BOVINE LEUKEMIA VIRUS

ABSTRACT

Enzootic bovine leukemia (EBL) is a naturally occurring, lymphoid cancer of cattle caused by Bovine Leukemia Virus (BLV). BLV infection is pandemic, and the prevalence of the infection in cattle herds can reach 60 to 90%. Only a fraction of the cattle that become infected with BLV develops malignant lymphoma, or persistent lymphocytosis, a benign proliferation of lymphoid cells. Thus, infection has been significant in the past primarily due to international trade restrictions placed on seropositive animals, and not because of malignant lymphoma. Because of cost prohibitive nature of any form of therapy of lymphoma in cattle and the high prevalence of infection, researchers have endeavored to develop vaccines that hold potential for future use. The discovery of the Human T Lymphocytotropic retroviruses (HTLV I, HTLV II, HIV) and their associated diseases (adult T cell leukemia, hairy cell leukemia, AIDS), as well as new animal lymphocytotropic retroviruses (bovine immunodeficiency-like virus or bovine lentivirus, feline immunodeficiency virus) has stimulated an ever-greater interest in EBL and BLV infection. This interest is prompted by the potential usefulness of this natural disease of animals as a model system to study newly recognized human and animal diseases, as well as by the persistent fear of BLV and other animal retroviruses as zoonotic agents. The general aspects of BLV infection have been reviewed here in order to summarize the current state of EBL research development.

Brief report.
The initial descriptions of leukemia in cattle appeared in German medical literature in 1871 (Leiserling 1871 mentioned in Johnson & Kaneene 1992). European cattle, probably infected with BLV, were imported from the shores of the Baltic Sea to the U.S. at the end of nineteenth century. The infection has spread in American and Canadian cattle and now is highly prevalent in many dairy herds in these countries. The direction of international trade on cattle changed, and high production American and Canadian dairy cattle, infected with BLV, may have contributed to the dissemination of BLV to cattle in other countries in Europe and South America, as attempts to upgrade those herds were being made. In Brazil, EBL was first described by Rangel & Machado (1943), who studied domestic animals cancer frequency in Minas Gerais State and noticed the occurrence of lymphosarcoma in cattle. However, the first official register of a clinical case
of EBL in Brazil was made by Merck et al. (1959), in Rio Grande do Sul State. Now, BLV is present all over the world, with the exception of the European Community and some other countries, which, since they had small herds and low prevalence, opted to eradicate BLV from those herds.

**History of Blv.**

The high prevalence of lymphosarcoma was observed in Swedish cattle. Fresh blood had been collected from a cow that was infected with *Babesia bovis* and was inoculated in healthy cattle as an immunizing agent against piroplasmosis in that country. This practice had been carried out for seven years when it first raised suspicion of the an infectious agent present in the blood of the donor cow being transferred to the recipients (Olson 1961). The development of "naturally" occurring lymphatic leukemia of unknown origin in a single flock of sheep at the same time called the attention of another group of researchers, who finally discovered that the sheep with leukemia had received injections of Babesia-infected blood of a cow from a herd with bovine leukemia several years prior to the leukemia epidemic (Enke et al. 1961). Latter, an experimental study resulted in the successful induction of lymphosarcoma in sheep with bovine lymphosarcoma material (Witmann & Urbanek 1969). A major finding came out in 1969 when it was reported that the leukocytes of some cattle produced virus particles similar to the particles from cells of other species with leukemia after in vitro passages of leukocytes for two to three days. The virus was isolated only from cows with adult lymphosarcoma, but not from cattle with sporadic bovine leukemia, calf with the calf form of leukemia, or animals with the skin form of leukemia. In herds with multiple cases of bovine leukemia, the virus was isolated from all cows with persistent lymphocytosis, a benign lymphoproliferative type of BLV infection (Miller et al. 1969).

