EPIDEMIOLOGY, INFECTION DYNAMICS AND EFFECTIVE CONTROL OF BOVINE LEUKEMIA VIRUS WITHIN DAIRY HERDS OF ARGENTINA: A QUANTITATIVE APPROACH

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EPIDEMIOLOGY, INFECTION DYNAMICS AND EFFECTIVE CONTROL OF BOVINE LEUKEMIA VIRUS WITHIN DAIRY HERDS OF ARGENTINA: A QUANTITATIVE APPROACH

Epidemiology, Infection dynamics and effective control of Bovine Leukemia Virus within dairy herds of Argentina: a Quantitative approach (Epidemiología, dinámica de la infección y control efectivo intra predial del virus de la Leucosis Bovina en rodeos lecheros de Argentina: Un enfoque cuantitativo)

Gustavo Enrique Monti

    Bovine Leukemia Virus (BLV) is a retrovirus that causes lymphomas (leucosis) and other disorders in cattle and it has a large economical impact on the livestock sector of many countries around the world. Bovine Leukemia has been reported in Argentina for the first time in 1968. Recently, a prevalence study in the whole country was performed showing an animal level prevalence of 33 % while 84 % of the sampled herds were positive. Given that the disease is more prevalent in dairy operations, the main objectives of this thesis were to provide an analytical framework to quantify several aspects related with the epidemiology and control of BLV in dairy herds from Argentina. Understanding and quantifying the transmission of BLV in dairy cattle is essential to the design of effective control strategies and BLV prevention in general. Two types of BLV isolates - Australian and Argentine - were present in dairy herds from different areas of Central Argentina and the phylogenetical tree clearly shows that Argentine isolates represent a separate and homogeneous group compared to other clusters. The low rate of non-synonymous substitutions compared to synonymous substitutions found in the analysis supports the hypothesis of purifying selection of *env*, *gag* and *pol* genes and several subunits; consequently, molecular evolution occurs under some functional constraint. It appears that for the Transmembrane Hydrophobic Region within the *env* gene - at least for the Argentine isolate - the host seems to drive the selective pressure and subtle natural variation in the structure resulted from host-pathogen interactions; hence it is a site that might be a good potential candidate for future functional studies. Simulation modeling showed that after introduction of only one infectious animal (either a heifer or adult cow) in a naive herd there is a high probability that an outbreak will occur and most likely the infection will then become endemic. Hence, if farmers want to eradicate BLV from their herds or keep their herds free of BLV, they should be extremely cautious in the addition of new animals to their herds. It was shown that although in larger herds it is possible to eradicate BLV, it takes longer and it demands more efforts in comparison with smaller herds. Simulation further showed that the time to achieve eradication was not significantly affected by a sampling frequency being either 6 or 3 months, resulting in lower costs for the farmer. The decision to adopt a specific BLV eradication strategy (combination of control measures, sampling frequency and test) that optimises the probability to achieve eradication and the time till eradication has been achieved depends heavily on the herd size. Also, early detection of infected calves dramatically reduces the time needed to achieve eradication. Our estimation of the properties (Sensitivity and Specificity) of diagnostic tests commonly used and available for surveillance showed that they are sufficiently high for being used in eradication strategies as simulated. Although several types of ELISA recently have been officially approved for surveillance purposes it does not incorporate the ELISA 108 in milk which can be advantageously used for routine screening.

To my parents who planted the seed and
To my beloved family: Ana, Melisa and Francesca
Who made it blossom

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

BOVINE LEUKEMIA VIRUS AND BOVINE LEUKOSIS

INTRODUCTION

Bovine Leukemia Virus (BLV) is an exogenous retrovirus that together with Human T-Lymphotropic Virus (HTLV) and Simian T-Lymphotropic Virus (STLV) belongs to the HTLV/BLV genus of family *Retroviridae* (Coffin *et al.,* 1997). BLV causes lymphomas and other disorders in cattle and the disease was for many years referred as Enzootic Bovine Leukosis due to the tendency of being clustered in geographical areas and herds.

Enzootic Bovine Leukosis is one of the most frequently diagnosed malignant neoplastic diseases in dairy cattle, and the virus has been found in milk of infected cows. In the early '70 this finding has raised concern about the role of BLV in neoplastic diseases in humans. Several studies have shown that there is no association between human lymphatic leukemia and BLV. No detectable antibodies to BLV was found in humans with high exposure to infected cattle and the absence of integrated BLV RNA into DNA of humans, provides evidence that there is a minimal risk of acquiring BLV and its related diseases (Johnson and Kaneene, 1991b, 1992; Miller and Van der Maaten, 1990).

The virus is distributed worldwide and produces a large economical impact on the livestock sector of many countries around the world (Johnson and Kaneene, 1992). The economic cost of Bovine Leukosis can be classified in direct and indirect costs. Direct costs are those costs associated with infection and impact more to commercial dairy producers. It includes treatment and diagnostic costs, reduced performance (especially when they show clinical signs), on-farm death losses, veterinary costs due to treatment and diagnosis, condemned carcasses and cost of early replacements (Johnson and Kaneene, 1992; Miller and Van der Maaten, 1990). Indirect costs are costs associated with: losses of revenues attributable to restrictions of export of cattle and cattle products (semen and embryos (Thurmond, 1987)), of blood collection, laboratory testing to fulfil export regulations, loss of genetic potential, an extended generation interval and costs to society for maintenance of regulatory agencies and research (Pelzer, 1997).

Under natural conditions, only cattle (*Bos taurus*), zebus (*Bos indicus*), buffaloes (*Bubalus bubalis*) and capybaras (*Hydrochoerus hydrochaeris*) have been found to be infected (Miller and Van der Maaten, 1990). Experimental infections have been most successful in sheep (Hoss and Olson, 1974). Goats and pigs do seroconvert but the pathologic effects of BLV infection either are more attenuated or do not occur in these species (Johnson and Kaneene, 1992). Persistent gp51 antibodies was found in deer, domestic rabbit and cat in a 56 month observation period whereas only one positive test was found in the dog, cottontail rabbit (*Silvilagus floridanus*) and the rat (Barthold *et al.,* 1976). Chimpanzees (Van der Maaten and Miller, 1976) and antelope are susceptible to the virus and monkeys develop a persistent infection (Schodel *et al.*, 1986). Infection of the chicken, chipmunk (*Tamias sibiricus*), deer, guinea pig, mouse, quail, domestic and wild rabbit, Sprague-Dawley rat, cat and dog also have been attempted but unsuccessfully (Johnson and Kaneene, 1992). All this information supports the hypothesis that BLV is spread and preserved primarily in cattle population and that infections in other species are accidental introductions (Miller and Van der Maaten, 1990).

VIROLOGY OF BLV

As all RNA viruses, the Bovine Leukemia Virus (BLV) genome is composed by a single strand of RNA that is duplicated as DNA by action of the enzyme Reverse Transcriptase. This process allows the virus to integrate with the genome of the host cells, producing a chronic infection. The target cells for BLV are mainly B-lymphocytes, but they can infect some T-lymphocyte populations as well (Stott *et al.*, 1991).

The life cycle of BLV is like most retroviruses and the steps are shown in Figure 1.

Figure 1. Retroviral replication cycle. Reproduced with permission of Cold Spring Harbor Laboratory Press**.**
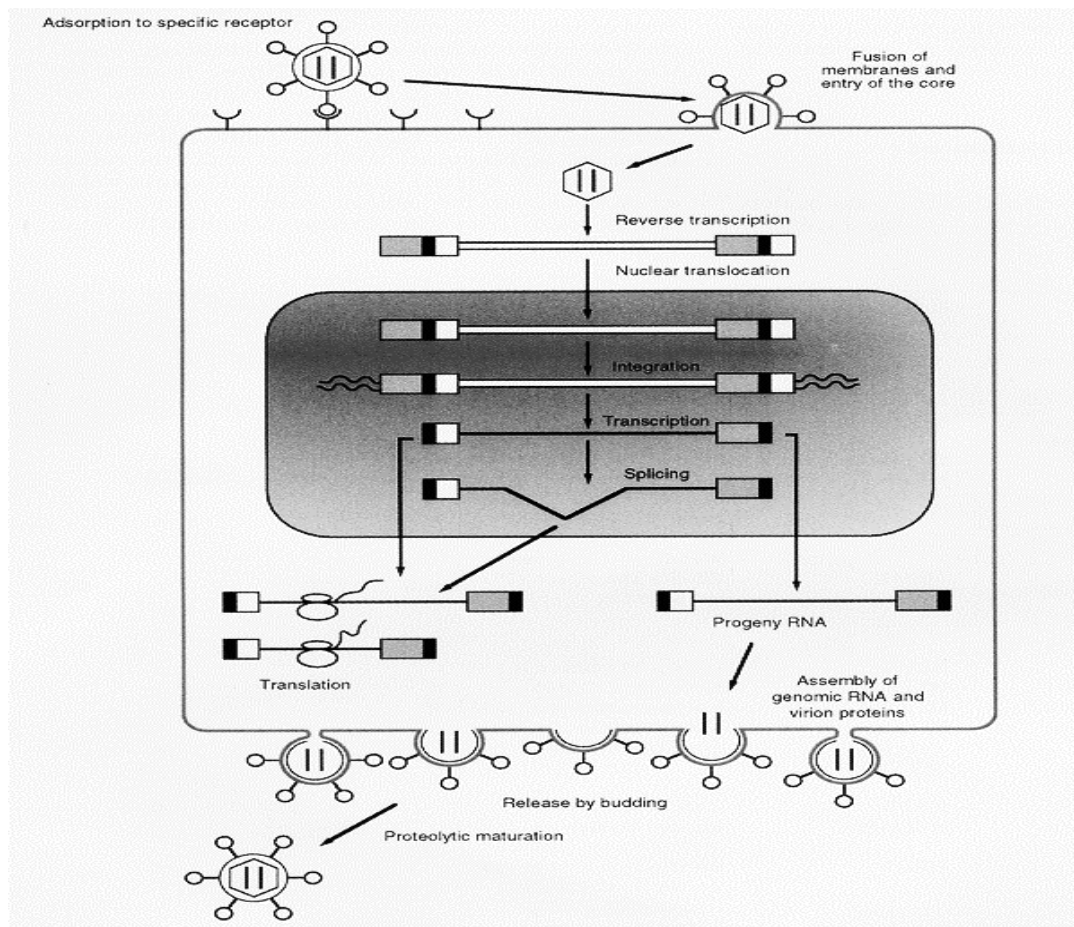
Virus envelope proteins (SU) recognise the surface of the target and the virus attaches the cell. Then, the virion is engulfed into the cell and the digestive enzymes of the host's cell remove the envelop. The virus RT makes a double-stranded DNA copy from the RNA and integrates with the host's cell nucleus and once it is integrated it uses the replication mechanisms from the host cell to produce more RNA (by cellular RNA polymerase II). Proteins are packed into new virions that bud from the cell, resulting in viremia and infection of other cells.

Unlike other retroviruses in which viremia readily occurs and can persist, BLV viremia occurs only in the early stages of infection (Gupta *et al.*, 1984). Unspliced or monospliced viral mRNAs are absent or weakly expressed, hence BLV infection is rarely replicative *in vivo* and the full understanding of the mechanisms that regulate the viral latency is still

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lacking (Merezak *et al.*, 2001; Schwartz and Levy, 1994). However, a non-immunoglobulin plasma factor that has been shown to participate in the viral synthesis, suppress the production of new virions *in vivo* (Gupta *et al.*, 1984). As a consequence, an infected host can clear itself from the virus only by destroying its own infected cells, but the immune system does not recognise these infected cells as being foreign. Therefore, infections with BLV become persistent and last lifelong. In contrast, culture of infected lymphocytes *in vitro* seems to unblock viral synthesis and it is used to produce virus (Miller and Van der Maaten, 1990).

The virus seems to be very unstable outside host cells and it can be destroyed by exposition to UV light, by freezing and thawing, heating at 56 °C for 30 minutes and to pasteurisation (Baumgartener *et al.*, 1976; Rubino and Donham, 1984). It can survive at least 2 weeks in refrigerated blood (4 °C) (Lucas, 1992). Transmission between individuals commonly resulted from exposition to provirus integrated in lymphocytes and not as a cell-free virus. This is an important feature because it implies that to minimise risk of transmission, for example by applying either hygienic measures or disinfecting procedures on contaminated objects, measures should be aimed at destroying infected lymphocytes and not only the virus.

Several structural proteins that are shown in Figure 2 compose the provirus.

Figure 2. Schematic cross section through a retroviral particle. The viral envelope consists of the lipid bilayer into which transmembrane (TM) and the surface (SU) components are inserted. Internal structural proteins are the matrix (MA) protein, capsid (CA) protein, and nucleocapsid (NC) protein. Other components are the reverse transcriptase (RT), integrase (IN) and the protease (PR). Reproduced with permission of Cold Spring Harbor Laboratory Press.
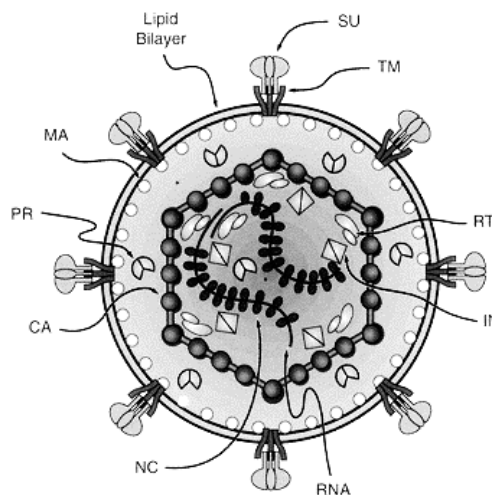
These proteins include the capsid proteins, the envelope proteins, the reverse transcriptase and the viral proteases encoded from the *gag, pol, env* and *prt* open reading frames respectively. The gag region of the viral genome encodes internal nonglycosylated structural proteins. They are the matrix (MA) protein (p15), capsid (CA) protein (p24), and nucleocapsid (NC) protein (p12). The proteins encoded by the *gag* gene are involved in the formation of the viral capsid (Schwartz and Levy, 1994). The *pol* region encodes the reverse transcriptase (RT) and also an integrase (IN) (Schwartz and Levy, 1994). The protease

(PR) is derived from the *prt* gene between *gag* and *pol*. The viral envelope is formed by a cell-derived lipid bilayer into which proteins encoded by the *env* region are inserted.

These proteins consist of the transmembrane (TM) (gp30) and the surface (SU) (gp51) components linked together by disulfide bonds. Gp51 ensures the recognition of the cellular viral receptor and monoclonal antibodies have been used to identify its eight antigenic sites (sites A through H) (Bruck *et al.*, 1982). Sites F, G and H are involved in determining infectivity of the virus and its ability to induce syncytia by target cells. N-terminal of gp 30 is inserted into the lipid bilayer and anchors the gp51/gp30 complex in the viral envelope and the infected cell membrane (Rice *et al.*, 1984; Sagata *et al.*, 1985). The gp30 intracytoplasmic portion presents a motif that is found in the signal-transduction molecules of the T cell and B cells receptors. Some of these proteins (envelope gp51 and core p24) are of especial interest because are used for diagnostic purposes.

The integrated BLV genome consists in a sequence of 8714 nucleotides long (Sagata *et al.*, 1985), the extremities present a sequence called LTR (Long Terminal Repeat) which is composed of three consecutive regions named U3, R and U5. At least seven alternatively spliced RNAs have been identified as well as eight open reading frames (orf) designated as *gag, prt, pol, env, tax, rex, RIII and GIV* (Figure 3).

Figure 3. Genomic organisation of Bovine Leukemia Virus (BLV). Boxes correspond to open reading frames.
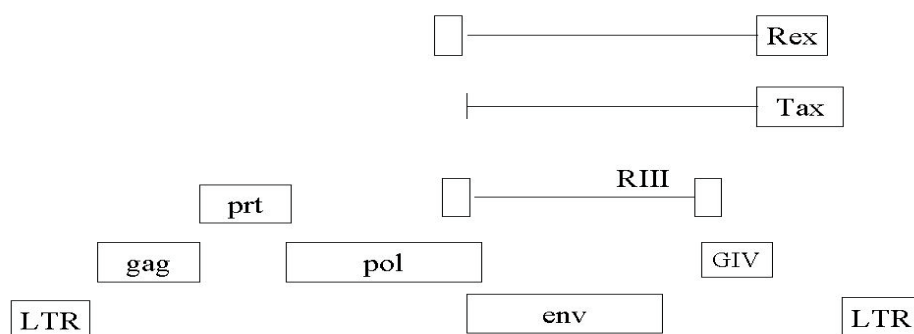
One feature that distinguishes BLV-HTLV genus from other retroviruses is the relatively low and slow genetic diversity. Nevertheless, at present four complete BLV genomes have been characterised: Belgian isolate (Rice *et al.*, 1985; Rice *et al.*, 1984), Japanese isolate (Sagata *et al.*, 1985), Australian isolate (Coulston *et al.*, 1990) and recently, Argentine isolate (Dube *et al.*, 2000).

## PATHOGENESIS OF THE DISEASE

The mechanism that makes BLV to induce tumor is not clear. No known oncogene could be derived from BLV-induced tumors (Johnson and Kaneene, 1991b). However, some studies indicate that Tax protein is the most probable viral element involved in lymphocyte transformation at least in the initiation of tumorigenesis (Schwartz and Levy,

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1994; Twizere *et al.*, 2000), but it may not be required to maintain the continuous production of tumor cells. Besides Tax protein, other viral proteins (*Rex, GIV, RIII* and even *env*) also are likely to play a role in BLV pathogenicity (Schwartz and Levy, 1994).

The schematic representation of the main phases of the disease is shown in Figure 4.

Cell-free viremia is only detectable in the first 2 weeks after infection due to the rise of the levels of a plasmatic non-humoral inhibition factor, produced by the host (Gupta and Ferrer, 1982). Then the animal develops a serologic immune response mainly to protein gp51 and p24 between 2 to 8 weeks post infection (Johnson and Kaneene, 1992). Antibodies against these proteins are present life-long but levels can oscillate, even below detection limits of commonly used serological tests. For example there is a decrease of antibodies levels due to physiological immunodepresion during peripartum (2 weeks prior and 4 posterior to calving) (Hopkins and DiGiacomo, 1997) or induced by other agents (persistent infected animals co-infected with Viral Bovine Diarrhoea) (Roberts *et al.*, 1988).

Figure 4. Proposed stages from Bovine Leukemia virus infection to clinical disease.
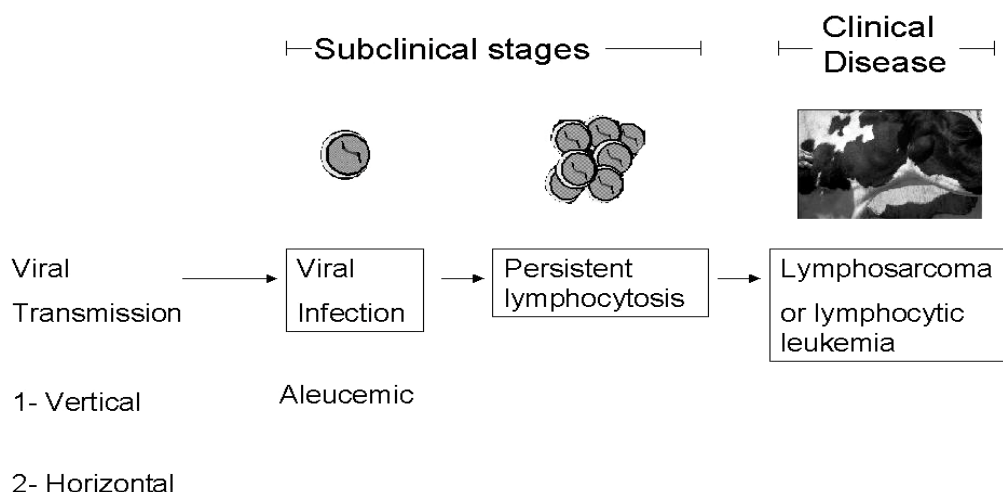
Most infected animals act as asymptomatic carriers and they are denominated aleucemics. A variable proportion Some of the infected animals (up to 70 %) start with a polyclonal expansion of mature circulating blood B-lymphocytes that are cytologically normal and cariotyped as normal, this is denominated Persistent Lymphocytosis (PL). Some animals shows an increase (towards B cells) of the ratio of B/T lymphocytes, but maintaining a normal level of total blood lymphocytes (Lewin *et al.*, 1988a). Then, the disease starts with the clinical expression and the symptoms are the development of a multicentric lymphosarcoma that can be detected between 1 to 8 years after infection and which occurs in 1 to 5 % of the infected population (Ferrer *et al.*, 1979). Whether clinical signs are present is largely related by the location of the tumours (lymph nodes, heart, abomasum, kidney, uterus, spinal canal and intestine). The most commonly reported signs are weight loss, agalactia, lymphoadenopathy, anorexia, chronic indigestion, melena, diarrhea, heart failure, and posterior paresis/ paralysis (Johnson and Kaneene, 1991a).

Important to mention is that only some animals develop all the stages of the disease and the sequence does not necessary represent early or late stages of the illness. For example, it has been shown that some animals can develop lymphosarcoma from the carrier-state without experiencing PL. Moreover, it has been reported, that the susceptibility of cattle to BLV infection, development of PL and lymphosarcoma is related, among other factors, to genetic traits of the host like bovine lymphocyte antigens (BoLA); leading to the concept of genetically susceptible/resistant individuals (Lewin and Bernoco, 1986; Lewin *et al.*, 1988b; van Eijk *et al.*, 1992; Xu *et al.*, 1994; Zanotti *et al.*, 1996).

TRANSMISSION

Infection has been confirmed when an infective inoculate was administrated via the most common routes of exposure: intradermal, intramuscular, intravenous, intraperitoneal, intranasal, intrauterine, rectal, aerosol and oral routes (Johnson and Kaneene, 1992).

Some biologic fluids from infected animals are potential sources of transmission of BLV, and their infectivity depends on the lymphocyte count of the fluid (Hopkins and DiGiacomo, 1997). Presence of BLV has been detected in blood, colostrum, milk, brochoalveolar lavage, urine, uterine flush fluids and occasionally in saliva and nasal secretions (Hopkins and DiGiacomo, 1997).

The virus is transmitted horizontally by infected lymphocytes (Meas *et al.*, 2002; Romero *et al.*, 1982) or vertically (Meas *et al.*, 2002; Piper *et al.*, 1979; Van der Maaten *et al.*, 1981b). Most of the time, contamination is iatrogenic and occurs when the animals are manipulated without hygienic care. High risk manipulations are injections, gouge dehorning, tattooing, ear tagging, castration, immunisations, venipuncture, teat removing and rectal examinations (Hopkins and DiGiacomo, 1997; Miller and Van der Maaten, 1990). Transmission by biting of insects has been report as well, especially bites from insects of the family *Tabanidae* (Hopkins and DiGiacomo, 1997; Johnson and Kaneene, 1992).

BLV does not seem to be transmitted by ova or embryo transfer but natural service might be a source of infection because blood may be transferred during copulation (Hopkins and DiGiacomo, 1997).

Prolonged physical contact between cattle is also a risk factor for transmission of BLV. Because the presence of cell-free virus is a rare event, direct contact between animals would be an insufficient mechanism of transmission without a mechanism for transferring of infected lymphocytes. The periparturient may be a period of increased risk of BLV transmission in cows and heifers (Hopkins and DiGiacomo, 1997). At parturition, dairy cows and heifers are often confined in maternity pens or paddocks for calving and postpartum care. During maternity confinement, opportunities exist for exposure to blood (Gatei *et al.*, 1989), tissues and uterine fluids from other cows in the calving area. Moreover, circulating antibodies to BLV decrease at parturition sometimes below the level of detection of serologic tests (Hopkins and DiGiacomo, 1997). Accordingly, viral expression could be increased in BLV-infected cows, increasing their potential infectivity.

Calves can be infected *in utero* (vertical transmission). Both virus and antibodies are detected in new-born calves, indicating that they are infected during the second half of gestation. However, this transplacental transmission mainly occurs when the mother is in the lymphocytosis stage (Piper *et al.*, 1979; Van der Maaten *et al.*, 1981b; Van der Maaten *et al.*, 1982). Furthermore, *in utero* infection with BLV does not result in a prolonged incubation period or a latent infection that can not be detected by serologic or virologic

methods at birth. Vertical transmission is unrelated to any specific age or parity of the cows (Thurmond *et al.*, 1983) and it is not related to successive pregnancies (Piper *et al.*, 1979).

Colostrum and milk are sources of infection (Hopkins and DiGiacomo, 1997) but also contain antibodies anti-BLV (Jacobsen *et al.*, 1989; Van der Maaten *et al.*, 1981a; Villouta *et al.*, 1990) and there is no relationship between detection of BLV and stage of lactation (DiGiacomo, 1992). Susceptibility to BLV oral infection probably is related to the brief time in which the intestinal mucosa allows permeability to large proteins or cells like lymphocytes and only can occur during the first three days of life (Romero *et al.*, 1983).

LABORATORY DIAGNOSIS

INDIRECT TESTS
Serological
The agar gel immunodiffusion test (AGID) (Miller and Van der Maaten, 1976) can be used to detect mainly antibody to viral antigens gp51 and in less extension to p15 and p24. The test is simple and practical and has been used widely (Johnson and Kaneene, 1992; Miller and Van der Maaten, 1990). One constraint is that it can not be used to detect antibodies in milk and like most serological tests it can not distinguish between antibodies passively transferred from the dam to those produced by an active infection.

Virus neutralisation tests (Balgava *et al.*, 1984) are based on the ability of antibodies to inhibit the effects of BLV in cell cultures. These include several variants like: syncytia inhibition (Johnson *et al.*, 1998) and early polykaryocytosis test (Ferrer and Cabradilla, 1978; Graves *et al.*, 1982; Guillemain *et al.*, 1978). A complement fixation test has been developed (Mammerickx *et al.*, 1977) but is less sensitive than AGID and another disadvantage is that many bovine sera are anticomplementary.

Radioimmunoassay (Bossmann *et al.*, 1989; McDonald and Ferrer, 1976) is very sensitive but special equipment is required and manipulation of radiolabelled reagents is required. Finally, Enzyme-linked immunosorbent assay (ELISA) (Mammerickx *et al.*, 1984a) has been adapted in several ways (to detect gp51 or p24) to detect BLV antibodies in serum and milk of individuals and of pooled samples as well (Mammerickx *et al.*, 1984b). Finally, immunoblotting techniques for detection of gp51 and p 24 have been developed (Bicka *et al.*, 2001; Choi *et al.*, 2002; Kittelberger *et al.*, 1996).

Haematological
It was one of the first methods used, it is based on 'keys' - that is lymphocytes counts for different age groups – that define 'normal'; 'suspected' and 'lymphocytosis' classifications (Mammerickx *et al.*, 1976; Ressang *et al.*, 1976).

DIRECT METHODS
Isolation and detection of BLV
Virus particles can be seen by electron microscopy in short-term cultures of lymphocytes (Bratu *et al.*, 1994; Solisch *et al.*, 1977). Cell culture methods of detection are based on the ability of the virus to induce syncytia in different cells monolayers (Jerabek *et al.*, 1978; Liebermann *et al.*, 1982; Van der Maaten and Miller, 1975).

Sheep inoculation (Lucas, 1992) is a sensitive method and currently several types of Polymerase Chain Reaction (PCR) methods have been developed: nested PCR of integrated virus DNA, real-time PCR, RT-PCR (Dube *et al.*, 1997; Fechner *et al.*, 1996; Kelly *et al.*, 1995; Klintevall *et al.*, 1994; Marquardt *et al.*, 1996).

PREVALENCE OF BOVINE LEUKEMIA VIRUS INFECTIONS IN ARGENTINA

Bovine Leukemia has been reported in Argentina for the first time, in 1968 (Sen *et al.*, 1968). Recently, an extended prevalence study in the whole country was performed (Trono *et al.*, 2001) showing an overall prevalence of 33 % of the tested cattle were infected and 84 % of the sampled herds. Stratification by prevalence level shows that 16 % of the herds did not show infected animals, approximately 20 % of the herds presented a prevalence less than 15 %, 17 % between 15 and 30 %, almost 50 % of the herds showed values greater than 30 %. Despite some differences in individual prevalence between different dairy areas, most areas can be regarded as heavily infected based on the proportion of infected herds. This is in agreement with other studies, though those studies were limited to specific areas of the country (Ghezzi *et al.*, 1997; Monti *et al.*, 2002). One of these studies (Esteban and Mettler, 1980; Ghezzi *et al.*, 1997) based on the region Mar y Sierra assessed changes over a period of several years and showed that 95 % of the farms where free of BLV in 1981 and 15 years later just 30 % of the sampled belonged to that category. It is the only study of this kind available and it is not clear whether the same effect has taken place in other areas.

# ARGENTINA AND THE DAIRY SECTOR

The Argentine Republic is bounded on the north by Bolivia and Paraguay; on the east by Brazil, Uruguay, and the Atlantic Ocean; on the south by the Atlantic Ocean and Chile; and on the west by Chile. The country occupies most of the southern portion of the continent of South America. The area of Argentina is 2.766.889 km² and it is the second largest South American country. Argentina comprises a diverse territory of mountains, upland areas, and plains. Eastward from the base of the Andean system, the terrain of Argentina consists almost entirely of a flat or gently undulating plain. The Pampas, treeless plains that include the most productive agricultural sections of the country, extend nearly 700.000 km².

Argentina and Uruguay are the only traditional dairy exporting countries of Latin America, thus their dairy production and processing sector has been focussed to increase milk production, to adopt new technology, etc. in order to obtain dairy products in sufficient quantity and of the quality required for export. Argentina is the second milk producer in Latin America with 9000 million litres per year and an average milk consumption of 109 litres/person/year. In addition, large dairy co-operative organisations (SANCOR, MILKAUT), private companies (Suc. Alfredo Williner, La Serenísima, Molfino) and big farms have a good economic capacity to promote research and help to

create technical and scientific organisations specialised in milk production and processing such as CITIL (Dairy Industry Technological Research Centre), CERELA (Lactobacillus Reference Centre) and others. The value of the total dairy production was U$S 4.800 mill. (1998). Argentina is the 6° world producer of milk powder, the 7° of cheese and 17° of butter. The main markets of those products are the Mercosur (Common Market of South American Countries) national markets, especially Brazil. In 1999 it has exported 256.000 tons of dairy products that represented US$ 377 millions 75% of it was due to 4 companies, 3 of them being located in the area under study.

The main dairy areas of Argentina are Central-Santa Fe Province (the one which the thesis is based on); South-Santa Fe Province; South-Córdoba Province; Center-Córdoba Province; Periurban-Buenos Aires; Mar y Sierras (South- Buenos Aires Province); Center Buenos Aires Province and Entre Ríos Province (Figure 5). The production system mainly used is semi-extensive on pasture supplemented with maize silage and moderate amount of cereals where animals are not confined in barns.

Figure 5. Geographical location of the main dairy areas of Argentina. (1) Central Buenos Aires; (2) Peri-Urban Buenos Aires; (3) Mar y Sierras; (4) Central Santa Fe; (5) South Santa Fe; (6 and 7) South Córdoba; (8 and 9) Center Cordoba and (10 and 11) Entre Ríos.
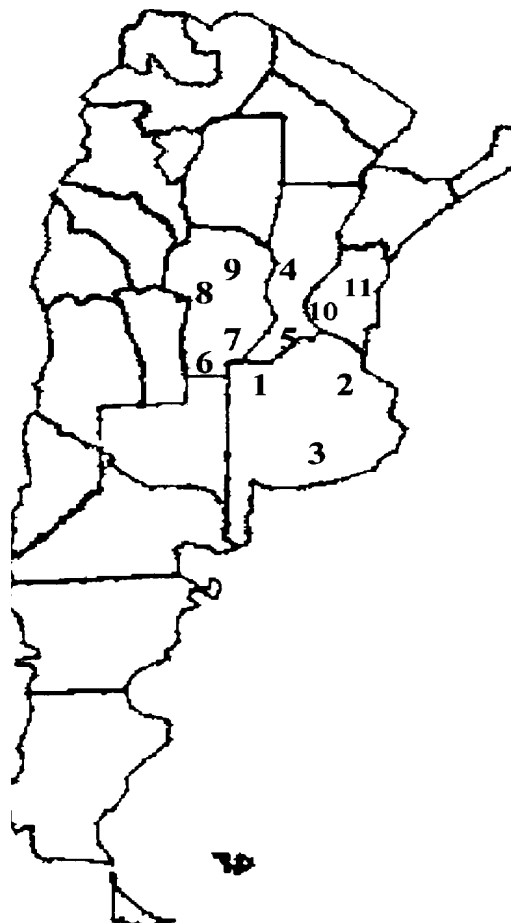
THE AREA UNDER STUDY AND THE PROBLEM OF BLV INFECTION

The Central Santa Fe dairy area comprises 2.500.000 hectares the main characteristics of it are summarised in Table 1.

Table 1. Main characteristics of the Central Milk Region, Argentina (MAGIC-Sta. Fe).

Total No. Farms	Kg. Fat/ Ha. / Year	Milk Production / lact. *(lt.)*	% Fat / % Prot.	Average No. of Milking-Cows	Average Farm Surface *(Ha)*
5.664	145	4.600	3.3 / 3.0	96	101

In this region 40% of country milk production is produced and the main dairy companies of the country (that commercialise 60 % of the domestic dairy products market) operates here. The area produces a volume of 7 millions litres/day and according to a very recent survey (2003) by researchers of Faculty of Agrarian and Veterinary Sciences of Universidad Nacional del Litoral, the composition and quality of the milk produced in this area is comparable to those of developed countries. Some of the main figures are: annual average contents of fat (3,59%), total protein (3.13%), lactose (4,78 %), total solid and non-fatty solids contents (12,2% y 8,62% respectively). *Staphylococcus aureus* was present but in limits that constitute no risk for human health. *Listeria monocytogenes*, salmonella sp., residues from plaguicides and heavy metals (like arsenic, cadmic, mercury and plumber) were not detected in any sample. In all cases the levels are in agreement to national and international standards for milk quality.

During the last years, many breeders from Santa Fe area have increased the trade of live animals (mainly reproducers) to Mercosur countries as well. Due to the good genetic level of the dairy cattle population, there is a potential world market of frozen embryos as well. Although it is well proven that BLV is not transmitted by embryos, international trade regulations (suggested by International Embryo Transfer Society) frequently require that exporting herds should be free of BLV-infected animals (Office International des Epizooties, 1998). Exporting contracts are risky because the low number of herds that are BLV-free. The endemic BLV situation might cause also a national, because the area under study is also one of the most important domestic suppliers of superior genetic replacements for dairies within the same area and other regions of the country. Consequently if a large number of herds simultaneously decide to substitute infected animals there would not be a sufficient number of BLV-free replacement animals at least in the early stages of the program. Hence this could constitute a potential bottleneck for a compulsory control program.

FACTS AND REASONS FOR A BLV-PROGRAM AND POTENTIAL CONTRIBUTIONS FROM THE THESIS

The primary dairy sector has evolved towards: reduction in the number of dairy farms, increasing herd sizes, increasing total production per farm and increasing individual cow production performance. All previous parameters can be interpreted as a significant improvement of the sector productivity but some of them (concentration of dairy units and bigger herds) also have increased the risk of spread of BLV.

Also, the area of study is the region with lowest prevalence of Brucellosis (Res. SENASA 150/2002, Res. SENASA 497/2002) and Tuberculosis (Res. 115/99, SENASA) of the country, with approximately 2800 (45 %) and 1500 (30 %) herds respectively, certified as free. For that reason Farmer's associations and Provincial Animal Health Services are interested in develop a compulsory pilot-control program in the area, instead of the current National Voluntary Control Program (RES. 337/94, SENASA).

This thesis aims to produce information that enhances understanding of the dynamics of BLV transmission and to identify critical factors that lead to effective control of BLV within premises. By that it will contribute to ensure the sustainability of the dairy sector in this important dairy area.

OUTLINE OF THE THESIS

The aim of the thesis is to increase the quantitative knowledge of various aspects of Bovine Leukaemia Virus (BLV, causative agent of Enzootic Bovine Leukosis) infection as it behaves in Argentine dairy herds. The acquired knowledge can be used to design new or improve existing control programs at herd or higher aggregation levels.

The thesis is organised in 8 chapters. The major aspects of each chapter are:

Chapter 1 comprises a description of the agent, the disease and the importance of the disease within the context of the dairy sector of Argentina.

