**SUPPLEMENTARY FILES**

**Analysis of immune-related microRNAs in cows and newborn calves**

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A. Supplementary methods

Reverse transcription - quantitative polymerase chain reaction (RT-qPCR)

Five immune-related miRNA were quantified using RT-qPCR assays that were performed in 5 steps (Figure S1) on the high-quality RNA samples.

Figure S1. RT-qPCR workflow.

*“A” in step 1 is the dilution factor when diluting total RNA for RT reaction; “B” in step 5 is the miRNA concentration (copies/µL) from Rotor-Gen 6000 software after the first normalization*

**Reverse transcription**

Total RNA was diluted with ultra-pure water for the reverse transcription (RT) reaction (approximately 2 ng/µL for RNA in blood and 10 ng/µL for colostrum/milk samples). Then, total RNA was transcribed into complementary DNA (cDNA) using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem, product P/N 4366596 and 4366597, USA) with the specific miRNA primers supplied by the company, and all steps followed by the manufacturer’s protocol. cDNA for each sample was synthesized in duplicate. Briefly, a master mix for RT reaction (13 to 14 µL/tube depending on the sample type) was prepared as per the protocol from the manufacturer, and then the diluted RNA (1 to 2 µL/tube) was added to the reaction tubes to give the final volume of 15 µL/tube. Specifically, 14 µL/tube master mix was added with 1 µL of diluted RNA blood sample and 13 µL/tube master mix was added with 2 µL of diluted RNA colostrum/milk (Table S1.1). The program for RT reaction was 30 minutes at 16oC, 30 minutes at 42oC, 5 minutes at 85oC and 5 minutes at 4oC. A negative reverse transcription control (NC-RT) was included by adding ultra-pure water instead of diluted total RNA and was synthesized at the same time as the samples.

Table S1.1. Reverse transcription reagents.

|  |  |  |
| --- | --- | --- |
| RT components | RNA from  Blood sample | RNA from  Colostrum/milk sample |
| Master mix  Pooled RT primer\* | 6 µL | 6 µL |
| dNTP mix (100mM total) | 0.3 µL | 0.3 µL |
| 10 x RT buffer | 1.5 µL | 1.5 µL |
| Multiscribe RT enzyme (50 U/µL) | 1 µL | 1 µL |
| RNase inhibitor (20 U/ µL) | 0.2 µL | 0.2 µL |
| RNase free water | 5 µL | 4 µL |
| Total master mix/reaction | 14 µL | 13 µL |
| Diluted total RNA\*\* | 1 µL of  2 ng/ µL [total RNA] | 2 µL of  10 ng/ µL [total RNA] |

*\* Pooled RT primer = 5 µL of each RT primer (miR-39-3p, miR-142-5p, miR-150, miR-155, miR-181a, miR-223) + 470 µL of RNase-fee water.*

*\*\* Diluted total RNA was used as the template for RT reaction, and the amount of diluted RNA in each sample type was optimised.*

Cel-miR-39-3p standard conversion for RT-qPCR

The miRNA cel-miR-39-3p is found in *Caenorhabditis elegans* and is not present in mammals. Cel-miR-39-3p was used as the template to create standard curves for qPCR and for normalization to correct for any technical variation during the RNA isolation, RT and qPCR. Cel-miR-39-3p concentration used for RT-qPCR was 5 picomole/µL (= 36.3 ng/ µL = 2.922 x 1012 copies/ µL), based on the conversion from http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr or following formula:

*Where: X = amount of amplicon (ng),*

*N = length of dsDNA, ssDNA or RNA amplicon,*

*a (g/mole) = average mass of 1 bp dsDNA, or ssDNA, or RNA, and*

*(a = 660 for dsDNA, a = 330 for ssDNA, a = 340 for RNA).*

To prepare the miR-39-3p for the qPCR standard curve, the cDNA of miR-39-3p was transcribed following the above RT steps. All steps to prepare cDNA of cel-miR-39-3p were performed as for the samples, however, instead of adding 1 µL of diluted total RNA, 1 µL of cel-miR-39-3p (5 picomole/µL) was added at RT reaction. One µL of 5 picomole/µL cel-miR39-3p was used as the starting material for the RT reaction, giving a cDNA concentration of cel-miR39-3p of 2922 x 109 copies in a total RT reaction volume of 15 µL or 194.8 x 109 copies/ µL.