**BLV.**

EBL is caused by a deltaretrovirus that preferentially infects B lymphocytes, but was also detected in T lymphocytes, monocytes and granulocytes (Schwartz et al. 1994). The virus particle has 80-130 nm of diameter and consists basically of two copies of single-stranded RNA that is 8,714 nucleotides length (Sagata et al. 1985), nucleoprotein p12, capsid protein p24, transmembrane glycoprotein gp30, envelope glycoprotein gp51, and several enzymes, including reverse transcriptase (Fields & Knipe 1990). RNA is converted to DNA by means of the reverse transcriptase enzyme. This enables BLV genome to become integrated into the DNA of the host cells where it can persist for the life of the host. The provirus is found integrated at a large number of sites in the cellular DNA. Like other retroviruses, the BLV provirus presents, on the extremities of its genome, a long terminal repeat (ltr) which is composed of three consecutive regions, U3, R and U5. The U3 region includes transcriptional regulatory elements. Between ltr are distributed genes for structural proteins gag and env, and for the viral enzymes prt (protease) and pol (reverse transcriptase, protease and integrase), besides overlapping, open reading-frames located adjacent to the env gene, that code for regulatory proteins (tax, rex, G3 and R4) (Buny 1988). Env gene codes for gp51, an associated surface molecule, and gp30, a protein that anchors the virion glycoprotein complex. Their localization at the surface of viral particle explains why they are the natural target for neutralizing antibodies. The viral envelope complex binds to the receptor of the target cell determining cellular tropism. This interaction leads to a process of fusion between the viral envelope and cellular membrane, allowing subsequent viral entry. The protein gp30 carries a fusion peptide at its amino-terminal end. The fusion process could be mediated by the oblique insertion of this peptide into the lipid bilayer of the cell membrane. There are eight epitopes in gp51, three of them (F, G and H) are target for monoclonal antibodies that result in virus neutralization and syncytium inhibition, and they correspond to the epitopes recognized by neutralizing antibodies of infected cattle. Each of the three epitopes is conformational type, and the glycosilation is important for recognition by antibodies (Mamoun et al. 1990). In contrast to the env gene of others lentiviruses, the variability of the BLV pol, env and ltr genes was below 6% and most of the nucleotide substitutions were conservative. Variations in env were not randomly distributed, but preferentially localized at the level of three conformational epitopes (F, G and H). Potential glycosilation sites and cysteine residues were highly conserved among the different BLV strains, what suggests the importance of both in obtaining an active biological form of gp51 (Mamoun et al. 1990, Willems et al. 1995, Molteni et al. 1996, Dube et al. 1997, Dube et al. 2000,
Camargos et al. 2002a, Licursi et al. 2003). The high degree of conservation in the BLV envelope sequences could result from high evolutionary constraints preserving the protein functions (Willems et al. 1995). Both tax and rex genes are essential for infectivity in vivo. The tax protein acts as a transactivator of transcription of BLV provirus and is also presumed to activate the transcription of cellular genes. High levels of transactivation activity correlate with viral infectious potential in vivo. In vitro, tax is able to immortalize rat embryo fibroblasts (REF) cells. The rex protein is a post-transcriptional regulator of viral expression required for the synthesis of structural genes (Kerkhofs et al. 1998). R3 and G4 functions are still unknown, although G4 protein exhibited oncogenic potential in vitro. They are not essential for infectivity in vivo, but their deletion greatly interferes with the efficiency of viral propagation and pathogenic potential (Kerkhofs et al. 1998, Willems et al. 2000).

The cellular receptor for BLV has no known physiological function and has no significant homology with sequences registered in the GenBank and EMBL data libraries (Ban et al. 1993). The widespread distribution of BLV receptor among mammals (murine, bovine, ovine, primate, caprine, canine, feline and swine) and its highly conserved sequence suggest that the molecule recognized by BLV plays a role in the physiology of many cell types. Elucidation of the reasons and mechanisms by which the virus is confined to a few cell types in vivo will require a much more detailed analysis (Ban et al. 1993, Ban et al. 1994).

BLV is inactivated by alcohol, ether and chloroform. It is more resistant to UV and X rays than other viruses, probably due to diploid genome, but does not survive for long time at ambient temperature. It is destroyed by freezing and thawing, heating at 56°C for 30 minutes and pasteurization. BLV survives in blood stored at 4°C for at least two weeks (Lucas 1992).

Public health.

Although BLV is able to infect cells from different species in vitro, including man (Dahberg 1988), natural infection was detected only in cattle, wild capybaras, buffaloes and sheep. Sheep are highly susceptible to tumor formation. Experimental infection with BLV, as indicated by persistent antibody production, has been reported in chimpanzees, macaques, pigs, guinea-pigs, rhesus monkey, antelope, domestic rabbits, rats, cats, dogs and deer. No evidence of production of BLV antibodies followed BLV inoculation in mouse, chipmunk, ground squirrel, Japanese quail or chicken (Lucas 1992, Johnson & Kaneene 1992, Suzuki & Ikeda 1998).

Virus particles have been found in milk of cows infected with BLV. This finding raised questions about the transmissibility of BLV to humans. Considering the degree of contact between humans and domestic cattle, and the fact that humans consume (bovine) milk and meat, the question of an association between human lymphoproliferative diseases and bovine leukemia has been raised. Besides, BLV infects human cell lines in vitro, it belongs to the same subfamily, harbors a similar genomic organization, and infects and transforms cells of the hematopoietic system much the same way as Human T Leukemia viruses 1 and 2 (Willems et al. 2000). Antibodies to BLV or BLV proviral DNA were not detected in people like veterinarians, slaughterhouse personnel, laboratory technicians and farmers, who had close contact with or drank raw milk from infected livestock. In addition, no association was observed between human leukemia/lymphoma and these presumed risk factors (Johnson & Kaneene 1991). Recently, using immunoblotting to test the sera of 257 humans to the BLV capsid antigen (p24), researchers detected at least one antibody isotype reactive with BLV in 74 % of the human sera tested. The specificity of the reactivity was demonstrated by competition studies and by ruling out cross-reacting antibodies to other chronic human viruses. These results do not necessarily mean that humans are actually infected with BLV; the antibodies could be a response to heat-denatured BLV antigens consumed in food. They do, however, suggest that further studies in this area could be important (Buehring et al. 2003).