Chapter 2 explores genetic diversity of the agent and spread of different variants in dairy farms from Argentina.

Chapter 3 presents the performance of available diagnostic tools in Argentina, especially in relation to a new developed enzyme-linked immunosorbent assay (ELISA 108/97) in milk and serum.

Chapter 4 evaluates the time from infection to seroconversion and the factors that affect the length of this interval.

Chapter 5 describes population dynamics of Bovine Leukaemia Virus in Argentine dairy herds and quantification of parameters that define transmission dynamics within herds from a field study.

Chapter 6 shows the results of transmission within herd a by quantitative simulation using mathematical modelling.

Chapter 7 evaluates the impact of different control measures and strategies on eradication of BLV within herds.

Chapter 8 presents the evaluation of the main outcomes of this thesis and a general discussion about them with some recommendations.

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CHAPTER 2

GENETIC DIVERSITY AND SPREAD OF BOVINE LEUKAEMIA VIRUS ISOLATES IN ARGENTINE DAIRY CATTLE

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SUMMARY

Effective tools for use in control programmes against Bovine Leukaemia Virus (BLV) infections require insight into the relationship between the variant structure of the Bovine Leukaemia virus and the spatial-temporal interaction of isolates and hosts. Our study showed the presence of two types of BLV isolates - Australian and Argentine - in dairy herds from various parts of Central Argentina; these isolates were characterised by RFLP on PCR amplicons, and some of them were confirmed by sequencing. One genotype (Argentine) was present in all herds, and the Australian genotype was found in two herds.

Phylogenetic analysis indicated four clusters. The first cluster was composed of the Argentine isolates and one from Brazil; the second was composed of several isolates found in European countries and one from Brazil; the third cluster was composed of BLV isolates found in Japan and Germany; the fourth cluster included American and Australian isolates and those from other countries.

The comparison of a number of synonymous and non-synonymous nucleotide substitutions using various BLV genes revealed purifying selection, suggesting that molecular evolution occurred under some functional constraint.

INTRODUCTION

Bovine Leukaemia Virus (BLV) is an exogenous retrovirus that, together with Human T-Lymphotropic Virus (HTLV) and Simian T-Lymphotropic Virus (STLV), belongs to the deltavirus genus of the *Retroviridae* family (Coffin *et al.*, 1997). Some of the cattle infected with BLV can suffer from lymphomas and/or a proliferation of B-lymphocytes-denominated Persistent Lymphocytosis, but the majority of BLV-infected cattle are healthy carriers of the virus (Miller and Van der Maaten, 1990).

The genetic structure of pathogen populations is important in many contexts (such as development of vaccines, resistance and treatments). However, the dynamics of virus strains are also of fundamental biological and epidemiological interest (Foxman and Riley, 2001). Moreover, an important goal in studying the genetic diversity of retroviruses is to gain a better understanding of the correlation between retrovirus variation and disease progression (Mansky, 1998). At present, four complete BLV genomes have been reported worldwide: Belgian isolate (Rice *et al.*, 1984; Rice *et al.*, 1985), Japanese isolate (Sagata *et al.,* 1985), Australian isolate (Coulston *et al.*, 1990) and recently, Argentine isolate (Dube *et al.*, 2000). Furthermore, knowing whether geographical patterns of variation in a particular trait are adaptations that arose through natural Darwinian selection or purifying selection could give valuable information to understand polymorphism variation and to determine candidate sites of the genotypes for functional studies.

In this study our aim was to characterise BLV isolates and their distribution in dairy herds in Argentina. In addition, we studied whether selective advantages of different genotypes could be introduced by purifying selection.

MATERIALS AND METHODS

STUDY POPULATION

Blood, serum and milk samples were collected from 445 lactating Holstein cows from nine farms located in the central region of Argentina. These herds were selected based on the owners' willingness to collaborate in this study. All lactating cows in five of these herds were sampled, while randomly-selected samples were taken from the other four herds. We also used results from two herds in this study that also were included in a two-year longitudinal study.

SEROLOGICAL ASSAYS

Each cow was screened with the ELISA 108 (milk and serum kit), described in (Gutierrez *et al.,* 2001), which is designed to detect antibodies against gp51. After testing these samples with the PCR, these cows were tested with the Agar Gel Immunodiffusion Test (Fac. Cs. Vet, UNLP) and a commercial ELISA (SERELISA BLV Ab Bi Indirect, Symbiotics).

PCR ASSAY

Oligonucleotide primers for PCR (MWG Biotech) were designed according to sequence data published elsewhere (Sagata *et al.*, 1985). Primers corresponding to the *env* gene (Rice *et al.*, 1985) were selected, and *env$_{5032}$* 5' - TCT GTG CCA AGT CTC CCA GAT A - 3', and *env$_{5099}$* 5' - CCC ACA AGG GCG GCG CCG GTT T - 3' were used as forward

primers. The reverse primers were env_{5521r} 5' - GCG AGG CCG GGT CCA GAG CTG G - 3', env_{5608r} 5' - AAC AAC AAC CTC TGG GAA GGG T - 3'. The sets env_{5099} and env_{5521} had been established and described previously (Naif *et al.,* 1990; Naif *et al.,* 1992).

DNA was obtained from frozen blood collected with EDTA and was extracted using the NucleoSpin Blood kit (Machery-Nagel). The first round of nested PCRs (Fechner *et al.*, 1996) was performed using env_{5032} / env_{5608r} as first primers; initial incubation of samples was at 72 °C for 2 minutes; denaturation took place at 94 °C for 2 minutes followed by 50 amplification cycles consisting of denaturation at 95 °C, 30 seconds, primer annealing at 58 °C, 30 seconds and extension at 72 °C for 1 minute; final extension took place at 72 °C for 4 minutes. The second round of amplifications was conducted using the second pair of primers (env_{5099} / env_{5521r}). The second round was the same as the first round except that the primer annealing temperature was changed to 72 °C. A known positive and negative control DNA sample was included in each test run, and samples showing a band migrating at 444 base pairs (bp) were considered as positive.

RESTRICTED FRAGMENT LENGTH POLYMORPHISM (RFLP) PROTOCOL

Integrated BLV provirus present in the DNA of circulating leukocytes from infected animals was compared by restriction endonuclease digestion. Randomly-selected samples (121) from amplicons of cows that tested positive for PCR were analysed by RFLP using a series of triple enzymes (*BamHI, PvuII* and *BclI*; Roche diagnostics GmbH) (Beier *et al.*, 2001). The test was done twice on 22 samples to assess repeatability of the results. RFLP was performed using 2.5 μl of the PCR product that was digested using 0.25 μl of each restriction enzyme, 1 μl of buffer and 7μl of distilled water. This mix was incubated for 2.5 hours at 37 °C for *BamHI* and *PvuII* and at 52 °C for *BclI*.

NUCLEOTIDE SEQUENCING AND ANALYSIS

In addition to RFLP analysis, 13 randomly-selected samples (of the initial 121 samples) that were characterised by RFLP were sequenced using *env*-forward-primer env_{5099} and *env*-reverse primer env_{5521r}; both primers were modified on the 5´-end with IRD800 fluorescent colour. A sequencing reaction was carried out using the ThermoSequenase fluorescent-labelled primer cycle sequencing kit with 7 deaza-dGTP. The reaction was composed of PCR-amplicons (same primers unmodified) diluted 1:10 as template DNA and sequence primer 1-2 pmol under the following conditions: 25 cycles using 95 °C, 30 seconds and 60 °C, 30 seconds.

Data collection and data analyses were carried out automatically using BaseImageIR™ software (V. 4.1). Multiple sequences were aligned using the ClustalW facility of DAMBE, V.4.0.84 (Xia and Xie, 2001). Pairwise comparison of nucleotide sequence and searches for homologous DNA sequences were performed with the program BLAST 2.1 available on the National Center for Biotechnology Information (NCBI) website (Altschul *et al.*, 1997) using all available sequence-database libraries. The sequences we obtained were compared with 15 sequences from GenBank® that were included in Table 1. Four sequences (1272 D/W, 1002 D, 505 DD/W and 1137NZ) belonging to a database from the Institute of Epidemiological Diagnostics in Wusterhausen (Beier, personal communication), were also included in the comparison.

Table 1. Bovine Leukaemia Virus isolates and genomic sequences

Isolate	Background Information	Accession no
	Sequences from GenBank®	
JAP	BLVCG , complete genome, Japan, 1985	K02120
AUST	Australian-BLV isolate, complete genome, Australia, 1990	D00647
ARG38	ARG38 Argentine isolate, complete genome, Argentina,2000	AF257515
D3	BLV isolate 3, env, partial cds, Germany,1997	U87872
D15	BLV strain cow 527, env, partial cds, Germany, 1997	AF007764
D18	BLV strain cow 134, env, partial cds, Germany, 1997	AF007763
D23	BLV isolate 23 env, partial cds, Germany,1997	U87873
VDM	VdM isolate from bovine tumor, env, complete cds, 1990	M35239
LB285B	LB285 isolate from bovine tumor, env, complete cds, Belgium, 1990	M35240,
FLK	FLK-BLV clone pBLV 913, env, complete cds, 1990	M35242
LB59F	LB59 isolate, cell line LB59Lyc , env, complete cds, France, 1990	M35238
IT2	North Italian isolate I2 isolated from blood leukocytes, env, partial cds, Italy, 1996	S83530
Bras1	BLV isolate 8513 isolated from blood leukocytes, env partial cds, Brazil, 2001	AF399702
Bras2	BLV isolate 30, env complete cds, Brazil, 2001	AF399703,
Bras3	BLV isolate 384, isolated from blood leukocytes, env complete cds, Brazil,2001	AF399704
GAGA	BLV from bovine tumour DNA gag, pol and env, complete cds, Belgium, 1985	M10987-K02251
	Sequences from our study	
SF2	Isolate BLV-ARGSF2, env partial cds, Argentina, 1999	AY178820
SF6	Isolate BLV-ARGSF6, env partial cds, Argentina, 1999	AY178818
SF7	Isolate BLV-ARGSF7, env partial cds, Argentina, 1999	AY178817
SF8	Isolate BLV-ARGSF8, env partial cds, Argentina, 1999	AF485773
SF11	Isolate BLV-ARGSF11, env partial cds, Argentina, 1999	AF485775
SF13	Isolate BLV-ARGSF13, env partial cds, Argentina, 1999	AY178819
ASF406	Isolate BLV- ASF406, env partial cds, Argentina, 1999	AF485774

Base composition bias was assessed by evaluating the effective number of codons (Nei and Kumar, 2000), and the distribution of sequence variation was analysed using mean genetic distances within and between clusters of isolates.

A powerful method of discriminating between positive Darwinian selection and neutral polymorphism is to compare the rates of synonymous (d_s) and non-synonymous (d_n) nucleotide substitutions per site (d_s/d_n) (Nei and Kumar, 2000). The null hypothesis is the neutral drift and equiprobable nucleotide mutations given the specific codon at each position (neutral theory). We analysed the d_s/d_n for complete alignment of *env* (isolates Bras2, Bras 3, Arg38, VdM, FLK, Jap, Aust, GAGA, LB59F and LB285B), *pro*, *pol* and *gag* (isolates Arg38, Jap, Aust and GAGA) genes and some target areas within them.

Based on overall and pairwise comparisons, the proportions of possible nucleotide substitutions for all sites for both synonymous and non-synonymous sites were calculated (Pamilo-Bianchi-Li-Method). Evidence of positive, neutral or purifying selection was

sought with a Z-test based on the number of synonymous substitutions per synonymous site (K_S) and the number of non-synonymous substitutions per non-synonymous site (K_N).

This test evaluated whether the null hypothesis $K_S = K_N$ can be rejected in favour of one of the alternative hypotheses (positive selection for $K_S < K_N$ or purifying selection for $K_S > K_N$). The analyses were conducted using MEGA version 2.1 (Kumar *et al.*, 2001).

PHYLOGENETIC ANALYSIS

Only seven of our sequences - ARGSF8, ARGSF11, ASF406, ARGSF2, ARGSF6, ARGSF7 and ARGSF13 - were used for phylogenetic reconstruction because the other six were indentical to these. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 and PHYLIP (Felsenstein, 1989). Phylogenetic trees were constructed by a neighbour-joining method using the Kimura Two-Parameter model (NJ) of nucleotide substitution (Saitou and Nei, 1987). The model assumes that there is independent change at all sites, but allows for a difference between transition and transversion rates. The reliability of the tree was assessed by 1000 bootstrap replications and using a bootstrap confidence value (P_B) of 70 % (Nei and Kumar, 2000).

RESULTS

DETECTION OF BLV INFECTED CATTLE.

Proviral DNA was detected in whole blood samples by PCR, and antibodies were detected in serum. Positive PCR reactions were found in 311 out of 445 animals (70 %), and 280 out of 311 (90 %) of these positive reactors also tested positive in a serological test. Moreover, 7 % (31 out of 445) of the animals had negative results in the serological test but positive results in the PCR test, probably due to a recent infection.

Table 2 shows the sero-prevalence and PCR results, stratified by county and herd.

Table 2. Distribution of BLV isolates by RFLP (n=121) and sequencing (n=13) according to geographical location and herd.

County	Herd	Prevalence	PCR +	RFLP (n=121)			Sequencing (n=13)	
		(%)[†]	n	Aust	Arg.	Indef.	Arg.	Aust.
Rafaela	3	60	8	0	7	0	1	0
	5	99	28	0	8	0	1	0
	7	99	2	1	1	0	0	0
G. Roca	4	99	89	0	13	0	2	0
	6	82	85	0	30	0	1	0
Susana	2	92	68	1*	31	0	4	0
	8	nd	3	0	3	0	nd	nd
B. Italia	1	4	25	1	21	1	3	1
Ataliva	9	85	3	0	3	0	nd	nd
Total			311	3	117	1	12	1

* Resulting classification did not match with sequencing [†]Herd prevalence based on results from serum Blocking ELISA 108 results nd = test not done. RFLP results

On most farms, high proportions of adult cows were infected. In addition, we found that some infected animals consistently showed negative results to serological tests after merging with the results of the longitudinal study.

The analysis of digestion products after using three restriction endonucleases from 121 samples showed that three samples were classified as Australian type according to the reference patterns (Beier *et al.*, 2001) of digestion products (*BamHI:* 316 bp and 128 bp; *PvuII:* 444 bp and *BclI:* 219 and 225 bp). Furthermore, 117 samples showed a different pattern: (*BamHI:* 316 bp and 128 bp; *PvuII:* 280 bp and 164 bp and *BclI:* 219 and 225 bp), which to our knowledge has not been published before. Using sequencing, we could confirm that they corresponded to Argentine isolates. Only one sample could not be classified as any known genotype based on RFLP.

The results of RFLP and sequencing, stratified according to county and herd, are shown in Table 2. One genotype (Argentine isolates) was present in all herds, but the other genotype (Australian isolate) was also present in two herds. Only a single RFLP result in Herd 2 – showing an Australian pattern but a nucleotide alignment that corresponded to ARG38 isolate - did not match the classification using sequencing. Therefore, correspondence between RFLP and nucleotide sequencing results was 92%. Samples with duplicate results in RFLP (22) matched the same characteristic pattern in both test runs (data not shown).

SEQUENCE ANALYSIS

The RFLP patterns were confirmed by sequencing 13 samples characterised by RFLP and comparing them with sequences available in GenBank®. Results from the search in BLAST showed that 12 of the nucleotide sequences we obtained exhibited high homology (E=0.0) with BLV Arg38 isolate; identities ranged from 99 to 100 %. The remaining sequence showed high homology (E=0.0) with Australian BLV isolate, also with high identity (99%).

As expected, single base changes were found in some of the isolates, variations among these sequences consisted of 20 nucleotide substitutions in total (6 % of the reported sequence), no insertions were observed and they seemed to be distributed randomly. Most of these substitutions were located in the portion of the sequence that belongs to the NH_2 half of the gp51 protein. A few differences in the effective number of codons used between isolates were detected, but these differences were not significant (P > 0.05).

The deduced amino acid sequences of some of our isolates and four known BLV isolates are compared in Figure 1.

It shows a conserved segment of gp51 (5154 to 5520) and resulted in four amino acid substitutions (67 % of total substitutions); three of them occurred in isolates from the Argentine group and one in our ASF 406. Most of these mutations were located within the peptide region that induces a CD8^{+} Cytotoxic T-Cell response of the host (Dube *et al.*, 2000) and the Proline-rich region (Johnston *et al.,* 2002).

One interesting result is that all Argentine isolates shared a substitution of Asparagine 31 (N) (neutral-polar side) for Aspartic Acid (D) (acidic) at the location of the second neutralising domain. This area was shown to be part of the receptor-binding surface, an area which seems to be critical for receptor binding and infection (Gatot *et al.*, 2002). The potential glycosylation sites and cysteine residues did not show variations.

Figure 1. Deduced amino acid sequences of some env proteins from some of our isolates compared to other published BLV strains: VdM - reference at top -, LB285B, ARG38, AUST and JAP. Bullets show identical amino acids with reference sequence; amino acid substitutions are indicated. Missing amino acids are represented by a hyphen (-). N-linked glycosylation sites are marked in small caps. The immunostimulatory epitope for CD8 T cells is underlined to indicate its beginning and end. "ND" stands for Neutralising Domains, and underlines indicate the beginning and end of the NDs. The framed part of the sequence represents the NH₂ half of the gp51 protein. G Conformational epitope G. The shaded background shows the Proline-rich area.

```
1                G     Second  ND              CD8⁺- T CELL RESPONSE EPITOPE
EPRCPYVGAD RFDCPHWDnA SQADQGSFYV nHQILFLHLK QCHGIFTLTW EIWGYDPLIT FSLHKIPDPP QPDFPQLnSD VdM
.......... HL........ .......... .......... .......... .......... .......... .......... LB285B
.......... .......... .......... D......... .......... .......... .......... .......... SF6
.......... .......... .......... D......... .......... .......... .......... .......... SF7
.......... .......... .......... D......... .......... ........T. .......... ...I...... SF8
.......... .......... .......... D......... .......... .......... .......... .......... ARG38
.......... .......... .......... .......... .......... .......... .......... .T........ ASF406
.......... .......... .......... .......... .......... .......... .......... .......... Aust
.......... .......... .......... .......... .......... .......... .......... .......... JAP


81                      THIRD   ND
WVPSVRSWAL LLnQTARAFP DCAICWEPSP PWAPEILVYn KT VdM
.......... .......... .......... .......... .. LB285B
.......... .......... .......... .....----- -- SF6
.......... .......... .......... .....----- -- SF7
.......... .......... .......... .....---- -- SF8
.......... .......... .......... .......... .. ARG38
.......... .......... .......... .....----- -- ASF406
.......... .......... .......... .......... .. Aust
.......... .......... .......... .......... .. JAP
```

PHYLOGENETIC ANALYSIS OF BLV ISOLATES

The final tree obtained by using the neighbour-joining distance method is represented in Fig. 2A and shows the distance between isolates and the grouping of isolates in 3 main clusters. Figure 2B represents the condensed tree obtained after bootstrapping and showed a tree that has low statistical support (P_B) values for some interior branches.

With this tree we can therefore emphasize reliable portions of the branching patterns (for topology), but branch lengths are not proportional to the number of nucleotide substitutions. Nevertheless, it shows that clusters observed in the original tree are well-supported. One cluster is composed of the Argentine isolates and one of the isolates from Brazil (Brazil 3).

The other cluster is represented by several isolates found in various European countries (Belgium (LB285B), Germany (D3, 1002, 1272 D/W), the northern region of France (LB59F)) and one isolate from Brazil (Brazil1). The third cluster included BLV isolates found in Japan and Germany (D15). The mean distance between isolates, estimated after 1000 replications of bootstrapping, was 0.028 ± 0.005 (SE).

Figure 2. A -Phylogenetic tree constructed using the method based on a 350 bp segment of the *env* region of BLV. B- Neighbour-joining consensus tree generated with MEGA2 software using a Kimura two-parameter model. Bootstrap analysis was applied with 1000 replications; values above 70 out of 100 are shown at the branch points.
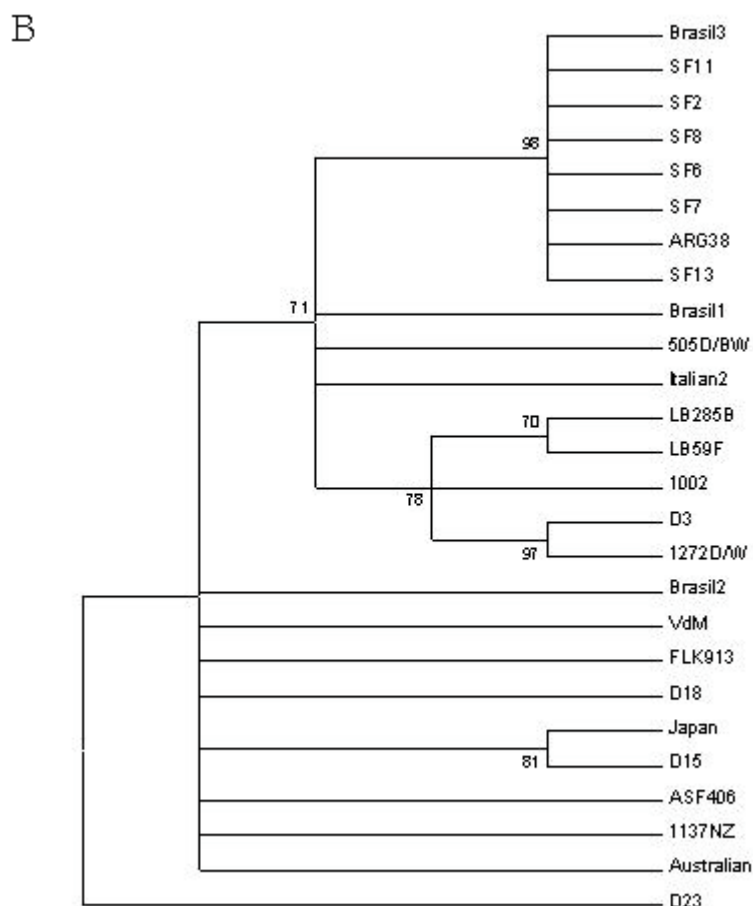
B
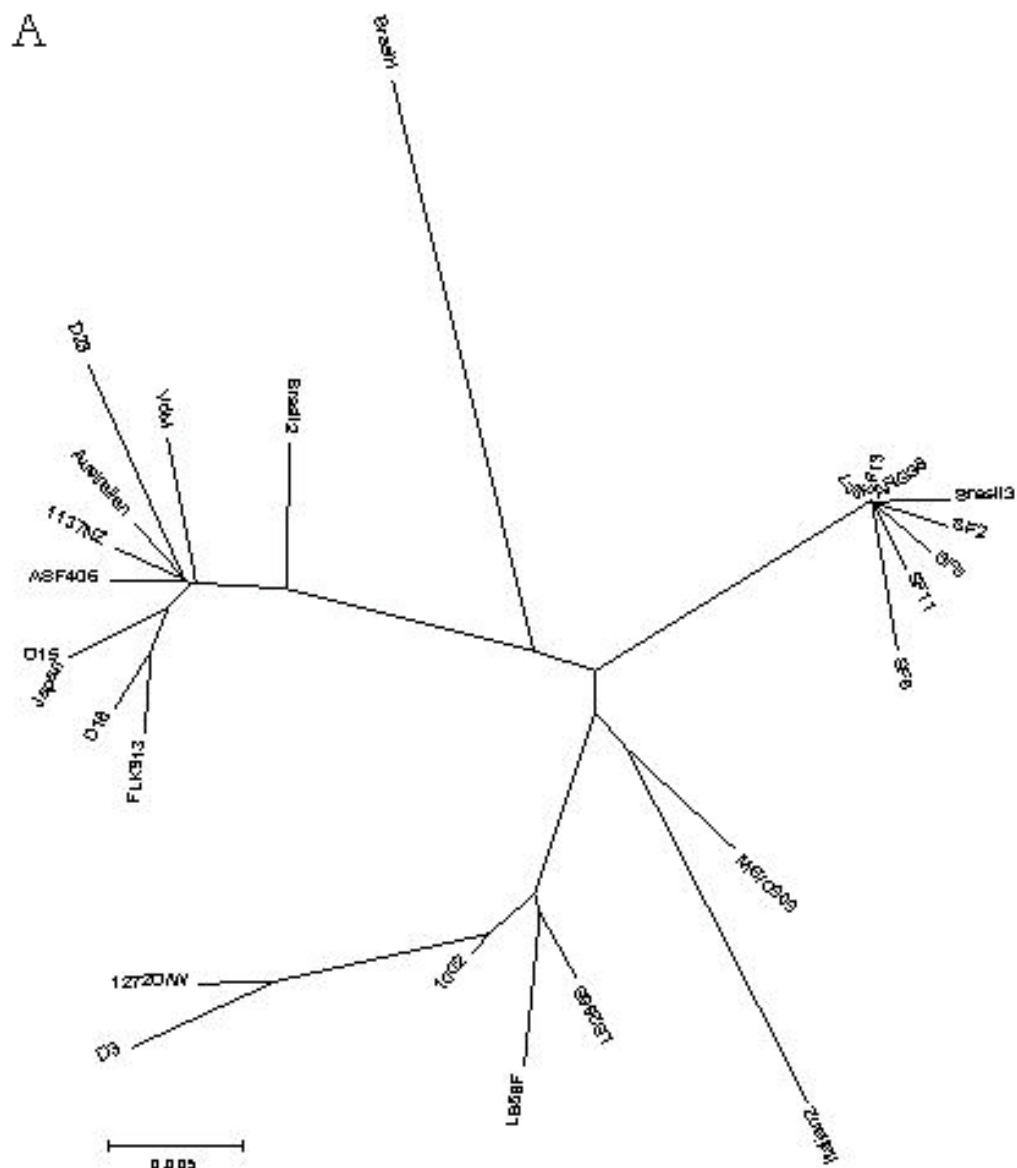
Table 3 summarizes variability within clusters. The higher variability of the European cluster seems to be a feature of this group; the larger number of subgroups in Fig. 2B, supported by bootstrapping, also reflects this variability.

Table 3. Mean net genetic distance (d) and SE (between brackets) within clusters of genotypes using Kimura 2-parameter model after 1000 bootstrapping replications.

Cluster	d	SE
Argentine	0.004	0.002
Japan	0.000	0.000
Europe	0.014	0.005

PROPORTION OF SYNONYMOUS AND NON-SYNONYMOUS CHANGES IN *ENV* AND *POL* SEQUENCES

Table 4 summarize the results of the analysis for estimating proportion of synonymous (d_s) and non-synonymous (d_n) nucleotide substitutions per site and the Z-test for selection testing for *gag, pro, pol* and *env* genes and several regions within them.

For *gag* sequences, the rates of synonymous nucleotide substitutions per site exceeded the non-synonymous rates; this difference was statistically significant. This pattern indicates purifying selection. The same tendency was observed for p15, p24 and p12. Similar results were observed for the protease.

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Table 4. Overall number of Synonymous (d_S) and Non-synonymous (d_N) nucleotide substitutions per 100 sites (±SE) calculated by Pamilo-Bianchi-Li Method and Codon-based Z-test for Tests of Selection, using different BLV genes.

Gene	Protein	Peptides	d_S	d_N	Z test
Gag			45.0 ± 3.7	35.1 ± 2.6	**
	p15		12.6 ± 2.8	2.4 ± 0.8	**
	p24		28.5 ± 3.4	14.8 ± 1.9	***
	p12		8.6 ± 3.0	0.74 ± 0.5	**
Pro			8.9 ± 2.1	2.3 ± 0.7	**
Pol			9.6 ± 0.8	1.8 ± 0.2	***
	RNase/RT		9.8 ± 0.9	2.1 ± 0.3	***
	Endonuclease		9.2 ± 1.3	1.2 ± 0.4	***
Env			6.1 ± 0.6	1.3 ± 0.2	***
	Leader Peptide		4.6 ± 2.8	2.3 ± 1.1	NS
	gp51		5.5 ± 0.9	1.3 ± 0.3	***
		Receptor-Binding Domain	5.9 ± 1.3	1.2 ± 0.4	**
		Neutralising Domains	3.8 ± 1.7	0.9 ± 0.5	NS
		CD8-T Cell	9.3 ± 4.9	0.0 ± 0.0	*
		TMHR*	4.6 ± 2.6	3.2 ± 1.1	NS
		T-Helper Cell	0.0 ± 2.9	0.0 ± 0.0	NS
	gp30		7.1 ± 1.2	1.2 ± 0.4	***
		GD21	8.9 ± 3.6	0.2 ± 0.2	**

Tests to reject the null hypothesis that $d_S = d_N$: *P<0.05; **P<0.01; ***P<0.001 NS = null hypothesis that $d_S = d_N$ * Transmembrane Hydrophobic Region

For *pol*, rates of nucleotide substitution were estimated for the whole gene and for two subunits (RNase/RT and endonuclease), and in each case, the rate of synonymous substitutions per site was significantly greater than non-synonymous substitutions, also indicating purifying selection. The pattern of nucleotide substitution seen in the case of the *env* gene did not differ from the others, although there are subtle differences in the statistical tests considering different subunits. For the main proteins (gp51 and gp30), the results lead to similar interpretations as for the other regions, but for the leader peptide the test indicates that there is no evidence to reject the neutral theory.

Considering that the gp51 protein encompasses several sites that are subject to strong interaction with the host's immunoresponse, several important epitopes were evaluated. The Z-test result from regions that cover the conformational (Receptor-Binding Domain) and the immunomodulator CD8-T cell epitopes also indicates probable purifying selection. In contrast, for the Neutralizing domains, T-Helper cells epitope and Transmembrane Hydrophobic Region (TMHR), there is no evidence that rejects the neutral theory.

The TMHR was the only site with rates of non-synonymous nucleotide substitutions per site that were greater than the synonymous rates, but the difference was not statistically significant. The results after conducting the pairwise comparison of all sequences indicate that for *gag, pro, pol* and *env*, all pairs are submitted to purifying selection. In contrast, the TMHR within the *env* gene showed that for some pairs of sequences the tests of Selection indicate positive selection (Table 5).

Table 5. Pairwise comparison of the number of Synonymous (d_S) and Non-synonymous (d_N) nucleotide substitutions per 100 sites (±SE) calculated by Pamilo-Bianchi-Li Method and Codon-based Z-test for Tests of Selection, for Transmembrane Hydrophobic Region of the BLV *env* gene

Pair of sequences	d_S	d_N	Z Test*
Arg38-LB59B	3.0 ± 3.5	16.1 ± 5.0	**
Arg38- Aust	3.0 ± 3.5	16.1 ± 5.0	**
Arg38 -FLK	3.0 ± 3.5	16.1 ± 5.0	**
GAGA-LB285F	3.0 ± 3.5	16.1 ± 5.0	**
Arg38-Jap	3.0 ± 3.5	16.1 ± 5.0	**
Arg38- VDM	3.0 ± 3.5	16.1 ± 5.0	**

Tests to reject the null hypothesis that $d_S = d_N$: ** $P < 0.05$

CASES OF PERSISTENTLY NEGATIVE SEROLOGICAL TESTS

In Herd 1, after matching results from cross-sectional and longitudinal studies, we found that three animals that had been selected for sequencing (ARGSF7, ARGSF8 and ASF406) were persistently infected but continually tested negative according to serological tests. Moreover, these animals tested negative for milk and serum ELISA 108 one year before and every two months for two years after the sample was taken that appeared positive in the PCR. They were consistently infected because the PCR was positive two years later and the RLFP results of the different samplings were also identical (results not shown).

The analysis of the predicted amino acid showed that two of these three sequences (ARGSF8 and ASF406) differed from the rest of the isolates in the peptide region that induces a CD8^{+} Cytotoxic T-Cell response of the host. For isolate ASF406, Proline (P) (hydrophobic-inflexible) changed to Threonine (T) (neutral-polar side) in position 84. For ARGSF8, Isoleucine (I) (hydrophobic-aliphatic) changed to Threonine (T) (neutral-polar side) in position 69 and Phenylalanine (F) (hydrophobic-aromatic non-polar) changed to Isoleucine (I) (hydrophobic-aliphatic, large non-polar) in position 82 (Fig. 1).

DISCUSSION

We studied the RLFP and sequence variation of BLV isolates obtained from various cattle herds in Argentina. Our results showed the presence of two types of BLV isolates - Australian and Argentine - in dairy herds from different areas of Central Argentina, which is one of the major dairy regions of South America. Our results complement other studies (Dube *et al.*, 2000) that report the presence of Argentine isolates in other geographical areas. These findings provide firm evidence that the Argentine isolate is the predominant isolate in cattle populations of Argentina. However, our phylogenetical analysis shows that variation occurs within the cluster of Argentina-like isolates, as indicated by the isolate from Brazil (Brazil 3) (Camargos *et al.*, 2002). This suggests that Argentine isolates are not geographically restricted to a single country. It is unknown if the presence of the Argentine genotype in Brazil represents a common native variant or if this is the result of introducing infected animals from Argentina. Nevertheless, the phylogenetical tree shows that the Argentine clusters are clearly different from other clusters.

We found only Argentine isolates in most of the herds we analysed except for two herds where both types of isolates were found. The fact that we ascertained both single and

multiple genotypes on individual farms agrees with previous findings (Camargos *et al.*, 2002; Fechner *et al.*, 1996; Licursi *et al.,* 2002; Portetelle *et al.*, 1989). Unexpectedly, we found that both farms with dual isolates were closed herds, i.e. they had not purchased replacement animals from other farms, therefore ruling out the possibility of introducing infected cattle. Although another study (Dube *et al.*, 2000) pointed out the closer relationship between the Argentine and Australian genotypes than reported in GenBank®, this fact cannot be used to attribute the presence of these genotypes to evolutionary linkages after a single introduction of an infected cow. Moreover, introduction of the virus via other vectors such as hematophagous insects from neighbouring farms or contaminated needles during mass inoculations (e.g. vaccination for foot and mouth disease) may also explain the presence of two variants.

It is known that variations in the genotype of retroviruses in general may be associated with changes in disease progression (Mansky, 1998) or in differences in infectivity between BLV strains, as was previously hypothesised (Bruck *et al.*, 1984; Dube *et al.*, 2000). In our study, we found a different distribution of isolates and differences in prevalence. Although our findings showed high rates of BLV infection within Argentine dairy herds, which is in line with previous studies (Alejo *et al.,* 2000; Trono *et al.,* 2001), we cannot conclude that the isolates in this study are representative strains that cause a high infection rate. The high prevalence probably reflects the lack of a compulsory control programme and not a high infectivity of the genotype present in the herd.

The phylogenetic data concerning genetic distances, based either on nucleotides or translated amino acids, showed relatively low divergence between various isolates, as previously reported (Dube *et al.*, 1997; Dube *et al.*, 2000; Mamoun *et al.*, 1990; Portetelle *et al.*, 1989). Nevertheless, the topology of phylogenetical tree clearly shows that Argentine isolates represent a separate and homogeneous group compared to other clusters. Although we could demonstrate a detectable immune response in most infected cattle, we found some persistently infected animals that did not show detectable antibody response. This confirms the findings of other studies (Blankenstein *et al.,* 1996; Fechner *et al.,* 1997). One study (Fechner *et al.,* 1997) suggests that this situation could be related to certain genotypes of the virus, but others (Licursi *et al.,* 2002) identified isolates identical to that study, and those animals showed a detectable immunoresponse. This could be explained by differences in the time after infection when the samples were taken or differences in immunocompetence between the animals. We also detected persistently infected and seronegative animals, but these animals had different BLV genotypes. We therefore concluded that occurrence of seronegative carriers is not related to one specific genotype.

We found one distinctive feature in two of these seronegative animals. The amino acids substitutions were mainly clustered in the segment that is associated with a CD8^{+} response of the host. Further study will be needed to determine if this affects the three-dimensional structure and subsequently the function of a protein, and to determine the possible relationship with seronegative carriers.

The low rate of non-synonymous substitutions compared to synonymous substitutions found in our analysis supports the hypothesis of purifying selection of *env*, *gag* and *pol* genes and several subunits; consequently, molecular evolution occurs under some functional constraint. Similar results (Biek *et al.*, 2003; Seibert *et al.*, 1995) were also found for other retroviruses. These results are consistent with the low divergence between isolates mentioned above and help to explain this distinctive feature of the BLV-HTLV genus.

Nevertheless, it appears that the TMHR is a site that might be a good potential candidate for future functional studies.

