INVESTIGATION OF GENETIC RESPONSES TO BOVINE LEUKEMIA VIRUS INFECTION IN BEEF AND DAIRY CATTLE

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ABSTRACT

The USDA estimated 78% of US beef operations and 89% of US dairy operations are seropositive for Bovine Leukemia Virus (BLV). Cattle infected with BLV may suffer reduced immune function and productivity. Animals with advanced BLV infections may develop lymphoma, leading to carcass condemnation at slaughter. Research has revealed growing evidence of host genetic markers associated with BLV infection. While research on BLV in beef cattle is limited, BLV research in dairy cattle is plentiful. In dairy cattle, evidence of an association between the Bovine Leukocyte Antigen (BoLA)-*DRB3* gene locus and proviral load (PVL) has been reported. However, these associations have not been explored in beef cattle. Chapter 1 investigates associations between *BoLA-DRB3* alleles and BLV PVL in upper Midwest beef cattle populations (n=157). Associations between BLV PVL and *BoLA-DRB3* alleles was determined. Alleles *DRB3**033:01 and *DRB3**002:01 were found to be associated with lower PVL and *DRB3**026:01 with higher PVL. Chapter 2 explores epigenetic relationships between dam and her calf. Epigenetics can be studied through microRNAs (miRNA), a small noncoding RNA species involved in post-transcriptional gene regulation. Variations in maternal environment have long been associated with fetal development and health. The study will measure differential expression of circulating miRNAs in calves born to BLV infected dams (n=24). The study found four miRNAs in 14-day old calves born to BLV infected dams. Literature indicates the miRNAs are associated with immunological response, skeletal muscle development, and cardiac development. The presented thesis provides some of the first insights of allelic and epigenetic regulations associated with BLV infections in cattle.

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LIST OF SYMBOLS AND ABBREVIATIONS

BLV: Bovine Leukemia Virus

BoLA: Bovine Leukocyte Antigen

CPM: counts per million

ELISA: enzyme linked immunosorbent assay

FC: fold change

FDR: false discovery rate

g: grams

GLM: generalized linear model

LC: lymphocyte count

LR: likelihood ratio

MHC: major histocompatibility complex

miRNA: microRNA

nt: nucleotide

OD: optical density

PVL: proviral load

qPCR: quantitative polymerase chain reaction

Tm: temperature

US: United States

WBC: white blood cell

β: beta

°C: degrees Celsius

±: plus or minus

≤: less than or equal to

CHAPTER 1

Identification of BoLA Alleles Associated with BLV Proviral Load in US Beef Cows

This chapter represents a manuscript currently under review in *Pathogens, Topical Collection "Bovine Leukemia Virus Infection"*.

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ABSTRACT

Bovine Leukemia Virus (BLV) causes Enzootic Bovine Leukosis, the most common neoplastic disease in cattle. Previous work estimates 78% of US beef operations and 38% of US beef cattle are seropositive for BLV. Infection by BLV in a herd is an economic concern for producers as evidence suggests an increase in cost and subsequent decrease in profit to producers. Studies investigating BLV in dairy cattle have noted disease resistance or susceptibility, measured by proviral load (PVL), associated with specific alleles of the bovine leukocyte antigen (BoLA)-*DRB3* gene. This study aims to investigate associations between *BoLA-DRB3* alleles and BLV PVL in beef cattle. Samples were collected from 157 Midwest beef cows. *BoLA-DRB3* alleles were identified and compared to BLV PVL. One *BoLA-DRB3* allele, **026:01*, was found to be associated with high PVL in relation to the average of the sampled population. In contrast, two alleles, **033:01* and **002:01,* were found to be associated with low PVL. This study provides evidence of a relationship between *BoLA-DRB3* alleles and BLV PVL in US beef cows.

KEYWORDS: Beef cattle; BLV; BoLA-DRB3; Bovine Leukemia Virus; disease progression; disease resistance

INTRODUCTION

Bovine Leukemia Virus (BLV) is a *delta retrovirus* and the etiological agent causing enzootic bovine leukosis in cattle. Approximately 89% of dairy and 78% of beef operations in the US have at least one BLV infected animal in the herd [1, 2]. Additionally, 38% of US beef cattle and 29% Midwest beef cattle were found to be seropositive for BLV [2, 3]. Transmission of BLV may occur with the reuse of hypodermic needles, direct contact, de-horning tools, examination sleeves, or by blood sucking insects [4, 5, 6]. Neighboring animals within an infected herd pose a significant risk of BLV transmission [7]. One infected animal can lead to multiple infected animals within the herd.

There are a range of clinical signs of BLV infection. Between 60 to 70% of infected animals remain aleukemic, having normal lymphocyte counts (LC) [8]. Approximately 30% infected animals progress to persistent lymphocytosis, characterized by an increased risk of infection by opportunistic pathogens [9]. A small percentage (2 to 5%) of infected animals develop lymphoma, leading to condemnation at slaughter of both dairy and beef animals [10, 11]. Malignant lymphoma accounts for 22% of the cause for condemnation at slaughter for beef and dairy cattle in the Great Lakes region of the US, and 13.5% for beef cattle in the US, a direct profit loss to producers [10, 11]. A quantitative polymerase chain reaction (qPCR) assay can be used to determine the concentration of BLV provirus in a blood sample, associating PVL with stage of disease, where animals with a greater PVL are indicative of a more severe infection and increased risk of transmission of the provirus infectious agent to herdmates [12].

Host genetics may play a role in BLV disease progression. The major histocompatibility complex (MHC) is composed of genes involved in antigen presentation to T-cells [13]. In cattle, the MHC gene region is termed bovine leukocyte antigen (BoLA). In cattle the MHC Class II *BoLA-DRB3* gene locus is highly polymorphic, with an identified 384 alleles [12, 14]. Multiple studies have linked variations in the *BoLA-DRB3* gene locus to levels of PVL in dairy cattle [15, 16]. The role of *BoLA-DRB3* alleles in BLV disease progression in beef cattle is largely unknown. The current study aims to identify the potential associations between *BoLA-DRB3* alleles and BLV PVL in a population of beef cows from the Midwest region of the US.

MATERIALS AND METHODS

Samples

All animals were approved for use by the Institutional Animal Care and Use Committee. Blood samples were collected from Angus, Simmental, and Angus x Simmental crossed beef cows aged 24 to 168 months (n=157) from 9 Michigan and Iowa beef cow-calf operations [2]. Immediately following blood sample collection, LC was assessed as previously described [17]. Cows with a known presence of BLV antibodies, tested by enzyme linked immunosorbent assay (ELISA) were selected. Whole blood was collected by coccygeal venipuncture from each selected cow and stored at -80°C in until DNA extraction.