Epidemiology.

Since infectious, free BLV is rarely produced in vivo, most susceptible cattle are thought to become infected by exposure to infected lymphocytes, and not by free virus. Thus, any way by which BLV-infected lymphocytes can be transferred from one animal to another is considered to be a potential means of transmission of virus. As little as 1.0 mL of blood from an infected animal was enough to cause infection in susceptible animals, after subcutaneous, intradermic, intramuscular or intravenous injection (Hopkins & DiGiacomo 1997). That way,
seropositive cattle with PL may be a major source of infection for susceptible cattle. Drugs by any route, castration, dehorning, supernumerary teat removal, tattooing, ear tagging, rectal palpation, tuberculosis testing, blood transfusion and pioplasmosis immunization are the main ways of BLV-infected lymphocytes are transferred to recipient animals (Hopkins & DiGiacomo 1997). The closeness of cattle provides ample opportunity for potential vectors (tabanids) to alternate feeding among animals and transmit infectious agents in the process, so transmission of BLV by vectors should be considered in tropical and subtropical areas. The transmission by milk or colostrum has not been demonstrated. In fact, colostral antibodies may reduce the risk of infection (Hopkins & DiGiacomo 1997). Semen with high lymphocyte count may, potentially, transmit BLV, mainly by natural service, since semen from artificial insemination normally do not have leukocytes, except if it is collected by manual massage of the bull's genital tract. Transplacental transmission occurred in approximately 3-6 % of gestations of seropositive dams that had shown persistent lymphocytosis (PL) or lymphosarcomas (Hübner et al. 1997). Embryo transfer from infected donors to seronegative recipients, (since preceded of embryo washings), was not associated with BLV transmission (Lucas 1992).

Serologic surveys revealed that BLV infection is widely disseminated throughout, with high prevalence rates in North America, South America, Africa, Asia and Australia. The higher prevalence has been observed in dairy herds, including in Brazil, where the virus is widespread (Moraes et al. 1996, Hopkins & DiGiacomo 1997, D’Angelino et al. 1998a, Molnar et al. 1999, Camargos et al. 2002b). In these reports, the prevalence of BLV infection was greater in cattle 24 months of age and older. It was observed low antibody prevalence in young cattle. The antibody prevalence threshold is located in cattle 24-month old, the age at which many join the milking herd. These data formed the basis for the hypothesis that the initiation of close physical contact and intensive management practices associated with increased human intervention in the herd, which represent important factors in the horizontal transmission of BLV. Beef cattle in Brazil and elsewhere have showed low antibody frequency (Hopkins & DiGiacomo 1997, Camargos et al. 2002b).

Pathogenesis.

The disease results from complex interplay between virus and animal with host-dependent factors being major determinants of pathogenicity. In cattle, most of the infected animals remain clinically healthy, the number of infected cells ranging around 1 % or less. A fraction of these infected animals will develop PL, when the proviral loads remain approximately constant over extended periods of time. PL is characterized by an increase in the number of B CD5 lymphocytes circulating in the blood stream, what is probably due to interactions between virus surface molecules and cell receptors, and increased levels of IL-10, IL-2 and IL-2 receptor. Similar to that occur in HTLV infections, where the tax protein transactivates IL-2 and IL-2 receptor (Trueblood et al. 1998). During this stage, the numbers of circulating B cells can remain stable over extended periods of time without any other severe clinical signs (Willems et al. 2000).