Another striking outcome is that our interpretation of the results from selection tests applied on the TMHR indicates that - at least for the Argentine isolate - the host influences this site. Additional data on other Argentine genotypes are lacking, making it difficult to evaluate determine if these results are consistent with this genotype cluster. To link selection forces and polymorphism, our results should be complemented with a study of the viral population within a given host that shows fixation of such mutation (Seibert *et al.*, 1995).

In conclusion, we characterized several BLV field isolates and their distribution in dairy herds in Argentina. Most of the isolates were represented in a BLV cluster, but point mutations did occur. We also detected seronegative carrier animals. Finally, we showed that selective advantages of different genotypes seem to be forced by purifying selection, and that this area could be an interesting topic for future functional studies.

ACKNOWLEDGEMENTS

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CHAPTER 3

EVALUATION OF A NEW ANTIBODY-BASED ELISA FOR THE DETECTION OF BOVINE LEUKOSIS VIRUS INFECTION IN DAIRY CATTLE

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SUMMARY

The objective of this paper was to validate a new blocking ELISA (designated ELISA 108) for detecting BLV infection in dairy cattle. Milk, serum and EDTA-blood samples were collected from 524 adult Holstein cows originating from 6 dairy herds in Central Argentina. The new Blocking ELISA (referred to as M108 for milk and S108 for serum samples) was compared with agar gel immunodiffusion (AGID), PCR and a commercial ELISA. Because there is currently no reference test capable of serving as a gold standard, the test sensitivity (SE) and (specificity) SP were evaluated by the use of a latent class model. The latent class model assumes that there is an underlying mixture of distributions for truly infected and non-infected animals and does not require a gold standard. Statistical inference was performed by classical maximum-likelihood and by Bayesian techniques. The maximum likelihood analysis was performed assuming conditional independence of tests, while the Bayesian approach allowed for conditional dependence. No clear conclusion could be drawn about conditional dependence of tests. Results with maximum likelihood (under conditional independence) and posterior Bayes (under conditional dependence) were practically the same. Conservative estimates of SE and SP (with 95% CI) for M108 were 98.6 (96.7; 99.6) and 96.7 (92.9; 98.8) and for S108 99.5 (98.2; 99.9) and 95.4 (90.9; 98.1), respectively. Results from testing several dilutions of international reference serum E4 in negative milk, showed that M108 gave positive results until a dilution which corresponds to the detection of one AGID-positive animal among 20 in a pooled milk sample. The ELISA 108 using either milk or serum to detect BLV-infected animals had comparable SE and SP to the official AGID and a commercial ELISA test, which are currently the most widely accepted tests for the serological diagnosis of BLV infection. Therefore, ELISA 108 can be used as an alternative test in monitoring and control programs.

INTRODUCTION

Bovine Leukemia Virus (BLV), a retrovirus that is widespread in the bovine population in many countries around the world, is the etiological agent of the lymphoproliferative disease known as enzootic bovine leukosis (EBL) (Lucas, 1992). BLV establishes a persistent infection in a sub-population of B-lymphocytes by integrating proviral DNA into the host cellular DNA (Lucas, 1992).

Since most of the infected cows develop specific antibodies (Lucas, 1992), many of the diagnostic tests to detect BLV infection aim at detecting antibodies in sera or milk from animals older than six months (Mammerickx *et al.,* 1985). The most frequently used serological tests are agar gel immunodiffusion (AGID)(Miller and Van der Maaten, 1977) and enzyme-linked immunosorbent assay (ELISA)(Beier and Siakkou, 1994; Klintevall *et al.,* 1991; Rosskopf *et al.,* 1994). Polymerase Chain Reaction (PCR) is an alternative that aims at the detection of integrated proviral DNA (Beier *et al.,* 1998; Eaves *et al.,* 1994; Fechner *et al.,* 1996) and is considered to be highly sensitive (Klintevall *et al.,* 1994; Mirsky *et al.,* 1993; Naif *et al.,* 1990). The readily obtained results and relative ease of implementation makes PCR an attractive alternative to serology (Blankenstein *et al.,* 1998).

A diagnostic test is a tool to indicate the presence or absence of a specific disease or condition in an individual from a specific population. Therefore, validation of a diagnostic test has important implications in many settings such us determination of true prevalence or probabilities of being infected for a given individual from a population, disease surveillance and monitoring, decision making and risk analysis (Greiner and Gardner, 2000). For that reason reliable estimates of test performance are required.

The sensitivity (SE) and specificity (SP) are often used to characterize the performance of a diagnostic test. SE and SP may be obtained in a simple way by cross-classifying the test results against the true infection status of the individuals as indicated by a reference test (often referred to as 'gold standard'). Conceptually, the results of the reference test can be used to discriminate animals into those that have the condition being tested for and those that do not (Swets, 1988). We will refer to the former group as 'disease positive' and the latter as 'disease negative'. In addition, 'test positive' or 'test negative' are defined as individuals that show either a positive or negative test result. Sensitivity is defined as the proportion of disease positive subjects that yield a test positive result and specificity as the proportion of disease negative subjects that yield a test negative result. It is assumed that the reference test is error-free (Swets, 1988). However, often, perfect categorization of the subjects is impossible, unethical or too expensive and one must settle for comparison with an imperfect 'reference' method. When the reference test is not imperfect (Greiner and Gardner, 2000; Walter and Irwig, 1988) misclassification will result in biased estimates of prevalence, and of SE and SP of the test(s) to be evaluated (Hui and Walter, 1980; Quade *et al.,* 1980; Walter and Irwig, 1988). When the true infection status cannot be precisely determined by a single reference test, SE and SP can still be estimated by the use of a latent class model using either maximum likelihood (Hui and Walter, 1980; Pouillot *et al.,* 2002; Qu and Hadgu, 1998) or Bayesian methodology (Dendukuri and Joseph, 2001; Enoe *et al.,* 2000; Georgiadis *et al.,* 2003).

When results from several tests are jointly analysed, it is important to know whether the tests are independent or dependent. Independence (dependence) between diagnostic tests relates to whether the tests measure unrelated (related) things given the true but unknown infection status. This is referred to as conditional independence (dependence). Conditional

dependence among test results might occur in either the infected or non-infected subpopulations (Gardner *et al.,* 2000). For example, in an infected animal the serologic response measured by 2 different tests will tend to follow a similar time-dependent pattern. False-negative test results on both tests might be more likely to occur early in the course of infection. It has been shown that ignoring conditional dependence may result in biased estimates of prevalence, SE and SP (Dendukuri and Joseph, 2001; Gardner *et al.,* 2000; Vacek, 1985). Conditional dependence could arise from other factors than infection status like cross-reaction with other antibodies from related infections which can (co-)exist within animals, or because different tests might measure the same antibody fraction or because some antibodies have affinity for other proteins present in the samples but different from the target. Conditional dependence is also of importance when control strategies comprise several tests (Gardner *et al.,* 2000).

A new ELISA that has operational advantages in comparison with the official AGID test was developed and may constitute another diagnostic tool for use in control programs. The objective of this paper was to estimate the SE and SP of the newly developed ELISA (for milk and serum samples) for detecting BLV infection in dairy cattle.

MATERIALS AND METHODS

ANIMALS AND HERDS

Milk, serum and EDTA-blood samples were collected from all lactating Holstein cows (n=524) originating from six dairy herds in the Central Dairy Area of Argentina, in October 1999. The seroprevalence of BLV in three of these herds had been previously assessed using AGID with the following results. One herd had no serological evidence of BLV infection, the second herd was a high prevalence herd (more than 50% of adult cattle seropositive), and the third represented a low prevalence herd (less than 5% of adult cattle seropositive). Three farms were of unknown BLV status. In Argentina, a proper random selection of herds is difficult because the BLV control program is voluntary and only few herds have enrolled. Also, it is quite difficult to find farms with a well-known history of disease status. The 6 purposively selected herds in the study represent typical dairy farms of the area in terms of herd size (between 150 to 300 animals), breed (Holstein) and management practices (not housed, 305-day milk production between 5000 to 6000 liters). Animals graze on pasture in rotational paddocks all year round and are milked twice a day.

SEROLOGICAL TESTS

AGAR GEL IMMUNODIFFUSION (AGID) TEST

AGID was performed using Argentina's official kit for BLV testing [a]. A one percent agar gel was prepared and mounted on a glass slide (13x 10-cm) with a punching design that allows 48 samples to be tested simultaneously. Slides were incubated in a humidified chamber, and were read at 24 and 48 hours. Two experienced technicians interpreted the test reactions under a light beam in a dark room. To avoid verification bias, the readings were made independently by both technicians and samples in which disagreement was observed were not re-tested to avoid review bias and they were considered as incoherent. Test results were reported as positive, negative or doubtful or incoherent when both observers did not agree. There were some samples were the technicians were unsure about the result and these results were scored as doubtful. The few incoherent and doubtful

results were excluded from the calculations. The kit information sheet did not contain any estimates of SE and SP, but the literature reports its SE and SP to be 98.5% and 69.8% respectively (relative to complement fixation)(Miller and Van der Maaten, 1977), 86% and 100% (relative to a commercial ELISA)(Martin *et al.*, 2001) and 85 % and 92.2 % (relative to radioimmunoprecipitation assay) (Jacobsen *et al.*, 1985).

BLOCKING ELISA 108 (MILK = M108, SERUM = S108)

The ELISA 108 was performed using a previously described protocol (Gutierrez *et al.*, 2001). A 96-well microplate[b] was coated with 100 μl/well of serum from a naturally infected cow diluted (1:10,000) in 20mM carbonate buffer and incubated for 16 h at 4ºC. After washing twice, BLV-gp51 capture was performed by incubating with a crude cell culture-derived antigen (FLK-BLV). Two washes were made with PBST washing solution and 100 μl of serum (diluted 1:2) or whole cow milk (diluted 1:9) was added and incubated for 16 h at 4ºC. Plates were washed four times and 50 μl/well of anti-gp51 monoclobal antibody[c] was added and plates were incubated for 90 min at 37ºC. Subsequently, 100 μl peroxidase-conjugated affinity-purified goat anti-mouse IgG (H+L)[d] used at a dilution of 1:10,000 was added and incubated for 45 min at 37ºC. Plates were washed four times with PBS only. A solution of Tetramethylbenzidine (TMB) was used as substrate and incubated for 30 min at room temperature. Reaction was stopped with 30μl/well of 2M H_2SO_4. Optical density (OD) at 450 nm was measured using a microplate reader[f].

Samples were tested in duplicate and in each plate the positive and negative control and the European Union (EU) international standard serum (E4) was included. The E4 represents a defined titer and it served for checking whether the positive control of the kit worked satisfactorily under different conditions (Wright *et al.*, 1993).

The percentage of inhibition (PI) of each test sample was calculated as:

$$PI = \frac{ODNC - ODSample}{OD\ NC - OD\ PC} * 100$$

where, ODNC = OD of negative control and ODPC = OD of positive control. Serum samples with PI $\geq$ 40% were considered as positive, those with PI < 35% were considered negative, and those with a value between them as doubtful (Gutierrez *et al.*, 2001). For milk samples different cut-off points were used ($\geq$ 52% as positive, < 47% as negative and between these values as doubtful). Doubtful results were not retested and were excluded from further analysis.

For validity of the results, two conditions had to be met on each plate. First, average ODNC must be $\geq$ 0.800 and blocking % calculated as 100*(ODNC – ODPC) / ODNC must be $\geq$ 80%. Second, duplicated PI values must relatively differ by less than 25%.

The estimated SE relative to radioimmuno-precipitation assay ranged from 96.4 % to 100 % and SP from 95 % to 100% (Gutierrez *et al.*, 2001).

The performance of the milk ELISA 108 was further assessed using Annex G to European Estates Community directive 88/406/EEC governing the diagnosis of Brucellosis and EBL. This directive states that for new milk ELISA aiming to detect BLV antibodies in pooled milk, serial dilutions of the international reference serum E4 in milk from non-infected cows should be tested. Therefore, dilution series 1:5000; 1:25000; 1:50000; 1:100000 and 1:200000 of E4 were tested. This series corresponds to one AGID-positive animal among 20, 50, 100, 200 and 400 cows, respectively.

SERELISA BLV BI (SER)[G]

The commercial indirect ELISA kit[g] was used according to the manufacturer's instructions and results were expressed as positive, negative or doubtful. When duplicate samples did not show the same result they were scored as inconsistent.

For any test, it was defined as missing value any missing result for a given test. These results originated when there was any inconvenience while running a given test and there was not enough material to perform it again.

PROVIRAL DETECTION BY NESTED POLYMERASE CHAIN REACTION (PCR)

PCR PRIMERS

Oligonucleotide primers for PCR were designed from published sequence data (Sagata *et al.,* 1985). Primers corresponding to the *env* and *ltr* genes were selected because this region is highly conserved among different BLV provirus isolates (Rice *et al.,* 1984). Forward primers were *env*5032 5' - TCT GTG CCA AGT CTC CCA GAT A - 3', and *env*5099 5' - CCC ACA AGG GCG GCG CCG GTT T - 3'. The reverse primers were *env*5521r 5' - GCG AGG CCG GGT CCA GAG CTG G - 3' and *env*5608r 5' - AAC AAC AAC CTC TGG GAA GGG T - 3'. The set *env*5099 and *env*5521 was established and described previously (Naif *et al.,* 1990; Naif *et al.,* 1992) The *ltr* primer sequences were: *ltr*B1 5' - TGT ATG AAA GAT CAT GCC GAC CTA G-3', *ltr*B506 5' - GGT CTC TCC TGG CCG CTA GA-3', *ltr*B42 5' - GTA AAC CAG ACA GAG ACG TCA GCT-3' and *ltr*B478 5' - GAA GGA GAG AGC GCG GGC-3'. All primers are commercially available [h].

PCR ASSAY

DNA was extracted from frozen EDTA-blood using NucleoSpin Blood kit[i]. The initial round of nested-PCRs (Fechner *et al.,* 1996) was performed using (*env5032 / env5608r*) as first primers; initial incubation of samples was at 72ºC for 2 minutes; denaturation at 94ºC for 2 minutes; then 50 amplification cycles consisting of denaturation at 95ºC, 30 seconds, primer annealing at 58ºC, 30 seconds and extension at 72ºC for 1 minute and final extension at 72ºC for 4 minutes. For *ltr* primers, conditions for first round (*ltrB1/ ltrB506*) were set similarly to those for *env* primers except that primer annealing was done at 62ºC. The second round of amplifications was carried out using the second pair of primers (*env5099 / env5521r*). The second round conditions were set as the first round except for primer annealing temperature that was switched to 72ºC. Conditions in the second round (*ltrB42/ ltrB478*) for annealing were set to 62ºC as well. A sample was considered as positive when any of the two PCR assays (env or ltr primers) tested positive.

Known positive and negative control DNA samples were included in each test run and samples showing a band migrating at 444 base pairs (bp) were considered as positive. Due to the very high analytical sensitivity of PCR, special precautions were taken to avoid contamination. All steps were carried out in separate rooms, all pipetting procedures were performed under plexiglas boxes and rooms were irradiated with UV light after each step.

STATISTICAL ANALYSIS

Cohen's Kappa (Landis and Koch, 1977) statistic was calculated as a global measure of agreement between test results. The Kappa statistic varies between 0 (chance agreement) and 1 (perfect agreement), where $0.00 < k < 0.20$ as "slight," $0.21 < k < 0.40$ as "fair,"

0.41<k<0.60 as "moderate," 0.61<k<0.80 as "substantial," and 0.81<k<1.00 as "almost perfect" agreement.

Test results (y) were analysed as binary: an individual was diagnosed as having the infection (positive test result; $y = 1$) or not (negative test result; $y = 0$). The cut-off points for the continuous signals (OD) of the ELISA 108 and SER tests, resulting in binary test results, were as suggested by the developers of ELISA 108 and manufacturers of SER. The true infection status of an individual, either infected (disease positive; $D = 1$) or not (disease negative; $D = 0$), was unknown.

The unknown infection status (D) can be introduced in the model as a latent variable (a variable that is not directly observed). This latent class ($D = 0$ or $D = 1$) model comprises a mixture of separate multinomial distributions for truly positive ($D = 1$) and negative ($D = 0$) animals. Joint probabilities for combinations of test results for $D = 0$ and $D = 1$ were expressed analogous to log-linear and multi-logistic models and were assumed to be the same across herds. Consequently, SE and SP were assumed constant across herds. Conditional independence between tests corresponds to a main effects model (model M1), while conditional dependence is represented by additional interaction terms between tests (model M2). A separate prevalence was introduced for each herd. Estimation was both by classical maximum likelihood (Pouillot *et al.,* 2002; Qu and Hadgu, 1998) and Bayesian inference (Enoe *et al.,* 2000; Georgiadis *et al.,* 2003).

Inference by maximum likelihood was performed with the TAGS (Test in the Absence of a Gold Standard) (Pouillot *et al.,* 2002) program. Since TAGS is not able to handle conditionally dependent tests, maximum likelihood estimates were derived under the assumption of conditional independence.

The Bayesian analysis was performed with the Gibbs sampler, as implemented in the WinBUGS program (Spiegelhalter *et al.,* 1999). In the Bayesian analysis, conditional dependence among tests (additional interaction terms) was included. In a Bayesian analysis, prior knowledge about the model parameters (SE, SP, covariance between tests) has to be supplied via prior distributions. Fairly uninformative priors were specified, that focussed on conditional independence or positive conditional dependence, as presented in Swildens *et al.,* 2004. Gamma and gaussian priors were used, to assess robustness of the results for choice of prior.

The priors were combined with the data for the latent class model into posterior distributions that summarise the current up-to date knowledge about the parameters. The medians of these posterior distributions were presented as point estimates, while the 2.5 and 97.5 percentile points were used as Bayesian 95% confidence intervals (credible intervals). Medians and percentile points were numerically evaluated by WinBUGS (Spiegelhalter *et al.,* 1999).

Finally, we also obtained results with PCR as a reference test, to illustrate the bias that may result from the use of an imperfect reference test. In a number of studies PCR failed to detect BLV provirus in cattle that tested positive in serum (Beier *et al.,* 1998; Cockerell and Rovnak, 1988; Eaves *et al.,* 1994). The nested PCR was included because several studies indicated that it performed better than AGID (Blankenstein *et al.,* 1998; Fechner *et al.,* 1996; Klintevall *et al.,* 1994) which is used as reference in many other studies. The superior performance of PCR stems from its ability for earlier detection of infected animals (Beier and Siakkou, 1994; Eaves *et al.,* 1994) and detection of as few as 4 (Naif *et al.,* 1990) to 10 copies of proviral BLV.

RESULTS

A summary of the test results is presented in Tables 1a and 1b.

Table 1a. Basic results from 5 diagnostic tests for detection of BLV infection (n = 524).

Result	PCR	M108	AGID	S108	SER
Positive	331	318	300	358	340
Negative	144	154	182	153	151
Sub Total	475	472	482	511	491
Doubtful	0	2 (2)*	9 (8)*	2 (2)*	1(0)*
Inconsistent Results	5	NA	2	NA	14
Missing Values	44	50	31	11	18
Total	524	524	524	524	524

*The figures in parenthesis represent the number of doubtful results that resulted in a positive PCR result.

Table 1b. Prevalence per farm included in the study, obtained by 5 diagnostic tests for detection of BLV infection (n = 524).

Test	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F
PCR	80.8 %	40.0 %	96.6 %	92.5 %	21.7 %	91.9 %
M108	82.7 %	50.0 %	96.6 %	100.0 %	0.0 %	91.9 %
AGID	78.8 %	40.0 %	96.6 %	94.6 %	0.9 %	85.1 %
S108	83.7 %	50.0 %	96.6 %	98.9 %	4.7 %	94.6 %
SER	81.7 %	50.0 %	96.6 %	98.9 %	1.9 %	90.5 %

The percentage of animals within each herd that tested positive ranged from 21 % to 97 % and from 0 % to 100 % for PCR and serological tests, respectively. Results of M108 and S108 were similar using either the standard E4 as positive control or the positive control of the kit (results not shown).

The kappa measures of agreement between M108, S108, PCR, SER and AGID are shown in Table 2. The M108, S108, SER and AGID test, all based on antibody detection, show similar kappa values, ranging from 0.81 to 0.93, suggesting strong agreement. The kappa values (ranging from 0.63 to 0.69) of PCR with the other tests suggest substantial agreement.

Table 2. The measure of agreement (kappa) and 95% confidence intervals among five diagnostic tests to detect BLV infection.

	S108	AGID	SER	PCR
M108	0.92 (0.88;0.96)	0.82 (0.77;0.0.88)	0.87 (0.82;0.92)	0.65 (0.57;0.72)
S108		0.81 (0.76;0.87)	0.93 (0.89;0.96)	0.69 (0.61;0.76)
AGID			0.84(0.79;0.90)	0.63 (0.55;0.70)
SER				0.67 (0.59;0.54)

The results of the maximum likelihood analysis (by TAGS assuming conditional independence) and of the Bayesian analysis (both under conditional independence and dependence; models M1 and M2 respectively) are presented in table 3. In M2 non-negative dependence (negative dependence was biologically not plausible) was allowed for with a gamma prior for the dependence parameters. Results from TAGS and from Bayesian analysis according to M1 and M2 are quite similar. The likelihood ratio test and correlation residuals provided by TAGS do not show a significant lack of fit of the independence model (p>0.032). In addition, different prior distributions yielded similar estimates of SE and SP (results not shown). SE and SP confidence intervals of the dependence parameters, obtained with a normal prior that allows for negative dependence as well, all contain the value 0 except for the pairs PCR–S108 and PCR–AGID. This also indicates that there is little evidence for conditional dependence. However, the confidence intervals for the interaction parameters for dependence (not shown), both for the gamma priors (non-negative dependence) and normal priors (negative or positive dependence), are fairly wide, indicating that the data do not offer a great deal of information with respect to conditional dependence between tests.

Therefore, we decided to present the (possibly more conservative) results from M2 as the final results for SE and SP. Estimated SE's of the ELISA tests are markedly higher than those of AGID (Table 3).

PCR performed quite poorly with respect to SE and SP (92.6 and 75.9 respectively) compared with the other tests. Finally, a look at some results with PCR as a reference (Table 3) showed much lower estimates for both SE and SP, than models that did not consider PCR as a reference test.

Results (based on 10 repeats) from testing several dilutions of international reference serum E4 in negative milk, showed that M108 gave positive results until a dilution of 1:5000 which corresponds to the detection of one AGID-positive animal among 20 in a pooled milk sample.

DISCUSSION

This paper described the evaluation of diagnostic performance of a new test for detecting BLV infection. Due to purposive selection the estimates of prevalence cannot be used to make inferences beyond this study, however the dairy herds used in this study were typical for the area. Results of kappa, maximum-likelihood and Bayesian analysis clearly show that under the conditions of this study, ELISA 108, either in milk or serum, performed at least as well as existing diagnostic tests. Hence, it can be incorporated as diagnostic tool in BLV control programs. For example, S108 can be preferentially used for certifying BLV- free status due to its high SE. Although the antibody level in milk is lower than in serum, M108 had a SE and SP as high as the official AGID test. Therefore, M108 could be preferred for routine screening of infected animals due to the operational advantages. Milk testing prevents the risk of spreading the virus during sampling as it is not common practice in Argentine to use a separate needle for each animal. However, under the conditions of this study the new ELISA test (S108, M108) performed as well as the existing ELISA test (SER), indicating that additional criteria such as kit costs, practicality, time to obtain results, etc. should be taken into account when deciding which test to use. In addition, bulk milk can be used, which makes M108 a powerful and cheap tool in large-scale epidemiological studies or monitoring programs. Our results using serial dilutions of

international reference serum E4 in negative milk showed that M108 may pick up a prevalence as low as 1 in 20 animals when milk is pooled. These preliminary results are in agreement with those of a previous study (Gutierrez *et al.,* 2001). They found that M108 detected positive bulk-tank samples in herds with 2 % prevalence. However, the number of herds in that study were low, therefore a more extensive study should be performed to conclude over the possibility of using M108 as a herd screening test.

Table 3. Estimated sensitivity (SE) and specificity (SP) with their 95 percent confidence intervals (conventional and TAGS models) and the median values with their 95 percent credible intervals of the posterior distribution (Bayesian models); for five diagnostic tests to detect BLV, based on methods with and without gold standard (considering samples without missing values n=414).

MODEL		TEST				
		PCR	M108	S108	AGID	SER
ML[†]	SE	92.8 (90.1; 95.6)	98.9 (97.5; 100.0)	99.6 (99.0; 100.0)	94.7 (91.6; 97.3)	99.4 (98.2; 100.0)
	SP	76.7 (69.6; 82.8)	97.6 (94.6; 100.0)	96.1 (91.9; 99.2)	98.5 (95.7; 100.0)	98.5 (96.2; 100.00)
M1[‡]	SE	92.9 (89.5; 95.5)	99.0 (97.4; 99.8)	99.7 (98.7;99.9)	94.7 (91.7; 96.9)	99.4 (98.0; 99.9)
	SP	77.0 (69.4; 83.6)	98.0 (94.6; 99.5)	96.5 (92.4; 98.8)	98.7 (95.8; 99.8)	98.7 (95.8; 99.8)
M2[§]	SE	91.6 (87.8; 94.5)	98.9 (96.4; 99.9)	98.4 (96.1; 99.7)	93.6 (90.2; 96.3)	97.6 (95.1; 99.3)
	SP	75.5 (67.4; 82.8)	100.0 (95.6; 100.0)	95.6 (90.3; 99.2)	98.2 (94.8; 100.0)	97.0 (92.7; 99.6)
GS[*]	SE	Ref.	88.9 (85.3; 92.5)	91.0 (87.8; 94.3)	86.4 (82.5; 94.0)	89.3 (85.7; 92.7)
	SP	Ref.	84.1 (77.5; 90.6)	83.9 (77.3; 90.5)	88.2 (82.5; 94.0)	84.9 (78.4; 91.3)

[*] Gold Standard (PCR reference)
[†] Maximum Likelihood estimation with TAGS
[‡] Bayesian conditional independence (M1: model 1)
[§] Bayesian conditional dependence assuming Gaussian distribution (M2: model 2)

Estimates of SE and SP relative to PCR were lower than previously reported for the same ELISA (Gutierrez *et al.,* 2001) and in comparison with other ELISAs (Beier and Siakkou, 1994; Klintevall *et al.,* 1991; Martin *et al.,* 2001). However, SE and SP estimates obtained by the latent class model, where no gold standard is required, were closer to those previously published. Part of the differences in the estimated SE and SP among different studies may be due to different testing conditions or use of different reference tests than PCR. An obvious reason for the low estimates of SE and SP using PCR as a reference, was the relatively poor performance of the PCR, as apparent from the analysis with the latent class model. Factors related to the PCR technique and factors related to the statistical methodology may explain this lower performance of PCR. First, the SE of the PCR is

highly dependent upon the design and execution of the primers used in the PCR as was shown in another study (Marsolais *et al.,* 1994). Second, circulating lymphocytes from seropositive, hematologically normal and asymptomatic cattle contain 1 to 3 proviral copies and the percentage of BLV infected cells has been found to be less than 10% (Cockerell and Rovnak, 1988; Mirsky *et al.,* 1996). These levels can be below the detection level of PCR but still might elicit a detectable immune response. This is consistent with our findings (results not shown) that herds with higher sero-prevalence tend to have a larger proportion of individuals yielding positive results in all serological assays but negative by PCR. A third reason for the relatively low SE could arise from the absence of provirus in circulating lymphocytes (Murtaugh *et al.,* 1991) while they can be sequestered in lymphoid tissues (Klintevall *et al.,* 1994) or in the mammary gland (Buehring *et al.,* 1994), while the animal is infected and produce an antibody response. In herds with an active infection one may expect such animals to be present.

The SP of PCR might be underestimated due to the way that models without gold standards estimate latent variables (true infection status). In these models, the true status of infection is obtained by consensus from all assay results; hence test results also influence what is 'true'. For example, recently infected animals detected by PCR but tested negative by most of the serological tests will be recorded as free of infection. Consequently, PCR results will be penalized as false positive, while in fact it is not true. Around 10% of our samples can be included in this category (results not shown) and a subsequent study (Monti *et al.,* 2005) showed that most of them seroconverted later, confirming that PCR might detect infection at an early stage.

We considered conditional dependence between both variants (milk, serum) of the new test and some of the existing serological tests for detecting BLV infections because combinations of tests are frequently used, e.g. due to regulations for international trade of animals and for confirming results. However, results from TAGS suggest that the model assuming conditional independence fitted the data well. Also, results (not shown) from Bayesian models (M2a-2, M2 and M2b) did not indicate marked dependence. Thus, dependence between serological tests cannot be supported with our data, but in view of the confidence intervals it cannot be ruled out either. Results under conditional independence and conditional dependence, both by maximum likelihood and by posterior Bayesian methods, were fortunately quite similar. Therefore, possible conditional dependence does not critically affect our conclusions with respect to the new ELISA tests.

In conclusion, the BLV ELISA 108 (either milk or serum) described in this paper has comparable SE and SP to the official AGID and a commercial ELISA, which are currently the most widely accepted tests for the serological diagnosis of BLV infection. Therefore, it can be incorporated as an alternative test in monitoring and control programs.

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CHAPTER 4

SURVIVAL ANALYSIS ON AGGREGATE DATA TO ASSESS TIME TO SERO-CONVERSION AFTER EXPERIMENTAL INFECTION WITH BOVINE LEUKEMIA VIRUS

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SUMMARY

Bovine Leukaemia Virus is a ubiquitous retrovirus that affects mainly cattle. Knowledge of the precise moment of infection is fundamental for identification and evaluation of factors related to BLV transmission. Systematic reviews and meta-analyses provide good evidence on the effects of medical interventions. The objectives were to estimate time to seroconversion after experimental infection using data from retrieved literature and to detect factors that may influence the length of that interval using survival analysis on pooled data. An analysis using aggregate data from 36 studies totalling 438 observations was performed. From this, 4 sets were created and analysed by interval-censored accelerated failure time models (AFT) with different distributions (Exponential, Weibull, log-logistic, lognormal and generalized gamma), and some variants of the Cox Model (Andersen-Gill, smoothing splines) with and without a frailty effect. The AFT gamma model fit best and the estimated median time to seroconversion in the null model was 57 days (95 % CI: 49; 75) using all data and 47 days (95 % CI: 39; 55) when only studies using experimental inoculation were considered. Some factors were consistently associated with time to seroconversion. These included exposure by animal-to-animal contact (resulting in a 7 fold increase in time to seroconversion compared to direct inoculation), diagnostic method to detect seroconversion (time to seroconversion was 1.4 times shorter when AGID was used compared to ELISA), and transmission by insect bites (biological media) delayed seroconversion 2.3 times compared transmission via needles or other inanimate media. After fitting a frailty Cox model, results showed that seroconversion in susceptible animals after infection using donors in which presence of virus before the experiment started was confirmed increased the hazard of seroconversion 2 times in comparison with donors in which virus presence was not confirmed before start of the experiment. Inoculation with blood decreased the hazard 2.5 times in comparison with lymphocyte suspensions. Heterogeneity due to different research groups was also present. Finally, a Cox model with smoothing splines contained 3 variables: research group, route of inoculation and a nonlinear spline for infective dose. In conclusion it can be stated some factors that influence the time to seroconversion were identified and quantified and that a moderate influence of research centre existed. These results may contribute to the estimation off the most probable times of infection in field conditions and in a better evaluation of control measures.

INTRODUCTION

Bovine Leukaemia Virus (BLV) is a ubiquitous retrovirus that mainly affects cattle. BLV integrates in DNA of B-lymphocytes and can produce a polyclonal expansion of B-cells that is manifested as persistent lymphocytosis and/or lymphosarcoma (Brunner *et al.*, 1997). Virus transmission mainly occurs horizontally via transmission of infected lymphocytes but also vertically from the dam to the calf (Evermann *et al.*, 1986; Kono *et al.*, 1983; Mammerickx *et al.*, 1987; Piper *et al.*, 1975; Piper *et al.*, 1979; Van der Maaten *et al.*, 1981b).

Previous studies have shown that management practices may influence the rate of contact between animals and between herds and hence increase or decrease the risk of BLV transmission (DiGiacomo *et al.*, 1985; DiGiacomo *et al.*, 1986; Henry *et al.*, 1987; Roberts *et al.*, 1981). To quantify the effects of (changes in) management practices in relation to BLV transmission by simulation or modeling, knowledge of the precise moment of infection is fundamental.

BLV infected individuals are routinely detected by serological testing and susceptible animals that become infected will seroconvert. Several factors may have an effect on the interval between infection and seroconversion: such as natural delay between infection and eliciting a detectable immune response (depending on the class of Ig), discrimination properties of the test used, infectious dose-response relationship and route of infection. However, even when the exact day of seroconversion is known, a retrospective determination of the precise moment of infection is not possible under field conditions (Lassauzet *et al.*, 1989). Therefore, experimental infection is a better way to estimate the time to seroconversion, as the time of exposure is better known. A previous study (Lassauzet *et al.*, 1989) estimated the median time to seroconversion for BLV as being 28 days. Time to seroconversion can be determined and analysed by parametric survival analysis, using several distributions. For BLV (Lassauzet *et al.*, 1989) a log-logistic model was used. Many studies designed for the analysis of time to detection are related to acquired immune deficiency syndrome (AIDS), which is induced by another member of the Retroviridae family. In those studies, Exponential, Weibull, log-normal and log-logistic were the parametric models most frequently used (Chiarotti *et al.*, 1994; Egger *et al.*, 2002; Lassauzet *et al.*, 1989; Muñoz and Xu, 1996; Tan *et al.*, 1996; Tassie *et al.*, 2002).

Several studies in which experimental infection with BLV was used and that have been published might serve as a basis for a meta-analysis. The rationale behind a meta-analysis is that pooled data could bring better results than individual reports due to larger number of observations, more repetitions of similar experiments and changing conditions, thus reducing uncertainty relative to results from a single study. Meta-analysis studies have provided good evidence of the effects of medical interventions but they are not free of bias, such as publication bias (Sterne *et al.*, 2002). Also, grouping several studies from one or more research centers from several countries, requires statistical adjustment to account for effects specific to the individual centers (Andersen *et al.*, 1997).

The objectives of this study were to estimate time to seroconversion after experimental infection and to detect factors that may influence the length of that interval using survival analysis on pooled data retrieved from literature.

MATERIAL AND METHODS

INCLUSION CRITERIA FOR STUDIES

The primary criterion for admission of studies was monitoring of BLV seroconversion using either Agar Gel Immunodiffusion (AGID) or ELISA as the diagnostic test. They were either transmission experiments or experiments in which infection was induced as a part of the experiment. The tests are by far the most frequently used tests in practice (Office International des Epizooties, 2000) and are designed to detect the gp51 envelope protein.

Duplication of studies was controlled in those cases where studies came from the same research group and where identification of individual animals was possible in the report. Observations from any study were discarded when the information was unclear. The target species was bovine and the unit of analysis was the individual animal.

To reduce subjectivity in the analysis, we extracted only information that was clearly stated in the original paper. If not clear, the observation for that particular variable was assigned a missing value. For one variable (age of the recipient animal) a new dichotomous variable (old vs. young) was created because in several original papers age was indicated as 'calves' or 'adult cows'.

LITERATURE REVIEW

Thirty-six studies totalling 438 observations published between 1975 and 1999 were recruited by computerized literature-retrieval using AGRIS, CAB, AGRICOLA, MEDLINE databases (starting from 1970 to 2002), covering English, Spanish, German, French, Portuguese and Italian language.

DESCRIPTION OF THE DATA

Table 1 summarises the retrieved studies and some characteristics of each of them. The factors (as variables) that might influence the time to seroconversion that were considered for the analysis and their definitions are summarised in Table 2.

The data were analysed using 4 sets. Set 1 included all animals (n = 438) but the analysis considered only variables that had no missing values (namely STUDY, GROUP, TYPETRA, AG and TEST). Subsequently, set 1 was split into 2 subsets. Set 2a included all studies where transmission was achieved by contact exposure with infected animals (n= 60) (variables STUDY, GROUP, AG and TEST). Set 2b (n = 378) consisted of those studies where transmission was achieved by exposure by inanimate inoculations or biological vectors (such as insect bites) (variables STUDY, GROUP, TEST, AGE, PARENT, EXP, DOSE, INTYPE and STATDON). Set 2c was based on set 2b but restricted to all observations with complete information on infective dose (n = 132).

Table 1. Description of various studies in which time to seroconversion after experimental infection with Bovine Leukemia virus was measured.

