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

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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. Bovine leukemia virus causes lymphoma in <5% of infected cattle, and increased lymphocyte counts (lymphocytosis) in about one-third. Recent research has shown that dairy cows infected with BLV have altered immune function associated with decreases in milk production and lifespan. 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 LC, these animals are thought to be responsible for most transmission of BLV in a herd. Milk or blood samples from adult cows in our 3 Midwestern dairy farm field trials were tested semiannually with ELISA for BLV antibodies, and ELISA-positive cattle were then re-tested using a blood LC and a quantitative PCR test for PVL to identify the animals presumed to be most infectious. Herd managers were encouraged to consider PVL and LC status when making cull decisions, and to segregate cows with the highest PVL and LC from their BLV ELISA-negative herd mates where possible. After 2 to 2.5 yr of this intervention, the incidence risk of new infections decreased in all 3 herds combined, from 13.8 to 2.2, and the overall herd prevalence decreased in all 3 herds combined from 62.0 to 20.7%, suggesting that this approach can efficiently reduce BLV transmission as well as 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 United States dairy cattle, with over 80% of United States dairy herds infected (USDA, 2008; LaDronka et al., 2018). Similar to the related human retrovirus human T-cell lymphotropic virus, BLV inserts proviral DNA into the host cell genome (primarily T-lymphocytes in human T-cell lymphotropic virus 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). In infected cattle, 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). The economic costs of BLV have been estimated to be \$43 million in lost milk production alone (Da et al., 1993), and, in 1995, \$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 predominantly to B-cell tropism (Paul et al., 1977a,b; Djilali et al., 1987) and subsequent clonal expansion (Gillet et al., 2013), BLV causes an increase in the absolute number of lymphocytes (lymphocytosis) in about one-third of infected cows at any time. Common practices in the dairy industry (e.g., reuse of needles,

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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 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) because of 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 United States makes this approach of removing all BLV-positive cows impossible for most farms without financial subsidies. A method is urgently needed to reduce BLV prevalence to a sufficiently low level so that all ELISA-positive cattle can affordably be culled.

In other retroviruses, such as the closely related deltaretrovirus human T-cell lymphotropic virus, the provirus is considered the infectious unit and the relative amount of integrated provirus in the host genome, known as proviral load (**PVL**), is associated with risk of transmission (Kaplan et al., 1996; Ureta-Vidal et al., 1999; Li et al., 2004). Although the mechanism is not clear, the infectiousness of a BLV-infected cow likely corresponds to PVL (Rodríguez et al., 2011). This is supported by studies in both Japan (Mekata et al., 2015) and Argentina (Juliarena et al., 2016), which found that cows with a low PVL were unlikely to spread BLV to herd mates. As well, removal of cattle with high BLV antigen expression reduced the incidence of new BLV infections in an Australian dairy herd (Molloy et al., 1994), and Gutiérrez et al. (2014) reported that dams with high PVL were more likely to transmit BLV to their calves.

We hypothesized that BLV within-herd transmission and prevalence could be reduced by identifying and removing (culling or segregation) ELISA-positive cows

with the highest PVL and LC. We conducted a 3-herd pilot intervention field trial, in which PVL and LC were measured in ELISA-positive cattle to identify the cattle that were presumably most infectious cattle so that they could be removed or separated from the herd's BLV-negative cattle with the objective of decreasing BLV transmission and prevalence in these herds.

MATERIALS AND METHODS

Herd Enrollment and Study Design

This study can be viewed as an intervention field trial, or as a case series of 3 herds attempting to control BLV by identifying the most infectious cattle for segregation or culling. Herd enrollment requirements were as follows: prevalence of antibodies against BLV via ELISA testing $\geq 40\%$; and herd managers who were willing to rapidly cull or segregate and subsequently cull cattle based on 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 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 3 weeks of their positive ELISA test. Quantitative results for PVL, LC, and anti-BLV antibody ELISA were compiled into a spreadsheet. The PVL results were further categorized as very high ($\geq 100,000$ copies/ 10^5 cells), high ($\geq 50,000$ to $<100,000$ copies/ 10^5 cells), moderate ($\geq 16,000$ to $<50,000$ copies/ 10^5 cells), or low (>0 to $<16,000$ copies/ 10^5 cells). Similarly, LC results were categorized as very high ($\geq 10.0 \times 10^9/L$), high (≥ 7.5 to $<10.0 \times 10^9/L$), or normal ($<7.5 \times 10^9/L$). Combined data were sorted by PVL and LC categories. 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 the ELISA-negative animals, given individual herd-management limitations. We consulted with herd managers to make a specific plan for each cow on the list (e.g., cull immediately, cull after calving, move to do-not-breed pen). In follow-up communications, we confirmed that herd managers were considering these plans when making culling and segregation decisions. However, all culling and segregation decisions were entirely at the discretion of the herd managers.