Animals PVL Quantification

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA quantity and quality was determined using the NanoDrop One/One^{c} (ThermoFisher Scientific, Austin, TX). Methods to determine PVL followed Pavliscak et al, 2021 [18], and PVL was reported as a ratio of BLV polymerase gene copies to Bos β-actin gene copies.

BoLA-DRB3 Allele Determination

The *BoLA-DRB3* exon 2 was amplified from each DNA sample. Following Lohr et al., 2022 [16], two master mixes were prepared with separate tagged primers specific to exon 2 of the *BoLA-DRB3* gene (Table 1.1). Separate tagged primers allowed for multiplex sequencing by combining the following in a master mix: 25µL 2X DreamTaq PCR Master Mix (ThermoFisher Scientific, Austin, TX), 0.5µL *DRB3.1F* or *DRB3.4F* forward primer, 0.5µL *DRB3.R* reverse primer, 20.5 µL water, and 3.5µL DNA for each reaction. All reactions were performed using Applied Biosystems 2720 thermal cycler 96 well (ThermoFisher Scientific, Austin, TX) with the following conditions: 95°C for 2 minutes, 34X (95°C for 30 seconds, 68°C for 30 seconds, 72°C for 30 seconds), then 72°C for 10 minutes. Amplicon size was confirmed by running a 1.5% agarose gel at 110V for 50 minutes.

Table 1.1 *BoLA-DRB3* Exon 2 primers

Primer	Direction	Sequence[1]	Length (nt)[2]	Tm[3]
DRB3.1F	Forward	ACACTGACGACATGGTTCTACA **TCGTGGAGCG** ATCCTCTCTCGCAGCACATTTCC	55	70.5
DRB3.4F	Forward	ACACTGACGACATGGTTCTACA **TGCCTGGTGG** ATCCTCTCTCGCAGCACATTTCC	55	70.8
DRB3.R	Reverse	TACGGTAGCAGAGACTTGGTCT TCGCCGCTGCACAGTGAAACTCTC	46	70

[1]Bold text of primer sequence highlights the unique barcode allowing two animals to be sequenced within a well. [2]Number of base pairs included in the primer. [3]Temperature at which the primer is optimal for PCR.

Following confirmation of amplicon size for each sample, DNA was sequenced by Illumina MiSeq. The *BoLA-DRB3* allele determination followed that of Lohr et al., 2022 [16] except heterozygous genotypes required at least 29% of the reads to align to the called reference allele and homozygous genotypes required at least 72% of the reads to align to the called reference allele.

Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc 2013, Cary NC). Proviral load was log transformed to stabilize variance and minimize skewness of residuals. The statistical model used to analyze the data was:

$$y_{ij} = \mu + \beta(x_i - \bar{x}) + bola_{j1} + bola_{j2} + e_i$$

where y is the response (log PVL) for ith cow having *BoLA-DRB3* genotype j= [j1j2]; β is the regression coefficient on cow age; x_i expressed as deviation from the mean cow age $\bar{x}$; $bola_{j1}$ and $bola_{j2}$ are the random effects of the 2 alleles j1 and j2 at the *BoLA-DRB3* gene locus; e_i is the environmental effect (or measurement error) related to the observation on the ith cow. Allelic effects at the *BoLA-DRB3* gene locus having allelic variance component σ^2_{bola}, were modeled as normally, independently, and identically distributed random additive effects within each cow. The combined variance due to the *BoLA-DRB3* gene locus was $2\sigma^2_{bola}$. The statistical model is similar to that shown in Saama et al., 2004 [19], which had random effect of *BoLA-DRB3.2* locus. Treating allelic effects as random is useful when there are some

alleles with low frequencies relative to other alleles in the population [20]. Allele **027:03* was removed from statistical analysis due to missing ELISA and PVL values.

RESULTS AND DISCUSSION

After enrolling cows with known BLV antibody presence, a qPCR assay revealed PVLs in the sampled beef population ranged from 0.00 to 2.54 BLV copies/Bos β-actin copies, with a mean equal to 0.52 and a median of 0.24. The animals with undetectable PVL were included in the analysis because a PVL of zero with a positive BLV ELISA result may indicate disease resilience by *BoLA-DRB3* alleles.

Lymphocyte counts were calculated as an average per allele. Previous publications have identified a correlation between BLV PVL and LC in addition to the observed association between BLV PVL and *BoLA-DRB3* alleles [16, 21, 22]. Therefore, it is likely there may be an association between *BoLA-DRB3* allele and LC. Future research may aim to identify the potential association between *BoLA-DRB3* allele and LC.

Alleles **009:02, *010:01, *011:01* have been associated with resistance to BLV disease progression in infected dairy cows. In contrast, alleles **012:01* and **015:01* have been associated with susceptibility to BLV disease progression, potentially leading to persistent lymphocytosis or lymphoma [16, 21]. Four out of these five alleles were also identified in the sampled beef population (Table 1.2).

Table 1.2 Estimated Allele Frequencies and Association between *BoLA-DRB3* Alleles and Bovine Leukemia Virus (BLV) Proviral Load (PVL) in Beef Cattle

Allele	Total Count[1]	Allele Frequency	Estimated Allelic Effect[2]	P-value[3]	Lymphocyte count (#/µL)[4]
*010:01	1	0.003	0.53	0.60	3934 ± 0
*001:01	5	0.016	1.52	0.68	6957.80 ± 2369.64
*011:01	1	0.003	0.93	0.95	6823 ± 0
*015:01	3	0.010	1.20	0.86	6033 ± 782
*016:01	4	0.013	0.95	0.96	4789.50 ± 625.97
*018:01	99	0.315	1.90	0.21	8494.57 ± 379.70
*002:01	92	0.293	0.33	0.04**	5628.72 ± 258.81
*026:01	66	0.210	2.55	0.08*	8394.68 ± 434.96
*032:01	6	0.019	1.31	0.79	8193.17 ± 1651.94
*033:01	10	0.032	0.08	0.01**	6531 ± 1236.41
*037:01	1	0.003	0.64	0.71	6602 ± 0
*048:02	4	0.013	1.54	0.65	5832.50 ± 405.59
*006:01	2	0.006	1.72	0.61	14475 ± 0
*007:01	7	0.022	1.40	0.68	5670.71 ± 336.23
*008:01	7	0.002	1.66	0.56	7296.43 ± 995.11
*009:01	3	0.010	1.01	0.99	7044 ± 1618
*009:02	2	0.006	1.76	0.60	9641 ± 0
*027:03	1	0.003	N/A	N/A	13574 ± 0

[1] Number of times the allele was identified in the US Midwestern beef cow population. [2] Estimated allelic effects are shown as deviations from the average PVL in the sampled population. [3] **P≤0.05, *P 0.05 ≤ P ≤ 0.10 [4]Lymphocyte count is shown with standard error.