ID	Reference	Year	Country	Individuals used [a]	Specie used	Test
1	Miller and V. d. Maaten	1975	USA	20	cattle	ID [b]
2	Baumgartener and Olson	1978	USA	3	cattle	ID
3	Straub	1978	Germany	19	cattle	ID
4	V. d. Maaten and Miller	1978	USA	9	cattle	ID
5	V. d. Maaten and Miller	1978b	USA	5	cattle	ID
6	Mammerickx *et al.*	1980	Belgium	6	cattle-sheep	ID
7	Miller *et al.*	1981	USA	5	cattle	ID
8	Roberts *et al.*	1981	UK	18	cattle-sheep	ID
9	V. d. Maaten *et al.*	1981	USA	3	cattle	ID
10	Kaaden *et al.*	1982	Germany	4	cattle	ELISA
11	Roberts *et al.*	1982	UK	16	cattle-sheep	ID
12	Severini *et al.*	1982	Italy	6	cattle	ELISA
13	V. d. Maaten *et al.*	1982	USA	6	cattle	ID
14	Kono *et al.*	1983	Japan	8	cattle	ID
15	Romero *et al.*	1983	Brasil	11	cattle	ID
16	Miller *et al.*	1985	USA	42	cattle	ID
17	Roberts *et al.*	1985a	UK	6	cattle	ID
18	Roberts *et al.*	1985b	UK	12	cattle	ID
19	Evermann *et al.*	1986	USA	8	cattle	ID
20	Roberts *et al.*	1986	UK	10	cattle	ID
21	Henry *et al.*	1987	USA	2	cattle-sheep	ID
22	Mammerickx *et al.*	1987	Belgium	22	cattle-sheep-goat	ID & ELISA
23	Walker *et al.*	1987	Australia	2	cattle	ID
24	Hopkins *et al.*	1988	USA	16	cattle	ID
25	Roberts *et al.*	1988	UK	16	cattle	ID & ELISA
26	Weber *et al.*	1988	USA	49	cattle	ID
27	Foil *et al.*	1989	USA	29	cattle-sheep	ID
28	Dimmock *et al.*	1991	Australia	6	cattle-sheep	ID
29	Fukuyama *et al.*	1993	Japan	7	cattle	ID
30	Hasselschwert *et al.*	1993	USA	21	cattle	ID
31	Kelly *et al.*	1993	USA-Can.	5	cattle	ID
32	Wentink *et al.*	1993	Holland	12	cattle	ID & ELISA
33	Klintevall *et al.*	1994	Sweeden	24	cattle	ID & ELISA
34	Kozaczynska	1996	Poland	4	cattle	ELISA
35	Yakobson *et al.*	1998	Israel	4	cattle	ID
36	Ungar Waron *et al.*	1999	Israel	2	cattle	ID

[a] after controlling for duplication and unclear information [b] ID = Agar Gel ImmunoDiffusion test

Table 2. Description of variables used for survival analysis in which time to seroconversion after experimental infection with Bovine Leukemia virus was measured.

Variable	Indicate	Definition
	Study Characteristics	
Study	*Study*	Published study identification
Group	*Research Group*	1 = Studies 8,11,17,18, 20 and 25 ; 2 = Studies 1, 4, 5, 7, 9, 13 and 16 ; 3 = Others
Typetra	*Transmission design*	1 = Experimentally infected ; 0 = Contact exposure with infected cow
Exp	*Type of experimental infection*	1 = Inoculation using an inanimate vehicle ; 0 = Mechanical using a biological vehicle
Isol	*How animals were kept after inoculation*	1 = Not isolated individually; 0 = Isolated individually
Test	*Diagnostic test used*	1 = AGID; 0 = ELISA
	Recipient characteristics	
Age	*Age of the bovine recipient*	1 = 1 to 24 months old; 0 = Older than 24 months
Iconf	*BLV recovered from recipients that had sero-converted*	1 = Yes; 0 = No
	Donor characteristics	
AG	*Virus confirmation of donor animal by any direct test*	1 = confirmed; 0 = tested but resulted negative
Statdon	*Status of Donor*	0 = Unknown; 1 = Infected and haematological normal / low infectious (by quantification of virus load); 2 = Persistent Lymphocytosis/ lymphoma / highly infectious (by quantification of virus load)
	Experimental Infection	
Route	*Considers only the two main infection routes*	1 = Intradermal ; 0 = Subcutaneous
Parent	*Considers all routes investigated and they are grouped into parenteral routes and the rest*	1 = EV [a] / SC [b] / ID [c]/ IM [d] ; 0 = Others
InType	*Type of inoculum used*	1 = Blood; 0 = Lymphocyte suspension
Dose	*Infection dose*	Reported from the original paper or calculated when it was reported in volume of blood and total number of lymphocytes/ml of blood

[a] Intravenous [b] Subcutaneous [c] Intradermal [d] Intramuscular

STATISTICAL ANALYSIS

Survival analysis (SA) was used to statistically evaluate the data, taking interval censoring into account. In SA, if the event is observed to occur, the time the event occurs (T) is recorded and the censoring indicator δ, is set to 1. If by the end of the period of observation the event has not been observed, the observation is considered to be right-censored, T takes the last observation time and δ would take the value of 0. If an animal is recruited at the start of the observation period but the event has already occurred, then the data would be left-censoring and T would be set to 0. If data are observed exactly or are right-censored there are numerous parametric, semi-parametric and non-parametric methods available for fitting model, hypothesis testing and estimation of effects of covariates.

However, there are situations where it is only known that the event has occurred at time T_i within an interval of time [L, R], where L ≤ T_i ≤ R, L being the lower limit of the time interval and R being the upper limit. This situation is known as interval censoring. Moreover, either right or left censoring might be treated as special cases of interval censoring where the upper or lower bounds respectively, are infinite (Lindsey and Ryan, 1998). A common example arises from the fact that the serological status of an animal is not measured continuously but at specific intervals. Another example is the monitoring of the remissions of symptoms of a given disease where patients are examined periodically. A common approach is to assume that the event occurs either in the beginning or at the end of the interval or at the midpoint, and dealing with this as an exact time. Then standard SA methods can be applied, but it has been shown that this approach can lead to biased results (Lindsey and Ryan, 1998). Although situations where interval-censoring survival analysis could be applied are relatively common in veterinary medicine this analytical approach has been largely overlooked, as evidenced by the fact that few references using this approach can be found in veterinary literature (Bishop *et al.*, 2000; Gibbens *et al.*, 2001; Radke, 2003).

Most studies that evaluated the time to seroconversion after exposure to BLV simply assessed the average of the intervals from the day of exposure to day of first sampling in animals that showed seroconversion. We used two approaches that took interval censoring into account: the standard parametric AFT (accelerated failure time) model (Allison, 1995) with interval-censored data (Bebchuk and Betensky, 2002; Collet, 1994) and a variant of the well-known semiparametric Cox Proportional Hazard Model (PH).

The most commonly used model is the PH model. Its use is preferred because estimation and inference about the parameters of interest are possible without assuming any form for the baseline hazard function, that is, it is not necessary to specify a survival distribution to model the effect of the explanatory variable on the time variable. However, this model is based on the PH assumption and if this assumption does not hold, the standard Cox PH model should not be used as it may lead to serious bias and loss of power when estimating or making inference about the effect of a given prognostic factor on time-to-failure. In this case, there are flexible methods to take into account the non-proportionality of the hazards (Orbe *et al.*, 2002).

The first technique we used to analyse the data was the Cox Proportional Hazard Model (Collet, 1994; O'Quigley and Stare, 2002; Therneau and Grambsch, 2000). A variant of this model (counting processes) that accounts for interval censoring with irregular intervals was adopted (Andersen and Gill, 1982), because not all the studies used equal sampling intervals. When the time scale is 'time since entry', the intensity process for the ith individual is:

$$\lambda_i = \lambda_0 (t) \; Y_i (t) \; \exp^{\beta X_i (t)} \tag{1}$$

where λ_i = is the hazard function for the ith individual; $\lambda_0(t)$ = the baseline hazard function; $Y_i (t)$ is the status of the individual up to time t, that takes the value of 1 when the ith individual is under observation and at risk at time t and 0 otherwise; $\beta x_i(t)$ = vector or coefficients of the explanatory variables x_1, x_2 …, x_i. Formula 1 is formally identical to the Cox model for survival data.

The AFT model is an important alternative to the PH in survival analysis. An AFT model can be written specifying a direct relation between survival time (or the logarithm of the survival time) and the explanatory variables. However, the main disadvantage of this

model is that a specific distribution is assumed for the duration, which in many cases is unknown.

The AFT model describes a relationship between the survivor functions of any two individuals. The general parametric formulation of the model assumes that the random variable t_i indicating the failure time satisfies:

$$\ln t_i = \mu + x_{ik}\,\beta_k + \sigma W \qquad (2)$$

where - t_i is the event time for the *ith* individual in the sample, μ is the intercept parameter, β_k is the vector of regression coefficients to be estimated, $x_{i1}, \ldots, x_{ik}$ are the values of k covariates for the same individual, σ is the scale parameter and the error term W follows a specified distribution. Thus, under this model, the explanatory variables either accelerate or delay the time to failure (seroconversion), depending on whether the value of $\exp(-x_k\,\beta)$ is lower or greater than unity. For dichotomous variables the exponential of β gives an estimation of the ratio of the median survival times of both categories.

As several studies were aggregated and they contributed more than one observation, heterogeneity was suspected. A source of heterogeneity specific to time-to-event outcomes will exist when the effect of an explanatory variable is not constant over time and the follow-up period varies across the trials included in the meta-analysis (Williamson *et al.*, 2002). Heterogeneity was investigated and taken into account by using either AFT or Cox model in which a frailty term was included (Therneau and Grambsch, 2000). Frailty models are basically random effect survival models. The concept of frailty may be used to explain the unaccounted heterogeneity, which leads to the differential time-to-failure patterns of members of a population (Keiding *et al.*, 1996).

The proportional hazard frailty model implies that the hazard function is fully determined by x, the observed covariate vector, although there may be unobserved covariates not represented in this model, which does not include a residual term. Therefore, a multiplicative term, ω, is introduced to the hazard rate, to account for the unobserved population heterogeneity. Then, the proportional hazard model as shown in (1) can be written as:

$$\lambda_i\,(t) = \lambda_0\,(t)\,\exp^{\beta X_i + \omega Z_i} \qquad (3)$$

where, each individual i, $i = 1,\ldots n$ is member of a single group j, $j = 1,\ldots, q$. X_i and Z_i, are the ith rows of covariates matrices X_{n*p} and Z_{n*q} respectively. X and β correspond to the fixed effects in the model and ω is a vector containing the q unknown random effects and Z is a design matrix – Z_{ij} equals 1 if subject i is a meber of family j, 0 otherwise. Here one assumes that the group specific effect for the *j*th group is represented by an unobservable random variable ω, which acts multiplicatively on the hazard rate for all individuals within the group. A gamma frailty model is equivalent to a penalised Cox model with a penalty function $(P(\omega)) = (1/\theta) \sum [\,\omega^2_i - \exp(\omega_i)]$. The ω_is are distributed as the logs of the iid gamma random variables and parameter θ is their variance. For this frailty distribution the correlation of observations within a group (Kendall's tau) can be calculated as $\theta\,/\,(2+\theta)$ (Therneau and Grambsch, 2000).

A parametric frailty survival model introduces an unobserved multiplicative effect ω on the hazard. Since the hazard function is non-negative, ω must be restricted to non-negative values. The linear regression (i.e. AFT model) is as (2):

$$\ln t_{ij} = \mu + x_{ij} \beta + \sigma W_{ij}$$

where t_{ij} - is the failure time of the ith individual (i=1, …, n) in cluster j (j=1, …, q), μ is the intercept parameter, β is the unknown vector of regression coefficients (that we want to estimate) and x_{ij} is the vector of observed covariates. We assume that the censoring time, the covariates and the random error W_{ij} are mutually independent. The random vectors $W_j = (W_{j1}, W_{j2} …., W_{jq})'$ j=1,…, q are independent of each other but for multiplicative failure time data their components (within-cluster errors) are generally not. The marginal independence approach estimates β while ignoring the possible correlation among components of W_j. In contrast, when including frailties, the possible correlation among failure times are explicitly modelled. Specifically, we assume that the hazard function of W_{ij}, conditional on a random variable ω_i, for j= 1…, q and i= 1,…, n is:

$$h_{ij}(t \mid \omega_j) = \omega_j h_0(t)$$

where h_0 is an unknown baseline hazard function independent of the covariates x_{ij} and ω_j are the frailties. Conditional on ω_j, W_{ij} are all independent. All within-cluster failure times share the same frailty term ω_j, which model common effects of the members of a cluster that are not explained by the available covariates. In addition, the frailty term ω_j may also model the heterogeneity of the individuals across the clusters. Note that the frailty ω_j effect act multiplicatively on baseline hazard function h_0 just as in the Cox proportional hazard model. We assume that ω_j's are an iid sample from a Gamma distribution.

In a Cox model, covariates have a proportional hazard structure. For a continuous variable, infection doses for example, this implicitly assumes the ratio between 10^7 and $2*10^7$ infection dose is the same that for $5*10^7$ to $6*10^7$. Threshold effects, both upper and lower are common in biological data (Therneau and Grambsch, 2000). To explore the correct functional form of the variable infective dose and keeping this variable as continuous instead of arbitrary split it into several categories, a more flexible Cox model with a smoothing spline was used. In the Cox model, the baseline hazard function is regarded as a high-dimensional nuisance parameter and is highly erratic (Royston, 2001). In addition, the behaviour of the hazard function by including the full information of infective dose was of interest because the hazard function directly reflects the time course of the process under study. An important second issue that was mentioned before is how to deal with non-proportional hazards, in this approach the functional form is directly modelled by using a special indicator function (smoothing splines). In addition, smoothing spline has better properties than regression spline with respect to locality of influence (Therneau and Grambsch, 2000).

To make the selection of the best model under conditions like those of this study (several models and data sets) more clear to the reader we proceeded as follows:
1. Choice between semi-parametric and parametric model
2. Testing model assumptions
3. Decision on which variables to incorporate into the model
4. Choice between frailty vs non-frailty model

1. Choice between semi-parametric and parametric model

Here we had the option to let the baseline hazard roam freely (semi-parametric) or to specify it (parametric). We analyzed both options, because previous information about the baseline was not known.

2. Testing model assumptions

A) For semi-parametric models

The key assumption of the Cox model is that the hazard function has a proportional structure (Collet, 1994) and two methods were used to evaluate the assumption of proportionality. First, assuming time-fixed covariates, the plot of the cumulative hazard against time was evaluated graphically (Collet, 1994). Second, assuming time-dependent coefficients, a chi-square test on the Pearson product-moment correlation between the scaled Schoenfeld residuals and time or some function of time (i.e. log t or rank of event times) for each covariate was performed (Therneau and Grambsch, 2000). The Efron method was used to handle ties.

B) For parametric models

Five distributions for W were evaluated: Exponential (Exp), Weibull (Wei), log-logistic (LogL), lognormal (LogN) and generalized gamma (Gam), considering models with and without covariates.

Overall goodness-of-fit of the various distributions was assessed by 4 criteria. First, the maximized log likelihood functions of the models without covariates were compared, where the greatest value suggests a better fit (Allison, 1995). Second, goodness-of-fit of nested models was evaluated by the likelihood-ratio statistic (Allison, 1995; Collet, 1994). A model is considered as nested within another model if the first model is a special case of the second. Exponential, Weibull, lognormal are all nested within the generalized gamma model. However, log-logistic is not nested in any of them, hence it can not be compared by the likelihood-ratio statistic. Therefore, Akaike's Information Criterion (Akaike, 1974), which is not a statistical test but an index that allows comparison of all fit models, was used as a third criterion. AIC results from combining the maximized log likelihood function of the model, the number of parameters and the number of covariates included in the model. Finally, fit models with covariates were compared visually, based on the graphs of the Cox-Snell residuals plotted against the estimated cumulative hazard rate, the distribution producing the most straight line fits the data best (Allison, 1995; Collet, 1994).

3) Decision on which variables to incorporate into the model

Either for AFT or Cox models, the model building strategy was based on (Collet, 1994) which suggested a mix of backward and forward procedures using a likelihood ratio test for assessing the goodness-of-the-fit of nested models.

4) Choice between frailty vs non-frailty

When the frailty effect was significant we used the frailty model, if not, a fixed effect model was used (Therneau and Grambsch, 2000).

In all models a detection-window of seven days was specified during which one could not detect seroconversion due to the natural delay between virus inoculation and minimal antibody detection level by any test.

The outcome variable was the time elapsed between day 0 of the experiment (day of inoculation for experimental inoculation designs or the day that susceptible and infected

animals started contact in animal-to-animal contact transmission designs) until the day that seroconversion was detected or end of observation (for those animals that did not seroconvert). The sampling schema of the experiment was used to assess the lower boundary of the interval.

Statistical analysis was performed using S-Plus and SAS (PROC LIFEREG and PROC PHREG) software.

RESULTS

3.1-ANALYSIS OF DATA USING ACCELERATED FAILURE TIME MODELS

Data set 1, based on all studies

The log likelihood of parametric AFT models with no covariates (intercept only) showed that of all analysed distributions the gamma model fit best (p<0.001) among the nested models. The estimated median time to seroconversion based on the gamma distribution was 57 days with a 95% confidence interval (CI) of (49; 75).

Evaluation of goodness-of-fit of nested models with covariates showed that the gamma model fit the data best based on the likelihood-ratio chi-square statistic (p< 0.001) and the gamma model also had the smallest AIC. Visual inspection of the Cox-Snell residuals against the estimated cumulative hazard rates (Figure 1) shows a reasonable good fit in the first half of the time axis for all models. However, in the tail of the time distribution, several models show less desirable fit except for gamma and log-logistic models. Based on all goodness-of-fit tests we conclude that the gamma model with frailty fits the data best.

The analysis reveals two significant variables (Table 3a): Type of transmission design (TYPETRA) and diagnostic test used (TEST). Experimental inoculation accelerates seroconversion approximately 7 times in comparison with animal-to-animal contact transmission design and ELISA detects seroconversion 1.4 times later (1/0.72) than AGID. The acceleration by experimental inoculation is also reflected in the predicted median times to seroconversion when using AGID (Table 4-Model 1): experimental 47 days and animal-to-animal contact 273 days.

Data set 2 (contact exposure, complete records)

The parametric AFT model with no covariates (intercept only) did not converge for the gamma model, hence, results are not conclusive to consider any distribution as best. Moreover, no statistical associations were found between covariates and time to seroconversion. Therefore, it is meaningless to calculate and discuss other goodness-of-the-fit methods. This set was not considered for further analysis.

Figure 1. Plots of Cox-Snell Residuals against estimated cumulative hazard rates for graphical assessment of goodness-of-the-fit of five Accelerate Failure Time interval-censoring frailty models, using data set 1 (n = 438).
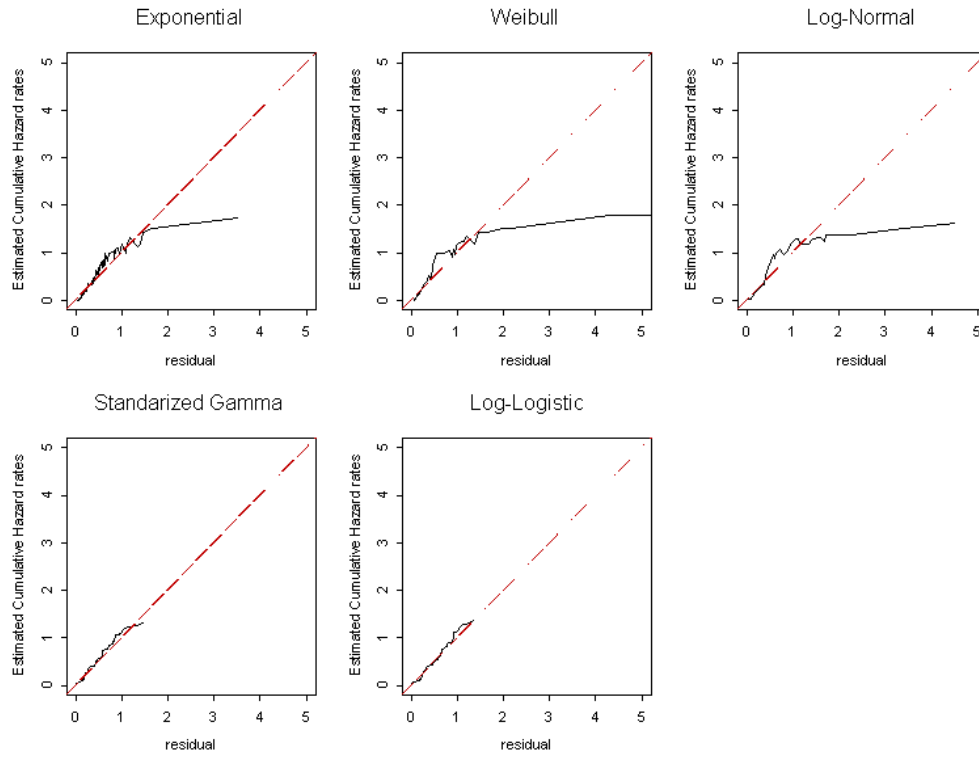
Table 3a. Analysis results, using an interval-censored AFT gamma model, of data set 1 (all data with complete variables (n = 438)).

Variable	Category	% sero-conversion (n)	Acceleration factor (e^{β})	95% CI of Acceleration factor	p - value
Transmission design	*Animal-animal contact*	16.7 (10/60)	ref.		
	Experimental inoculation	71.2 (269/378)	6.8	5.5; 8.3	<0.01
Diagnostic test used	*AGID*	63.1(239/379)	ref.		
	ELISA	67.8 (40/59)	0.7	0.6; 0.9	<0.01
Intercept	5.383				
Shape	- 2.406				
scale	0.622				

log-likelihood =-810.3; AIC = 1631

Data set 3 (inoculation, complete records)

Results of the fitting procedures on models without covariates were comparable to those of dataset 1, the gamma model fit significantly better than the others. The estimated

median time to seroconversion was 47 days with a 95 % CI of (39; 55). Results of the analysis of models with covariates are shown in Table 3b.

Table 3b. Analysis results, using an interval-censored AFT gamma model, of dataset 2b (all data with complete variables (n = 378)), only coefficients of variables with a p<0.05 are shown.

Variable	Category	% sero-conversion (n)	Acceleration factor (e^{β})	95% CI of Acceleration factor	p - value
Parenteral routes	*Others*	60.3 (41/68)	ref.		
	EV+IM+SC+ID	73.1 (225/308)	1.3	1.1; 1.6	0.01
Type of experimental infection	*Biting of insects*	38.6 (17/44)	ref		
	needles or inanimate media	75.2 (251/334)	2.3	1.9; 2.8	<0.01
Diagnostic test used	*AGID*	71.5 (228/319)	ref.		
	ELISA	67.8 (40/59)	0.8	0.6; 0.9	0.02
Intercept	4.402				
shape	-2.496				
Scale	0.544				

log-likelihood = -740.7; AIC = 1493

As for set 1, the gamma model fit best according to each of the 4 criteria. Inoculation routes EV, IM, SC and ID increased seroconversion interval 1.33-fold and diagnostic test used reveals a similar effect as in dataset 1 (ELISA 1.3 times longer (1/0.76) than AGID). Performing inoculation via needles or other inanimate media increases the seroconversion interval 2.3-fold versus biting of insects (biological media). Table 4-Model 2 shows the predicted median times to seroconversion ranging from 39 to 117 days.

3.2- ANALYSIS OF DATA USING COX PROPORTIONAL HAZARD MODELS

The stratified models using data sets 1 and 3 did not hold the assumption of proportional hazards, checked either by the cumulative hazard plot (assuming a time-fixed effect of the variables) and scaled Schoenfeld residuals (assuming a time-dependent effect of the variables). Therefore, standard Cox proportional hazard model was excluded for further analysis.

Table 4. Predictions of median times to seroconversion from interval-censored Accelerated-Failure-Time gamma models, for the different combinations of categories of the variables included in the fit model. Model 1 represents estimations using, data set 1 (all data with complete variables (n = 438)). Model 2, represents estimations using dataset 2b (all data with complete variables (n = 378).

Model 1				Model 2				
Variable predictors		Predicted median time (days)	SE Predicted median time (days)	Variable predictors			Predicted median time (days)	SE Predicted median time (days)
Transmission Design	Diagnostic Test			Parenteral routes	Type experimental infection	Diagnostic Test		
Experimental inoculation	*ELISA*	55	8	*EV+IM + SC+ID*	*needles or inanimate media*	*Elisa*	51	8
Experimental inoculation	*AGID*	47	5	*EV+IM +SC+ID*	*needles or inanimate media*	*AGID*	39	5
Animal-animal contact	*ELISA*	NA	NA	*EV+IM +SC+ID*	*Biting of insects*	*Elisa*	117	19
Animal-animal contact	*AGID*	273	37	*EV+IM +SC+ID*	*Biting of insects*	*AGID*	89	12
				Others	*needles or inanimate media*	*Elisa*	68	11
				Others	*needles or inanimate media*	*AGID*	52	7

NA= no-observation available

3.3- ANALYSIS OF DATA USING COX PROPORTIONAL HAZARD MODELS WITH FRAILTY TERM TO ACCOUNT FOR HETEROGENEITY DUE TO STUDY CONDITIONS.

Data set 1, including all studies with complete information variables

This analysis shows that two variables are significantly related to the time to seroconversion (Table 5-model 1). Experimental transmission using inoculation increases the risk of seroconversion 8 times in relation to animal–to-animal contact exposure. Exposure by donors that were positive to BLV by any direct test before the beginning of experiment increases the hazard almost 2 times in comparison with donors in which virus

was not confirmed (AG). The correlation of subjects within-groups is 21.6 % and the explained variance by the frailty term is 14.8 %.

Table 5. Results after fitting a Cox model (Anderson–Gill model) using Research Group as frailty term, using dataset 1 (all data with complete variables (n = 438)) (Model 1) and dataset 3 (all data with complete variables (n = 378)) (Model 2).

	Variables	**Category**	**% sero-conversion (n)**	**HR** *	**95% CI**	**p-value**
Model 1	Transmission design	*Animal-animal*	16.7 (10/60)	ref.		
		Experimental inoculation	71.2 (269/378)	8.5	4.4;16.8	<0.01
	Virus confirmation of donor animal	*Virus Absent donor*	52.5 (52/99)	ref.		
		Virus present donor	66.7 (227/339)	1.7	1.2;2.4	<0.01
	Frailty (Research Group)			-	-	<0.01
	Theta (θ) = 0.55					<0.001§
Model 2	Diagnostic Test used	*ELISA*	67.8 (40/59)	ref.		
		AGID	71.5 (228/319)	1.5	0.9;1.9	0.05
	Type of inoculum used	*Blood*	65.3 (154/236)	ref.		
		Lymphocyte suspension	81.2 (82/101)	2.5	1.8;3.6	<0.01
	Virus confirmation of donor animal	*Virus Absent in donor*	59.5 (50/84)	ref.		
		Virus present in donor	74.2 (218/294)	1.8	1.2;2.7	0.02
	Type of experimental infection	*Biting of insects*	38.6 (17/44)	ref.		
		Infection performed via needles or inanimate media	75.2 (251/334)	5.4	3.0;9.8	<0.01
	Frailty (Research Group)			-	-	<0.01
	Theta (θ) = 0.73					<0.001§

*Hazard Ratio; § Wald test

Data set 3 (inoculation, complete records)

Four variables remained in the final model (Table 5-model 2). The hazard of detecting seroconversion is 1.5 times increased when AGID is used compared to ELISA. In addition, exposure by lymphocyte suspensions increases the hazard of seroconversion 2.5 times in comparison with inoculation with whole blood. Exposure by donors being positive to BLV by any direct test at the start of the experiment increases the hazard of seroconversion almost 2 times in comparison with negative donors. Using inanimate vehicles increases the hazard 5.4 times in comparison to inoculation through bites of insects. The autocorrelation of subjects within-groups is 26.7 % and the explained variance by the frailty term is 12.7 %.

3.4- EFFECT OF INFECTIVE DOSE ANALYSED BY MEANS OF A COX MODEL WITH SMOOTHING SPLINES

Because in the previous model the variable representing the infectiousness of the donor animal contains a category with 'unknown' status for the infective dose, the set was re-analysed considering high and low infectious categories only. The final model contained 3 variables, research group, route of inoculation and a nonlinear spline for infective dose (Wald test p <0.001 and 2 df.). In this model, after Group stratification, animals inoculated via routes (EV+IM+SC+ID) have a risk of sero-conversion that is increased 7 times (95 % CI 1.5; 29.9) in comparison with animals inoculated via other routes. In addition, the nonlinear spline term was significant (P< 0.05). Figure 2 shows the functional form of the infective dose.

Figure 2. Functional form of infective dose after fitting a Cox model with smoothing splines based on data set 4 after re-categorising the variable AG in donors with only high/low infectious status (n = 122). On the horizontal axis, ticks mark at each location of a data point.
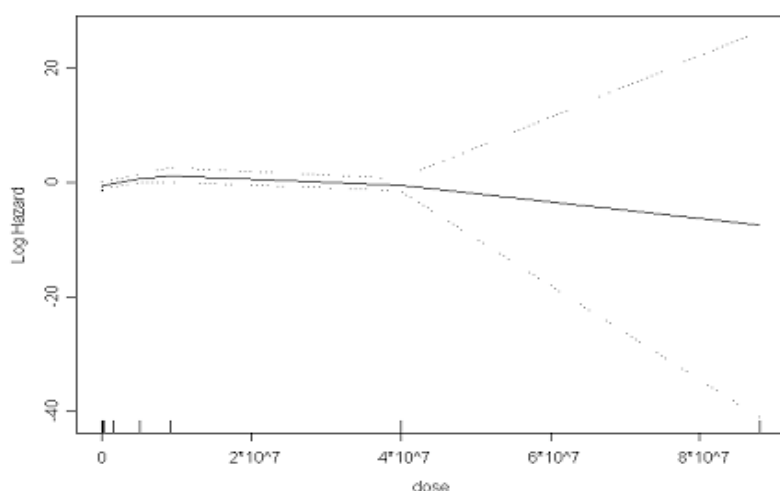
The shape of the hazard function shows a slightly increased risk of seroconversion up to a value of 10^7 lymphocytes, after that point an increase in dose seemingly does not increase or even decrease the risk of seroconversion but wider confidence intervals were observed, due to fewer observations.

DISCUSSION

The purpose of this study was to estimate the time to seroconversion for BLV infections using a meta-analysis. Our estimates of the time to seroconversion (57 days, SE 6 days) using an interval censored AFT frailty model were longer than calculated in the study of Lassauzet *et al.*, (1989) (28 days, SE 1 day). Some reasons might explain the difference: first, we included studies that used animal-to-animal contact transmission and second we included more studies and observations in the analysis. Animals that were infected by contact transmission tend to show a delayed seroconversion time when

compared to animals infected by inoculation, hence, the median time until seroconversion is extended. When excluding experiments using contact exposure and insect bites the time to seroconversion was 39 days (Table 4). A third reason could be related to the type of distribution and the type of model we fit.

In our study a gamma model fit the data best, while in the study of Lassauzet et al., (1989) a log-logistic model was used. No other studies using either BLV or other retroviruses-caused diseases of veterinary interest were available for comparison. Most of the published studies that investigated various parametric models to analyse time to detection are related to other retroviruses such as acquired immune deficiency syndrome (AIDS). In those studies, Exponential, Weibull, log-normal and log-logistic were the parametric models most frequently used (Chiarotti et al., 1994; Egger et al., 2002; Lassauzet et al., 1989; Muñoz and Xu, 1996; Tan et al., 1996; Tassie et al., 2002). They all belong to the generalized F family of models that the gamma model includes also. The gamma model has preference over the other models as it has one extra parameter and its hazard function can take on a variety of shapes that other models can't (Allison, 1995) and which might explain the better fit to the data.

Our analysis shows the large difference in time to seroconversion that exists between animal-to-animal contact transmission and inoculation exposure (Table 4-model 1, between 47 and 57 days and over 300 days respectively). Abt et al, (1976) reported that up to 18 months post contact only 3 % of the animals at risk were infected but 58 % became infected up to 4 years. This might be either due to a delay in eliciting a detectable humoral response as a consequence of continuous exposure to low infective doses or to the fact that the true infection date is not the first day that infectious and susceptible animals are kept together. Therefore, we included the uncertainty of the true infection date by using interval-censored models in which the infection date is approximated between the date of first contact and the day that infectious and susceptible animals were separated. Animal-to-animal transmission experiments need a long follow-up period as most (80%) of the animals that seroconverted in animal-to-animal transmission belonged to studies that were extended for more than 2 years (data not shown).

Diagnostic tests have a different ability to detect antibodies which influences the measurement of the time to seroconversion as is clearly shown from results of our study. However, a shorter detection time for AGID test than for ELISA was unexpected, because ELISA tests are described to have a relatively higher sensitivity in comparison with AGID (Have and Hoff-Jorgensen, 1991; Martin et al., 2001), so one would expect the opposite trend. Although the number of observations tested with AGID was greater than for ELISA, the proportion of censoring was very similar in both groups (data not shown), therefore a sound explanation for this finding is difficult.

Lassauzet et al., (1989) discussed that the incubation time for BLV might be influenced by virulence of the agent, infective dose, route of inoculation and host resistance, which agrees with the results of our study. When focusing on inoculation, the most important route for BLV transmission (Ferrer, 1979; Hopkins and DiGiacomo, 1997), our results indicate that some factors (EXP and TEST) are consistently associated with seroconversion using either an AFT or a frailty Cox model. Other variables (AG, PARENT and INTYPE) are significant in only one of both models. Inoculations by insect bites resulted in an increased seroconversion time in comparison with other inoculation methods. This may be due to a lower number of transferred lymphocytes as all trials used interrupted meals. The virus may also be partly inactivated in the gut of the insect resulting

in a reduced 'final effective infective dose'. Parenteral routes seem to accelerate seroconversion as shown by the analyses using the AFT model and the Cox model with smoothing splines. A possible explanation is that virus can access the bloodstream quicker and hence trigger host's humoral immune response faster. Lassauzet *et al*, (1989), suggested no association between inoculation routes and time to seroconversion. The difference with our study may arise from the fact that we used a multivariable analysis, instead of a univariable, that adjusts for other variables that are included in the model. Also, in our analysis we pooled all parenteral routes (IM, SC, EV and IP) in one category, thus increasing the power of the analysis.

Considering the effect of the BLV antigen status in donor cows, we found inconsistent results. The analyses based on AFT gamma model and the Cox model with smoothing splines showed no significant association while the association was significant using a Cox Andersen-Gill model (Tabel 5 & 6). Other studies also found a delayed antibody response when BLV antigen-negative donors were used (Buxton and Schultz, 1984; Lassauzet *et al.*, 1989; Miller *et al.*, 1985).

Using blood as infectious material, compared with using a lymphocyte suspension, seroconversion only delays significantly in the frailty Cox model and the Cox model with smoothing splines. This association can be explained by the presence of neutralising antibodies in whole blood which has been shown by others (Gupta and Ferrer, 1982; Miller *et al.*, 1985; Ohishi *et al.*, 1990). Another explanation could be that during the process of lymphocyte purification the neutralising blocking factor was sufficiently diluted or even completely removed, then virus could integrate in DNA of target cells easier. Nevertheless, natural infections most likely will be produced with blood and not with isolated lymphocytes, revealing scarce relevance when considering transmission in the field.

As expected the infective dose has an effect on time to seroconversion as shown from results of the Cox model with splines. The functional form of the hazard ratio for infective dose suggests that this relationship is not linear. This is consistent with the non-significant effect of infectious dose in models that assume a linear relationship (results not shown). This indicates that the onset of the humoral response of the infected host is linear with the dose of infection up to certain threshold level and then steadily declines.

Including several studies from the same centres may induce heterogeneity of data which may add a non-constant effect to the time-to- event outcomes (Williamson *et al.*, 2002). Several options are available to account for heterogeneity depending on the type of model used. In AFT models significant heterogeneity was present although the magnitude of the estimations did not change significantly. However, the frailty Cox models showed a moderate effect of the research centre and it accounted for 15 % of the overall variation using set 1 and similar results were found when analysing dataset 3.

