RELATIONSHIP BETWEEN PROVIRAL LOAD, MHCII DRB3 ALLELES, AND THE EFFECT OF IMMUNIZATIONS ON PROVIRAL LOAD IN BOVINE LEUKEMIA VIRUS INFECTED DAIRY CATTLE

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ABSTRACT

Bovine leukemia virus (BLV) is the causative agent of a life-long disease in cattle. In dairy cows, it has been shown to decrease productivity, negatively affect immune response to novel pathogens and vaccinations, and in turn leads to increased culling rates. This dissertation has shown that by inoculating cows with a novel antigen, proviral load (PVL) may increase and pose risk to uninfected herdmates. This dissertation has also shown that Michigan dairy cows have a limited major histocompatibility complex II (MHCII) repertoire of alleles. The alleles direct the genetically inherited immune response to pathogens through antigen recognition among the binding site. The lower the repertoire of alleles, the less likely pathogens are to be recognized among a population. Together, these results may suggest the need for new BLV herd management strategies that could include further segregation by BLV PVL status, as well as the opportunity to genetically select cattle based on their genetically inherited immune response.

This thesis is dedicated to my parents, David and Annemarie Eichberger; to my sisters, Nicole and Julia Eichberger; and to my husband, Lukas Reynolds.

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LIST OF ABBREVIATIONS

AL	Aleukemic
APCs	Antigen presenting cells
BA	Bovine beta actin
BLV	Bovine leukemia virus
BLV+	Bovine leukemia virus ELISA-positive
BLV-	Bovine leukemia virus ELISA-negative
BHV-1	Bovine herpes virus type-1
BoLA	Bovine leukocyte antigen
BSA	Bovine serum albumin
CBC	Complete blood count
cDNA	Copy DNA
DDA	Dimethyldioctadecylammonium bromide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
gDNA	genomic DNA
HPL	High proviral load
IUPAC	International Union of Pure and Applied Chemistry
KLH	Keyhole limpet hemocyanin
LPL	Low proviral load
MHCII	Major histocompatibility complex II
OD	Optical density
PBS	Phosphate-buffered saline

PL	Persistent lymphocytosis
PVL	Proviral load
TMB	Tetramethylbenzidine substrate

CHAPTER 1: INTRODUCTION

Bovine Leukemia virus (BLV) is a delta retrovirus that causes a latent disease in cattle, enzootic bovine leukosis, which may develop into persistent lymphocytosis (PL) or lymphosarcoma [1-3]. BLV is known to primarily infect B cells of cattle and can reduce levels of both total and antigen specific antibodies resulting in a compromised immune system [1, 2, 4]. BLV can also affect some function of T cells [5-8]. These properties of BLV may lead to increased infections, reduced responses to vaccines, lower cow longevity and decreased milk production [1, 9].

Some BLV-positive (BLV+) cattle defined by enzyme linked immunosorbent assay (ELISA), although not clinical, can still spread the virus within herds. BLV+ cattle can also be further defined by the number of lymphocytes circulating in their peripheral blood. Aleukemic (AL) cattle have a normal or slightly elevated lymphocyte count relative to uninfected cattle. Persistent lymphocytotic (PL) cattle (~30% of BLV+ cattle) have an extremely elevated lymphocyte count, while 5% of BLV+ cows will progress to or develop clinical lymphosarcoma [10]. The loss of milk production in combination with lymphosarcoma results in an annual economic loss of $525 [11]. BLV infection of B cells and T cells have been individually studied, along with the importance of each cell type in cattle's immune response [1, 13-15]. While BLV infection of B cells can readily be explained by the presence of BLV expressing proteins, the effect of BLV on T cell function may not be as clear. Importantly, previous studies in our lab and other literature have confirmed that BLV targets B cells, thus rendering them dysfunctional [1-3, 16].

Through designed studies and use of collected data, the aim of this dissertation is to explore the genetically determined immune response that may be responsible for a cows BLV

disease progression, as well as how vaccines targeting other pathogens may also influence BLV proviral load (PVL).

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CHAPTER 2: BoLA DRB3 MHCII EXON 2 ALLELE ASSOCIATED WITH MICHIGAN HOLSTEIN CATTLE BLV DISEASE STATUS AND PROVIRAL LOADS

Abstract

Bovine leukemia virus (BLV), a delta retrovirus that primarily infects cattle, is the causative agent of the latent disease enzootic bovine leukosis. BLV primarily infects the B cell, an immune cell that is responsible for producing antibodies following antigen stimulation. Nearly 80% of dairy operations in the United States have at least one cow that is seropositive for BLV. Recent studies have investigated possible genetic attributions that may influence a dairy cow's resistance or susceptibility to disease progression through genetic variation in the major histocompatibility complex II (MHCII) bovine leukocyte antigen (BoLA) DRB3 Exon 2. This study aims to explore the MHCII BoLA DRB3 Exon 2 allelic associations of 135 Michigan Holstein dairy cows with proviral load (PVL) and BLV disease status. Allele 0902 was found to be significant among BLV-ELISA negative (BLV-) cows. Allele 0101 was trending towards significance among cows with a low PVL.

Introduction

Bovine leukemia virus (BLV) is a delta retrovirus responsible for causing a latent disease in cattle, enzootic bovine leukosis. Transmission has been attributed to reused hypodermic needles, direct contact with infected animals, dehorning, reused examination sleeves, and possibly by biting insects [1-3]. Once cattle are infected, the virus primarily targets B cells where it can establish a latent infection. Once a B cell is infected, the virus will insert a provirus into the host genome, which allows the virus to cause lifelong infection as a retrovirus, rendering the B cell dysfunctional [4, 5]. Many infected animals show no clinical signs of the infection and show only low amounts of proviral load (PVL). PVL is the measure of the BLV provirus copies

that have been integrated into the host genome relative to a single copy host gene, bovine beta actin (BA), that is expressed in all cells. Animals with low proviral load (LPL), are designated as aleukemic (AL). About one third of infected animals will develop persistent lymphocytosis (PL). PL is defined by high lymphocyte counts with concomitant increases in PVL. Only 5% of infected animals will progress to lymphosarcoma [4, 6, 7].

BLV infection may lead to increased secondary infections, reduced responses to vaccines, shorter cow longevity and decreased milk production [4, 8-11].Lymphosarcoma is a major cause for condemnation of cattle carcasses at slaughter and a large portion of these are likely due to BLV [7]. These effects among herds where BLV is present were estimated to decrease annual milk production value by $59 per cow [12]. Previous studies have estimated BLV to infect 40% of cattle in the United States, while >80% of herds are infected with at least one enzyme-linked immunosorbent assay (ELISA) determined BLV-positive (BLV+) cow [13, 14].

A large number of studies have shown that infection with BLV can lead to a dysfunctional immune system. B cells have been studied most intensely since they are the primary cell targeted by BLV. Some studies suggest that BLV-infected cattle suffer from a significant reduction of B cell proliferation compared to BLV-ELISA negative (BLV-) cows [15]. To further corroborate, a 2017 study showed that B cells from ELISA BLV+ cows had reduced activation following immune stimulation compared to BLV- cows [16]. As a result, infected B cells may result in reduced levels of antibodies, leading to a compromised immune system [4, 16, 17]. It has been reported that surface and circulating levels of IgM expression, which are antibodies important for immune response against secondary infections, and that surface expression of major histocompatibility complex II (MHCII) on $CD5^+$ B cells are reduced

in BLV+ cows. [4, 18-20]. These circulating levels of IgM were shown to be reduced in response to the specific antigens *L. hardjo, L. Pomona* and bovine herpes virus type-1 (BHV-1), which are common pathogens that dairy cattle are vaccinated against, again suggesting a reduced immune response [4]. In contrast, a study suggests that surface IgM levels in BLV+ cattle are elevated [16].

The immune pathway directed by MHCII on antigen presenting cells (APCs), such as B cells, and macrophage cells may influence BLV disease progression. Because helper T cell activation ($CD4^+$) is directly dependent on the expression and function of MHCII, it is important to discuss the effects BLV has on T cells. Although BLV has not been shown to alter MHCII surface expression on T cells [21], recent studies have determined that the mean florescent intensity of MHCII on B cells is elevated in BLV+ cows relative to BLV- cows [4]. Further, *in vitro* studies suggest that cultured T cells isolated from BLV+ cattle have altered responses to immune stimulants compared to BLV- cattle. For example, one study showed that PL cattle had $CD4^+$ T cells expressing significantly higher levels of FoxP3, which is known to reduce T cell activation and proliferation [22], while another suggests that BLV+ cows had significantly lower percentages of circulating $CD4^+$, $CD8^+$ and $\gamma\delta$ T cells [4, 23-24]. A more recent study which focused on Vitamin A restriction in diets in BLV+ cows suggests that BLV negatively affects T cell phenotypes, or expression of CD markers. The proportion of $CD4^+$, $CD3^+$ and $CD8^+$ T cells was negatively correlated with BLV PVL [25]. This abundance of data suggests that an infected BLV cow may have compromised T cell reactivity. T cell activity is known to depend on MHCII and MHCI reaction cascade via $CD4^+$ and $CD8^+$ T cells, respectively.