Similar to what has been identified in dairy cattle, allele **002:01* was associated with low PVL in the sampled population of Midwest beef cows [16, 22]. Animals with allele **002:01* were found to have approximately one-third of the PVL in comparison to the average of the sampled population (Table 1.2). Additionally, animals with allele **033:01* were found to have a PVL less than one-tenth of the sampled population average (Table 1.2). To date, no publications have associated allele **033:01* to BLV PVL in beef or dairy cattle.

Allele **026:01* has been reported at frequencies between 1 to 3% in populations of Baggara, Kenana, and Butana cattle [23]. However, in the current study, allele **026:01* was present at a higher frequency (20.36%), and animals with the allele were found to have a BLV PVL approximately 3 times greater than the average of the sampled population (Table 1.2). Allele **026:01* may potentially associate with greater susceptibility for BLV disease progression in beef cattle. A lower population frequency of **026:01* may

indicate a decreased likelihood for disease progression in BLV infected beef herds. In dairy cattle, allele **026:01* has not been associated with BLV PVL.

In the present study, 18 of the known 384 *BoLA-DRB3* alleles were identified (Table 1.2). The relationship between BLV PVL and *BoLA-DRB3* alleles can be observed in Figure 1.1, where the estimated allelic effect is the deviation from the average PVL at the allele from the average PVL of the sampled population (0.52 BLV copies/Bos β-actin copies). Publications observing *BoLA-DRB3* alleles in dairy cattle have found a similar number of alleles in populations approximately double in size [16]. The greater allelic diversity observed in beef cows may be a result of the differences in effective population size between the beef and dairy industries [24, 25]. The allelic diversity in the study population could be increased further with a larger population of animals from various regions outside the Midwest US. Additionally, the sampled population is limited to Angus and Simmental breeds. Greater diversity in beef breed may also increase allelic diversity.

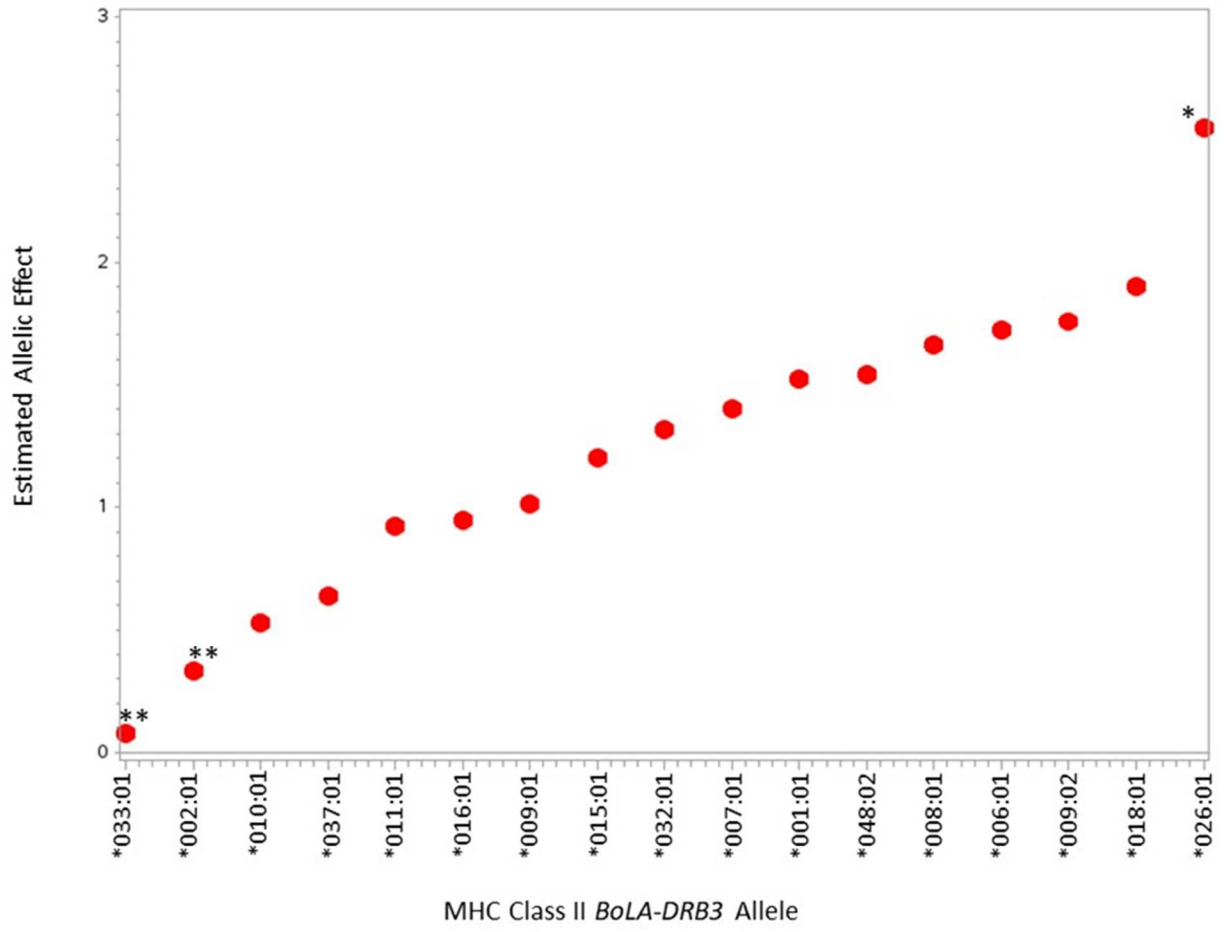

Figure 1.1 Estimated Effect of MHC Class II *BoLA-DRB3* Alleles on BLV Proviral Load (PVL) in Beef cattle. Red dots indicate the *BoLA-DRB3* allele estimated allelic effect as a deviation from the population average PVL. Asterisks represent a significance with ** $P \leq 0.05$ and * $0.05 \leq P \leq 0.10$.

CONCLUSIONS

Novel associations have been found between *BoLA-DRB3* alleles and BLV PVL in the sampled population of US Midwest beef cows. Further research is needed to include a larger, more diverse population. Additionally, it may be valuable to longitudinally measure PVL in a BLV infected beef population and determine *BoLA-DRB3* alleles. Doing so would achieve a comparison of *BoLA-DRB3* alleles to disease progression. With more evidence, the beef industry may consider selecting cattle for breeding that have resistance to BLV disease progression and are less infectious to their herdmates as measured by PVL.

REFERENCES

1. U.S. Department of Agriculture, Animal and Plant Health Inspection Service. Bovine Leukosis Virus (BLV) on U.S. Dairy Operations, 2007. October 2008.