Tumor cells are monoclonal or oligoclonal for the site of BLV integration. No evidence has been found so far for a common integration site for BLV provirus in different proviruses (Lucas 1992). They are not necessarily preceded by PL and develop two to five years post infection, most frequently in animals from three to eight years of age. The way it occurs remains unclear. BLV probably has a role in the initial process, however it is not necessary for maintenance of the tumor state in transformed cells, since a large percentage of the tumors have proviruses in which much of the provirus is deleted (Willems et al. 2000). The synthesis of viral proteins might have been required for transformation, and the subsequent deletion of the provirus would have provided a selective advantage to a rare clone, responsible for the generation of the tumor (Willems et al. 2000). The lack of viral expression could confer a selective advantage to the cell harboring a defective provirus. An infected cell, once transformed, would be hiding in the absence of viral gene expression. This suggests a strategy for escaping the host immune system during pathogenesis (Willems et al. 1995). The consistent presence of the 3' half of the provirus has led to the conclusion that a protein, namely tax, is mandatory to maintain cells in the neoplastic state. Cell transformation could be caused by transitory expression of tax gene coding for regulatory non-structural proteins which increase transcription of 14-3-3 and genes involved in cell growth control, finally leading to tumor formation.
(Toma et al. 1990). In vivo, approximately half of the solid tumors induced by BLV in cattle contain a mutant p53 gene (tumor suppressor gene). These mutations interfere with essential p53 functions required for transactivation and suppression of cell growth. In contrast, very few mutations were found in B cells from cows with PL, and none of the uninfected cattle tested harbored a mutant p53 gene. These observations indicate that alterations of p53 frequently occur at the final stage of BLV-induced leukemia in cattle. Since genetic modifications are very rare events, one could speculate that the requirements of p53 mutations in cattle significantly extend the latency period preceding the onset of full transformation. Inactivation of p53 gene by mutation appears to be one of the critical events associated with tumor formation in cattle (Willems et al. 2000).

None of 13 sheep inoculated with recombinant plasmids containing BLV proviral DNA with mutations in G4 alone or in G4 and R3 developed leukemia after more than eight years of incubation. Whether none of these sheep will ever develop leukemia is currently unknown. Anyway, the pathogenic potential of the R3 and G4 deletants is drastically reduced in sheep, a host highly susceptible to BLV-associated disease (Willems et al. 2000).

PL is not observed in sheep, but the number of infected cells in the peripheral blood rises more gradually until the onset of leukemia, the levels of circulating lymphocytes being above 10,000/mm³ and up to 500,000/mm³. Leukemia in sheep is not an obligatory step preceding the tumor phase. In a significant number of cases, lymphosarcoma can develop in the absence of any proliferation of lymphocytes in the circulating blood. However, since the probability of tumor development is greater in animals harboring higher levels of circulating lymphocytes, PL in cattle, or leukemia in sheep, may thus be considered pre-tumoral stages. The latency period before the onset of the disease in sheep is shorter than in cattle and leukemia occurs usually one to four years after infection in sheep. In addition, the frequency of virus-induced pathology is much higher in sheep, almost all the infected animals dying within their normal lifetime, compared to only about 5% in cattle. The fact that sheep are not natural hosts for BLV might explain why virus-induced pathology is more acute in this species. Since coevolution between BLV and sheep could not occur, the ovine host, which is genetically related to cattle, could not adapt itself to the pathogen. In sheep, the p53 tumor suppressor gene does not appear to be altered at any stage of the disease, indicating that the inhibition of p53 by mutation of the gene is not a mandatory step for leukemogenesis in sheep. This could explain the differences in the frequencies of tumor formation between cattle and sheep. It also appears that interactions between BLV and its ovine host have not reached the equilibrium that has been achieved in cattle (Willems et al. 2000). Another difference in BLV pathogenesis observed in sheep is apoptosis inhibition. Apoptosis is an active program leading to the destruction of cells in which abnormalities have occurred, as is the case with cells infected by viruses, or with tumoral cells. The inhibition of this process alters the equilibrium between proliferation and cell death, modifies homeostasis within an organism, and can ultimately lead to leukemia. In sheep, protection against apoptosis seems to be acquired at the early asymptomatic stages and persists throughout the leukemic and tumor phases. The precise nature of this protective factor is currently unknown. It could, for example, be a cellular cytokine, or the viral tax protein. High levels of ex vivo apoptosis are observed in cattle, which might be correlated to a decreased susceptibility to leukemia in vivo (Willems et al. 2000). The lack of spontaneous transmission within herds and shorter latency period in sheep made this species a possible model system for a related oncovirus, like HTLV-1, which induces leukemia in humans.

**Signs and lesions.**

BLV infections are mostly asymptomatic. Approximately 30% of infected animals develop PL and in 0.5% to 5.0% of four or more year old bovines a very aggressive expansion of a transformed clone evolves into massive tumors (Kerkhofs et al. 1998). Clinical signs in animals that develop tumors depend on the particular organ or organs involved. One or more superficial lymph nodes may be enlarged and these can be felt as lumps beneath the skin, especially in the neck and hind flank areas. However, when the internal lymph nodes are the only ones affected, diagnosis may be more difficult. Tumors can occur in the uterus, mesenteric, retrobulbar, pre-scapular and sub-iliac lymph nodes, the right auricle of the heart, abomasum, spleen, lungs, kidney, urinary tract and spine (Sparling 2000). Clinical signs may include inappetance, indigestion,
chronic bloat, displaced abomasum, diarrhea, constipation, lameness, paralysis, weight loss, exoftalmia, weakness, or general debility, and sometimes neurological manifestations. Cattle with lymphosarcomas almost invariably die, either suddenly or weeks or months after the onset of clinical signs. At necropsy, lymph nodes and a wide range of tissues are found to be infiltrated by neoplastic cells (Toma et al. 1990).