A specific region of the gene encoding MHCII in cattle, referred to as *BoLA-DRB3,* are polymorphic with 384 identified alleles in the DRB3 Exon 2 alone [26]. *BoLA DRB3* encodes for various peptide binding grooves along MHCII which allow for presentation of antigens to T cells. This antigen presentation interaction between MHCII and T cells results in a cascade of immune responses to the presented antigen. This study was designed to identify associations between a cows' BLV disease status, PVL (if BLV+), and MHCII alleles within samples obtained from Michigan dairy herds.

BLV has been eradicated in 22 countries across the world since the 1980s [14]. The United States, Canada, Japan and many countries in South America have yet to eradicate the virus. Other countries, such as Japan, Myanmar and Argentina have begun studying the genetic associations of BLV infection and immune phenotypes in combination with management strategies based on PVL [27-36]. More recent studies have also began investigation of the genetic associations between BLV infection and variation of BoLA alleles [37, 39]. PVL is the measure of the BLV provirus copies that have been integrated into the host genome relative to a single copy host gene, bovine beta actin, that is expressed in all cells. These studies have identified that major histocompatibility complex II (MHCII) BoLA DRB3 alleles are associated with BLV susceptibility or resistance within a BLV+ cows' PVL [29, 37].

Methods

Sample Collection and preparation

Samples of serum and buffy coat derived from whole blood were derived from a previous study focused on the genetics of Johne's Disease in Holstein dairy cows across the state of Michigan from 8 dairy farms (n=135) [38]. Genomic DNA (gDNA) samples were prepared from buffy coats and stored at -80°C. The serum samples were utilized to determine if the cows were

either BLV-positive or BLV-negative (BLV-) by ELISA. All BLV-ELISA-positive (BLV+) gDNA samples were normalized to 35ng/µl prior to PVL analysis.

Proviral load quantification

All gDNA samples determined to be BLV+ were amplified to quantify BLV PVL. BLV PVL was determined using the SS1 assay (CentralStar Cooperative) and were run in triplicate according to assay manufacturer (CentralStar Cooperative). Briefly, copy number of BLV proviruses was determined relative to a single copy host gene control, bovine Beta Actin (BA), and was calculated as a ratio of the BLV PVL copy to the BA copy. The BLV and BA standards were created by serial dilution of 10^6 and ending at 10^1. 19µl of Master Mix (IDT PrimeTime) was added to each standard well, while 11µl of BLV or BA standard was added to each standard well. Standards were run on each 96-well plate. 17µl of Master Mix was added to all other sample wells and 31µl sample gDNA was added to each assigned well. In the no-template control well, 201µl of Master Mix was added with no sample. 19µl of Master Mix was added to the positive control well along with 1µl of positive control gDNA. Once prepared, each plate was run using the ABI 7500 qPCR instrument for amplification of the BLV PVL (Thermo Fisher Scientific). For 40 cycles, the instrument was programmed as follows: 95C x10min, 95C x15sec, 60C x1min, 60C x1min.

Amplification and Sanger sequencing of the MHCII Exon 2 BoLA DRB3 gene

All samples, regardless of BLV status, were prepared for amplification and sequencing of BoLA (MHCII) DRB3 Exon 2. Primer sequences were obtained from the literature [29] and were confirmed 100% match to Bos Taurus BoLA using NCBI BLAST nucleotides 100-122 in GenBank sequence ID LR797980.1 (DRB3FWD) and nucleotides 268-283 in GenBank sequence ID KF918698.1 (DRB3REV). The primer pair is as follows: DRB3FWD: (5'-

CGCTCCTGTGAYCAGATCTATCC-3') and DRB3REV: (5'-CACCCCCGCGCTCACC-3'). All PCR amplification took place in an Applied BioSystems Thermocycler and were run in duplicate. Any samples that contained more than 1000ng/µl of gDNA were diluted 1:10 with nuclease free water. The reaction mixture contained 5µl of Taq buffer (New England BioLabs), 1µl of 10mM dNTPs (Invitrogen), 1µl of both the forward and reverse primer, 5µl of template (gDNA sample), and 0.25µl of *Taq* Polymerase (New England BioLabs) and brought up to 50µl volume by adding nuclease free water. According to *Taq* Polymerase requirements, an initial duration of 94°C x30s and 35 cycles as follows: 95°C x20sec, 58°C x15sec, 68°C x1min and a final extension of 72°C x5min. Amplification products were observed and documented while under blue light transillumination following electrophoresis in a 1.2% agarose gel stained with ethidium bromide in TAE buffer.

Following PCR amplification, samples were purified using the Wizard SV Gel and PCR Clean-Up System per manufacturer protocol (Promega). All samples were checked for amplification quality and quantity using the Nanodrop ND 1000 Spectrophotometer.

After confirming quality and quantity of DNA (20-200ng/ul, A260/280 ~1.85), samples were prepped for Sanger sequencing to be done by an external source, Genewiz. Per Genewiz protocol, samples were pre-mixed with the purified PCR product and either DRB3FWD or DRB3REV primers mentioned above. 10ng of purified PCR product in a volume of 10µl with 5µl of primer at a concentration of 25pmol was added to a 96-well plate for a total of 15µl per reaction. Samples were sealed and shipped overnight to Genewiz with dry ice to ensure sample integrity.

https://www.genewiz.com/Public/Resources/Sample-Submission-Guidelines/Sanger-Sequencing-Sample-Submission-Guidelines

Analysis of Sanger sequencing results

Following Sanger Sequencing, sample data were uploaded to MacVector (software version 18.2) to confirm base pair assignment and manually annotate any possible discrepancies utilizing IUPAC (International Union of Pure and Applied Chemistry) nomenclature. Sequences were manually edited based on electropherogram results displayed in MacVector. When necessary, areas containing single nucleotide polymorphisms were denoted using IUPAC nomenclature. Sequencing samples were then uploaded to NCBI BLAST software and individually compared to all possible combinations of alleles. Samples with 100% matches to homozygous or heterozygous genotypes listed in the proprietary reference database (generously provided by Dr. Bonnie Mallard, University of Guelph, Ontario, Canada) were noted and visually confirmed via subject and query alignment comparison of IUPAC nomenclature within NCBI BLAST results and recorded.

Statistical Analysis

We tested for association between each of the BoLA DRB3 alleles with BLV disease status or BLV proviral status (PVL > 0) using Fisher's exact test, which derives P values from the hypergeometric distribution. The test considers a 2x2 contingency table that tallies counts of four categories of alleles, including 1) presence of the BoLA DRB3 allele in BLV+ cows; 2) absence of the BoLA DRB3 allele in BLV+ cows; 3) presence of the BoLA DRB3 allele in BLV- cows; and 4) absence of the BoLA DRB3 allele in BLV- cows. The odds ratio of an allele in BLV+ and BLV- cows were also calculated to estimate disease status associations.

Results

BLV disease status and allelic associations

Of the gDNA samples, 135 cows with known BLV disease status were viable for PVL determination and Sanger Sequencing. Due to sample quality or quantity of gDNA needed to determine PVL and/or Sanger Sequencing, 37 samples were not included in analysis. Of the 135 samples, 83 cows were BLV+ and 52 cows were BLV- as previously determined by ELISA. Of the 384 known BoLA MHCII Exon 2 alleles, 15 alleles were identified in the sample population of Michigan dairy cows (Table 1). No alleles were associated with ELISA and PVL BLV+ cows, while allele *0902 was associated with BLV- cows (p=0.02123), and allele *2703 was trending towards significance in BLV- cows (p=0.1383) (Table 2).

BLV+ cows and allelic associations to PVL

BLV+ cow samples had PVL determined and were then split between high PVL (HPL) and low PVL (LPL). HPL cows were defined to have a ratio equal to or greater than 1 copy of BLV provirus per BA, while LPL cows were determined to have less than 1 copy of BLV provirus per BA. Of the viable BLV+ samples, 17 cows were determined to have HPL and 29 cows had LPL for a total of 46 cows. Alleles *0101, *0501 and *1501 were determined to be trending towards a significant frequency among LPL cows (p=0.1152, p=0.1535 and p=0.2266, respectively).

Allelic suggestion of dominance or recessive affect

Two models were performed among all BLV+ and BLV- alleles. The dominance model was performed to determine if having no copy (0) one (1) or two (2) copies of the allele, being heterozygous or homozygous, respectively, influenced disease status (summary table not provided). The model suggests that allele *0902 is dominant (p= 0.02046), while allele *1001 is

trending towards being a dominant allele (p= 0.196). The recessive model had no significant or trending findings.