Kellogg Biological Station Herd. The Michigan State University W. K. Kellogg Biological Station

(KBS) Pasture Dairy Center is an automated milking, rotational pasture grazing, freestall 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 1 of 2 pastures and routed to separate robotic milking machines. The ability to make management changes (including selection of cows to be culled) was limited, because this was 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, which received larvicide in their feed) and the herd used a variety of dehorning methods (polling, burn, and gouge) for heifer calves. Within approximately 3 mo 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, LC, or both 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 1 of the 2 pastures and milking lines, and by the end of the second year was able to move all the highest PVL and LC cows into 1 group. Over the course of the study, the herd manager reported relatively constant overall cull rates (approximately 30%) 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. The prevalence of BLV at enrollment (spring 2016) was 66%. Herd H enrolled approximately 6 mo later than the other 2 herds, and therefore participated for a shorter period (2 yr). 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 the herd owner was 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 cull rates similar to previous years (approximately 45%).

Herd J. Herd J is a Michigan freestall dairy with approximately 200 milking cows. At enrollment (fall 2015), BLV prevalence was 64%. This herd already had a policy of single-use needles and sleeves, as well as on-premises fly control. Cows with the highest PVL, LC,

or both were culled as soon as possible, and the herd owner almost immediately began moving the remaining cows with the highest PVL and LC into a separate pen. Within 6 mo, they had established one pen with the highest PVL cows and another 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 ongoing commingling occurred in dry and fresh pens. Herd J experienced higher-than-average cull rates in 2016 and 2017 (approximately 67%, up from approximately 36%) 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 (Grand Ledge, MI). Milk samples were first analyzed for milk components (e.g., fat, protein, somatic cells) and then tested for antibodies against BLV using 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 Michigan State University 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 that were 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 Michigan State University Institutional Animal Care and Use Committee.

ELISA Test for Anti-BLV Antibodies

A modified ELISA test (IDEXX Laboratories, Inc., Westbrook, ME) to detect antibodies against BLV as described by Erskine et al. (2012c) was performed at NorthStar. Briefly, aliquots of DHI milk samples or serum and 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 the addition of an enzyme substrate. Color development of positive controls was used to standardize reaction times, and the reaction was stopped by adding 0.5 N H₂SO₄. Results were reported as corrected 450 nm 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) either on-farm or at the Michigan State University BLV laboratory. Briefly, 80 μ L of blood was added to 20 μ L of K-stain (Advanced Animal Diagnostics) and mixed well. Then, 10 μ L of the mixture was pipetted onto a slide and immediately placed in the analyzer. The machine measured total white blood cell concentration and percent lymphocytes, and calculated LC.

Proviral Load Test

The PVL test was conducted using the CoCoMo BLV quantitative PCR 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). Eluted DNA was normalized to 30 ng/ μ L, and 150 ng of genomic DNA was added to each quantitative PCR using the BLV-CoCoMo primer set, FAM BLV probe (RIKEN Genesis, Tokyo, Japan), and TaqMan Gene Expression Master Mix (Life Technologies, Carson, CA).

Data Analysis

The 2 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 mo 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 semiannual time period. Changes in within-herd BLV prevalence over time were evaluated using the χ^2 test for trend by individual herd and across all 3 herds using OpenEpi 3.01 (Dean et al., 2013).

Incidence risk (proportion) was calculated for each inter-test period of approximately 6 mo. 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. We considered ELISA-positive cows to be always positive, so they were 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 half the number of these cows absent at the semiannual test at the end of the period (withdrawals, C):

$$\begin{aligned} \text{Corrected at-risk population} = \\ \text{starting at-risk population} - (0.5 \times C). \end{aligned}$$

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 spanned the missed semiannual test period, and the specific period when the infection occurred could not be identified. To avoid bias, each “missed” newly BLV ELISA-positive cow (D) was considered to have contributed 1 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:

$$\begin{aligned} \text{Corrected number of new infections} = \\ \text{number of new infections} + (0.5 \times D); \end{aligned}$$

$$\begin{aligned} \text{Corrected at-risk population} = \\ \text{starting at-risk population} - (0.5 \times C) + D. \end{aligned}$$

The incidence risk for new infections over time was evaluated using the χ^2 test for trend by individual herd and across all 3 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 United States. 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 were compared using Fisher’s mid-*P* exact test (Dean et al., 2013).

RESULTS

Semiannual BLV Prevalence

All 3 herds experienced statistically significant decreases in BLV prevalence over the course of this study

Figure 1. Within-herd and combined 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. J = herd J; KBS = Kellogg Biological Station; H = herd H.

