

DECREASING PERINATAL BOVINE LEUKOSIS VIRUS
INFECTION IN CALVES

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INFECTION IN CALVES

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DEDICATION

I would like to dedicate this dissertation to my family. My family has grown since I completed my Masters and embarked on the PhD. Dad, Tracy, Kevin, Chris, and Kelly all of the stuff I said the last time still holds true. Mom....I still miss you and carry your memory with me in all that I do.

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ABSTRACT

Bovine leukosis virus is an oncogenic retrovirus of cattle that causes lymphosarcoma in a proportion of infected individuals. Currently the United States estimates are that 44% of dairy cattle and 10% of beef cattle are infected with the virus. Many states have voluntary control programs in place, but no mandatory or federal programs currently exist. This is dramatically different from many other industrialized, cattle producing countries that have government controlled, mandatory control and eradication programs in place. Many of these countries in Europe, Australia, and New Zealand have dropped the prevalence of viral infection to negligible levels.

The inability to accurately detect infection in calves by serologic test methods due to the interference of colostral immunoglobulin has led to US control programs focusing on the identification of infected adults. The ease at which serologic methods can be used in adults has also removed attention from the potential role that persistent lymphocytosis may play on an infected premise. The goal of this body of work was to develop alternative methods that may be utilized on heavily infected farms to help in the control of BLV infection. The studies presented here focus on the utility of diagnostic tests in the identification of infected calves and the identification of adults with persistent lymphocytosis.

CHAPTER 1

BOVINE LEUKOSIS VIRUS INFECTION IN CATTLE

RETROVIRUSES AND THEIR CLASSIFICATION

The Retroviridae family of viruses is a group of RNA viruses. The discovery of Human immunodeficiency virus (HIV) and the recognition of its devastating effects have focused attention on retroviruses for the past 25 years. Retroviral virions are spherical, enveloped viruses that are 80 – 100nm in size. There are 4 main genes that encode for the virion proteins present in all retroviruses. They are 5'- *gag*, *pro*, *pol*, *env*-3'. Some families may have additional genes encoding additional proteins required for viral function. One unique feature of the retroviruses is the reverse transcriptase and integrase encoded by the *pol* gene. Viral replication begins with the reverse transcription of the viral RNA to cDNA by the reverse transcriptase. During this process RNA is digested and serves to prime positive sense cDNA synthesis on the negative sense DNA transcripts. The integrase is responsible for the incorporation of the viral DNA into the host genome, making the virus a permanent component of the host genome.

BLV-HTLV group

Bovine leukemia virus (BLV) is a retrovirus of the BLV- HTLV group. In addition to the standard retroviral genes, BLV also contains additional genes *tax* and *rex* which are found in overlapping reading frames following the *env* gene. Both genes are necessary for gene expression. The genome structure, nucleotide sequence of the provirus, and amino acid sequence of the structural and nonstructural proteins are very similar to that of human T cell lymphotropic virus (HTLV-1 and HTLV-2). Both BLV and HTLV-1 have a stable genome, distinctly unlike the more notable human retrovirus, HIV. Both viruses lack a chronic viremia, have a long “latent” period, and lack preferred sites of proviral integration (Kettmann et al., 1980; Grégoire et al., 1984). Despite the lack of preferred proviral integration sites, the tumors generated by both viruses in a single individual are typically monoclonal and have a single integration site (Kettmann et al., 1980; Kettmann et al., 1983). Both BLV (Kettmann et al., 1980) and HTLV-1 (Franchini et al., 1984) escape the immune response by low levels of viral replication. In BLV it appears that replication is blocked at the transcriptional level, but the mechanism has not been completely elucidated (Gupta et al., 1984; Tajima and Aida, 2000; Merezak et al., 2001; van den Heuvel et al., 2005).

Cattle are infected with BLV through the transfer of blood and blood products that contain infected lymphocytes. Once infected cattle will develop a persistent lifelong antibody response; primarily to the gp51 envelope protein and the p24 capsid protein. B lymphocytes harbor the integrated provirus, but rarely

express viral proteins on their cell surface. The exact site of viral replication and expression that drives the immune response remains elusive. One study suggested that this site was the spleen and regional lymph nodes (Van Der Maaten and Miller, 1978). This was refuted in a later study by the same group (Van Der Maaten et al., 1982). Another group has suggested that this site may be the mammary epithelial cells (Buehring et al., 1994). There is also evidence that cells of the monocyte/macrophage lineage may be able to harbor the virus (Schwartz et al., 1994; Doménech et al., 2000), as is the case with HTLV-1 (Hoffman et al., 1992; de Revel et al., 1993). While much is known about the transmission, epidemiology, and outcome of viral infection, the sequence of molecular events immediately post-infection that allows BLV to elude the immune system remains unclear.

In cattle infected with BLV only 1 of every 25,000 – 50,000 peripheral blood lymphocytes expresses viral proteins or has viral mRNA present in the cell cytoplasm (Mirsky et al., 1996). This block of viral expression appears to be at the transcriptional level as no viral products including proteins, or RNA is detectable in most circulating cells (Kettmann et al., 1980). This block of viral expression is crucial, allowing the majority of BLV-infected cells to escape the surveillance of the host immune system. Once infected with BLV, cattle mount a strong, persistent immune response; making it clear that there are some infected cells that must express viral antigens on a consistent basis. The source of the antigenic stimulus that drives the immune response remains controversial.

OUTCOME OF INFECTION WITH BLV

There are 3 main outcomes in cattle infected with BLV. The vast majority of animals will remain persistently infected with no outward signs of infection. There is debate as to whether BLV infection causes measurable production losses. Increased culling rate (Brenner et al., 1989; Pollari et al., 1993) and decreased milk yields (Brenner et al., 1989; D'Angelino et al., 1998) have been attributed to BLV infection. However, some studies contradict these claims (Wu et al., 1989; Rhodes et al., 2003). Approximately 29% of BLV-infected cattle will develop persistent lymphocytosis, while less than 5% of BLV-infected cattle will develop lymphosarcoma (Ferrer et al., 1979b).

Persistent lymphocytosis

There are many reasons that cattle may develop lymphocytosis that are not BLV-associated. In addition a proportion of BLV-infected cattle with lymphosarcoma will develop lymphocytosis in association with their tumor process. The term persistent lymphocytosis (PL) refers to a benign elevation in lymphocyte count that accompanies BLV infection in a proportion of infected animals. Approximately 29% of cattle infected with BLV will develop persistent lymphocytosis and approximately 95% of animals with persistent lymphocytosis are BLV positive (Ferrer et al., 1979b). Persistent lymphocytosis is sometimes referred to as a preneoplastic syndrome, but there is no convincing evidence that PL cattle have an increased risk of developing lymphosarcoma. The lymphocytes

present in PL are not neoplastic although they may have mild reactive changes consistent with normal blood smears in cattle. Lymphocytosis associated with BLV infection is defined as an elevation in lymphocyte count that is 3 or more standard deviations above the mean (Marshak et al., 1968). In general demonstration of lymphocytosis at 2 testing periods over 60 – 90 days apart is accepted as persistent.

Persistent lymphocytosis is considered to be a benign condition associated with BLV infection. For this reason it is often overlooked. However, these cows may serve as a greater reservoir of infection on a farm. The increased lymphocyte count is attributed to a 45-fold increase of infected CD5+ and a 99-fold increase in infected CD5- B cells (Mirsky et al., 1996). In addition it has been suggested that cows with PL may be a greater risk for passing BLV infection on to their calves *in utero* (Lassauzet et al., 1991; Agresti et al., 1993) and may suffer from decreased milk production, (Da et al., 1993) and alteration of milk components (Da et al., 1993; Motton and Buehring, 2003).

There is good evidence that genetics are involved in the development of PL (Xu et al., 1993), but the expression is considered to be multifactorial. The time from infection to the development of PL is unknown. In one experimental infection trial 5/16 inoculated calves developed persistent lymphocytosis (Miller et al., 1972). Three calves developed persistent lymphocytosis at 4-6 months and 2 at 13 months post inoculation. There is no work currently available that has evaluated the development of PL in naturally infected animals.

Lymphosarcoma

The development of lymphosarcoma occurs in 1-5% of BLV-infected cattle (Ferrer et al., 1979b). Clinical signs associated with the development of lymphosarcoma are highly variable, as the affected organ will dictate the predominant clinical signs. Animals with BLV associated lymphosarcoma will commonly show lesions in the central or peripheral lymph nodes leading to lymphadenopathy. Lesions of the abomasum may lead to signs of cranial abdominal pain, melena, or abomasal outflow obstruction. Pelvic limb paresis progressing to paralysis can occur in animals with extradural spinal lesions. Retrobulbar lesions will cause protrusion of the globe resulting in exposure keratitis and eventually proptosis. Lesions of the right atrium may be mild and undetectable clinically, or may present with arrhythmias, murmurs, or heart failure. Lesions of the uterus may present as cases of reproductive failure. Lastly, lesions of the internal organs typically will involve the spleen, liver, or kidneys and ureters. Lesions of the spleen are often initially asymptomatic, but may result in rupture of the spleen and exsanguination into the peritoneal cavity. Lymphosarcoma of the liver is often asymptomatic. Disease of the kidney and ureter can lead to abdominal pain and the subsequent development of hydroureter or hydronephrosis and clinical signs associated with renal failure.

DISEASE TRANSMISSION

The transmission of BLV occurs through the movement of infected lymphocytes from infected to naïve animals. Cell free virus had not been

documented *in vivo*. Infection may become established by various routes. Inoculation of blood-origin lymphocytes or whole blood from a BLV-infected animal by subcutaneous (Roberts et al., 1982a; Evermann et al., 1986), intramuscular (Evermann et al., 1986; Kelly et al., 1993), intravenous (Evermann et al., 1986; Klintevall et al., 1997), intradermal (Roberts et al., 1982a; Evermann et al., 1986), intrauterine (Roberts et al., 1982a), or intratracheal (Roberts et al., 1982a) routes have all caused infection in BLV negative animals. Intravenous inoculation of infected kidney culture cells (Ungar-Waron et al., 1999) has also been shown to easily transmit viral infection. In one study inoculation with a cell free supernatant from BLV positive cell culture lymphocytes was used to infect a calf (Miller et al., 1972).

Nasal secretions, bronchoalveolar lavage fluid, and saliva have been evaluated for the presence of provirus and their ability to transmit BLV. Provirus within lymphocytes has been isolated in the cellular fraction of bronchoalveolar lavage fluid, but not from the cell free fraction (Roberts et al., 1982b). Sheep and cattle inoculated with infected bronchoalveolar lavage fluid became infected but only 6/9 cattle and 1/6 sheep infected in this manner has detectable BLV in their bronchoalveolar lavage fluid (Roberts et al., 1982b).

Bovine leukosis virus has been found in the nasal secretions of infected cattle by some researchers (Lucas et al., 1993; Roberts et al., 1982b), but not by others (Miller and Van Der Maaten, 1979). In one study, both sheep and cattle in contact with animals that shed the virus in the nasal secretions did not become infected despite documented infectivity of the nasal secretions (Lucas et al.,

1993). Infectivity of saliva has been documented (Ressang et al., 1982). In all cases the saliva from BLV positive animals was injected into naïve animals. In this unnatural setting the secretion was infectious. In one study, sheep that were in contact with a BLV cattle that shed virus in the saliva remained BLV negative. However ¼ calves in contact converted to BLV positive status (Ressang et al. 1982). It is clear that some BLV positive animals will have infectious provirus present in respiratory secretions. However, natural contact studies suggest that this is a minor route of transmission in the majority of cases.

A study that examined the infectivity of urine and feces found both to be noninfectious when injected into naïve sheep (Ressang et al., 1982). In this study the urine samples were spun in a centrifuge and the supernatant was used as the inoculum. The fecal samples were diluted sonicated, and spun in a centrifuge. The supernatant was utilized as the inoculum. Both of these processing methods may have actually disrupted or removed potentially infectious cells from the inoculums. Injection of 100 mls of urine from BLV positive animals into the peritoneum of susceptible sheep did not result in viral transmission (Miller and Van Der Maaten, 1979). Very little work has been done on the infectivity of feces and urine. There is no evidence to support that these excretions are routinely infectious.

Reproductive tissues and fluids such as embryos, semen, and uterine fluids also have been evaluated for the presence of BLV provirus and their ability to transmit the virus. Researchers have found conflicting results. One epidemiologic study found natural service using BLV infected bulls to be a risk

factor for transmission (Ritter, 1965). Another demonstrated the infectivity of semen from one BLV infected bull (Lucas et al., 1980). In this study the bull was collected using rectal massage and between 2-10 ml was injected intraperitoneally into sheep. Three of 11 sheep were infected with BLV by this route. In another study BLV status of the sire was found to have no effect on the BLV status of the progeny using either natural service or artificial insemination (Baumgartener et al., 1978). This is supported by a study that evaluated the risk of artificial insemination on a dairy herd. No increase in transmission could be attributed to the use of artificial insemination (Thurmond et al., 1983a). Infected fresh chilled semen from 4 BLV infected bulls was found to be non infectious in the 8 sheep which were intraperitoneally inoculated (Miller and Van Der Maaten, 1979). This is supported by a study that intraperitoneally inoculated 32 sheep with 2-8 mls of pooled ejaculates from BLV infected bulls after freezing in liquid nitrogen (Kaja and Olson, 1982). In this study none of the sheep developed BLV infection. In addition Embryos collected from infected cows that were placed into negative recipients failed to produce BLV infection in either the recipient cow or the offspring (Eaglesome et al., 1982; Kaja et al., 1984). In all studies animals were exposed to the virus by an unnatural route, typically intraperitoneal injection. It is clear that some bulls shed potentially infectious BLV in semen. It is also documented that cows can become infected via intrauterine inoculation of infected lymphocytes (Roberts et al., 1982a). Based on this information, it is possible for cows to become infected by use of a BLV infected bull. It appears that some methods of collection, rectal massage vs. electroejaculator, may be

more significant risks than others and some processing methods such as freezing semen in liquid nitrogen, may be protective.