Discussion

Michigan dairy cows have limited DRB3 BoLA MHCII genetic diversity

Of the 384 known BoLA DRB3 alleles, 15 alleles were identified in this study. The sampled Holstein dairy herds, which vary in location across the lower peninsula of Michigan, each likely have unique breeding protocols. As such, this suggests that the Michigan Holstein cows included in this study have decreased genetic variance in the BoLA DRB3 gene, which directs the genetically inherited immune response to pathogens because it encodes for the MHCII biding site on antigen presenting cells [43]. This in turn dictates the binding affinity of an antigen to MHCII. This binding affinity then directs the immune pathway to either enlist a strong, moderate, or insufficient immune response to the pathogen. Thus, because only 15 alleles were determined across this study population, it can be concluded that Michigan Holstein cows may have a limited immune repertoire that is dictated by the present MHCII alleles.

Other studies in Michigan and the Midwest region of the US have found similar findings. Two studies both identified only 18 BoLA DRB3 alleles, which consisted of 1 (n=558) and 3 (n=574) breeds each. [37, 39]. In contrast a study performed outside of the US was able to identify 71 alleles among Myanmar cattle (n=294) among 17 breeds [40]. These breed differences in US and Myanmar studies further highlights the lack of diversity in US cattle that may stem from controlled breeding programs. Due to this Myanmar study's vast inclusion of breeds, this may contribute to the increased diversity in DRB3 BoLA alleles in contrast to the US studies that had limited breeds studied. Our current study also further highlights that US cattle have limited genetic diversity of the DRB3 BoLA alleles, which may have been caused by

the selective genetic pressure across the US dairy and beef industry. It is important to note that the BoLA DRB3 allele is not included in genetic selection and testing prior to breeding. Rather, selection in breeding programs is often based on production and meat quality. As genes related primarily to production traits are under selection in dairy breeding programs, genes associated with other traits, like immune response, may or may not be under selective pressure.

Allelic associations and frequencies among BLV+ and BLV- cows

Of the 15 alleles identified, allele *0101 had a frequency of 15% among BLV+ cattle, but 8% among BLV- cattle. Although not statistically significant with either disease status (BLV+ or BLV-), this allele was found to be trending towards significance with the highest frequency among LPL cattle at 27.5% (p= 0.1152). This suggests that allele *0101 may provide protection against BLV infection and could be classified as a resistant allele. Another study among cattle found that allele *0101 is not statistically associated with HPL or LPL cattle. Rather, the frequency was almost identical between the two designated groups [29].

Allele *0902 was significantly associated among BLV- cows (p=0.02123) with a frequency of 1.5% in BLV- cows, and not identified in any BLV+ cows. Similarly, another Michigan study found a frequency of only 0.6% (n=157) among beef cattle [39]. A separate study among Midwest dairy cows found a 20% frequency of allele *0902 which was also associated with the lowest PVL cows [37] . Allele *0902 has increasingly been found to be internationally associated with LPL cows and is typically referred to as a BLV resistant allele [28, 29, 37, 41, 42]. Some studies suggest that once infected, cattle with the *0902 allele will progress to LPL and generally do not progress to HPL and have a lower rate of lymphosarcoma [29, 33, 34].

Allele *1501, often referred to as a BLV susceptibility allele, was not found to be statistically significant among this study's BLV+ cows (p= 1.0) [42, 44]. Within BLV+ cows, allele *1501 had the highest frequency among HPL cows at 35% and 22% among LPL cows, the second highest behind allele *0101, with no statistical significance (p= 0.2266). This data is similar to that of a Michigan study of beef cattle where no statistical significance for allele *1501 among BLV+ cows was found [39]. In contrast, a separate study found that among Midwest BLV+ cows, the cows with the highest PVL were associated with allele *1501 and *2703 [37].

Allele *0501 showed no significant frequencies between BLV+ or BLV- cows (p= 1.0). However, it may be trending towards significant association among LPL cows with a frequency of 8.6% (p= 0.1535). Although this allele does not prevent infection, it may have a protective effect against viral replication that leads to a HPL. The statistical analysis of this data suggests that a larger sample population would be needed to draw a definitive conclusion of this potential protective effect. This may suggest that allele *0501 is a BLV resistant allele. To date, this is the first study to identify this DRB3 BoLA allele among Michigan dairy cattle.

BoLA DRB3 resistant allele shows dominance in Michigan dairy cows

A dominance and recessive model were created to determine if BoLA DRB3 alleles may be dominant or recessive among the sample population. If an allele was determined to be dominant, the cow could possess in one or two copies of the allele, resulting in either a heterozygous or homozygous genotype. If an allele was determined to be recessive, a cow would possess two copies of the allele as a homozygous genotype. No alleles were shown to be recessive. However, allele *0902 was shown to be dominant (p= 0.0205). Because this allele is suggested to be resistant to BLV infection across this study and others, it can therefore be

suggested that if a cow has at least one copy of allele *0902, it could be protected from BLV disease progression, or resistant to HPL when infected. This further suggests that allele *0902 could be genetically selected for protection from BLV [28, 29, 37, 41, 42].

Conclusion

Further associations have been made between BoLA DRB3 alleles, BLV status, and PVL in Michigan Holstein cows. The authors acknowledge limitations among this study's population size, therefore, we suggest further research to include a larger population among Michigan Holstein dairy cows prior to considering breeding programs including the BoLA DRB3 allele. With several studies now highlighting the decreased immunological variety of these alleles among Michigan dairy cows, a meta-analysis may provide further clarification and insight to these corresponding findings. With such a study, Michigan dairy producers may consider selecting cattle with BLV resistant alleles in aim of decreasing herd BLV PVL prevalence.

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APPENDIX

Table 1 Estimated BoLA DRB3 allele frequencies and association with BLV disease status in 135 Michigan Holstein dairy cows.

Allele	Allele Count BLV+[1]	Allele Frequency in BLV+ cows	Allele Count BLV-[2]	Allele Frequency in BLV- cows	Odds ratio	P Value[3]
***0101**	42	0.25	23	0.22	1.19	0.66100
***0902**	0	0	4	0.04	0	0.02123**
***1001**	14	0.08	6	0.06	1.50	0.48150
***1101**	33	0.20	15	0.14	0.33	0.32650
***140101**	14	0.08	8	0.08	1.10	1.00000
***1501**	41	0.25	26	0.25	0.98	1.00000
***2703**	8	0.05	10	0.1	0.48	0.13830*
***0501**	2	0.01	1	0.009	1.23	1.00000
***0601**	0	0	1	0.009	0	0.38520
***1201**	6	0.04	5	0.05	0.74	0.75390
***1801**	1	0.006	1	0.009	0.63	1.00000
***1601**	3	0.02	3	0.03	0.62	0.67890
***1506**	1	0.006	0	0	Inf[4]	1.00000
***0701**	1	0.006	0	0	Inf[4]	1.00000
***3101**	0	0	1	0.009	0	0.38520
Sum	166	1.0	104	1.0	N/A	N/A

[1]Number of times the allele was identified in Michigan BLV+ dairy cow sample population (n=166). [2]Number of times the allele was identified in Michigan BLV- dairy cow sample population (n=104). [3] **$p \leq 0.05$, ** $0.05 \leq p \leq 0.15$. [4] Infinite.

Table 3 Estimated BoLA DRB3 allele frequencies and association between BLV proviral status in 135 Michigan BLV+ Holstein dairy cows.

Allele	Total HPL allele count[1]	Frequency of alleles among HPL cows	Total LPL allele count[2]	Frequency of alleles among LPL cows	Odds Ratio	P Value[3]
***0101**	4	0.12	16	0.28	0.35	0.1152 **
***1001**	1	0.03	3	0.05	0.56	1.0000
***1101**	10	0.30	11	0.19	1.77	0.3061
***140101**	2	0.06	2	0.03	1.74	0.6245
***14011**	2	0.06	1	0.02	3.51	0.5525
***1501**	12	0.35	13	0.22	1.87	0.2266
***1601**	1	0.03	0	0	1.72	1.0000
***2703**	1	0.03	3	0.05	0.56	1.0000
***1801**	1	0.03	0	0	0.37	0.3696
***0501**	0	0	5	0.09	0	0.1535 **
***0601**	0	0	1	0.02	0	1.0000
***1201**	0	0	1	0.02	0	1.0000
***1506**	0	0	1	0.02	0	1.0000
***1507**	0	0	1	0.02	0	1.0000
SUM	34	1	58	1	N/A	N/A

[1]Number of times the allele was identified in Michigan HPL Holstein dairy cow sample population (n=34). [2]Number of times the allele was identified in Michigan LPL Holstein dairy co w sample population (n=58). [3] **$p \leq 0.05$, ** $0.05 \leq p \leq 0.15$.

