FIELD STUDIES ON THE CONTROL OF BOVINE LEUKEMIA VIRUS IN DAIRY COWS

By

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ABSTRACT

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Bovine leukemia virus (BLV) is an oncogenic deltaretrovirus of cattle that is estimated to infect more than 40% of U.S. dairy cows and cost the dairy industry hundreds of millions of dollars per year. BLV inserts a DNA copy (provirus) into the host genome and can cause leukemia and lymphoma in 1-5% of infected animals, while around 30% develop a non-malignant lymphocytosis, often coupled with high proviral load. BLV infection in dairy cows has also been associated with a number of economically important effects, such as decreased milk production, longevity, and immune function. The primary goal of this dissertation was to develop management protocols that can be implemented on U.S. dairy operations. We also aimed to further characterize *in vivo* immunologic effects of BLV infection, especially at mucosal junctions, hypothesizing that BLV+ cows would have lower concentrations of IgA antibody.

To test the effect of BLV infection on total IgA concentrations, we analyzed the milk, saliva, and serum of BLV+ cows with varying lymphocyte count and proviral load profiles for comparison with BLV- herd mates. We found that BLV+ cows exhibited numerically lower concentrations of IgA in both milk and saliva, but not serum, in comparison to BLV- cows. Although the observed decreases were not statistically significant, the biological effects remain unknown. We also observed that the lowest concentrations of IgA were seen in the milk and saliva of BLV+ cows without lymphocytosis or high PVL, and these differences were trending toward significance. Our data, if confirmed, suggests that BLV may be disruptive to the immunology of mucosal junctions.

We developed and implemented three field trials to evaluate management protocols for control of BLV in dairy herds. The first trial investigated the frequently suggested management intervention of improved medical hygiene by changing from shared needles and reproductive sleeves to single-use items. In this field trial, BLV- herd mates were assigned to control (re-use) or intervention (single-use) groups and the incidence risk was calculated semi-annually. Although we saw no difference in the risk of new infections in our study, medical hygiene may still play a role in the transmission of BLV as well as other diseases. This study also identified an increase in incidence risk in the summer exposure period.

Meanwhile, our second field trial was a novel intervention which used milk ELISA screening to identify BLV+ cows for further characterization of their lymphocyte count (LC) and proviral load (PVL). In this manner, we identified cows thought to be the most infectious to their susceptible herd mates, and worked with herd managers to create a priority removal plan to consider when culling cows or implementing segregation measures. Overall, the three enrolled herds significantly reduced both BLV prevalence and incidence risk over a 2 to 2.5 year period. In this study, no significant associations with season were observed.

Our final field trial aimed to demonstrate that milk ELISA testing could be used in herds with low BLV prevalence in order to identify and remove the few remaining BLV+ animals and achieve eradication of the virus. Two herds that were able to remove all BLV+ adult cows were generally successful, though the young stock (first lactation heifers) were a reservoir for reintroduction. In addition, we observed 5 first lactation heifers in one herd which had previously tested negative on serum ELISA but later tested positive on milk ELISA after entering the milking herd. This agrees with reports of latent BLV infections, and provides further evidence that long-term surveillance and further research on the infection dynamics of BLV are needed.

This dissertation is dedicated to Ruth, who planted the seed, and Christine, who watered it; and to Bobbi and Phyllis: how I wish I could share this with you.

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KEY TO ABBREVIATIONS

BLV: bovine leukemia virus

BoLA: bovine leukocyte antigen

DHIA: dairy herd improvement association

EBL: enzootic bovine leukosis

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

EU: European Union

gp51: BLV glycoprotein 51

HTLV-1: human T cell lymphotropic virus type I

IgA: immunoglobulin A

IgG: immunoglobulin G

IgM: immunoglobulin M

LC: lymphocyte count

MHCII: major histocompatibility complex type II

miRNA: microRNA

OD: optical density

p24: BLV capsid protein

PCR: polymerase chain reaction

PVL: proviral load

qPCR: quantitative polymerase chain reaction

SEM: standard error of the mean

CHAPTER 1: Introduction

Abstract

Bovine leukemia virus (BLV) is an oncogenic deltaretrovirus of cattle with worldwide distribution. Although most infected animals are considered asymptomatic, around 30% are persistently leukemic (elevated lymphocyte count), and a small percentage (≤5%) develop lymphosarcoma. Due to this low percentage of clinically affected animals, few countries outside Western Europe have adopted control measures, allowing BLV infection to spread unchecked. With ongoing global trade restrictions of infected animals (and their reproductive material), and studies continuing to identify and confirm subclinical health effects of BLV infection that result in economic losses – particularly for dairy producers, evidence-based strategies to reduce the prevalence of BLV infection are more important than ever.

Viral Identification

Bovine leukemia, or enzootic bovine leukosis (EBL), is a naturally occurring infectious disease of cattle caused by bovine leukemia virus (BLV). The clinical disease – lymphoma – was first described in Eastern Europe in 1871 (Schwartz and Lévy, 1994), and over the next decades, many theories were put forth as to why these tumors appeared in geographic clusters and in some herds, but not others (Bendixen, 2006). Although an infectious cause was highly suspected, it was nearly a century before Miller, et al. identified virus-like particles, similar in morphology to C-type leukemia viruses of other species, that were induced from lymphocytes taken from cattle from a highly affected (multiple tumorous animals) herd as well as from cattle inoculated with blood or tumor suspensions from affected cattle (Miller et al., 1969). The virus was further described by Kettmann et al. (1976), in biochemical studies that characterized the agent as an exogenous RNA virus, and its genome was sequenced by Sagata et al. in 1985. Since that time, the genome – particularly the structure of the *env* gene – has been used to identify and investigate a growing number of viral genotypes that have been found throughout the world (Polat et al., 2016).

Diagnosis

Infection with BLV induces the generation of antibodies to virus envelope glycoproteins (primarily gp51) and capsid proteins (primarily p24) which can subsequently be detected in a variety of immunoassays to diagnose infection (Reichel et al., 1998; EFSA AHAW Panel, 2015). Time to seroconversion after infection has been shown to depend on the number of infected cells an animal is exposed to as well as the diagnostic test used (Klintevall et al., 1994; Nagy et al., 2007a), but a positive antibody test is currently the standard for identifying infected animals

(EFSA AHAW Panel, 2015). After infection, antibody levels typically increase over time (Portetelle et al., 1978; Bex et al., 1979; Kono et al., 1982), though variations may be associated with immunologic status (Schwartz and Lévy, 1994) and very high or very low antibody levels have been associated with a poor prognosis (Portetelle et al., 1978; Kettmann et al., 1980; Kono et al., 1982). PCR tests for the BLV provirus have been developed for research use (Eaves et al., 1994; Jimba et al., 2012; Saepulloh and Sendow, 2015) and frequently detect infection earlier than serologic tests (Klintevall et al., 1994; Nagy et al., 2007a), but are less commonly used for diagnosis due to the cost and expertise necessary to achieve accurate results. Complicating detection efforts is viral latency, a common characteristic of many retroviruses (Lieberman, 2016) that has been reported in experimental BLV infection (Klintevall et al., 1994).

Pathology

Host Range and Susceptibility

As with many retroviruses, infection with BLV is considered to be life-long due to the integration of the virus into the DNA of infected cells (Kettmann et al., 1976; Coulston et al., 1991), primarily B-lymphocytes (Kenyon and Piper, 1977; Paul et al., 1977b; a). Other cells, including T-lymphocytes (Stott et al., 1991; Schwartz et al., 1994), monocytes (Heeney et al., 1992; Schwartz et al., 1994), granulocytes (Schwartz et al., 1994), and mammary epithelial cells (Buehring et al., 1994) have occasionally been reported to be susceptible to BLV proviral integration. Few natural hosts other than cattle have been identified: the closely related zebu, water buffalo, and yak (EFSA AHAW Panel, 2015) are the primary reservoirs outside of domestic cattle. However, many other species, including sheep, goats, and rabbits have been experimentally infected (Schwartz and Lévy, 1994; EFSA AHAW Panel, 2015), and both

alpacas (More et al., 2017) and members of the cavy family (Smith et al., 2017) are reportedly susceptible. BLV is thus suspected to sporadically infect a wide variety of species, but this has rarely been investigated directly (EFSA AHAW Panel, 2015). Evidence for differences in individual-animal susceptibility or resistance to BLV infection, disease progression, and lymphoma has been mixed in herd-level surveys (Abdalla et al., 2013), though a growing body of work has identified genetic elements (Croshaw et al., 1963; Baumgartener et al., 1978; Lewin, 1989) such as bovine major histocompatibility complexes (BoLA alleles) – particularly BoLA-DRB3 – which may be associated with these differences (Xu et al., 1993; Juliarena et al., 2008; Miyasaka et al., 2013).

Lymphosarcoma and Lymphocytosis

The development of lymphoma/lymphosarcoma, despite being the first recognized sequela of the virus, only occurs in a small percentage (≤5%) of infected cattle (Bendixen, 2006; Tsutsui et al., 2016). Despite this fact, lymphomatous tumors are the most common reason for carcass condemnation of dairy cows at slaughter in the US (White and Moore, 2009), representing an economic burden for producers (Pelzer, 1997; Ott et al., 2003; Rhodes et al., 2003). A subclinical stage of the disease was proposed initially by European veterinarians who observed an association between multiple-tumor herds and herds with a high number of cattle with an elevated lymphocyte count (lymphocytosis; Bendixen, 1963; Marshak et al., 1963). Experimental data has borne this out, and it is now recognized that approximately 30% of infected cattle will develop persistently elevated lymphocyte counts (Lewin, 1989; Kabeya et al., 2001). Up to 70% experience an expansion in the B-cell population, increasing the ratio of B- to T-cells, and sometimes resulting in an inversion of the B- to T-cell ratio (Lewin et al., 1988). While

lymphocytosis appears to be a marker of immune compromise (Trainin et al., 1996), it is not predictive of tumorigenesis, as not all lymphomatous cattle have lymphocytosis (Lewin, 1989). In a report on 112 bovine lymphoma cases by Burton et al., (2010), only 25% had lymphocytosis. However, Lewin (1989) reported that cattle with an inverted B- to T-cell ratio provided the background population for animals that developed tumors. As mentioned above, genetics are an area of focus for understanding susceptibility and resistance to infection, disease profiles, and tumor development. BoLA alleles, specifically, have been associated variously with increased or decreased risk for lymphocytosis (Lewin and Bernoco, 1986; Stear et al., 1988; Xu et al., 1993) and lymphoma (Aida, 2001; Nikbakht Brujeni et al., 2016).

Proviral Load

Research of BLV provirus integration into the host cell genome indicates that BLV is capable of integrating at multiple sites in the genome and that multiple copies of provirus can be integrated into a single genome (Onuma et al., 1982). Newer quantitative PCR (qPCR) tests provide further insight by permitting the determination of proviral load (PVL), generally defined as the number of copies of provirus detected per some denominator (e.g. microgram of extracted genomic DNA (Juliarena et al., 2007)). In addition to improved sensitivity over serologic assays (Klintevall et al., 1994; Fechner et al., 1996), several authors have proposed using PVL as a measure of transmission efficiency (Gutiérrez et al., 2011; Rodríguez et al., 2011). This methodology is commonly used for monitoring the risk of transmission for humans infected with the structurally and functionally related retrovirus human T-cell lymphotrophic virus 1 (HTLV-1; Kaplan et al., 1996; Percher et al., 2016). Ohno et al. (2015) reported a strong correlation (r=0.855) between BLV PVL and lymphocyte numbers in dairy cattle, and previous studies have demonstrated that

cattle with high lymphocyte counts are more efficient at transmitting BLV (Buxton and Schultz, 1984; Mammerickx et al., 1987). Experimentally, Molloy et al. (1994) were able to reduce incidence of new BLV infections by selectively culling cows based on viral antigen expression, which they associated with PVL. More recently, Gutiérrez et al. (2014) reported increased periparturient transmission of BLV in cows with high PVL, and other investigators have demonstrated that cows with low PVL present a low transmission risk to their susceptible herd-mates (Mekata et al., 2015; Juliarena et al., 2016).

Sub-clinical Effects and Economic Impact

The sub-clinical (i.e. non-tumorous) effects of BLV are varied, but higher culling rates in BLV-infected cattle have been consistently reported (Pollari et al., 1993; Trainin et al., 1996; Bartlett et al., 2013). Altered immune responses may help explain this trend (for a review, see Frie and Coussens, 2015) such as recent reports of diminished response to vaccination in BLV-infected cattle (Erskine et al., 2011; Frie et al., 2016, 2017). Trainin et al. (1996) also observed diminished humoral responses in infected animals, including failure to clear *Trichophyton* infections, and Emanuelson et al. (1992) found that BLV infection was associated with the incidence of infectious diseases such as mastitis. Perhaps more immediately significant, despite somewhat mixed evidence (Pelzer, 1997), is the effect of BLV infection on milk yield in dairy cattle, as decreased production is an economic issue for the dairy producer, and may result in premature culling of cows (Da et al., 1993). Although some studies have found no effect (Heald et al., 1992; Tiwari et al., 2007) or even a positive effect of BLV infection on milk production (Pollari et al., 1992), the majority of studies have determined that BLV infection is associated with reduced milk yield (Wu et al., 1989; Norby et al., 2016; LaDronka et al., 2018).

Complicating these findings are reports that genetic factors that increase milk yield potential may be associated with susceptibility to BLV (Da et al., 1993; Abdalla et al., 2016). Another economic factor of concern is decreased milk fat production by BLV-positive cows (Wu et al., 1989; Pollari et al., 1992) where, again, lymphocytotic cows show the greatest decline in milk quality (Pollari et al., 1992; Da et al., 1993).

The total economic impact of BLV is difficult to calculate, and is likely to be underreported due to unidentified costs and unrecognized subclinical effects (Pelzer, 1997). Various reports estimate economic effects to be $1500 to $5000 per genetically valuable cow, $2500 to over $10,000 per 100 dairy cows/year, and over $400 per EBL case (Rhodes et al., 2003), $85 to $250 million (to U.S. dairy producers; Ott et al., 2003), and often depend on uncertain factors such as market pressures (Losinger, 2006) and herd-level prevalence (Rhodes et al., 2003). Many countries and regions around the world are reporting moderate (China (Yang et al., 2016), Columbia (Benavides et al., 2013; Úsuga-Monroy et al., 2015), Costa Rica (Jiménez et al., 1995), Iran (Mousavi et al., 2014; Nekoei et al., 2015), Japan (Murakami et al., 2013), Mongolia (Ochirkhuu et al., 2015), Philippines (Polat et al., 2015), South Africa (Ndou et al., 2011), Turkey (Şevik et al., 2015), West Africa (Walrand et al., 1986)) to high (Argentina (Monti et al., 2005), Canada (Nekouei et al., 2015), USA (USDA, 2008; LaDronka et al., 2018)) and often increasing prevalence. Meanwhile areas that have implemented BLV-control programs, such as the EU, continue to tighten trade restrictions to protect their herds (The European Commission, 2016).

Transmission

Inter-herd transmission appears to be almost exclusively via animal movement (Bendixen, 2006; EFSA AHAW Panel, 2015). Intra-herd transmission between herd-mates is a far more complicated question, as both vertical and horizontal transmission of BLV have been described throughout the literature, although in utero transmission appears to be rare (Thurmond et al., 1983a; Lassauzet et al., 1991a). Viral particles are unstable (Rodríguez et al., 2011; de Brogniez et al., 2016), so cell-to-cell transmission is considered to be the primary mechanism for the spread of BLV among host cells in early stages of infection, much like other retroviruses (Sattentau, 2010).

Although some studies have reported BLV provirus detection in semen (Dus Santos et al., 2007; Sharifzadeh et al., 2011), experimental administration of semen from BLV-positive bulls to susceptible sheep (Kaja and Olson, 1982) and artificial insemination with semen from BLV-positive bulls has not been associated with BLV-infection (Monke, 1986). Detection of BLV in semen is therefore most likely due to contamination of smegma with BLV-containing lymphocytes (Choi et al., 2002; Benitez et al., 2019). In addition, neither experimental exposure of embryos to BLV nor insemination of oocytes with BLV-containing semen resulted in detectable BLV (Bielanski et al., 2000). The most likely time of dam-to-offspring transmission is the periparturient period, either through blood transfer during parturition (Nagy et al., 2007b) or through ingestion of infectious cells in milk (Ferrer et al., 1981). Colostrum has been reported both as a risk factor for transmission (Hopkins and DiGiacomo, 1997) and to have protective effects due to the passive transfer of anti-BLV antibodies (Nagy et al., 2007b). Evidence for the latter is seen in both experimental studies (Nagy et al., 2007b) and in natural transmission

settings (Kobayashi et al., 2010), such as a Queensland, Australia herd where no new BLV infections in young animals were detected in calves fed colostrum from their BLV-positive dams (Dimmock et al., 1991).