Results from our study emphasise the importance of not relying on only one commonly used method such as the Cox proportional hazard model when there is no previous information. A more refined exploration is needed to get insight in how hazard functions change over time. Moreover, in our study the Cox model failed the assumption of proportionality in a number of cases, which is not surprising for a meta-analysis where the follow up period may differ between studies (Keene, 2002). Studies with shorter follow-up will tend to show hazard ratios more discrepant from unity when compared to those with longer follow-up and ignoring these issues could lead to biased conclusions (Lindsey and Ryan, 1998; Orbe *et al.*, 2002). Some authors suggest that the accelerated failure time model as a better alternative because it readily provides estimates for the ratios of medians. The

median times are a robust summary and they allow quantifying size of effect on the time axis (Keene, 2002). Recently it has been shown that AFT models are more robust to mis-specification (such as using an incorrect distribution) than semi-parametric-proportional hazard models because of their log-linear form (Hutton and Monaghan, 2002). Next to estimation of the effects of covariates, parametric models such as AFT have the advantage that information about the hazard function can be obtained. Their main disadvantage is that a specific distribution for the time duration is assumed, which in most cases is unknown (Orbe *et al.*, 2002). Finally, one aspect that must be taken into account is that the results under the different methodologies are not directly comparable. The PH model explains the effect of covariates on the hazard function, while the AFT models measure the direct effect of the covariates on the time to event occurrence (Orbe *et al.*, 2002).

CONCLUSIONS

When all types of experimental infections are considered, the median time to seroconversion is 57 days and the main factor that influences time to seroconversion is the type of transmission used. When only studies using experimental inoculation are considered the median time is estimated as 48 days, using a gamma model. A moderate influence induced by research centres can not be neglected and some factors that influence the time to seroconversion were identified.

These results may contribute to calculate the most probable times of infection in field conditions resulting in a better evaluation of control measures.

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CHAPTER 5

EVALUATION OF NATURAL TRANSMISSION OF BOVINE LEUKAEMIA VIRUS WITHIN DAIRY HERDS OF ARGENTINA

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SUMMARY

Bovine Leukemia Virus (BLV) is a retrovirus that causes lymphomas and other disorders in cattle and has a large economical impact on the livestock sector of many countries around the world. For the development of effective BLV control strategies, quantitative information transmission of BLV in cattle herds is needed. The purpose of this study was to describe patterns of seroconversion to BLV and to estimate main parameters needed for future model building. A longitudinal observational study was carried out between February 1999 and November 2001 in seven commercial dairy farms in Argentina and some of them were known to be endemically infected with BLV. Foremilk samples (n=7,961) from all lactating cows (n=1,535) in each herd were collected at average every 2 months during milk control sampling and tested with a blocking ELISA test. At the end of the study 4 herds were entirely bled. Time interval parameters (age at first calving and culling) were analyzed using a parametric survival model with shared frailty. Time till infection was analyzed using a Bayesian interval-censoring survival analysis approach and the infection transmission parameter (β) was estimated by a generalized linear model (GLM) with a log link and an offset variable and a Poisson error term. The reproduction ratio was calculated as well.

1,000 cows tested positive and 494 tested negative. In 41 animals we found a pattern that consisted of one positive result followed by 4 to 12 negative results. 15 % of the animals that tested positive showed a pattern that consisted of several positive test results with few intermediate negative results. 7 % of the negative test samples from infected animals occurred around parturition. For cows that seroconverted, the predicted median age-at infection was 4.6 years. The proportion of positive calves for infected herds was as high as for infected cows. Infected herds also showed a large proportion of infected breeding heifers. Peaks in the overall average incidence per season-year were observed during autumn and spring.

Under the conditions of this study, natural transmission of BLV was observed and results reveal that the period around parturition is a high risk period. Also, heavily infected herds seem to have and increased proportion of young stock infected. The overall β was estimated as 2.9 year $^{-1}$ (95 % CI 1.9; 3.7) and combined with a relatively long infectious period it resulted in a high reproductive ratio (R_0 =8.9). Therefore, a high effectiveness of control measures should be achieved to eradicate the disease.

INTRODUCTION

Bovine Leukemia Virus (BLV) is an retrovirus that together with Human T-Lymphotropic Virus (HTLV) and Simian T-Lymphotropic Virus (STLV) belongs to the Deltavirus genus of family *Retroviridae* (Coffin *et al.,* 1997). BLV causes lymphomas and other disorders in cattle (Miller and Van der Maaten, 1990). Due to the tendency of being clustered in geographical areas and herds, the disease was for many years referred to as Enzootic Bovine Leucosis. BLV has a large economical impact on the livestock sector of many countries around the world.

BLV is transmitted horizontally by infected lymphocytes (Meas *et al.,* 2002; Romero *et al.,* 1982) or it is transmitted vertically (Meas *et al.,* 2002; Piper *et al.,* 1979; Van der Maaten *et al.,* 1981). Most of the time, infection is iatrogenic and occurs when the animals are treated without adequate hygienic care e.g. when injected (Wilesmith, 1979), dehorned, (DiGiacomo *et al.,* 1985; DiGiacomo *et al.,* 1987; Lassauzet *et al.,* 1990) tattooed, (Parodi *et al.,* 1985) ear-tagged, castrated, bled (Amaddeo *et al.,* 1982) at teat removal and at rectal examinations (Divers *et al.,* 1995; Hopkins *et al.,* 1991; Lucas *et al.,* 1985; Pelgrim *et al.,* 1993; Wentink *et al.,* 1993). Transmission by biting of insects has been reported as well (Bech Nielsen *et al.,* 1978; Buxton *et al.,* 1982a; Buxton *et al.,* 1982b; Kaaden *et al.,* 1982; Ohshima *et al.,* 1981), especially with insects of the family *Tabanidae* (Buxton *et al.,* 1985; Foil *et al.,* 1988; Manet *et al.,* 1989b).

For the development of effective BLV control strategies, quantitative information on transmission of BLV in cattle herds is needed such as length of the infectious period, probability of transmission given exposure, etc. This information is currently not available in literature.

The purpose of this study was to describe patterns of seroconversion to BLV and to estimate main parameters needed for future model building. This information can then be used in simulation models to evaluate various scenarios, which may result in hypotheses about which aspects are important for a control strategy

MATERIAL AND METHODS

STUDY POPULATION

A longitudinal observational study was carried out between February 1999 and November 2001 in seven commercial dairy farms (denoted A to G) in Argentina. Although these herds were selected based on owner's willingness to collaborate in the study, they are considered typical dairy farms of the area in terms of herd size, breed and management policies. The main characteristics of the herds are summarized in Table 1. Animals graze on rotational paddocks all year round and are milked twice a day. It was known that herd B, D, E and F were endemically infected with BLV. During the study, farmers did not receive information on the infection status of their animals.

DATA COLLECTION

Data were obtained from farmer's records and the Milk Control Association, which supplied information on dates of birth or purchase, breed, calving, dry-off and culling or death. Information on pregnancy status and health-related problems was recorded during

regular (between 3 to 8 weeks) visits of the veterinarian responsible for monitoring herd reproduction.

Table 1. Main characteristics of herds used during the study.

Herd	Mean# lactating cows ±SD	Breed	305-day milk production
A	105 ± 9	Holstein	5,880 ± 1,556 lt.
B	73 ± 22	Holstein & Holstein x Jersey	5,810 ± 1,491 lt.
C	130 ± 17	Holstein	6,020 ± 1,454 lt.
D	126 ± 19	Holstein	6,020 ± 1,460 lt.
E	95 ± 14	Holstein	5,070 ± 1,400 lt.
F	40 ± 8	Holstein	5,670 ± 1,235 lt.
G	93 ± 19	Holstein	4,662 ± 1,091 lt.

COLLECTION OF SAMPLES AND DIAGNOSTIC ASSAYS

Foremilk samples (5 ml) from all lactating cows in each herd were collected at average every 2 months during milk control sampling. This milk was used to determine the infection status of animals. All samples were transported on ice in cool boxes to the lab, where they were stored at –20 °C until processing. At the end of the study four herds (B, C, F, G) were entirely bled, while in Herd (A) only lactating and dry cows, were bled.

Serum and milk samples were tested using a blocking ELISA 108 (Gutierrez *et al.,* 2001), with an estimated sensitivity of 98.9 % (CI: 96.4 %; 99.9) and a specificity of 98.0 % (95% CI: 94.6; 99.5) (Chapter 3). Serum samples were considered positive when their percentage of inhibition (PI) was more than 40 % of the standard positive, inconclusive when the PI was between 35 and 40 % and negative when the PI was less then 35%. The cut-off points used for milk samples were slightly higher, over 52 % for being considered positive and inconclusive when between 47 and 52 %.

DATA PROCESSING & ESTIMATION OF PARAMETERS

Age structure

In dairy farms, animals usually are kept in groups to optimize and facilitate management. To keep the analysis relatively simple but representative of the general situation, 3 age classes were considered. Group 1 includes all females from birth until 180 days of life. Group 2 includes all females over 181 days but not yet introduced in the milking herd (2 months before the expected day of calving). Group 3 includes all pregnant heifers within 2 months of calving and all cows that had calved at least once.

Age at first calving and culling

Because some of the parameters to be estimated has as unit time, we assessed the potential relationship of those variables and BLV status, using a parametric survival model

with shared frailty to account for heterogeneity between individuals clustered within-herd (Therneau and Grambsch, 2000). Briefly, a parametric frailty survival model introduces an unobserved multiplicative effect ω on the hazard. Since the hazard function is non-negative, ω must be restricted to non-negative values. The linear regression form is as:

$$\ln t_{ij} = \mu + x_{ij}\,\varphi + \sigma\,W_{ij}$$

where t_{ij} is the failure time of the ith individual ($i=1, …, n$) in herd j ($j=1, …, q$), μ is the intercept parameter, φ is the unknown vector of regression coefficients (that we want to estimate) and x_{ij} is a vector of observed covariates. The random vectors $W_j = (W_{j1}, W_{j2} …., W_{jq})'$ $j=1,…, q$ are usually assumed to be independent of each other but for multiplicative failure time data, their components (within-herd errors) are generally not. The marginal independence approach estimates φ while ignoring the possible correlation among components of W_j. In contrast, by including frailties, the possible correlation among failure times are explicitly modelled. Specifically, we assume that the hazard function of W_{ij}, conditional on a random variable ω_i, for $j= 1…, q$ and $i= 1,…, n$ is:

$$h_{ij}\,(t\,|\,\omega_j) = \omega_j\,h_0(t)$$

where h_0 is an unknown baseline hazard function independent of the covariates x_{ij} and ω_j are the frailties. Conditional on ω_j, W_{ij} are all independent. All within-herd failure times share the same frailty term ω_j, which models common effects of the members of a herd that are not explained by the available covariates. In addition, the frailty term ω_j may also model the heterogeneity of the individuals across herds. We assume that ω_j's are an iid sample from a Gamma distribution.

The effect of herd on the age at infection and on the age of sero-positive young stock was assessed by the log-rank test for equality of survival distributions (Kaplan-Meier estimation). Because the follow-up period covered a relatively long time, the age at infection might be related to changes in prevalence over time. Therefore, the study was divided in two periods of approximately one year each and the effect of year on the age at infection was tested using a log-rank test.

PROBABLE TIME TO INFECTION AND CRITERIA FOR DETERMINING INFECTED ANIMALS

An individual was considered as infected when it showed 2 or more positive milk test results during the follow-up period. In the special case that from a given animal only 2 test results were available and one was positive it was considered as infected as well; when one of the results was doubtful and the other negative, the animal was considered as non-infected.

The date of observed seroconversion is not the time of infection. For cows that sero-converted during the follow-up period, the most likely time of infection can be deduced from the last observation of a negative serological result and the time of the first positive observation, t'_1 and t'_2 respectively. We used a Bayesian approach based on (Lassauzet et al., 1989) using the concept of interval-censoring survival analysis. Briefly, let T_α represent the time till seroconversion for an animal after start of follow-up and assume that $T_\alpha\ \varepsilon(t'_1, t'_2)$. Then, if θ represents the current time till infection, it follows that

$$\Pr(T_\alpha \, \varepsilon(t'_1, t'_2] \mid \theta) = \Pr(T_\alpha - \theta \, \varepsilon \, (t'_1 - \theta, t'_2 - \theta] \mid \theta) \tag{1}$$

The quantity $T_\alpha - \theta$ is the time till seroconversion from experimental infections, which was derived from a previous study (Monti and Frankena, 2004). Assuming that $T_\alpha - \theta$ has a distribution with survival function S_t, then (1) is:

$$\Pr(T_\alpha \, \varepsilon \, (t'_1, t'_2] \mid \theta) = S(t'_1 - \theta) - S(t'_2 - \theta) \tag{2}$$

and follows a gamma distribution.

We reflect prior knowledge or uncertainty about θ in the form of a density $D(\theta)$, where $D(\theta) \geq 0$ and $\int_0^\infty D(\theta)d\theta = 1$. Then the posterior density for θ, $L < \theta \leq U$, where L and U are the lower and upper bounds of the time till infection given the data, takes the following expression:

$$D(\theta \mid Tser \in (t1, t2]) = D(\theta)\{S(t1 - \theta) - S(t2 - \theta)] / \int_L^U D(\theta)\{S(t1 - \theta) - S(t2 - \theta)\}\,d\theta$$

We obtain the point estimate of θ by finding the value θ' or the middle value if many, that maximizes the function and we consider it as the estimate of the most probable time till infection.

DETERMINATION OF TRANSMISSION PARAMETERS

Infection rate parameter

We assumed that new infections are generated by a Poisson process within a SI stochastic model. In this model individuals can either be Susceptibles (S) or Infectious (I). New infections are assumed to occur at the rate $(\beta \, S.I)/N$, where β is the infection rate parameter and N the total number of individuals. To obtain the value of the infection rate parameter β we also assume the following conditions:

Animals are susceptible during their whole life;
There is no age-dependent susceptibility;
An infected individual remains infectious until removal or dead with equal infectiousness during this period;
The coefficient of transmission is constant over time
Hence, the number of new infections (C) in the interval (t_1, t_2), follows a Poisson distribution with a mean:

$$E\{c(t_1, t_2)\} = \int_{t1}^{t2} \lambda(\tau)d\tau$$

Given the data obtained from the longitudinal study, the number of new cases per sampling period was known and since S, N and I are known, the coefficient β can be estimated. We used a Generalized Linear Model (GLM) with a log link, and *Log* (S.I/N) as offset variable. Then, we have approximately:

$$\log(E\{C(t_i, t_{l+1})\}) = \log(\beta) + \log(1/2 * \{S(t_i) * I(t_i) / N(t_i) + S(t_{i+1})I(t_{i+1}) / N(t_{i+1})\} * \Delta t) \qquad \textbf{(3)}$$

Transmission parameter β was estimated for each farm using equation (3). To estimate the 95 % confidence interval for log β, we used the standard error (se) as:

$$\log \beta \pm t_{1-\alpha} * \sqrt{\text{var}(\log \beta)} = \log \beta \pm 1.96 * se$$

where $t_{1-\alpha}$ is the two –side confidence coefficient assuming a normally distributed variable.

The residual deviance of the model and the plot of the deviance residuals against fitted values were used to evaluate goodness of the fit of the model.

REPRODUCTION RATIO (R_0)

The basic reproduction ratio (R_0) is a key parameter in transmission theory as it defines a threshold condition that determines whether an infectious disease will spread in a susceptible population when the disease is introduced into it. The basic reproductive ratio is defined as the average number of secondary cases produced by one infected individual during the individual's entire infectious period when the pathogen is first introduced (Diekmann *et al.,* 1990). Then the reproductive number can be estimated by the following formula:

$$R_0 = \beta * 1/\gamma$$

where $1/\gamma$ is the average infectious period.

We estimated $1/\gamma$ as the difference between the median age at infection and the life expectancy for BLV infected cattle because animals remain infected for the rest of their lives.

RESULTS

DESCRIPTIVE ANALYSIS

7,961 milk samples and 1,009 blood samples from 1,535 animals were taken. From all animals, 1,000 of them tested positive and 494 tested negative to milk samples. The remaining animals (n = 41) showed a pattern that consisted of one positive result followed by 4 to 12 negative results. In addition, we observed that 15 % of the animals that were tested positive showed a pattern that consisted of several positive test results with few intermediate negative results. This type of response has been described for BLV-infected animals that are co-infected with BVDV (Roberts *et al.,* 1988; Roberts *et al.,* 1989). 1.7 % of the negative results of infected animals were from samples taken during peripartum time (4 weeks previous to 2 weeks after calving).

The median age at infection by herd and by year of study was different by log-rank test for equality of survival distributions (χ^2 = 90.2, 9 d.f., p < 0.001). For cows that seroconverted, the predicted median age at infection was 4.6 years. The median age-at infection was different between herds as indicated by log-rank test for equality of survival distributions (χ^2 = 48.1, 5 d.f., p < 0.001).) Also the median age at infection in the first

year of the study was different (higher) than in the second year (log-rank test for equality of survival distributions ($\chi^2 = 55.7$, 1 d.f., $p < 0.001$)).

Seroprevalence and median age at infection for the three categories of animals are shown in Table 2. The prevalence in calves of herds B and G was as high as the prevalence of infected cows. Infected herds also showed a large proportion of breeding heifers infected (group 2). For the age of infection there is no clear association with prevalence.

The ELISA test used could not distinguish between passively-transferred antibodies to those actively produced after infection. Therefore, sero-positive animals of group 1 were excluded from calculations because a positive test result can not always be attributed to infection.

Table 2. Distribution of serological results in 3 age groups of 4 selected farms.

Age Group	Prevalence of Positive sero-reactors (%)				Median age for sero-positive animals (years)			
	Herd B	Herd C	Herd F	Herd G	Herd B	Herd C	Herd F	Herd G
Group 1: 0 - 180 days	71.4	0.0	nd (*)	100.0	NC	NC	nd (*)	NC
Group 2: 181 days till introduction in milking herd	57.1	0.0	46.1	40.3	1.7	NC	2.5	1.2
Group 3: Adult cows & pregnant heifers	77.2	0.0	99.9	99.5	3.4	NC	4.7	5.5

(*) nd= not sampled   NC = not calculated

TEMPORAL PATTERN OF SEROLOGICAL REACTIVITY

The evolution of the prevalence and estimated incidence rates across the follow-up period for each farm is shown in Figure 1 and 2 respectively. The prevalence curves show a steadily increasing trend (herds B, E and G) or small oscillations at high (herds D and F) or low (herds A and C) levels.

Figure 1. Sero-prevalence by herd during follow-up period (February, 1999 to November, 2001)
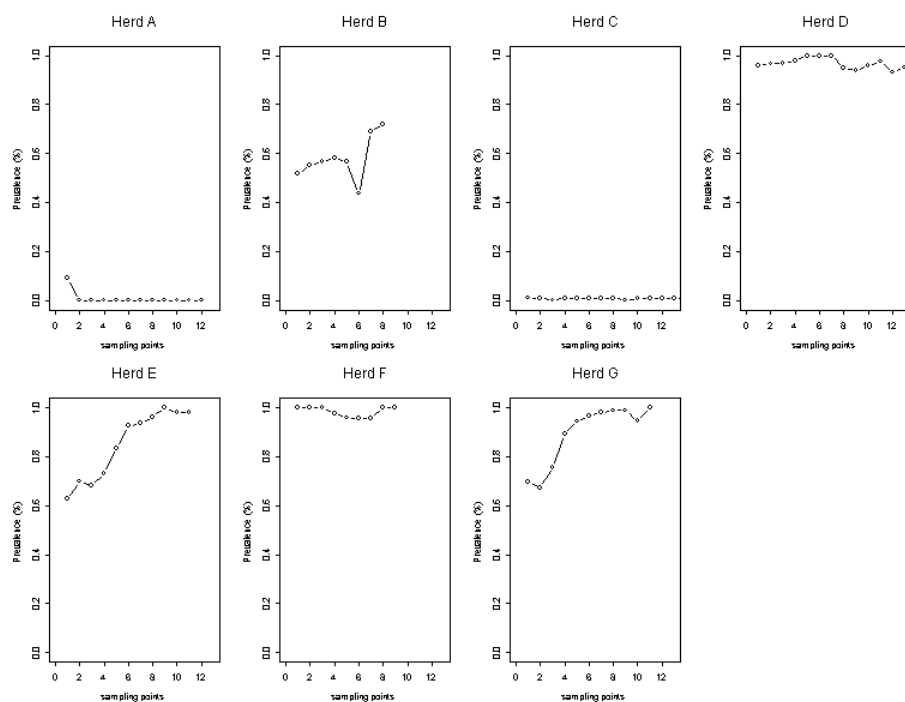
Figure 2. Incidence rate by herd during follow-up period (February, 1999 to November, 2001)

81
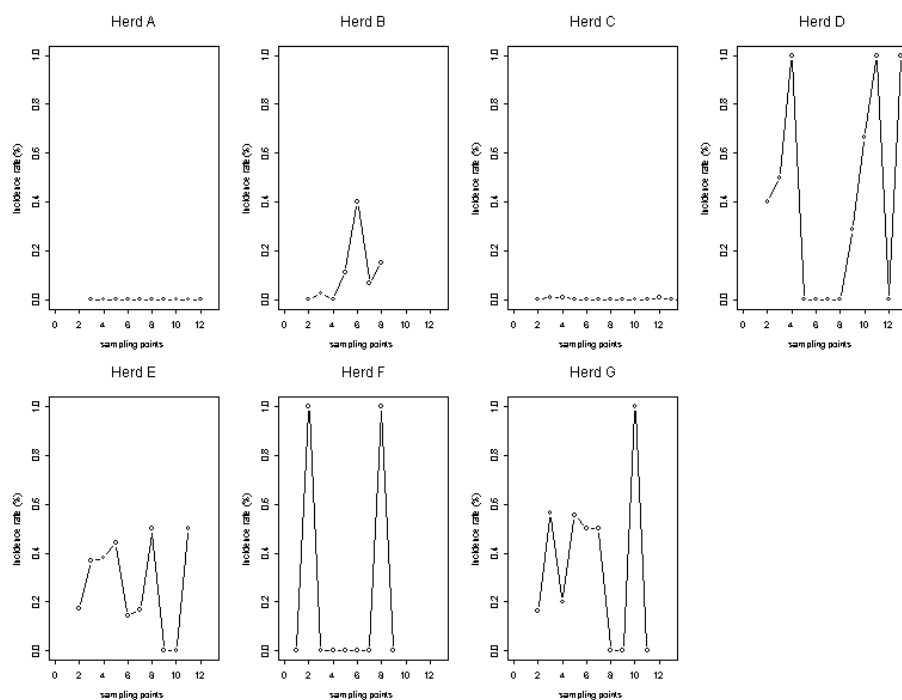
Tendencies of incidence rates could be spliced in 2 halves for herds B, E, and G representing the first and second year of the follow-up interval. First, the curve reflects a steady increase of the incidence in the first period, followed by high oscillations in the second half. This shift in the shape started when prevalence was larger than 70 %. In highly infected herds (D, F) the oscillations may reflect the very few susceptible individuals. Finally, herds A and C represent a situation of low transmission between individuals.

Figure 3 shows a plot of the overall average incidence per season-year, peaks during autumn and spring across years were observed.

Figure 3. Seasonal Average Incidences rates.
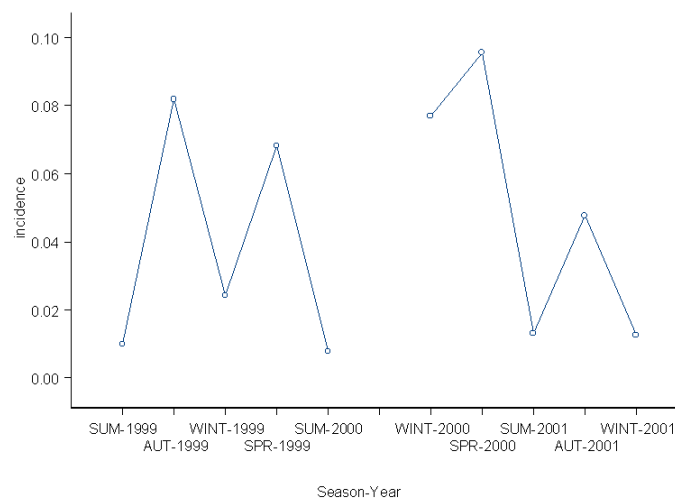
SEROLOGICAL STATUS AND CULLING

Cows that were culled during the follow-up period had a median age of 9.1 years (3322 days). BLV positive cows had a median age of 8.3 years (3044 days) and for BLV negative cows the median age was 9.1 years (3327 days) but the difference was not statistically significant (p= 0.248).

The median age at first calving was 2.8 years (1010 days). No statistical difference (p = 0.49) was present between BLV positive cows (2.8 years) (1012 days) and BLV negative cows (2.7 years) (1002 days).

TRANSMISSION PARAMETERS

The date of infection was estimated for each newly infected cow in our dataset and it was used as such for the calculation of the transmission parameter (assuming that BLV infected animals become infectious on a short term).

The overall estimated β was 2.89 year $^{-1}$ and the matching 95 % CI equaled (1.89; 3.74). The estimated βs and respective 95 % CIs by herd are shown in Table 3. When we compare the intervals by herd we see that they extensively overlap, therefore it can be concluded that although BLV prevalence differed between herds infectiousness did not differ much. Residual deviance of all models and the plot of the deviance residuals against fitted values (not shown) indicate adequate model fit.

The periods during which cattle were assumed to be infectious are shown in Table 3 and we estimated the reproductive ratios based on averages of them. R_0 was estimated as 8.88 (95% CI: 5.40; 12.74).

Table 3. Estimated transmission rate and their 95 confidence interval for transmission of BLV and the average estimated infectious period of BLV infected cattle by herd, within 6 dairy herds of Argentina.

Herd	Transmission rate parameter (Year $^{-1}$) (95 % CI)	Average estimated infectious period (years)	R_0
A	nd	nd	nd
B	2.22 (0.22; 4.22)	3.96	8.79 (0.87; 16.72)
C	1.20 (0.0; 2.60)	4.24	5.08 (0.00; 10.99)
D	3.08 (2.41; 3.81)	2.88	9.86 (6.93; 10.97)
E	2.92 (2.34; 3.49)	1.95	4.72 (4.57; 6.83)
F	4.15 (3.50; 4.81)	3.81	15.81 (13.32; 18.29)
G	2.86 (1.99; 3.73)	3.38	9.02 (13.32; 12.63)

DISCUSSION

The purpose of this study was to describe patterns of seroconversion to BLV and to estimate main parameters needed for future model building.

Although infected individuals produce a permanent antibody response we found patterns of alternate positive and negative results as were reported in previous studies (Roberts *et al.*, 1988, 1989). This may reflect the situation where levels are below the detection level of the test. We did not test for presence of BVDV antigen, but in most of the herds where this pattern was present, farmers recognized the presence of BVDV infected animals within their herds. We also found patterns of negative results when infected cows were sampled around parturition as reported before (Bause and Schmidt, 1980; Burridge *et al.,* 1982; Ebertus *et al.,* 1987; Tekes, 1994). Although this situation has important implications for management decisions from single results of a test, it did not interfere with the aims of our study because several tests result from these cows were available.

The figures that represented the changes of sero-prevalence and incidences showed that two main types of disease changes over time were present in our study. One that characterize low level of disease or the start of an outbreak and the other, representing a more advanced phase of the outbreak starting from moderate to heavily infected herds. Incidence results from either heavily infected herds or from herds that started from a situation of moderate infection force in the second half of the follow up period may reflect an oscillatory move to an endemic steady-state level of infection. Because we observed the incidence for a relatively short period of time it is not possible to estimate whether this state persists (i.e. quasi-stationary endemic state (Diekmann and Heesterbeek, 2000) or conversely if it is a transitory state.

When looking at patterns of new infections over time we observed peaks during autumn and spring, which coincide with major calving patterns as well. Previous studies reported peaks in summer that could be related with an increased activity of haematophagous insects (Manet *et al.,* 1989a) but others failed to find any seasonal trend (Thurmond *et al.,* 1983). As a consequence of the way of selecting herds we can not extend our results to the whole population. Therefore, the possibly calving-pattern-related peaks need further investigation into the underlying mechanism. The peripartum could represents a high risk for BLV infection due to:

Depressed BLV immunoglobulin levels in infected cows at parturition (Bause and Schmidt, 1980; Burridge *et al.,* 1982; Tekes, 1994) . This alteration in the immune system and stress associated with parturition and early lactation may increase the susceptibility of uninfected cows (Pollari *et al.,* 1993). In addition, infected cows could become more efficient shedders if loss of immune-mediated suppression caused increased viral expression or higher levels of infected cells in the peripheral blood and discharges at parturition.

More intensive manipulation of cows around calving. Therefore if proper hygienic measures are not taken, the probability of spread is increased.

An increased spread of liquids and materials during the moment of calving and some hours after, that might contain infected lymphocytes thus, facilitating transmission; and

Prevailing management systems in Argentina, heifers and cows are calving together in the same paddock, and usually are sharing the same paddock for at least 2 to 8 weeks.

Under the conditions of this study the prevalences in heifers were higher than reported before (Trono *et al.,* 2001) but due to the herd selection procedure and the sample size it can not be extrapolated to a more general situation and this point remains open for further research. The estimated age at infection in young stock seems not to be related to the prevalence in adult cattle but our estimations were based on a single test moment and therefore these results should be taken cautiously. More extensive investigations of the dynamics of the disease in young stock are also needed to clarify potential associations.

Age at infection in adult cattle varied between herds reflecting either different introduction paths, different routes of transmission or different times since introduction. However, we could not relate a definite cause that could explain these differences.

Our estimations of age at first calving are in agreement with other reports from the country (Vicentini, 1990) but due to the lack of sound information we cannot compare our results of longevity. A report from the Argentine Holstein Breeders Association (ACHA, 1999) mentioned that breeding-registered cows have a productive lifetime of 5 lactating periods.

Although the age at culling was lower in BLV infected cattle in comparison with non-infected cattle this difference was not significant and it is in accordance with previous studies (Heald *et al.,* 1992; Rhodes *et al.,* 2003).

Characteristics of the disease - life-long infectiousness and relatively low mortality rate-combined with a rather long lifetime of the animals plus the relative young age that animals get infected explains the large estimates of the length of the infectious periods. However, another aspect of crucial importance is to assess whether the infectiousness remains constant over time. From our study we couldn't obtain evidence for that and it is an aspect for further research. Moreover, only few studies (Buxton and Schultz, 1984; Kramme *et al.,* 1995) looked into this aspect and using proxy markers of infectivity indicated that although a few days after infection there is an increased infectivity, virus load (defined as amount of

virus present in peripheral blood mononuclear cells) remains relatively constant at least until 3 years post infection.

We don't have any previous reference of the infection rate parameter to compare with but we think that it is a conservative value because data used for calculation was obtained from farms that had an initially moderate to high prevalence when the follow-up started.

We estimated R_0 as 8.8, and no previous estimations for BLV are available for comparison but our estimation is similar to R_0 estimations from another retrovirus (HIV) which ranges between 9-12 (Anderson and May, 1991). Although we choose for a very simple model for estimating R_0, its magnitude shows that a high degree of control is necessary to eradicate bovine leukaemia.

CONCLUSIONS

Under the conditions of this study, natural transmission of BLV was observed and results reveal that the period around parturition is an important risk period and that heavily infected herds seems to have an increased proportion of young stock infected. Also we estimate the infection transmission parameter as relatively high and combined with a long infectious period this resulted in a high reproductive ratio. Therefore, a high effectiveness of the control measures should be achieved to eradicate the disease.

ACKNOWLEDGEMENTS

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CHAPTER 6

TRANSMISSION OF BOVINE LEUKAEMIA VIRUS WITHIN DAIRY HERDS BY SIMULATION MODELLING

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SUMMARY

In Argentina, Bovine Leukemia Virus (BLV) infection is common in dairy herds. The country currently has a National Voluntary Control Program but relatively few farms have enrolled. However, there is an increased interest of authorities and farmers to implement regional compulsory programs but there is scarce quantitative information of the transmission of BLV in cattle herds. This information is a prerequisite to develop effective BLV control strategies. Mathematical modelling offers ways of integrating population level knowledge and epidemiological data to predict the outcomes of intervention scenarios. The purpose of the current paper is to gain understanding about the dynamics of the transmission of BLV in dairy herds from Argentina by simulation and to compare various BLV transmission models to select the one that is most appropriate. The hypothetical herd is conceptually described in terms of BLV status as a population of individuals that are protected by maternal antibodies (M), that are susceptible (S), that are in the latent period (E) or that are infectious (I). BLV is spread by horizontal and vertical transmission. We used an age-structured population model and within-herd transmission was simulated by Monte-Carlo techniques. The next generation approach has been used for the systematic computation of the basic reproduction ratio (R_0). Parameter values for disease transmission were derived from previously published data; rates of entry, exit or transition between age groups were calculated based on our previous study, observational data, expert opinions and literature. With these parameters values the probability of a minor outbreak was estimated to be 10 %, the probability of extinction was estimated as less than 0.001 % and the expected time to extinction as more than 80 years. The probability of a minor outbreak and changes in prevalence were different when the index case was an adult cow compared to introduction by a heifer. Prediction of prevalences from MSI models fit the data satisfactorily. R_0 was estimated as 9.5. The sensitivity analysis on R_0 showed that all measures directed to reduce the transmission rate are potentially effective given operational control measures

An important prediction of these models is that, even in a relatively small, closed dairy herd, the time-scale for a BLV outbreak may be as long as several years and within-herd control of BLV requires intensive efforts.

INTRODUCTION

In Argentina, Bovine Leukemia Virus (BLV) infection is common in dairy herds. In some areas (and especially in the Central Milk Region of Santa Fe Province) the proportion of infected herds is high (approximately 80 %) and the within-herd prevalence ranges approximately from 40 to 50 % (Ghezzi *et al.,* 1997; Trono *et al.,* 2001). Currently, Argentina has a National Voluntary Control Program (SENASA, 1994) but relatively few farms have enrolled. However, there is an increased interest of authorities and farmers to implement regional compulsory programs but there is scarce quantitative information of the transmission of BLV in cattle herds which is needed to develop effective BLV control strategies.

The virus can spread by both natural and iatrogenic vectors that transfer blood (lymphocytes) from infected to non-infected animals (Ferrer, 1979). Vertical transmission of BLV may occur post-natally through milk, colostrum, and by dam-to-calf contact and some calves born to BLV infected dams will already have been infected *in utero* (Piper *et al.,* 1979).

To develop successful interventions and to assign resources effectively, it is essential to understand the dynamics of the transmission and to have quantitative information of factors related to it (De Jong, 1995). Mathematical modelling offers ways of integrating population level knowledge and epidemiological data to predict the outcomes of intervention scenarios. Deterministic models are commonly used for describing epidemics and are suitable when population sizes are relatively large. However, they are less suitable to simulate epidemics in relatively small populations, like within dairy herds, because disease transmission is fundamentally a stochastic process (Diekmann and Heesterbeek, 2000). For small population sizes differences between individuals and random effects are important and stochastic models are needed to describe such situations.

One of the fundamental questions of mathematical modelling is to find threshold conditions that determine whether or not an infectious disease will spread in a susceptible population when the disease is introduced into it. The threshold conditions are characterised by the so called Reproduction Ratio (R_0) (Anderson and May, 1991) a dimensionless parameter which encapsulates the biological details of different transmission mechanisms, such that if $R_0 < 1$, the modelled infection goes extinct, and if $R_0 > 1$, the infection may spread in the population. Due to the stochastic nature of the infection process there will be some probability that minor outbreaks will take place and infection will go extinct by chance. In our previous study (Chapter 5), using data obtained from an observational study we estimated the value of R_0 as 8.8.

The purpose of the current paper is to gain understanding about the dynamics of the transmission of BLV in dairy herds from Argentina by simulation and to compare various BLV transmission models to select the one that is most appropriate. Such a model can serve as a tool to evaluate the effect of several control strategies on the dynamics of transmission.

MATERIAL AND METHODS

INITIAL MODEL OVERVIEW AND DESCRIPTION OF INFECTION PROCESS

The hypothetical herd is conceptually described in terms of BLV status as a population of individuals that are protected by maternal antibodies (M), that are susceptible (S), that are in the latent period (E) or that are infectious (I). BLV is spread by contact between

infected and susceptible individuals (horizontal transmission) and by infected dams giving birth to infected newborn calves (vertical transmission). Both types of transmission depend on the size of the susceptible subpopulation and transmission parameter β. The transmission parameter represents the probability per unit of time that a contact between an infectious individual with a susceptible one will result in the infection of the latter. In addition, once an animal becomes infected it remains infected for life and it will always be a potential source of infection (Miller and Van der Maaten, 1990).