CHAPTER 3: KLH IMMUNIZATION OF BLV+ COWS SHOWS ROBUST IMMUNE RESPONSE AND SUGGESTS DAIRY PRODUCERS CONSIDER ALTERNATIVE HOUSING MEASURES

Abstract

Bovine leukemia virus (BLV) is a delta retrovirus and the causative agent of a chronic infection in dairy and beef cattle. BLV is known to negatively affect the cattle's immune system and may reduce antibody response to novel pathogens following exposure. Dairy cows are commonly exposed to pathogens in their natural environment and must be able to mount an immune response. This study aims to determine if a BLV ELISA-positive (BLV+) cow is capable of mounting a robust immune response. Exposure to the novel pathogen keyhole limpet hemocyanin (KLH) was used to simulate an exposure to a novel antigen, such as infection with a foreign animal disease, without causing disease in Holstein cows (n=17). Proviral load (PVL), or the measure of amount of provirus in BLV+ cows, antibody response to KLH and complete blood counts were tracked over a 50-day time period. Statistical differences were not detected between treatment groups for anti-KLH IgM and complete blood counts. However, statistical differences were detected between the KLH exposed treatment group and control group for anti-KLH IgG. It was concluded that this study may provide biologically significant evidence that could suggest that dairy producers wishing to control BLV among their herds may need to consider alternative animal housing measures.

Introduction

Bovine Leukemia virus (BLV), a delta retrovirus, is the causative agent of a chronic infection that can affect dairy and beef cattle. A cattle's host immune system typically cannot clear the infection, and there are currently no vaccines available to prevent infection or the

spread of BLV [1-3]. BLV will enter a host cell where viral reverse transcriptase produces a copy DNA (cDNA) that is integrated into the host genome as a provirus. After infection with BLV, the majority of cattle will remain asymptomatic (aleukemic; AL). However, some cattle will progress to persistent lymphocytosis (PL), and a rare percentage will develop leukemia or lymphoma (lymphosarcoma). Shortly after infection, BLV replicates, stimulating robust antibody and cytotoxic T cell responses that persist for the life of the cow. Once antibody response is developed, BLV will typically exist in a latent stage, which prevents the immune system from detecting its presence, with little to no sign of infection. Following development of the initial immune response, BLV proviruses enter a latent state with little production of BLV encoded proteins.

North American cattle have increasingly become infected with BLV, with 38% of beef and over 80% of dairy operations now infected [4]. Within American dairy herd infection rate has been estimated at 46.5% (n=4,120), while Canadian infection rates have similarly been estimated at 45% [4, 5]. A total of 22 countries were successful in eradicating BLV decades ago by use of strict control programs [6]. BLV therefore represents a significant barrier for export of animals to these markets. Further, lymphosarcomas are the cause of 13% of beef carcass and 26% of dairy carcass condemnation at slaughter [7]. As a serious threat to milk production in the United States, BLV has been estimated to cause annual losses over $520 million [8].

In previous studies, BLV infection of cows ranging from 50-101 months in age has been associated with a significantly reduced IgM and notable IgG2 levels following a booster dose of the commercial vaccine Bovishield Gold (Zoetis Animal Health) [9]. Further negative effects of BLV infection have been associated with decreased immune responses to keyhole limpet hemocyanin (KLH), a novel antigen used to simulate immune responses [10]. In both studies,

results showed that BLV affects the B cells, the primary target of BLV, and decreases their antibody production function. Several studies have increasingly shown that BLV dysregulates B cells in enzyme-linked immunosorbent assay (ELISA) BLV-positive (BLV+) cattle in comparison to ELISA BLV-negative (BLV-) cows [11-13]. Other studies have linked BLV to infection of T cells and monocytes [14, 15]. BLV infection has been associated with decreased circulating T cells [16, 17] and changes in T cell response to mitogenic stimulation, which further indicates BLV dysregulates the immune system [17, 18]. Although there is much literature supporting the immune dysregulation by BLV, there is little addressing any correlation between BLV infection and the increased risk posed by infectious diseases or decreased response to vaccines.

Previous studies within our group have addressed this knowledge gap. After booster vaccination, dairy cows that were BLV+ exhibited a strong reduction in serum and plasma IgM antibodies against *Leptospiria* and bovine herpesvirus type-I (BHV-1) compared to BLV- cows [9]. Further, BLV+ cows had a significant reduction of serum IgG2 antibodies which are known to be important in controlling infections. These preliminary results were similar to another study of BLV+ cows in response to the novel antigen keyhole limpet hemocyanin (KLH) in combination with a common vaccine adjuvant dimethyldioctadecylammonium bromide (DDA)[10]. In this preliminary data, BLV+ cows had increased levels of proviral load (PVL); some up to 20-fold following a secondary challenge with KLH.

As seen in these two studies, it is likely that BLV+ cows will create a robust, albeit reduced immune response to vaccinations and novel pathogens relative to BLV- herdmates. However, we suspect it is likely that their PVL will increase in response to vaccination if an infected B cell is stimulated by the vaccine. Novel management strategies to control BLV focus

on identifying high PVL cows and removing them from the milking herd. Neighboring cows known to be BLV+ have shown to pose a risk of transmitting BLV to uninfected cows [19-22] . High PVL cows have been suggested as the most likely to spread BLV infection to uninfected herd mates [23-25]. In support of this idea, studies on commercial farms where the highest PVL cows are culled, the incidence of BLV within some herds was significantly reduced [26]. If vaccinating cows or exposure to novel pathogens truly increases PVL, this may be an important factor for producers to consider when controlling for BLV. The current study was specifically designed to directly address the effect of vaccination/immunization on BLV PVL in naturally infected cows in a commercial setting.

Material and Methods

Cow and KLH+DDA and DDA inoculation

Two cohorts consisting of 17 BLV+ Holstein cows (ELISA OD unavailable) in total were selected prior to the study. Additionally, PVL was determined to verify BLV infection. BLV PVL was determined 6 months prior cohort 1 animal enrollment and 8 months prior to cohort 2 animal enrollment. Each cohort received identical treatment performed at separate time points. Cohort 2 was enrolled immediately following the completion of Cohort 1. Each cohort had 3 treatment groups that were given one of three injections; KLH+DDA, DDA only, and bovine serum albumin (BSA) only as negative control (Table 3).

Each cohort received their respective primary injection on day 0 (d0) and secondary injection on d29 of 1.5mL KLH+DDA cocktail, 1.5mL of DDA cocktail, or 1.5mL of 5% BSA. The KLH+DDA cocktail contained 200ug KLH (Sigma) in 0.75 mL 1x phosphate-buffer saline (PBS) containing 5% BSA and 0.75mL 20mg/mL adjuvant (DDA) in 1x PBS as previously described [10]. The DDA cocktail consisted of 0.75 mL 20mg/mL DDA in 1x PBS and 0.75mL

of 5% BSA. The control group received 1.5mL of 5% BSA in 1x PBS. Treatments were subcutaneously injected into the right side of the neck for d0 and left side of the neck on d29. All protocols were reviewed and approved by the Michigan State University Institutional Animal Use and Care Committee (AUF# PROTO201900110).

Whole blood, serum, and buffy coat isolation

Whole blood was collected via coccygeal venipuncture on d0 prior to primary treatment and on days 7, 14, 21, 28. The secondary treatment was administered on d29 and whole blood was collected on days 4, 8, 11, 15 and 22 after secondary treatment (days 32, 36, 39, 43, 50 after primary treatment). Blood for PVL quantification was collected in Vacutainer blood collection tubes (Becton Dickinson) containing the anticoagulant Ethylenediaminetetraacetic acid (EDTA). Blood for antibody quantification was collected in Vacutainer blood collection tubes (Becton Dickinson) for serum isolation. Serum was isolated via manufacture recommendation (Becton Dickinson). Aliquots of serum were stored at -80˚C for later quantification of anti-KLH antibodies.

BLV PVL Quantification

Genomic DNA (gDNA) was extracted from whole blood and quantified as previously described [10]. Briefly, 20µl of Proteinase K from Qiagen (DNeasy Blood and Tissue Kit) was added to a 1.5mL Eppendorf tube that contained 100µL of buffy coat and 100µL of 1xPBS. 200µL of Buffer AL (Qiagen) was added, mixed thoroughly by pulse vortexing, and incubated at 56˚C for 10 minutes. 200µL of 96% ethanol was added to the sample and mixed thoroughly by pulse vortexing. The mixture was pipetted into the DNeasy mini-spin column in a 2mL collection tube and spun at 8000rpm for 1 minute. Flowthrough was discarded along with the collection tube. A new 2mL collection tube was placed under the DNeasy mini-spin column, and

500μL of Buffer AW1 was added and then spun at 8000rpm for 1 minute. Flowthrough and the collection tube were discarded. The DNeasy mini spin column was placed in a new 2mL collection tube, and 500μL of Buffer AW2 was added, and then spun at 14,000rpm for 3 minutes. Flowthrough and the collection tube were once again discarded. The DNeasy mini-spin column was placed in a clean 1.5mL Eppendorf microcentrifuge tube and 200μL of Buffer AE was pipetted directly onto the DNeasy membrane. This was incubated for 1 minute at room temperature and then spun at 8000rpm for 1 minute. Immediately following, the extracted gDNA sample quality and concentration were analyzed and recorded using the Thermo Scientific Nanodrop 1000.