These routes of transmission still only account for a small number of cases (6-16%; Hopkins and DiGiacomo, 1997), even in herds with high prevalence of BLV infection. Horizontal transmission has been demonstrated through a variety of common management practices that can result in blood transfer such as tattooing (Lucas et al., 1985) or gouge dehorning without disinfecting equipment (DiGiacomo et al., 1985, 1987; Lassauzet et al., 1990), reuse of hypodermic needles and obstetric sleeves (Roberts et al., 1980; Divers et al., 1995; Hopkins and DiGiacomo, 1997), and natural breeding (Lassauzet et al., 1991b; Erskine et al., 2012). The role of hematophagous insects is unclear and may be geographically dependent, though experimental (Buxton et al., 1985; Perino et al., 1990), observational (Bech-Nielsen et al., 1978), and epidemiological (Erskine et al., 2012; Kobayashi et al., 2014; Ohno et al., 2015) evidence seems to indicate these vectors may play a role. Close contact between animals has also been identified in many studies as a risk factor (Kono et al., 1983; Lassauzet et al., 1991b; Ohno et al., 2015), though the exact mechanism of transmission has not been elucidated. Recent studies detecting BLV proviral DNA in salivary and nasal secretions (Yuan et al., 2015) as well as vaginal secretions and feces (Yang et al., 2016) may provide some insight into contact as a risk factor, but no experimental evidence – aside from failed attempts to inoculate sheep with saliva (Dimmock et al., 1991) – has yet been reported.

Herd-Level Risk Factors

Although effective, test-and-cull and test-and-segregate programs are expensive and burdensome to farmers, particularly in herds with a high prevalence of infected animals. Control programs focusing on management risk factors are therefore the next logical step, yet identifying cost-effective strategies for control of BLV is challenging. Some risk factors are clearly more important than others in influencing transmission, as has been demonstrated in studies of tattooing and gouge dehorning mentioned above. Unfortunately, the level of contribution of other management factors to the risk of BLV transmission remains unclear.

Medical Hygiene

Reuse of obstetric sleeves for rectal palpation has been identified as a risk factor for BLV transmission in some (Hopkins and DiGiacomo, 1997; Erskine et al., 2012) but not all (Kobayashi et al., 2010) epidemiologic studies. However, BLV has been transmitted experimentally in this manner (Hopkins et al., 1988; Kohara et al., 2006), and significant associations with sleeve re-use were observed in both a commercial dairy herd (Divers et al., 1995) and in a study performed at a teaching university (Hopkins et al., 1991). Similar hematogenous transmission of BLV through reuse of hypodermic needles has been reported in cattle (Wilesmith, 1979; Roberts et al., 1980), as has the transmission of other pathogens (Reinbold et al., 2010; Darpel et al., 2016). Hypodermic needle sharing is a known risk factor for transmission of HTLV-1 (Robert-Guroff, 1986) and has been identified as a risk factor for BLV infection in epidemiologic studies (Hopkins and DiGiacomo, 1997; Erskine et al., 2012). However, a number of other studies have shown no statistical association between reuse of hypodermic needles and BLV infection (Thurmond et al., 1983b; Lassauzet et al., 1990;

Kobayashi et al., 2010), and Weber et al. (1988) were unable to transmit BLV experimentally to susceptible sheep by the reuse of hypodermic needles. The true impact of this common management practice in the transmission of BLV therefore remains unclear.

Colostrum and Milk

As previously discussed, feeding of colostrum is varyingly associated with infection and protective effects in both experimental and natural transmission settings, while feeding pooled, untreated milk is more frequently identified as a risk factor for BLV infection (Romero et al., 1983; Dimmock et al., 1991; Kanno et al., 2014) than not (Nekouei et al., 2015). Best practice recommendations, therefore, are based either on an abundance of caution (feed only BLV-negative or pasteurized colostrum or milk) or on the desire to prevent transmission to calves (feed colostrum from BLV-positive cows). Recent work by Kanno et al. (2014) indicates that lymphocytes from colostrum from BLV-positive cows that undergoes a 24-hour freeze-thaw cycle are not infectious to sensitive sheep. This may be a more easily attainable protective measure for small farmers than investment in a pasteurizer (Pelzer, 1997) and may be less likely than pasteurization to denature antibodies (Argüello et al., 2003; McMartin et al., 2006).

Hematophagous Insects and Seasonal Transmission

The contribution of blood-sucking insects to BLV transmission, commonly investigated by qualitative means such as subjective descriptions of fly numbers (Kobayashi et al., 2010) or self-reported regular use of fly control (Erskine et al., 2012), is supported in some cases by increased incidence in summer months (Bech-Nielsen et al., 1978; Kobayashi et al., 2010; Tsutsui et al., 2010). However, other studies show that seasonal changes in housing (i.e. increased close

contact in the winter (Wilesmith et al., 1980; Sargeant et al., 1997) and calf-rearing practices (Sargeant et al., 1997; Monti et al., 2007) are more significant. Clearly, individual herd management practices as well as geographical locations and climate factors can influence the temporal dynamics of BLV transmission.

Control Programs

Test and Cull Programs

Prior to an understanding of intra-herd risk factors and identification of an etiologic agent, early control programs were based on the observation that affected herds often were associated by contact, usually through transfer of breeding animals (Marshak et al., 1963; Bendixen, 2006). Hematologic testing (for lymphocytosis) was included in programs such as that implemented in Denmark in 1959 (Bendixen, 2006). Briefly, upon identification of an affected (lymphoma) animal, the herd would be screened for leukocytosis. If lymphocytotic animals were identified, the herd would be quarantined and these cows were culled. Although this eliminated new tumor cases over a five year period, cows continued to develop leukemia and therefore such programs were considered unable to eradicate the disease (Bendixen, 2006). National programs focused on depopulation of affected herds saw success in reducing or eradicating the disease (EFSA AHAW Panel, 2015), while programs that attempted to control the disease by eliminating only lymphocytotic cows saw similar results to the Danish experiment and were unable to prevent the spread of the disease (EFSA AHAW Panel, 2015). The discovery of a population of cattle with normal lymphocyte count and a high proviral load (Juliarena et al., 2007) may at least partially explain why this approach failed. When serologic testing became available, the increased

sensitivity made test and slaughter of individual animals more effective (Nuotio et al., 2003; Batho et al., 2008).

Management Practice Modification

In some cases, culling of all serologically positive cattle is not desirable or may not be economically feasible. Segregation of BLV-positive animals until they can be eliminated and replaced with BLV-free cattle has been successfully used to eliminate herd-level BLV prevalence (Shettigara et al., 1989). However, strict adherence to management protocols to prevent contact between BLV-positive and susceptible cattle – including disinfecting or discarding equipment and supplies used on BLV-positive animals – is necessary and must be maintained for long periods of time, as lapses prior to eradication of the disease quickly result in new infections (Johnson et al., 1985; Shettigara et al., 1989).

Other control programs have been developed based exclusively on management practices other than segregation, such as treatment of colostrum/milk and preventing blood transfer via dehorning equipment, needles, and sleeves. Although some of these programs have decreased the prevalence of BLV in herds with high starting prevalence (Ruppanner et al., 1983; Sprecher et al., 1991), they have not been universally successful (Gutiérrez et al., 2011).

Conclusion

European-style ‘test-and-remove’ control measures for BLV require low initial prevalence of infection (Nuotio et al., 2003; Batho et al., 2008) and/or commitment to high-cost test and cull programs (Gottschau et al., 1990; Bendixen, 2006; EFSA AHAW Panel, 2015). Mixed

approaches have shown success particularly with the addition of strict segregation of BLV-positive animals and adherence to management protocols designed to reduce intra-herd transmission (Ruppanner et al., 1983; Shettigara et al., 1989; Sprecher et al., 1991), though these efforts require a financial investment (Pelzer, 1997; Gramig et al., 2010) as well as committed herd managers (Johnson et al., 1985; Gramig et al., 2010; Maresca et al., 2015) and are most successful with national-level implementation (Acaite et al., 2007).

Breeding programs to leverage the genetic factors related to susceptibility or resistance to BLV infection and disease progression have been proposed for the control of BLV (Lewin and Bernoco, 1986; Xu et al., 1993; Forletti et al., 2013). However, genetic associations between BLV susceptibility and desirable production traits such as milk production, fat yields, and somatic cell count have been identified (Wu et al., 1989; Abdalla et al., 2016). Consequently, if high genetic potential cows also have higher genetic susceptibility to BLV as reported by Wu et al. (1989), selecting cows with decreased genetic susceptibility to BLV may have negative effects on production. Careful consideration of breeding selection programs and their potential long-term consequences on the dairy industry is therefore necessary (Rauw et al., 1998).

New PCR detection techniques, particularly quantitation of proviral load, open up new possibilities for improving BLV-control programs, and will continue to gain importance for diagnostic use as vaccine development (Barez et al., 2015) continues. Further research to elucidate the role and significance of both herd- and animal-level risk factors is ongoing and necessary in order for producers and veterinarians to design cost-effective (Gramig et al., 2010)

BLV control plans, particularly in countries like the U.S. that do not have national reporting, control, or support programs.

Specific Aims

Bovine leukemia virus (BLV) is highly prevalent in U.S. dairy herds and has been found to negatively impact immune function. Associations between BLV status and declines in production outcomes, which result in millions of dollars of economic losses, have also been reported and may be related to this immune dysfunction. The goal of this dissertation was to investigate approaches for on-farm control of bovine leukemia virus infection and better characterize the effect of BLV on the immune function of naturally infected dairy cows through the following specific aims:

Aim 1: To investigate associations between BLV profiles and total IgA concentrations in the milk, saliva, and serum of BLV-positive dairy cows.

Aim 2: To investigate the role of common-use hypodermic needles and reproductive examination sleeves in the transmission of BLV.

Aim 3: To examine test-and-removal protocols for feasibility in U.S. dairy herds.

Sub-aim 3.1: To develop and test an approach for high BLV prevalence herds in which the cows thought to be the most infectious are selectively removed from the herd.

Sub-aim 3.2: Evaluate the utility of a test-and-remove method of BLV control using milk ELISA results.

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CHAPTER 2: Immunoglobulin A levels are altered in milk and saliva, but not serum, of bovine leukemia virus antibody-positive dairy cows

This chapter represents a manuscript in preparation for submission for publication.
Authors who contributed to this study were: Vickie J. Ruggiero, Monika M. Dziuba, Catherine Wilson, Ashley Greenlick, Paul C. Bartlett, Paul Coussens.

Abstract

The objective of this study was to characterize total IgA concentrations in serum, milk, and saliva of cows with bovine leukemia virus (BLV). BLV is a widespread retroviral infection among U.S. dairy cattle that disrupts the immune function of infected animals. The impact of this immune disruption can be varied and costly in dairy cows, resulting in decreased milk production, longevity, and vaccine response. Recent research has identified differences in IgM antibody levels and responses in BLV-ELISA positive versus BLV-ELISA negative cattle. These differences were particularly significant in cattle with lymphocytosis - a condition that affects approximately one third of BLV-infected cattle and which is characterized by clonal expansion of the B-lymphocyte population. This condition is often, but not always, associated with BLV proviral load (PVL), a presumptive measure of infectivity and disease progression. In this study, total IgA concentrations were evaluated in milk, saliva, and serum of dairy cows with varying BLV ELISA, lymphocyte count, and PVL status. Average total IgA concentrations were 33.6% lower in milk samples and 23.7% lower in saliva samples from BLV-positive cows, although neither of these results were statistically significant. No statistically significant differences were observed in cows with lymphocytosis or high proviral load although there was an unexpected trend toward markedly lower concentrations in BLV-ELISA positive cows without these profiles. No remarkable differences were observed in total IgA concentrations in serum samples, regardless of BLV profile. This is the first report to date investigating IgA concentrations in milk and saliva in the context of BLV infection, and further investigation is needed to more extensively characterize the effects of BLV infection on IgA.

Key Words: enzootic bovine leukosis, lymphyocytosis, proviral load, immunoglobulin A, antibody

Introduction

Bovine leukemia virus (BLV) is an oncogenic deltaretrovirus of cattle. Infected animals may develop lymphosarcoma (about 5%) and/or lymphocytosis (about 30%) (Kabeya et al., 2001), and immune dysfunction (Frie et al., 2016; Blagitz et al., 2017). BLV is primarily transmitted horizontally by transfer of infected cells. In commercial dairy cattle, this is commonly the result of management practices that result in blood-to-blood contact or by ingestion of unpasteurized milk (Romero et al., 1983; Dimmock et al., 1991; Erskine et al., 2012). BLV predominantly infects B-lymphocytes, and immortalization and clonal expansion (Gillet et al., 2007) of these cells results in the lymphocytosis that develop in approximately one third of infected cattle. Once introduced into a cell, BLV inserts itself into the DNA of that host cell (Johnston et al., 2002) and this proviral DNA can be identified and measured by PCR and qPCR. This quantification (termed proviral load; PVL) is used in the closely related retrovirus HTLV-1 (human T-cell lymphotropic virus) as an indicator of infectivity and disease progression (Kaplan et al., 1996; Li et al., 2004; Percher et al., 2016), and there is mounting evidence that BLV PVL can be used in the same way (Rodríguez et al., 2011; Gutiérrez et al., 2014; Mekata et al., 2015; Juliarena et al., 2016).

The economic cost of BLV has long been assumed to be limited almost exclusively to morbidity and mortality related to malignancy, and despite low incidence of this disease, lymphomatous tumors remain the top cause of condemnation for dairy cattle carcasses (White and Moore, 2009). However, the economic impact of 'subclinical' BLV has been gaining recognition. Recent work by the BLV research group at Michigan State University (MSU) has described decreased longevity (Bartlett et al., 2013), and milk production (Norby et al., 2016) in BLV-ELISA

positive (BLV+) dairy cows as compared to their BLV-ELISA negative (BLV-) herd-mates, in agreement with other investigators around the world (Emanuelson et al., 1992; Nekouei et al., 2016; Yang et al., 2016).

One proposed mechanism for these effects, recently reviewed by Frie and Coussens (2015) is altered immune responses in BLV+ cattle. Numerous authors have reported a BLV-associated decrease in indicators of systemic immunity (IgG2, IgM), altered activation of immune cells, and disrupted T:B cell ratios, particularly in response to vaccination (Trainin et al., 1996; Erskine et al., 2011; Frie et al., 2016, 2017). Conditions with infectious causes are of economic concern in the dairy industry, and increased incidence of mastitis, hoof problems, and other mucosal diseases (Emanuelson et al., 1992) as well as failure to clear ringworm infection (Brenner et al., 1989; Trainin et al., 1996) have been associated with BLV infection. Despite these reports, the effects of BLV infection on indicators of mucosal immunity are not well characterized.

In many species, IgA is considered the primary immunoglobulin in mucosal immunity. While IgA, IgM, and IgG concentrations have been described in cattle serum and secretions (Butler et al., 1972; Duncan et al., 1972; Guidry et al., 1980), we found no works to date which have examined IgA concentrations in the context of BLV infection. In addition, previous works have reported relationships between immunoglobulin levels and BLV-ELISA and/or lymphocyte count (LC) status, but not PVL. The aim of this pilot project was to characterize the potential relationship between total IgA in milk, saliva, and serum and BLV-ELISA, LC, and PVL status in dairy cows.

Materials and Methods

Study Design

Milk, saliva, and serum samples for this study were collected from lactating dairy cows greater than two years of age with known BLV ELISA status (as positive, BLV+; or negative, BLV-) at the Michigan State University (MSU) W. K. Kellogg Biological Station (KBS) Pasture Dairy Center. This herd is an automated milking rotational pasture grazing/free-stall dairy with approximately 160 cows in the milking herd (~90% Holsteins and 10% Friesians). Forty cows were sampled with the goal of representing each ELISA and LC or PVL category (described below), based on previous PVL and LC results determined as part of another research study. Samples of milk, saliva, and whole blood were collected contemporaneously. Approximately six months later, an additional 16 cows (10 additional BLV ELISA- and 6 additional BLV ELISA+) were selected and sampled in the same manner for a total of N=56 enrolled cows.

Milk, Saliva, and Blood Sample Collection

Milk was collected into untreated 50 mL conical tubes and aliquots were frozen at -80C after collection. Saliva samples were collected using a SalivaBio Children's Swab (Salmetrics, Carlsbad, CA, USA), centrifuged at 1500 x g for 15 minutes, and frozen (at -80C). Protease Inhibitor (Pierce, Thermo Fisher Inc., Waltham, MA, USA) was added to saliva samples to prevent protein degradation. Blood samples were collected into a clot activator/polymer gel evacuated tube and a K2 EDTA-treated evacuated tube. Blood in clot tubes was rendered to serum for immunoglobulin ELISA testing. Blood collected into EDTA-treated tubes was separated into aliquots and used for PVL testing and leukocyte counts. Animal procedures for

this study were reviewed and approved by the MSU Institutional Animal Care and Use Committee.

ELISA Test for Immunoglobulins

Commercial antibody ELISAs (Bethyl Laboratory, Inc., Montgomery, Texas, USA) were optimized for each sample type. The optimal dilution for IgA in serum was 1:640 and 1:250 in milk. Saliva samples were normalized based on total protein concentrations using a Bicinchoninic acid assay (ThermoFisher, Inc., Pittsburg, PA) according to the manufacturer's protocol. The optimal dilution for IgA in saliva was 1:10,000 after protein normalization. All samples were measured at 450nm using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA, USA) to determine optical density (OD) values. These OD values were compared to the standard curve run on each plate to calculate a total antibody concentration. Individual animal samples were run in duplicate; mean concentrations are reported in ug/mL.

Lymphocyte Count Determination

Anticoagulated (EDTA) blood samples were collected to measure total and differential leukocyte counts. The first set of samples were analyzed using an automated blood leukocyte differential test (QScout BLD, Advanced Animal Diagnostics, Morrisville, NC, USA) at the MSU BLV Laboratory. This machine was not available at the time of the second sample collection, therefore blood samples were submitted for leukocyte analysis at the MSU Veterinary Diagnostic Laboratory (Lansing, MI, USA). LC results were dichotomized based on the Laboratory reference range: High (≥7,500; HLC), or Normal (<7,500/μL; nLC).