POPULATION STRUCTURE

We used an age-structured population model because disease can be transmitted either horizontally or vertically and infected animals remain a source of infection for the rest of their lives. Therefore, it is important to account for demographic changes in the population, for example, the rate of culling and the frequency of calving. In dairy herds, animals are usually kept in several age categories to optimise and facilitate management. Therefore, the population is heterogeneous and different contact patterns could be associated with different age groups.

In a standard management practice, representing a 'typical' farm of the area, the age classes can be defined as:

Group 1 (calves): includes all females from birth until 180 days of life.

Group 2 (heifers): includes all females from 181 days till they are introduced into the adult category (2 months before the expected date of calving).

Group 3 (adults): includes all pregnant heifers within 2 months of calving and all cows (dry, maternity and lactating).

It is assumed that groups are kept separately and no mixing occurs between groups while homogeneous random mixing occurs within groups. It is also assumed that the herd is closed and new animals enter by birth only, animals leave the herd due to sale or death. The hypothetical herd consisted only of females because in dairy enterprises male calves are either sold after birth or if reared they are moved to another production unit independent from the females.

MODEL SELECTION

A scheme of the initial model is presented in Figure 1.

Figure 1. A three-age-class model, showing the routes within compartments. The external bound represents the limits of the farm.
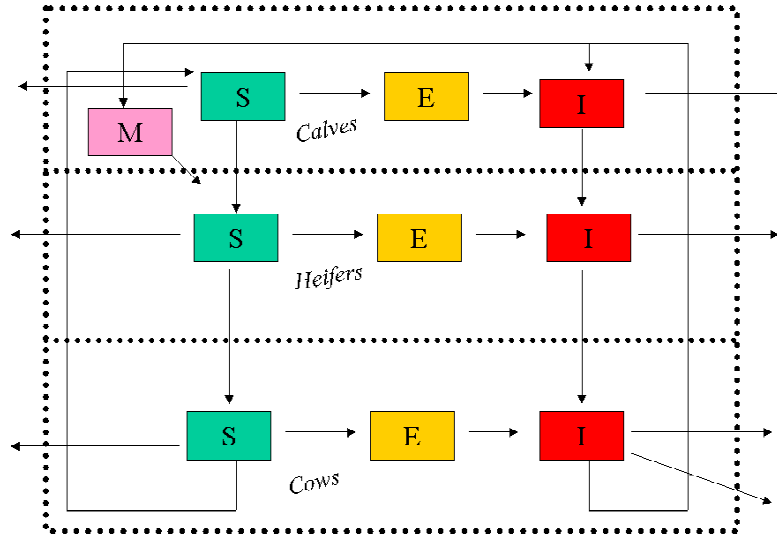
M= calves with maternal antibodies.
S= susceptibles
E= animals in latent period.
I= infectious animals.

The resulting model (MSEI) was used as reference and then compared with simpler models (that did not include some of the assumptions) to evaluate efficiency. Within-herd transmission was simulated by Monte Carlo techniques and Table 1 describes all events and their probabilities that were considered. We choose the smallest time scale (12 hours) (which is related to transmission of disease) because in our model we include two biological processes (disease transmission and demographic changes) that have very different time dynamics. The chance of a simultaneous jump of 2 events (neither related to disease transmission nor demographic) was suficiently low to be neglected (1e-6) and we simulated a total time-horizon of 30 years.

The first step was to evaluate two aspects of the disease dynamics to decide whether or not they should be included in the model:

- the time-delay induced by the latent period (i.e. the period from where the animal get infected until it became infectious, status E);
- the temporal immunity acquired by calves born from infected cows through passive transfer of antibodies.

When a virus is introduced in a naïve population there will be few infective animals for a certain period, hence demographic stochasticity could lead to extinction of the infective agent. The probability of such extinction is defined as the probability of a minor outbreak (p_{minor}).

Table 1. Description of possible events occurring in the MSEI stochastic model.

Event	Transition	Transition rate	Probability
Susceptible to Latent	$(S_i, E_i) \longrightarrow (S_i - 1, E_i + 1)$	$V_3(S_i, E_i) = \beta * S_i * I_I / (S_i + I_I)$	$\beta * S_i * I_I / (S_i + I_I) * \Delta t$
Latent to Infectious	$(E_i, I_i) \longrightarrow (E_i - 1, I_i + 1)$	$V_4(E_i, I_i) = \varepsilon * E_i$	$\varepsilon * E_i * \Delta t$
Birth of Susceptible calf from Susceptible dam	$(S_i, I_i) \longrightarrow (S_i + 1, I_i)$	$\lambda_1(S_i, I_i) = b * S_i$	$b * S_I * \Delta t$
Birth of Susceptible calf from Infectious dam	$(S_i, I_i) \longrightarrow (S_I + 1, I_i)$	$\lambda_2(S_i, I_i) = b * p * I_i$	$b * p * I_I * \Delta t$
Birth of calf Protected by Maternal Antibodies from Infectious dam	$(M_i, I_i) \longrightarrow (M_i + 1, I_i)$	$\lambda_2(M_i, I_i) = b * p * I_i$	$b * p * I_I * \Delta t$
Bi7rth of Infectious calf from Infectious dam	$(S_i, I_i) \longrightarrow (S_I, I_i + 1)$	$\lambda_3(S_i, I_i) = b * q * I_i$	$b * q * I_I * \Delta t$
Death of S individual not related to BLV	$(S_i, I_i) \longrightarrow (S_I - 1, I_i)$	$\mu_1(S_i, I_i) = \mu_1 * S_i$	$\mu_1 * S_I * \Delta t$
Death of I individual not related to BLV	$(S_i, I_i) \longrightarrow (S_I, I_I - 1)$	$\mu_2(S_i, I_i) = \mu_2 * I_i$	$\mu_2 * I_I * \Delta t$
Death of M individual not related to BLV	$(M_i, I_i) \longrightarrow (M_i - 1, I_i)$	$\mu_3(M_i, I_i) = \mu_3 * M_i$	$\mu_3 * M_I * \Delta t$
Death of I individual related to BLV	$(S_i, I_i) \longrightarrow (S_I, I_I - 1)$	$\mu_4(S_i, I_i) = \mu_4 * I_i$	$\mu_4 * I_I * \Delta t$
Transfer of M to Susceptible Age group 2	$(M_i, S_{i+1}) \longrightarrow (M_i - 1, S_{I+1} + 1)$	$\Psi_{MS}(M_i, S_{I+1}) = Ti * M_i$	$Ti * M_I * \Delta t$
Transfer of S to other Age Class	$(S_i, S_{I+1}) \longrightarrow (S_I - 1, S_{I+1} + 1)$	$\Psi_{IS}(S_i, S_{I+1}) = Li * S_i$	$Li * S_I * \Delta t$
Transfer I to other Age Class	$(I_i, I_{i+1}) \longrightarrow (I_I - 1, I_{I+1} + 1)$	$\Psi_{II}(I_i, I_{I+1}) = Li * I_i$	$Li * I_I * \Delta t$

However, the virus still can go extinct after a first outbreak when a combination of chance events will drive the agent to fade-out and this was defined as the probability of extinction. In stochastic models in which the population of infected individuals cannot grow unlimited, the endemic state can only be quasi-stationary, which means that it could persist for a long time (Diekmann and Heesterbeek, 2000). The expected time till extinction is an indicator that denotes over which time scale the quasi-stationary state is a reasonable description. For assessing p_{minor}, probability of extinction and expected time till extinction for BLV, we used a Monte Carlo simulation. We examined the effects of the various disease models (MSEI, MSI, SI, SEI), and herd size (100, 200 and 400 animals) on these indicators by running 1000 iterations and using a time horizon of 80 years.

VALIDATION OF THE MODEL

The validation of the model was performed by running the model several times (1000 simulations) but using demographic parameters and starting conditions of some of the herds that participated in a longitudinal study (Chapter 5). Afterwards, for each herd, from each serial of runs (n= 1,000) we obtained a prevalence prediction with the respective 95% CI, from prevalence at day 0. Finally, we compared predicted values with the observed field prevalence using a goodness-of-fit test.

CALCULATION OF REPRODUCTIVE NUMBER

R_0 is usually defined as the average number of secondary cases produced by a "typical" infected (assumed infectious) individual throughout its infectious period when the disease is first introduced into a population consisting solely of susceptible individuals. This non-dimensional quantity cannot be computed explicitly in many cases because the mathematical description of what is a "typical" infectious individual is difficult to quantify in populations with high degree of heterogeneity (Castillo-Chavez *et al.,* 2002). The next generation approach (Diekmann and Heesterbeek, 2000) can be used for the systematic computation of R_0.

In our study, the host population consists of various types of individuals (different age groups and infected via horizontal or vertical transmission routes). For the calculation of R_0 and for seeking simplicity, instead of considering all 3 age groups, we considered group 1 and 2 as one group (replacements or young stock).

A key element is that we regard only generations of infected individuals that at the moment of being infected are distributed over all the possible age groups. Then, a linear positive operator that will supply the next generation of infected animals conditional to the present generation can be build-up (Diekmann *et al.,* 1990). This operator (K) is obtained by multiplication of transition matrix G (which represents the demographic dynamics) by infectivity matrix I (which represents the transmission of the disease). K is defined as k_{ij}, to be the expected number of new cases that have h-state i (host-state i) at the moment they become infected, caused by one individual that was itself infected while having h-stage j, during the entire period of infectiousness. In our case it can be represented by:

$$K = \begin{pmatrix} \dfrac{\beta_2 S_a}{(\phi + \mu_2) N_a} & \dfrac{S_a \lambda \beta_2}{(\lambda + \mu_1) N_a (\mu_2 + \phi)} \\[2em] \dfrac{\alpha q}{\phi + \mu_2} & \dfrac{\alpha \lambda q}{\lambda + \mu_1} + \dfrac{S_r \beta_1}{(\mu_1 + \lambda) N_r} \end{pmatrix}$$

Where,
S_j = Susceptible (j = a for adults and j = r for replacements)
β_i = transmission parameter (i = 1 for adults and i = 2 for replacements)
q = probability of born infected
α = probability of a parturition resulting in a female calf to be born alive
λ = replacement-to-adult transfer rate
φ = per capita BLV-induced death rate
μ_j = mortality rate (not specific to BLV) (j = 1 for replacements and j = 2 for adults)

N_j = total number of animals in a given age-group (j = a for adults and j = r for replacements)

R_0 is found through computation of the dominant eigenvalue of the next-generation matrix

at the disease or infectious-free equilibrium.
Consequently,

$$R_0 = \frac{1}{2}B + \frac{1}{2}\sqrt{B^2 + 4A}$$

with,

$$B = \frac{\alpha \lambda q}{\lambda + \mu_1} + \frac{S_r \beta_1}{(\mu_1 + \lambda)N_r}$$

$$A = \frac{\alpha q}{\phi + \mu_2} \frac{S_a \lambda \beta_2}{(\lambda + \mu_1)N_a(\mu_2 + \phi)}$$

To explore the relative impact of single factors on R_0 a sensitivity analysis was performed by changing values for single parameters (keeping the rest unchanged) and assessing the percentage of change in R_0.

Calculations were performed using Mathematica ®.

PARAMETERS VALUES

Parameters values for disease transmission were derived from previously published data (Chapter 5) (Table 2) and were assumed to be constant over time and equal for the 3 age-groups.

Mortality related to BLV infection was only considered for the adult-age group because the time needed to develop tumours is longer than the rearing period from calf to adult (Ristau *et al.*, 1987).

Rates of entry, exit or transition between age groups were calculated based on our previous study, observational data, expert opinions and literature (Table 2).

Table 2. Parameter values used in the simulations.

Parameter	Categories	Default values	References
Related to transmission and disease			
Transmission parameter (β)		2.8	Chapter 5
Probability of being born infected (q)		0.05	(Piper *et al.,* 1979)
Per capita BLV-induced mortality rate (φ)		0.05	(Miller and Van der Maaten, 1990)
Latent period		7 days	(Miller and Van der Maaten, 1990)
Maternal antibodies protection period		6 months	
Related to population demographics			
Population size (N)		100, 200, 400	
Mortality rate(μ)	*Adults*	0.34	Experts opinion
	Heifers	0.08	
	Calves	0.10	
Probability of birth alive a female calve (α)		0.39	Experts opinion
Replacement-to-adult transfer rate (λ)		0.39	
Surviving and growing (from stage *j* to *j* + 1) rate (G$_j$)	*Adults*	0.10	
	Heifers	0.39	
	Calves	1.85	

Furthermore, all rates were assumed to be constant over the observation period. Therefore, the probability of growing to the next age-group depends on the time spent in the current age-group but independent of the time spent in the previous group. The problem is to obtain information on the probabilities P$_j$ (probability of growing) and G$_j$ (probability of survival) from information on stage duration.

To do this, we separate the processes of survival and growth, both of which appear in P$_j$ and G$_j$, (Caswell, 2001) by defining:

σj = survival probability (1-mortality rate for the age-group)
Ti = residence time (average time that an animal will stay in each age group)
ν = 1 (for represent that age distribution within an age group is stable) and
λj = probability of growing from *j* to *j + 1* given survival) and it was estimated as:

$$\lambda_j = \frac{\left(\dfrac{\sigma_i}{\upsilon}\right)^{t_i} - \left(\dfrac{\sigma_i}{\upsilon}\right)^{t_{i-1}}}{\left(\dfrac{\sigma_i}{\upsilon}\right)^{t_i} - 1}$$

In terms of these parameters (Caswell, 2001),

$P_j = \sigma_j (1 - \lambda_j)$ probability that an individual in age class j will survive from t to $t + 1$ and staying in stage j

$G_j = \sigma_j \lambda_j$. Probability of surviving and growing from stage j to $j + 1$.

The entry rate of newborns was calculated as offspring per cow per time unit and it was expressed in terms of female offspring per cow per year. It was estimated based on the standardised (calving interval/ 365) calving rate per year common in the area, adjusted by the number of stillbirth, calves dead at birth and newborns that die in the first hours after calving (which is estimated as 7 %).

For assessing the impact of the different parameters of R_0 we performed a sensitivity analysis by doubling or halving parameters values one by one.

RESULTS

DEFAULT MODEL AND PARAMETERS SETTINGS

First, we evaluated the MSEI model using an average dairy population (n=200) in which initially one infectious individual is introduced in the adult group while the rest of the herd is susceptible. Monte Carlo simulations showed that p_{minor} was approximately 10 % and the median time to fade-out for minor outbreaks was 64 days with an inter-quartile range of 58 days. The highest prevalence reached for a minor outbreak before going extinct was 3 %.

Figure 2a gives the probability distribution of the prevalence after one year, post introduction and shows a prevalence peak between 0 and 5 %. After the second year (Figure 2b) the most frequent prevalence was around 30 % and after 5 years (Figure 2c) between 85 to 95 %. At year 30 (Figure 2d), the prevalence distribution indicates that a quasi-stationary distribution has been reached as it did not change significantly onwards. Additionally, we extended the simulation for another 50 years, starting from the 'quasi-stationary distribution' and no extinction occurred. Because for practical applications the results were clear, simulations were not further extended for a more precise estimation. If the probability of extinction would have been 1/1000 1 iteration in which BLV went extinct should have been found with 95% certainty. Because this did not occur the probability of extinction is smaller than 0.001 % when starting from the quasi-stationary distribution. Following the same reasoning, the approximation of the expected time to extinction is longer than 80 years.

Figure 2. Probability distribution of prevalence after introduction of an infectious individual in a totally susceptible population of 200 animals after 1, 2, 5 and 30 years.
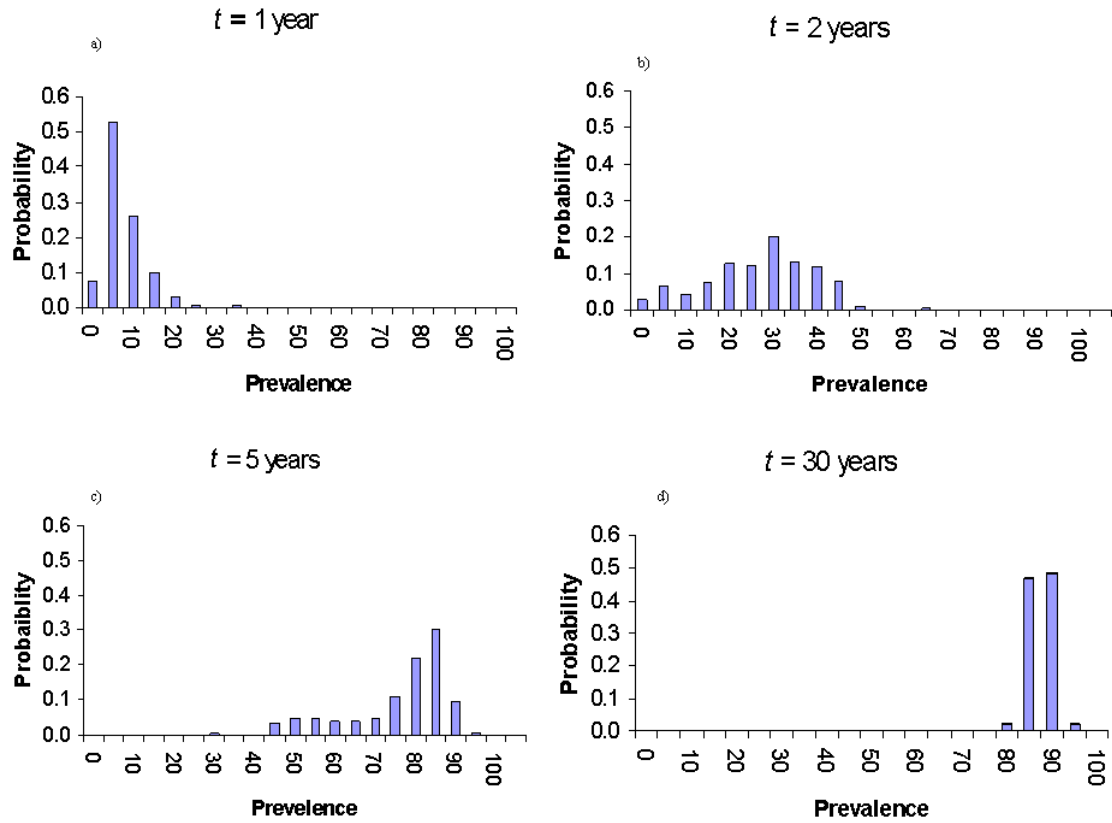
EFFECT OF MODEL TYPE, HERD SIZE AND INITIALLY INFECTED GROUP ON P_{MINOR}

Table 3 shows the estimations of p_{minor} and the extinction times after introduction of BLV for the various models. When protection by maternal antibodies-state was included in the model, the probability of a minor outbreak was somewhat increased but differences between models were not statistically significant ($p > 0.05$).

However, when considering the impact of population size on the time till extinction after introduction of BLV, using the MSEI model, the outcomes show that the p_{minor} are quite similar but there are some statistically significant differences between herd sizes 100 and 400 (Table 4).

Table 3. Probability of minor outbreaks (p_{minor}) and median time till extinction for 4 infection models (range and interquartile range (IQR) are also presented).

Model	P_{minor} (%)	Median time till extinction (days) *	IQR (days)	Range (days)
SI	9.0	66.5	27	29-131
SEI	7.5	50	84.5	13-168
MSI	12.0	89	103.5	2-361
MSEI	9.5	63.5	58	17-334

*Kruskal-Wallis chi-square test on extinction times (two sided) = 0.4779, df = 3, p-value = 0.93

Finally, p_{minor} and time till extinction were estimated when the introduction of an infectious individual is in the group of heifers. Simulation showed a p_{minor} of approximately 1 % and it went extinct in 65 days.

Table 4. Probability of minor outbreaks (p_{minor}) and median time till extinction for 4 herd sizes using a MSEI model (range and interquartile range (IQR) are also presented).

Herd Size	P_{minor} (%)	Median time till extinction (days) *	IQR (days)	Range (days)
100	8.0**	53	71	5-220
200	11.4	143	179	1-566
400	12.4**	74	411	1-452
800	8.9	48	124	1-786

*Kruskal-Wallis chi-square test on extinction times (two sided) = 9.4049, df = 3, p-value = 0.024
** Chi-square test on difference between proportions (reference row is based on size 200).

TRANSMISSION DYNAMICS OF MAJOR OUTBREAKS

As for the probability of minor outbreaks, the progress of larger outbreaks also depends on the group where the virus was introduced first. Figure 3 shows results of Monte Carlo simulations after an index case is introduced in age group 3 (adults).

Figure 3. Distribution of prevalence for age-groups 2 and 3, obtained by Monte Carlo simulations, after 1, 3 and 5 years posterior to introduction of a BLV infectious animal in the adult age group.
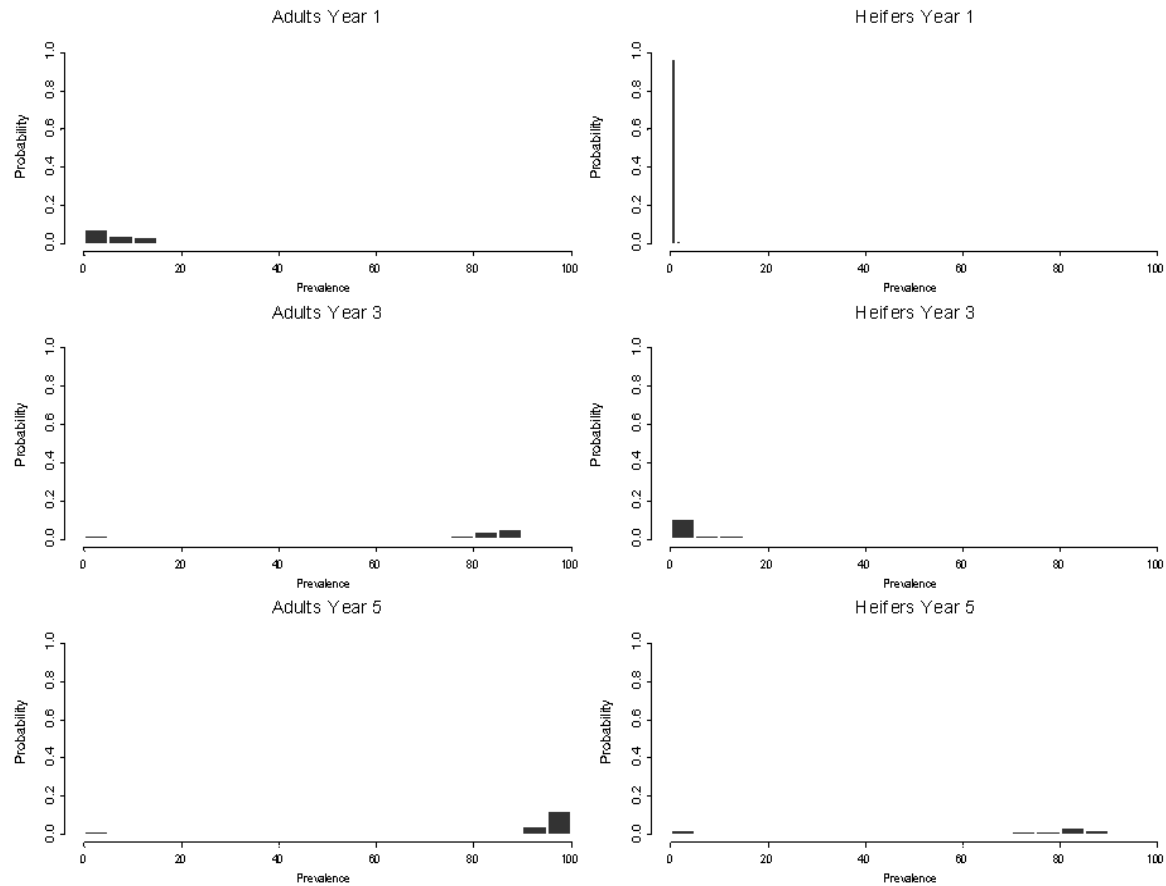
At the end of the first year most realisations showed prevalences under 20 %. As expected (in the model no cross-infection from adult stock to young stock was assumed) within young stock the prevalence is very low (it started by vertical transmission and then some horizontal transmission may have occurred). After the third year, the prevalence within the adult group increases dramatically (most runs were over 65 %). Prevalence, in the replacements group also increased but moderately (less than 20 %). Finally, after 5 years, almost all cows and a great proportion of heifers are infected.

However, if an index case is introduced in age group 2 (replacements or heifers) (Figure 4), the prevalence within adults is lower (less than 15 %) and higher in the replacement group at a one year time scale.

After the third year, the prevalence in the adult group increased dramatically and it looks quite similar to the distribution in Figure 3. The prevalence within young stock increased dramatically and it is higher than in the previous scenario. Finally, after 5 years, in both age groups almost all cattle seem to be infected. Thus, the overall impact is larger when the virus is introduced in young stock.

Figure 4. Distribution of age-group prevalence, obtained by Monte Carlo simulations, after 1, 3 and 5 years posterior to introduction of a BLV infectious animal in the heifers age group.
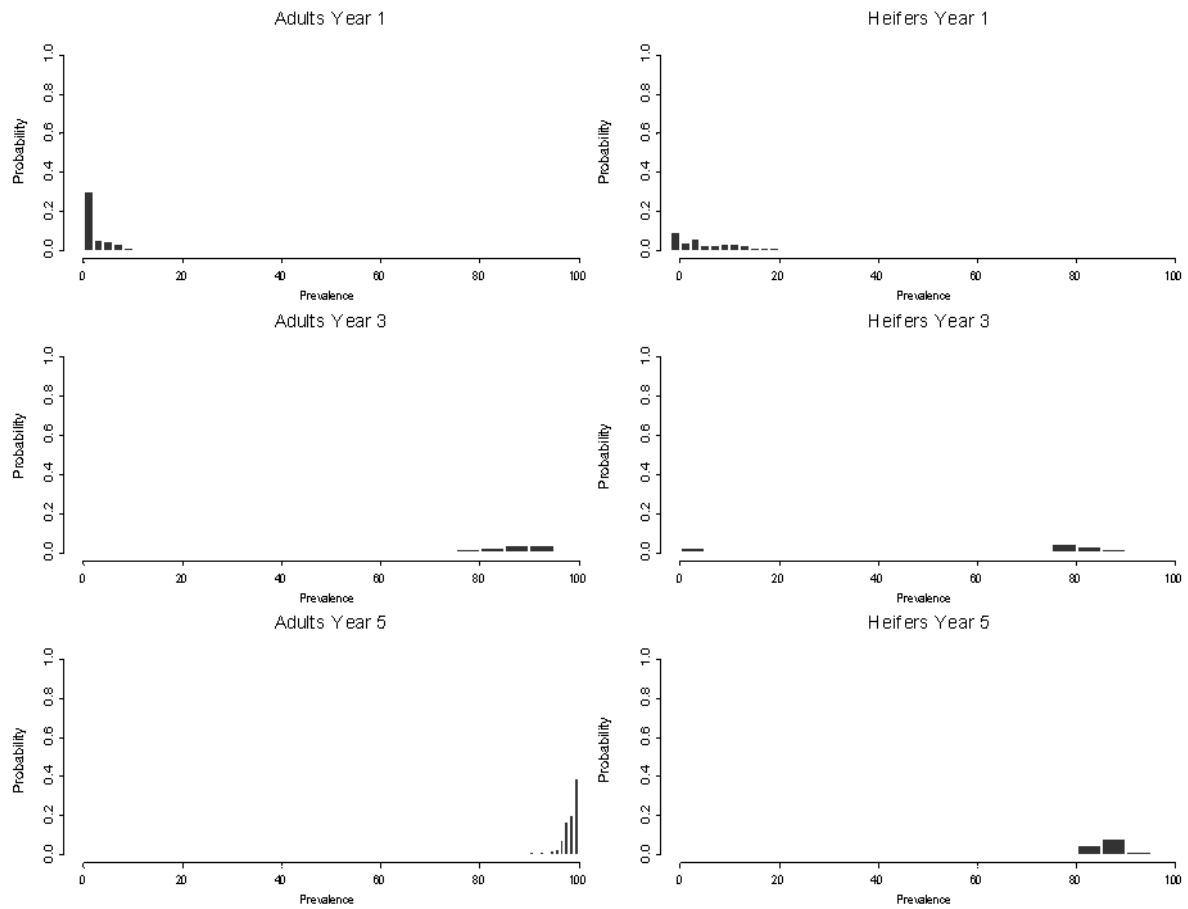
REPRODUCTION RATIO R_0

The resulting matrix that corresponds to the ' next generation operator' can be represented as:

From to	Adult	Young Stock
Adult	9.33	7.43
Young Stock (Group 1 + 2)	0.07	5.77

In other words, under the assumptions of this approach, it was estimated that on a per generation basis, one infected adult will infect 9 other adults and will produce far less than one infected calf during its infectious reproductive period. In addition, an infected young stock will infect about 6 other young stock during its rearing period and another 7 to 8 during its reproductive period. The R_0 was estimated as 9.5.

Table 5 summarises how R_0 changes when we vary parameter settings used for R_0 estimation. From this table we infer that the most influential parameters are: the coefficient of transmission, the removal rate of adults, the mortality rate due to BLV and in a lesser extent the replacement rate from heifers to adults (which reflects the age at first calving of the heifers). For the parameter values used and assumptions stated, elimination is more difficult to achieve by the removal of infected cases.

Table 5. Sensitivity analysis on parameters used to calculate R_0.

Parameter	variation	Value	R_0	% of change
Coefficient of Transmission (β)	default	2.8	9.5	
	doubled	5.6	18.8	+ 97.9 %
	halved	1.4	4.8	- 49.5 %
Proportion of calves infected from mothers (q)	default	5 %	9.5	
	doubled	10 %	9.6	+ 1.1 %
	halved	2.5 %	9.4	- 0.32 %
Removal of Adult (γ)	default	25 %	9.5	
	doubled	50 %	5.9	- 37.9 %
	halved	12.5 %	16.1	+ 69.5 %
Heifers transition to adult age (ó)	default	39 %	9.5	
	doubled	78 %	9.5	- 0.4 %
	halved	18.5 %	10.3	+ 8.4 %
Maternity Function (b)	default	39 %	9.5	
	doubled	78 %	9.6	+ 1.8 %
	halved	18.5 %	9.4	- 1.1 %
Mortality rate due to BLV (α)	default	5 %	9.5	
	doubled	10 %	8.1	- 14.7 %
	halved	2.5 %	10.3	+ 8.4 %
Mortality rate not related to BLV (replacements) (μ)	default	10 %	9.5	
	doubled	20 %	9.4	- 1.1 %
	halved	5 %	9.5	+ 0.4 %

Of the options for within-herd transmission of BLV considered here, some reduction may be achieved by the slaughter of calves born to infected cows (vertical transmission).

MODEL FIT

Figure 5 shows several plots of the model predictions with the observed field data obtained in other studies. Most field observations are within the limits of the 95 % CI of the predictions, indicating that simulations mimicked field variations reasonably well.

Figure 5. Validation of model estimations with field data from 5 herds.
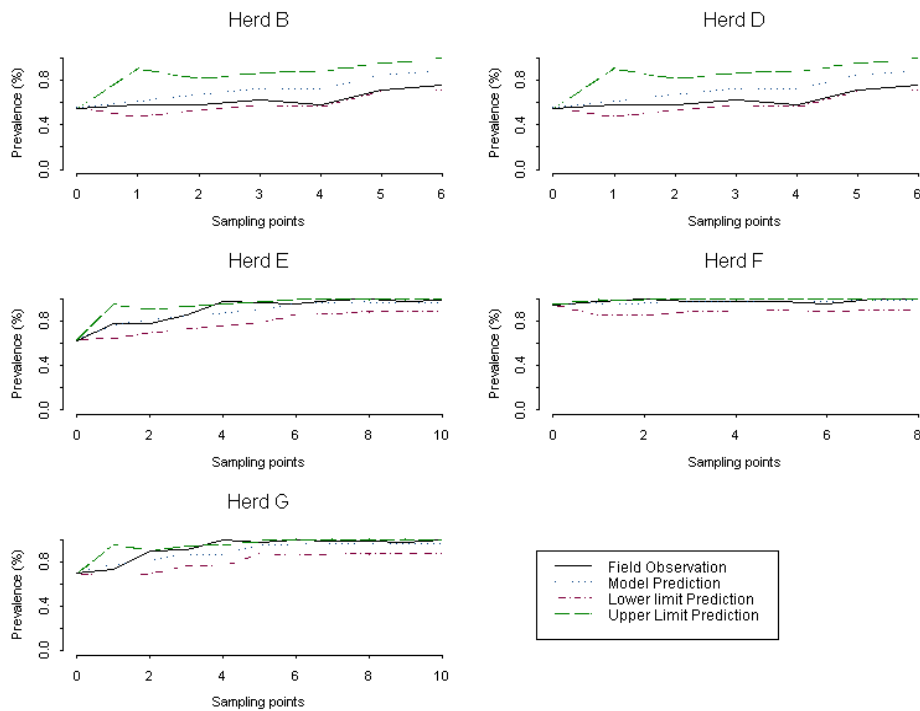
DISCUSSION

In this study we assessed, by Monte Carlo simulation, the transmission model that fits best to the course of a BLV infection in Argentine dairy farms and estimated the probability and time till extinction of BLV. Regarding the latter, the effect of herd size and in age group in which the index case was present were explored further.

After evaluating the fit of the models it shows that both the MSEI and the MSI model predict satisfactorily disease transmission as obtained under field conditions. Because inclusion of the latent period did not improve the fit further the MSI model was selected for evaluation of the impact of control measures on transmission. Unfortunately, there are no other modelling studies based on BLV to compare our results with.

Our results indicate that once an infectious individual is present there is a high probability that the disease persists in the herd. However, if it goes extinct, then in most cases it will go extinct relatively fast.

The probability that the disease can fadeout after extensive spreading is very low which can be interpreted as one should not expect getting rid of the disease without any control measure. In addition, such a high probability of persistence implies that great effort should be put on prevention of introduction e.g. by testing all purchased animals and keeping them in quarantine until definite test results are available. In addition, the high persistence of the infection after introduction of BLV is not only important for the single herd but also increases the probability of between-herd transmission when no proper preventive measures are taken. Results indicate that introduction of infection in the heifers group augment - compared to introduction in adult cow - the chances of persistence. Although these concepts were not evaluated quantitatively before, it has been qualitatively pointed out in previous studies (Gottschau *et al.,* 1990; Johnson and Kaneene, 1991).

Simulations started with one infectious animal but no particular transmission path that has lead to that primary infection was considered, therefore we can not assign the physical introduction to an infected animal. In closed herds, the virus can be introduced by haematophagus insects or by use of blood-contaminated needles or other instruments (Esteban *et al.,* 1988; Rogers *et al.,* 1988). Such a risk arises from e.g. the way the Foot-and-Mouth-Disease vaccination scheme is implemented or when practitioners don't take proper care of their instruments or via blood-sucking insects (Buxton *et al.,* 1985).

We estimated R_0 as 9.5 and no previous estimations for BLV are available for comparison. Our estimation is similar to R_0 estimations for another retrovirus infection (HIV) which ranges between 9-12 (Anderson and May, 1991).

The sensitivity analysis showed that all measures directed to reduce the transmission rate are potentially effective given operational control measures. Previous studies pointed out different ways to decrease transmission such as, reducing the use of mechanical vectors contaminated with blood (DiGiacomo *et al.,* 1987; Evermann *et al.,* 1986; Hopkins and DiGiacomo, 1997), insect control (Buxton *et al.,* 1985) or physical contact between infected and susceptible individual (Brunner *et al.,* 1997). In addition, longevity of adult cattle is desirable for production goals, but has a negative impact on BLV persistence as it increases the length of the infectious period. The natural mortality rate also seems to have an important influence on the length of the infectious period.

Our analysis suggests that reduction of vertical transmission will be relatively ineffective since most cases arise through horizontal transmission, as has been suggested before (Hopkins and DiGiacomo, 1997).

CONCLUSIONS

An important prediction of these models is that, even in a relatively small, closed dairy herd, the time-scale for a BLV outbreak may be over 80 years.

One objective of this analysis was to illustrate aspects of BLV epidemiology and control for biologically plausible sets of assumptions and parameter values. The detailed results are obviously sensitive to these assumptions and parameter values. However, the key conclusions appear to be robust: the time-scale of BLV outbreaks within an Argentine dairy herd may be very long and become endemic, and within-herd control of BLV requires intensive efforts.