BLV PVL was determined using the SS1 Assay (CentralStar Cooperative) per manufacturer protocol. The assays were run on an ABI-7500 Real-Time PC System (Applied Biosystems). BLV copy number, along with bovine beta Actin (BA) as the host gene control were calculated as previously described [27]. Briefly, the PVL ratio was calculated by determining the ratio between the BLV PVL copies and BA copies and are reported as a ratio of the BLV PVL copy to the BA copy. BLV and BA standards were created by serial dilution beginning with 10^6 and ending with 10^1, which were ran on each plate to minimize cross-plate differences. On a 96 well plate, 19μL of Master Mix were pipetted into each standard well, while 17μL of Master Mix were pipetted into each sample well. 1μL of either BLV or BA standard were pipetted into the appropriate wells, and 3μL of extracted gDNA were pipetted into their respective wells. 20μL of Master Mix was pipetted into the no-template control well of each plate. The positive control well consisted of 19μL of Master Mix and 1μL of the positive control DNA. The 96-well plate was then ran using the ABI 7500 qPCR instrument (Thermo Fisher Scientific) to amplify the BLV PVL. The instrument was programmed to cycle 40 times as

follows: 95˚C x10 minute, 95˚C x15seconds, 60˚C x1minute, 60˚C x1minute. The results were then compared to standard curves to estimate the PVL of each sample before determining the ratio of the BA gene to BLV provirus, as mentioned above. PVL of each cow was determined to either be low (< 1 BLV copy/BA copy or high (≥ 1 BLV copies/BA copy).

Anti-KLH Antibody Quantification

Serum was aliquoted and stored at -80˚C on each sample day. To limit differences between plates, each cow's samples were analyzed on the same plate for each timepoint. Anti-KLH antibody quantification was performed using serum collected on days 0, 7, 14, 21, 28, 32, 36, 39, 43, and 50. 90-well flat-bottom plates with high binding affinity (Thermo Fisher Scientific) were incubated overnight at 4˚C with 100µL of 1µg/mL KLH in 50mM carbonate/bicarbonate buffer, as previously described [10]. Following, the plates were washed 3X with wash buffer (0.05% Tween-20 in 1X PBS) and blocked with 2% heat-inactivated horse serum in wash buffer for 1h at 37˚C. Plates were then washed 5x with wash buffer. 10µL of serum (diluted 1:200 in blocking buffer) were added to wells in duplicates for each sample timepoint and incubated for 1h at room temperature, then washed 5 times with wash buffer. 100µL of anti-bovine IgM or IgG conjugated to horseradish peroxidase (Integrated DNA Technologies) diluted 1:10,000 in blocking buffer was added to each well and then incubated for 1h at room temperature. Plates were washed 5 times with wash buffer. 100µL of Tetramethyl Benzidine substrate (TMB) substrate (Thermo Scientific) was added to each well and incubated in the dark at room temperature for 15min. 100µL of stop solution (2M H2SO4) was added to each well. Plates were immediately measured for optical density at 450nm using a Spectra-Max M5 microplate reader (Molecular Devices).

Whole Blood cell quantification

Whole blood was collected for each sample date. Utilizing a rapid-result hematology analyzer (Advanced Animal Diagnostics, QScout), a leukocyte differential was reported. To begin, whole blood was pulse vortexed. Following, 7µL of whole blood was pipetted onto a cassette slide (Advanced Animal Diagnostics). Manufacture protocol was then followed. Reports were exported and analyzed as described below.

Statistical analysis

Statistical analysis was preformed using the GLIMMIX model fit with repeated measures with days and BLV as fixed effects utilizing SAS 9.4 (SAS Institute). Antibody, complete blood count (CBC) and PVL were analyzed with a model fit with repeated measures with days and BLV as fixed effects. Data that did not have a normal distribution was log transformed. Although treatment and day effects were detected, they were not included in the data interpretation and conclusions. Rather, the interaction of treatment-by-day was considered for this analysis to best assess a cows antibody response following stimulation. Following pairwise comparisons of treatment-by-day, a tukey *post hoc* was performed. Spatial power was used to mean separation by day. A *post hoc* Bonferroni correction was used for pairwise comparisons that were log transformed. Significance was determined as $p < 0.10$.

Data was analyzed with the following SAS model:

```
proc glimmix  data=all2; class trt day cow;
title" Trait  = &dep";
model &dep = trt|day;
random day/ subject=cow(trt) type=sp(pow)(day) residual;
lsmeans trt day /adjust=tukey lines;
lsmeans trt*day/plot=meanplot(sliceby=trt join);
slice trt*day /sliceby = day lines adjust=bon;
run; ods graphics off;
%mend;
```

Results

Anti-KLH IgM antibody testing among BLV+ cows

To illustrate the humoral immune response to KLH in KLH+DDA cows, the relative quantities of anti-KLH IgM and IgG were measured. The anti-KLH IgM optical density (OD) of cows treated with the KLH+DDA challenge did now show a significant overall effect in treatment-by-day. Further, when each sample date's anti-KLH IgM OD were calculated relative to d0, and sample days 28-50 were calculated relative to d28, no significant overall effect was detected in treatment-by-day.

Anti-KLH IgM antibody response

No statistical differences were detected among the anti-KLH IgM antibody response following treatment. To visualize numerical differences, OD was presented relative to d0 means for each treatment group (Figure 1).

To track changes following only the secondary challenge injection on d28, anti-KLH IgM OD was calculated relative to d28. To visualize numerical differences, OD was presented relative to d28 means for each treatment group (Figure 2).

BLV+ cows show a significant anti-KLH IgG antibody response

Overall treatment-by-day significance was found on days 14, 21, 28, 32, 36, 39, 43, and 50 for anti-KLH IgG ($p<0.0001$) (Figure 3). Following development of IgM responses, isotype switching typically leads to development of IgG antibodies. We therefore wished to study if this immunization protocol elicited an IgG response among the KLH+DDA treatment group in comparison to the DDA and no treatment group.

PVL response following immune stimulation by KLH

Although no statistical significance was detected, the average PVL of the KLH+DDA group doubled from a d0 PVL average of 0.644 (standard error +/- 0.134) to a d50 PVL of 1.175 (standard error +/- 0.601) (Figure 4). Meanwhile, the DDA treatment group PVL started at a d0 average PVL of 0.851 (standard error +/- 0.142) and ended the trial at d50 with an average PVL of 0.811 (standard error +/- 0.048). Further, the control treatment group d0 average PVL began at 0.710 (standard error +/- 0.199) and decreased at the end of the trial at d50 with an average PVL of 0.581 (standard error +/- 0.048).

QScout complete blood count

No significant data was found when comparing treatment-by-day across the BLV+DDA, DDA only and control groups (data not shown).

Discussion

Robust Anti-KLH IgM response suggests BLV+ cows can mount antibody response to a novel antigen

The KLH+DDA immune system challenge reveals that although BLV+ cows with PVL may have suppressed immune systems, they are still able to produce an antibody response. Although not statistically significant, there was indeed a robust antibody response demonstrated by the anti-KLH IgM results (Figure 1). It can also be concluded that the BLV+ cows with PVL in this challenge are indeed responding to KLH (KLH+DDA treatment group) rather than DDA (Figure 1), a question posed by a previous study in our group [10]. Since the DDA and Control cows were not exposed to KLH, it was expected that they would not show an anti-KLH IgM response. The DDA only and control group cows did show a minimal response in OD titers over time; however, this is likely attributed to IgM's non-specific binding properties, and is displayed in Figure 1 with minimal differences across the duration of the challenge.

From this data, it can be inferred that when BLV+ cows with PVL are challenged with a novel antigen they will likely produce a robust antibody response, which was modeled by KLH (Figure 1). Further, a secondary challenge with a novel antigen will stimulate a secondary antibody response (Figure 2). This idea can also apply to vaccinations and their boosters. Although BLV+ cows with PVL may produce a robust antibody response to novel antigens or vaccinations, it is still important for producers to consider PVL increase following immune stimulation (see *PVL and lymphocyte count pose new implications to herd management)*.