The miR-39-3p standard cDNA was diluted in serial 10-fold dilutions to create a standard curve. Each dilution was aliquoted into Eppendorf tubes and stored at -80oC. Aliquots were thawed once for use and then discarded to avoid repeated freeze-thaw cycles. The cDNA for the standard curve was amplified at the same time as the samples for each PCR amplification.

**Quantitative polymerase chain reaction (qPCR) steps**

Quantitation by real-time PCR was performed using a Corbett Robotics system (CAS Robotics 4 v4.9.1) to load the PCR reagents into Gene-Disc 100 well rings, and the PCR ring was placed into a thermal cycler (Rotor-Gen 6000, Corbett Research, 2 PLEX). The reagents used for the qPCR step were from a TaqMan Universal Master Mix II, no uracil-N-glycosylase (UNG) kit (Applied Biosystems) and all steps followed the protocol of the manufacturer with modifications for the template amount that were optimised. The cDNA prepared from each of the samples was diluted at a ratio of 1:10 with RNase-free water and used as the template for the qPCR reaction. A negative reverse transcription control (NC-RT) was included. Briefly, a PCR master mix was prepared by mixing 7.5 µL of TaqMan Universal Master Mix II, no UNG 2x and 0.38 µL of TM primer 20X for each reaction. The master mix was added to each well of the ring, and the cDNA template was added to a final volume of 15 µL (Table S1.2). A positive control, which was pooled total RNA from 35 blood samples on day 1, was included in each PCR run as a reference to compare between runs (Table S1.3).

Table S1.2. PCR reagents.

|  |  |  |
| --- | --- | --- |
| Reagents | Standard solution Volume/tube (µL) | Sample Volume/tube (µL) |
| TagMan master mix, no UNG | 7.5 | 7.5 |
| TM primer (specific primer) | 0.38 | 0.38 |
| Ultra-pure water | 0 | 1.12 |
| Template | 7.12 µL  Serial dilution of cDNA of  (miR39-3p) | 6 µL  Diluted (1:10) cDNA of sample |
| Total volume | 15 | 15 |

*Note: The volume of reagents and template for qPCR were optimised*

The PCR was for 40 cycles as follows: hold (95oC for 10 minutes), cycling (95oC for 15 seconds, then 60oC for 60 seconds).

Table S1.3. Copy number of positive control for qPCR.

|  |  |
| --- | --- |
| **miRNAs** | **Positive control (copies/µL)** |
| miR-39-3p | 29,126,698 |
| miR-142-5p | 298,685 |
| miR-150 | 564,873 |
| miR-155 | 80,857 |
| miR-181a | 29,259 |
| miR-223 | 29,824,376 |

**Normalization**

The RT-qPCR data were normalized to determine the original miRNA concentration. Two steps of normalization were carried out. The first normalization step used Rotor-Gene 6000 software (Corbett, Life Science, Australia). A standard curve with a high coefficient of correlation (R2 > 0.99) and a high efficiency (from 0.9 to 1.1) was used for all runs. Results from the first 10 cycles of real-time amplification were removed from the analysis, and “auto Threshold” was used to obtain the baseline. After obtaining the copy number from the Rotor-Gen 6000 software, the second step of normalization was to calculate the original miRNA concentration based on the internal cel-miR-39-3p concentration added at the RNA extraction step plus the RNA and cDNA dilutions for the RT and PCR, respectively.

The copy number of miRNAs in the sample was calculated based on the RT-qPCR workflow (Figure 1). The equations for the calf blood and colostrum samples were as follows:

Blood sample: [miRNA at step 1] =

Colostrum sample: [miRNA at step 1] =

*Where:*

*[miRNA at step 1]: miRNA concentration at step 1*

*A is a dilution factor when diluting total RNA for RT reaction*

*B is copy number from software after 1st normalization*

As the miRNA assay was performed with separate RT and PCR assays, there were two different efficiencies for the assay. The final starting miRNA concentration for each sample was calculated using the concentration of 1 µL cel-miR-39-3p (5 picomole/µL) (equal to 2.922 x 1012 copies) added to each sample prior to the RNA isolation step as the reference.