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CHAPTER 7

IMPACT OF DIFFERENT CONTROL STRATEGIES ON BOVINE LEUKAEMIA VIRUS TRANSMISSION WITHIN DAIRY HERDS

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SUMMARY

Bovine Leukemia Virus (BLV) is a retrovirus that belongs to the *Retroviridae* family and causes lymphomas and other disorders in cattle. In herds infected with BLV economic losses occur due to death, carcass condemnation at slaughter, veterinary services, but also by limitation of trade (export) of animals for life. In Argentina, BLV infection is common, mainly in dairy herds and the country has a National Voluntary Control Program but relatively few herds have enrolled. The purposes of the current paper are: a- to assess which are the most important measures that a successful program should include, b- to explore the potential effectiveness of different BLV control strategies, and c-to determine the time required for achieving eradication. Estimations were based on an age-structured stochastic mathematical model of the dynamics of BLV infection within a dairy herd. Several control measures: corrective management, removal of infected animals (and aspects related like frequency of removal, accuracy in removal, testing (sub)population), herd size and control strategies (corrective management alone or combined with test and removal of positive reactors, removal of positive reactors alone) were evaluated as well as specific control strategies for different herd sizes. The effect of corrective management was analysed by assuming that it reduces the baseline transmission parameters. Results were evaluated in terms of efficacy, which is defined as probability of eradication and which is obtained as the percentage of simulations that ended with eradication of BLV within the time limit of 40 years and efficiency, which is defined as mean time needed to achieve eradication. All the control measures that were considered had a substantial impact on efficacy and efficiency. There is also a substantial impact on efficiency by reducing the transmission baseline parameter values and the proportion of animals included in the testing scheme. Important differences in model outcomes were found when comparing low (n=100) or average (n=200) with large herd size (n=800). Although adopting corrective management measures only produced a significant decrease in prevalence over the course of the outbreak, it is neither effective nor efficient. The combination of removal of infected animals and corrective management is the favoured strategy. For small and average herds the optimal strategy is testing twice a year animals older than 6 months, followed by removal of positive reactors and adopting concurrently, corrective management measures that ensure an effective reduction of transmission of at least 25%. For large herds, the farmer should adopt a more stringent control program. The conclusions are that, depending on the right choice of control strategy, the time-scale of BLV eradication within a dairy herd may be very long when infection does become endemic, and within-herd eradication of BLV will, especially for large herds, require intensive effort and only be effective over a time-scale of several years. The model supports a policy that considered the use of combined control measures to control the spread of the disease.

INTRODUCTION

Bovine Leukemia Virus (BLV) is a retrovirus that belongs to the *Retroviridae* family (Coffin *et al.,* 1997) and causes lymphomas and other disorders in cattle (Miller and Van der Maaten, 1990). The virus can spread by both natural and iatrogenic vectors that transfer blood (lymphocytes), mainly post-natally and horizontally (Ferrer, 1979). Vertical transmission of BLV may occur post-natally through milk, colostrum, and by dam-to-calf contact and some calves born to BLV infected dams will already have been infected *in utero* (Piper *et al.,* 1979).

In herds infected with BLV economic losses occur due to death, carcass condemnation at slaughter, veterinary services, but also limitation of trade (export) of animals for life (Johnson and Kaneene, 1991; Pelzer, 1997).

In Argentina, BLV infection is common, mainly in dairy herds. In some areas (and especially in the Central Milk Region of Santa Fe Province) the proportion of infected herds is high (approximately 80%) and the within-herd prevalence ranges from 40 to 50% (Ghezzi *et al.,* 1997; Trono *et al.,* 2001). Currently, Argentina has a National Voluntary Control Program (SENASA, 1994) but relatively few herds are participating. However, there is an increased interest among authorities and farmers in implementation of regional compulsory programs. It is imperative to develop control strategies on a herd basis because of the economic implications of BLV infection, the absence of treatment or effective vaccination, combined with a high prevalence.

Several countries have eradicated the disease using a policy of test and cull of positive reactors (Knapen *et al.,* 1993; Schmidt, 1987). However, this approach meets resistance by most farmers in Argentina due to the high within herd prevalence and the currently poor economic climate Argentine dairy farms have to operate in. However, there are other approaches that can be practical, realistic and appealing to livestock producers and that have been reported also as successful, such as test and segregation of positive reactors and/or combined with additional corrective management measures (Brenner *et al.,* 1988; Ruppanner *et al.,* 1983).

Because transmission is a function of biological and environmental factors, control programs may be successful in one region/country but not in others. Therefore, a control program must be based on the general knowledge of BLV transmission in addition with locally acquired quantitative information of conditions affecting the transmission of BLV. All available information can be combined in mathematical models and the progress (or regress) of the disease can be evaluated in both time and space. Mathematical modelling has been used to design and evaluate surveillance and control strategies for various diseases (Buijtels *et al.,* 1997; Klinkenberg *et al.,* 2003; Morris *et al.,* 2001), especially when formal field experimentation is impossible for logistical or ethical reasons. Therefore, mathematical modelling is a suitable tool to determine which strategy is best for BLV eradication by comparing different alternatives keeping other conditions similar.

We developed an age-structured stochastic mathematical model of the dynamics of BLV infection within a dairy herd (Chapter 6). The purposes of the current paper are:

I. to assess which are the most important measures that a successful program should

   include,
II. to explore the potential effectiveness of different BLV control strategies, and
III. to determine the time required for achieving eradication.

MATERIAL AND METHODS

MODEL FOR SIMULATION OF TRANSMISSION WITHIN HERD

The full description of the transmission model can be found elsewhere (Chapter 6) and only a brief summary is provided here. The model can be described as stochastic and age-structured. The hypothetical herd was conceptually described in terms of BLV status as a population of individuals that were either protected by maternal antibodies (M), susceptible (S), or infectious (I). BLV was propagated through contact between infected and susceptible individuals (horizontal transmission) and by infected parents giving birth to infected newborn individuals (vertical transmission). Both types of transmission depended on the size of the susceptible subpopulation and the transmission parameter β. In addition, once an animal became infected it remained infectious for life (Miller and Van der Maaten, 1990).

In dairy farms populations usually are broken down into several age categories to optimize and facilitate management; we mimicked this by dividing the population in three age classes, which were defined as:

Group 1 (calves): includes all females from birth until 180 days of life.

Group 2 (heifers): includes all females from 181 days until pregnant heifers are introduced to adult category (2 months before the expected day to calving).

Group 3 (adults): includes all pregnant heifers previous to calving and all cows (dry, maternity and lactating).

It is assumed that groups are kept separately and no mixing occurs between groups but mixing within groups is at random. Also we assume that transfer of category occurs at a fixed age and once transferred animals will not return to the previous category.

We considered a closed herd in which new animals were born on the farm while animals could leave the herd due to culling, sale or death. In addition, we assumed that the herd consists of females only because in dairy enterprises males are either sold after birth or if reared they are moved to a production unit independent of the females.

POTENTIAL CONTROL MEASURES

The potential impact of control measures was explored:

1. How important is it to avoid exposure to virus or to increase hygiene?

There is evidence that several management procedures and mechanical vectors affect effective transmission of BLV but quantitative information of the reduction in the baseline transmission rate by controlling these factors is lacking. Therefore, we adopted a generic approach by assuming that the farmer applied corrective management measures that altered the subsequent rate of infection, thus reducing the baseline value of the transmission rate with either 25, 50, 90 or 99 %.

2. Which age group of the herd should be tested?

It is often recommended that testing schemes should be applied to animals older than 6 months, because available serological tests can not distinguish between maternal antibodies and antibodies produced after infection. At the age of six months passively acquired antibodies have been eliminated (Thurmond et al., 1982). However, there are other tests available (such as PCR (Kuzmak et al., 1999), immunoblotting (Choi, 2002) or ELISA for detecting antibodies against the core protein of the virus (p24)(Kittelberger et al., 1999)) that are able to detect infected calves. Despite the fact that these tests are not yet

commercially available and are more expensive it was considered as a potential alternative. Therefore, in our simulations we considered programs that include the testing of the whole herd (antibodies in all animals and confirmation with direct method in calves) or only animals older than 6 months (antibodies).

3. How important is it to accurately detect the source of infection?

We evaluated this aspect by varying the sensitivity (91%, 95% and 99%) of the test that is used to detect infected animals when only animals older than 6 months were tested. When calves were screened we considered that a series of 2 tests where applied (one indirect method and one direct method) and we considered 91 % as final sensitivity for the serial procedure.

4. How important is the frequency of removal of infected cattle?

We evaluated this aspect by simulation of several control strategies (see next paragraph) applied on various testing frequencies (once a year, every 6 months and every 3 months).

5. How important is herd size?

This was evaluated by simulation of several control measures applied on various herd sizes. A small herd consisted of a farm with 100 animals (50 cows, 38 replacements and 12 calves); an average or typical herd consisted of a farm with 200 animals (100 cows, 75 replacements and 25 calves) and a large herd consisted of a farm with 800 animals (400 cows, 300 replacements and 100 calves).

Outcomes for a specific measure were derived by making separate runs (1,000) with all possible combinations of values of the other measures; subsequently an average over these separate runs was calculated.

CONTROL STRATEGIES OR SCENARIOS

Several control strategies or scenarios were modelled and they consisted of either single control measures or a combination of them. To assess which alternative control strategies a farmer could favour, we compared the following control strategies:

A-Test and slaughter/segregation of positive reactors

Test and slaughter of positive reactors has been used extensively in Europe, and thus most reports of its success have come from there ((Mammerickx *et al.,* 1978; Roberts and Bushnell, 1982; Schmidt, 1982). Segregation and confining of serologically positive reactors to another unit or keep them together in the same farm unit but managed separately has also proven to be effective in Israel (Brenner *et al.,* 1988) and USA (Johnson *et al.,* 1985; Miller and Van der Maaten*,* 1978; Shettigara *et al.,* 1989). Then removal of the infected animals from the herd is done at the normal culling rates or at the moment the farmer considers it convenient.

We assumed that the removal/segregation of positive animals was done immediately after testing. Within the model segregation and culling were treated identically as no contact and no subsequent transmission from segregated animals to the animals remaining in the farm is assumed.

B- Implement corrective management alone

Corrective management is aimed to reduce transmission between animals (DiGiacomo *et al.,* 1987; Hopkins *et al.,* 1991; Sprecher *et al.,* 1991) and hence to eliminate the disease. This strategy does not use any testing and infected and non-infected animals are not

segregated. Some measures to correct management are use of single needles, disinfecting non-disposable equipment, change in dehorning system, one sleeve per cow for rectal palpation, etc. The measures adopted can vary from taking several actions at the same time to only a few that are thought to be the most important for a specific farm situation.

C-Testing and removal/segregation of positive reactors supplemented with implementation of corrective management

This approach is a combination of the previous strategies, where surveillance is done by serological testing, followed by removal of positive reactors and simultaneous implementation of corrective management (Brunner *et al.,* 1997; Ruppanner *et al.,* 1983).

COMPARISON OF CONTROL STRATEGIES
Finally, we assessed the outcomes of the control strategies and all combinations of measures per herd size category to evaluate which is the best approach that farmers can follow.

We assumed the sensitivity of the test applied on animals older than 6 months to be 95% because tests that are available now have sensitivities around that value (Monti et al, 2005). All removed animals were replaced by susceptible animals raised within the farm (if there was enough supply) otherwise from external sources (assumed to be unlimited).

In all simulations, the initial number of infected animals was derived from the endemic state situation (equilibrium situation where the prevalence is more or less constant) based on the quasi-stationary distribution obtained in our previous study (Chapter 6). An infection level of 95% in cows, 85 % in replacements and 10% in the calves characterised this situation.

Outcomes for a specific control strategy were derived by making 1,000 separate runs for each combination of strategy and herd size in a time-horizon of 40 years; subsequently an average over these separate runs was calculated.

The model used for all simulations was developed in Mathematica ® but automated using @Risk ®.

EVALUATION OF PROGRAM'S RESULTS
We have numerically evaluated the impact of control strategies and control measures by Monte-Carlo simulation. The criteria used for comparing the strategies were:
- Efficacy, which is defined as probability of eradication and which is obtained as the percentage of runs that ended with eradication of disease within the time limit of 40 years.
- Efficiency, which is defined as mean time needed to achieve eradication. It is calculated as the mean of the time elapsed between the starting of the program till the test-round in which all cattle in the herd resulted as free of infection (in those iterations that eradication was achieved) and for those iterations in which eradication was not achieved, 40 years were added.

RESULTS

MODEL OUTCOMES: POTENTIAL CONTROL MEASURES
Model outcomes for several control measures are summarized in Table 1.

Table 1. Efficacy and efficiency of several control measures using test and removal of positive reactors plus corrective management.

Variable	Category	Probability of eradication (%)	Mean eradication time (years)
Effective reduction of baseline transmission rate	0.00 (equivalent to Test&Removal-only)	91.8	6.3 (6.2; 6.4)
	0.25	99.9	2.0 (2.0; 2.1)
	0.50	100.0	1.2 (1.2; 1.2)
Tested population	All herd	99.8	1.4 (1.3; 1.4)
	Only animals older than 6 months	94.7	4.9 (4.9; 5.0)
Test sensitivity	91 %	96.0	4.4 (4.4; 4.5)
	95 %	97.6	2.9 (2.9; 3.0)
	99%	98.1	2.1 (2.0; 2.1)
Testing frequency	Every 12 months	95.0	5.1 (5.1; 5.2)
	Every 6 months	96.7	2.7 (2.7; 2.8)
	Every 3 months	100.0	1.7 (1.6; 1.7)
Herd Size	100	100.0	1.2 (1.2; 1.3)
	200	98.8	2.0 (1.9; 2.1)
	800	93.4	6.0 (5.8; 6.1)

All the control measures that were considered resulted in a substantial impact on efficacy and efficiency. High testing frequency and a 50% effective reduction of transmission baseline parameter values resulted in eradication in all simulations. There is also a considerable effect of testing the whole herd and of herd size.

There is also a substantial impact on efficiency by reducing the transmission baseline parameter values and the proportion of animals included in the testing scheme. Additionally, testing frequency and sensitivity of the test used seems to be critical when it is applied once a year or when it is lower than 95 %, respectively.

Important differences in model outcomes were found when comparing low (n=100) or average (n=200) herd sizes vs. large herd (n=800). These results indicate that eradication is more difficult and takes longer time to be achieved with increasing herd size.

These differences are more apparent when we inspect the cumulative distribution of the outcomes (Figure 1), for example when the 90% percentile is considered, there are larger differences between categories in time to achieve eradication than for the median.

Figure 1. Effects of specific control measures and conditions on efficacy and efficiency. Simulations started in the quasi-stationary endemic state and reached a time horizon of 40 years. Results are expressed as the cumulative proportion of runs that achieve eradication over time.
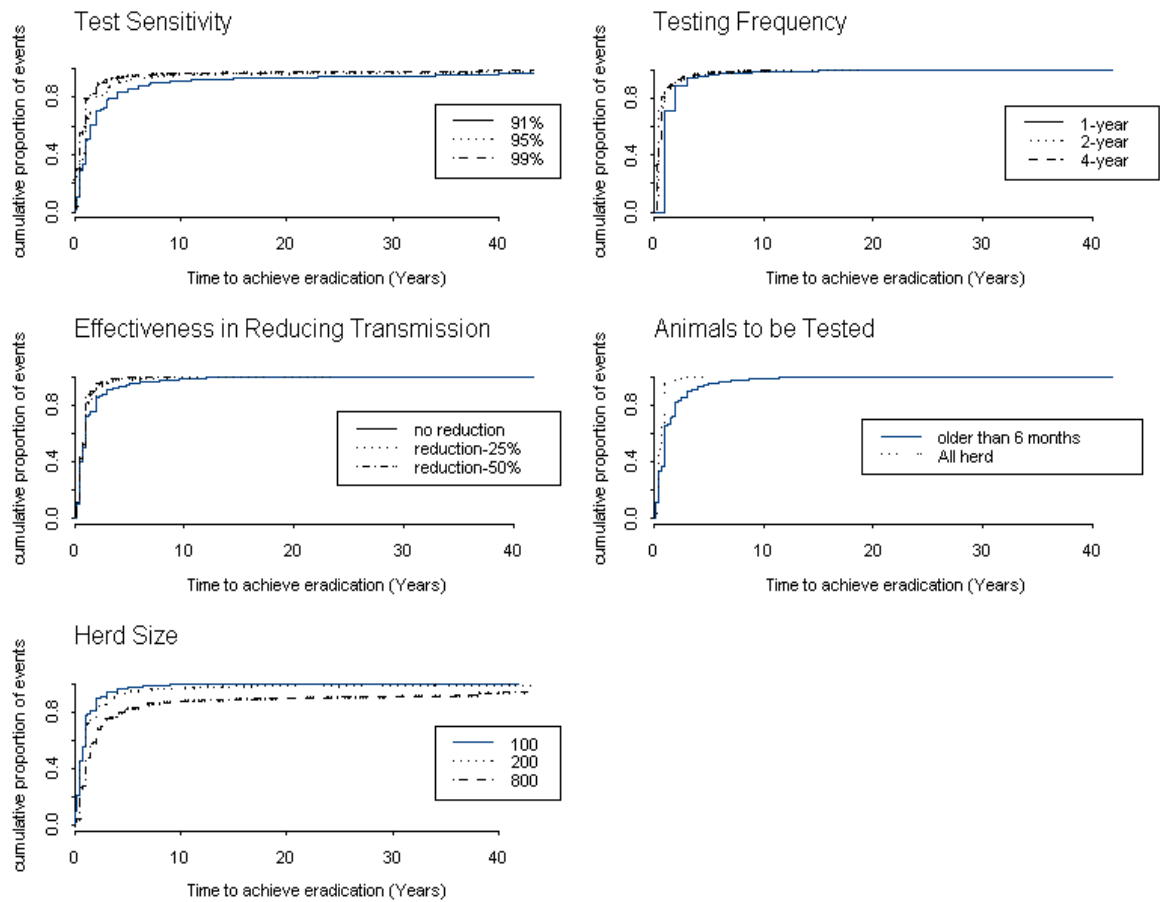
MODEL OUTCOMES: CONTROL STRATEGIES OR SCENARIOS

Table 2 and Figure 2 show the impact of control measures using the standard model for a closed herd where only measures that reduced the baseline transmission parameter values are applied (no test and removal).

Although reduced transmission results in a significant decrease in prevalence over the course of the outbreak (Figure 2) adopting corrective management measures only is neither effective (only 1,914/12,000 = 16% of the number of simulations ended in eradication) nor efficient (the time till eradication is very long, on average almost 29 years, Table 3).

Table 2. Effects on BLV within-herd transmission of corrective management-only control strategy, starting in the quasi-stationary endemic state, for different herd sizes.

Effective reduction of transmission (%)	Herd size	Probability of eradication (%)	Mean eradication time (years)
0.25	100	1.9	29.7[++]
	200	0.0	40.0[**]
	800	0.0	40.0[**]
0.50	100	2.5	29.6[++]
	200	0.0	40.0[**]
	800	0.0	40.0[**]
0.90	100	2.8	29.6[++]
	200	0.0	40.0[**]
	800	0.0	40.0[**]
0.99	100	92.7	20.7[++]
	200	70.4	26.2[++]
	800	21.4	29.5[++]

**All runs were censored at 40 years   ++ based on extinct runs only

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Figure 2. Changes in BLV prevalence after implementing a corrective management strategy (assuming a decreased baseline transmission rate of 25, 50, 90 and 99%), after starting in the quasi-stationary endemic state, by 1000 runs of Monte Carlo simulations over a time horizon of 40 years.
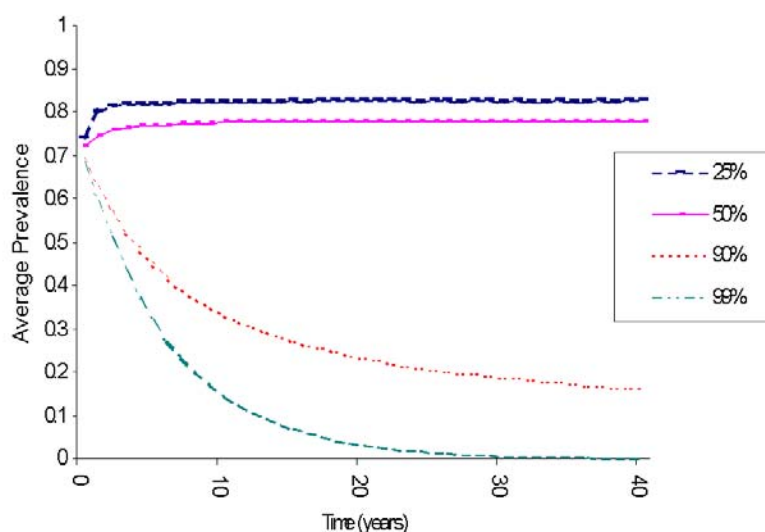
Moreover, prevalence may continue to rise (for reduced transmission baseline parameter values of 25 and 50) (Figure 2) for some time (2 years) after a control measure has been introduced. However, although the time till eradication is too long from a practical point of view, eradication is possible when the reduction in the baseline transmission value is 99%, especially for small or average herd sizes.

The combination of removal of infected and corrective management is the favoured strategy (Table 3) since it achieves eradication in almost 100 % of the runs but also reduces significantly the time to achieve eradication. Figure 3 shows the cumulative distribution of outcomes over time for the control strategies.

Table 3. Results from comparing three strategies to eradicate BLV after 1000 runs of Monte Carlo models, over a time horizon of 40 years, after starting in the quasi-stationary endemic state. Results expressed in mean delay time needed to achieve eradication with the 95% CI.

Strategy	Probability of eradication (%)	Mean eradication time (years)
Corrective management (only)	16.0	28.8 (28.7; 28.8)
Test and removal of positive reactors (only)	91.8	6.3 (6.2; 6.4)
Test and removal plus Corrective management	99.9	1.6 (1.6; 1.6)

Figure 3. Cumulative proportion of runs that achieve eradication over time after considering different BLV control strategies: test-and-removal positive reactors plus corrective management (T&R+CM), test-and-removal positive reactors (T&R) and corrective-management-only (CM). Simulations started in the quasi-stationary endemic state and they were extended to a time horizon of 40 years.
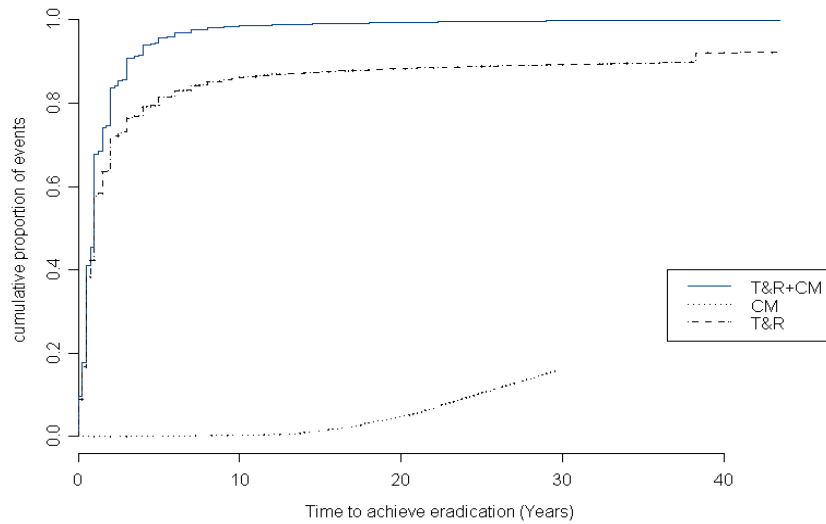
COMPARISON OF CONTROL STRATEGIES IN DIFFERENT SCENARIOS

For small herds (n = 100)

Figure 4a-c shows the cumulative distribution of outcomes over time after applying different control strategies and alternative testing frequencies.

Eradication of BLV in small herds could be certainly achieved within one year (75% cumulative proportion). For that, testing twice a year animals older than 6 months, followed by removal of positive reactors and adopting concurrently, corrective management measures that ensure an effective reduction of transmission of at least 25% is needed. The time to achieve eradication can be speeded up if - instead of a selective part - the whole herd is tested and/or a more effective corrective management is adopted (for example 50 % reduction of baseline transmission rate).

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Figure 4. Cumulative proportion of runs that achieve eradication over time after considering different BLV control strategies in a herd of 100 animals: test-and-removal positive reactors plus corrective management that reduced baseline transmission 25%(T&R+CM25); test-and-removal positive reactors plus corrective management that reduced baseline transmission 50%(T&R+CM50); test-and-removal positive reactors (T&R) and corrective-management-only (99% of reduction of baseline transmission rate) (CM). Simulations started in the quasi-stationary endemic state and they were extended to a time horizon of 40 years. A) When testing only animal older than 6 months. B) When testing the whole herd. C) When testing with different frequency.
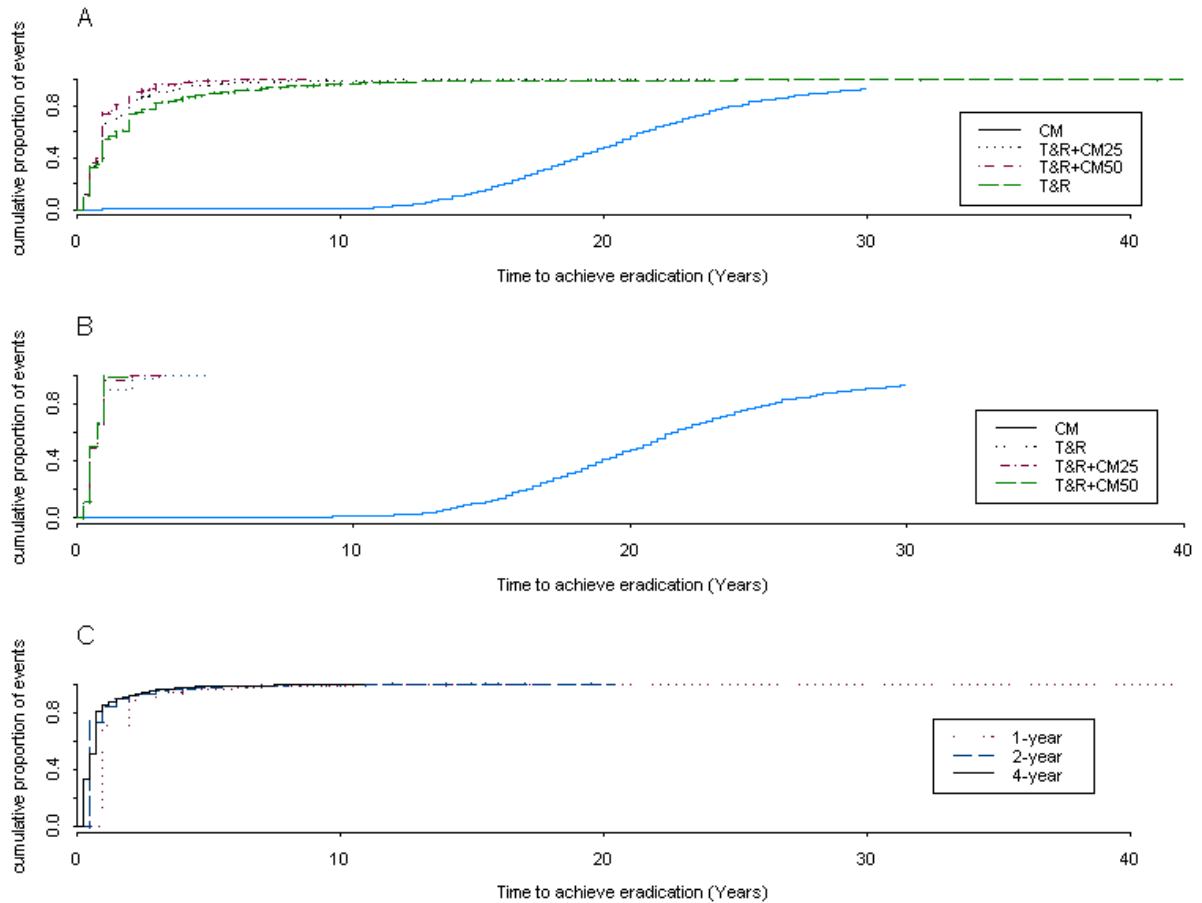
For average-sized herds (n =200)

For herds of average size, essentially the same results were obtained, although the time-scale is prolonged in comparison with size 100, for the same set of conditions (Figure 5a-c). However, time till eradication is similar to herd size 100, if the farmer adopts a program that includes a more frequent testing schedule.

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Figure 5. Cumulative proportion of runs that achieve eradication over time after considering different BLV control strategies in a herd of 200 animals: test-and-removal positive reactors plus corrective management that reduced baseline transmission 25%(T&R+CM25); test-and-removal positive reactors plus corrective management that reduced baseline transmission 50%(T&R+CM50); test-and-removal positive reactors (T&R) and corrective-management-only (99% of reduction of baseline transmission rate) (CM). Simulations started in the quasi-stationary endemic state and they were extended to a time horizon of 40 years. A) When testing only animal older than 6 months. B) When testing the whole herd. C) When testing with different frequency.
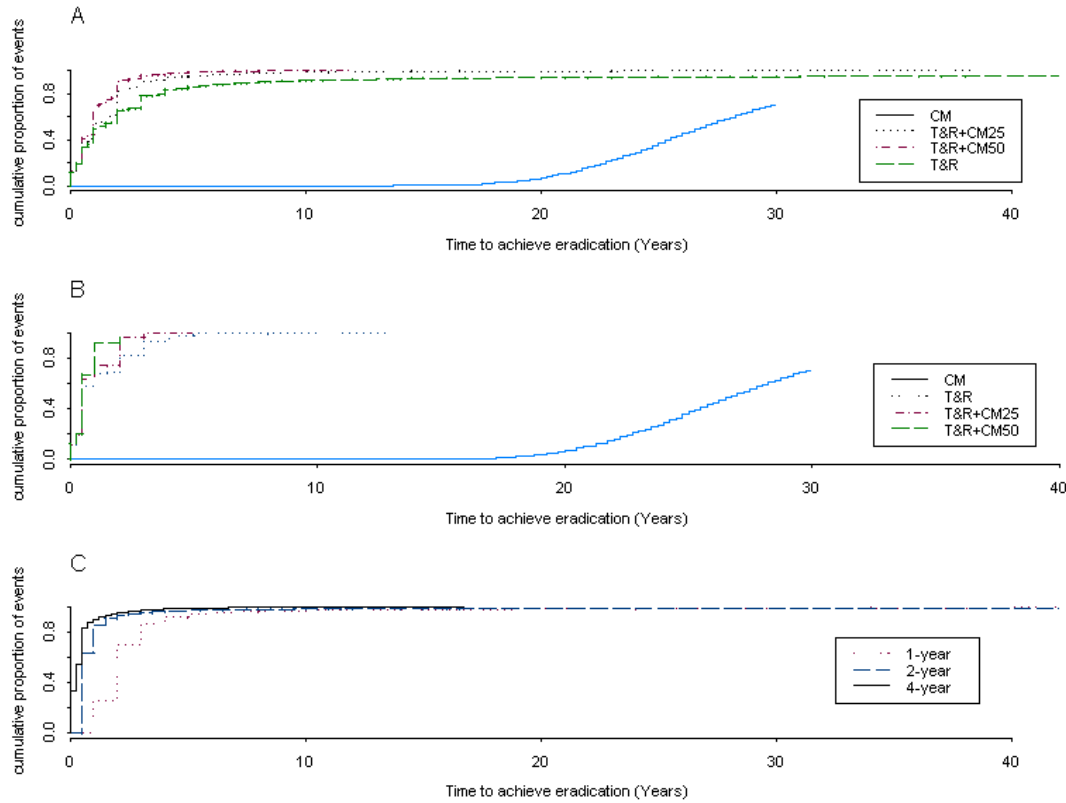
For large herds (N= 800)

Figure 6a-c shows the cumulative distribution of outcomes over time after applying different control strategies and alternatives testing frequencies in large herds. The same strategies as for smaller herd size were superior, although the time-scale to achieve eradication is much longer (eradication time will be delayed to 2-3 years). However, still it is possible to eliminate infection at similar time as for other herd sizes, but then the farmer should adopt a more stringent control program. Such a program should include testing the whole herd for surveillance supplemented with more severe corrective-management measures that ensures a reduction of the baseline transmission rate of at least 50 %.

Figure 6. Cumulative proportion of runs that achieve eradication over time after considering different BLV control strategies in a herd of 800 animals: test-and-removal positive reactors plus corrective management that reduced baseline transmission 25%(T&R+CM25); test-and-removal positive reactors plus corrective management that reduced baseline transmission 50%(T&R+CM50); test-and-removal positive reactors (T&R) and corrective-management-only (99% of reduction of baseline transmission rate) (CM). Simulations started in the quasi-stationary endemic state and they were extended to a time horizon of 40 years. A) When testing only animal older than 6 months. B) When testing the whole herd. C) When testing with different frequency.
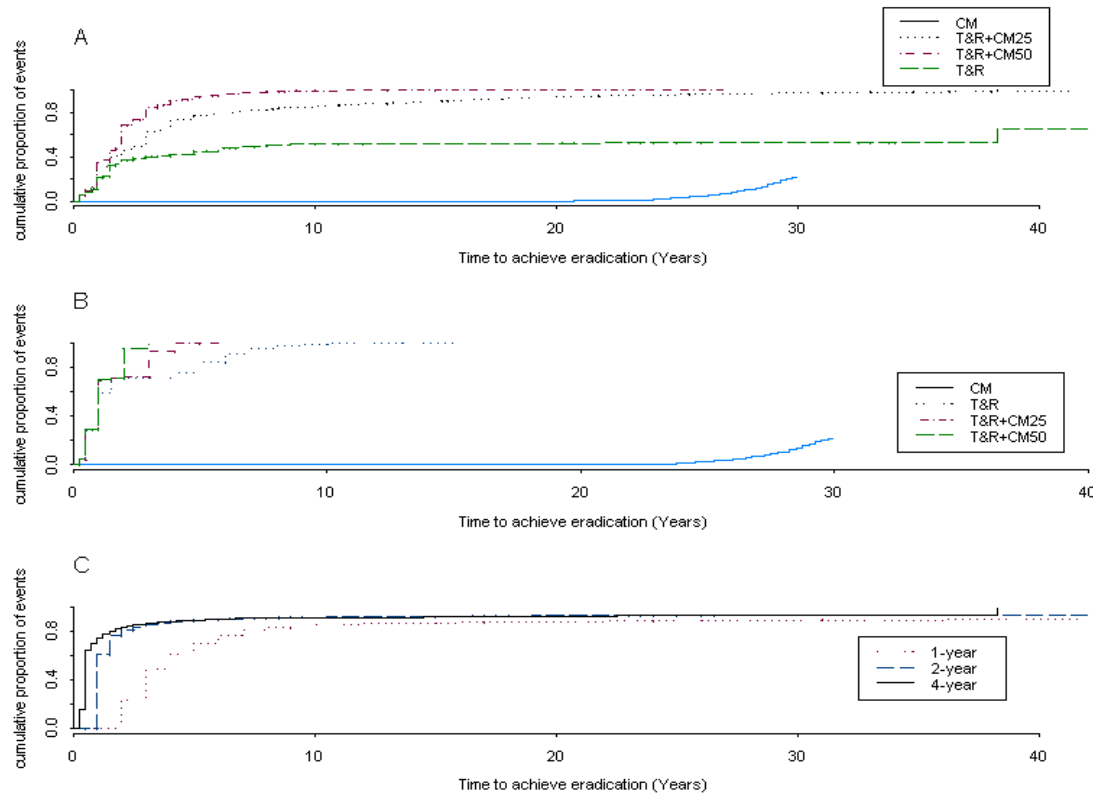
DISCUSSION AND CONCLUSION

One objective of this study is to illustrate aspects of BLV control by using simulation modelling, for biologically plausible sets of assumptions and parameter values. The detailed results are obviously sensitive to these assumptions and parameter values and it is important to emphasize that there is uncertainty about both. It should be noted that the patterns of BLV eradication were subject to stochastic effects, therefore, not only a time effect was incorporated but also stochasticity that resulted in variability for outcomes.

However, an important prediction of these models is that, even in a relatively small, closed dairy herds, although eradication of BLV is possible it may take as long as one year to several decades, depending on certain aspects.

Reduction of horizontal transmission alone is not potentially effective as a single control measure when the initial prevalence is high and this is in agreement with other studies (Johnson *et al.,* 1985; Ruppanner *et al.,* 1983). However, in the cited studies authors explain the failure by a less strict application of the measures after some time (which constitutes a disadvantage of this approach). Our results shows that even in the hypothetical ideal

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scenario of successful reduction of transmission (99%) and keeping it constant for 40 years, the probability of eradication is comparatively low and many years are needed to achieve eradication. Although this result is disappointing, corrective management alone as control strategy, is the cheapest strategy to implement, hence the economic feasibility of this strategy remains to be established. Nevertheless, our results show that implementation of corrective management alone, helps in controlling the level of infection when substantial reduction in the baseline transmission rate (more than 90 %) is achieved and this is in agreement with previous studies (Johnson *et al.,* 1985; Ruppanner *et al.,* 1983). Moreover, our analysis shows that the addition of corrective management to test and removal of infected individuals provides extra benefits by shortening the time needed for eradication.