2. Benitez, O. J., Norby, B., Bartlett, P. C., Maeroff, J. E., & Grooms, D. L. (2020). Impact of bovine leukemia virus infection on beef cow longevity. Prev Vet Med, 181, 105055.

3. U.S. Department of Agriculture, Animal and Plant Health Inspection Service. Bovine Leukosis Virus (BLV) in U.S. beef cattle. February 1999.

4. Erskine, R. J., Bartlett, P. C., Byrem, T. M., Render, C. L., Febvay, C., & Houseman, J. T. (2012a). Association between bovine leukemia virus, production, and population age in Michigan dairy herds. J Dairy Sci, 95(2), 727-734.

5. Ramirez Vasquez, N. F., Villar Argaiz, D., Fernandez Silva, J., Londono Pino, J., Chaparro Gutierrez, J. J., & Olivera Angel, M. E. (2016). Seroprevalence and risk factors of several bovine viral diseases in dairy farms of the San Pedro de los Milagros, Antioquia, Columbia (Vol. 11, pp. 15-25). CES Med. Vet. Zootec.

6. Kobayashi, S., Tsutsui, T., Yamamoto, T., Hayama, Y., Kameyama, K., Konishi, M., et al. (2010). Risk factors associated with within-herd transmission of bovine leukemia virus on dairy farms in Japan. BMC Vet Res, 6, 1.

7. Kobayashi, S., Tsutsui, T., Yamamoto, T., Hayama, Y., Muroga, N., Konishi, M., et al. (2015). The role of neighboring infected cattle in bovine leukemia virus transmission risk. J Vet Med Sci, 77(7), 861-863.

8. Ferrer, J. F., Marshak, R. R., Abt, D. A., & Kenyon, S. J. (1978). Persistent lymphocytosis in cattle: its cause, nature and relation to lymphosarcoma. Ann Rech Vet, 9(4), 851-857.

9. Bendixen, B. H. (1960). Leucosis in cattle in Denmark. Pathogenesis and Endemic Status (Vol. 67, pp. 57-63): Deutsche Tierarztliche Wochenschrift.

10. White, T. L., & Moore, D. A. (2009). Reasons for whole carcass condemnations of cattle in the United States and implications for producer education and veterinary intervention. J Am Vet Med Assoc, 235(8), 937-941.

11. Rezac, D. J., Thomson, D. U., Siemens, M. G., Prouty, F. L., Reinhardt, C. D., & Bartle, S. J. (2014). A survey of gross pathologic conditions in cull cows at slaughter in the Great Lakes region of the United States. J Dairy Sci, 97(7), 4227-4235.

12. Panei, C. J., Takeshima, S. N., Omori, T., Nunoya, T., Davis, W. C., Ishizaki, H., et al. (2013). Estimation of bovine leukemia virus (BLV) proviral load harbored by lymphocyte subpopulations in BLV-infected cattle at the subclinical stage of enzootic bovine leucosis using BLV-CoCoMo-qPCR. BMC Vet Res, 9, 95.

13. Janeway, C. A. J., Travers, P., & Walport, M. (2001). Immunobiology: The Immune System in Health and Disease (5th ed.). New York: Garland Science.

14. Maccari, G., Robinson, J., Ballingall, K., Guethlein, L. A., Grimholt, U., Kaufman, J., et al. (2017). IPD-MHC 2.0: an improved inter-species database for the study of the major histocompatibility complex. Nucleic Acids Res, 45(D1), D860-D864.

15. Kobayashi, T., Inagaki, Y., Ohnuki, N., Sato, R., Murakami, S., & Imakawa, K. (2019). Increasing Bovine leukemia virus (BLV) proviral load is a risk factor for progression of Enzootic bovine leucosis: A prospective study in Japan. Prev Vet Med.

16. Lohr, C. E., Sporer, K. R. B., Brigham, K. A., Pavliscak, L. A., Mason, M. M., Borgman, A., et al. (2022). Phenotypic selection of dairy cattle infected with Bovine Leukemia Virus demonstrates immunogenetic resilience through NGS-Based genotyping of BoLA MHC Class II Genes. Pathogens, 11(1).

17. Hutchinson, H. C., Norby, B., Droscha, C. J., Sordillo, L. M., Coussens, P. M., & Bartlett, P. C. (2020). Bovine leukemia virus detection and dynamics following experimental inoculation. *Res Vet Sci, 133*, 269-275.

18. Pavliscak, L. A., Nirmala, J., Singh, V. K., Sporer, K. R. B., Taxis, T. M., Kumar, P., et al. (2021). Tracing viral transmission and evolution of Bovine Leukemia Virus through long read oxford Nanopore Sequencing of the proviral genome. Pathogens, 10(9).

19. Saama, P. M., Jacob, J. B., Kehrli, M. E., Freeman, A. E., Kelm, S. C., Kuck, A. L., et al. (2004). Genetic variation in bovine mononuclear leukocyte responses to dexamethasone. J Dairy Sci, 87(11), 3928-3937.

20. Van Arendonk, J. A. M., Bink, M. C. A. M., Bijma, P., Bovenhuis, H., DeKoning, D. J., & Brascamp, E. W. (1999). Use of phenotype and molecular data for genetic evaluation of livestock. (pp. 60-69): Visions for Animal Breeding and Genetics.

21. Lo, C. W., Borjigin, L., Saito, S., Fukunaga, K., Saitou, E., Okazaki, K., et al. (2020). BoLA-DRB3 polymorphism is associated with differential susceptibility to Bovine Leukemia Virus-Induced lymphoma and proviral load. Viruses, 12(3).

22. Takeshima, S. N., Ohno, A., & Aida, Y. (2019). Bovine leukemia virus proviral load is more strongly associated with bovine major histocompatibility complex class II DRB3 polymorphism than with DQA1 polymorphism in Holstein cow in Japan. Retrovirology, 16(1), 14.

23. Salim, B., Takeshima, S. N., Nakao, R., Moustafa, M. A. M., Ahmed, M. A., Kambal, S., et al. (2021). BoLA-DRB3 gene haplotypes show divergence in native Sudanese cattle from taurine and indicine breeds. Sci Rep, 11(1), 17202.

