastitis. GO and KEGG pathway analysis showed that predicted target genes were significantly enriched in immunity.

Author contributions SJL and MCC contributed to concept design. HBH and WDX performed the animal experiments. MCC, BWL, and YS performed the RNA and protein isolation and western blot. MCC, HBH, and XBJ performed data analysis. MCC, SYC, and JW prepared the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

All experiments were carried out according to the Regulations for the Administration of Affairs Concerning Experimental Animals published by the Ministry of Science and Technology, China in 2004 and approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University, China.

Conflict of interest The authors declare that they have no conflict of interest.

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