The majority of BLV transmission is horizontal. Close contact between BLV negative and BLV positive cattle has been thought to be a risk factor (Straub, 1978; Maas-Inderwiesen et al., 1978; Thurmond et al., 1983a). Many common farm practices have been implicated in viral transmission including tattooing, dehorning, rectal palpation, injections, and blood collection. It is possible that vectors such as tabanids and other large biting flies also may transmit the virus. Vertical transmission may occur transplacentally from an infected dam to her fetus, intrapartum by contact with infected blood, or postpartum from the dam to the calf through the ingestion of infected colostrum.

Contact

Close contact has been considered to be a substantial risk factor for the transmission of BLV (Straub, 1978; Maas-Inderwiesen et al., 1978; Thurmond et al., 1983a). The concept of contact transmission has been supported in one study which found an increased transmission during the winter in a herd that was extensively managed on pasture during the summer and intensively managed in a barn during the winter (Wilesmith et al., 1980). However at least one study has found animal density played no role in increased transmission (Lassauzet et al, 1990a). Survey demographic data also supports contact, as BLV is more common in large dairy herds and small beef herds (NAHMS 1997; NAHMS 1999), both of which are typically more closely housed than their counterparts,

large beef herds and small dairy herds. The exact reasoning behind this increased risk has not been elucidated. It has been demonstrated that infectivity of contaminated blood is time (Buxton et al., 1985) and dose (Evermann et al., 1986) dependent. It seems likely that crowding of animals would enhance the ability of larger quantities of provirus to move from animal to animal more efficiently when they are closer together.

Animal processing

Many veterinary husbandry practices that occur on farms have been implicated as risk factors for BLV transmission. Tattooing BLV negative sheep after BLV positive calves has been shown to be an effective method in transmitting BLV (Lucas et al., 1985). Tattoo inks have not been demonstrated to have any virucidal activity (Lucas et al., 1985). Gouge dehorning also has been demonstrated to be a risk factor (Lassauzet et al., 1990b; DiGiacomo et al., 1985). One study found an increased risk of BLV infection in calves that were dehorned by gouge methods when compared to non-dehorned calves (Lassauzet et al., 1990b). The risk almost doubled when a calf was dehorned after a calf that was BLV positive. Another study evaluated 2 methods dehorning compared to a non dehorned negative control group (DiGiacomo et al., 1985). Within 3 months of dehorning 1/19 control calves, 7/22 gouge dehorned, and 0/14 gouge dehorned followed by cautery and instrument disinfection had seroconverted to BLV positive status. One study attributed a decrease in BLV prevalence in a herd to changing dehorning methods from gouge dehorning of

older calves to cauterization of young calves during a time where colostral origin antibodies may aid in calf protection (DiGiacomo et al., 1987).

Injections

Since blood is the major source of infectious provirus, injections seem a likely risk. Early investigations point to intravenous blood sampling as a risk factor for BLV transmission. One study found animals in which a blood sample was obtained immediately following a BLV positive animal had an 8 fold greater risk of becoming infected with BLV (Wilesmith et al., 1978). This group attributed 80 - 90% of BLV transmission during the study period to blood sampling with multi-use needles. Another study evaluating BLV infection in 52 herds found 293 BLV positive animals after a herd test (Maas-Inderwiesen, 1978). All positive animals were removed from their herds immediately. On a follow up test 3 months later an additional 114 animals were found to be BLV positive. Of these 78 animals had been housed next to a BLV positive animal and 49 had been sampled immediately after a BLV positive animal. This gave rise to the suspicion that needle transmission of BLV may be important.

Unlike multi-use bleeding needles, there is little to support injection needles as a risk factor for BLV transmission. One study evaluated the ability of tuberculin needles and tuberculosis testing as a mode of transmission (Roberts et al., 1981). Fifteen cows and fifteen sheep that were tuberculin tested after a BLV positive cow failed to become infected. When tuberculin needles were intentionally contaminated with BLV positive blood and then used for tuberculin

testing, 3/3 cows and 3/3 sheep became infected. Other studies have found no association between brucellosis vaccination (Lassauzet et al., 1990b) and other routine vaccinations (Thurmond et al., 1983c) with BLV infection. It has also been found that cleaning multi-use needles with cotton wool in between animals may prevent proviral transmission (Roberts et al., 1981). In this study needles were contaminated with blood from a BLV positive cow, wiped clean and used to tuberculin test sheep. They found that wiping needles with cotton wool prevented infection in 3/3 animals tested while cotton wool and water and cotton wool and alcohol only prevented infection in 2/3.

Contamination of blood based vaccines has also been shown to potentially transmit the virus (Rogers et al., 1988). In one case 13,959 doses of a tick fever vaccine was generated with the use of blood from a BLV infected calf that had a negative AGID test prior to vaccine production. In herds where the contaminated vaccine was used the BLV prevalence in vaccinated cattle was 62% in dairy and 51.8% in beef compared to prevalences of 6.1% and 1.5% in non-vaccinated animals in the same herds.

Rectal Palpation

Routine rectal examinations for the determination of health or pregnancy status using multiple use sleeves have been implicated as a potential risk for BLV transmission. Early studies laid the groundwork demonstrating that blood from a BLV positive animal may be infectious when deposited in the rectum of an uninfected animal. In one study transmission occurred in both cattle and sheep

receiving inoculums of 500ml and 50ml blood per rectum, respectively (Henry et al., 1987). Another study placed 2 ml of infected blood on a rectal sleeve and a rectal examination was performed to simulate blood contamination more consistent with that obtained on a routine rectal examination (Hopkins et al., 1988). Each examination was 30 seconds in length and was performed once weekly for three weeks. In all cases animals became BLV positive within 5 weeks. In contrast one study found that the probability of seroconverting to BLV positive status following routine rectal examination was 0.034 and was not associated to the prevalence of infection at the time of rectal palpation (Lassauzet et al., 1989b).

Perhaps the most convincing study to date is one that evaluated transmission in a dairy herd over a 22 month period (Divers et al., 1995). In this study seronegative cattle were housed with seropositive cattle and identified only by neck chain. In this herd one group of cattle had rectal sleeves changed between cows and the other had no sleeves changed between rectal examinations. At the end of the study period cows palpated in the no sleeve change group had a 2.8 fold greater risk of BLV infection than the sleeve change group.

Insects

Blood sucking insects have been considered a risk for the spread of BLV infection in cattle (Bech-Nielsen et al., 1978). Epidemiological studies are split on this point. One large study in France evaluated the incidence and prevalence of

BLV infection in conjunction with horsefly density and geographical distribution (Manet et al., 1989). This study found a correlation between the incidence of BLV infection and the density of horsefly population. They also found a seasonal pattern with new infections higher during the times of peak horsefly activity. Another study demonstrated a seasonal effect with increased transmission occurring during the summer months. This combined with the identification of infected lymphocytes on the mouthparts of horse flies that had been feeding BLV-infected cows led the group to conclude that Tabanids were a substantial risk for BLV transmission (Bech-Nielsen et al., 1978). A United States study evaluating incidence of infection on a 200 cow dairy over a 15 month period found no evidence of a seasonal effect of BLV transmission (Thurmond et al., 1983c) This is supported by a study conducted in a summer grazing and winter housed dairy which did not demonstrate an increase in proviral transmission during the grazing season (Wilesmith et al., 1980).

Experimental studies highlight the potential for blood-sucking insects to serve as a mechanical vector (Buxton et al., 1982; Buxton et al., 1985; Ohshima et al., 1981). When inoculated subcutaneously after ingesting a blood meal the mouthparts from mosquitoes, stable flies, horn flies, horse flies and deer flies all have the capability of infecting cattle and sheep with BLV (Buxton et al., 1982; Buxton et al., 1985). In these studies sheep would consistently become infected when inoculated with lower numbers of infected mouthparts than cattle. Infectivity is also time dependent as mouthparts that were injected one hour or more after a blood meal failed to transmit the virus (Buxton et al., 1985).

When more natural routes of insect exposure are examined, transmission is not as clear. In a study designed to test the interrupted feeding behavior of stable flies, 4 BLV negative calves remained negative after exposure to 75 bites from stable flies that had previously fed on a BLV positive cow (Buxton et al., 1985). An additional study designed to simulate natural exposure placed BLV negative and positive cattle in an enclosure that only allowed animal contact by way of the flies. In this study no transmission of the virus occurred (Buxton et al., 1985). A study that evaluated tabanid flies was able to demonstrate viral transmission in 2/3 lambs that received 131-140 fly bites over a 4 day period (Ohshima et al., 1981)

Summarizing the literature, it is clear that biting insects that have multiple blood meals on different hosts in a short period of time pose the greatest risk. Large numbers of bites from a heavily infected host over a short period of time are required for insect transmission to occur. This makes the total risk of BLV transmission from biting insects small.

Transplacental

Vertical transmission of BLV across the placenta occurs in a proportion of BLV infected cattle. However, the rate at which this occurs is not clear. The rate of transplacental transfer in the published literature is highly variable, ranging from 0.0 – 26 % (Meas et al., 2002; Van Der Maaten et al., 1981b; Jacobsen et al., 1983; Kono et al., 1983; Piper et al., 1979) None of the studies documented that samples were truly precolostral using a quantitative test for passive transfer.

While only one study drew samples from observed parturitions (Jacobsen et al., 1983). In addition, many of these studies were performed in unnatural settings including a herd selected for a high prevalence of enzootic bovine leukosis (Piper et al., 1979) and another in which the pregnant cows were experimentally infected with the virus (Van Der Maaten et al., 1981b).

One potential cause for the wide range in the literature that has not been sufficiently evaluated is that all BLV positive cows may not have the same risk of passing the virus to their fetus. One study found *in utero* infection to be more likely in cattle with a blood lymphocyte count above 12,000 cells/ μ L during pregnancy and in those that developed malignant lymphoma (Lassauzet et al., 1991). This is supported by 2 other studies. One found that 33% of fetuses in cows with lymphosarcoma were positive for BLV prior to birth (Ohshima et al., 1981). Another suggests that transplacental infection may be more common in cows with persistent lymphocytosis (Agresti et al., 1993). In this study 5/18 calves born to PL cows were BLV provirus positive at birth while 0/25 calves born to BLV positive aleukemic cows were infected at birth. Another study found a higher rate of transplacental transmission in cows that were co-infected with BLV and bovine immunodeficiency virus when compared to cows only infected with BLV (Meas et al., 2002).

An association between *in utero* infection and calf sex has been noted by one group (Thurmond et al., 1983a). In this study bull calves were more commonly infected *in utero* than heifer calves. Calf sex has not been evaluated in most *in utero* studies and a male predilection has not been demonstrated in

studies on postnatal BLV infections (Baumgartener et al., 1975; Evermann et al., 1980; Burrige et al., 1981). One study found no association of *in utero* infection with dam age, dam parity, breed, or time of seroconversion (Thurmond et al., 1983a). Another study found no association between *in utero* infection and seropositivity to p24 antibodies (Lassauzet et al., 1991).

It appears that transplacental transmission of BLV in cattle is an uncommon event. It may be that cattle with higher lymphocyte counts, higher proviral loads, and those concurrently infected with other retroviruses may pose greater risks to their calves.

Colostrum and milk

The effect of colostrum on calf BLV status has been previously investigated. In one study the rate of colostrum transmission can be derived from the data although determining a colostrum rate of transfer was not the intended purpose of the work. The data presented suggested colostrum infection rates of 7% were present (Piper et al., 1979). Two divergent conclusions have been drawn from the current body of literature. One suggests that colostrum is a risk factor for the transmission of BLV to neonatal calves (Miller and Van Der Maaten, 1979; Ferrer and Piper, 1981). The other suggests that the antibody present in colostrum serves a protective role in preventing infection in the neonate (Van Der Maaten et al., 1981a; Lassauzet et al., 1989a). Infected lymphocytes are present in both colostrum and milk of BLV infected cattle and it has been shown that the oral ingestion of infected serum origin lymphocytes can transmit the virus early in

life (Baumgartner et al., 1976; Van Der Maaten et al., 1981a). It has also been shown that the cellular component of colostrum and milk is capable of transmitting the virus when injected parenterally (Chung et al, 1986; Miller and Van Der Maaten, 1979). Despite these being unnatural routes of exposure, these studies highlight the potential for the transmission of the virus through infected colostrum and milk. This is particularly concerning during the first 24 hours of life because the neonatal gut will allow the absorption of macromolecules and cells.

Despite the potential for colostrum being a mechanism for BLV transmission, several studies suggest that the maternal antibody present in colostrum may have a protective role against BLV infection (Van Der Maaten et al., 1981a; Lassauzet et al., 1989a). One study demonstrated that calves fed blood origin lymphocytes in BLV antibody positive colostrum did not develop persistent BLV infection. The control group which was fed a smaller number of infected lymphocytes in BLV negative colostrum seroconverted within 2 months (Van Der Maaten et al., 1981a). In the same study, calves that were injected intradermally with a dose of infected lymphocytes that was 10-fold lower than the BLV antibody positive colostrum-fed study group were not as uniformly protected as the oral challenge group (Van Der Maaten et al., 1981a). Another study attributed the low level of transmission via colostrum and milk in calves with continual contact with BLV positive animals to colostral antibodies (Ferrer and Piper, 1981). Perhaps the most convincing study demonstrated that BLV antibody negative calves were 2.0 – 2.7 times more likely to be infected with BLV

when they left the hutches than calves that acquired BLV antibodies via colostrum (Lassauzet et al, 1989a).

The ability of milk to harbor and transmit BLV has been investigated (Chung et al., 1986; Baumgartener et al, 1976; Rubino and Donham, 1984; Roberts et al., 1983). The majority of the studies utilized milk from BLV negative cows and added infected blood origin lymphocytes (Baumgartener et al, 1976; Rubino and Donham, 1984; Roberts et al, 1983). Infectivity on all of these studies was determined by injection of the suspension into sheep. Only one study actually evaluated the ability of milk origin lymphocytes to transmit the virus through intraperitoneal injection (Chung et al., 1986). In all studies, sheep that were injected with milk or milk/lymphocyte suspensions developed infection with BLV. These studies make it clear that milk is a potentially infectious fluid, but epidemiologic studies do not support colostrum and milk as a major source of transmission (Lassauzet et al, 1989a). In addition studies evaluating routes of exposure do not support oral exposure of BLV as a major route of infection (Roberts et al, 1982a).