24. de Araujo Neto, F. R., Vieira, D. A., Santos, D. J. A., Pessoa, M. C., Borquis, R. R. A., de Oliveira, H. N., & Marques, L. F. A. (2020). Population structure of Simmental beef cattle using pedigree analysis. Trop Anim Health Prod, 52(3), 1513-1517

25. Makanjuola, B. O., Miglior, F., Abdalla, E. A., Maltecca, C., Schenkel, F. S., & Baes, C. F. (2020). Effect of genomic selection on rate of inbreeding and coancestry and effective population size of Holstein and Jersey cattle populations. J Dairy Sci, 103(6), 5183-5199

CHAPTER 2

Calf microRNA Abundance Associated with Dam BLV Infection Status

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ABSTRACT

MicroRNAs are a small, noncoding RNA species that are involved in post-transcriptional gene regulation. Research provides evidence that miRNAs influence immune development in calves. Bovine leukosis is a disease in cattle caused by Bovine Leukemia Virus (BLV) and leads to increased susceptibility to opportunistic pathogens. A 2007 USDA survey estimated 89% of dairy operations in the US had at least one animal that was seropositive for BLV. No research has addressed the potential influence that a maternal BLV infection may have on gene regulation through differential expression of miRNAs in progeny. Blood samples from 14-day old Holstein calves born to BLV infected dams were collected. Antibodies for BLV were assessed by ELISA. The amount of BLV provirus, or PVL was assessed by qPCR. RNA was extracted from samples for isolation and miRNA sequencing. Calf microRNA abundance was evaluated to discover differential expression of microRNAs in calves born to BLV infected dams. Four microRNAs (bta-miR-1, bta-miR-133a, bta-miR-133b, bta-miR-206) were downregulated in calves born to BLV infected dams. The four miRNAs appear to be involved in gene regulation of immunological responses and muscle development. These findings provide evidence for the effects that dam BLV infection status may have on development of progeny.

INTRODUCTION

In cattle, environmental impacts in dam health while pregnant can impact the progeny's productive life. For example, heat stress in pregnant dams has been found to decrease lifespan and milk yield in both daughters and granddaughters [1]. Further, variation in maternal nutrition during pregnancy causes reduced immunity and increased risk of obesity in progeny [2, 3]. Arsenault et al. (2014) found that pups born to virally infected dams experienced decreased growth rates and reduced immune signaling up to day ten of life [4]. Given the importance that early calf health has on productive potential, it is important to consider the effects of maternal environment on progeny.

Epigenetic studies may aid to further investigate influences of the intrauterine environment on progeny development. MicroRNAs (miRNA) are a non-coding RNA species, ranging from 18 to 24 nucleotides in length, that play a role in epigenetic regulation through post transcriptional gene regulation [5]. MiRNAs function by repression of target genes in various ways. Complementary binding to mRNA can either provoke cleavage or translational repression of the targeted mRNA [6]. Additionally, research suggests miRNAs can cause deadenylation of a target mRNA, leading to instability and repression of the targeted mRNA [7].

Bovine Leukemia Virus (BLV) is a *delta retrovirus* infecting cattle and the etiologic agent causing enzootic bovine leukosis [8]. Infection by BLV presents in several stages within the host. About 60 to 70% of BLV infected animals remain asymptomatic, 30% develop persistent lymphocytosis, and 2 to 5% develop malignant lymphoma [9]. Dairy producers may suffer increased cost and decreased profit due to BLV infection in the herd. Infected animals that develop persistent lymphocytosis are susceptible to infection by other opportunistic pathogens, increasing vet costs for the producer [10]. Additionally, malignant lymphoma leads to carcass condemnation at slaughter, a direct profit loss to producers [11, 12]. Infection by BLV can be found by a quantification of proviral load (PVL) tested by a qPCR assay, where a greater PVL is indicative of disease progression [13].

In 2008, the United States Department of Agriculture (USDA) estimated 89% US dairy operations were seropositive for BLV, having at least one infected animal in the herd [14]. Bovine Leukemia Virus is a

bloodborne pathogen. Therefore, cattle residing on farms with at least one BLV infected animal are at risk for spreading the virus by blood transfer through, but not limited to, the reuse of examination sleeves, hypodermic needles, and dehorning tools [15, 16, 17].

While research continues to explore the effects of BLV on dairy cattle and the dairy industry, studies exploring the potential effects that dam BLV infection has on early development of progeny is nonexistent. The present study aims to identify differences in miRNA expression in calves born to BLV infected dams. . Results provide evidence of potential alterations in development in calves born to BLV infected dams. Specifically, differential expression of miRNAs involved in gene regulation of immunological response, cardiac development, and skeletal muscle development of calves born to BLV infected dams was observed.

MATERIALS AND METHODS

Samples

The use of all animals in this study was approved by the Institutional Animal Care and Use Committee. All study dams were enrolled in a larger study, which required supplementation of choline at pre-parturition for approximately 30 days. Treated dams were supplemented with 30g (n=10) or 45g (n=6) of choline per day, and control dams were fed no choline (n=8). All study calves were purebred Holstein calves. Twelve treatment calves were born to BLV PVL-positive dams, and 12 control calves were born to BLV PVL-negative dams (Table 2.1). All calves were fed Bovine IgG Colostrum 200 (Saskatoon Colostrum Company, Saskatoon, SK, Canada) to supplement maternal colostrum, followed by Calf's Choice Total HiCal (Saskatoon Colostrum Company, Saskatoon, SK, Canada) throughout the study period of three weeks. Two blood samples were collected via jugular venipuncture from all calves into a PAXgene Blood RNA tube (Becton Dickinson, Franklin Lakes, NJ, USA) and a vacutainer K2 EDTA tube (Becton Dickinson, Franklin Lakes, NJ, USA) at 14 days of age. Samples in PAXgene blood RNA tubes were stored at 4°C for 16 to 24 hours, then stored at -80°C for further analysis. Blood stored in vacutainer K2 EDTA tubes was immediately used to determine LC, then transferred to the laboratory for white blood cell (WBC) preservation.

Table 2.1 Summary Data of Study Animals

Calf	Sex[1]	Calf ELISA OD[2]	Dam BLV Status[3]
1	M	2.507	Negative
2	F	2.253	Negative
3	M	2.441	Negative
4	F	2.585	Negative
5	F	1.246	Negative
6	M	2.461	Negative
7	F	1.952	Negative
8	M	2.628	Negative
9	F	2.257	Negative
10	M	2.756	Negative
11	M	2.246	Negative
12	M	2.765	Negative
13	F	2.061	Positive
14	F	2.622	Positive
15	M	3.093	Positive
16	M	1.611	Positive
17	F	2.720	Positive
18	F	2.820	Positive
19	F	2.596	Positive
20	F	2.325	Positive
21	M	2.232	Positive
22	F	1.927	Positive
23	F	2.052	Positive
24	M	2.909	Positive

[1] M or F describing a male or female calf enrolled in the study. [2] ELISA optical density describing the optical density exhibited by anti-BLV antibodies. [3] Dam BLV status as determined by SS1 qPCR assay.

White Blood Cell Preservation

In brief, red blood cell lysis (ThermoFisher Scientific, Waltham, MA, USA) was diluted to 1x concentration and 1.5mL was added to microcentrifuge tubes containing 500µL of whole blood. All aliquots were inverted for 10 minutes at 20°C, then centrifuged at 500xg for 5 minutes at 20°C. The top plasma layer and lysed red blood cells were removed, avoiding the WBC pellet. The described steps were repeated once more to achieve optimal purification. Finally, 500µL of RNAlater (ThermoFisher Scientific, Waltham, MA, USA) was added to each sample, and samples were incubated overnight at 4°C before long term storage at -80°C. Preserved WBCs were used for PVL determination.