Proviral Load Test

The proviral load was measured using the CoCoMo BLV quantitative polymerase chain reaction (qPCR) method (Jimba et al., 2010). Genomic DNA was extracted from EDTA anticoagulated whole blood samples (Wizard Genomic DNA Purification Kit; Promega Corporation, Madison, WI, USA). Eluted DNA was normalized to 30 ng/uL, and 150 ng of genomic DNA was added to each qPCR using the BLV-CoCoMo primer set, FAM BLV probe (RIKEN genesis, Tokyo, Japan), and TaqMan Gene Expression Master Mix (Life Technologies, Carson, CA, USA). PVL was dichotomized as: High ($\geq$50,000 copies per 10^5 cells; HPVL), or Low (<50,000 copies; LPVL).

Data Analysis

The principal study endpoints were the comparison of total IgA in milk, saliva, or serum between groups defined by 1) ELISA results alone, 2) LC category, and 3) PVL category. Between group differences in mean total antibody concentration were evaluated by a two-tailed student T-test.

Results

Milk Immunoglobulins

Mean IgA concentrations in milk were 33.6% lower in BLV+ cows (N = 37) compared to BLV- cows (N = 19, P = 0.2429; Figure 1a), and lowest in BLV+ cows with nLC (34.7%, N = 24, P = 0.2521) or LPVL (36.1%, N = 24, P = 0.2371; Figure 1c). Within BLV+ cows, mean IgA concentrations in milk were nearly equivalent between nLC (N = 24) and HLC (N = 13) groups (P = 0.8554; Figure 1b) and between LPVL (N = 24) and HPVL (N = 9) groups (P = 0.6659; Figure 1c).

Saliva Immunoglobulins

Mean IgA concentrations in saliva were 23.7% lower in BLV+ cows (N = 36) compared to BLV- cows (N = 19, $P = 0.4511$; Figure 2a). Within BLV+ cows, mean IgA concentrations in saliva differed more markedly; saliva IgA concentrations were 57.2% lower in nLC (N = 23) cows compared to HLC (N = 13) cows ($P = 0.1267$; Figure 2b) and the decrease in concentration in nLC cows compared to BLV- cows trended toward significance (48.5%, $P = 0.1003$), while the concentration in HLC cows was 20.4% higher compared to BLV- cows ($P = 0.6777$). Even more dramatically, the average total saliva IgA concentration in HPVL cows (N = 9) was 56.0% higher than BLV- cows ($P = 0.3816$; Figure 2c). The concentration in LPVL cows (N = 23) was also markedly lower than both BLV- cows (45.9%, $P = 0.1170$) and HPVL cows (65.3%, $P = 0.1087$).

Serum Immunoglobulins

Mean IgA concentrations in serum were slightly numerically higher in BLV+ cows (4.3%, N = 37) compared to BLV- cows (N = 19, $P = 0.6258$; Figure 3a). There was minimal difference between mean concentrations in BLV+ nLC (N = 24) and HLC (N = 13) groups ($P = 0.6301$; Figure 3b) and LPVL (N = 24) and HPVL (N = 9) groups ($P = 0.6296$; Figure 3c).

Discussion

The objective of this study was to investigate total IgA concentrations in the milk, saliva, and serum of BLV-ELISA positive cows with different levels of lymphocytes and proviral load. BLV-infection-associated disruption of antibody levels has been reported in previous studies from our group (Erskine et al., 2011; Frie et al., 2016, 2017) and others (Trainin et al., 1996;

Puentes et al., 2016). However, these studies were primarily concerned with serum antibody levels, and did not examine antibody levels in milk or saliva, nor did they assess IgA. Although our study found no statistically significant differences in total IgA concentrations across groups, we observed a markedly lower concentration of IgA in both the milk and saliva of BLV+ cows that was even more pronounced in BLV+ nLC or LPVL cows.

IgA exists primarily as a dimer in secretory form in bovine secretions (Porter and Noakes, 1970; Butler, 1971). It has been suggested that BLV inhibits the J-chain (Brym and Kamiński, 2016), which joins IgA molecules together and is necessary for IgA assembly, secretion, and cross-membrane transport (Korhonen et al., 2000; Hurley and Theil, 2011). Therefore, we expected the majority of any BLV-associated effects on IgA to be identified in either milk or saliva, or both, rather than serum. As a result, our finding that serum IgA concentrations did not differ in BLV+ cows regardless of sub-population was unsurprising. Although the total milk IgA concentrations in BLV+ cows were not statistically significantly different from BLV- cows, we observed an overall decrease of 33.6% compared to BLV- cows. In agreement with the results in milk samples, total saliva IgA concentrations were 23.7% lower in BLV+ cattle as compared to BLV- cows. These results are consistent with other reports of immune disruption in BLV-infected cows.

Contrary to expectations, cows categorized as nLC or LPVL had the lowest average IgA concentrations in both milk and saliva. This result agrees with other investigators who report that BLV infection results in immune disruption even in aleukemic cows (Isaacson et al., 1998; Konnai et al., 2013). The higher concentrations observed in HLC and HPVL cows may be

explained by the presence of higher numbers of antibody-producing B-cells in this lymphoproliferative profile, and Isaacson et al. (1998) reported that BLV infection may result in a non-specific activation of B-cells. In addition, cytokine disruptions such as increased IL-2 in persistently HLC cattle may result in a proinflammatory shift (Frie and Coussens, 2015) and increased secretion of immunoglobulins from B-cells (Collins and Oldham, 1995).

This study was carried out in naturally infected cows from a single dairy herd where the herd manager was actively participating in a program to reduce BLV by targeting cows with HPVL and HLC (Ruggiero, et al., in review). We therefore could not control for the duration of BLV infection, and at the time of our sample collections, this herd had been systematically removing cows with the most extreme PVL and LC results. As a result, cows were selected for this study on the basis of availability, which resulted in low sample numbers, particularly in the HLC and HPVL groups. Because these disease profiles are associated with immune disruption (Trainin et al., 1996; Farias et al., 2016), the effect of these advanced stages of BLV on antibody levels may have been underestimated in this study. Immunoglobulin concentrations are also highly variable based on levels of inflammation, seasonal husbandry (Kociņa and Antāne, 2012), and reproductive cycles (Guidry et al., 1980), which were not addressed in the current study design. In particular, high levels of individual variation in saliva IgA concentrations have been reported by other investigators (Duncan et al., 1972) and saliva proteins, including immunoglobulins, are rapidly degraded in saliva (Ng et al., 2003). While we attempted to minimize variability due to degradation by treating saliva samples with a proteasome inhibitor and storing them at -80C, samples were collected from a functioning production facility where immediate freezing and/or centrifugation was not possible. Future research is needed to better characterize the effect of

BLV infection on IgA in saliva and milk as well as to investigate the potential biological implications of the differences observed in this study.

Conclusions

In this study, mean total immunoglobulin A concentrations in milk, saliva, and serum were characterized in relation to BLV profiles defined by ELISA, lymphocyte determination, and proviral load assays. Mean IgA concentrations were decreased in milk and saliva of BLV+ cows, but not in serum. Although no differences were statistically significant, these results agree with the growing consensus that BLV infection results in altered immune systems in cattle. Many pathogens of importance in the dairy industry enter via mucosal junctions and are mediated by IgA, therefore understanding the effect of BLV on this important immunoglobulin could result in improved dairy health. This report provides a useful starting point for examining the effect of BLV infection on an immunoglobulin that is important for mucosal immunity in cattle.

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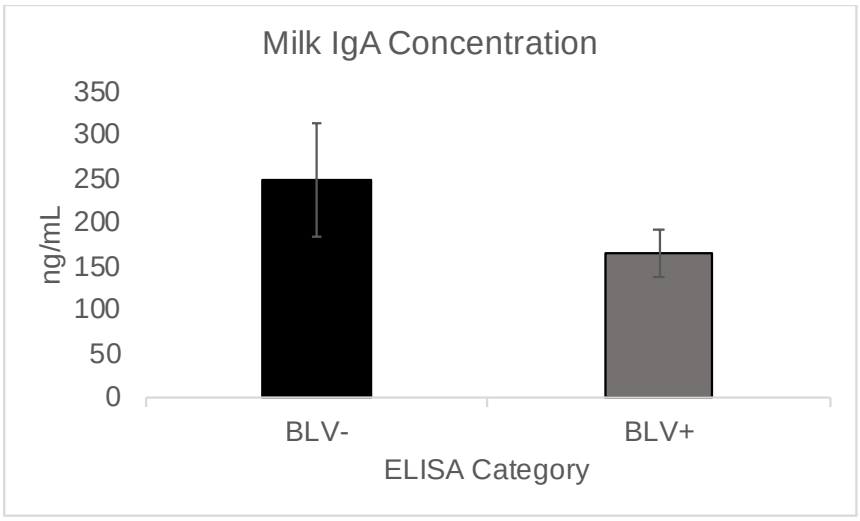
APPENDIX

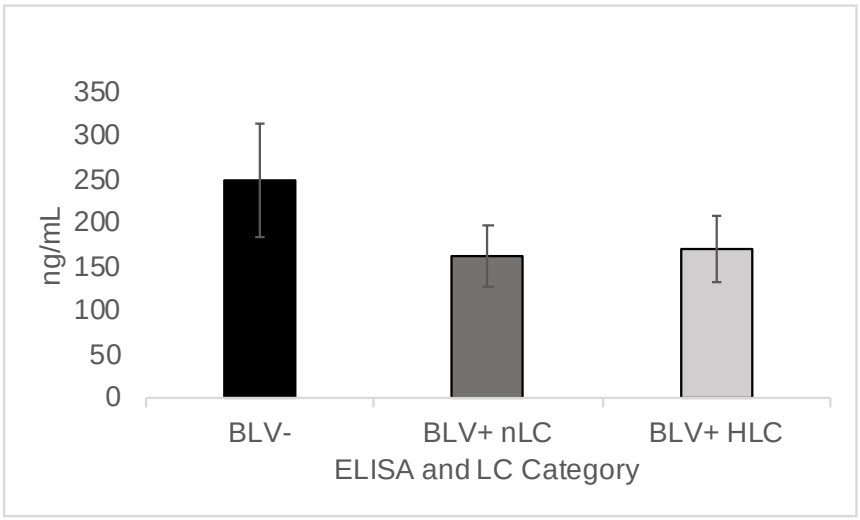
Figure 2.1. Comparisons of average total IgA concentrations in milk.

(A) BLV+ cows (grey bars, N = 37) exhibited a lower average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.2429$. (B) BLV+ nLC cows (grey bars, N = 24) exhibited a lower average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.2521$ and HLC cows (light grey bars, N = 13), $P = 0.8761$). (C) BLV+ LPVL cows (grey bars, N = 24) exhibited a lower average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.2371$ and HLC cows (light grey bars, N = 9), $P = 0.6659$). Error bars represent the SEM.

A)


Milk IgA Concentration
350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+
ELISA Category

B)


350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+ nLC
BLV+ HLC
ELISA and LC Category

C)

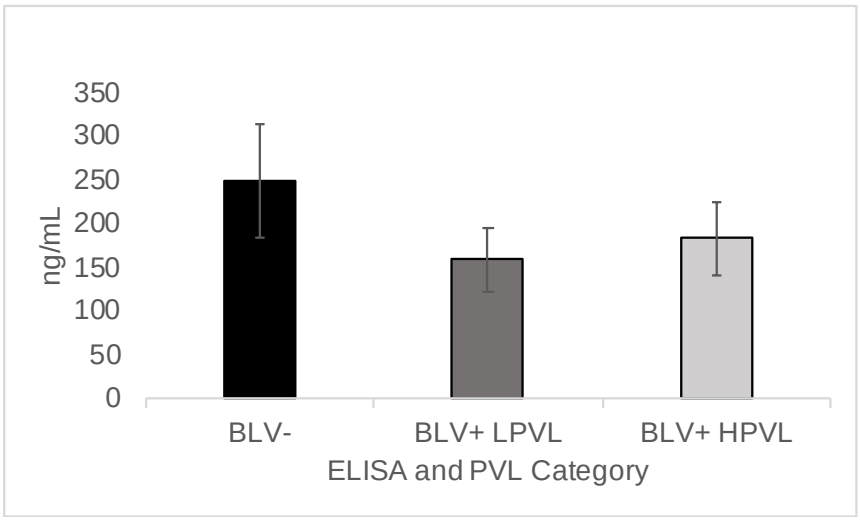
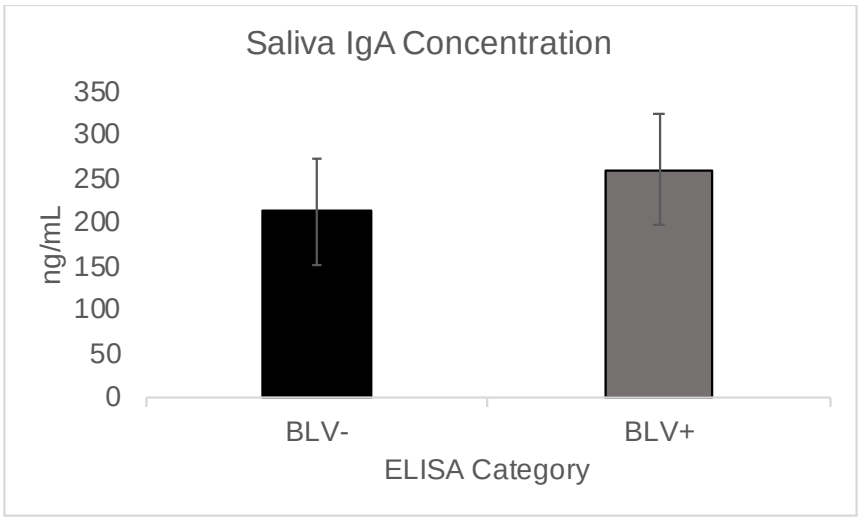

350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+ LPVL
BLV+ HPVL
ELISA and PVL Category

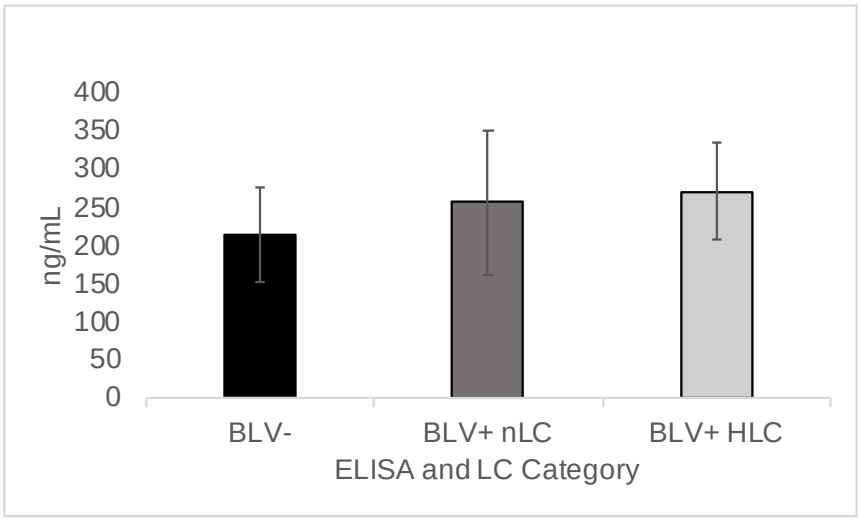
Figure 2.2. Comparisons of average total IgA concentrations in saliva.

(A) BLV+ cows (grey bars, N = 36) exhibited a lower average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.4511$. (B) BLV+ nLC cows (grey bars, N = 23) exhibited a lower average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.1003$ and HLC cows (light grey bars, N = 13), $P = 0.1267$). (C) BLV+ LPVL cows (grey bars, N = 23) exhibited a lower average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.1170$ and HLC cows (light grey bars, N = 9), $P = 0.1087$). Error bars represent the SEM.