AVAILABLE TESTS FOR BLV INFECTION

Many tests have been developed to detect BLV in infected cattle. The earliest tests were hematologic assays that indirectly detected infected animals. After the virus was identified, serologic assays and virus or provirus detecting assays were developed.

Hematologic assays

Lymphocytosis – Lymphocytosis is not a true test for BLV status. This test arose from the recognition that farms that had lymphosarcoma also had a proportion of cows that maintained a consistently high blood lymphocyte count (Table 1) (Bendixen, 1959). With this rudimentary understanding of the disease process many European countries began successful control programs. The development of serologic tests dramatically improved both sensitivity and specificity for detection of infected cattle. One study suggests that BLV prevalence may be underestimated by up to 10-fold using hematologic techniques (Maas-Inderwiesen et al., 1978)

Hematologic assays have fallen out of favor for more accurate testing modalities. Despite this, the detection of lymphocytosis may still have some utility in BLV control and eradication. Approximately 29% of BLV positive animals will develop a persistent lymphocytosis consisting of virally infected cells. A BLV positive, hematologically normal cow typically has approximately 2% of its lymphocytes infected with the virus (Mirsky et al., 1996). In contrast, a BLV positive animal with persistent lymphocytosis can be expected to have approximately 39% of its lymphocytes infected with the virus (Mirsky et al., 1996). Cattle with persistent lymphocytosis may serve as a larger reservoir of infection on a farm with a greater number of infected lymphocytes per unit of blood.

Antibody tests

After viral exposure it typically takes 6 - 14 weeks to develop a detectable antibody response to BLV (Evermann et al., 1986). All commercial test kits rely on the detection of gp51 and/or p24 antibodies. Most of the tests currently on the market are gp51 tests. Cross-reactions to the serologic assays have not been documented, nor has infection without antibody production. Positive serology results are consistently present in cattle infected with BLV and cattle not infected with BLV are nearly uniformly negative with regard to their serologic status. However, false-positive and false negative tests do occur under predictable situations. False-positive tests are most commonly seen in calves that receive colostrum from infected dams (Ferrer and Piper, 1981; Van Der Maaten et al., 1981a; Lassauzet et al., 1991). The maternal origin antibody may be detectable for up to 6 months (Burrige et al., 1982a). False negative test results are seen most commonly in periparturient cows (Burrige et al., 1982b). It is postulated that during colostrogenesis it is possible for a cow to move enough serum antibody into the mammary gland that BLV antibody levels drop below a detectable range (Burrige et al., 1982b). This phenomenon has been documented using the AGID and has not been tested using more sensitive serologic test methods. As always, collection and labeling error or laboratory error may play a minor role in generating false positive or false negative test results.

Interpretation of sensitivity and specificity data on serologic BLV tests should be approached with some caution. Reported data for all of the serologic

methods has similar ranges in both sensitivity and specificity. However, the sensitivity of the assays is clearly different when evaluated in comparative studies (Martin et al., 2001; Choi et al., 2002). Currently, there is not one test that is accepted as the gold standard and all available test modalities present at least minor problems that may affect sensitivity and specificity of the test. Based on technology alone, the western immunoblot should be the most accurate followed by the ELISA and finally the AGID. Comparative studies have supported this ranking (Choi et al., 2002). Despite this, the ELISA is probably the most common test run to determine BLV status and the AGID is still the required test for many import/export situations.

AGID – The agar gel immunodiffusion test relies on the precipitation of antigens for test results. The agar gel immunodiffusion was an early antibody detection test for determining BLV status. Early tests that relied on the detection of p24 lacked the sensitivity of the newer tests that identify gp51. The gp51 test has a reported test sensitivity of 0.946 – 0.985 and a specificity of 0.964 – 0.998 (Jacobsen et al., 1985; Monke et al., 1992). This test has an incubation time of 48-72 hours. Reading at the latter test time is responsible for the higher reported sensitivity and specificity data (Monke et al., 1992).

ELISA – There are 2 ELISA test kits currently on the market in the United States. Both tests detect the gp-51 surface glycoprotein. Manufacturer reported sensitivity and specificity are similar for the two tests are similar. Side by side comparisons have not been performed. This test takes less time to run than the AGID, typically under 1 hour. Most test kits require the use of a plate reader,

restricting their use to laboratory settings. The improved sensitivity of the ELISA conforms to the World Health Organization's requirements for use on pooled samples of up to 10 animals. The ELISA has also been documented to perform similar to serum on milk samples (Çarli et al., 1993).

Western immunoblot – Recent work suggests that this test is more sensitive and specific than both the ELISA and the AGID. However, this study used the method as the gold standard in the study so sensitivity and specificity data were not generated (Choi et al., 2002). Previous studies on the western blot suggest a sensitivity of 0.974 of and a specificity of 0.994 (Kittelberger et al., 1999). The western blot only gives reliable results for p24 antigen (Walker et al., 1987; Kittelberger et al., 1996). The gp51 antigen appears to be degraded during the electrophoresis portion of the procedure. This may affect overall sensitivity of the test in that gp51 is the major antigen that cattle respond to immunologically. Historically responses to p24 were not reliably detected. This may be linked to the overall sensitivity of earlier methodologies.

Antigen tests

Polymerase chain reaction – PCR relies on the amplification of specific DNA sequences unique to the BLV provirus. Amplification products can then be visualized in a 2% agarose gel by ethidium bromide staining and ultraviolet transillumination. Polymerase chain reaction assays are relatively new to BLV diagnostics. There are multiple methodologies using different primers yielding some differences in sensitivity (0.627 – 0.984) and specificity (0.89 - 1.0) (Eaves

et al., 1994; Nagy et al., 2003). Since the PCR amplifies the DNA present in the sample, conversion to PCR positive status has been postulated to occur more rapidly than seroconversion (Kelly et al., 1993; Klintevall et al., 1994). This has not held true in all studies (Nagy et al., submitted b). The detection of provirus instead of antibody and the ability to detect small quantities of provirus makes this test viable in neonates, periparturient cows, and potentially in animals with recently acquired infections that have yet to seroconvert. The current cost of the PCR is too expensive for routine use of the test.

STATUS OF THE UNITED STATES

The lack of an official BLV surveillance program has made estimating national prevalence of viral infection difficult. In 1975 a survey involving 4,394 dairy cattle in 100 herds and 2,794 beef cattle in 50 herds found BLV infection present in 66% of the dairy and 14% of beef herds (Baumgartener et al., 1975). Overall, 10.2% of the dairy cattle and 1.2% of the beef cattle tested were positive. In this study dairy herds with less than 50 cows tended to have the highest prevalence of infection. However, very few herds had over 100 cows. Another study evaluating prevalence in herds in 5 states found a BLV prevalence of 28.2 % in dairy and 2.6% in beef herds (House et al., 1977). This study found no association of prevalence to herd size. Production type, age, and previous cases of lymphosarcoma in the herd were associated with BLV prevalence. Prevalence was higher dairy herds, older cows and in herds that had experienced at least one case of lymphosarcoma.

In 1996 the National Animal Health Monitoring System (NAHMS) conducted a study on the prevalence of BLV in United States Dairy Herds (NAHMS, 1997). The study included data from randomly selected herds with 30 cows or more. Twenty states were involved and the study represented 79% of the US dairy cattle population at that time. The study revealed that 89% of herds had BLV-infected cows on the premises and the prevalence of BLV infection in US dairy cattle was estimated to be 44%. Of infected herds 16.9% had 75% or more of the cows infected and 44% of herds had at least 50% of the cows infected.

In 1997 NAHMS conducted a similar study in United States beef herds. The study included data from 2713 operations in 23 states (NAHMS, 1999). The total numbers of animals enrolled were not high enough to be representative of the national beef cattle population. However, these data are the best we have to date on beef cattle in the US. In this study, 38% of herds had BLV-infected cattle and 10% of all animals tested were infected with the virus. Unlike dairy, the majority of infected beef herds had low prevalence of infection with the herd. Of herds tested 56% had less than 25% of the cattle infected.

At slaughter, mature cows are second to bob veal in the percentage of condemnations, ranging from 2.11 – 2.77% per year from 1998-2002 (USDA:FSIS Animal disposition reporting system). Malignant lymphoma was the single highest cause of cow condemnations every year in these years (Table 2).

TRADITIONALLY RECOMMENDED CONTROL PROGRAMS

The original control and eradication programs for BLV were focused on identification and culling of hematologically positive cattle (Bendixen, 1959). Utilizing these methods many countries were thought to have substantially decreased BLV prevalence prior to the identification of the virus and development of more accurate serologic tests. Serology has better sensitivity and specificity in identification of BLV infected cattle. Once serologic tests became available programs centered on the culling of serologically positive cattle.

In some herds the prevalence of BLV infection makes culling all positive cattle cost prohibitive. In this light test and segregation programs evolved. The earliest serology based test and segregation program documented success of this method in the adults, but failure in the calves of a single herd (Van Der Maaten and Miller, 1979). In this herd segregation of positive and negative animals over a 12 month period led to only 4/27 new infection, with 2 of them purchased animals of unknown status. In the calves, 6/8 calves converted to seropositive status over a 7 month period. A control herd that tested without segregation found 9/16 cows seroconvert during a time period in which the test herd only saw 1/18, suggesting the positive effect of segregation. The same researchers demonstrated similar results in a research facility and a commercial breeding service.

An experiment evaluating a test and segregation protocol on 6 commercial dairy herds demonstrates the potential for these programs (Shettigara et al., 1989). In this study six herds were tested using the gp51 AGID at 6 month

intervals. This study involved segregation of BLV negative and BLV positive cattle by a minimum of 200 yards. An additional group to quarantine cows of unknown status was also required. New additions were tested 30 days prior to entry and remained in the quarantine herd until a second negative test at least 60 days from entry into the quarantine herd. In 3 herds with initial prevalences of 7.1%, 2.8%, and 7.1%, herd negative status was achieved after one test and segregate cycle. In 2 herds with initial prevalence of 7.9% and 12.2%, herd negative status was achieved after 3 test and segregate cycles. In the last herd with an initial prevalence of 24.5%, the first herd negative test was achieved at 4 test and segregation cycles. However, problems with keeping the groups separate led to additional reactors and complete negative status was not achieved for a total of 9 test and segregation cycles.

An experiment on test and cull in 5 herds demonstrated the ability to use the AGID in test successfully in BLV eradication (Mammerickx et al., 1978). In this study 5 herds with varying prevalence all achieved BLV free status within 3 test and cull cycles. Two herds with prevalences of 2% and 10% achieved negative status after 1 cycle, 1 herd with an initial prevalence of 22% achieved negative after 2 cycles and 2 herds with prevalence of 37% and 56% achieved negative status after 3 cycles.

In addition to test and cull or test and segregation programs, managemental changes without segregation and culling practices have been evaluated in a commercial dairy herd (Sprecher et al., 1991). In this study single use needles, single use rectal sleeves, disinfection of tattoo pliers in between

calves, electric cautery for dehorning, heat treatment of colostrum, and cessation of dump milk feeding were instituted. Within the 2 year study the prevalence of BLV in all cohorts decreased. Overall prevalence in the age groups studied (0-1st parturition) decreased significantly from 0.44 to 0.17.

Selective culling based on *in vitro* antigen expression from infected cows has also been evaluated (Molloy et al., 1994). These researchers found that culling of BLV positive cows with the highest *in vitro* antigen expression had the ability to considerably decrease BLV transmission on farms. Cows with higher antigen expression were also found to have higher lymphocyte counts and higher proviral loads based on serial dilution PCR.

PROBLEMS WITH TRADITIONAL CONTROL PROGRAMS

Based on this information, it is clear that test and segregate and test and cull programs can be effective, even in high prevalence herds. However, the lack of indemnity, the high prevalence of nation wide infection, and the lack of mandatory control programs, cripples BLV control at the level of the farm. In addition control programs rely heavily on serologic detection of BLV which is adequate in adults, but unreliable in calves.

There is currently no incentive, financial or otherwise, to entice a commercial cattleman to eradicate BLV in their herd. National prevalence is so high that it is almost as likely to buy a BLV positive cow as a BLV negative cow during the purchase of a cow of unknown status. Since the negative outcome of viral infection is so rare, many people have trouble finding the incentive in being

BLV free. Test and cull programs can become financially crippling in herds with a BLV prevalence over 10%. Test and segregation programs are extremely laborious. If strict adherence to the segregation procedures is not followed, failure to control viral infection in the herd may result (Shettigara et al., 1986). Even programs that routinely survey cattle for BLV can be foiled by the latent period. The time from viral infection to serologic detection can be as long as 90 days if the infectious inoculum is small (Evermann et al., 1986). This lag time is sufficiently long to allow for the accrual of new infections even if positive animals are removed from the herd once detected. The isolation of purchased additions from the rest of the herd can be problematic in the lag time to positive tests. Failure to isolate and repeatedly test can result in program failure (Shettigara et al., 1986). The rapid results documented in the early control and eradication studies are misleading. In the majority of cases, studies required testing over multiple years to become BLV free. Compliance was often a problem in commercial herds, resulting in delayed attainment of BLV free status or lost status. The Finnish eradication program is an example of the complexities of test and cull programs. Despite a stringent test and slaughter program with an indemnity in place, it took 30 years to completely eradicate BLV from Finland (Nuotio et al., 2003).

Traditional control programs rely on serology to detect infected individuals. On the surface, this appears to be adequate when you consider that once a cow becomes infected with the virus they develop a life long antibody response to BLV. However, these programs do not have an adequate way to address

calfhood infection. Calves that have received colostrum from infected cows will test positive to BLV serologic tests due to the absorption of colostral origin antibodies (Piper et al, 1979; Burridge et al, 1982a). Detection of this maternal origin antibody may last as long as 6 months (Burridge et al., 1982a). In most commercial dairy herds calves leave individual calf hutches for small group housing by 8 weeks of age. This loss of quarantine may allow for viral spread through a group of animals if there is a BLV positive calf present at the time the calves are moved to group housing. Problems associated with identifying BLV infection in neonatal calves have shifted the focus of control programs to the adult herd. This may be a critical financial loss to the farmer as substantial investment in time and materials is invested in each heifer calf prior to identification of infected replacements.