BLV antibody, and BLV PVL Determination

Gp51 antibody capture ELISA test kit (IDEXX Laboratories, Inc., Westbrook ME, USA) was used to determine anti-BLV antibodies from calf plasma samples as previously described by Hutchinson et al., 2020 [18] (Table 2.1). Additionally, both colostrum supplements were assayed for anti-BLV antibodies via milk ELISA.

To determine BLV PVL, DNA extraction was performed from frozen WBC lysates of all 14-day old calf samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). To determine BLV PVL for both dam and calf samples, the SS1 qPCR assay (CentralStar Cooperative, Lansing, MI, USA) was used as previously described [19].

RNA Extraction, Library Preparation, and Sequencing

RNA was isolated from stored PAXgene blood RNA tubes by first centrifuging all tubes at 3000xg for 10 minutes, decanting the supernatant, and adding 4mL of RNase free water. The pellet was vortexed to dissolve into solution, and the centrifugation step was repeated. The supernatant was decanted once again. Total RNA was extracted using the mirVana miRNA Isolation kit following manufacturers protocol (ThermoFisher Scientific, Waltham, MA, USA).

Small RNA libraries were prepared using the NEBNext Small RNA Library prep kit (New England Biolabs, Ipswich, MA, USA) following manufacturer's protocol. RNA quality and quantity was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

The 24 pooled samples were sequenced using a single S1 flow cell on the Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA). Approximately 37 million single-end reads of 100nt were obtained for each sample.

Small RNA processing

Adapter sequence (AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC) was removed from the raw sequencing reads by using cutadapt v1.18 [20]. Reads with low quality bases (Phred score < 20) were trimmed using the dynamictrim function of SolexaQA++ v3.1.7.1 [21]. Reads with lengths shorter than 17 bases were discarded after adapter and quality trimming, and the remaining reads were aligned to the

bovine genome (ARS-UCD1.2) [22] using miRDeep2 v0.0.8 [23]. The parameter allowing a configuration file was used to allow all files to be processed together. Reads aligned to known bovine miRNAs from miRBase (release 22 [24]) were quantified using the default settings of miRDeep2 in which only one mismatch was allowed within the read. In addition, only reads that fit the specified default setting region, namely the length of the miRNA (≥17-≤25 nt) and 7 nucleotide flanking regions (i.e. ≤2 nt upstream and ≤5 nt downstream of the miRNA) were counted. Reads mapped to miRNAs with multiple precursors were only counted once for each read per miRNA.

miRNA Sequence Analysis

Differential expression analysis was performed with RUVseq 1.24.0 in conjunction with EdgeR v3.32.1 [25, 26]. The betweenLaneNormalization function of EDAseq v2.24.0 was used for full-quartile normalization and the RUVg function of RUVseq was implemented to identify a set of *in-silico* empirical control genes with consistent expression across all treatments to remove factors of unwanted variation [26, 27]. After normalization, a negative binomial GLM approach and a likelihood ratio test were implemented with function glmLRT of edgeR to identify differentially expressed miRNAs [24]. Five differential expression tests were performed: (1) calves born to BLV-Positive dams vs calves born to BLV-Negative dams; (2) calves born to BLV-Positive Choline-Control dams vs BLV-Negative Choline-Control dams; (3) calves born to BLV-Positive Choline-Control dams vs BLV-Positive Choline-30 g/day dams; (4) calves born to BLV-Positive Choline-Control dams vs BLV-Positive Choline-45 g/day dams; and (5) calves born to BLV-Positive Choline-30 g/day dams vs BLV-Positive Choline-45 g/day dams. MicroRNAs with a p-value and false discovery rate ≤ 0.05 were considered significant and used for downstream analysis.

RESULTS AND DISCUSSION

All colostrum supplements and calves were positive for the presence of anti-BLV antibodies via ELISA (Table 2.1). Samples from study calves were all BLV PVL-negative, indicating no detectible levels of BLV provirus. Therefore, it is likely the calves obtained anti-BLV antibodies from consumption of colostrum supplements containing BLV antibodies.

Of the 1,094,501,679 read counts obtained from sequencing, 886,018,917 mapped to known bovine reference miRNAs. Two miRNAs (bta-miR-11997, bta-miR-11985) were differentially expressed in progeny born to dams that were infected with BLV and supplemented with 30g of choline per day. No miRNAs were differentially expressed in calves born to BLV infected dams supplemented with 45g of choline per day. Additionally, no miRNAs were differentially expressed in calves born to BLV infected dams fed 30g and 45g of choline per day. Further investigation is necessary to understand possible gene targets and downstream responses in calves born to BLV infected dams supplemented with 30g of choline per day.

Regardless of the dam's choline treatment, four miRNAs (bta-miR-1, bta-miR-206, bta-miR-133a, and bta-miR-133b) were significantly downregulated in calves born to BLV infected dams (Table 2.2). Normalized read counts for each significant miRNA is provided in Table 2.3. Previous publications have found miR-133b and miR-206 are coregulated with genes responsible for IL-17 production in circulating human and mouse T-cells [28]. IL-17 is a cytokine specific to fight extracellular bacteria and fungi during an immune response. Downregulation of bta-miR-133b and bta-miR-206 in the current study suggests possible hindered expression of genes responsible for IL-17 production, or impaired immunological response. It is not uncommon for calves to be infected with E. coli on dairy operations, leading to diarrhea and even death [29, 30]. An impaired immunological response to fungi or bacterial infections in calves born to BLV infected dams may lead to a poor outcome for calves infected with bacteria, such as E. coli.

Table 2.2 Differential Expression of miRNAs in Calves Born to BLV Infected Dams

miRNAs	logFC[1]	logCPM[2]	LR[3]	p-Value	FDR[4]
bta-miR-1	-6.548	5.506	29.893	4.57E-08	4.70E-05
bta-miR-206	- 5.793	3.979	25.800	3.79E-07	0.000
bta-miR-133a	-4.006	3.022	16.220	5.64E-05	0.019
bta-miR-133b	-4.337	0.284	13.760	2.08E-04	0.043

[1] log fold change in differential expression. [2] log counts per million. [3] likelihood ratio. [4] False discovery rate.