A)


Saliva IgA Concentration
350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+
ELISA Category

B)


400
350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+ nLC
BLV+ HLC
ELISA and LC Category

C)

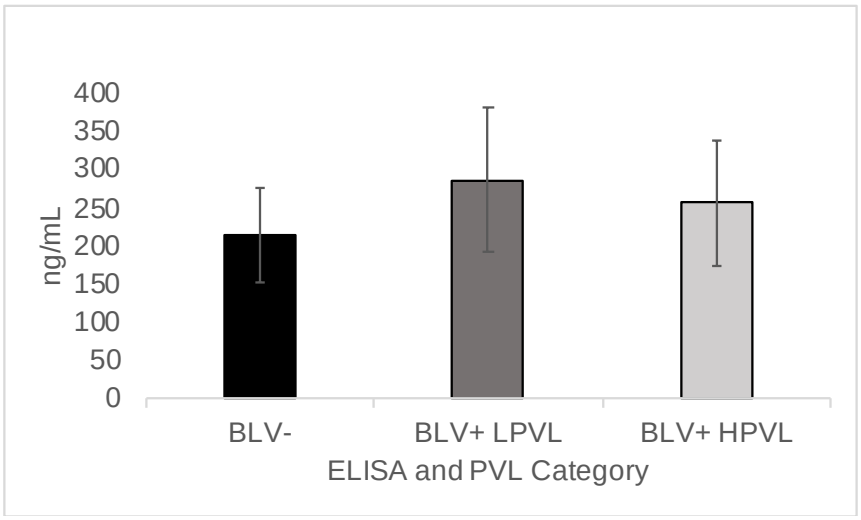
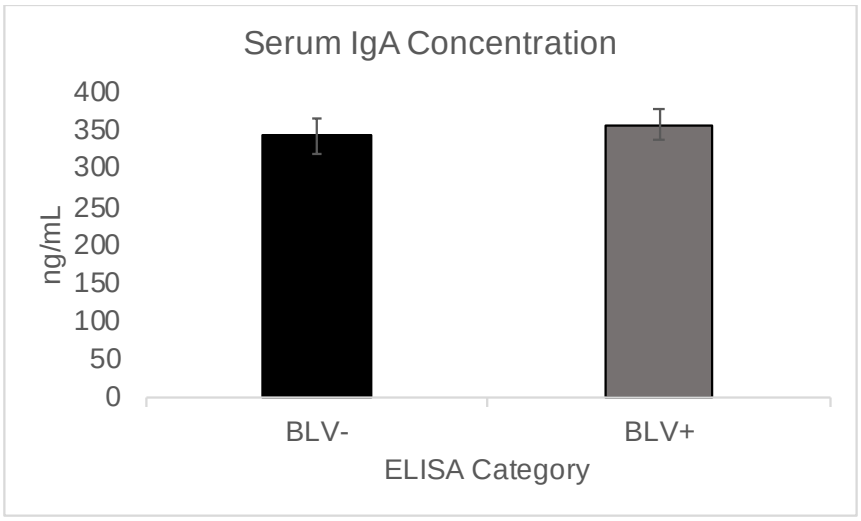

400
350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+ LPVL
BLV+ HPVL
ELISA and PVL Category

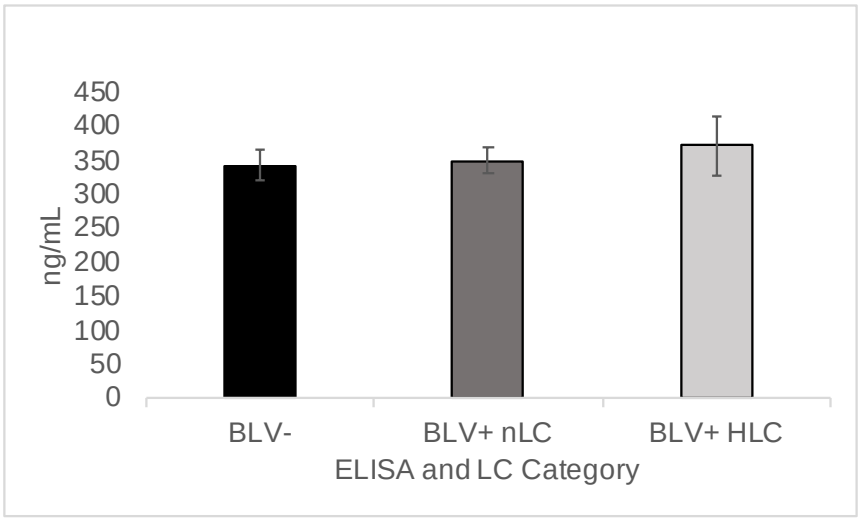
Figure 2.3. Comparison of average total IgA concentrations in serum.

(A) BLV+ cows (grey bars, N = 37) exhibited a similar average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.6258$. (B) BLV+ nLC cows (grey bars, N = 24) exhibited a similar average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.8213$ and HLC cows (light grey bars, N = 13), $P = 0.6301$). (C) BLV+ LPVL cows (grey bars, N = 24) exhibited a similar average total IgA concentration compared to BLV- cows (black bars, N = 19), $P = 0.4172$ and HLC cows (light grey bars, N = 9), $P = 0.6296$). Error bars represent the SEM.

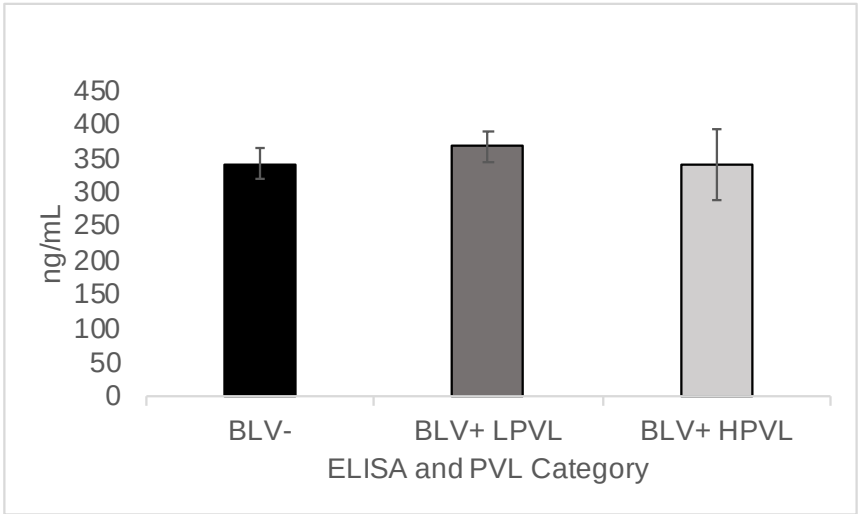
A)


Serum IgA Concentration
400
350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+
ELISA Category

B)


450
400
350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+ nLC
BLV+ HLC
ELISA and LC Category

C)


450
400
350
300
250
200
150
100
50
0
ng/mL
BLV-
BLV+ LPVL
BLV+ HPVL
ELISA and PVL Category

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CHAPTER 3: Single-use hypodermic needles and obstetric sleeves failed to reduce bovine leukemia virus transmission in three dairy herds.

This chapter represents a manuscript in review at *Bovine Practitioner*.
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Abstract

Our objective was to determine the utility of single-use hypodermic needles and reproductive examination sleeves in reducing the transmission of bovine leukemia virus (BLV) in dairy herds. Numerous epidemiological studies have identified re-use of needles and exam sleeves as significant risk factors for BLV. Adopting a practice of single-use needles and exam sleeves is a commonly suggested management change for reducing BLV transmission. We conducted a field trial on three Midwestern commercial dairy herds that had not been employing single use of needles or sleeves as part of their herd health protocol. Additionally, each of the herds had a BLV prevalence among adult cows of at least 20%. BLV milk-ELISA negative cows were randomly assigned to receive shared needles and sleeves with their ELISA-positive herd mates (controls) or to always receive a new single-use needle and new exam sleeve (intervention group). The risk of new infections was determined by semi-annual BLV milk-ELISA testing. The risk of new BLV infections was not statistically different between the two groups. Medical hygiene to prevent bloodborne transmission is still recommended, but in these three herds, re-use of needles and sleeves did not appear to be a major route of BLV transmission. Further studies are recommended.

***Key Words*:** enzootic bovine leukosis, incidence, medical hygiene, bloodborne, seasonal

Introduction

Bovine leukemia virus (BLV) is a retrovirus of cattle that causes significant economic losses in the U.S. dairy industry. In addition to causing cancer in about 5% of infected cattle (Burny et al., 1988), the virus disrupts the immune system and results in decreased milk production (Emanuelson et al., 1992; Da et al., 1993; Norby et al., 2016; Yang et al., 2016), decreased cow longevity in the herd (Emanuelson et al., 1992; Erskine et al., 2012a; Bartlett et al., 2013; Nekouei et al., 2016), and decreased response to vaccination (Erskine et al., 2011; Frie et al., 2016; Puentes et al., 2016). Over 20 nations worldwide have eradicated the virus, and many other nations have eradication programs (EFSA AHAW Panel, 2015). In the U.S., however, BLV is present in over 90% of dairy herds (LaDronka et al., 2018), and the average herd prevalence has increased to about 43% (USDA, 2008; LaDronka et al., 2018). The economic losses due to BLV in the U. S. have been estimated at $525 million in 1995 (Ott et al., 2003), and a more recent estimate of BLV costs revealed a loss of $283 per milking cow annually (Bartlett et al., 2017, 2018).

BLV is transmitted by transfer of infected cells, primarily lymphocytes (Paul et al., 1977; Mammerickx et al., 1987). Epidemiological studies have identified a number of common management practices as risk factors for BLV transmission in commercial dairy herds (Erskine et al., 2012b; Kobayashi et al., 2014; Nekouei et al., 2015). Most identified risk factors relate to bloodborne transmission. Transmission of BLV via reproductive/rectal sleeves has been demonstrated in both experimental (Hopkins et al., 1988; Kohara et al., 2006) and observational studies (Hopkins et al., 1991; Divers et al., 1995). Direct evidence for BLV transmission by re-use of needles is perhaps less clear. For example, Roberts et al., (1980) reported no transmission

from re-use of tuberculin needles during routine tuberculosis (TB) testing, but the same authors were able to transmit BLV in this way when the needle was intentionally contaminated with "a minute quantity" of blood.

Although adopting single-use needles and exam sleeves incurs costs in employee and veterinary time as well as supplies, both are commonly recommended to control BLV transmission (Brunner et al., 1997; Rodríguez et al., 2011; Kuczewski et al., 2019). To determine the impact of re-use of hypodermic needles and reproductive examination sleeves in the transmission of bovine leukemia virus, we implemented a field trial in three Midwestern commercial dairy herds to compare the new BLV infection rates among two randomly-assigned treatment groups.

Materials and Methods

Herd Enrollment and Study Design

Herd enrollment requirements were as follows: 1) Current management practices where common needles and sleeves were re-used regularly, 2) Herd prevalence of antibodies against BLV by testing of milk by ELISA $\geq$ 20%, 3) Herd managers who were willing to follow the study protocol described below.

Individual cow milk testing to detect antibodies against BLV by ELISA was conducted on all lactating cows at enrollment to identify BLV-negative cows and thus susceptible to infection. BLV-negative cows in each herd were enrolled and randomly assigned to the control or intervention group, approximately balanced on days in milk. The control group cows received needles and sleeves shared with other control cows and with BLV-positive cows. The

intervention group cows were marked by the herd manager with additional ear tags, leg bands, and/or chalk and always received a new single-use needles and sleeves. All cows intermingled freely with each other and with their ELISA-positive herdmates as per the herd's standard management practices. Subsequent individual cow milk testing was conducted semiannually, as close as possible to each November 1 and May 1. After each semiannual test, we reviewed the protocol with the herd managers and asked if any mistakes had been made in the single-use needle and sleeve protocol. One herd was excluded from the study after one year when they became convinced that single-use needles and sleeves was a good management practice and, unbeknownst to us at the time, began using this procedure for all of their cattle.

Herd "P". Herd P was a Michigan free-stall dairy that milked about 220 cows with a starting BLV ELISA prevalence of 25.3% (58/223) at enrollment in Fall 2014. This herd participated for one year until Fall 2015.

Herd "W". Herd W was a Wisconsin free-stall/pasture organic dairy milking about 350 cows with a starting BLV ELISA prevalence of 74.4% (262/352) at enrollment in Fall 2014. This herd participated for 2.5 years, until Spring 2017; however, the semi-annual test scheduled for May 2016 was inadvertently missed due to a communication failure with the laboratory.

Herd "F". Herd F was a Michigan free-stall dairy (with non-lactating cows on pasture) milking about 320 cows. The starting BLV ELISA prevalence was 53.5% (169/316) at enrollment in Spring 2015. This herd participated for 2 years, through Spring 2017.

Milk Sample Collection

Routine milk samples were collected by DHIA technicians into containers with standard DHIA preservative (bronopol/natamycin), transported to the NorthStar Cooperative Michigan Laboratory (Grand Ledge, MI, USA; NorthStar), and tested for antibodies against BLV via ELISA. Procedures for this study were reviewed and approved by the MSU Institutional Animal Care and Use Committee.

ELISA Test for Anti-BLV Antibodies

A modified ELISA test to detect antibodies directed against BLV as described by Erskine et al. (2012c) was performed at NorthStar. Aliquots of milk samples were diluted (1:30) and added to 96-well BLV-coated ELISA plates. After washing, BLV antibodies were detected by reaction with horseradish-peroxidase-labeled antibodies to bovine immunoglobulin with addition of an enzyme substrate. Standardized reaction times were determined by color development of positive controls, and the reaction was stopped by addition of 0.5 N H_2SO_4. Results were reported as corrected 450nm optical density (OD) measurements (raw sample OD - negative control OD). Milk samples with a corrected OD > 0.1 were considered positive for anti-BLV antibodies.

Data Analysis

The principal study endpoint based on ELISA results was incidence risk of new infections, calculated for the previous 6 months from semiannual test results. Incidence risk (proportion) was calculated for each treatment group at each inter-test period of approximately six months. For each inter-test period, the population was closed. Only enrolled, ELISA-negative cows at the beginning of a period were considered at risk of acquiring a new infection. A new infection was

defined as an at-risk cow that converted to a positive ELISA result, and positive cows were excluded from all future at-risk populations. The at-risk population for each inter-test period was estimated by calculating a corrected at-risk population (Ruggiero, in review): the number of enrolled, BLV ELISA-negative cows at the semiannual test at the start of the period, minus one half the number of these cows absent at the semiannual test at the end of the period. In rare cases where cows were inadvertently not sampled at a semiannual test point (despite remaining in the herd) and subsequently identified as BLV-positive on the following semiannual test (e.g. Cow X tested negative at test 1, was not tested at test 2, and tested positive at test 3), analogous adjustments were made to the 12-month period. Briefly, each 'missed' newly BLV ELISA-positive cow was considered to have contributed one new infection over the total inter-test period and was included in the at-risk population of each semiannual test period associated with the missed test. The incidence risk for new infections between groups was evaluated using the Fisher mid-P exact test in individual herds (each herd considered a strata) and in all three herds combined in a stratified analysis (OpenEpi 3.01; Dean et al., 2013).

Each period between semiannual tests approximated summer (May through October) or winter (November through April) months at risk. The incidence risk was calculated for each season using the corrected at-risk population and the definitions of a new infection as described above. Due to the missed semi-annual test in Herd W, only one summer season could be measured in that herd. The incidence risks of new infections in the summer and winter exposure periods that could be measured were compared using the Fisher mid-P exact test as described above (Dean et al., 2013).

Results

Semiannual Incidence Risk of New Infections Between Groups

The ELISA test results were used to calculate the incidence risk of new infections during each inter-test period for each treatment group. The treatment groups did not differ significantly in their risk of new BLV infections ($P = 0.378$) and were 20.0 for the controls and 22.7 for the intervention group (Table 1).

Seasonal Incidence Risk of New Infections

The combined new infection risk for the three-herd combined analysis by season was higher during summer periods of exposure ($P = 0.036$; Table 2). This appears to be primarily driven by Herd W, where the new infection risk was more than twice as high in the one summer exposure period that could be measured (40.8 vs 16.8; $P = 0.001$). The difference in seasonal new infection risk was not statistically significant between summer and winter in Herd F (24.5 and 23.6, respectively; $P = 0.788$) and Herd P, although the risk in Herd P was numerically higher in the winter (9.4 vs 6.2 in the summer; $P = 0.357$).

Discussion

This field trial examined the impact of a commonly recommended management procedure for BLV control in dairy herds. We used within-herd controls to ensure other management practices were consistent between control and intervention cows. However, this required that the herd managers and staff know which cows were assigned to the intervention group, thus preventing a blind study. Although our data did not show a statistically significant difference between our two treatment groups in the risk of new BLV infections, the use of single-use needles and exam

sleeves, along with other interventions, has been part of several successful BLV control programs (Ruppanner et al., 1983; Kaja et al., 1984; Johnson et al., 1985; Sprecher et al., 1991). In addition, blood transmission via needles and sleeves is a plausible mechanism of BLV transmission in cattle (Roberts et al., 1980; Hopkins et al., 1991; Divers et al., 1995).

The risk of new infections varied among herds and tended to be higher in the herds with higher BLV prevalence, consistent with other reports (Dimmock et al., 1991; Lassauzet et al., 1991; Hopkins and DiGiacomo, 1997). The overall semi-annual risk of new infection among all enrolled cows was 7.9 in Herd P (~25% prevalence), 24.2 in Herd F (~54% prevalence) and 32.1 in Herd W (~75% prevalence). The risk of new infections was fairly stable in Herd F throughout their participation, while the risk of new infections in Herd W was more variable.

The seasonal risk of new infection was higher in the summer period of exposure, consistent with reports that associate the presence of blood-sucking insects with BLV incidence (Bech-Nielsen et al., 1978; Onuma et al., 1980). Herd W was the major contributor to this result; seasonal risk of new infections was essentially equivalent in Herd F and numerically higher in winter in Herd P. Herd W was an organic dairy, and cows spent much of their summer on pasture; both of these management approaches may have influenced seasonal risk of infection. In contrast, our research group reported a numerically higher risk of new infections in winter in a different field trial, except for one tie stall/pasture herd which had numerically higher risk of new infections in summer (Ruggiero, in review). Individual herd management practices, including calving practices and housing, as well as the presence of blood-sucking insects, likely impact BLV

infection and may explain the inconsistency in reported seasonal patterns of BLV infection (Wilesmith et al., 1980; Thurmond et al., 1983; Sargeant et al., 1997).

Although single-use needles and exam sleeves have been part of successful BLV control programs, this management practice is rarely the sole change implemented. It is possible that other practices associated with BLV incidence, such as housing practices (Sargeant et al., 1997; Ohno et al., 2015) and feeding of pooled milk (Dimmock et al., 1991; Kanno et al., 2014) may contribute more significantly to BLV transmission than re-use of needles and sleeves, or there could be a synergistic effect when multiple risk-associated practices are in use. Recent reports indicate that BLV transmission may be disproportionately attributable to a small number of highly infectious cows (Rodríguez et al., 2011; Gutiérrez et al., 2014; Ruggiero, in review). The presence (or absence) of these cows may have affected the transmission risk in each herd and in each exposure period.