CHAPTER 2

USE OF A POLYMERASE CHAIN REACTION ASSAY TO DETECT BOVINE LEUKOSIS VIRUS IN DAIRY CATTLE

INTRODUCTION

Bovine leukosis virus (BLV) is a retrovirus of cattle belonging to the BLV-HTLV group. This oncogenic virus is transmitted from infected cattle to naïve cattle via blood-to-blood transfer. Transmission can occur during the course of daily farm activities such as tattooing (Lucas et al., 2001) and dehorning (DiGiacomo et al., 1985; Digiacomo et al., 1987), and by reuse of contaminated rectal sleeves (Henry et al., 1987; Hopkins et al., 1988; Divers et al., 1994) or hypodermic needles (Wilesmith et al., 1979). Transmission by tabanid flies has also been documented (Manet et al., 1989; Ohshima et al., 1981).

Serologic methods for diagnosis of BLV infection, such as agar gel immunodiffusion test (AGID) and ELISA, are commonly used to identify infected animals. These tests are sensitive (ELISA, 0.98; AGID, 0.946 to 0.985) and specific (ELISA, 1.0; AGID, 0.964 to 0.998) for detection of infection (Jacobsen et al., 1985; Monke et al., 1992; Field testing data set for USDA license, VMRD Inc, Pullman, WA). Despite the availability of tests that are both sensitive and specific for BLV, diagnosing infections in neonates and periparturient cows remains

problematic (Burridge et al., 1982a; Evermann and Jackson, 1997; Ferrer et al., 1977; Johnson and Kaneene, 1991a). Passive transfer of colostral immunoglobulin from BLV-positive cows to their offspring may cause false-positive serologic test results, which persist for the first 6 months of life (Burridge et al., 1982a; Burridge et al., 1982b). Consequently, testing by serologic methods is not reliable in post-colostral neonatal calves, severely limiting our ability to cull infected calves prior to substantial financial investment in those animals.

Relying on serologic tests to determine BLV status of recently purchased cattle is also problematic. Seroconversion may not develop for as long as 90 days following experimental viral exposure (Evermann et al., 1986). The inability to detect BLV in recently exposed cattle forces prolonged isolation of recently purchased cattle to assure that BLV-positive animals are not introduced to the herd. Most farms lack the facilities and the commitment to impose on-farm quarantine periods of long duration.

Polymerase chain reaction (PCR) assays may eliminate these concerns, because PCR can detect BLV provirus despite the presence of maternal antibody (Agresti et al., 1993). Polymerase chain reaction assays detect small quantities of virus, potentially leading to fewer false negative tests in recently exposed animals. The purpose of the study reported here was to evaluate the use of a PCR assay to detect BLV in an established dairy herd with a high prevalence of BLV.

MATERIALS AND METHODS

Cows – 223 adult dairy cows owned by the University of Missouri Foremost dairy were used in this study. The study was approved by the Institutional Animal Care and Use Committee.

Laboratory specimens – Blood samples for ELISA and PCR assay were collected simultaneously. Samples for ELISA were collected into tubes without anticoagulant. Serum was separated and stored at 4°C for further analyses. If an initial ELISA result was negative, a second ELISA was performed approximately 95 days later to confirm that the cow was not infected with BLV.

Samples for PCR assay were collected into tubes containing EDTA. Samples were stored at 4°C and processed within 48 hours. Lymphocytes were isolated by use of a commercially available erythrocyte lysis (EL) buffer (Erythrocyte lysis buffer, Qiagen Inc, Valencia, CA). Lymphocytes were washed 3 times with EL buffer and stored at –70°C for subsequent analysis. The DNA was extracted by use of a commercially available kit (QIAamp DNA mini kit, Qiagen Inc, Valencia, CA) following the manufacturers' protocol.

ELISA – A commercial assay (Bovine leukemia virus antibody test kit, ELISA, VMRD Inc, Pullman, WA) was used to detect of BLV antibodies. A positive test result was defined as a sample-to-positive ratio (S:P) > 0.5 and a negative test result was defined as a S:P ≤ 0.5, with S:P calculated as

$$\text{S:P} = \frac{(\text{sample absorbance} - \text{mean negative control absorbance})}{(\text{positive control absorbance} - \text{mean negative control absorbance})}$$

PCR assay – Forward (5'-TGG CTA TCC TAA GAT CTA CTG-3') and reverse (3'-AGA GGG AAC CCA GTC ACT GTT-5') primers were selected on the basis of a published report (Klinteval et al., 1994). The DNA sequences were amplified in a 25- μ l reaction mixture containing 0.5 μ M of each primer and 1.0 unit Taq (HotStarTaq, Qiagen Inc, Valencia, CA) DNA polymerase in the manufacturer's buffer, which contained 2.0mM MgCl₂ and 0.2mM (each) dNTPs. The reaction was performed in a programmable thermocycler.(Perkin-Elmer 9700, Perkin-Elmer Inc, Shelton, CT) An initial incubation of 12 minutes at 95°C was followed by 10 cycles of denaturation at 95°C for 30 seconds, annealing at 70°C for 30 seconds, and extension at 72°C for 90 seconds. Annealing temperature was reduced by 1°C each cycle. An additional 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 90 seconds were performed, followed by a final extension at 72°C for 7 minutes. Amplification with BLV-specific primers yielded a product of 330 base pairs. Amplification products were isolated in a 2% agarose gel and stained with ethidium bromide (Sambrook et al., 1989). Each batch was performed with known positive and negative samples as controls.

Data analysis – Sensitivity, specificity, predictive values of positive and negative tests and percentage of cows correctly classified by PCR assay were calculated with standard epidemiologic methods (Tyler and Cullor, 1989). A BLV-positive cow was defined as having a positive ELISA result. A BLV-negative cow was defined as having 2 sequential negative ELISA results. Cows with initial negative ELISA results that were positive on the second test were not included.

True positive, true negative, false positive, and false negative rates were also calculated. Ninety-five percent confidence intervals (CI) were calculated for sensitivity and specificity (Daniel, 1999). Positive predictive values (PPV) and negative predictive values (NPV) at all possible prevalences were calculated as:

$$\text{PPV} = \frac{(\text{sensitivity} \times \text{prevalence})}{([\text{sensitivity} \times \text{prevalence}] + \{[1 - \text{specificity}] \times [1 - \text{prevalence}]\})}$$

$$\text{NPV} = \frac{(\text{specificity} \times [1 - \text{prevalence}])}{([\text{specificity} \times [1 - \text{prevalence}]] + [\text{prevalence} \times [1 - \text{sensitivity}]])}$$

False negative test rates for serial tests were calculated as:

$$\text{False negative rate} = (1 - \text{sensitivity})^{\text{number of tests (smith, 1995)}}$$

RESULTS

Polymerase chain reaction and ELISA results were positive for 121 cows and were negative for 43 cows. Fifty-nine cows had a positive ELISA result, but a negative PCR assay result. No cows had a negative ELISA result and a positive PCR assay result. Sensitivity and specificity for the PCR assay were 0.672 (95% CI, 0.611, 0.733) and 1.00, respectively. A CI was not calculated for specificity because performance of the PCR assay resulted in no false positives. Prevalence of BLV in this herd was 0.807. Positive and negative predictive values were 1.00 and 0.421, respectively. The percentage of cows correctly

classified by PCR assay was 73.5%. The PPV and NPV at all possible prevalences of infection for cows infected with BLV were determined (Figure 1).

In populations with an initial prevalence of BLV of 5%, 10%, and 15%, serially testing each animal twice by use of the PCR assay can decrease prevalence to 0.5%, 1.1%, and 1.6%, respectively (Figure 2). Testing each animal 3 times will decrease prevalence to 0.2%, 0.4%, and 0.5%, respectively. Cattle that have positive PCR assay results are removed after each test.

DISCUSSION

In this study, use of a PCR assay was evaluated in a dairy herd with a high prevalence of BLV. Specificity of the PCR assay in determining BLV status in adult cows in this herd was ideal; hence PPV was ideal. The PPV of the PCR assay was independent of prevalence because specificity was 1.00. Consequently, a positive test result by use of PCR assay indicates that the cow tested is infected with BLV. Sensitivity of the PCR assay used in this study was low. Consequently, NPV was also low, making the PCR assay unreliable in herds with a high prevalence of BLV. The NPV is dependent on prevalence and is improved in populations with lower prevalence of disease.

Performance of the PCR assay was promising and it may provide a useful adjunct to traditional testing programs. However, in most circumstances serologic tests will be superior to the PCR assay because higher sensitivity (> 95%) will expedite identification of BLV-infected animals. Potential applications of the PCR assay may be restricted to 3 specific situations in clinical practice. First, the PCR

assay could be used to detect BLV in neonatal calves with passive transfer of maternal antibodies against BLV. Second, the PCR assay could be used as a confirmatory test in valuable animals that may otherwise be culled as a result of a positive serologic test. Third, the PCR assay could be used to identify cattle that were recently exposed to BLV, before seroconversion develops. However, to our knowledge, there is no available data to support the hypothesis that a PCR assay will detect BLV-positive cattle before seroconversion.

Polymerase chain reaction assay also may be of use in neonatal calves. Calf BLV-status cannot reliably be determined by use of serologic tests until 6 months of age (BurrIDGE et al., 1982a; BurrIDGE et al., 1982b). This is undesirable because infected and noninfected calves would likely be commingled at 8 weeks of age permitting amplification of low prevalence infections. A strategy to remove BLV-positive cattle solely on the basis of serologic status may be unsuccessful because of the prolonged duration that may be required for seroconversion following exposure to BLV. Despite being seronegative, infected calves may continue to be a source of BLV throughout the incubation period. As new seropositive carriers are removed, new incubationary carriers will have already been added to the group and will serve as reservoirs for infection.

The PCR assay can identify BLV in calves with maternally acquired antibody. Calves may be tested before commingling, eliminating the potential for horizontal transmission. Detection of infection by use of the PCR assay in experimentally infected cows suggests that the incubation period for BLV may be as short as 1 week (Kelly et al., 1993). Therefore, the initial PCR assay could be

completed at 2 weeks of age. Retesting BLV-negative calves at 5 weeks of age would detect calves that may have been incubating BLV when the initial test was performed. This diagnostic strategy would facilitate early removal of BLV-positive calves. The shortened interval between infection and a confirmed diagnosis would eliminate incubationary carriers, assuring that only BLV-negative heifers enter the milking herd. Raising BLV-negative replacement heifers is crucial to decreasing BLV prevalence within a herd.

Serial testing could be used to increase the accuracy of testing procedures when applying a PCR assay to BLV control programs. Serial PCR assays can decrease the rate of false negative tests. In populations with low prevalence of BLV, 2 consecutive tests will reduce the false negative rate to an acceptable level, whereas 3 consecutive tests will reduce prevalence to a clinically unimportant level. In populations with high prevalence of BLV, serial testing could improve the ability to identify all infected cattle.

Early removal of BLV-positive calves is important to dairy herd management. Rapid seroconversion to BLV-positive status once heifers reach the milking herd has been reported (Piper et al., 1979). This may represent exposures that developed before heifers entered the milking herd, indicating that neonate and juvenile transmission of BLV may be more important than previously recognized. The PCR assay may be of use in detecting BLV in recently exposed cattle and neonatal calves. In both of these groups, results of serologic testing may be inaccurate. However, serologic methods of BLV detection are more sensitive for routine screening of adult cattle.

CHAPTER 3

ASSOCIATION BETWEEN THE STRENGTH OF SEROLOGIC RECOGNITION OF BOVINE LEUKOSIS VIRUS AND LYMPHOCYTE COUNT IN BOVINE LEUKOSIS VIRUS-INFECTED COWS

INTRODUCTION

Bovine leukosis virus (BLV) is an oncogenic retrovirus of cattle. Transfer of lymphocytes from infected cattle to naïve cattle spreads the virus. Many common management practices including gouge dehorning (DiGiacomo et al., 1985; Digiacomo et al., 1987), ear tagging, and tattooing (Lucas et al., 2001) have been implicated as mechanisms of viral transmission (Johnson and Kaneene, 1991b; Thurmond, 1991). Alternatively, the virus may also be transmitted by insects (Buxton et al., 1982; Buxton et al., 1985; Manet et al., 1989; Ohshima et al., 1981), natural service (Johnson and Kaneene, 1991b), transplacental transmission from dam to the fetus (Van Der Maaten et al., 1981b; Thurmond et al., 1983a; Piper et al., 1979; Jacobsen et al., 1983), and ingestion of infected colostrum (Ferrer and Piper, 1981). The 1996 National Animal Health Monitoring System's Dairy 96 study confirmed that 89% of U.S dairy herds were endemically infected with BLV (NAHMS, 1997). Of these positive herds 75% and 44% had herd prevalences above 25% and 50% respectively.

Among infected cattle, a subset will develop persistent lymphocytosis (PL), a benign lymphoproliferative condition. The estimated prevalence of PL in BLV infected cattle is 29% (Ferrer et al., 1979a). PL is more prevalent in cattle of certain lineages. Persistent lymphocytosis rates of up to 88% have been documented within some familial groups (Ferrer et al., 1979a). The presence of PL has not been definitively associated with the development of clinical lymphosarcoma (Ferrer et al., 1974; Abt et al., 1975). However, PL may still be clinically important because the virus is transmitted through infected lymphocytes (Gotze et al., 1956). A logical assumption is that cattle with lymphocytosis pose a greater risk to the herd. Cows with PL may be more important reservoirs for BLV transmission because their blood contains higher concentrations of infected lymphocytes than PL-negative cattle. Consequently blood from PL-positive, BLV-positive cows is likely to be more infectious than blood from PL-negative, BLV-positive cows.

Early European eradication programs premised upon the removal of cattle with PL and experimental infection trials both have substantiated that increased infectivity is associated with increased lymphocyte counts (Bendixen, 1959; Ferrer, 1979). Prior to establishing the etiologic agent of enzootic bovine leukosis or the development of sensitive and specific serologic assays, many European eradication programs focused on the presence of lymphocytosis to target cows for culling. Programs that advocated the slaughter of PL-positive cows and those that focused on segregation of PL-positive cows both had success (Bendixen, 1959). Results of experimental infection trials have substantiated this theory by

correlating lymphocyte dose with time to seroconversion and more importantly proportion of cattle that become infected after challenge. Results of recent studies substantiate that PL-positive cattle are more important as reservoirs of BLV. The increase in lymphocyte count seen in PL-positive, BLV-infected cows is attributable entirely to proviral-infected lymphocytes (Mirsky et al., 1996).