Table 2.3 Normalized Read Counts of Differentially Expressed miRNAs in Study Calves

Calf	Dam BLV Status[1]	bta-miR-1	bta-miR-206	bta-miR-133a	bta-miR-133b
1	Negative	1.39	0.73	1.60	0.16
2	Negative	0.38	0.34	0.14	0.07
3	Negative	0.73	0.19	1.12	0.16
4	Negative	6.71	7.28	10.55	1.35
5	Negative	0.52	0.43	0.12	0
6	Negative	975.66	333.18	145.26	22.21
7	Negative	0.28	0.07	0.24	0
8	Negative	9.2	0	0	0
9	Negative	0.45	0.11	0.14	0
10	Negative	1.04	0.37	0.44	0
11	Negative	92.00	18.40	73.60	0
12	Negative	0.40	0.17	0.24	0.02
13	Positive	0.45	0.49	1.14	0.13
14	Positive	0.93	0.88	3.48	0.46
15	Positive	1.03	0.34	0.39	0.03
16	Positive	0.51	0.38	0.17	0.04
17	Positive	0.59	0.59	0.32	0.05
18	Positive	1.00	0.19	0.23	0.04
19	Positive	0.77	1.17	1.50	0.21
20	Positive	0.30	0.25	0.21	0
21	Positive	0.72	0.42	0.48	0.09
22	Positive	0.85	0.33	0.33	0.03
23	Positive	0.59	0.30	0.30	0.07
24	Positive	4.72	2.07	2.81	0.25
Total[2]		**1,101.22**	**368.68**	**244.81**	**25.37**

[1] Dam BLV status as determined by SS1 qPCR assay. [2] Total normalized count of each differentially expressed miRNA.

In human and mouse orthologs, all four identified miRNAs belong to a gene cluster. MiR-1 and miR-133a are believed to be transcribed together, and miR-206 and miR-133b are transcribed together. Human and mouse orthologs of the four miRNAs have been demonstrated to play roles in cardiac and skeletal muscle growth and differentiation. Specifically, miR-1 targets the Hand2 transcription factor, which is necessary for cardiac development [31]. MiR-1 also targets HDAC4, which functions to repress muscle growth [32]. MiR-133 seems to oppose the function of miR-1 by targeting the SRF gene, which functions to activate muscle growth [33]. Further, miR-206 indirectly upregulates MyoD, leading to muscle cell differentiation [34]. Recognizing that downregulation of miRNAs suggests upregulation of target genes, it

may be that Hand2, HDAC4, SRF, and MyoD are upregulated in the study population at 14 days of life. This finding suggests that the presence of BLV infection in dams influences skeletal muscle and cardiac growth and development in their progeny – potentially due to misregulation of these target genes. Studies in mice reveal that miR-1 and -133a are involved in cardiac growth and development, in addition to their role in skeletal muscle growth and development. When gene clusters for miR-1 and miR-133a were removed, no embryos survived [35]. Upon post-mortem examination, these embryos exhibited developmental heart defects [36]. The data demonstrates a connection between BLV infection in the dam and impairment of gene expression involved in cardiac development of her progeny.

CONCLUSION

Publications have identified the impacts a dam's viral infection status has on the progeny's growth and immunological development [4]. Other environmental factors affecting the dam, such as heat stress and nutrition have been associated with impaired productive life of progeny [1, 2, 3]. The mechanisms by which the intrauterine environment may impact the life of progeny can be studied through identifying epigenetic regulation patterns including investigating DNA methylation patterns, histone modification, or miRNA expression. The present study identifies differences in miRNA expression in progeny born to BLV infected dams in an effort to obtain evidence of potential misregulation of genes in these calves. MicroRNAs have been shown to play a role in early calf development [35, 36]. In the current study, four differentially expressed miRNAs (bta-miR-1, bta-miR-133a, bta-miR-133b, bta-miR-206) were found to be downregulated in calves born to BLV infected dams. MiR-133b and miR-206 were found to coregulate with IL-17 production in T-cells [27]. Studies have associated all four downregulated miRNAs with skeletal muscle and cardiac development in human and mouse orthologs [31, 32, 33, 34]. Study findings indicate that dam BLV infection during gestation may impact the progeny's immunological response as well as cardiac and skeletal muscle development, though the connection is unclear. Future studies are necessary to obtain additional data and investigate this connection.

MicroRNA expression fluctuates depending on stage of life, immune regulations, and multiple other functions within an organism [37]. The study obtained a single time point of miRNA expression at 14

days of age. Therefore, longitudinal studies are necessary to assess the differential expression of miRNAs in calves to achieve a more thorough understanding of the epigenetic effects of dam BLV infection through her progeny's life span. It may also be valuable to collect animal metadata on disease status and growth throughout life to further investigate the potential role miRNAs play in development and productive life of progeny. Additional future work may investigate other means of epigenetic regulation, including DNA methylation patterns and histone modifications.

REFERENCES

1. Laporta, J., Ferreira, F. C., Ouellet, V., Dado-Senn, B., Almeida, A. K., De Vries, A., et al. (2020). Late-gestation heat stress impairs daughter and granddaughter lifetime performance. *J Dairy Sci, 103*(8), 7555-7568

2. Moriel, P., Piccolo, M. B., Artioli, L. F., Marques, R. S., Poore, M. H., & Cooke, R. F. (2016). Short-term energy restriction during late gestation of beef cows decreases postweaning calf humoral immune response to vaccination. *J Anim Sci, 94*(6), 2542-2552.

3. Alfaradhi, M. Z., & Ozanne, S. E. (2011). Developmental programming in response to maternal overnutrition. *Front Genet, 2*, 27.

4. Arsenault, D., St-Amour, I., Cisbani, G., Rousseau, L.-S., & Cicchetti, F. (2014). The different effects of LPS and poly I:C prenatal immune challenges on the behavior, development, and inflammatory responses in pregnant mice and their offspring (Vol. 38, pp. 77-90). Elsevi: Elsevier, Inc.

5. Legoff, L., D'Cruz, S. C., Tevosian, S., Primig, M., & Smagulova, F. (2019). Transgenerational Inheritance of Environmentally Induced Epigenetic Alterations during Mammalian Development. *Cells, 8*(12).

6. Dykxhoorn, D. M., Novina, C. D., & Sharp, P. A. (2003). Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol, 4*(6), 457-467.

7. Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., et al. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science, 312*(5770), 75-79

8. Aida, Y., Murakami, H., Takahashi, M., & Takeshima, S. N. (2013). Mechanisms of pathogenesis induced by bovine leukemia virus as a model for human T-cell leukemia virus. *Front Microbiol, 4*, 328.

9. Bendixen, H.J., 1960a. Leucosis in cattle in Denmark. II. Pathogenesis and endemic status. Deutsche Tierarztliche Wochenschrift, 67, 57-63

10. Erskine, R. J., Bartlett, P. C., Byrem, T. M., Render, C. L., Febvay, C., & Houseman, J. T. (2012b). Herd-level determinants of bovine leukemia virus prevalence in dairy farms. *J Dairy Res, 79*(4), 445-450.