Even though we did not see an effect on BLV transmission in this trial, implementing a practice of single-use needles and exam sleeves improves medical hygiene and likely reduces blood transfer of many pathogens such as anaplasmosis (Reinbold et al., 2010). The cost of each needle and sleeve has decreased to less than $.10 USD each, so implementing single-use needles and sleeves is much less costly than in prior decades. Adopting single-use needles and exam sleeves should be recommended as part of most comprehensive disease control programs regardless of the impact on BLV transmission, which still requires further investigation to determine if the results reported here are repeatable.

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APPENDIX

Table 3.1. Combined risk of new infections in enrolled groups overall and by herd.

Group	Herd P			Herd W		
	New Infections	Cows at Risk	Incidence Risk	New Infections	Cows at Risk	Incidence Risk
Control	11	134.5	8.2	35	130	26.9
Intervention	11	142.5	7.7	46	122	37.7

Group	Herd F			All Herds Combined		
	New Infections	Cows at Risk	Incidence Risk	New Infections	Cows at Risk	Incidence Risk
Control	42	175.5	23.9	88	440	20.0
Intervention	45	184	24.5	102	448.5	22.7

Mid P exact 2 tail p-value: Herd P, $P = 0.893$; Herd W, $P = 0.134$; Herd F, $P = 0.921$; all herds, $P = 0.378$.

Table 3.2. Combined seasonal risk of new infections in enrolled groups overall and by herd.

Season	Herd P			Herd W		
	New Infections	Cows at Risk	Incidence Risk	New Infections	Cows at Risk	Incidence Risk
Winter	14	148.5	9.4	17.5	104	16.8
Summer	8	128.5	6.2	37.5	92	40.8

Season	Herd F			All Herds Combined		
	New Infections	Cows at Risk	Incidence Risk	New Infections	Cows at Risk	Incidence Risk
Winter	29.5	125	23.6	61	377.5	16.2
Summer	57.5	234.5	24.5	103	455	22.6

Mid P exact 2 tail p-value: Herd P, $P = 0.357$; Herd W, $P = 0.001$; Herd F, $P = 0.788$; all herds, $P = 0.036$.

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CHAPTER 4: Controlling bovine leukemia virus in dairy herds by identifying and removing cows with the highest proviral load and lymphocyte counts

This chapter represents a manuscript in review at the *Journal of Dairy Science*.
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Abstract

The objective of this field trial was to reduce bovine leukemia virus (BLV) transmission and prevalence in commercial dairy herds using proviral load (PVL) and lymphocyte count (LC) measurements as indicators of the most infectious animals for culling or segregation. BLV inserts proviral DNA into host cell genomes and the provirus concentration may be quantified as proviral load. BLV causes lymphoma in <5% and increased lymphocyte counts (lymphocytosis) in about one third of infected cattle, and recent research has shown that dairy cows infected with BLV have altered immune function associated with decreases in milk production and lifespan. More than 20 nations have eradicated BLV from all their dairy cattle during past decades. In contrast, the U.S. has experienced an increase in BLV prevalence to over 40% of all dairy cows, raising concerns about global trade, animal welfare, herd health, consumer perceptions, and production efficiency. Recent findings show that a minority of infected cattle have PVL concentrations in blood and other body fluids of over 1,000 times that of other infected cattle. In combination with a high lymphocyte count, these animals are thought to be responsible for most transmission of BLV within the herd. Their removal via culling or segregation may be an important critical control point to diminish the many direct and indirect routes of BLV transmission. Milk or blood samples from adult cows in our three Midwestern dairy farm field trials were tested semiannually for BLV ELISA antibodies, and ELISA-positive cattle were then retested using a blood lymphocyte count and a qPCR test for PVL to identify the animals presumed to be most infectious so they could be culled or segregated. The incidence risk of new infections and overall herd prevalence decreased in all three herds, suggesting that this approach can reduce BLV prevalence. This is encouraging because a very low prevalence of BLV

infection would make it economically feasible to cull the remaining ELISA-positive cattle, as was achieved in national eradication programs in other countries decades ago.

***Key Words*:** enzootic bovine leukosis, incidence, prevalence, proviral load

Introduction

The prevalence of bovine leukemia virus (BLV) has now surpassed 40% in U.S. dairy cattle, with over 80% of U.S. dairy herds infected (USDA, 2008; LaDronka et al., 2018). Similar to the related human retrovirus human T-cell lymphotrophic virus (HTLV), BLV inserts proviral DNA into the host cell genome (primarily T-lymphocytes in HTLV or B-lymphocytes in BLV), progressively altering cell maturation and function to eventually disrupt the host's immune system (Callahan et al., 1976; Esteban et al., 1985; Frie et al., 2016). Immune dysfunction is thought to facilitate development of neoplasia (lymphoma and leukemia), as well as decreases in milk production (Ott et al., 2003; Erskine et al., 2012a) and cow longevity (Brenner et al., 1989; Bartlett et al., 2013; Nekouei et al., 2016) in infected cattle. Economic costs of BLV have been estimated at $43 million in lost milk production alone (Da et al., 1993), and, in 1995, at $525 million across the industry before considering veterinary expenses or potential lost trade dollars (Ott et al., 2003). Rhodes et al. (2003) estimated a mean cost of $412 per case of lymphosarcoma and $6,406 annually per 100 cows. By comparison, mastitis costs have been estimated at around $450 per case (Heikkilä et al., 2012; Rollin et al., 2015) or $200 per milking cow annually (Cha et al., 2011).

Due to predominately B cell tropism (Paul et al., 1977a; b; Djilali et al., 1987) and subsequent clonal expansion (Gillet et al., 2013), BLV causes a lymphocytosis in about 1/3 of infected cows at any one time. Common practices in the dairy industry (e.g. re-use of needles, obstetric sleeves, or dehorning and surgical equipment) risk transfer of blood containing infected lymphocytes and are associated with increased BLV prevalence (Hopkins and DiGiacomo, 1997; Erskine et al.,

2012b). Biting flies and hematophagous insects have also been identified as potential transmission vectors (Bech-Nielsen et al., 1978; Buxton et al., 1982).

Historically, producers and veterinarians recognized that herds with high BLV tumor burdens also had increased numbers of cows with a lymphocytosis, and an age-adjusted lymphocyte count (**LC**) became the first diagnostic tool for detection of BLV (Bendixen, 1963). Control programs for BLV were quick to adopt newly developed antibody detection tests (agar-gel immunodiffusion and ELISA) that are now the standard tests approved for BLV status certification by the World Organisation for Animal Health (OIE, 2018) due to their high sensitivity and specificity (Klintevall et al., 1991; Simard et al., 2000; Monti et al., 2005). Successful BLV control programs in other countries have focused on rapid culling, or segregation until culling, of all ELISA-positive cattle (Nuotio et al., 2003; Acaite et al., 2007; EFSA AHAW Panel, 2015). However, national and within-herd prevalence of BLV was typically low when these control and eradication efforts were initiated (Batho et al., 2008). Unfortunately, the high prevalence of BLV in the U.S. makes this approach of removing all BLV-positive cows impossible for most farms without financial subsidies. Therefore, a method to reduce BLV prevalence to a sufficiently low level whereby all the ELISA-positive cattle can affordably be culled is urgently needed.

In other retroviruses, such as the closely related deltaretrovirus HTLV, the provirus is considered the infectious unit and consequently we assume the same is biologically plausible for BLV. Therefore, the infectiousness of a BLV-infected cow likely corresponds to the relative amount of integrated provirus within the host genome, known as proviral load (PVL), and PVL may be

associated with risk of transmission, similar to HTLV (Ureta-Vidal et al., 1999; Li et al., 2004). Studies in both Japan (Mekata et al., 2015) and Argentina (Juliarena et al., 2016), found that cows with a low PVL were unlikely to spread BLV to herd mates. Additionally, removal of cattle with high BLV antigen expression reduced the incidence of new BLV infections in an Australian dairy herd (Molloy et al., 1994).

We hypothesized that BLV within-herd transmission and prevalence can be reduced by identifying and removing (culling or segregation) ELISA-positive cows with the highest PVL and LC. Therefore, we conducted a three herd pilot intervention field trial in which PVL and LC were measured in ELISA-positive cattle to identify the presumably most infectious cattle for preferential removal or separation from the herd's BLV-negative cattle.

Materials and Methods

Herd Enrollment and Study Design

Herd enrollment requirements were as follows: 1) Prevalence of antibodies against BLV via ELISA testing ≥ 40%, 2) Herd managers who were willing to rapidly cull or segregate and subsequently cull cattle based upon BLV diagnostic data.

Individual cow milk testing to detect antibodies against BLV via ELISA was conducted twice yearly on the milking herd, as close as possible to each November 1 and May 1. If cows were not lactating on the test day, whole blood samples were collected instead of milk and serum or plasma was harvested to test for antibodies against BLV via ELISA (Walsh et al., 2013). Cows found to be ELISA-positive had EDTA anticoagulated blood samples collected for PVL and LC

testing within three weeks of their positive ELISA test. Quantitative results for PVL and LC and anti-BLV antibody ELISA results were compiled into a spreadsheet. The PVL was further categorized as: Very High (≥100,000 copies/10^5 cells), High (≥50,000 to <100,000), Moderate (≥16,000 to <50,000), or Low (>0 to <16,000). Similarly, LC results were categorized as: Very High (≥10,000 lymphocytes/μL), High (≥7,500 to <10,000/μL), or Normal (<7,500/μL). Combined data were sorted by PVL and LC categories. All culling and segregation decisions were entirely at the discretion of the herd managers. Herd managers were encouraged to target cows with the highest PVL and LC for culling, and to separate those that could not be immediately culled from ELISA-negative animals, given the individual herd management limitations.

"KBS" Herd. The Michigan State University (MSU) W. K. Kellogg Biological Station (KBS) Pasture Dairy Center is an automated milking rotational pasture grazing/free-stall dairy with approximately 160 cows in the milking herd (~90% Holsteins and 10% Friesians). The starting BLV ELISA prevalence was 58% at enrollment (fall 2015). Cows in this herd were assigned to one of two pastures and routed to separate robotic milking machines. There were limitations to management changes that could be made (including selection of cows to be culled) because this is a research herd.

At enrollment, the KBS Herd had a policy of single-use hypodermic needles and reproductive exam sleeves. Fly control consisted of pour-on treatment (except heifer calves that received larvicide in the feed) and the herd used a variety of dehorning methods (polling, burn, and gouge) for heifer calves. Within approximately three months of enrollment, the herd manager

and the herd veterinarian implemented washing of gouge dehorning and hoof trimming tools to reduce the chance of blood carryover between cattle. Cows with the highest PVL and/or LC were culled as soon as possible. Although complete physical separation between cows that were ELISA-negative and those with the highest PVL and LC was not possible, the herd manager prioritized moving these targeted cows to one of the two pastures/milking lines and by the end of the second year was able to move all the highest PVL and LC cows into one group. Over the course of the study, the herd manager reported relatively constant overall cull rates compared with pre-trial years.

Herd "H". Herd H is a Southwestern Wisconsin tie-stall/pasture dairy with approximately 70 milking cows. Following an outbreak of lymphoma in this purebred Holstein herd, the herd owner became highly motivated to control BLV. There was a 66% BLV prevalence at enrollment (spring 2016). Herd H enrolled approximately six months later than the other two herds, and therefore participated for a shorter period (2 years). At enrollment, this herd had a policy of single-use needles and sleeves and an active fly control program. Cows with the highest LC and PVL were culled as soon as possible. Although they were unable to separate the remaining cows based on BLV test results, they began moving cows with the highest PVL, LC, or both to the end of the milking line in mid-2017. The owner began constructing additional fencing around the same time to increase opportunities for separation of cows on pasture based on PVL and LC. Over the course of the study, the herd owner reported similar cull rates compared with previous years.

Herd "J". Herd J is a Michigan free-stall dairy with approximately 200 milking cows. The BLV prevalence was 64% at enrollment (fall 2015). This herd already had a policy of single-use needles and sleeves as well as on-premises fly control. Cows with the highest PVL and/or LC were culled as soon as possible, and the herd owner almost immediately began moving the remaining highest PVL and LC cows into a separate pen. Within six months, they had established one pen with the highest PVL cows and another pen with lower PVL cows mixed with ELISA-negative cows. By the spring of 2017, they were able to move all ELISA-positive milking cattle to a single pen, although there was ongoing commingling in dry and fresh pens. Herd J experienced higher than average cull rates in 2016 and 2017 due to an effort to reduce herd size because of lower feed yields resulting from drought.

Milk and Blood Sample Collection

Routine milk samples were collected by DHI technicians or herd managers into containers with the standard DHI preservative (bronopol/natamycin) and transported to the NorthStar Michigan Laboratory (NorthStar) located in Grand Ledge, MI, USA. Milk samples were analyzed for milk components (fat, protein, somatic cells, etc.) first, and then tested for antibodies against BLV via ELISA. Blood samples from dry cows were collected into a clot activator/polymer gel evacuated tube and a K2 EDTA-treated evacuated tube and transported to NorthStar for ELISA (using harvested serum) and the MSU BLV Laboratory for PVL testing (using DNA extracted from an aliquot of EDTA blood) and blood counts (performed on a second aliquot of EDTA blood), respectively. Blood samples from cows positive for anti-BLV antibodies via ELISA were collected into a K2 EDTA-treated evacuated tube and processed for PVL and blood counts as

above. Animal procedures for this study were reviewed and approved by the MSU Institutional Animal Care and Use Committee.

ELISA Test for Anti-BLV Antibodies

A modified ELISA test (IDEXX Laboratories, Inc., Westbrook, ME, USA) to detect antibodies directed against BLV as described by Erskine et al. (2012c) was performed at NorthStar Cooperative Inc. Briefly, aliquots of DHIA milk samples or serum/plasma (from nonlactating animals) were diluted (1:30) in sample buffer and added to 96-well BLV-coated ELISA plates. After washing, BLV antibodies were detected by reaction with horseradish-peroxidase-labeled antibodies to bovine immunoglobulin with addition of an enzyme substrate. Color development of positive controls was used to standardize reaction times, and the reaction was stopped by addition of 0.5 N H_2SO_4. Results were reported as corrected 450nm optical density (OD) measurements (raw sample OD - negative control OD). Milk samples with a corrected OD > 0.1 and serum or plasma samples with a corrected OD > 0.5 were considered positive for anti-BLV antibodies.

Lymphocyte Count Determination

Anticoagulated (EDTA) blood samples were collected to measure total and differential leukocyte counts using an automated blood leukocyte differential test (QScout BLD, Advanced Animal Diagnostics, Morrisville, NC, USA) either on-farm or at the MSU BLV Laboratory. Briefly, 80 µL of blood was added to 20 µL of K-stain (Advanced Animal Diagnostics, Morrisville, NC, USA) and mixed well. Ten µL of the mixture was pipetted onto a provided slide and

immediately placed in the analyzer. The machine measured total white blood cell concentration and percent lymphocytes and calculated the LCs.

Proviral Load Test

The proviral load was measured using the CoCoMo BLV quantitative polymerase chain reaction (qPCR) method (Jimba et al., 2010). In brief, genomic DNA was extracted from EDTA anticoagulated whole blood samples using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). Eluted DNA was normalized to 30 ng/uL, and 150 ng of genomic DNA was added to each qPCR using the BLV-CoCoMo primer set, FAM BLV probe (RIKEN genesis, Tokyo, Japan), and TaqMan Gene Expression Master Mix (Life Technologies, Carson, CA, USA).

Data Analysis

The two principal study endpoints based on ELISA results were 1) prevalence at each semiannual test and 2) incidence risk of new infections calculated for the previous 6 months using semiannual test results.

Within-herd BLV prevalence was calculated at each semiannual test as the number of cows with a positive ELISA result divided by the number of cows sampled at that semi-annual time period. Changes in within-herd BLV prevalence over time was evaluated using the Chi square test for trend by individual herd and across all three herds using OpenEpi 3.01 (Dean et al., 2013).

Incidence risk (proportion) was calculated for each inter-test period of approximately six months. For each inter-test period, the population was considered closed. Only ELISA-negative cows at the beginning of a period were considered at risk. A new infection was defined as an at-risk cow that converted to a positive ELISA result. ELISA-positive cows were considered to be always positive and were therefore permanently removed from the at-risk population for new infections. The at-risk population for each inter-test period was estimated by calculating a corrected at-risk population as follows: the number of BLV ELISA-negative cows at the semiannual test at the start of the period, minus one half the number of these cows absent at the semiannual test at the end of the period (withdrawals, *C*). As an equation:

Corrected at Risk Population = Starting at Risk Population – (0.5 x C)

Rarely, cows were inadvertently not sampled at a semiannual test point (despite remaining in the herd) and subsequently identified as BLV-positive on the following semiannual test (e.g. Cow X tested negative at test 1, was not tested at test 2, and tested positive at test 3). Due to the missing test data, the at-risk period for that cow spans the missed semiannual test period, and the specific period when the infection occurred cannot be identified. To avoid bias, each 'missed' newly BLV ELISA-positive cow (*D*) was considered to have contributed one new infection over the total inter-test period (e.g. 0.5 to the first semiannual period, 0.5 to the second) and was included in the at-risk population of each semiannual test period associated with the missed test. As equations:

Corrected Number of New Infections = Number of New Infections + (0.5 x *D*)

Corrected at Risk Population = Starting at Risk Population – (0.5 x *C*) + *D*

The incidence risk for new infections over time was evaluated using the Chi square test for trend by individual herd and across all three herds using OpenEpi 3.01 (Dean et al., 2013).