While systematic culling of lymphocytotic cows may rid a herd of BLV, this approach is not as practical for herds with a high incidence of BLV-infected cows. It is logical to attempt to lower the total virus load on the farm by culling the cattle that contribute the most to the overall viral load. Whole herd sampling for lymphocyte count is difficult due to the laborious nature of this laboratory procedure. Alternatively, ELISA sample-to-positive ratio (S:P) may potentially serve as a substitute to measure infectiousness. Multiple serum samples for S:P are easier to process and obtain timely results than blood lymphocyte counts, particularly because ELISA methods have become automated.

If the strength of the serologic response to BLV and lymphocyte counts are directly related, cattle with a higher S:P with respect to BLV infection would be presumed to be more important reservoirs of infection. These cows would then be targeted for earlier removal in eradication programs. Prior studies have failed to identify associations between serologic recognition by use of the agar gel immunodiffusion (AGID) assay and blood lymphocyte counts (Itohara et al., 1985). Current ELISA technology is better adapted to the exploration of this hypothesis because unlike AGID assays, S:P more closely approximates a continuous endpoint as a measure of serologic recognition. The purpose of the

study reported here was to determine whether the strength of serologic recognition of BLV by use of ELISA was associated with blood lymphocyte counts.

MATERIALS AND METHODS

Cows – Initially, adult cows at the University of Missouri Foremost Dairy were tested for BLV status by use of a serum ELISA. All cows that tested positive for BLV (n=161, 146 Holstein and 15 Guernsey) were included in the study.

ELISA – A commercial assay was used to detect BLV antibodies (Bovine Leukemia Virus Antibody Test Kit, HerdChek, IDEXX Laboratories, Inc., Westbrook, ME). Briefly, the assay used a microtitration format in which BLV antigens were bound in the microassay wells. Antibodies within the test sample formed a complex with the bound viral antigens during incubation of the sample. The sample was washed and an anti-bovine IgG:Horseradish peroxidase conjugate was added, which bound to bovine antibody attached to the wells. Unbound conjugate was removed by washing. An enzyme substrate and chromogen tetramethylbenzidine were added. The absorbance at 620 nm was measured spectrophotometrically. A positive test result was defined as S:P >0.5, with S:P calculated as follows:

$$S:P = \frac{(\text{Sample absorbance} - \text{negative control absorbance})}{(\text{positive control absorbance} - \text{negative control absorbance})}$$

Lymphocyte counts – Blood samples were collected in EDTA-containing tubes. Total leukocyte counts were determined with an automatic cell counter (Coulter 880 automatic cell counter, Hialeah, FL). Blood films were prepared from fresh EDTA-treated blood and stained on an automatic slide stainer (Ames Hematek 1000 slide stainer, Bayer Corporation, Elkhart, IN) with Wright's-Giemsa stain. Differential leukocyte counts were completed via microscopic examination and total lymphocyte concentration was calculated as the product of total WBC count and lymphocyte proportion determined by differential cell counts.

Data analysis – Sample-to-positive ELISA values were compared between lymphocytotic and nonlymphocytotic cows by use of 1-way ANOVA. Lymphocytosis status was defined by use of the Bendixen method (Bendixen, 1959). Thereafter, a forward stepwise regression model was constructed to evaluate the association between the dependent variable, blood lymphocyte concentration and the independent variables. The independent variables included, S:P, age in months, and the interaction of the preceding 2 terms. A p value of 0.05 was required for variables to enter the model. At each step, the variable with the lowest p value was added to the model. Multiple transformations of the dependent and independent variables were performed to determine which model best predicted S:P. The model with the highest r^2 was chosen as the final model.

A dataset was constructed that contained lymphocyte counts and S:P to compare the effects of 3 strategies of removing BLV positive cows from an

endemically infected herd. These strategies included random culling of infected cattle, culling based on S:P, and culling based on lymphocyte count. Initially, the data set was sorted in descending order on the basis of S:P. Lymphocyte counts were summed and the percentage contribution of each cow to the herd total lymphocyte count was calculated. A cumulative frequency distribution was constructed to evaluate each cow's contribution to the total lymphocyte pool on the basis of S:P. Likewise, a similar cumulative distribution was created on a data set, which was sorted from the highest to lowest lymphocyte count. These distributions were depicted graphically. Linear regression models were developed that predicted cumulative contribution to herd total lymphocyte as a function of either S:P or lymphocyte count using the described data sets ordered on S:P or lymphocyte count.

RESULTS

Mean S:P between lymphocytotic (2.58 ± 0.36) and nonlymphocytotic (2.38 ± 0.39) cows varied significantly ($P < 0.003$). The regression model which best-predicted lymphocyte count was as follows:

$$\text{Lymphocyte count} = 2304.52 + (-38.06) \text{ age in months} + (2827.06) \text{ S:P}$$

Age and S:P were both significantly associated ($P < 0.001$) with lymphocyte count ($r^2=0.091$).

Cumulative group lymphocyte numbers ordered by lymphocyte count and S:P are illustrated (Figure 3). Cows with a higher S:P contributed more to the total group lymphocyte load, but this increased contribution was not substantial. Cows with high lymphocyte counts were scattered throughout the range of S:P.

DISCUSSION

In the original Danish eradication program, BLV infected herds were identified by detection of lymphocytotic cows (Bendixen, 1959; Radostitis et al., 1994). Once identified, the herd was placed on a mandatory control program. Herds on the program were restricted from contact with negative herds. Sale of cattle was prohibited with the exception of slaughter, and all contagious material from the farm was controlled. Cows were then culled or segregated until lymphocytosis could no longer be detected in the herd. By using this simple program, national BLV infection dropped to a negligible amount (Radostits, 1994)

In cattle infected with BLV, as determined by ELISA (Bovine Leukemia Virus Antibody Test Kit, HerdChek, IDEXX Laboratories, Inc., Westbrook, ME), S:P and lymphocyte count are related. While the association was significant ($P < 0.001$), the magnitude of the association is so small that it should be considered of minimal biological relevance. Since the identification of BLV as the causative agent in enzootic bovine leukosis, control programs have focused on limiting transmission of the virus and culling antibody-positive cows. The concept of targeted culling on the basis of lymphocyte count to reduce whole farm virus load

was lost as the hematologic indicators of infection gave way to serologic test methods, which offer greater sensitivity.

Work has been done demonstrating that BLV can be eradicated from infected herds by use of the AGID test. One study revealed that eradication from a high-prevalence herd (>30% infected) can be achieved within 90 days (Shettigara et al., 1986). However, strict adherence to the protocol was necessary. Herds that had even minor noncompliance problems had difficulty eradicating the virus. The study protocol included entire-herd testing at 0, 1, 3, 6, 12, 18, 24, and 30 months. Reactors were to be immediately isolated 200 meters from the negative herd and removed from the farm within 30 days. Replacement cattle were tested 30 days prior to purchase and within 60 days of being on the premises. All new introductions were to be isolated until negative test results were obtained. No contact was allowed with cattle from other herds. While this protocol clearly allowed for the eradication of the virus, it is not practical for many commercial farms. Isolation of cattle requires a separate facility and increased personnel hours to care for the cattle until their removal from the farm. The rigorous culling of cattle can be financially devastating. The prices that farmers currently obtain for cull cows will not cover what they must spend to replace the cow with a noninfected cow. The requirement for no contact with cattle from other herds will require all cattle that attend shows, exhibitions, sales, or move to breeding facilities to be isolated and have 2 negative test results before returning to the herd. These strict protocols are unrealistic for many farmers and the aggressive culling is financially limiting.

Unfortunately, in herds that have a high incidence of BLV infection, the systematic culling of cows on the basis of a positive test status is economically prohibitive. Presently, control is focused on preventing further transmission of the virus between cows. The random culling of cows on the basis of antibody-positive status would result in similar proportions of virus being removed from farms. For example, if 10% of the cows were culled, 10% of the infectious virus would be removed from the herd; if 20% were culled, 20% of the virus would be removed.

Culling cows purely on the basis of S:P should be approached cautiously because many high-S:P cows are not lymphocytotic. Conversely, high lymphocyte counts were observed in cows with a more modest S:P. Culling 10%, 20%, and 30% of cattle with the highest S:P would remove 12.4%, 23.3% and 32.7% of infected-lymphocyte load within a herd, respectively. This is better removal of virus load than that achieved by random culling, but the advantage gained is less than substantial.

Alternatively, culling 10%, 20% and 30% of cattle with the highest lymphocyte count in a BLV-positive herd would remove 23.9%, 40.1% and 52.4% of infected-lymphocyte load within a herd. This means that culling BLV-positive cows with high lymphocyte counts may serve as a means to control the amount of infectious virus on a farm. This type of targeted culling program in a herd with a high prevalence of BLV infection could decrease the amount of virus present for the at-risk population, while allowing for more economically acceptable culling of cattle with a positive serologic test.

CHAPTER 4

TIMING OF ELISA SEROCONVERSION AND ACQUISITION OF POSITIVE POLYMERASE CHAIN REACTION ASSAY RESULTS IN CALVES EXPERIMENTALLY INFECTED WITH BOVINE LEUKOSIS VIRUS

INTRODUCTION

Bovine leukosis virus (BLV) is an oncogenic retrovirus of the BLV/HTLV group. Approximately 44% of US dairy cattle (NAHMS, 1997) and 10% of US beef cattle (NAHMS, 1999) are BLV infected. Cattle infected with BLV mount immune responses to both viral surface and core antigens. Consequently, calves that ingest colostrum from BLV infected cows will have positive BLV serologic test results, regardless of their infection status. Previous studies indicate that these positive serologic assay results may persist for as long as 6 months (Burridge et al., 1982a)

Transmission of BLV in the perinatal period can occur *in utero* (Van Der Maaten et al., 1981b; Thurmond et al., 1983a; Piper et al., 1979; Jacobsen et al., 1983), or through the ingestion of infected colostrum (Piper et al., 1979). *In utero* and colostrum infection of the fetus and neonate has been investigated and transmission is estimated to be 3%-18% and 12% respectively (Van Der Maaten et al., 1981b; Thurmond et al., 1983a; Piper et al., 1979; Jacobsen et al., 1983;

Ferrer and Piper, 1981). Infection during parturition has been postulated to occur, but has not been investigated in depth. A recent study observed a 33 % transmission rate in colostrum deprived calves born to BLV infected cows (Nagy et al, submitted). In a control group of colostrum fed calves born to BLV infected dams, transmission rate was 0%. It was postulated that the exposure to the virus in the colostrum-deprived group occurred at parturition.

Blood inoculation of naïve calves by intradermal, intravenous, intramuscular, and subcutaneous routes efficiently transmits BLV (Evermann et al., 1986). Seroconversion after blood inoculation has been documented to occur as early as 3 weeks and as long as 14 weeks (Evermann et al., 1986). Time to seroconversion appears to be dose-dependent (Evermann et al., 1986). One study demonstrated that seroconversion measured by the AGID lags behind PCR identification of provirus by 2-4 weeks (Kelly et al., 1993). Another study comparing the AGID, ELISA, and PCR found PCR recognition to occur between days 7-56 depending on challenge dose with 3 animals remaining negative by all test methods (Klintevall et al., 1994). Seroconversion lagged behind PCR conversion in all calves; however, data was not presented to determine if the timing was similar for all calves. Most studies comparing serology and PCR detection of BLV infection use the AGID as the serologic test comparison. The purpose of this study was to determine the time to proviral and antibody detection by ELISA in calves after experimental infection. We hypothesized that PCR conversion would precede seroconversion.

MATERIALS AND METHODS

Calves – Eight colostrum deprived, Holstein bull calves 6 weeks of age or older were used for the study. Each calf had 5 sequential, weekly negative ELISA and PCR test results prior to being enrolled in the study.

Infection procedure – Each calf was injected with fresh whole blood in ACD from a BLV positive, non-persistent lymphocytosis cow. The inoculum for each calf contained approximately 2,000,000 lymphocytes.

Laboratory specimens – Blood samples for ELISA and PCR assays were collected from calves prior to inoculation and weekly thereafter for 7 weeks by jugular veinipuncture. Samples for ELISA were collected into tubes with no anticoagulant and serum was harvested after centrifugation. Samples for PCR assay were collected into tubes containing EDTA. Samples were stored at 4°C and processed within 48 hours. Lymphocytes were isolated from the whole blood samples by use of a commercially available erythrocyte lysis (EL) buffer (Erythrocyte lysis buffer, Qiagen Inc, Valencia, CA). Lymphocytes were washed 3 times with EL buffer and stored at –70°C for subsequent analysis. The DNA was extracted by use of a commercially available kit (QIAamp DNA mini kit, Qiagen Inc, Valencia, CA) following the manufacturer’s protocol.

ELISA – A commercial assay (Bovine leukemia virus antibody test kit, ELISA, VMRD Inc, Pullman, WA) was used to detect of BLV antibodies. A positive test result was defined as an optical density > the mean of the positive controls and a negative test result was defined as an OD < the mean of the

negative controls. For test validation the mean OD of the positive controls was ≥ 0.250 and the mean OD of the negative controls was ≤ 0.200 .

Polymerase chain reaction assay – Forward (5'-TGG CTA TCC TAA GAT CTA CTG-3') and reverse (3'-AGA GGG AAC CCA GTC ACT GTT-5') primers were selected on the basis of a published report (Klintevall et al., 1994). The DNA sequences were amplified in a 25- μ l reaction mixture containing 0.5 μ M of each primer and 1.0 unit Taq (HotStarTaq, Qiagen Inc, Valencia, CA) DNA polymerase in the manufacturer's buffer, which contained 2.0mM MgCl₂ and 0.2mM (each) dNTPs. The reaction was performed in a programmable thermocycler (Perkin-Elmer 9700, Perkin-Elmer Inc, Shelton, CT) An initial incubation of 12 minutes at 95°C was followed by 10 cycles of denaturation at 95°C for 30 seconds, annealing at 70°C for 30 seconds, and extension at 72°C for 90 seconds. Annealing temperature was reduced by 1°C each cycle. An additional 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 90 seconds were performed, followed by a final extension at 72°C for 7 minutes. Amplification with BLV-specific primers yielded a product of 330 bp. Amplification products were isolated in a 2% agarose gel and stained with ethidium bromide (Sambrook et al., 1989). Each batch was performed with known positive and negative samples as controls.