Significant Anti-KLH IgG responses in BLV+ cows with PVL

IgG is an antibody produced to neutralize various immunogens, such as KLH, with a high affinity and may activate the complement cascade. The statistically significant antibody response shown between KLH+DDA treatment group and the DDA and control group clearly demonstrates that BLV+ cows with PVL are capable of mounting a significant antibody response (Figure 3). A previous study in our group also concluded similar results and showed that BLV+ cows, when challenged with an immune stimulant, were significant when compared to d0 and similar to that of BLV- cows [10].

This data is important to consider in regards to vaccinations. Routine commercial vaccinations stimulate the immune system similar to that of a novel immune stimulant such as KLH. Following the administration of the first round of a commercial vaccine, a cow's immune system will first respond with IgM from day 7-14. Following, IgG is produced as a long-term antibody starting between day 14-21 after vaccination. This is precisely modeled in this data (Figure 1, Figure 3). This study was limited in that BLV- cows were not included. However, a previous study reviewed the immune response of BLV infected cows to commercial vaccines

and found that, although infected with BLV, cows were able to produce robust IgG responses following primary and secondary commercial vaccinations [9].

PVL and lymphocyte count pose new implications to herd management

KLH was used to simulate a novel pathogen exposure to BLV+ cows with PVL and monitor changes in PVL. As shown in Figure 4, the KLH+DDA treatment group showed a 2-fold increase in PVL from the beginning to the end of the trial. This data, although not statistically significant, may be biologically relevant. Dairy farms naturally have many immunological risks to cows such as pneumonia, *salmonella*, bovine viral diarrhea, *Leptospira*, bovine alpha herpesvirus-1, *campylobacter* and other microbes that may cause disease [28]. Animals are vaccinated against many of these pathogens. If immune stimulation by infection or vaccination increases PVL, segregation may be needed to prevent spread of BLV to negative herd mates.

This data may be important regarding BLV management within dairy herds. It has recently been suggested that dairy farms split high and low PVL cows within herds since high PVL cows have been shown to produce virus more frequently, which poses a risk to uninfected animals. Further, a study also suggests culling cows with the highest PVL due to the resultant decrease in herd prevalence [26]. As seen with the current study, a BLV+ cow's PVL following a novel immune response could alter a cows PVL status from low (< 1 BLV copy/BA copy) or high (≥ 1 BLV copy/BA copy). Further, some farms include low PVL cows with their negative herd mates since studies have suggested that low PVL cows pose minimal risk of disease transmission [29, 30]. However, our data suggests that BLV-infected cows, when immune-stimulated by a novel pathogen, may cross from low PVL to high PVL as demonstrated by the KLH+DDA groups 2-fold increase in PVL. A previous study determined that if BLV- cows are placed next to BLV+ cows with PVL, the BLV- cows are at 12 times higher risk of contracting

the virus compared to cows not exposed to BLV+ cows with PVL [19]. Thus, when a BLV+ cow's PVL increases due to immune stimulation and is housed with BLV- animals, it may pose a disease transmission threat to uninfected animals. In comparison to our data, the PVL and lymphocyte proliferations were steadily increasing with no sign of reduction following the 50-day immune challenge. This may suggest that BLV+ cows with PVL should be segregated from BLV- cows, even if an infected cow is deemed a low-transmission risk with an initially low PVL.

To the authors' knowledge, this is the first study to track CBC and BLV PVL in such an immune challenge, and a novel immune response was seen among the lymphocyte count over the course of the study. It has previously been shown that lymphocyte count positively correlates with a BLV+ cow's PVL (r=0.855)[31]. During the limited time of our study, we noted variations in lymphocyte count and a numerical increase in PVL. The exact mechanism of BLV PVL increase has not yet been determined. It has been suggested that infected B cells may survive longer, which could explain why in response to a novel infection, the amount of provirus also increases [32]. Considering that BLV provirus infects B cells, which are lymphocytes, these results may indicate that as infected B cells proliferate in response to a novel infection such as KLH+DDA, the PVL increases as well.

Limitations of this study

The lack of statistical significance may be due to the number of cows enrolled in this study. Further, each cow biologically has variance that cannot be controlled. Specifically, each cow was naturally infected with BLV. We cannot determine with confidence when each cow was infected and their stage of disease progression. Although we can normalize data to minimize this variance, each cow still has a unique immune response, including PVL, that may or may not be

dictated by their current stage of BLV infection. Thus, it would be beneficial to expand this study to include more cows, as well as selecting cows based on their proviral loads.

Conclusion

This study aimed to address the effect of vaccination/immunization on BLV PVL in naturally infected cows in a commercial setting. This was also the first study to track BLV+ cows with PVL CBC every 3-7 days in response to a novel antigen challenge. It was found that BLV+ cows with PVL are capable of producing a robust antibody response. Although not significant, the PVL average was shown to double at the end of the trial while lymphocyte counts increased. Dairy producers strongly committed to eradication of BLV in their herds may wish to consider housing uninfected cows separately from low PVL cows (specifically at times of vaccination).

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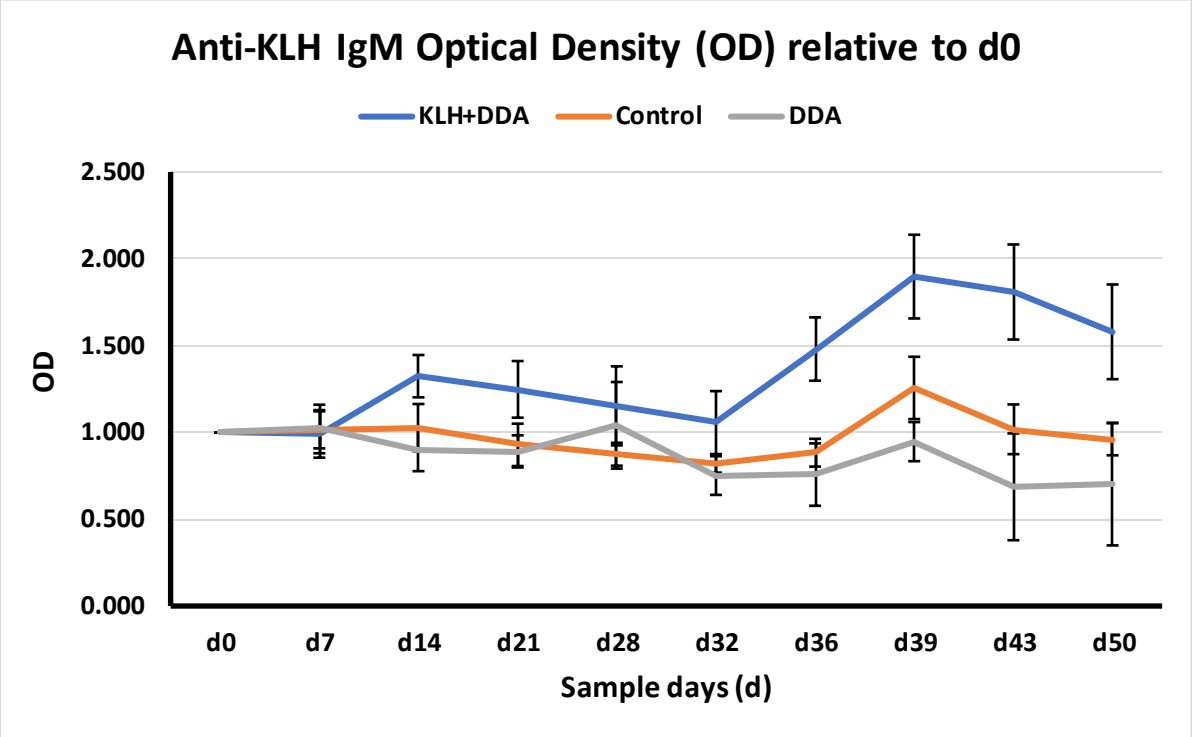
APPENDIX

Table 3 Study Treatment groups, cohorts, and summary data

	Treatment Group	Cow	Age (months)	BLV[4] PVL[5] when selected for enrollment	d0 PVL
Cohort 1	BSA[1] (Control)	5337	56	0.819	1
		5365	41	1.37	1
	DDA[2]	5389	51	1.94	1
		73374	70	N/A*	1
		86409	37	N/A*	1
	KLH[3]+DDA	5393	50	0.568	1
		5347	44	1.5	0.999
		5241	64	0.75	1
		5310	71	2.44	0.594
Cohort 2	BSA[1] (Control)	86101	52	0.083	0.155
		5499	41	0.316	0.687
	DDA[2]	5536	37	0.105	1
		5031	93	0.28	0.983
		5322	55	0.414	0.986
	KLH+DDA	5503	40	0.401	0.304
		5277	30	0.21	0.202
		5492	42	0.294	0.407