Each semiannual test approximated summer (May through October) or winter (November through April) months at risk in the Midwestern U.S. The incidence risk was calculated for each season using the corrected at-risk population and new infection calculation definitions described above. The incidence risks of new infections in summer and winter exposures were compared using the Fisher mid-P exact test (Dean et al., 2013).

Results

Semiannual BLV Prevalence

All three herds experienced statistically significant decreases in BLV prevalence over the course of this study (Figure 1). Within-herd point prevalence decreased in Herd J from 63.8% to 13.9% ($P < 0.001$) after 2.5 years on-study. The KBS Herd prevalence decreased from 58.2% to 27.4% ($P < 0.001$), after 2.5 years on-study. Prevalence in Herd H was 65.8% at enrollment, increased to 73.1% at the second semiannual test, then declined at each remaining semiannual test for an overall decline in prevalence to 45.3% ($P < 0.001$) during the 2 years on-study. An overall decreased ($P < 0.001$; Table 1) BLV prevalence over the duration of the pilot intervention was observed for combined data from the three herds.

Semiannual Incidence Risk of New Infections

In addition to monitoring herd BLV infection prevalence, the ELISA test was used to calculate the incidence risk of new infections during each inter-test period. All three herds together experienced an overall decrease ($P < 0.001$; Table 2) in new infections (Figure 2). The incidence risk for Herd J decreased from 17.8% in the first 6 months of intervention to 0.9% in the final reported 6 months ($P < 0.001$). The incidence risk for the KBS Herd during the first 6 months was 9.6%, remained stable (9.5%) over the following inter-test period, and then increased (13.8%) before dropping sharply to about 4% (4.2 and 4.0%) for the final two periods ($P = 0.069$). The incidence risk for Herd H was 13.6% in the first 6 months, dropped to 0 in the subsequent inter-test period, then remained under 4% (3.9 and 3.4%) for the final two periods ($P = 0.116$).

Combined new infection risk for all three herds was not statistically different between seasons, but tended to be higher during winter periods of exposure ($P = 0.349$; Table 3). New infection risk was also not statistically different between seasons in individual herds. Herd H experienced more new infections during the summer months (8.3% vs 2.2%), while both Herd J (2.3% vs 5.3%) and the KBS Herd (6.7% vs 8.5%) experienced more new infections during winter months.

Distribution of PVL and LC by Category

A descriptive crosstabulation presenting the distribution of categorical PVL and LC values (Table 4) shows a trend toward higher PVL in cows with higher LC. As shown in Figure 3, 74.4% of BLV antibody positive cows with LC in the “Normal” category also had PVL categorized as

"Low". Meanwhile, 90.9% of cows with "Very High" LC had either "High" or "Very High" PVL.

Discussion

This pilot intervention field trial appears to be the first time selective culling and segregation of the likely most infectious cattle, along with other management practices, has been used to reduce BLV transmission since 1994 in Australia (Molloy et al., 1994). Despite differences in herd size and management, all three herds experienced decreased within-herd BLV prevalence over the course of our study. Herd J had a free-stall barn where cows could be assigned to specific pens and this herd experienced the greatest decrease in prevalence, from 63.8% to 13.9%, over 2.5 years. Increased culling in this herd during the intervention was a likely contributor to the markedly decreased BLV prevalence. In the event a producer is downsizing, prioritizing removal of high PVL and LC cows may allow for rapid reduction of BLV which, in turn, may aid in improving milk production efficiency in the remaining herd.

In contrast, the KBS Herd had cows grouped onto one of two pastures in the summer and one of two sides of a free-stall barn in the winter, each side associated with a separate robotic milking unit. This allowed contact both among cows within each group and also between cows in the two different groups, particularly in the milking barn. The herd manager worked to move the highest PVL and LC cows to one of the two groups, which may have helped reduce overall BLV transmission by reducing close contact of more infectious cows with their BLV-negative herd-mates (about half the herd). During 2.5-years of participation in the intervention field trial, the BLV prevalence in the KBS Herd decreased by more than half, from 58.2% to 27.4%, without an

increased culling rate. We estimated the annual costs of BLV infection for this herd at $283 per milking cow using a partial budget tool (Bartlett et al., 2017, 2018).

Herd H was a pasture and tie-stall herd, making physical segregation difficult. In a study of six tie-stall dairies in Japan, BLV-negative cows were at more than 12 times greater risk of becoming infected if an adjacent cow was BLV-positive (Kobayashi et al., 2015). Therefore, the herd manager began moving the highest PVL and LC cows to the end of the milking line to enhance the degree of separation. Over a shorter period (2 years), Herd H experienced a decrease in BLV prevalence from 65.8% to 45.3%, a relative decline of nearly one third.

Overall, the enrolled herds had varying abilities to cull and segregate cows based on PVL and LC. We communicated regularly with herd managers to review semiannual results and asked them to develop a plan to, at minimum, cull or separate the highest priority cows. We also checked in with herd managers between test dates to update these plans and address any questions about BLV control that may have arisen. Culling decisions rely on many factors; consequently, herd managers may not always select the highest PVL and LC cows for culling. All decisions were ultimately made by herd managers, and the intensity of culling and segregation in relation to PVL and/or LC was entirely up to the herd manager in each herd and was dependent on the individual herd management practices and abilities, including physical limitations and economic considerations. The intensity of culling directly impacts prevalence and could indirectly impact incidence. Of the three herds, Herd J most intensively culled and segregated cows with the highest PVL and LC, and thereby experienced the most rapid and significant reduction in BLV prevalence and incidence.

The risk of new infections decreased in all three enrolled herds over the course of this study. Because these herds were not evaluated prior to initiating the intervention field trial, the first new infection risk period was also the first six months on-study for each herd. Therefore, the baseline (pre-trial) new infection risk was likely underestimated for each of these herds because we do not have pre-intervention data. Both the J and H Herds experienced large decreases in new infections in the second inter-test period. The new infection risk in the KBS Herd remained at about 10% for the first two inter-test periods; therefore, management changes made in this herd (washing gouge dehorning and hoof trimming equipment) did not appear to decrease BLV transmission. These management changes were applied within the first three months of the trial and had no effect on reducing new infection risk during subsequent inter-test periods. In fact, a small (from 6 to 8.5) increase in new infections combined with additional withdrawals during the third inter-test period increased the new infection risk to about 14%. This increase occurred during the winter exposure months and prior to the herd manager moving cows with the highest PVL and LC to one robotic milking unit and pasture. However, following this segregation, the new infection risk in the KBS Herd decreased sharply to just over 4% in the next inter-test period. Management practices were surveyed in all three herds at enrollment and changes were recorded during the course of the study. However, if management practices had been changed immediately prior to study enrollment, such changes could potentially influence the apparent effect of the intervention program.

The trend overall for our three field trial herds was toward more new infections over the winter inter-test period, but this was not statistically significant. Although also not statistically

significant, Herd H was the only herd to have a numerically higher rate of new infections in summer rather than winter, despite employing a fly control program. Though ELISA test results generally become positive within 2 to 3 weeks following BLV infection, this may take up to 3 months due to individual variability (Evermann et al., 1986; Klintevall et al., 1994; Mammerickx et al., 2010). Therefore, there is a possibility of animals that had not developed sufficient antibody concentrations by the test date being misclassified using ELISA. However, the time of our testing was selected intentionally to minimize this risk, given that the typical biting fly season in the Midwest is late May to late August. Thus, our finding suggests that biting flies present only during the summer may not cause much BLV transmission in at least some herds in this part of the Midwestern U.S.

Although antibody testing is a widely accepted diagnostic tool for BLV infection, there remains a chance for false positive and false negative results. In a herd-level control program, the prevalence of BLV is important to consider because of its effect on the predictive value of the test. While prevalence is high, the negative predictive value of the test is decreased, which increases the chance of keeping a cow that is BLV-antibody positive but ELISA-negative in the herd, or not further screening her for PVL and LC. However, only approximately 1/3 of BLV positive cows have high PVL and LC. Therefore, a misclassified ELISA-negative cow is unlikely to be among those in the herd with high PVL and LC. As herd prevalence decreases, the likelihood of false-negative results also decreases but the chance of false-positive results increases. This does not jeopardize the effectiveness of a control program, but may lead to increased costs (PVL and LC testing; culling of a BLV-free cow).

This pilot intervention trial focused on milking cows and did not directly address BLV infection in young stock. However, all three herd managers quickly recognized that incoming heifers needed to be tested before introduction into the milking herd. Although outside of the scope of the pilot intervention, we accommodated a request from Herd H management to ELISA test blood from pregnant heifers in September 2016, April 2017, and April 2018. We also began ELISA testing blood from pregnant heifers and/or ELISA milk testing at each animal's first DHI test in Herd J and the KBS Herd after the last sampling of the pilot intervention. The prevalence of BLV in incoming heifers may have a significant and variable effect on BLV control from herd to herd. In fact, Herd H had a BLV-ELISA positive prevalence of ~30% in heifers entering the milking herd at the second testing period, causing a subsequent overall increase in BLV prevalence at their next semiannual test. Future BLV control programs could utilize PVL or LC concentrations in young stock to aid in determining which animals to breed or cull.

Herd managers of all three enrolled herds received both PVL and LC testing data on ELISA-positive cows because our intervention was focused on removing cows with the highest PVL and LC counts. Ohno et al. (2015) reported a correlation of r=0.855 between PVL and LC in 749 Japanese dairy cattle. No statistical correlation was conducted on the PVL and LC data collected for this study because each unique cow included in our data set (ELISA positive, PVL positive, LC result reported; n=343) contributed at an average of 2.2 time points (n=764). However, the distribution of PVL and LC in the three herds enrolled in this study appears similar to that reported by Ohno, which indicates that future studies on BLV control programs might use either of these measures to identify the presumably most highly infectious cows for removal. Early BLV control programs used LC as a diagnostic tool to inform culling decisions, but in at least

one Danish dairy herd, a control program of culling on the basis of semi-annual LC testing was unable to eradicate BLV after 10 years (Flensburg, 1976). Other programs for BLV control may consider using LC as a screening tool, with subsequent PVL testing to assist with identification of animals for targeted removal.

Although nearly 1,000 PVL and LC tests were performed over ~3 years, no control herds were enrolled and results of this pilot field trial for BLV control should be repeated by evaluating additional herds over multiple geographic locations with a variety of herd sizes and management styles before proposing optimal recommendations that will be effective across these differences. The largest herd in this pilot intervention program had approximately 200 milking cows; a potential limitation is therefore scalability of this approach in larger herds.

Although we cannot separate the effects of the culling, segregation, and management changes in this intervention, there was a similar overall response in all 3 herds where the consistent component was culling the highest PVL and LC cows. Therefore, we believe that targeting the highest PVL and LC cows for culling and segregation could become a viable way for producers to reduce herd BLV prevalence to a level where traditional ELISA-based test and cull approaches can be employed to achieve eradication. This pilot field intervention trial is being continued and expanded with the goal of reaching a low level of BLV prevalence that would enable herd managers to economically cull all remaining ELISA-positive cows and achieve a BLV-negative herd status.

Future work is needed to estimate the total impact and implementation cost of this type of control program, particularly since no commercial BLV qPCR test for PVL is currently available in the U.S. However, our unique approach of using routine DHIA milk samples as a herd-wide BLV screening strategy is more convenient and inexpensive than the herd-wide serum testing used in other control programs, and the most substantial cost to the herds in this study was culling of cows due to high PVL and LC.

Conclusion

Dairy producers enrolled in this pilot BLV control program were able to significantly decrease BLV herd prevalence and new infection risk after 24 to 30 months of participation.

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APPENDIX

Table 4.1. Within-herd point prevalence of bovine leukemia virus (BLV) by semiannual test.

	Herd J			KBS Herd		
Herd Test	BLV +	BLV -	Prevalence	BLV +	BLV -	Prevalence
0[1]	127	72	63.8%	96	69	58.2%
1	110	88	55.6%	81	72	52.9%
2	76	99	43.4%	71	74	49.0%
3	49	112	30.4%	69	82	45.7%
4	34	136	20.0%	61	102	37.4%
5	23	142	13.9%	46	122	27.4%

	Herd H			All Herds Combined		
Herd Test	BLV +	BLV -	Prevalence	BLV +	BLV -	Prevalence
0[1]	50	26	65.8%	273	167	62.0%
1	49	18	73.1%	240	178	57.4%
2	47	27	63.5%	194	200	49.2%
3	31	34	47.7%	149	228	39.5%
4	34	41	45.3%	129	279	31.6%
5	-	-	-	69	264	20.7%

BLV + (ELISA antibody); BLV - (ELISA antibody)

[1]Baseline test at enrollment.

Extended Mantel-Haenszel chi square for linear trend: Herd J, $P < 0.001$; KBS Herd $P < 0.001$; Herd H, $P < 0.001$; all herds, $P < 0.001$.

Table 4.2. Incidence risk for new bovine leukemia virus (BLV) infections by semiannual test period for each herd.

Herd Test	Herd J			KBS Herd		
	New Infections	At Risk	Incidence Risk	New Infections	At Risk	Incidence Risk
1[1]	12	67.5	17.8	6	62.5	9.6
2	2	78.5	2.5	6	63	9.5
3	1	83.5	1.2	8.5	61.5	13.8
4	2	97	2.1	3	71	4.2
5	1	114	0.9	3.5	88.5	4.0

Herd Test	Herd H			All Herds Combined		
	New Infections	At Risk	Incidence Risk	New Infections	At Risk	Incidence Risk
1[1]	3	22	13.6	21	152	13.8
2	0	16.5	0.0	8	158	5.1
3	1	26	3.8	10.5	171	6.1
4	1	29.5	3.4	6	197.5	3.0
5	-	-	-	4.5	202.5	2.2

[1]First semi-annual test after enrollment. Extended Mantel-Haenszel chi square for linear trend p-values: Herd J, $P < 0.001$; KBS Herd, $P = 0.069$; Herd H, $P = 0.116$; all herds, $P < 0.001$.

Table 4.3. Comparison of seasonal incidence of new bovine leukemia virus (BLV) infections.

	Herd J			KBS Herd		
Exposure Period	New Infections	Cows at Risk	Incidence Risk	New Infections	Cows at Risk	Incidence Risk
Summer	4	175.5	2.3	9	134	6.7
Winter	14	265	5.3	18	212.5	8.5

	Herd H			All Herds Combined		
Exposure Period	New Infections	Cows at Risk	Incidence Risk	New Infections	Cows at Risk	Incidence Risk
Summer	4	48	8.3	17	357.5	4.8
Winter	1	46	2.2	33	523.5	6.3

Summer season of exposure: inter-test period from approximately May to November; Winter season of exposure: inter-test period from approximately November to May. Mid-P exact 2-tail p-value: Herd J, $P = 0.130$; KBS Herd, $P = 0.584$; Herd H, $P = 0.236$; all herds, $P = 0.349$.

Table 4.4. Summary distribution of categorical proviral load (PVL) and lymphocyte count (LC) results in bovine leukemia virus (BLV) antibody positive cows.

	Herd J			KBS Herd		
PVL Category	Normal LC	High LC	Very High LC	Normal LC	High LC	Very High LC
Low	149	26	5	110	21	5
Moderate	28	21	7	12	11	0
High	18	27	45	7	21	46
Very High	0	2	15	1	1	14

	Herd H			All Herds Combined		
PVL Category	Normal LC	High LC	Very High LC	Normal LC	High LC	Very High LC
Low	38	2	0	297	49	10
Moderate	21	10	0	61	42	7
High	15	32	30	40	80	121
Very High	0	5	19	1	8	48

Data was restricted to samples that were ELISA positive, PVL positive, and had a LC result reported; N=764 samples. Some cows contributed repeated measures (N=343 unique cows).

Figure 4.1. Within-herd bovine leukemia virus (BLV) antibody prevalence by ELISA over time.

Prevalence was calculated as the number of BLV-ELISA positive cows divided by the total number of cows tested at each semiannual test point.

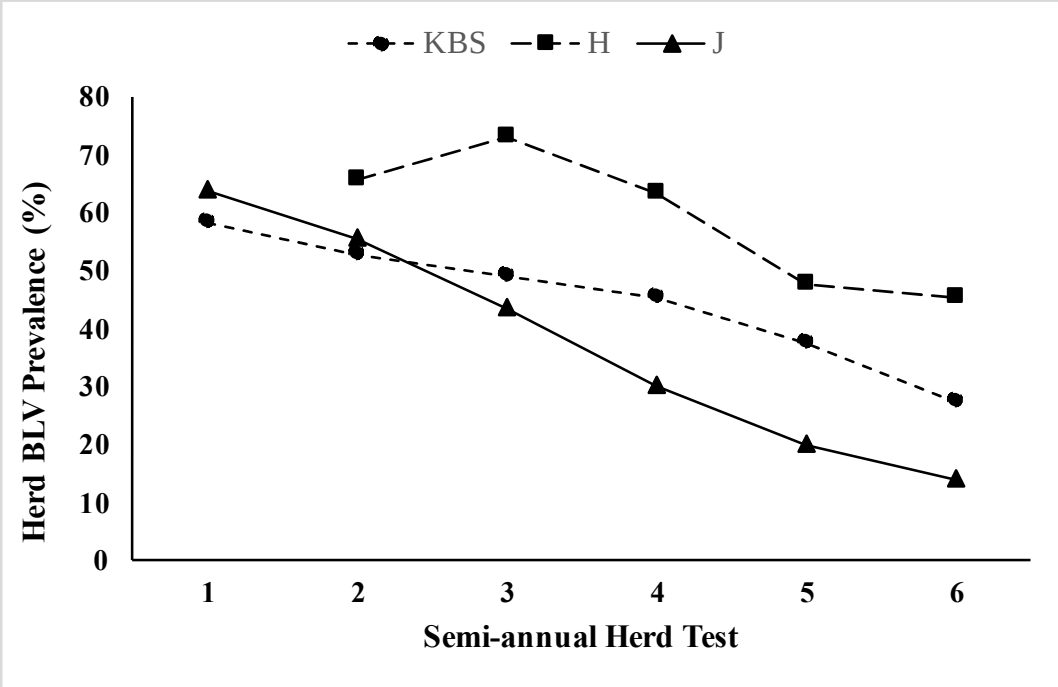


Figure 4.2. Incidence risk for new bovine leukemia virus (BLV) infections over time.