**Immunity.**

Almost all infected bovines develop antibodies that persist for their whole lives but they are not able to prevent tumor formation (Johnson & Kaneene 1992). Humoral immunity plays a major role in primary retroviral infection, however, in general, antibodies alone are considered to be incapable to eliminate viral genome integrated into host DNA without antibody-dependent cell-mediated cytotoxicity (Oshishi & Ikawa 1996). Both humoral and cell-mediated immunity (CMI) are known to be induced in natural BLV infection, and they play an important role in the protection of the host against infection. Particularly, CMI against BLV antigens contributes to the suppression of BLV replication, leading to the delay of disease progression. Production of prostaglandin E₂ and changing of macrophage functions contribute to the suppression of CMI responses in animals showing clinical signs of EBL. Tumor necrosis factor α (TNF-α) may be involved in the elimination of BLV at the early stages of infection, when receptor I (RI) predominates in B cells. When the cells are stimulated by TNF-α, apoptosis may be induced and, consequently, BLV-infected cells will be cleared. When, as a result of some changing, which include the shift of cytokine profiles, B cells infected with BLV express more receptors II than receptors I of some of the infected animals will develop PL and leukemia. Thus TNF-α and its receptor may play an important role in protection against EBL (Kabeya et al. 2001).

Cytokine imbalances may contribute to disease pathogenesis. Although IL-2 is mainly known as a T cell growth factor, it can also promote BLV-infected B cells to proliferate. In addition, IL-2 increases the expression of viral protein and IL-2 receptors in B cells from cows with PL. These developments suggest that IL-2 contributes to the development of BLV-induced PL (Trueblood et al. 1998).

BLV showed a reduction of IgM-producing cells in the spleen and lymph nodes. Calves infected experimentally had lower levels of secretory IgM and reduction of T lymphocytes in the peripheral blood. BLV-infected animals showed diminished responsiveness to newly encountered antigens. Cows naturally infected with BLV produced IgG with impaired structural or biological reactivity. A correlation was found between BLV infection and lack of spontaneous recovery from *Trichophyton verrucosum* infection (Trainin et al. 1996).

CMI response to BLV antigens was suppressed in correlation to disease progression. Animals with PL and tumor formation present decreased humoral and cellular immune responses which is probably a result of viral protein immunosuppression, high levels of IL-10, T-helper cell inhibition and alterations on LB/LT ratio (Olič & Splitter 1996).

It has been shown that polymorphism in the major histocompatibility complex class II (MHC-II) was associated with disease resistance against experimental BLV infection. There was a significant difference between PL and healthy groups in the aminoacid sequence in the DRB3 region of bovine leukocyte antigen (BoLA) class II. It is expected that a breeding strategy based on the MHC haplotype can be developed to produce BLV-resistant animals (Lewin & Bernoco 1986, Zanotti et al. 1996, Kabeya et al. 2001).

**Diagnosis.**

Tumors may develop in peripheral lymph nodes, in which case the condition is easily recognized, but they may be confined to internal organs resulting in ill-defined signs. Abdominal tumors are sometimes detected by rectal palpation during pregnancy examination (Lucas 1992).

**Laboratory tests.**

*Hematological*

Herd's with a high incidence of lymphosarcoma often contain many clinically normal cattle with persistent lymphocytosis. The development of lymphosarcoma is often preceded by a period in which the animal has PL without any clinical signs of disease. Hematological methods were the main diagnostic tools for several years, and various "keys" were developed relating lymphocyte counts and age presenting maximal values above that an animal was considered to have persistent lymphocytosis. The
percentage of B-lymphocytes in normal cattle varies from 18% to 28%. In BLV-infected cattle with PL the percentage of lymphocytes can increase to as high as 70%. In clinically normal BLV-infected cattle without lymphocytosis, the B-lymphocytes are increased to 40% to 50%. PL is defined as an increase in the lymphocytes number by three or more standard deviations above the mean, according to breed and age group, maintaining this level for at least three months (Modena 1984, Lucas 1992, Radostitis 1994).

Microscopic lesions.

Nodular or diffuse infiltrations of lymphoid cells can be seen in affected organs. Based on cellular morphology, the lymphosarcoma is classified as a round cells tumor. The cells do not have intercellular junctions, being isolate disposed. Neoplastic cells show one or more of the following characteristics: increased nucleus-to-cytoplasm ratio, lost of chromatin, presence of macronucleus (usually multiple), pleomorphism (cells in the same development stage presenting different forms), intranuclear vacuolization, anisocytosis (presence of cells of different sizes) and anisokaryosis (cells with nucleus of different sizes) (Oliveira et al. 1999).

Virus isolation.