B. Supplementary Tables

Supplementary Table S1. Immune-related miRNAs in dam colostrum day 0 to 3 postpartum.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| miRNAs | Day 0 (n = 12) | | Day 1 (n = 11) | | Day 2 (n = 12) | | Day 3 (n = 12) | |
| LSM | SE | LSM | SE | LSM | SE | LSM | SE |
| miR-142-5p | 402.1a | 50.4 | 129.0b | 52.7 | 31.3b | 50.4 | 28.6b | 50.4 |
| miR-150 | 488.8a | 56.9 | 78.0b | 71.7 | 34.1b | 68.7 | 105.2b | 68.7 |
| miR-155 | 2,036.2a | 171.2 | 695.3b | 216.0 | 424.6b | 206.8 | 412.3b | 206.8 |
| miR-181a | 483.7a | 56.9 | 379.2a | 71.7 | 138.8b | 68.7 | 21.6b | 68.7 |
| miR-223 | 97,970.0a | 11,240.0 | 44,470.0b | 14,180.0 | 15,030.0b | 13,570.0 | 11,950.0b | 13,570.0 |

*Units = 106 copies/µL.*

*LSM = least square mean (was estimated from a mixed linear model in SAS), SE = standard error.*

*a,b LSM within a row with different superscripts differ (P < 0.05).*

Supplementary Table S2. Concentration of immune-related miRNAs in colostrum and milk.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| miRNAs | Dam colostrum day 0  (n = 12) | | Pooled colostrum  (n = 5) | | Bulk tank milk  (n = 25) | |
|  | LSM | SE | LSM | SE | LSM | SE |
| miR-142-5p | 402.1a | 84.3 | 398.4ab | 130.5 | 131.4b | 58.4 |
| miR-150 | 618.9 | 149.7 | 174.6 | 231.9 | 274.1 | 103.7 |
| miR-155 | 2,399.2a | 301.5 | 1,125.4b | 467.1 | 179.6b | 208.9 |
| miR-181a | 451.2a | 72.3 | 491.4a | 112.0 | 31.7b | 50.1 |
| miR-223 | 67,190.0 | 19,940.0 | 115,600.0 | 30,890.0 | 52,430.0 | 13,820.0 |

*Units = 106 copies/µL.*

*LSM = least square means (was estimated from a mixed linear model in SAS), SE = standard errors.*