Incidence risk was calculated for each consecutive semi-annual inter-test study period beginning with the baseline enrollment milk test and the first semi-annual herd test

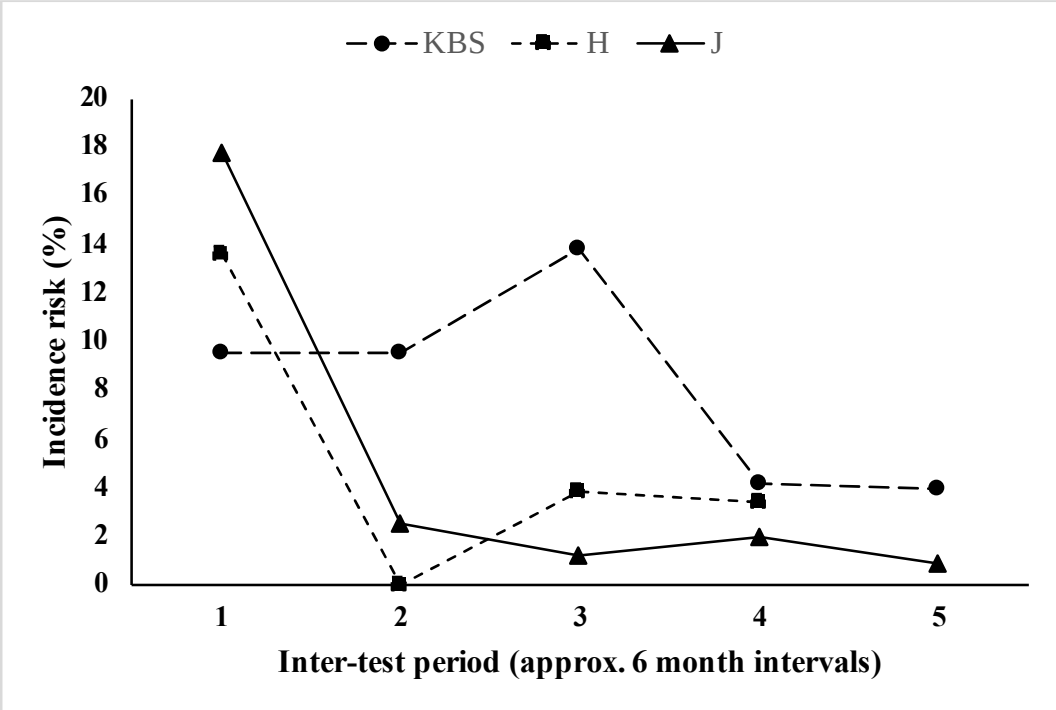
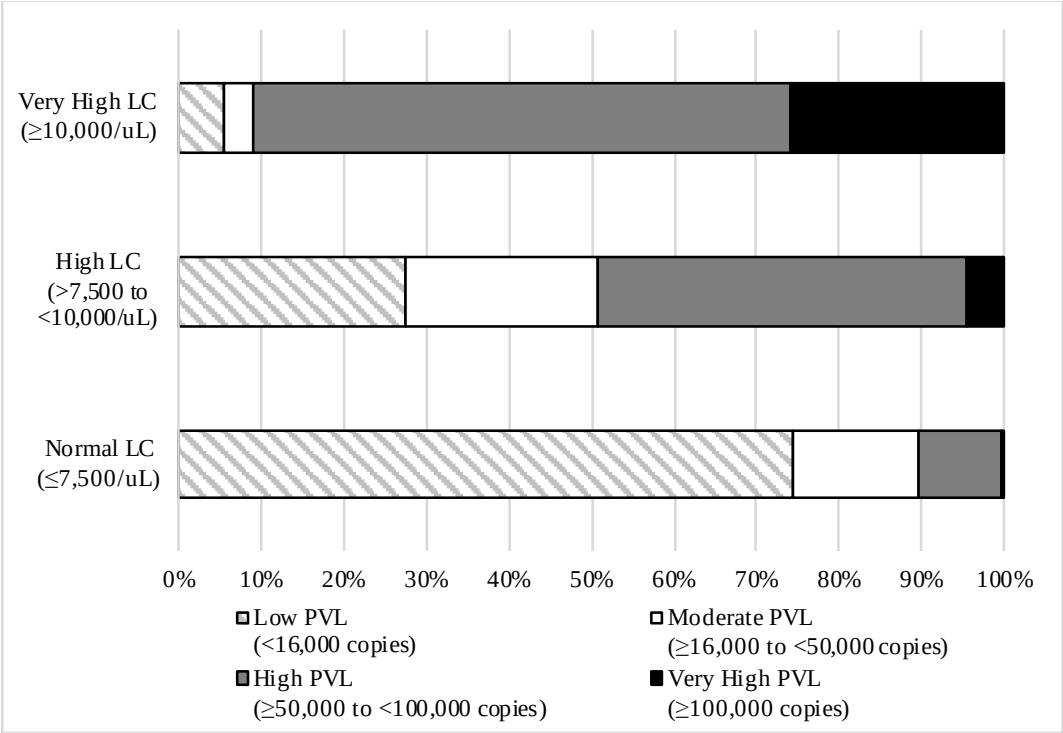


Figure 4.3. Row percentages of the distribution of proviral load (PVL) and lymphocyte count (LC) categories for all herds.

Data was restricted to samples that were ELISA positive, PVL positive, and had a LC result reported; N=764 samples. Some cows contributed repeated measures (N=343 unique cows).



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CHAPTER 5: Control of bovine leukemia virus in three U.S. dairy herds by culling ELISA-positive cows

This chapter represents a manuscript in submission for publication.

Authors who contributed to this study were: Vickie J. Ruggiero, and Paul C. Bartlett.

Abstract

The objective of this trial was to evaluate a test-and-cull approach to controlling bovine leukemia virus (BLV) in U.S. dairy herds with a low BLV prevalence. Despite worldwide distribution of the virus, 21 nations have eradicated BLV from their dairy cattle and are currently considered 'BLV-free'. In contrast, the U.S. has attempted no industry-wide BLV control programs, and has experienced an increase in BLV prevalence among dairy cows to about 40%. This raises concerns about production efficiency, herd health, and sustainability. In a pilot field trial with three Midwestern-U.S. dairy herds, a test-and-cull approach using ELISA screening of milk samples was successful in reducing BLV prevalence in two herds. In the third herd, BLV prevalence increased following the introduction of infected heifers that were raised at an out-of-state calf raising facility. This trial demonstrated that a test-and-cull approach to BLV control can be successful in U.S. dairy herds with low BLV prevalence, but ongoing surveillance is necessary to prevent re-introduction of the virus.

Introduction

Currently, 21 nations have eradicated BLV from their dairy herds and others have implemented eradication programs (Anonymous, 2010; Animal Health Committee, 2013; More et al., 2017). Eradication has been achieved by testing blood or milk for BLV antibodies, followed by culling of the animals that test positive. Occasionally, BLV-antibody positive animals were temporarily segregated from the rest of the herd until they could eventually be culled. These programs were primarily implemented in countries with low BLV prevalence (Batho et al., 2008). The objective of our study was to evaluate the efficacy of this approach in U.S. dairy herds with a similarly low initial BLV prevalence.

The prevalence of BLV in U.S. dairy cattle has been increasing over the past decades, with the most current report estimating that 94% of herds and 42% of dairy cows are positive for BLV antibodies (LaDronka et al., 2018) using a lactation-stratified 40-cow sample termed a "BLV Herd Profile" (Erskine et al., 2012). Although about 6% of the herds in this study were BLV free, this is lower than USDA-NAHMS reports that found ~16%, and a 2010 Michigan study that found 13% of herds were free of BLV (USDA, 1997, 2008; Bartlett et al., 2014). This observation supports the theory that herds which eliminate BLV should be able to keep their herds from being re-infected if they are careful to not introduce infected animals.

Historically, the major economic impetus for BLV control has been the prevention of lymphoma (lymphosarcoma), as other impacts of BLV have only recently been recognized. Lymphoma affects an estimated 5% of infected cattle (Burny et al., 1988; Rhodes et al., 2003), and in the U.S., lymphoma is the most common reason for post-mortem carcass condemnation at slaughter

(White and Moore, 2009). However, approximately 30% of ELISA-positive cattle have a lymphocytic leukosis (lymphocytosis) which is accompanied by immune suppression (Callahan et al., 1976; Esteban et al., 1985; Frie et al., 2016). This immune dysfunction may be the cause for the epidemiologic observations that BLV-antibody positive cows have decreased milk production (Nekouei et al., 2016; Yang et al., 2016; LaDronka et al., 2018) and a shortened lifespan (Bartlett et al., 2013; Nekouei et al., 2016). A 2003 economic analysis found the annual cost of BLV to the dairy industry to be $525 million lost annually (Ott et al., 2003) and a more recent, informal estimate by our research group showed that the cost to the W.K. Kellogg Biologic Station Pasture Dairy Center was $379.92 per infected cow yearly (Bartlett et al., 2017, 2018). In comparison, mastitis costs to the dairy industry have been estimated at $450 per case (Heikkilä et al., 2012; Rollin et al., 2015) or $200 per milking cow annually (Cha et al., 2011). Another impact of BLV is that the export of U.S. animals and animal products has become more difficult, with some countries such as those within the EU requiring that animals come from BLV-free herds and be tested for BLV prior to introduction (EFSA AHAW Panel, 2015). Epidemiologic studies have shown an association between BLV infection and infectious conditions such as mastitis, respiratory diseases, and gastrointestinal diseases, as well as delayed recovery from some infections (Emanuelson et al., 1992), making animal welfare issues a concern both due to the risk of lymphoma and from immune dysfunction. Public health issues related to BLV are still being investigated, and public perception of the dairy industry could be impacted by these reports (Miller and Van der Maaten, 1982; Buehring et al., 2014). Taken together, there is a strong case for controlling BLV in the U.S. Therefore, we designed a pilot BLV-control program to determine if dairy herds under U.S. management conditions with a <5%

prevalence of BLV antibodies could achieve eradication by periodic BLV ELISA testing of milk samples followed by selectively culling or segregating antibody-positive cows.

Materials and Methods

Herd Enrollment and Study Design

Herd enrollment requirements were as follows: 1) BLV-antibody prevalence by ELISA ≤5%, 2) Herd managers who were willing to cull ELISA-positive cattle or at least temporarily segregate them for eventual culling. All lactating cows in the milking herd were tested by milk ELISA at least yearly and sometimes dry cows and young stock were tested by serum ELISA prior to entering the milking herd. The timing of BLV testing was customized to each herd's management practices, resources, and level of engagement, with the goal of minimizing the amount of time an identified antibody-positive cow might be in contact with BLV-susceptible herd mates. Results of ELISA testing were immediately given to the herd managers, whom we encouraged to cull the cows that were positive for BLV antibodies, or separate them from BLV-negative animals if they could not be immediately culled.

Herd "M": Herd M was a free-stall dairy with 150 milking cows at enrollment in August 2015. Individual-cow milk BLV ELISA testing was carried out approximately every six months. Additional tests were conducted on milk samples from cows that were dry at the semi-annual testing day, or on cows that we expected to be dry at the upcoming semi-annual test day, and from freshened (1st lactation) heifers that had entered the milking herd between semi-annual tests, as described in Table 1.

Herd "R": Herd R was a free-stall dairy with 850 milking cows at enrollment in July 2015. Individual-cow milk BLV ELISA testing was carried out yearly. Additional tests were conducted on milk samples from freshened (1st lactation) heifers and cows that had been dry at the previous annual test, as described in Table 2.

Herd "S": Herd S was a free-stall dairy (except for transition heifers that were on bedded pack) with 350 milking cows at enrollment in December 2014. Individual-cow milk BLV ELISA testing was carried out yearly. Additional tests were conducted on milk samples from cows that were dry at the annual test day, or that were going to be dry at the upcoming annual test day, and from freshened (1st lactation) heifers that had entered the milking herd between semi-annual tests. The herd managers were highly motivated to control BLV and conducted additional interim serologic testing of dry cows and heifers, and testing of bulk tank milk samples as described in Table 3.

Blood and Milk Sample Collection

Routine milk samples were collected by DHI technicians into containers with bronopol/natamycin preservative and transported to a NorthStar Cooperative, Inc. Laboratory (Grand Ledge, MI, USA or Kaukauna, WI, USA). Milk samples were analyzed for milk components (fat, protein, somatic cells, etc.) first, and then evaluated for BLV antibodies via ELISA. If the cow to be tested was not lactating, blood samples were collected into clot activator/polymer gel evacuated tubes and transported to the NorthStar Cooperative Michigan Laboratory for testing. The animal procedures for this study were reviewed and approved by the MSU Institutional Animal Care and Use Committee.

ELISA Test for BLV Antibodies

A modified ELISA (IDEXX Laboratories, Inc., Westbrook, ME, USA) for BLV antibodies (Erskine et al., 2012) was performed by a NorthStar Cooperative commercial diagnostic laboratory. Aliquots of milk samples were diluted 1:30 (individual animal) or 1:2 (pooled/bulk tank). Alternatively, diluted (1:30) serum from nonlactating cows was used. Briefly, samples were added to 96-well BLV-coated ELISA plates and washed. BLV antibodies were detected by reaction with horseradish-peroxidase-labeled antibodies to bovine immunoglobulin with addition of an enzyme substrate. Reaction times were standardized by color development of positive controls, and the reaction was stopped by addition of 0.5 N H_2SO_4. Results were reported as corrected 450nm optical density (**OD**) measurements (raw sample OD - negative control OD). Milk samples with a corrected OD > 0.1 and serum samples with a corrected OD > 0.5 were considered positive for anti-BLV antibodies.

Results

Herd M: Herd M had a starting BLV prevalence of 2.2% and culled all known BLV-antibody positive ("positive") cows after their first test of all lactating cows in the herd in August 2015 (Table 1). The testing protocol for this herd was approximately semi-annual milk testing of all lactating cows, with additional tests of cows that were newly lactating since the last semi-annual test, i.e. cows that had been dry at the semi-annual test and 1st lactation heifers that entered the milking herd after calving.

The first additional test in November 2015 identified one positive cow. This animal was still in the herd at the semi-annual test of all milking cows in February 2016, and was the only positive animal identified. It was subsequently culled in March 2016. For the remainder of the intervention, the only positive animals identified were 1st lactation heifers, one at each semi-annual test, that had recently entered the milking herd.

Herd R: Herd R had a starting BLV prevalence of 3.2% in July 2015 (Table 2). The herd manager culled some positive animals from the herd and moved any remaining positive animals to a single pen to segregate them from the negative cattle. The testing protocol for this herd was similar to Herd M, semi-annual milk tests of all lactating cows, with additional tests of cows that were newly milking since semi-annual tests.

The first interim test in September 2015 identified five positives, all of which were 1st lactation heifers. At the next test of all lactating cows in February 2016, BLV prevalence had increased to 3.67% with 31 positive cows. Of these, fourteen were 1st lactation heifers, twelve were positive at a prior test, and five previously BLV-antibody negative cows were now positive. An interim test of 1st lactation heifers in May 2016 identified a 10% prevalence in this age group and the herd manager indicated that they were no longer able to move all positive animals to the segregated pen or cull them. The BLV prevalence at the semi-annual test of all lactating cows in August 2016 increased to >5%. At this point, the herd was no longer within the inclusion criteria for the study and the August 2016 test concluded their participation.

Herd S: Herd S had a starting prevalence of 2.3% (Table 3) in December 2014 and culled all positive animals shortly after testing. The herd manager wanted to be proactive, so nearly 70 cows and heifers that were due to enter the milking herd after calving in the next six months were tested by serum ELISA in February 2015. Two additional positives were identified and subsequently sold. The testing protocol occurred approximately on an annual basis for all lactating cows, with quarterly interim tests for cows that initiated lactation between the semi-annual tests. Bulk milk tank samples were tested for BLV antibodies at unstructured intervals as an exploratory surveillance method.

Interim tests in March and July (including a July bulk tank sample) were negative for BLV antibodies, while two positive animals were identified in October 2015 and were subsequently culled. Only one animal was positive for BLV antibodies at the January 2016 milk test of all lactating cows, and one was positive at the April 2016 interim test, both of which were culled. A bulk tank sample in April 2016 was also negative. In July 2016, two positive animals were identified at the interim milk test, and the bulk tank samples was positive. Both positives were culled. We conducted serum testing of heifers due to calve in the upcoming six month period in September 2016, with no positives identified. At the October 2016 interim milk test, there were three positive animals, which were then culled, and the bulk tank sample remained positive as well.