Virus can be detected by in vitro cultivation of mononuclear cells from blood collected with anticoagulant. Fetal bovine lung (FBL) cells should be used. Virus causes syncytia to develop in the cell sheet. The p24 and gp51 antigens can be detected in the supernatant of the cultures by radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot or agar gel immunodiffusion (AGID), and the presence of BLV particles can be demonstrated by electron microscopy (OIE 2000).

Serological tests.

The methods most widely used are the AGID and ELISA. ELISA can also be used to detect antibodies in milk. Infection with the virus in cattle is lifelong and gives rise to a persistent antibody response. Antibodies can be first detected 3-16 weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear. Serum samples from animals more than six-month old can be sent to antibodies detection. Calves less than six-month old may have colostrum antibodies that tend to protect them against infection. Cows in the periparturient period, 30 days before or after delivery, which present negative results, should be retested after this period because of a shift of antibody from the dam's circulation to their colostrum (Reichel et al. 1998). The antibodies most readily detected are those directed towards the virus antigens gp51 and p24. Most AGID tests and ELISAs in routine detect antibodies to the glycoprotein gp51, as these appear earlier.

Depending on the stage of the infection, the pathogenesis of BLV in cattle may involve fundamental differences in the host-viral relationship, including the number of cells infected, the number of copies of integrated provirus per cell, regulation of expression of viral antigens, induction of the antiviral immune response, and the monoclonal or polyclonal proliferation of lymphocytes, which finally influence the diagnostic tests results (Cockerell & Rovnak 1988).

Serological tests for individual serum samples should detect OIE E4 BLV International Standard Serum diluted 1:10. For pooled samples of milk, the E4 standard serum must give a positive result when diluted 250 times more than the number of individual samples that form the pool. Where serum samples are tested as a pool, E4 standard serum must test positive when diluted ten times more than the number of individual serum samples included in the pool (OIE 2000). The interpretation of serologic tests is presented in table 1.

Agar gel immunodiffusion (AGID) test.

The AGID test is a specific, but not very sensitive test for detecting antibodies in serum samples from individual animals. The AGID is simple and easy to perform and has proven to be highly useful and efficient as a basis for eradication schemes. It does, however, require skilled observers. By this method, antibodies may be detected in serum three weeks post infection. The initial appearance of the antibodies may depend upon the dose of virus received by the host as well as other peculiarities of the immune system of each animal. Seropositivity cannot be correlated with susceptibility to tumor formation. AGID-gp51 test indicates freedom from exposure to BLV from three to 12 weeks prior to collection of the serum sample. False positive or false negative reactions may occur in some calves up to six or seven months of age. These calves usu-
<table>
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<th>Age</th>
<th>Serologic result</th>
<th>Last contact with infected animal</th>
<th>Interpretation</th>
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<tr>
<td>Less than 7 months</td>
<td>Positive</td>
<td></td>
<td>*If borne from an infected dam: it is not possible to distinguish by serology if the antibodies result from infection or from colostrum. Retest the animal after it completes 7 months old, or uses PCR as diagnostic test. *If borne from a seronegative dam, or did not drink colostrum from seropositive dams, it is infected.</td>
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<td>More than 7 months</td>
<td>Negative</td>
<td>Less than 3 months</td>
<td>Retest 3 months after last contact with infected animal.</td>
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<td></td>
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<td>More than 3 months</td>
<td>It is not infected.</td>
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<tr>
<td>More than 7 months</td>
<td>Positive</td>
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<td>It is infected.</td>
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<td>Negative</td>
<td>Less than 3 months</td>
<td>Retest 3 months after last contact with infected animal.</td>
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ally will have consumed colostrum from their respective dams, which are seropositive. Cows may have undetectable antibodies in the presence of infection during the periparturient period, two to six weeks pre and postpartum. Some cattle may not develop high levels of precipitating antibody after infection (Trono et al. 2001).

**Enzyme linked immunosorbent assay (ELISA).**

The ELISA method is especially recommended for serological examinations because of its high sensitiveness and specificity, ease of interpretation, and high automatization requiring a minimal time for setup. Its main shortcoming is the possibility of nonspecific reaction occurrence. The use of virus-specific monoclonal antibodies greatly enhances the specificity of ELISA (Nguyen & Maes 1993, Kozaczynska 1999). ELISA permits the use of milk or serum pooled samples and could potentially be used to monitor herd-level disease status, and to identify herds where sub-clinical disease is present. The capability of detecting antibodies in milk would be of extreme practical value, since it would not only reduce the stress on the cattle during the process of collecting samples, but also the requirements for laboratory personnel and equipment to process serum samples (De Boer et al. 1989, Sargeant et al. 1997).