Data Analysis – Mean and median times to PCR and seroconversion were calculated. At each sampling time the sensitivity of PCR and ELISA in the detection of early post-infection events was calculated. At each sampling time the cumulative sensitivity, defined as the proportion of calves with at least one

positive test using the assay under consideration, was calculated. The proportion of calves identified as infected by cumulative weekly testing results using the ELISA and PCR were compared at each sampling time using a Fischer's exact test. An approximation of ELISA sensitivity in known infections was calculated by dividing the number of positive tests by the number of total tests in calves with previous positive ELISA results. A similar calculation was performed for the PCR assay.

RESULTS

All 8 calves became infected with BLV. Each calf had a minimum of 4 positive ELISA test results and 1 positive PCR results prior to the completion of the study. The total weeks to PCR and seroconversion are presented (Table 3). In 5 calves PCR conversion for BLV status preceded seroconversion. Seroconversion preceded PCR conversion in 2 calves. In 1 calf both tests became positive at the same test date. Once antibodies were detected, animals were consistently seropositive for the remainder of the study, equivalent to a sensitivity of 1.00. Six calves had at least 1 false negative PCR test after the initial positive result. After initial positive PCR results 20 of 30 total tests were positive, analogous to a sensitivity of 0.67 in the detection of documented infections. The proportion of calves identified as infected by cumulative weekly testing results using ELISA and PCR were not significant at any of the sampling times and only approached significance ($P = 0.08$) at 2 week post-infection (Table 4).

DISCUSSION

In this study, the ability of PCR to detect early infection with BLV after experimental inoculation was evaluated. Positive test results were observed earlier using the PCR. Four of 8 calves had positive test results within 2 weeks post-infection and no positive ELISA results were observed until week 3. The ranges in times to seroconversion and proviral detection by PCR were similar to that reported in adult cattle with low challenge exposure. Mean and median conversion with the PCR was faster than with the ELISA. However, the difference in conversion time is probably not clinically important. In addition, 3 animals demonstrated positive antibody prior to proviral detection by PCR. At least 1 false negative PCR test result after initial provirus detection was present in 6 calves. This is consistent with the reported sensitivity of the assay (Nagy et al., 2003). In contrast no calves had a negative ELISA after their first positive ELISA result.

The PCR holds few advantages over ELISA based serology for early identification of infected adult animals. Assessing all possible measures of sensitivity, either cumulative sensitivity with serial testing programs or point sensitivity, the ELISA will detect a higher proportion of infected adults than will the PCR. Cost and the ease with which the ELISA is adapted to mass sample processing also support the routine use of serology to identify infected cattle.

If this infection model is representative of natural exposure, the time to seroconversion may be shorter than previously thought. It is possible that the ELISA assay is able to detect infections prior to the AGID assays that were used in previous studies. Alternatively, the challenge dose may be higher than that

present in natural exposure. The larger challenge dose may result in shorter times to seroconversion and earlier detection of infection. If the model is truly representative, the results of this study suggest that a single negative ELISA test result at 5 weeks after a quarantine period, should provide sufficient assurance that the animal is truly BLV negative.

Despite the failure of the PCR to identify all animals prior to seroconversion, some clinical utility to the use of PCR remains. The PCR may still be used as a confirmatory test in animals of exceptional value that may otherwise be culled due to a positive serologic test. Additionally, testing premised upon viral identification may become critically important if vaccines are developed and marketed for BLV. Under these circumstances vaccines may produce positive serologic tests results and more definitive viral detection methods may become necessary.

The PCR also may play a role in the identification of infection in neonatal calves that have absorbed BLV antibodies through colostrum. The ability to identify provirus will allow for the identification and removal of infected calves prior to commingling and processing events that allow for the amplification of low level infections. It should be noted that serial weekly testing detected BLV infection in 50% of calves within 2 weeks post-infection and 100% of calves by 5 weeks post-infection. On most modern dairy farms calves typically housed singly in calf hutches until 8-10 weeks of age. Under these conditions the potential for horizontal transmission is negligible. We envision the development of programs in which calves infected transplacentally, at parturition, or by ingestion of

colostrum may be detected using the serial PCR testing cycles and culled, producing BLV negative cohorts with minimal risk for horizontal transmission.

This strategy is particularly attractive because recent studies have suggested that the ingestion of colostrum from positive cows may have profound effects in the prevention of parturition associated BLV transmission. In this study provision of frozen colostrum from BLV positive cows resulted in a 0% neonatal transmission rate. While, colostrum deprived calves born to infected cows had a 30% neonatal transmission rate. These results suggest that either depriving calves of colostrum or providing colostrum from BLV – negative cows may in fact be counterproductive in the development of comprehensive BLV control strategies.

The ability to raise BLV negative replacement heifers is crucial to control and eradication programs in endemically infected herds. Serology is superior to other test methods for routine identification of infected animals. To date the suggested utilities attributed to PCR have not gained widespread use.

CHAPTER 5

ENHANCED PERIPARTURIENT TRANSMISSION OF BOVINE LEUKOSIS VIRUS IN COLOSTRUM – DEPRIVED CALVES

INTRODUCTION

Bovine leukosis virus (BLV) is an oncogenic retrovirus that is highly homologous to human T cell lymphotropic virus 1 and 2 (HTLV-1, HTLV-2). Approximately 44% of dairy cattle and 10% of beef cattle in the United States are BLV infected (NAHMS, 1997; NAHMS 1999). The primary disease manifestation of BLV infection is the development of lymphosarcoma in approximately 5% of BLV infected cattle (Ferrer et al., 1979b). Any practice that transfers lymphocytes from infected to naïve cattle potentially spreads the virus. Dehorning (DiGiacomo et al., 1985; Lassauzet et al., 1990), tattooing (Lucas et al., 1985), rectal palpation (Henry et al., 1987; Hopkins et al., 1988; Divers et al., 1994), and bleeding for diagnostic tests (Wilesmith 1978; Maas-Inderwiesen, 1978) have been implicated in viral transmission. Arthropod vectors (Bech-Nielsen, 1978; Buxton et al., 1982; Ohshima et al., 1981; Buxton et al., 1985; Manet et al., 1989), colostrum ingestion (Ferrer and Piper, 1981), and transplacental transmission (Van Der Maaten et al., 1981b; Thurmond et al., 1983a; Piper et al.,

1979; Jacobsen et al., 1983) also have been incriminated as modes of transmission.

The relationship between calf infection status and colostrum ingestion is not entirely clear. Two divergent conclusions have been drawn from previous studies. The first suggests that colostrum ingestion may transmit BLV to neonatal calves (Miller and Van Der Maaten, 1979; Ferrer and Piper, 1981). The second suggests that colostral antibodies protect against neonatal infection (Van Der Maaten et al., 1981a; Lassauzet et al., 1989a). Infected lymphocytes are present in both colostrum and milk of BLV infected cattle (Parfanovich et al., 1978; Ferrer et al., 1981; Kenyon et al., 1982). Oral ingestion of infected blood origin lymphocytes can transmit the virus early in life and the cellular component of colostrum and milk is capable of transmitting the virus when injected parenterally (Van Der Maaten et al., 1981a; Baumgartener et al., 1976). Despite this being an unnatural route of exposure, these studies highlight the potential for the transmission of the virus through infected colostrum and milk. This is of particular concern during the first 24 hours of life when the neonatal bovine gut permits the absorption of intact macromolecules and cells.

MATERIALS AND METHODS

Animals – 12 colostrum – deprived Holstein calves 6 weeks of age or older and 20 colostrum – fed Holstein calves were used in the study. All calves were born to BLV infected cows. All colostrum – deprived calves had 6 weekly ELISA and PCR tests for BLV antibody and provirus completed. All colostrum –

fed calves were fed colostrum derived from BLV positive cows and had positive serum ELISA test results after ingestion of colostrum. Thereafter, colostrum – fed calves had ELISA and PCR tests for BLV antibody and provirus performed every other week until 2 consecutive negative ELISA tests were achieved or until 1 positive PCR tests were achieved.

Laboratory specimens – Blood samples were collected by jugular veinipuncture for ELISA and PCR assays for bovine leukosis virus. Enzyme linked immunosorbant assay samples were collected into tubes with no anticoagulant. Polymerase chain reaction assay samples were collected into tubes containing EDTA. All samples were stored at 4°C and processed within 48 hours. Lymphocytes were isolated by use of a commercially available erythrocyte lysis (EL) buffer (Erythrocyte lysis buffer, Qiagen Inc, Valencia, CA). Lymphocytes were washed 3 times with EL buffer and stored at –70°C for subsequent analysis. The DNA was extracted by use of a commercially available kit (QIAamp DNA mini kit, Qiagen Inc, Valencia, CA) following the manufacturers' protocol.

ELISA – A commercial assay (Bovine leukemia virus antibody test kit, ELISA, VMRD Inc, Pullman, WA) was used to detect of BLV antibodies. A positive test result was defined as an optical density > the mean of the positive controls and a negative test result was defined as an OD < the mean of the negative controls. For test validation the mean OD of the positive controls was \geq 0.250 and the mean OD of the negative controls was \leq 0.200.

Polymerase chain reaction assay – Forward (5'-TGG CTA TCC TAA GAT CTA CTG-3') and reverse (3'-AGA GGG AAC CCA GTC ACT GTT-5') primers were selected on the basis of a published report (Klintevall et al., 1994). The DNA sequences were amplified in a 25- μ l reaction mixture containing 0.5 μ M of each primer and 1.0 unit Taq (HotStarTaq, Qiagen Inc, Valencia, CA) DNA polymerase in the manufacturer's buffer, which contained 2.0mM MgCl₂ and 0.2mM (each) dNTPs. The reaction was performed in a programmable thermocycler (Perkin-Elmer 9700, Perkin-Elmer Inc, Shelton, CT). An initial incubation of 12 minutes at 95°C was followed by 10 cycles of denaturation at 95°C for 30 seconds, annealing at 70°C for 30 seconds, and extension at 72°C for 90 seconds. Annealing temperature was reduced by 1°C each cycle. An additional 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 90 seconds were performed, followed by a final extension at 72°C for 7 minutes. Amplification with BLV-specific primers yielded a product of 330 bp. Amplification products were isolated in a 2% agarose gel and stained with ethidium bromide (Sambrook et al., 1989). Each batch was performed with known positive and negative samples as controls. This procedure has been previously described (Nagy et al., 2003).

Data Analysis –The proportion of calves that converted to BLV positive status was calculated for each group. In the colostrum – deprived group, calves were classified as infected when a positive test result by either assay was obtained. In the colostrum fed group, calves were classified as infected or true positives when a positive PCR was obtained. The proportion of calves becoming

infected with BLV was compared among the 2 treatment groups using a Fischer's exact test.

RESULTS

In the colostrum deprived group, 4/12 (33%) calves converted to BLV positive status. Each BLV positive colostrum–deprived calf had a minimum of 2 positive ELISA tests and 1 positive PCR tests by the end of the study. In the colostrum fed group, 0/20 (0%) calves converted to BLV positive status. The proportion of calves that became infected with BLV was significantly higher in the colostrum deprived group ($p=0.01$).

DISCUSSION

In this study, the difference in BLV prevalence in BLV antibody positive colostrum-fed and colostrum-deprived calves was evaluated. Prevalence of infection was higher in the colostrum-deprived calves suggesting that the administration of BLV positive colostrum reduces the risk of infection relative to deprivation. Based on previous studies the sensitivity of the serial testing protocols employed should exceed 99% (Nagy et al., 2003). It should be noted that similar methodology determining the source of neonatal infection has been used in numerous human studies.

It is impossible to determine the exact timing of viral exposure in the colostrum deprived calves. It is unlikely that the exposure occurred *in utero* because a similar infection rate would be expected in the colostrum fed calves.

Additionally, reported *in utero* transmission rates are dramatically lower than the transmission rates observed in the present study, ranging from 0 – 20% (Van Der Maaten et al., 1981b; Jacobsen et al., 1982; Kono et al., 1983; Piper et al., 1979). Most studies report BLV transmission rates from ranging from 0 – 4% (Van Der Maaten et al., 1981b; Jacobsen et al., 1982). This disparity suggests that the vast majority of transmission observed in the present study occurred during or after birth. The negative PCR and serology results in the early postpartum period substantiate this conclusion. The difference in transmission rates between colostrum-fed and colostrum-deprived calves eliminates colostrum transmission as a source of infection. Hence, we are left with the conclusion that parturition results in exposure to BLV and administration of colostrum greatly ameliorates the risk of infection. The mechanism of this protection may be passively transferred humoral immunity or the transfer of immunologically active lymphocytes via colostrum.

Vertical transmission is thought to be a critical factor in maintenance of HTLV-1 in affected populations. Studies of vertical transmission have documented rates as high as 21 and 33% (Tsuji et al., 1990; Ando et al., 2003). The majority of this transmission is thought to occur through prolonged breast feeding of greater than 6 months (Furnia et al., 1999; Tsuji et al., 1990). One study that followed infants of infected mothers found that maternal origin HTLV-1 antibody disappeared in positive infants by 6 months of age. In these infants infection origin antibodies began to appear after 6 months and the number of positive infants rose until 2 years after which infection status of the child

remained static (Tsuji et al., 1990). In a study using PCR to detect provirus, the earliest estimate of infection was 5.8 months (Furnia et al., 1999). It is important to recognize that this is a presumptive date that was derived by splitting the time from the last negative test result to the first positive test result so this infection may have actually occurred several months earlier or later than this estimate.

In humans, breastfeeding has long been considered a risk factor for the transmission of retroviral diseases including HTLV-I and II, and HIV (Tsuji et al., 1990; Read, 2003; Newell, 2004). However, it should be noted that similar to the study presented here, a number of studies support a protective role of breastfeeding against retroviral transmission (Coutsoudis et al., 1999; Hisada et al., 2002; Iliff et al., 2005). One recent study found a decreased risk of HIV transmission in infants that were exclusively breastfed when compared to those that were predominantly breast fed or had mixed feedings from multiple mixed sources (Iliff et al, 2005). Infants with mixed feeding sources had a 4.03, 3.79, and 2.60 greater risk of being HIV infected at 6, 12, and 18 months, respectively when compared to infants that were exclusively breastfed. Another study found transmission rates of 21.3%, 24.1%, and 14.6% in 3-month-old infants that had no breast feeding, mixed source feeding, and exclusive breast feeding, respectively (Coutsoudis et al., 1999). When evaluated as a group, these studies suggest that high serum immunoglobulins in the neonate at the time of retroviral exposure is likely protective. Additionally, these studies suggest that as these maternally derived colostral and milk immunoglobulins decline the risk of

contracting BLV, HIV, or HTLV in the respective species, increases (Ando et al., 2003; Iliff et al., 2005).