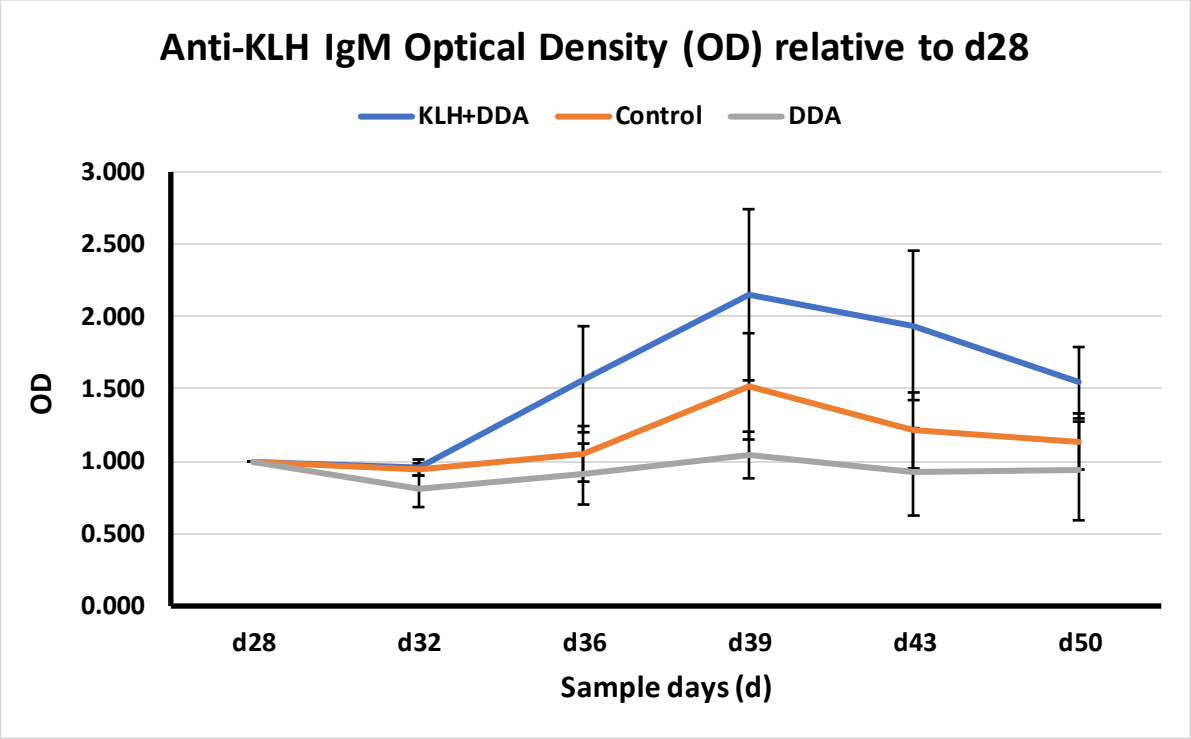
[1] Bovine serum albumin [2] dimethyldioctadecylammonium bromide [3]Keyhole limpet hemocyanin [4] bovine leukemia virus [5] Proviral load *BLV PVL was unavailable for these cows, however, cows were known to be ELISA BLV+. Cows were enrolled in two trial timepoints where treatments were identical. Cohort 2 was initiated following the completion of Cohort 1. Cows were randomly assigned to one of the following treatment groups: KLH+DDA, DDA or BSA which served as the control.

Figure 1. Anti-KLH IgM Optical Density (OD) Relative to d0



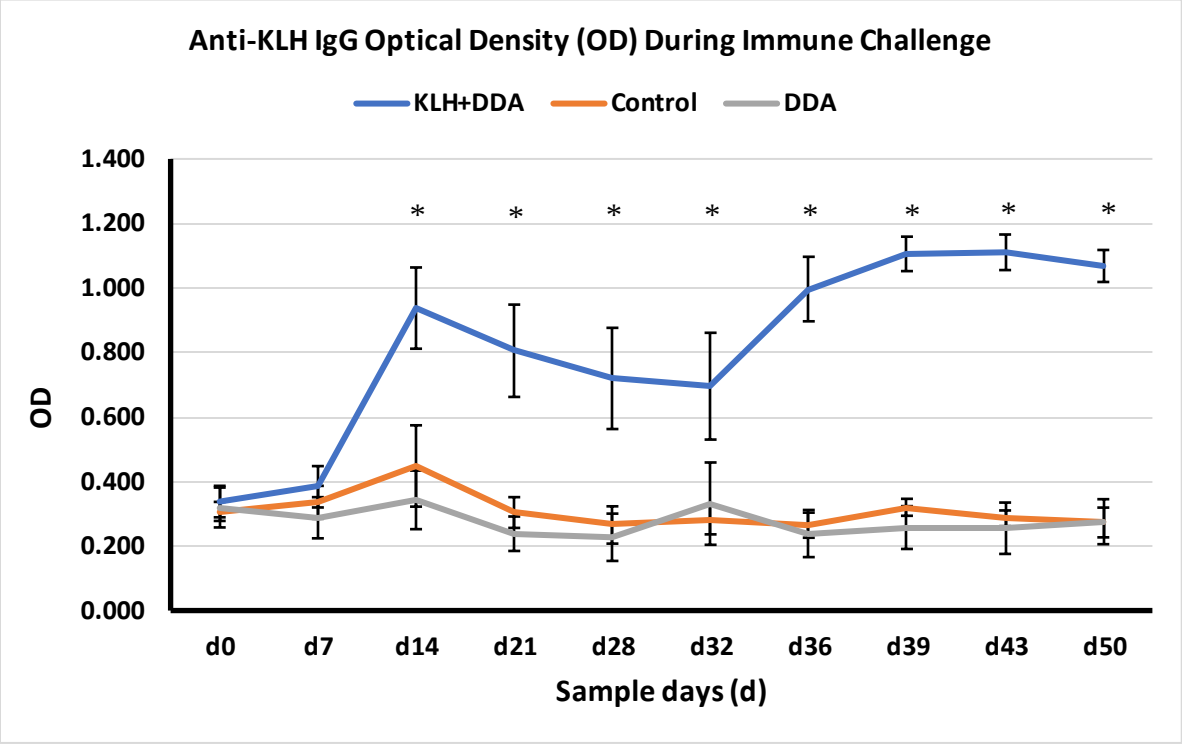
Anti-KLH IgM antibody response among three treatment groups: KLH+DDA, DDA-only, and control relative to their d0 OD values. Error bars (standard error of the means) are shown for each treatment group.

Figure 2. Anti-KLH IgM Optical Density (OD) relative to d28



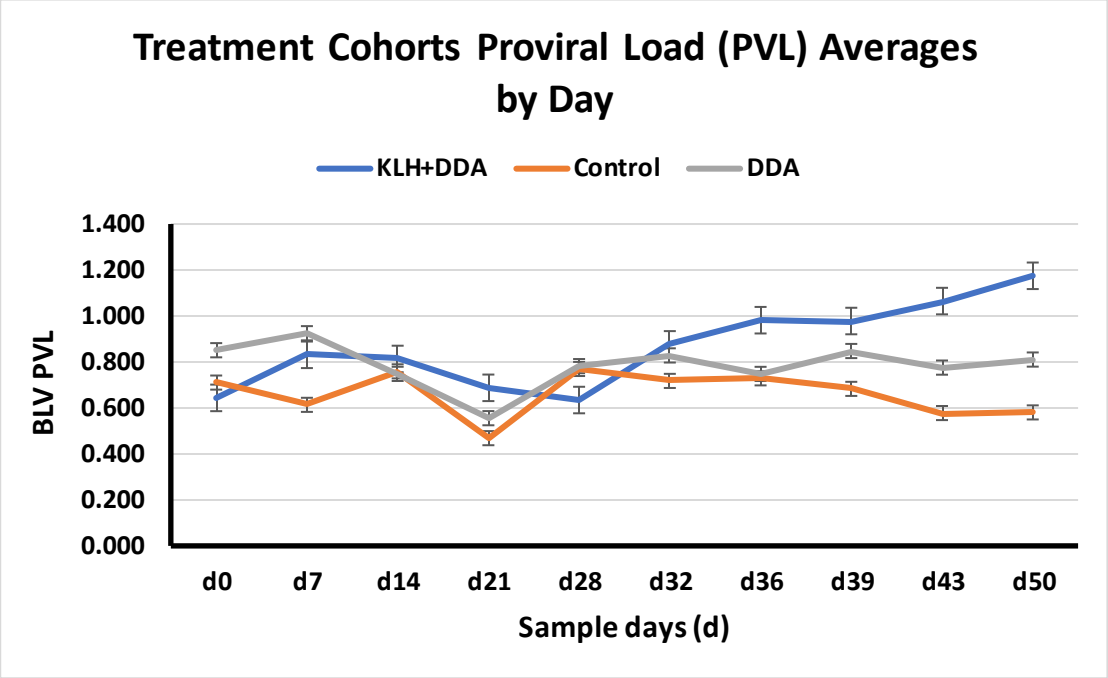
Anti-KLH IgM antibody response among three treatment groups: KLH+DDA, DDA-only, and control relative to their d28 OD values. Error bars (standard error of the means) are shown for each treatment group.