**Immunoblotting.**

A single serological method, which is able to identify serum antibodies against several viral proteins, may be expected to give useful information on the true infection status of questionable serological reactors by AGID or ELISA. Electrophoretic immunoblotting fulfills these requirements (Kittelberger et al. 1996). Immunoblotting using p24 as antigen has been suggested as a suitable confirmatory serologic test. Unfortunately, immunoblotting is a labor-intensive technique that many laboratories are not willing to tackle. One of the most difficult obstacles to successful immunoblotting is to prepare reproducible batches of antigen, which may be solved by using commercially available recombinant antigen that can be easily standardized (Kittelberger et al. 1999, Bicka et al. 2001, Choi et al. 2002).

**Syncytia inhibition assay.**

This test involves specific antibody-mediated inhibition of BLV-induced cytopathic effects in an indicator cell line. Fetal lamb kidney (FLK) cells, which are persistently infected with BLV, are capable of inducing syncytia formation in the human B-lymphoblastoid cell line (Raji), following co-cultivation. Furthermore, it was found that pre-incubation of FLK cells with sera obtained from cows infected with BLV, but not from BLV-negative cows, would effectively inhibit syncytia formation, providing the basis for a new way to screen cows for BLV infection. This assay presented results, which were comparable to Western blot and AGID (Johnson et al. 1998).

**Molecular tools.**

**Polymerase chain reaction (PCR).**

The general use of AGID and ELISA is frequently hampered by the discovery that BLV infected cattle can be found with low, transient, or even without detectable BLV antibodies (Cockerell & Rovnak 1988, Eaves et al. 1994). It is therefore important to determine the BLV status by direct detection of BLV proviral DNA. The use of PCR to detect BLV provirus has been described by various workers (Ballagi-Pordany et al. 1992, Belak et al. 1993, Beier et al. 1998). Primers constructed to match the gag, pol and env regions of the genome have all been used with variable success. Double (nested) PCR based on primer sequences from the env gene, coding for gp51, followed by gel electrophoresis and staining is the most rapid and sensitive method (Beier et al. 1998). The test is applicable to the detection of BLV infection in individual animals in the following circumstances: young calves with colorectal antibodies, tumor cases (for differentiation between sporadic and infectious leukosis), new infections (before development of antibodies to BLV), cases of weak positive or uncertain results in ELISA or AGID, systematic screening of cattle in progeny-testing stations (before introduction into insemination centers), and for cattle used for production of vaccines (ensuring that they are BLV free). PCR is not suitable as a herd test, but may be used as an adjunct to serology as a confirmatory test (OIE 2000, Rola & Kuzmak 2002, Kuckelburg et al. 2003).

**Differential diagnosis.**

Lymphosarcoma are found in young animals in the absence of BLV and it is generally classified as sporadic bovine leukosis (SBL). Three forms of SBL exist. The juvenile form in calves under six months of age involves lymph nodes, liver, spleen
and bone marrow. Few signs are seen in this form during the first part of the disease, in spite of marked enlargement of the superficial lymph nodes. As the disease progresses, internal organs, such as the heart and liver, become affected and this leads to death of the animal. The tymic form is seen in animals 6-30-month old. There is massive tumor formation in the thymus and tumorous changes are also seen in the nodes of the neck and thorax. The condition is fatal. The cutaneous form is a rare condition occurring in animals 18 months to three years of age, in which nodular lymphocytic neoplasia is seen in the skin. The first signs are urticaria-like nodules in the skin, especially around the neck, back and thighs. The nodules have a diameter of 1-2 cm. These become encrusted with thick scabs, and alopecia and hyperkeratosis follow. If the animal survives, apparent recovery takes place in several weeks. However, the remission is temporary and lesions develop again, but at this time with general lymph node involvement resulting in death. SBL does not appear to be transmissible (Willems et al. 2000). In some animals with SBL, antibodies to BLV or proviral DNA were detected, suggesting that the role of BLV in SBL should be reexamined (Jacobs et al. 1992). Besides SBL, differential diagnosis should be made with bovine tuberculosis, Jhöne’s disease (lymph node enlargement, digestive form), rabies (nervous system lesions), and pericarditis and endocarditis, when the heart is affected (Radostitis 1994).

Economic impact.

The economic costs of EBL can be categorized as direct and indirect. Direct effects are those associated with infection: lost of production in clinically and subclinically infected animals, veterinary treatment and diagnosis of EBL, and replacement costs associated with death or culling. Indirect costs are costs associated with loss of revenues due to restrictions to export of cattle and cattle products, such as semen and embryos, and costs to society for maintenance of regulatory agencies and research (Pelzer 1997).