Currently, targeted antiviral protocols are the cornerstone of mother-to-child HIV prevention protocols. These are not without risks, as some protocols have been associated with increased postpartum drug resistance in the mother (Eshleman and Jackson, 2002). The targeted administration of high levels of anti-HIV antibodies to these protocols may afford additional protection to exposed infants or provide prophylaxis without selection for maternal drug resistance.

In this study the administration of colostrum from BLV positive cows exerted substantial protection against infection following parturition associated exposure. This information is critical when considering calf centered control programs for BLV infection. Recommendations to feed colostrum from BLV negative cows when available are commonplace (Brunner et al., 1997). These recommendations stem from the assumption that colostrum is infectious. However, the results of this study suggest that efforts to feed colostrum from BLV negative dams or the use of colostrum replacers have the potential to increase neonatal transmission of BLV.

CHAPTER 6

DECAY OF COLOSTRALLY DERIVED BOVINE LEUKOSIS VIRUS IMMUNOGLOBULINS IN NEONATAL CALVES

INTRODUCTION

Bovine leukosis virus (BLV) is an oncogenic retrovirus of cattle that shares a high degree of homology with human T cell lymphotropic virus 1 and 2 (HTLV-1, HTLV-2). The virus primarily infects B lymphocytes; although T lymphocytes also may be infected (Williams et al., 1988; Mirsky et al., 1996). Transfer of lymphocytes from infected to naïve cattle spreads the virus. Many common practices including dehorning (DiGiacomo et al., 1985), tattooing (Lucas et al., 1985), rectal palpation (Hopkins et al., 1988; Divers et al., 1994), and bleeding for diagnostic tests (Wilesmith et al., 1978) have been implicated in viral transmission. Arthropod vectors (Buxton et al., 1985; Manet et al., 1989), colostrum ingestion (Straub, 1982), and transplacental infection (Van Der Maaten et al., 1981b; Jacobsen et al., 1983; Miller et al., 1979; Ferrer and Piper, 1981) also have been incriminated as important modes of transmission. Close contact with BLV infected animals is considered a primary risk factor in the development of persistent BLV infection (Straub, 1978).

In commercial herds with a low prevalence of BLV infection, control and eradication programs are straightforward and easily implemented. Adult and juvenile cattle (> 6 months of age) are tested using available serologic assays and cattle with positive test results are removed from the herd. Such programs will generally have remarkable progress causing dramatic decreases in the prevalence of BLV infection. In herds with a high prevalence of BLV infection, test and slaughter programs are economically untenable, particularly in the absence of state or federal indemnity programs.

Despite the presence of testing modalities that are both sensitive and specific for BLV, identifying neonatal infection with the virus has been problematic (Burridge et al., 1982a; Evermann and Jackson, 1997; Ferrer et al., 1977; Johnson and Kaneene, 1991a; Monke et al., 1992). The issue which is most problematic is the passive transfer of antibodies that occurs from the dam to the neonate upon the ingestion of colostrum. The majority of tests detect BLV indirectly by testing for the presence of antibody (Johnson and Kaneene, 1991c). Once a calf has ingested colostrum from a positive dam; these tests are rendered useless in determining calf virus status. It was once thought that the advent of PCR would eliminate this concern because the PCR would detect integrated BLV provirus despite the presence of maternal antibody (Agresti et al., 1993; Brandon et al., 1991; Eaves et al., 1994; Kelly et al., 1993; Marsolais et al., 1994; Naif et al., 1992; Sherman et al., 1992). However, inadequate sensitivity and high test cost associated with the PCR decrease the clinical utility of this test.

In addition to recognition of infected animals, understanding the decay of colostral origin BLV antibodies would be important to determine appropriate vaccination protocols for calves should a vaccine become available. Colostral antibody decay curves for BLV antibody have not been established using ELISA methodology although they have been established using the AGID (Thurmond et al., 1982; Johnson et al., 1987). It is possible that differences exist due to the increased sensitivity of the ELISA.

The objective of this study is to generate a decay curve for antibodies recognizing BLV in neonatal calves fed colostrum from BLV-infected cows. The curve will allow more accurate interpretation of BLV serology in youngstock, creating an additional tool for identifying active BLV infection in calves less than 6 months of age.

MATERIALS AND METHODS

Calves – Seven heifer calves born to BLV positive dams were enrolled in this study. Each calf was bottle fed pooled colostrum from BLV positive cows ad libitum divided into 2 2-liter feedings.

Sample collection – Blood samples for ELISA and PCR assay were collected prior to colostrum ingestion. Samples for ELISA were collected into tubes without anticoagulant. Serum was separated and stored at 4°C for further analyses. Samples for PCR assay were collected into tubes containing EDTA. Samples were stored at 4°C and processed within 48 hours. Additional serum

samples were collected at day 2, 30, 60, and then every 2 weeks until 2 consecutive negative test results were obtained.

Serum ELISA recognizing BLV - A commercial assay (Bovine leukemia virus antibody test kit, ELISA, VMRD Inc, Pullman, WA) was used to detect of BLV antibodies. A positive test result was defined as an optical density > the mean of the positive controls and a negative test result was defined as an OD < the mean of the negative controls. For test validation the mean OD of the positive controls was ≥ 0.250 and the mean OD of the negative controls was ≤ 0.200 .

Blood PCR recognizing BLV - Forward (5'-TGG CTA TCC TAA GAT CTA CTG-3') and reverse (3'-AGA GGG AAC CCA GTC ACT GTT-5') primers have been selected based on a previously published report (Klintevall et al., 1994). The DNA sequences were amplified in a 25 μ l reaction mixture containing 0.5 μ M of each primer, 1.0 units HotStarTaq in the manufacturer's buffer containing 2.0 mM MgCl₂ and 0.2 mM (each) dNTPs. The reaction was performed in a programmable thermocycler. An initial incubation of 12 minutes at 95°C will be followed by ten cycles of denaturation at 95°C for 30 seconds, annealing at 70°C for 30 seconds, and extension at 72°C for 90 seconds. The annealing temperature was reduced by 1°C each cycle. An additional 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 90 seconds was performed followed by a final extension at 72°C for 7 minutes. Amplification with the BLV-specific primers yielded a product of 330 bp. Amplification products were visualized in a 2% agarose gel by ethidium bromide staining and ultraviolet transillumination (Sambrook et al.,

1989). Each sample was performed in tandem with known positive and negative control samples. This technique has been validated in populations of known positive and negative status (Nagy et al., 2003).

Data analysis – For each calf a log linear regression line was calculated from the data using a statistical software package. The half life of BLV antibodies in each calf was the calculated using the following equation:

$$T_{1/2} = [\log_{10}(10^a/2) - a]/b$$

Where a is the y-intercept and b is the slope of the regression line. The mean, median, standard deviation, and 95% confidence interval of the half-life were calculated. For each calf a linear regression line was calculated to determine the slope of the antibody decay curve and the standard deviation of said slope. Mean slope and standard deviation of slope was calculated. Anticipated decay curves were constructed for calves premised upon known initial (48 hr) BLV ELISA optical density and calf age at the time of sampling.

RESULTS

All calves were BLV negative by 105 days. The calculated half-life of BLV antibodies in the calves ranged from 31 – 76 days with a mean of 48.5 days, a median of 47.3 days, and a standard deviation of 15.6 days. The 95% confidence interval for the half-life mean was (34.1, 62.9). The serum BLV antibody decay is depicted in figure 4.

DISCUSSION

Disease control and eradication requires accurate, definitive tests that are able to identify infected animals early in the course of infection. Historically, BLV control and eradication programs have not addressed neonatal calves because of the inability to assess BLV status in calves that have ingested colostrum from BLV-positive cows. Prolonged false positive tests due to the interference of colostrally acquired BLV antibody limits the use of the AGID and ELISA. While, the PCR allows for accurate identification of infected animals in the presence of maternal antibody, the current cost of a BLV PCR limits use of the test. Determining the natural decay of colostrally derived BLV antibodies in neonatal calves may allow for earlier identification of infected calves using serologic methods.

The time for all calves to reach BLV negative status was in between the 2 AGID based studies previously reported (Thurmond et al., 1982; Johnson et al., 1987). The range of calculated half lives in the current study is broader and higher than initially expected. The published decay of colostral IgG1 in calves ranges from 18 – 22 days, with BLV specific antibody decay falling at 25.8 and 36 days (Thurmond et al., 1982; Johnson et al., 1987). Several factors may account for the differences seen. The present study used optical density test values from the ELISA while previous BLV antibody decay rates have been determined using the AGID. It has also been postulated that variability may be due to the variability of BLV antibodies in individual cow colostrum. Studies have documented increased time to test negative status in calves that received

colostrum from cows with higher BLV titers on the day of parturition (Johnson, et al., 1987) and in calves with initial high colostral antibody titers (Lassauzet et al., 1990c). Immunoglobulin decay should be constant. For that reason time to test negative status may vary by total amount of immunoglobulin ingested at birth, but half life should not.

With the clinical availability of PCR the current study was also able to have a more definitive definition of a negative calf by looking for detectable provirus rather than relying on serial negative serotests. In addition decay of colostral immunoglobulins is curvilinear. Each calf in the study reported here had a minimum of 5 ELISA tests while previous work included some subjects with as few as 3 tests (Thurmond et al., 1982; Johnson et al., 1987). The increased number of test points will more accurately reflect the curvilinear nature of antibody decay extending the calculated half-life.

In the current study the development of a decay curve for colostrally derived BLV antibodies in calves will allow for estimation of BLV status in the face of colostral antibodies. Calves with half lives substantially longer than 48 days are likely to have endogenous anti-BLV antibodies production. This is suggestive of active infection with BLV. The practical utility of this model remains unproven.

CHAPTER 7

CONCLUSIONS

Performance of the PCR assay is promising and it may provide a useful adjunct to traditional testing programs. However, in most circumstances serologic tests will be superior to the PCR assay because higher sensitivity (> 95%) will expedite identification of BLV-infected animals. Potential applications of the PCR assay may be restricted to 2 specific situations in clinical practice. First, the PCR assay could be used to detect BLV in neonatal calves with passive transfer of maternal antibodies against BLV. Second, the PCR assay could be used as a confirmatory test in valuable animals that may otherwise be culled as a result of a positive serologic test. Use of the PCR to identify cattle that were recently exposed to BLV seemed like an initial promise of this modality, but did not outperform serology to the point that it would have a lot of clinical utility.

Serial testing could be used to increase the accuracy of testing procedures when applying a PCR assay to BLV control programs. Serial PCR assays can decrease the rate of false negative tests. In populations with low prevalence of BLV, 2 consecutive tests will reduce the false negative rate to an acceptable level, whereas 3 consecutive tests will reduce prevalence to a clinically unimportant level. In populations with high prevalence of BLV, serial

testing could improve the ability to identify all infected cattle. However, serologic methods of BLV detection are more sensitive for routine screening of adult cattle.

Historically the detection of lymphocytosis has been used to drop BLV prevalence to negligible levels in infected herds. However, the development of more sensitive and specific tests had limited the use of this potentially powerful diagnostic tool. The random culling of cows on the basis of antibody-positive status would result in similar proportions of virus being removed from farms. For example, if 10% of the cows were culled, 10% of the infectious virus would be removed from the herd; if 20% were culled, 20% of the virus would be removed. Culling 10%, 20%, and 30% of cattle with the highest S:P would remove 12.4%, 23.3% and 32.7% of infected-lymphocyte load within a herd, respectively. This is better removal of proviral load than that achieved by random culling, but the advantage gained is less than substantial. Alternatively, culling 10%, 20% and 30% of cattle with the highest lymphocyte count in a BLV-positive herd would remove 23.9%, 40.1% and 52.4% of infected-lymphocyte load within a herd. This means that culling BLV-positive cows with high lymphocyte counts may serve as a means to control the amount of infectious provirus on a farm. This type of targeted culling program in a herd with a high prevalence of BLV infection could decrease the amount of provirus present for the at-risk population, while allowing for more economically acceptable culling of cattle with a positive serologic test.

APPENDIX

Table 1 – Bendixen hematologic key for the classification of cattle as leukotic (Bendixen, 1959).

Age in years	Group I Normal	Group II Dubious	Group III Leucaemic
0-1	< 10,000	10,000 – 12,000	> 12,000
1-2	< 9,000	9,000 – 11,000	> 11,000
2-3	< 7,500	7,500 – 9,500	> 9,500
3-4	< 6,500	6,500 – 8,500	> 8,500
4-5	< 5,000	5,000 – 7,000	> 7,000

Table 2 – Summary of slaughter data for 1998 – 2002 for condemnations due to lymphosarcoma.

Year	Slaughter category	Number slaughtered	Number condemned	Lymphosarcoma Condemnations
1998	Bulls and stags	615,888	1,608	209
	Steers	16,202,791	14,144	204
	Cows	5,886,745	130,470	20,907
	Heifers	10,567,435	10,902	133
Annual total		32,718,559	157,124	21,453
1999	Bulls and stags	601,652	1,877	96
	Steers	16,647,224	18,348	141
	Cows	5,342,619	112,993	19,479
	Heifers	11,088,609	22,057	94
Annual total				
2000	Bulls and stags	619,616	2,012	97
	Steers	17,457,463	23,436	209
	Cows	5,269,576	142,874	24,070
	Heifers	11,789,720	20,592	164
Annual total				
2001	Bulls and stags	706,958	2,202	125
	Steers	18,363,668	17,339	314
	Cows	6,214,474	165,881	28,315
	Heifers	12,355,710	12,801	214
Annual total				
2002	Bulls and stags	610,130	1,484	102
	Steers	15,621,561	11,988	276
	Cows	5,175,861	143,484	25,037
	Heifers	9,996,325	8,915	121
Annual total				
Total				

Table 3 – Time to seroconversion and the detection of provirus by PCR in calves experimentally inoculated with BLV.