(Figure 1). Within-herd point prevalence decreased in herd J from 63.8 to 13.9% ($P < 0.001$) after 2.5 yr in the study. The KBS herd prevalence decreased from 58.2 to 27.4% ($P < 0.001$), after 2.5 yr in the study. Prevalence in herd H was 65.8% at enrollment, increased to 73.1% at the second semiannual test, and then declined at each remaining semiannual test for an overall decline in prevalence to 45.3% ($P < 0.001$) during the 2 yr of the study. An overall decreased BLV prevalence ($P < 0.001$) over the duration of the pilot intervention was observed for combined data from the 3 herds.

Semiannual Incidence Risk of New Infections

In addition to monitoring herd BLV infection prevalence, we used the ELISA test to calculate the incidence risk of new infections during each inter-test period. All 3 herds together experienced an overall decrease in new infections ($P < 0.001$; Figure 2). The incidence risk for herd J decreased from 17.8% in the first 6 mo of intervention to 0.9% in the final reported 6 mo ($P < 0.001$). The incidence risk for the KBS herd during the first 6 mo 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 2 periods ($P = 0.069$). The incidence risk for herd H was 13.6% in the first 6 mo, dropped to 0 in the subsequent inter-test period, then remained under 4% (3.9 and 3.4%) for the final 2 periods ($P = 0.116$).

Combined new infection risk for all 3 herds was not statistically different between seasons, but tended to be higher in the winter ($P = 0.349$; Table 1). 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%), and both herd J (2.3 vs. 5.3%) and the KBS herd (6.7 vs 8.5%) experienced more new infections during the winter months.

Distribution of PVL and LC by Category

A descriptive cross tabulation presenting the distribution of categorical PVL and LC values (Table 2) 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 that selective culling and segregation of the cattle likely to be most infectious, 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 3 herds experienced decreased within-herd BLV prevalence over the course of our study. Herd J had a freestall 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 yr. The increased culling in this herd because of downsizing during the intervention was likely another contributor to the markedly decreased BLV prevalence in this herd. Thus, in the event a producer is downsizing, prioritiz-

Figure 2. Incidence risk for new bovine leukemia virus infections over time. Incidence risk was calculated for each consecutive semiannual inter-test study period, beginning with the baseline enrollment milk test and the first semiannual herd test for each herd and for all herds combined. J = herd J; KBS = Kellogg Biological Station; H = herd H.

Table 1. Comparison of seasonal incidence of new bovine leukemia virus infections¹

Exposure period ²	Herd J				KBS herd ³				Herd H				All herds combined			
	New infections	Cows at risk	Incidence risk		New infections	Cows at risk	Incidence risk		New infections	Cows at risk	Incidence risk		New infections	Cows at risk	Incidence risk	
Summer	4	175.5	2.3		9	134	6.7		4	48	8.3		17	357.5	4.8	
Winter	14	265	5.3		18	212.5	8.5		1	46	2.2		33	523.5	6.3	

¹Mid-*P* exact 2-tail *P*-values: herd J, *P* = 0.130; KBS herd, *P* = 0.584; herd H, *P* = 0.236; all herds, *P* = 0.349.

²Summer season of exposure: inter-test period from approximately May to November; winter season of exposure: inter-test period from approximately November to May.

³KBS = Kellogg Biological Station.

Table 2. Summary distribution of categorical proviral load (PVL) and lymphocyte count (LC) results in bovine leukemia virus antibody-positive cows¹

PVL category	Herd J				KBS herd ²				Herd H				All herds combined			
	Normal LC	High LC	Very high LC		Normal LC	High LC	Very high LC		Normal LC	High LC	Very high LC		Normal LC	High LC	Very high LC	
Low	149	26	5		110	21	5		38	2	0		297	49	10	
Moderate	28	21	7		12	11	0		21	10	0		61	42	7	
High	18	27	45		7	21	46		15	32	30		40	80	121	
Very high	0	2	15		1	1	14		0	5	19		1	8	48	

¹Data were restricted to samples that were ELISA-positive, PVL-positive, and had a reported LC result; n = 764 samples. Some cows contributed repeated measures (n = 343 unique cows).

²KBS = Kellogg Biological Station.

Figure 3. Row percentages of the distribution of proviral load (PVL) and lymphocyte count (LC) categories for all herds. Data were restricted to samples that were ELISA-positive, PVL-positive, and had a reported LC result; $n = 764$ samples. Some cows contributed repeated measures ($n = 343$ unique cows). J = herd J; KBS = Kellogg Biological Station; H = herd H.

ing removal of cows with high PVL and LC may allow for rapid reduction of BLV which may, in turn, aid in improving milk production efficiency in the remaining herd.