There are different opinions about the economic impact of the BLV infection. Wu et al. (1989) and Heald et al. (1992) did not observe any influence of BLV infection on milk production or reproductive performance. For others, seropositive cows produce less milk and have poor reproductive performance (Reinhardt et al. 1988, Emanuelson et al. 1992). Brenner et al. (1989) related that seropositive animals are prematurely culled and produce less milk than seronegative ones. It seems that BLV infection affects the immune system of a cow to such an extent that it ceases to be productive enough to be kept in the herd and, in most cases, the animal is culled before any signs of illness associated with persistent immunodeficiency become apparent (Trainin et al. 1996). In Brazil, D’Angelino et al. (1998b), evaluated milk and reproductive performance of a dairy herd and observed reduction in milk production in seropositive cows. Da et al. (1993), predicted annual losses for dairy cattle in U.S. in 42 millions dollars. Recently, Ott et al. (2003) observed that herds with test-positive cows produced 218 kg (i.e. 3%) less milk per cow than herds with no test-positive cows. The average reduction in annual production was US$ 59 per cow for test-positive herds relative to test-negative herds. For the dairy industry as a whole, BLV seropositivity was associated with loss to producers of US$ 285 million, and US$ 240 million for consumers. Most of this US$ 525 million industry loss was due to reduced milk production in test-positive herds.

Another causes of economic losses are: cost of trip and examination fee, treatment for vague signals (oral fluids and injectable vitamins), blood count, serology, withholding of milk if pharmaceuticals were administered, inaccurate diagnosis and premature culling, loss of production associated with premature culling, if the culled cow was pregnant, fetal wastage (cost of semen, number of services), death of cattle with lymphosarcoma, condemnation of carcasses at slaughter (Pelzer 1997).

Control of the disease.

Appropriate control measures in any particular situation depend on factors such as prevalence of infection, impact of restrictions on ability to sell cattle, husbandry practices, economic and political considerations. Importers often require animals to be free of virus, which is one reason why many countries are developing programs of BLV control, such as eradication or attested herd schemes. In Brazil, there is no official control program for BLV. Slow evolution, high number of infected, asymptomatic animals, ill defined economic impact, lack of federal or state indemnity programs are some factors that make control very difficult. The control is based on farmer’s education, identification, segregation and/or slaughter of infected animals. For more detailed information on disease control please refer to

**Treatment and vaccination.**

*Up to now there is no treatment available.*

Eradication programs have never been considered as being economically feasible when the prevalence is high. Since the high prevalence increases the exposure risk of seronegative cattle in a herd, vaccine studies were initiated to provide some means of keeping susceptible cattle from becoming infected. A BLV vaccine must meet the requirements of being noninfectious, nononcogenic, should not interfere with the serological diagnostic tests and should be able to induce BLV-specific CMI responses.

Experiments using inactivated BLV, fixed infected FLK cells, and purified gp51 have indicated that these vaccine candidates give only short-term protection. It was also found that vaccination of cattle with live cells from a BL3 cell line, established from an animal with sporadic bovine leukosis, resulted in short-term protection (Theilen et al. 1982).

Portetelle et al. (1991) found that vaccinia virus expressing both components of the virus envelope (proteins gp51 and gp30) protected seven out of eight sheep from BLV infection, while the six controls became infected. One sheep vaccinated was infected at challenge and presented extremely low levels of neutralizing antibodies. These data suggested the involvement of antibodies in protection against primary infection with BLV. However, antibodies do not seem to be effective in eliminating the virus or in protecting BLV-infected animals from progressing to disease. Vaccination in sheep with a recombinant vaccinia virus (rVV) expressing BLV gp51 induced protection, although it was achieved without production of detectable levels of neutralizing antibodies. CMI is thought to play an important role in suppression of viral replication and elimination of virus-infected cells. From the point of view of public health, reduction of the source of the virus by rVV vaccination will lower the prevalence of the disease (Ohishi et al. 1991, Ohishi & Ikawa 1996).

Two types of live attenuated BLV variants, BLV DX, with R3 and G4 deleted and BLV 6073, with a mutation in the transmembrane gp30 gene were evaluated in cattle and sheep challenged with heterologous wild-type BLV. Four months after challenge, the protection of the vaccinated animals was effective in contrast to unvaccinated controls. However, long-term protection (18 months after challenge) was observed only in six out of seven animals, one of the vaccinated cattle being infected 12 months after challenge. A second prospective approach investigated the injection of naked plasmid DNA that encodes the BLV envelope gene under the control of the cytomegalovirus promoter (pCMVenv). Two sheep were injected with plasmid DNA, the challenge virus infection was delayed but could not be completely abrogated. These results demonstrate that vaccines based on live attenuated viruses and naked DNA injections are able to delay BLV infection, although complete protection cannot be achieved. In addition, these data cast light onto the need to perform long-term vaccination trials, because superinfection challenge can occur even after apparent protection for the first 12 months (Kerkhofs et al. 2000, Reichert et al. 2000).

It must be emphasized that most vaccination trials concerning retroviruses of large animals generally involve few animals per experimentation group and should thus be interpreted with caution. Despite these advances in knowledge, there is as yet no vaccine available commercially for the control of EBL. The mechanisms of retrovirus persistence and replication constitute a formidable challenge to vaccine development in both veterinary and human research field.

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