Calf number	Weeks to first positive test	
	PCR	ELISA
1	2	3
2	3	5
3	5	4
4	5	3
5	4	4
6	2	4
7	2	3
8	2	3
Mean	3.125	3.625
Median	2.5	3.5

Table 4 – Proportion of calves identified as infected by cumulative testing using the ELISA and PCR in calves experimentally infected with BLV.

Time	Cumulative sensitivity	
	PCR	ELISA
Week 1	0.00	0.00
Week 2	0.50	0.00
Week 3	0.63	0.50
Week 4	0.75	0.88
Week 5	1.00	1.00
Week 6	1.00	1.00
Week 7	1.00	1.00

Figure 1 – Positive (solid line; PPV) and negative (dotted line; NPV) predictive values of a polymerase chain reaction (PCR; sensitivity, 0.672; specificity, 1.00) assay for all possible prevalences of infection for cows infected with bovine leukosis virus (BLV).

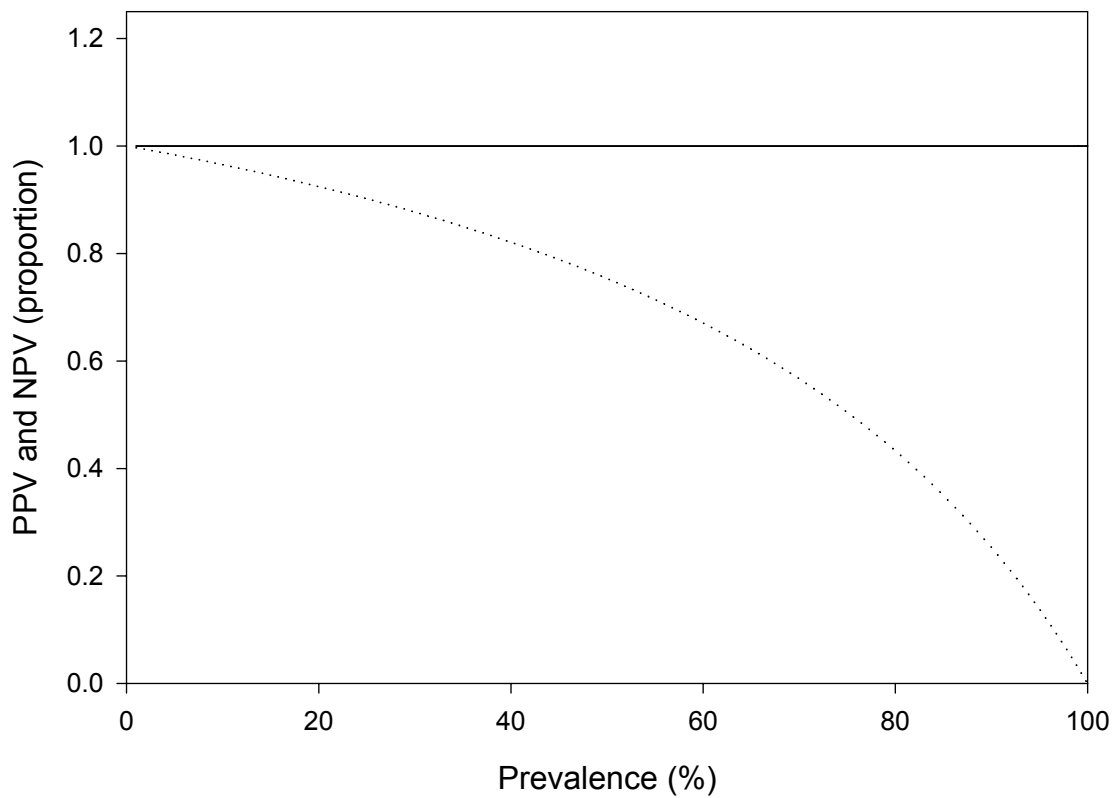


Figure 2 – Theoretical estimates of prevalence for cattle serially tested via PCR assay for BLV infection status in 3 populations with varying initial prevalence. Prevalence of 5% (closed circles), 10% (open circles), 15% (closed triangles).

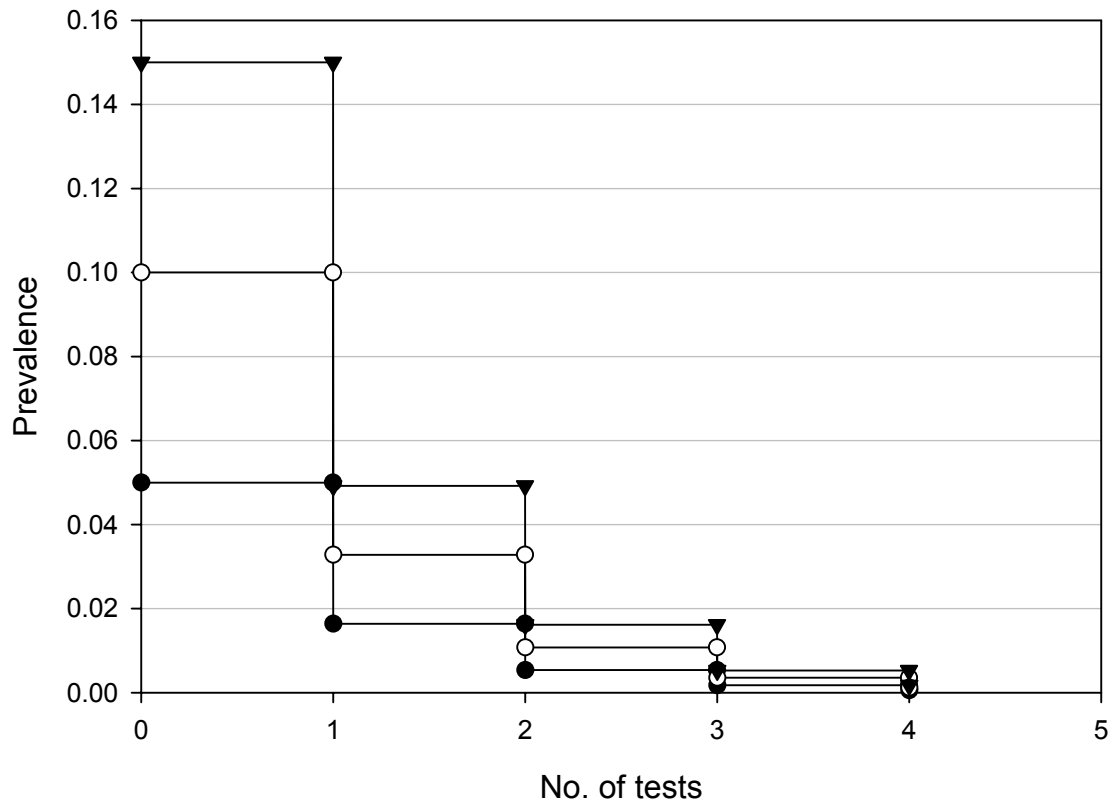


Figure 3 – Graph of the cumulative proportion of herd total lymphocyte count versus the cumulative proportion of the herd that would be removed by targeted culling versus random culling in a herd of cattle with bovine leucosis virus (BLV) infection.

- a- Targeted culling of BLV positive cows based on lymphocyte count from highest to lowest.
- b- Targeted culling of BLV positive cows based on S:P ELISA from highest to lowest.
- c- Random culling of cows with positive results of ELISA for BLV.

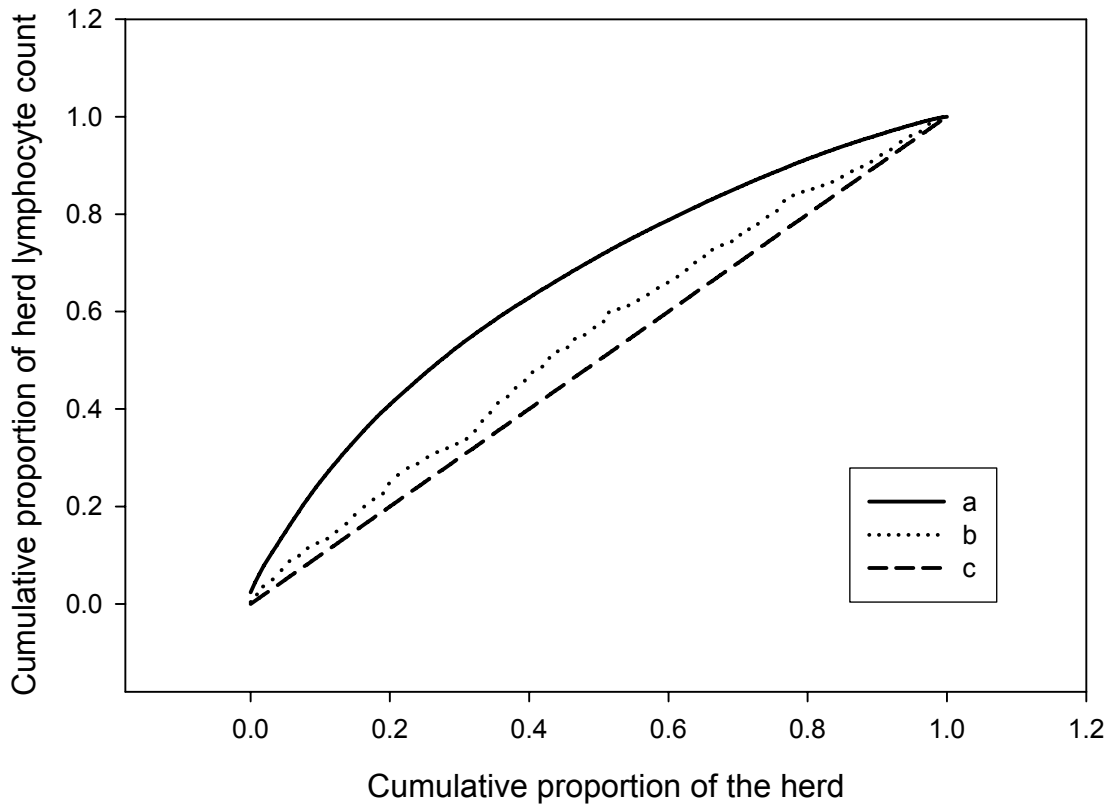
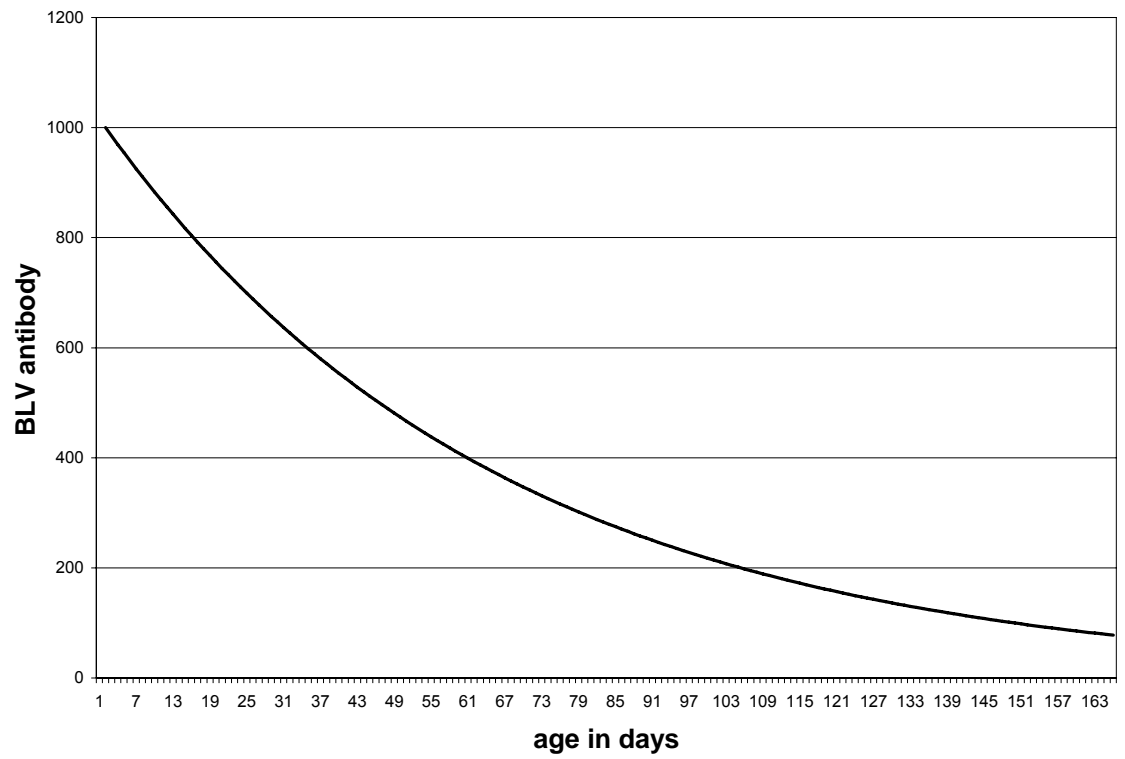


Figure 4 – Theoretical decay curve of colostral origin BLV antibodies in neonatal calves.



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VITA

Dusty Nagy was born on January 4, 1970 in Baltimore, MD. On February 14 she was adopted by Fred and Dorothy Weaver. Dusty was raised in Columbia, MD and remained there until going to Colorado for college. Dusty spent 3 semesters at Colorado State University and 3 semesters at the University of Maryland prior to entering veterinary school at the New York State College of Veterinary Medicine. Upon graduating from veterinary school, Dusty completed an internship and residency in food animal medicine, surgery, and production medicine at the University of Missouri. During this time she also completed a MS in veterinary medicine and surgery and became board certified in the American College of Veterinary Internal Medicine.

On April 1, 2000, Dusty married Jesse K. Nagy and shortly thereafter moved to Urbana, Illinois where she worked on the faculty in the Department of Veterinary Clinical Medicine at the University of Illinois, College of Veterinary Medicine. Currently, Dusty is on the faculty in the Department of Veterinary Medicine and Surgery at the University of Missouri, College of Veterinary Medicine. She resides in Columbia, MO with her husband Jesse, daughter Peyton, son Aiden, and the family pets Rypien, Gibbs, Girth, and Mot.