Figure 3. Anti-KLH IgG Optical Density (OD) During Immune Challenge



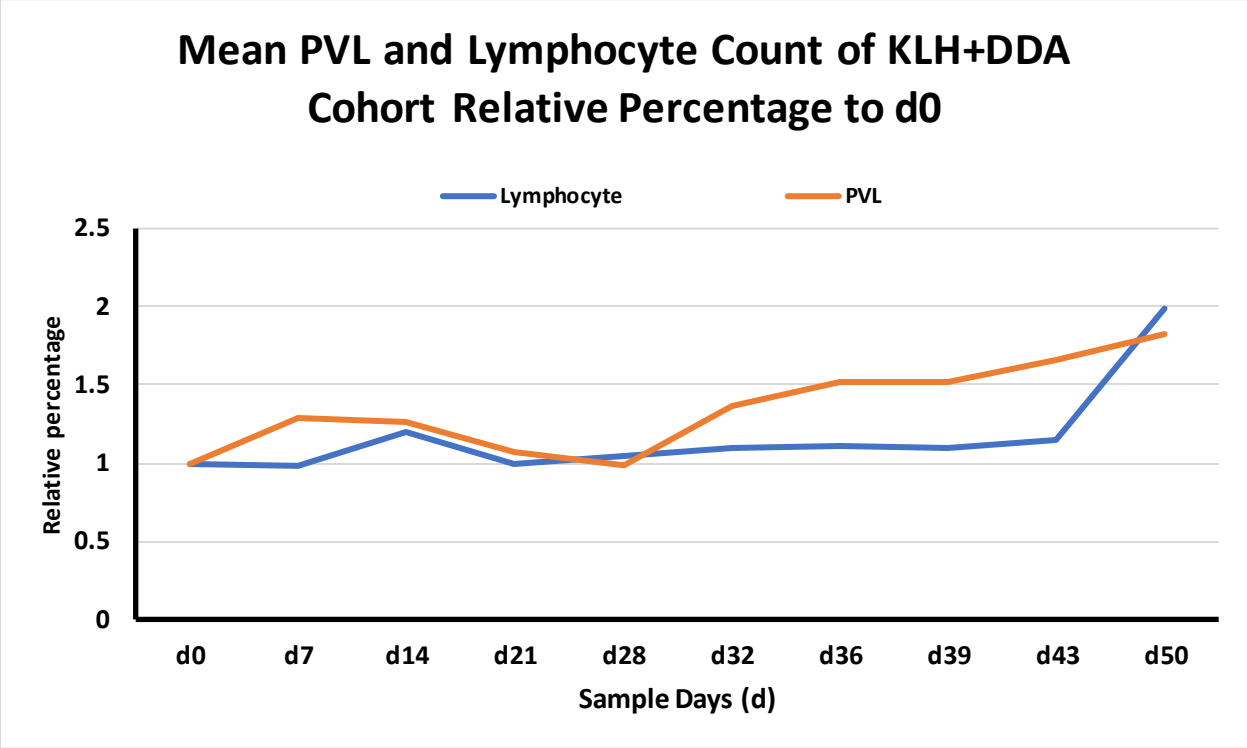
Anti-KLH IgG antibody response among three treatment groups: KLH+DDA, DDA-only, and control OD values. Error bars (standard error of the means) are shown for each treatment group. Asterisks represent significance with * $p<0.0001$.

Figure 4. Treatment Cohorts Proviral load (PVL) Averages by Day



PVL response among three treatment groups: KLH+DDA, DDA-only, and control following primary and secondary immune challenge according to treatment group. Error bars (standard error of the means) are shown for each treatment group.

Figure 5. Mean PVL and Lymphocyte Count of KLH+DDA Cohort Relative Percentage to d0



Mean PVL and Lymphocyte count of KLH+DDA relative percentage to d0.

CHAPTER 4: FUTURE DIRECTIONS OF BOVINE LEUKEMIA VIRUS RESEARCH

Introduction

Bovine leukemia virus (BLV) is a lifelong infection in cattle and may negatively affect BLV-infected cows' milk production, negatively impact immune responses, and increase culling rates. It can be difficult to control the transmission of BLV on dairy farms, as it has been shown to transmit through transfer of infected lymphocytes carried on fomites such as needles, sleeves, and dehorning equipment. Dairy farms are currently encouraged to cull high proviral load (PVL) cows, as they have been shown to increase the risk of disease transmission to uninfected herd mates. However, culling all high PVL cows is not always financially feasible. Thus, dairy farms are also encouraged to separate low PVL and uninfected cows from high PVL cows.

It is important to note that some farm BLV management protocols, restricted by either available space or other farm operations, house BLV ELISA-negative (BLV-) cows and low PVL cows together. Given the data presented in Chapter 3, housing low PVL cows with uninfected cows may pose more risk than research originally suggested. This new data may suggest amending the current BLV management strategies to house BLV- cows and low PVL cows separately. However, as mentioned previously, such separation could pose strain to dairy farms where not feasible.

The data in Chapter 2 suggests that by selectively breeding cows with BLV-resistant alleles, BLV disease management strategies could theoretically begin before a cow is born by incorporating genetic selection of BLV-resistant BoLA alleles. More research must be done before such conclusions can be made.

In conclusion of Chapters 2 and 3, I suggest future research efforts in response that link the BoLA DRB3 Exon 2 allele genetic capability in fighting infection, as well as investigating the trend of PVL after a novel secondary immune challenge to determine if PVL decreases, plateaus, or continues to increase.

Future directions

Future research of BLV+ cow immune response to novel pathogens

The data presented in Chapters 2 and 3 warrant further research. Due to the limitations of each study (low animal numbers), I would suggest a larger sample population be included determined by a power-analysis possibly using data presented herein. Since the results of Chapter 3 showed increasing PVL and lymphocytes over a 50-day immune challenge, I would suggest extending the sample period to perhaps 90 days post-primary exposure. This would allow for an extended study to track the immune system challenge to capture any extended response since, in general, the adaptive immune system may take up to 90 days to return to homeostatic levels. In Chapter 3, we were not able to determine if the KLH+DDA treatment group average PVL that doubled did indeed continue to rise, plateau, or decrease. By extending the timeline from 50 to perhaps 90 days, this study may be able to capture these results.

I would suggest a study that has three treatment groups, and 2 sub-treatment groups within each treatment group. Treatment would consist of KLH+DDA, DDA, and control. Within each treatment group, 2 groups of high and low PVL cows would be included. This would allow researchers to assess the variability of initial PVL, unlike in Chapter 3.

Future research of BLV and BoLA DRB3 alleles

The results of Chapter 2 are in general agreement with other similar studies, whether they are international, national, or regional to the Midwest and Michigan. The common consensus of each study warrants research to consider including the BoLA DRB3 alleles in selective breeding programs on dairy farms. However, before such a breeding strategy suggestion can be made, I believe a meta-analysis is warranted to include cow breed, lactation, PVL status, BLV status, BoLA DRB3 alleles, and geographical location in the analysis.

There have now been 3 studies among the Midwest region regarding the BoLA DRB3 alleles, and each of them also considers a cow's BLV PVL. By combining each of these study's data into a meta-analysis, researchers have the opportunity to more conclusively define which alleles are resistant or susceptible to BLV infection and high PVL rates.

Expected results

Following an extended trial of the immune system challenge, I would expect the PVL of HPL cows to slightly increase relative to their initial status, but not a significant amount. This is because HPL cows may already have most lymphocytes infected with BLV provirus. However, I expect LPL cows to have a significant PVL increase that would normalize to a higher PVL status than prior to the immune challenge.

CHAPTER 5: OVERALL CONCLUSIONS

Bovine leukemia virus (BLV) poses challenges to a cow's immune system and in turn leads to decreased milk production and increased culling rates. The objective of our studies were to determine what BoLA DRB3 alleles Michigan dairy cows possess and how they may or may not correlate to a cows BLV proviral load (PVL) and disease status. We also sought to determine if a cow's immune system, when infected with BLV, is capable of producing a robust antibody response and how the PVL may or may not be affected. Together, both of these studies evaluated a cow's immune system when infected with BLV.

In Chapter 2, it was shown that Michigan dairy cows have a limited BoLA DRB3 allele variance. However, the data did show that some Michigan dairy cows possess alleles that suggest disease resistance and susceptibility. These results in combination with other similar studies of Midwest cows suggest the possibility of genetically selecting animals based on BoLA DRB3 as an alternative measure to BLV disease management.

In Chapter 3, it was shown that BLV+ dairy cows with PVL are able to produce robust antibody responses to novel pathogens. However, in doing so, the amount of BLV PVL may increase, and thus pose risk to uninfected herdmates and concern for BLV management strategies.