Subsequently, we began monthly serum tests of pregnant heifers with a due date within six months. In November, December, and January, two positives were identified – one in December and one in January – and sold. At the January 2017 milk test of all lactating cows, five cows

were positive, as was the bulk tank sample. Notably, all five had been antibody-negative on serum tests as heifers. The January 2017 milk test was the final test of all lactating cows for this study, but we conducted the final three monthly serum tests on heifers in February, March, and April 2017, as had been agreed upon with the herd manager. Only one positive animal was identified on these tests (in February 2017) and she was sold. The herd prevalence in Herd S dropped from 2.3% to 0.3% after the first year of this program. Although the prevalence increased to 1.5% in the second year due to the first lactation heifers, it was lower compared to the starting prevalence.

Discussion

The test-and-removal approach to BLV eradication has been successful in many other nations, most of which started their control programs with low BLV prevalence (Batho et al., 2008; EFSA AHAW Panel, 2015). These programs used serum testing almost exclusively, which is inconvenient for dairy producers. Our results demonstrate that this approach also works for milking herds in the U.S. which have similarly low BLV prevalence, primarily using milk samples already being collected for milk component testing. However, a limitation of milk testing is that the inflow of infected heifers quickly re-introduces BLV into the milking herd. U.S. herds which can reduce their prevalence to less than 5% may consider eradicating BLV from their herds by removing all ELSA-positive cows, but need to ensure that incoming heifers do not re-introduce the infection as was seen the current study.

In just 19 months, Herd M appeared to nearly eradicate BLV. However, ongoing monitoring will be necessary before complete eradication can be claimed. Similarly, Herd S appeared close to having eradicated BLV from the milking herd, but incoming infected young stock prevented

complete elimination during the 29 months of their participation. Herd R discovered a relatively high BLV prevalence from heifers raised off-premises. This led to a progressive increase in herd BLV prevalence when the herd manager could not cull or segregate this large number of incoming BLV-positive animals.

The results of Herd S demonstrated an additional concern for BLV eradication: that of possible latent infections, in which a BLV infection – perhaps from calf-hood – remains sequestered and dormant until later in life. This is consistent with mechanisms seen in other retroviruses that avoid immune detection (Bolinger and Boris-Lawrie, 2009) and the potential for latent BLV infections has been speculated about since early PCR tests were sometimes unable to detect BLV provirus in antibody-positive animals (e.g. Murtaugh et al., 1991). Klintevall et al. (1994) reported a calf which, after experimental infection with BLV, appeared to have maintained the virus in a latent state, sequestering provirus in the spleen and having no detectable circulating antibodies. The five first lactation heifers in Herd S that were BLV-antibody positive in January 2017 had previously tested negative for BLV-antibodies and no other animals in the herd were known to be BLV-positive. This suggests that these heifers had latent infections that became active after entering the milking herd. Therefore, it is clear that monitoring heifers for BLV is important both before and shortly after they enter the milking herd is an important consideration for BLV control programs.

The test and cull of all ELISA-positive cows method to control BLV in the three study herds was only economically feasible because they started the program with a low BLV prevalence. U.S.

dairy herds with BLV prevalence closer to the national average (>40%) will probably need to reduce their prevalence to a point where test and removal is economically feasible.

Conclusions

Depending on the herd, test and cull by using milk ELISA as means to reduce or eliminate BLV from dairy herds with low BLV prevalence may be effective. However, average BLV prevalence in U.S. dairy herds is over 40%. Therefore, future BLV research is needed to determine cost-effective methods of reducing BLV prevalence to a point where test and cull may be cost-effective.

Abbreviations

BLV: Bovine leukemia virus

ELISA: Enzyme-linked immunosorbent assay

OD: Optical density

PVL: Proviral load

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APPENDIX

Table 5.1. Bovine leukemia virus (BLV) antibody testing schedule and results for Herd M.

Test Date	Test Type	Description	Results[1]
August 2015	Herd (milk)	All cows in the milking herd	3/138 (2.2%)
November 2015	Additional (milk)	Cows dry at August test and fresh heifers[2]	1/42
February 2016	Herd (milk)	All cows in the milking herd[3]	1/153 (0.6%)
March 2016	Additional (milk)	Cows dry at February test and fresh heifers	0/13
August 2016	Additional (milk)	Cows projected to be dry at late August test	0/16
August 2016	Herd (milk)	All cows in the milking herd[4]	1/143 (0.7%)
October 2016	Additional (milk)	Cows dry at August test and fresh heifers	0/18
February 2017	Herd (milk)	All cows in the milking herd[4]	1/150 (0.7%)
March 2017	Additional (milk)	Cows dry at February test and fresh heifers	0/16

[1] BLV-antibody positive ("positive") over total tested, with point prevalence (%) for herd tests.
[2] Excludes five cows tested in August 2015 that were inadvertently retested in November
[3] The one positive cow was the same cow identified at the November 2015 test; subsequently culled.
[4] The one positive was a first lactation heifer; subsequently culled.

Table 5.2. Bovine leukemia virus (BLV) antibody testing schedule and results for Herd R.

Test Date	Test Type	Description	Results[1]
July 2015	Herd (milk)	All cows in the milking herd	27/849 (3.2%)
September 2015	Interim (milk)	Cows dry at July test and 1st lactation heifers[2,3,4]	5/136
February 2016	Herd (milk)	All cows in the milking herd[2,5]	31/844 (3.7%)
May 2016	Interim (milk)	Test of 1st lactation heifers[2,6]	22/218
August 2016	Herd (milk)	All cows in the milking herd[2,7]	46/849 (5.4%)

[1] BLV-antibody positive ("positive") over total tested, with point prevalence (%) for herd tests.
[2] Heifers had comingled with animals from other herds at the out-of-state calf-raising facility.
[3] All five positive animals were 1st lactation heifers.
[4] Excludes one cow tested in July 2015 that was inadvertently re-tested in September.
[5] Five positive cows were new infections in previously negative animals, fourteen were 1st lactation heifers, and twelve were previously positive animals.
[6] Includes ninety-four 1st lactation heifers previously tested in February, eleven of which were positive at that time.
[7] Seven positive cows were new infections in previously negative animals, nine were 1st lactation heifers, and thirty were previously positive animals.

Table 5.3. Bovine leukemia virus (BLV) antibody testing schedule and results for Herd S.

Test Date	Test Type	Description	Results[1]
December 2014	Herd (milk)	All cows in the milking herd	8/343 (2.3%)
February 2015	Interim (serum)	Cows and heifers due to calve in the next six months[2]	2/69
March 2015	Interim (milk)	Cows dry at the December test	0/10
July 2015	Interim (milk)	All cows and heifers that calved since the March test	0/27
	Bulk tank (milk)		Negative
October 2015	Interim (milk)	All cows and heifers that calved since the July test	2/43
January 2016	Herd (milk)	All cows in the milking herd	1/342 (0.3%)
April 2016	Interim (milk)	All cows and heifers that calved since the January test	1/39
	Bulk tank (milk)		Negative
July 2016	Interim (milk)	All cows and heifers that calved since the April test	2/31
	Bulk tank (milk)		Positive
September 2016	Interim (serum)	Heifers due to calve in the next six months	0/38
October 2016	Interim (milk)	All cows and heifers that calved since the July test	3/30
	Bulk tank (milk)		Positive
November 2016	Interim (serum)	Heifers due to calve in the next six months	0/10
December 2016	Interim (serum)	Heifers due to calve in the next six months	1/14
January 2017	Interim (serum)	Heifers due to calve in the next six months	1/14
January 2017	Herd (milk)	All cows in the milking herd[3]	5/343 (1.5%)
	Bulk tank (milk)		Positive
February 2017	Interim (serum)	Heifers due to calve in the next six months	1/35
March 2017	Interim (serum)	Heifers due to calve in the next six months	0/12
April 2017	Interim (serum)	Heifers due to calve in the next six months	0/8

[1] BLV-antibody positive ("positive") over total tested, with point prevalence (%) for herd tests.
[2] Excludes one cow tested in December 2014 that was inadvertently re-tested in February.
[3] All five positive animals had previously tested negative on serum as heifers.

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CHAPTER 6: Conclusions and Future Directions

Specific Aims

This dissertation investigated a novel aspect of the effect of bovine leukemia virus (BLV) infection on the immune system of U.S. dairy cows and several approaches to the on-farm control of BLV transmission and prevalence in U.S. dairy cows via the following specific aims:

Aim 1: To investigate associations between BLV profiles and total IgA concentrations in the milk, saliva, and serum of BLV-positive dairy cows.

Aim 2: To investigate the role of common-use hypodermic needles and reproductive examination sleeves in the transmission of BLV.

Aim 3: To examine test-and-removal protocols for feasibility in U.S. dairy herds.

Sub-aim 3.1: To develop and test an approach for high BLV prevalence herds in which the cows thought to be the most infectious are selectively removed from the herd.

Sub-aim 3.2: Evaluate the utility of a test-and-remove method of BLV control using milk ELISA results.

Conclusions

Aim 1

For Aim 1, we specifically investigated the total IgA concentration in milk, saliva, and serum of cows with various BLV profiles based on ELISA, lymphocyte count (LC) and proviral load (PVL). A growing body of evidence has demonstrated that BLV negatively impacts the bovine immune system (Frie and Coussens, 2015), resulting in potentially significant health impacts to the animal (Brenner et al., 1989; Trainin et al., 1996) and diminished economic output for the dairy industry (Ott et al., 2003). Many of the major pathogens of concern in dairy cows enter via mucosal junctions, yet no studies to date had examined any potential effects of BLV on IgA, the primary immunoglobulin of mucosal secretions. We hypothesized that total IgA concentrations would be decreased in BLV ELISA+ cows compared to BLV ELISA- cows. We also hypothesized that cows with high LC or high PVL would have decreased total IgA concentrations compared to BLV ELISA- cows.

Our results showed that total IgA concentrations in serum were essentially equivalent in all cows regardless of BLV profile. However, total IgA concentrations in milk were 33.4% lower in BLV ELISA-positive cows, although the difference was not statistically significant. Similarly, total IgA concentrations were 23.7% lower in the saliva of BLV ELISA-positive cows, although this again did not reach statistical significance. Other research has shown that cows with markedly elevated LC and/or PVL have higher degrees of immunologic disruption (Trainin et al., 1996) and we anticipated observing similar results in this study. Contrary to our expectations, differences in total IgA concentrations between cows with BLV profiles based on LC or PVL showed a trend toward lower concentrations in cows without leukocytosis or high PVL. One

possible explanation may be that any decrease in IgA secretion is offset by higher total numbers of B-cells; research recently reviewed by Frie and Coussens (2015) indicates that other elements of immune dysfunction in BLV-positive cows could also play a role in this finding.

Our study was conducted on samples from only one herd and that herd had eliminated cows with the most extreme BLV profiles as part of our BLV control program (sub-aim 3.1). Therefore, our study may have underestimated the impact of BLV-associated high LC and/or high PVL profiles on total IgA concentrations, and we could not control for the duration of infection. Investigation of the potential biological impact of these alterations in antibody concentrations was beyond the scope of this study and should be studied along with attempts to repeat our other findings. Overall, our results indicate that immune disruption in BLV infected cows may be even more widespread than reported and more work is needed to determine the degree to which IgA disruption affects both health and production outcomes.

Aim 2

To investigate Aim 2, we designed a field trial wherein BLV-negative cows were identified by milk ELISA screening and assigned either to receive the herd's standard management practice of common (shared) hypodermic needles and reproductive sleeves (control) every time, or to the intervention group which received a new hypodermic needle and new reproductive sleeve for every usage. By using within-herd controls, all other management practices should have been balanced between treatment groups. However, this approach required intervention cows to be identified to the herd managers and staff, so the study was not blinded. We calculated the risk of new infections semi-annually with a testing schedule that also allowed us to also approximate a

‘winter’ and ‘summer’ seasonal exposure period. In one herd, the herd manager decided that this medical hygiene approach was a good management practice and began single-use needles and sleeves to all cows in the herd; this herd was excluded from our analysis. In the three remaining enrolled herds, there was no statistical difference in the risk of new infections between the control and intervention groups. There was, however, a significant increase in the risk of new infections in the summer season. This was primarily driven by one of the three herds; each herd alone had a different seasonal profile: one herd had a numerically higher risk of new infections in the winter season, while there was no seasonal difference in the third herd.

Re-use of needles and sleeves is frequently identified as a management practice associated with increased BLV incidence and prevalence in epidemiologic studies and blood-contaminated needles and sleeves have been used to transmit BLV experimentally. The results of our field trial, if repeated, may indicate that re-use of needles and sleeves may not play a primary role in BLV transmission in some herds, or that other aspects of BLV transmission (e.g. individual cow susceptibility or, conversely, infectivity) may interact synergistically with this management practice. Although we did not see a difference in the risk of new infection with this intervention, this does not preclude medical hygiene as an important aspect of BLV transmission, and regardless of its role in BLV transmission, good medical hygiene is important for preventing the transmission of other pathogens (Reinbold et al., 2010; Darpel et al., 2016).

Aim 3

For Aim 3, we first investigated test-and-remove (culling and/or segregation) approaches to BLV control. More than 20 nations, mostly in the EU, have enacted successful BLV eradication

programs based on screening herds for BLV-positive animals and subsequently culling them from the herd (EFSA AHAW Panel, 2015). Most of these programs were implemented in herds starting with low BLV prevalence (Batho et al., 2008); therefore we wanted to determine if this approach could be successful in U.S. dairy herds with similarly low prevalence. To accomplish this, we designed a 'test-and-cull' field trial where milk BLV ELISA screening was used to identify animals presumptively infected with BLV so they could be culled from the herd. Herd managers agreed to cull BLV-positive cows, or segregate them from un-infected herd mates until they could be culled. As expected, BLV prevalence in two of the three enrolled herds decreased immediately following implementation of this protocol. In one herd, however, heifers were raised out of state, and as they returned it became clear that this was an ongoing source of BLV infection. Although the starting prevalence of this herd was 3.2%, one group of heifers returned with 10.1% prevalence. Unable to cull or segregate such large numbers of animals, the overall herd prevalence increased to over 5% and the herd was withdrawn from the study. This lesson about the importance of BLV dynamics in the young stock was reinforced by the experience of the other two herds, where the test-and-cull approach eliminated BLV from the adult cows, but the virus was re-introduced as heifers (presumably infected as calves) entered the milking herd. One herd was willing to approach BLV control in the young stock more aggressively, and screened heifers with BLV ELISA testing of serum. However, even this additional testing did not prevent re-introduction of BLV to the milking herd; some heifers which tested negative at the early screening then tested positive for BLV at a subsequent milk test. We suspect that this is evidence of latent BLV infections, an immune-avoidance mechanism which is common to many retroviruses, reinforcing the need for ongoing surveillance as a part of any BLV control program.

The prevalence of BLV in the U.S. dairy industry has been steadily increasing, and a recent survey by our research group found that more than 90% of dairy herds had at least one BLV-positive cow and within-herd prevalence was over 40% (LaDronka et al., 2018). As a result, culling all BLV ELISA-positive cows is economically impossible for most U.S. dairy herds. We designed and tested a novel test-and-remove approach to BLV control that focused on preventing virus transmission by targeted removal of the most infectious cows, using proviral load and lymphocyte count as a measure of infectivity. All three herds enrolled in our field trial experienced declines in BLV incidence risk as well as overall prevalence. In contrast to our medical hygiene field trial, there was no significant seasonal effect, although two of the three herds had numerically higher BLV incidence risk in the winter exposure period. Management factors that may change seasonally in some herds, particularly housing and calving practices, likely strongly influence any observed seasonal trends in BLV infection (Onuma et al., 1980; Thurmond et al., 1983a). The most important factor for success in our targeted approach appeared to be the ability to prevent, either by culling or segregation, close contact between the highest PVL and LC cows and susceptible herd mates.

Future Directions

The work described in this dissertation indicates that total IgA concentrations may be disrupted in the secretions of BLV-positive cows. In addition to studies confirming this finding, assessing total IgA concentrations in BLV-positive cows with more extreme PVL and LC profiles would provide additional evidence to determine the immunologic effects of BLV infection. This investigation also evaluated only the total IgA concentrations; the effect of BLV-infection on antigen-specific IgA antibodies was beyond the scope of this study and should be investigated.

We examined total IgA concentrations in only two secretions – lacteal (milk) and oral (saliva). IgA is also present in vaginal secretions, and is the major immunoglobulin of nasal and lacrimal secretions (Duncan et al., 1972). It is likely that IgA plays a meaningful role in immunologic protection at these mucosal junctions. Further studies to characterize IgA concentrations in these secretions in relation to BLV infection are therefore warranted. Similarly, although IgA is a minor component in colostrum compared to IgG1 (Butler et al., 1972), passive immunity transfer to calves from BLV-infected dams may be affected by altered IgA levels in colostrum, and there have been no studies on this topic at the time of this writing.

While our test-and-remove field studies demonstrated the potential utility of these approaches for the control of BLV in dairy herds, a better understanding of BLV infection dynamics in young stock is needed. The phenomenon of latent BLV infection is poorly understood and especially insidious in the context of BLV control. Based on the results of our field trial, we speculate that latent BLV may become active close to or shortly after calving, potentially resulting in re-introduction of the virus into an otherwise BLV-negative milking herd. Also unknown at this time is the risk to the calves of cows with reactivated BLV; it would be highly informative to investigate the dynamics of BLV re-activation, particularly with regard to infectivity to both offspring and susceptible herd mates.

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