11. White, T. L., & Moore, D. A. (2009). Reasons for whole carcass condemnations of cattle in the United States and implications for producer education and veterinary intervention. *J Am Vet Med Assoc*, 235(8), 937-941.

12. Rezac, D. J., Thomson, D. U., Siemens, M. G., Prouty, F. L., Reinhardt, C. D., & Bartle, S. J. (2014). A survey of gross pathologic conditions in cull cows at slaughter in the Great Lakes region of the United States. *J Dairy Sci*, 97(7), 4227-4235.

13. Panei, C. J., Takeshima, S. N., Omori, T., Nunoya, T., Davis, W. C., Ishizaki, H., et al. (2013). Estimation of bovine leukemia virus (BLV) proviral load harbored by lymphocyte subpopulations in

BLV-infected cattle at the subclinical stage of enzootic bovine leucosis using BLV-CoCoMo-qPCR. *BMC Vet Res, 9*, 95.

14. U.S. Department of Agriculture, Animal and Plant Health Inspection Service. Bovine Leukosis Virus (BLV) on U.S. Dairy Operations, 2007. October 2008.

15. Erskine, R. J., Bartlett, P. C., Byrem, T. M., Render, C. L., Febvay, C., & Houseman, J. T. (2012a). Association between bovine leukemia virus, production, and population age in Michigan dairy herds. *J Dairy Sci, 95*(2), 727-734.

16. Ramirez Vasquez, N. F., Villar Argaiz, D., Fernandez Silva, J., Londono Pino, J., Chaparro Gutierrez, J. J., & Olivera Angel, M. E. (2016). Seroprevalence and risk factors of several bovine viral diseases in dairy farms of the San Pedro de los Milagros, Antioquia, Columbia (Vol. 11, pp. 15-25). CES Med. Vet. Zootec.

17. Kobayashi, S., Tsutsui, T., Yamamoto, T., Hayama, Y., Kameyama, K., Konishi, M., et al. (2010). Risk factors associated with within-herd transmission of bovine leukemia virus on dairy farms in Japan. *BMC Vet Res, 6*, 1.

18. Hutchinson, H. C., Norby, B., Droscha, C. J., Sordillo, L. M., Coussens, P. M., & Bartlett, P. C. (2020). Bovine leukemia virus detection and dynamics following experimental inoculation. *Res Vet Sci, 133*, 269-275.

19. Pavliscak, L. A., Nirmala, J., Singh, V. K., Sporer, K. R. B., Taxis, T. M., Kumar, P., et al. (2021). Tracing viral transmission and evolution of Bovine Leukemia Virus through long read oxford nanopore sequencing of the proviral genome. *Pathogens, 10*(9).

20. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J*. 2011;17(1):10–12.

21. Cox M. P., Peterson D.A., Biggs PJ. SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics*. 2010. September 27;11:485.

22. Rosen B.D., Bickhart D.M., Schnabel R.D., Koren S., Elsik C.G., Tseng E., Rowan T.N., Low W.Y., Zimin A., Couldrey C., Hall R., Li W., Rhie A., Ghurye J., McKay S.D., Thibaud-Nissen F., Hoffman J., Murdoch B.M., Snelling W.M., McDaneld T.G., Hammond J.A., Schwartz J.C., Nandolo W., Hagen D.E., Dreischer C., Schultheiss S.J., Schroeder S.G., Phillippy A.M., Cole J.B., Van Tassell C.P., Liu G., Smith T.P.L., Medrano J.F.. De novo assembly of the cattle reference genome with single-molecule sequencing. Gigascience. 2020 Mar 1;9(3):giaa021.

23. Friedlander M.R., Mackowiak S.D., Li N., et al. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res*. 2012. January;40(1):37–52

24. Kozomara A., Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2013;42(D1):D68–D73

25. Robinson M.D., McCarthy D.J., Smyth G.K.. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010; 26(1):139–40.

26. Risso D., et al. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat Biotechnol.* 2014; 32(9):896–902.

27. Risso D., Schwartz K., Sherlock G., Dudoit S. (2011). "GC-Content Normalization for RNA-Seq Data."*BMC Bioinformatics*, **12**(1), 480.

28. Haas, J. D., Nistala, K., Petermann, F., Saran, N., Chennupati, V., Schmitz, S., Korn, T., Wedderburn, L. R., Förster, R., Krueger, A., & Prinz, I. (2011). Expression of miRNAs miR-133b and miR-206 in the Il17a/f locus is co-regulated with IL-17 production in αβ and γδ T cells. *PloS one*, *6*(5), e20171.

29. Worley, J. N., Flores, K. A., Yang, X., Chase, J. A., Cao, G., Tang, S., et al. (2017). Prevalence and Genomic Characterization of Escherichia coli O157:H7 in Cow-Calf Herds throughout California. *Appl Environ Microbiol, 83*(16).

30. Viidu, D. A., & Mõtus, K. (2022). Implementation of a pre-calving vaccination programme against rotavirus, coronavirus and enterotoxigenic Escherichia coli (F5) and association with dairy calf survival. *BMC Vet Res, 18*(1), 59.

31. Zhao, Y., Ransom, J. F., Li, A., Vedantham, V., von Drehle, M., Muth, A. N., et al. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell, 129*(2), 303-317.

32. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., et al. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet, 38*(2), 228-233.

33. Kim, H. K., Lee, Y. S., Sivaprasad, U., Malhotra, A., & Dutta, A. (2006). Muscle-specific microRNA miR-206 promotes muscle differentiation. *J Cell Biol, 174*(5), 677-687.

34. Wystub, K., Besser, J., Bachmann, A., Boettger, T., & Braun, T. (2013). miR-1/133a clusters cooperatively specify the cardiomyogenic lineage by adjustment of myocardin levels during embryonic heart development. *PLoS Genet, 9*(9), e1003793.

35. Do, D. N., Dudemaine, P. L., Fomenky, B. E., & Ibeagha-Awemu, E. M. (2018). Integration of miRNA and mRNA Co-Expression Reveals Potential Regulatory Roles of miRNAs in Developmental and Immunological Processes in Calf Ileum during Early Growth. *Cells, 7*(9).

36. Jacometo, C. B., Alharthi, A. S., Zhou, Z., Luchini, D., & Loor, J. J. (2018). Maternal supply of methionine during late pregnancy is associated with changes in immune function and abundance of microRNA and mRNA in Holstein calf polymorphonuclear leukocytes. *J Dairy Sci, 101*(9), 8146-8158.

37. Kim, D., Grün, D., & van Oudenaarden, A. (2013). Dampening of expression oscillations by synchronous regulation of a microRNA and its target. *Nat Genet, 45*(11), 1337-1344.