*a,b LSM within a row with different superscripts differ (P < 0.05).*

**Supplementary Table S3. Level of immune-related miRNAs in three calf Groups within one week after birth.**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| miRNA | Time | Group A  (n = 12) | | Group B  (n = 12) | | | Group C  (n = 11) | | | All calves  (n = 35) | |
| Mean | SE | Mean | SE | Mean | | SE | Mean | | SE |
| miR-142-5p | Day 0 | 22.7 | 3.7 | 23.8 | 3.7 | 19.9 | | 3.4 | 22.2z | | 2.3 |
| Day 1 | 17.6 | 3.7 | 16.9 | 3.7 | 19.9 | | 3.4 | 18.1zw | | 2.3 |
| Day 2 | 39.3 | 3.7 | 27.3 | 3.7 | 35.3 | | 3.4 | 33.9y | | 2.3 |
| Day 3\* | 14.3 | 3.7 | 12.5 | 3.7 | 11.9 | | 3.4 | 12.9w | | 2.3 |
| Day 7 | 58.9a | 3.7 | 36.1b | 3.7 | 34.9b | | 3.4 | 43.5x | | 2.3 |
| miR-150 | Day 0 | 51.3 | 7.7 | 72.0 | 7.7 | 83.1 | | 8.1 | 68.4x | | 4.6 |
| Day 1 | 70.2 | 7.7 | 77.2 | 7.7 | 79.4 | | 8.1 | 75.5x | | 4.6 |
| Day 2 | 43.0 | 7.7 | 50.8 | 7.7 | 46.9 | | 8.1 | 46.9y | | 4.6 |
| Day 3\* | 22.6 | 7.7 | 27.3 | 7.7 | 20.4 | | 8.1 | 23.5z | | 4.6 |
| Day 7 | 49.3 | 7.7 | 51.5 | 7.7 | 37.6 | | 8.1 | 46.4y | | 4.6 |
| miR-155 | Day 0 | 8.8 | 1.7 | 9.2 | 1.7 | 12.8 | | 1.8 | 10.2y | | 1.0 |
| Day 1 | 16.0 | 1.7 | 15.2 | 1.7 | 21.1 | | 1.8 | 17.3x | | 1.0 |
| Day 2 | 8.6 | 1.7 | 5.9 | 1.7 | 7.1 | | 1.8 | 7.2yz | | 1.0 |
| Day 3\* | 3.2 | 1.7 | 2.4 | 1.8 | 2.5 | | 1.8 | 2.7w | | 1.0 |
| Day 7 | 5.5 | 1.7 | 5.1 | 1.7 | 4.7 | | 1.8 | 5.1zw | | 1.0 |
| miR-181a | Day 0 | 3.4 | 0.6 | 3.0 | 0.6 | 5.1 | | 0.5 | 3.8y | | 0.3 |
| Day 1 | 7.6 | 0.6 | 8.0 | 0.6 | 8.5 | | 0.5 | 8.0x | | 0.3 |
| Day 2 | 1.5 | 0.6 | 0.6 | 0.6 | 0.6 | | 0.5 | 0.9zw | | 0.3 |
| Day 3\* | 0.3 | 0.6 | 0.2 | 0.6 | 0.2 | | 0.5 | 0.2w | | 0.3 |
| Day 7 | 2.2 | 0.6 | 2.1 | 0.6 | 1.2 | | 0.5 | 1.8z | | 0.3 |
| miR-223 | Day 0 | 4,410.7 | 484.1 | 4,753.7 | 484.1 | 6,092.3 | | 505.6 | 5,056.8x | | 282.7 |
| Day 1 | 4,874.0 | 484.1 | 5,650.6 | 484.1 | 5,241.8 | | 505.6 | 5,255.9x | | 282.7 |
| Day 2 | 2,578.6 | 484.1 | 1,909.6 | 484.1 | 1,956.5 | | 505.6 | 2,153.7y | | 282.7 |
| Day 3\* | 473.8 | 484.1 | 496.7 | 505.6 | 441.0 | | 505.6 | 471.6z | | 286.8 |
| Day 7 | 1,354.3 | 484.1 | 1,269.1 | 484.1 | 1,035.5 | | 505.6 | 1,224.9xy | | 282.7 |

*Units = 109 copies/µL.*

*Group A = calves fed own dam colostrum, Group B = calves fed foster cow colostrum, Group C = calves fed only a bottle of pooled colostrum. Mean was predicted from a mixed linear model using the asreml model in R, SE = standard error.*

*\* Day 3, Group B had 11 samples instead of 12 samples.*

*a,b,c Mean within a row of each time point with a different superscript letter are significantly different (P < 0.05).*

x, y, z, w *Mean within an “All calves” column with a different superscript letter are significantly different (P < 0.05).*

**Supplementary Table S4**. **Spearman correlations between individual miRNAs in dam colostrum and corresponding calf blood over time.**

|  |  |  |
| --- | --- | --- |
| MiRNA | Day 1 dam colostrum*a*  & day 2 calf blood*b* | Day 2 dam colostrum*a*  & day 3 calf blood*b* |
| miR-142-5p | -0.13 | -0.09 |
| miR-150 | 0.29 | 0.24 |
| miR-155 | 0.12 | 0.44 |
| miR-181a | -0.33 | 0.32 |
| miR-223 | 0.08 | 0.08 |

*an = 12 samples from Group A dams*

*bn = 24 samples from Group A & B calves fed colostrum from corresponding Group A dam*