In contrast, the KBS herd had cows grouped onto 1 of 2 adjoining pastures in the summer and 1 of 2 sides of a freestall 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 2 groups, particularly in the milking barn. The herd manager worked to move the cows with the highest PVL and LC to 1 of the 2 groups, which may have helped to reduce overall BLV transmission by reducing close contact between more infectious cows and their BLV-negative herd mates (about half the herd). During 2.5 yr of participation in the current study, 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 to be \$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 6 tie-stall dairies in Japan, BLV-negative cows were at more than 12 times higher risk of infection if an adjacent cow was

BLV-positive (Kobayashi et al., 2015). Therefore, the herd manager began moving the cows with the highest PVL and LC to the end of the milking line to enhance the degree of separation. Over a shorter period (2 yr), herd H experienced a decrease in BLV prevalence from 65.8 to 45.3%, a relative decline of almost one-third.

Overall, the enrolled herds had varying abilities to cull and segregate cows based on PVL and LC. Segregation was applied differently in each herd, and cows were segregated for a variable length of time before being culled, so it was not possible to separate the effect of segregation and culling in these herds. We communicated regularly with herd managers to review semianual results and asked them to develop a plan to cull or separate the highest priority cows, at minimum. We also checked in with herd managers between test dates to update these plans, confirm that they were acting on these plans, and address any questions about BLV control. Culling decisions rely on many factors; consequently, herd managers may not always select the cows with the highest PVL and LC for culling. All decisions were ultimately made by herd managers; the intensity of culling and segregation in relation to PVL, LC, or both was entirely up to the herd manager in each herd and was dependent on individual herd-management

practices and abilities, including physical limitations and economic considerations. The intensity of culling directly affects prevalence and could indirectly affect incidence. Of the 3 herds, herd J undertook the most intensive culling and segregation of cows with the highest PVL and LC, and therefore experienced the most rapid and significant reduction in BLV prevalence and incidence.

The risk of new infections decreased in all 3 enrolled herds over the course of this study. Because these herds were not evaluated before initiating the intervention field trial, the first new infection risk period was also the first 6 mo in the study for each herd. Therefore, the baseline (pre-trial) new infection risk was likely underestimated for each of these herds, because we did 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 2 inter-test periods, so 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 in the first 3 mo of the trial and had no effect on reducing new infection risk during subsequent inter-test periods. In fact, a small increase in new infections (from 6 to 8.5) combined with additional withdrawals during the third inter-test period increased the new infection risk to about 14%. This increase occurred during the winter months and before the herd manager moved cows with the highest PVL and LC to a single 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 3 herds at enrollment, and changes were recorded during the course of the study. However, if management practices had been changed immediately before study enrollment, such changes could have influenced the apparent effect of the intervention program.

The trend overall for our 3 field trial herds was toward more new infections over the winter inter-test period, but this finding 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. The ELISA test results generally become positive within 2 to 3 wk after BLV infection, but could take up to 3 mo due to individual variability (Evermann et al., 1986; Klintevall et al., 1994; Mammerickx et al., 1980). Therefore, animals that had not developed sufficient antibody concentrations by the test date could have been 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 United States.

Although antibody testing is a widely accepted diagnostic tool for BLV infection, false-positive and false-negative results are possible. 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, and this 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 one-third 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 3 herd managers quickly recognized that incoming heifers needed to be tested before introduction into the milking herd. Although it was outside the scope of our pilot intervention, we accommodated a request from herd H management to conduct ELISA tests on blood from pregnant heifers in September 2016, April 2017, and April 2018. We also began conducting ELISA tests on blood from pregnant heifers, milk at each animal's first DHI test, or both 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 approximately 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 use PVL or LC concentrations in young stock to aid in determining which animals to breed or cull.

Herd managers of all 3 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 cow 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 3 herds in this study appeared similar to that reported by Ohno, indicating that future studies on BLV control programs might use either of these measures to identify the cows that are presumably most highly infectious 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 semiannual LC testing was unable to eradicate BLV after 10 yr (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 almost 1,000 PVL and LC tests were performed over approximately 3 yr, 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. As well, the largest herd in this pilot intervention program had approximately 200 milking cows, so a potential limitation is scalability of this approach in larger herds.

Although we cannot separate the effects of the culling, segregation, and management changes in this intervention, we found a similar overall response in all 3 herds, where the consistent component was culling the cows with the highest PVL and LC. Therefore, we believe that targeting the cows with highest PVL and LC for culling and segregation could become a viable way for producers to reduce herd BLV prevalence to a level at which 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 BLV-negative herd status.

Future work is needed to estimate the total effect and implementation cost of this type of control program, particularly because no commercial BLV quantitative PCR test for PVL is currently available in the United States. However, our unique approach of using routine DHI 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.

CONCLUSIONS

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 mo of participation.

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