Of the options considered in this study, a substantial improvement in efficacy (but not by 100%) may be achieved by the slaughter of positive reactors. Clearly, our results show that either the physical separation or removal of the infected animals is the key of success for any BLV eradication program and this view is shared by others (Ferrer, 1982). Eradication of the disease - mainly in Europe - has been largely based on this strategy (Bendixen*,* 1987; Kettmann *et al.,* 1994; Mammerickx *et al.,* 1978; Shettigara *et al.,* 1986). However, in the context of the Argentine dairy sector such a strategy is difficult to be adopted. Argentine farmers - in contrast to European farmers - do not receive any monetary compensation for the animals to be culled and farmers carry all the costs of the program. Past experience in controlling other diseases showed that most of the benefits coming from the eradication are transferred and captured by other stakeholders of the sector. Moreover, the high prevalence of the disease would force removal of a substantial number of animals that will entail a significant reduction in milk production and drainage of genetic resources. Finally, previous studies (Trono *et al.,* 1997; Trono *et al.,* 2001) estimated not only a relatively high prevalence but also a high proportion of herds being infected. Therefore it is possible that a sufficient number of non-infected animals would just not be available for replacement in a situation of massive removal of infected animals.

Our results show that surveillance followed by segregation of positive reactors could yield similar to test and slaughter results if the isolation is fully successful in avoiding transmission between both groups (infected/non-infected) (Miller and Van der Maaten*,* 1978). Previous studies (Brenner *et al.,* 1988; Shettigara *et al.,* 1989) demonstrated that a minimum distance of at least 200 m. apart between groups is sufficient for avoiding cross-transmission. In Argentina, many dairy farms are constituted by more than one-operation unit. This structure facilitates the adoption of this strategy because it only needs a re-distribution of animals. However, for those dairy farms that do not have such a decentralised structure, this control strategy poses operational challenges. From experience from others (Brunner *et al.,* 1997) absolute reduction of horizontal transmission could be difficult to maintain over an extended period as we considered (40 years).

Although in this study we did not investigate combinations of strategies over time, our results supports the conviction that important improvement in effectiveness is possible by combining control options. For example it is a realistic alternative to change to slaughter of positive reactors only, at low infection level, after the application of a more stringent strategy in a relatively short time.

Results show that eradication is possible by testing only a part of the herd (animals older than 6 months). However, a more effective control program should take special care of early detection and effective segregation of infected young stock. Our previous study

(Chapter 6) suggests that although vertical transmission is not significant it will be relatively important for maintaining infection within the herd since more cases will arise through horizontal transmission. However, because the identification of the infected individuals in this age group should be done at expenses of a more expensive test and more difficult to implement other aspects (e.g. an economic evaluation or logistics) should be included for taking a final decision.

Results for the model provided a means of assessing the relative merits of potential strategies for eradication of BLV when applied to herds of different size. First, for all models the effects of size were always significant. Secondly, the effect of herd size has important implications for eradication of BLV but this association has not been documented yet. For small and medium herd sizes, our results are conclusive in pointing to a control strategy that includes surveillance at least twice a year on all animals of the herd, followed by removal of positive reactors coupled with corrective management.

Finally, the results of this study show that the sensitivity of test used for surveillance has an important effect either in the efficiency or efficacy of the control strategy only when sensitivity is below 95 %. However, previous studies (Gutierrez *et al.*, 2001, Monti *et al.*, 2005; Trono *et al.*, 2001) estimated that the sensitivity of the commonly available kits (ELISAs for serum or milk and agar gel immunodiffusion test) is at least 95%.

The key conclusions appear to be robust: the time-scale of BLV eradication within a dairy herd may be very long when infection does become endemic, depending on the choice of control strategy and within-herd eradication of BLV will, especially for large herds, require intensive effort and only be effective over a time-scale of several years. The model supports a policy that considers the use of combined control measures to control the spread of the disease.

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CHAPTER 8

GENERAL DISCUSSION

Bovine Leukemia has been reported in Argentina for the first time in 1968 (Sen *et al.,* 1968). Recently, a prevalence study in the whole country was performed (Trono *et al.,* 2001) showing an animal level prevalence of 33 % while 84 % of the sampled herds were positive. Given that the disease is more prevalent in dairy operations (Trono *et al.,* 2001), the main objectives of this thesis were to provide an analytical framework to quantify several aspects related with the epidemiology and control of BLV in dairy herds from Argentina. Understanding and quantifying the transmission of BLV in dairy cattle is essential to the design of effective control strategies and BLV prevention in general.

The thesis consists of 7 chapters. Chapter 1 presents an overview of the pathogen, the disease and the main characteristics of the dairy sector in Argentina. Chapter 2 provides insights about molecular characterisation and spread of the virus, phylogenetical relationships between isolates and an analysis of molecular evolution of the virus. Diagnostic tests are means to detect infected animals for screening or surveillance purposes or in observational studies, hence, it is essential to know their accuracy in terms of sensitivity and specificity (Chapter 3). Chapter 4 offers a statistical approach to calculate the most probable time of becoming infected with BLV when data of repeated serological screening are available. Results of a longitudinal study without any control interventions that aimed to follow-up the population dynamics and spread of BLV infection are described in Chapter 5. Chapter 4 and 5 serve as a source of estimation for parameter values needed to build-up a transmission model (Chapter 6). A stochastic age-structured model was developed (Chapter 6) to simulate the spread of the disease and to explore whether or not certain stages of the disease should be included, and to compare the dynamics of the disease after different starting situations. Moreover, the probabilities and time to extinction after the virus was introduced in a naive population were estimated. Finally, with the transmission model several alternatives for eradication of the disease were simulated and compared (Chapter 7). The impact of various control measures on the probability to achieve eradication, in a time-horizon of 40 years, was assessed as well as the time till eradication.

In the current chapter, results from previous chapters are discussed with a focus on herd level. Finally, implications for a national or regional program and directions for future research are considered.

MAIN FINDINGS OF THIS THESIS
1. Results from this thesis showed the presence of two types of BLV isolates - Australian and Argentine - in dairy herds from different areas of Central Argentina and the phylogenetical tree clearly shows that Argentine isolates represent a separate and homogeneous group compared to other clusters.
2. Although a detectable immune response in most infected cattle could be demonstrated, some persistently infected animals did not show a detectable antibody response.
3. The low rate of non-synonymous substitutions compared to synonymous substitutions found in the analysis supports the hypothesis of purifying selection of *env*, *gag* and *pol* genes and several subunits; consequently, molecular evolution occurs under some functional constraint. Nevertheless, it appears that for the Transmembrane Hydrophobic Region within the *env* gene - at least for the Argentine isolate - the host seems to drive the selective preasure and subtle natural variation in the structure resulted from host-pathogen interactions), hence is a site that might be a good potential candidate for future functional studies.

4. BLV ELISA 108 (either milk or serum) described in this paper has a sufficiently high sensitivity and specificity to be incorporated as an alternative test in monitoring and control programs.

5. When all types of experimental infections are considered, the median time till seroconversion is 57 days and the main factor that influences time till seroconversion is the type of transmission used. When only studies using experimental inoculation are considered the median time is estimated as 48 days.

6. Under the conditions of this study, natural transmission of BLV was observed and results reveal that the period around parturition is an important risk period and that heavily infected herds seem to have an increased proportion of young stock infected. The infection transmission parameter was estimated as relatively high and combined with a long infectiousness period this resulted in a high reproductive ratio (9.5).

7. An important prediction of our transmission model is that, even in a relatively small, closed dairy herd, the time-scale for a BLV outbreak may be as long as several decades.

8. The time-scale of BLV eradication within a dairy herd may be very long when infection does become endemic. Depending on the right choice of control strategy, the within-herd eradication of BLV will, especially for large herds, require intensive efforts and only be effective over a time-scale of several years. Results support a policy that consists of the use of combined measures to control the spread of the disease.

MOLECULAR EPIDEMIOLOGY OF BOVINE LEUKEMIA VIRUS

An important goal in studying the genetic diversity of retroviruses is to gain a better understanding of the correlation between retrovirus variation and disease progression (Mansky, 1998). Chapter 2 shows the relatively low diversity of BLV and demonstrated that Argentine isolates constitute a distinctive cluster and this contributes to the understanding of the evolution of the virus. Furthermore, it pointed out a target area (Transmembrane Hydrophobic Region within *env* gene) for further functional studies. This area may provide more insight in the evolution of the genetic structure which could be of help in the development of a vaccine.

The application of molecular techniques to heterogeneous organisms improves our ability to sub-classify these organisms into epidemiological meaningful groups (Foxman and Riley, 2001). In addition, the use of a simple and accurate protocol for molecular identification of variants as the one used in Chapter 2, will enable us to study and quantify transmission within and between herds. For example, it will contribute to a better understanding of the various ways of direct transmission. Also, most herds that we sampled showed the predominance of one isolate and this could result from differences in transmission capacity between isolates and molecular identification can help to test this hypothesis of differential transmission capacity. Such knowledge not only will increase the understanding of BLV transmission but also will improve the effectiveness of BLV control programs and can be used for a better communication to farmers, veterinarians and animal health officers.

Another striking outcome of this study is that not all infected animals will produce a detectable immune response which has an impact on serologic surveillance programs and on certification of freedom of the disease. However, due to the purposive selection procedure that we used for selecting the herds it is not appropriate making inferences to

the general population. Therefore, it is essential to quantify this phenomenon of sero-negative carriers in the population, before justifying a change to antigen-based methods to detect the infection. An economic evaluation should also be carried out to quantify the increased testing costs that such a test would demand.

PROSPECTS FROM MODELLING

The study of infectious diseases represents one of the oldest and richest areas in mathematical biology. As new epidemics, from AIDS to bovine spongiform encephalopathy (BSE: mad cow disease) to food-and-mouth disease, affect human and animal populations around the world, mathematical models are essential to inform decision-makers (Levin, 2002). In veterinary medicine models have been developed for several diseases (BVD (Cherry *et al.,* 1998); FMD (Durand and Mahul, 2000); Respiratory disease in cattle (Hurd and Kaneene, 1995); CBPP (Lesnoff *et al.,* 2004) and IBR (Vonk Noordegraaf *et al.,* 1998)).

Disease models are simplifications of a real situation and details, which are not determining the dynamics of the disease, are excluded (Busenberg and Cooke, 1993). Modeling also involves making assumptions that characterize the contact structure. Some of the important assumptions (Diekmann and Heesterbeek, 2000) are related to:

1. the contact process,
2. the mixing of susceptible and infectious animals,
3. the probability that a contact between an infectious and a susceptible animal leads to transmission.

In mathematical modelling of disease transmission there is always a trade-off between simple models, which only explain the general qualitative behaviour, and more complex models which attempt to incorporate more details. Such complex models can make short term quantitative predictions but are generally difficult to solve analytically (Brauer, 2002). Given the fact that there was no previous model for BLV, the approach was to begin with simple models to establish broad principles and subsequently bring in a more detailed structure.

The basic structure of the model is presented in Chapter 6 where transmission dynamics were simulated and preliminarily validated with field observations (Chapter 5). This basic model provides a simple analytical framework but by using stochastic simulation it may show the natural variability of the transmission process and its intrinsic randomness. A stochastic compartmental model was preferred for three reasons. The first was the need to take the demographic stochasticity (i.e. chance fluctuations associated with the fact that individual hosts are discrete units rather than fractional ones) into account (Diekmann and Heesterbeek, 2000). The second was the fact that this study deals with small populations, hence, individual differences and random effects are important (Busenberg and Cooke, 1993). The third was that for small compartment size the behaviour of the compartment size may be strongly influenced by random perturbations (Brauer, 2002).

Several control programs have proven their effectiveness in field trials, but it is impractical to test all possible control scenarios. However, using simulation modeling the merits of control measures or strategies can be examined and extended to hypothetical situations or scenarios. For example, at the moment there is no vaccine available for BLV, though a number of trials with experimental vaccines have been performed but results are not yet conclusive (Altaner *et al.,* 1991; Cherney and Schultz, 1996; Mateo *et al.,* 2001;

Ohishi and Ikawa, 1996; Ohishi et al., 1997; Reichert et al., 2000). From our estimation of the reproductive ratio ($R_0 = 7.5$) in Chapter 6, we can infer that the vaccine coverage needed to protect the population is high ($93\% = 100*(1- 1/R_0)$) assuming a 100% efficacy. Alternatively, further simulation can be performed to assess if vaccination with such a vaccine could be used economically to eliminate BLV from an endemic situation like the current one in Argentina. Another alternative that needs further study and which could be included in future models is genetic selection of more resistant cattle. Susceptibility of cattle to BLV infection, development of PL and lymphosarcoma are related to genetic traits of the host like bovine lymphocyte antigens (BoLA) which leads to the hypothesis of genetic differences in susceptibility (Lewin and Bernoco, 1986; Lewin et al., 1988; van Eijk et al., 1992; Xu et al., 1994; Zanotti et al., 1996).

VALIDITY OF THE BLV SIMULATION MODEL

One of the most critical aspects in the development and application of infectious disease models is its validation, due to their complexity and potential for nonlinear behavior. Model validation is usually done by testing the model on another independent data set. Unfortunately, we could not perform an extended external validation of our models (either for transmission or control) due to the lack of such an independent dataset. However, the goal of model-based evaluation should not be to replace the decision maker, but rather to redefine the terms of the debate using quantitative methods that make the beliefs and values, that are in the center of all difficult choices, explicit (Paltiel, A., 2000).

CURRENT ARGENTINE BLV CONTROL PROGRAM

The current directives (SENASA 337/94 and SAGPyA 128/1) (SAGPyA, 2001; SENASA, 1994) for BLV control in the country aim to obtain the certified free status that allows farmers for example to export breeding cows. These norms are expressed in a manual of regulations and procedures (Manual de Procedimientos Leucosis Bovina, Dirección de Luchas Sanitarias, Dirección Nacional de Sanidad Animal; 2004) which has 2 aims:
1. To offer an official procedure with standardised strategies for controlling BLV to farmers,
2. To indicate and point out control measures to be adopted by farmers when being part of a future national or regional eradication program.
The procedure starts with testing the whole herd with AGID or ELISA and when results are negative the herd is retested twice and when both are again negative the herd is certified-free. A yearly retest is done to assure the free status. When infected animals are present, the herd can be classified as Herd Class A (prevalence less than 15 %); Herd Class B (prevalence between 16 and 30%) or Herd Class C (prevalence higher than 30 %). When BLV infected animals are present, the directive suggests surveillance of all animals older than 6 months by AGID or ELISA and the farmer can choose for elimination or segregation of positive reactors. When he chooses for removal, the whole herd should be retested twice with an interval; of 2-3 months. If both retests are negative then the herd can be accredited.
When the farmer decides not to remove reactors the herd will not be certified as free. The directive considers several actions depending on the initial prevalence but in general a strategy of test and segregation of positive reactors complemented with corrective

management measures is advised. It also indicates the surveillance of animals that tested negative each 3 months.

FUTURE SECTORAL TRENDS AND RELATIONS WITH BLV INFECTION

AT NATIONAL OR REGIONAL LEVEL

A study performed in one of the dairy areas of the Argentina (Ghezzi *et al.,* 1997) estimated that the proportion of infected herds was 4.5% during 1979-81. In a later screening (1994-95), the estimate increased to 69 %. Finally, in 1999, a national survey (Trono *et al.,* 2001) estimated that 84 % of the herds were infected. From these figures it can be deduced that the prevalence level is far from stable and that the situation has deteriorated in the last decades. The voluntary program that started in 1994 was not successful as few farmers enrolled (SENASA (Animal Health Services), personal communication) and, considering the increasing prevalence, the program did not have any impact. Therefore, one conclusion is that only a more aggressive control policy will improve the situation.

Since the end of the '80, important structural, technological and productive transformations have taken place in the Argentine dairy production sector. As a consequence, a steady increase in production and productivity has been achieved (SAGPyA, 1999). This increment in the national production was characterised by a significant reduction of the number of herds (28% between 1988 and 1996), that was compensated by an increment in the number of dairy cows (17%). Hence, herd size increased with 63% and the individual production per cow increased with 25% (Schneider *et al.,* 2000).

These results are considered to be a positive change for many stakeholders of the sector. However, the increment of herd size has negative consequences for the persistence of BLV infection. Chapter 7 showed that although in larger herds it is possible to eradicate BLV, it takes longer and it demands more efforts in comparison with smaller herds.

AT FARM LEVEL

In the previous section the implications derived from the increased size of farms were discussed. However, at farm level, the consequences of this process are related to the way the herd increases in size. Common practice is that farmers increase their herds by buying complete herds from other farmers that went out of business or by incorporating replacements from other sources than their own stock. Given the high prevalence of BLV infection, these practices imply higher risks of spreading and/or acquiring the disease which was demonstrated by previous studies (Casal *et al.,* 1990; DiGiacomo *et al.,* 1986). In Chapter 6 it was shown that after introduction of only one infectious animal (either a heifer or adult cow) in a naive herd there is a high probability that an outbreak will occur and most likely the infection will then become endemic. Hence, if farmers want to eradicate BLV from their herds or keep their herds free of BLV, they should be extremely cautious in the addition of new animals to their herds. The recommendation here is to adopt a policy consisting of screening all additions before they enter the herd or keep them isolated from the herd until results demonstrate a negative status. Results from Chapter 3 indicate that serum ELISA 108 is the best option available, given its high sensitivity.

The other way farmers usually expand their herds is by retaining the excess of replacements raised in their own premise and reducing voluntary culling. If BLV is present

in the herd, these actions will result in an increase of R_0 (Chapter 5), by an increase of the length of the infectious period.

A farmer may decide to monitor all cattle added to his herd or keep his herd closed, but the risk of introducing BLV is not reduced to nil as the infection may also be acquired from contiguous herds by haematofagous insects, improper hygiene of needles in cases of massive vaccination campaigns (for example Foot-and-Mouth-Disease) or iatrogenically by veterinarians. The last two ways are important because these transmission routes can be easily avoided. Practitioners and FMD Vaccination Committees should implement more mitigating measures (e.g. change or disinfecting sleeves or needles between cows, hand hygiene products or procedures when contaminated with blood) to reduce the risk of iatrogenic transfer by infected fomites.

FUTURE OPPORTUNITIES OF BLV CONTROL IN ARGENTINE DAIRY FARMING: SHOULD THE PROGRAM BE COMPULSORY

POSSIBLE CONSEQUENCES AND RISKS OF A COMPULSORY PROGRAM

All the aspects discussed in the previous section made the author hesitate about the convenience of having a voluntary program like the current. At present, it is not clear if a farmer who decides to go for freedom of BLV infection will obtain a clear benefit or will be exposed to a larger economical risk (given the costs of keeping free (tests, labour, etc)) compared with just doing nothing. In addition, the fact that there is a relatively low number of non-infected herds also implies a high risk of loosing the status (Brunner *et al.,* 1997). When the program would be made compulsory and extended to the whole country, the current figures indicate that the number of non-infected replacements is insufficient.

As mentioned before, the movement of animals between premises constitutes an important way to spread the disease between farms. Next to that, farmers seldom check for BLV infection (and many other diseases) before introducing an animal and it is a common believe among farmers that the benefits of a larger milk production scale overwhelm the potential risk of losses. Here, massive communication of such risks is a point for improvement as well. Therefore, if a compulsory program is implemented, a clear directive should be added to the present one. For example by including interdiction, limitations in movements or more strict and clear rules that regulate movement of animals from infected premises. Another aspect that could be included is the need of screening for infection before addition to the herd, at least when animals are coming from a non-certified herd.

In Argentina control of disease is not state funded, farmers have to pay the costs themselves. Therefore, only those farmers that export breeding cows or those herds with a high incidence of clinical cases would profit from the program. Hence, for the major part of the commercial dairies a control program will represent an economic burden, which could bring conflicts with authorities.

POSSIBLE MODIFICATIONS TO THE CURRENT PROGRAM

Simulation modelling of control strategies have been developed for several diseases (Buijtels *et al.,* 1997; Klinkenberg *et al.,* 2003; Morris *et al.,* 2001) with the aim to evaluate various strategies, from vector control measures to use of vaccines (Lodos *et al.,* 2000). However, there is no previous study that involves such an approach for BLV.

Model outcomes were used to assess the relative values of potential strategies for control and eradication of BLV at farm level (Chapter 7). The lack of massive adherence of farms to the program at national level stimulated the studies described in this thesis to find alternative control strategies that motivate farmers to participate. Studies in this thesis have their focus on farm level, and extrapolating conclusions from this study to a regional or national level is not scientifically sound. However, due to the lack of studies at a higher aggregation level, the outcomes of this thesis could be used to improve those aspects related to within-farm control of the current national eradication program as will be pointed out in the next paragraphs.

Our estimation of the properties (sensitivity and specificity) of diagnostic tests commonly used and available for surveillance (Chapter 3) shows that they are sufficient for being used in eradication strategies as simulated in Chapter 7. Although several types of ELISA have recently been approved officially for surveillance purposes (SAGPyA, 2001) it does not incorporate the ELISA 108 in milk. This test is very convenient to be used in dairies because it facilitates and reduces cost of sampling by making use of milk control testing and bulk tank sampling (Forschner *et al.,* 1988; Hayes *et al.,* 1997).

Another option not included in the current program is the use of pooled samples. Our estimations reveal that the ELISA in bulk milk has a sufficient sensitivity as it is able to detect 1 positive cow per 20. This is an interesting option to decrease the costs of testing especially when the prevalence is high. This approach has been successfully used in New Zealand (Burton *et al.,* 1997)

Moreover, simulation outcomes showed that sampling frequency could be 6 instead of 3 months, without significantly affecting the time to achieve eradication, resulting in lower costs for the farmer.

Results from Chapter 7 showed that the decision to adopt a specific BLV eradication strategy (combination of control measures, sampling frequency and test) that optimises the probability of achieving eradication and time to achieve eradication depends heavily on the herd size. In literature several eradication strategies have been discussed (Brunner *et al.,* 1997; Burton *et al.,* 1997; Pelzer and Sprecher, 1993; Sprecher *et al.,* 1991) but all of them assumed that the efficacy and/or efficiency are constant for any herd. Moreover, most of the field control studies were performed within one (Ferdinand *et al.,* 1979; Meszaros *et al.,* 1994) or a few herds (Mammerickx *et al.,* 1978) which frequently were experimental farms (Johnson *et al.,* 1985; Ruppanner *et al.,* 1983).

Early detection of infected calves dramatically reduces the time needed to achieve eradication. However, this approach will imply a higher initial investment in terms of more costs related to sampling and testing and the use of more sophisticated tests (such as PCR, immunoblotting or ELISA to virus proteins) that are likely to be more expensive (more sophisticated equipment, reagents, costs, etc).

Mathematical epidemiologists have frequently assumed a perfect world (a world in which there are no complicating variables or random variability in behaviour), where response times are not particularly relevant, where perfect isolation is possible and where farmers are all well informed and compliant with government policy (Castillo-Chavez *et al.,* 2003). These scenarios are simple to model. However, it can be difficult to maintain segregation strategies or adherence to strict management practices for very long times for all farms in the real world because intervention strategies are implemented in a very complex and dynamic environment. Eradication of a disease within a farm is ultimately successful when it is cost-effective. Although this thesis did not include any economic

evaluation, it provides rational information that should constitute the basis for that analysis.

In addition, social aspects associated with disease transmission and control may probably have a significant impact (Castillo-Chavez *et al.,* 2003). Hence, to incorporate distinctive sociological aspects of farmers (such as educational background, life goals, etc) might constitute an aspect to be assessed in further research. The addition of more realistic elements, however, may require unrealistic assumptions and therefore, numerical and theoretical results need to be observed cautiously until the underlying model assumptions are verified.

POTENTIAL WAY TO IMPLEMENT A COMPULSORY BLV PROGRAM

In Argentina there is no state refunded option for control of BLV. Under this condition the following 3 steps approach can be considered. The first step is to make an accurate estimation of the number of heifers that would be needed to replace the infected cattle and identification of all herds that are selling purebred animals. The following step would be to implement a compulsory regional eradication program with the aim of obtaining a sufficient number of non infected cattle for supplying replacements and should include all that farms that provide replacements. Raising calves free of BLV is relatively easier than raising free adult cows because of practical implementation (most farmers raise replacements in separate units to adult cattle) (Pelzer and Sprecher, 1993; Sprecher *et al.,* 1991). The stimulus to focus on calves might be the opportunities of future business and the creation of a future free area that will be competitive for exportation as well. The third step is the extension of the program to commercial farms. A great part of the infected animals will not show any significant negative effect on their (re)productive performance and because of the good genetic background of the Argentine dairy cattle, one transient alternative is the creation of 'garbage' farms. These farms receive infected animals from farms enrolled in the program and are allowed to cull animals for slaughter only. This system might save a lot of resources, it will smooth the economic impact of massive removal and it will avoid a large economical disturbance in the production chain. Due to the production characteristics, economical relevance and size, the area under study (Central Santa Fe) constitutes a good candidate for such a plan.

FUTURE RESEARCH

For decisions about control strategies an economic evaluation needs to be performed. Benefits from current and future opportunities in the market of embryos, semen and export of breeding animals, should be realistically estimated. Also the inclusion of calves and their testing scheme (for example at 2 or 4 months of age) should be economically evaluated.

More alternatives for eradication should be explored (like vaccination, strategic use of genetic selection for resistance) and one important area to proceed is the combination of strategies that are more effective in reducing prevalence with those that are less costly to the farmer (including economical evaluations), for example start with corrective management to reduce prevalence and once a certain level is achieved then apply test and removal.

Other aspects that should be further investigated are related to the test and replacement strategy. E.g. relaxation of the assumption of instantaneous replacement of all positive

reactors needs knowledge about the impact of a delay in removing them. Secondly, more should be known about the effect of using home-bred or bought-in replacements.

Computer simulation of the transmission and intervention processes can improve our understanding of how the system generates and propagates risk and how to most effectively manage it. One gap in the literature is the lack of quantitative information about the ways to reduce effectively the baseline transmission rate. This information could be easily incorporated in our model and by extra simulations we can quantify the effects on transmission, hence, it would result in more precise recommendations about corrective management.

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SUMMARY

Bovine Leukemia Virus (BLV) is a retrovirus that causes lymphomas (leucosis) and other disorders in cattle. Leucosis has a large economical impact on the livestock sector of many countries around the world. In herds infected with BLV economic losses occur due to death, carcass condemnation at slaughter, veterinary services, but also by limitation of trade (export) of animals for life. Under natural conditions, only cattle (*Bos taurus*), zebus (*Bos indicus*), buffaloes and capybaras have been found to be infected. Experimental infections have been successful in sheep.

Bovine Leukemia has been reported in Argentina for the first time in 1968. Recently, a prevalence study in the whole country was performed showing an animal level prevalence of 33 % while 84 % of the sampled herds were positive. Stratification by prevalence level shows that 16 % of the herds did not show infected animals, approximately 20 % of the herds presented a prevalence less than 15 %, 17 % between 15 and 30 %, almost 50 % of the herds showed values greater than 30 %. Despite small differences in prevalence between dairy areas exist, most areas can be regarded as heavily infected based on the proportion of infected herds.

The area of study (Santa Fe center) is the region with lowest prevalence of Brucellosis (Res. SENASA 150/2002, Res. SENASA 497/2002) and Tuberculosis (Res. 115/99, SENASA) of the country, with approximately 45 % and 30 % of the herds respectively, certified as free. Given that these important diseases are under control Farmer's associations and Provincial Animal Health Services are interested in develop a compulsory pilot-control program against BLV in the area, instead of the current National Voluntary Control Program (RES. 337/94, SENASA). Given that the disease is more prevalent in dairy operations, the main objectives of this thesis were to provide an analytical framework to quantify several aspects related with the epidemiology and control of BLV in dairy herds from Argentina. Understanding and quantifying the transmission of BLV in dairy cattle is essential to the design of effective control strategies and BLV prevention in general.

MOLECULAR EPIDEMIOLOGY OF BOVINE LEUKEMIA VIRUS

Chapter 2 describes the presence of two types of BLV isolates - Australian and Argentine - in dairy herds from different areas of Central Argentina and the phylogenetical tree clearly shows that Argentine isolates represent a separate and homogeneous group compared to other clusters. An immune response could be demonstrated in most infected cattle, but some persistently infected animals did not show a detectable antibody response (see also Chapter 5).

The low rate of non-synonymous substitutions compared to synonymous substitutions found in the analysis supports the hypothesis of purifying selection of *env*, *gag* and *pol* genes and several subunits; consequently, molecular evolution occurs under some functional constraint. It appears that for the Transmembrane Hydrophobic Region within the *env* gene - at least for the Argentine isolate - the host seems to drive the selective pressure and subtle natural variation in the structure resulted from host-pathogen interactions; hence it is a site that might be a good potential candidate for future functional studies.

QUANTITATIVE EVALUATION OF DIAGNOSTIC TESTS, ESTIMATION OF TIME FROM INFECTION TILL SEROCONVERSION AND FIELD TRANSMISSION

For modeling purposes estimates for several input parameters were needed. In **Chapter 3** BLV ELISA 108 (either milk or serum) is evaluated and it shows that this diagnostic test has a sufficiently high sensitivity (larger than 97%) and specificity (larger than 96%) to be incorporated as an alternative test in monitoring and control programs.

Chapter 4 shows that when all types of experimental infections are considered, the median time till seroconversion is 57 days and the main factor that influences time till seroconversion is the type of transmission used. When only studies using experimental inoculation are considered the median time is estimated as 48 days.

Chapter 5 deals with natural transmission of BLV and results reveal that the period around parturition is an important risk period and that heavily infected herds seem to have an increased proportion of young stock infected. The infection transmission parameter was estimated as relatively high and combined with a long infectiousness period this resulted in a high reproductive ratio (R_0) (8.8).

The basic information obtained in chapters 3 to 5 was used in the subsequent chapters.

PROSPECTS FROM MODELLING

An important prediction of our transmission model is that, even in a relatively small, closed dairy herd, the time-scale for a BLV outbreak may be as long as several decades (**Chapter 6**). R_0 was estimated as 9.5. The sensitivity analysis on R_0 showed that all measures directed to reduce the transmission rate are potentially effective given operational control measures (Chapter 6).

The time-scale of BLV eradication within a dairy herd may be very long when infection does become endemic. To optimization the time needed to achieve eradication, a strategy that includes several control measures is necessary. Depending on the choice of control strategy, the within-herd eradication of BLV will, especially in large herds, require intensive efforts and only be effective over a time-scale of several years (**Chapter 7**).

DISCUSSION AND CONCLUSIONS

In the general discussion the results are discussed with respect to the initial objectives. Simulation modeling showed that after introduction of only one infectious animal (either a heifer or adult cow) in a naive herd there is a high probability that an outbreak will occur and most likely the infection will then become endemic. Hence, if farmers want to eradicate BLV from their herds or keep their herds free of BLV, they should be extremely cautious in the addition of new animals to their herds. It was shown that although in larger herds it is possible to eradicate BLV, it takes longer and it demands more efforts in comparison with smaller herds. Simulation further showed that the time to achieve eradication was not significantly affected by a sampling frequency being either 6 or 3 months, resulting in lower costs for the farmer. The decision to adopt a specific BLV eradication strategy (combination of control measures, sampling frequency and test) that optimises the probability to achieve eradication and the time till eradication has been achieved depends heavily on the herd size. Also, early detection of infected calves dramatically reduces the time needed to achieve eradication.

Our estimation of the properties (Sensitivity and Specificity) of diagnostic tests commonly used and available for surveillance showed that they are sufficiently high for

being used in eradication strategies as simulated. Although several types of ELISA recently have been officially approved for surveillance purposes it does not incorporate the ELISA 108 in milk which can be advantageously used for routine screening.

SAMENVATTING

Het Bovine Leukemie Virus (BLV) is een retrovirus dat lymfekliertumoren (leucose) en andere aandoeningen bij runderen kan veroorzaken. Leucose is een grote economische schadepost voor de rundveesector in veel landen. De economische schade voor met BLV geïnfecteerde bedrijven bestaat naast sterfte, afkeuring van karkassen in het slachthuis en kosten voor veterinaire handelingen, ook uit export restricties voor levende dieren. Natuurlijke infecties met BLV treden alleen op bij runderen (*Bos taurus*), zebus (*Bos indicus*), buffels en capybaras ( ). Experimenteel zijn ook schapen te infecteren.

Bovine Leucouse is in 1968 voor het eerst gerapporteerd in Argentinië. Recentelijk is een prevalentie onderzoek uitgevoerd over heel Argentinië waaruit bleek dat de prevalentie op dierniveau 33% bedroeg terwijl 84% van de onderzochte bedrijven positief werd bevonden. Stratificatie naar prevalentie niveau liet zien dat op 16% van de bedrijven geen enkel geïnfecteerd dier werd gevonden, dat ongeveer 20% van de bedrijven een prevalentie had van minder dan 15%, 17% tussen de 15 en 30%, en op bijna 50% van de bedrijven waren meer dan 30% van de dieren besmet. Ondanks kleine regionale verschillen in prevalentie kan gesteld worden dat de meeste regio's zwaar besmet zijn op basis van het percentage geïnfecteerde bedrijven.

De regio waarin het onderzoek beschreven in dit proefschrift heeft plaatsgevonden (centraal Santa Fe) is de regio met de laagste prevalentie van brucellose (Res. SENASA 150/2002, Res. SENASA 497/2002) en tuberculose (Res. 115/99, SENASA), respectievelijk 45 % and 30 % van de bedrijven is gecertificeerd als zijnde vrij van deze aandoeningen. Het feit dat deze 2 belangrijke aandoeningen onder controle zijn, was aanleiding voor boerenorganisaties en de provinciale gezondheidsdienst om een verplicht bestrijdingsprogramma voor BLV te ontwikkelen, wat het bestaande vrijwillige nationale bestrijdingsprogramma (RES. 337/94, SENASA) zou kunnen vervangen. Omdat leucose het meest voorkomt op melkveebedrijven was de hoofddoelstelling van dit proefschrift om verscheidene aan de epidemiologie en bestrijding van BLV gerelateerde aspecten te kwantificeren. Kwantitatieve kennis van de transmissie van BLV is essentieel om effectieve bestrijdingsstrategieën te ontwikkelen.

MOLECULAIRE EPIDEMIOLOGIE VAN HET BOVINE LEUKEMIE VIRUS

Hoofdstuk 2 beschrijft het voorkomen van 2 types van BLV isolaten – het Australische and Argentijnse – in melkveekoppels in verschillende gebieden in Centraal Argentinië en de phylogenetische stamboom toont duidelijk aan dat het Argentijnse isolaat een separate en homogene groep is vergeleken met andere clusters. Een immuun response werd aangetoond in de meeste geïnfecteerde dieren, maar sommige persistent geïnfecteerde koeien lieten geen detecteerbare antistof respons zien (zie ook hoofdstuk 5).

De lage mate van niet-synonieme substituties vergeleken met synonieme substituties ondersteund de hypothese van zuiverende selectie van de *env*, *gag* en *pol* genen en verscheidene subunits; dientengevolge vind moleculaire evolutie plaats onder functionele beperkingen. Het blijkt dat voor de "Transmembrane Hydrophobic Region" binnen het *env* gen - tenminste voor zover het het Argentijnse isolaat betreft – de gastheer de selectiedruk bepaalt en dat natuurlijke variatie in de structuur is het resultaat is van gastheer-pathogeen interacties. Daarom is dit gedeelte van het genoom een goede kandidaat voor verder functioneel onderzoek.

KWANTITATIEVE EVALUATIE VAN DIAGNOSTISCHE TESTEN, SCHATTINGEN VAN DE TIJDDUUR TUSSEN INFECTIE EN SEROCONVERSIE EN VAN TRANSMISSIE IN HET VELD

Voor het modelmatig onderzoek waren schattingen van diverse input parameters vereist. In **hoofdstuk 3** wordt de BLV ELISA 108 (zowel in melk als in serum) geëvalueerd en het blijkt dat deze test een voldoende hoge gevoeligheid (groter dan 97%) en specificiteit (groter dan 96%) heeft om te worden gebruikt als alternatieve test in monitoring en bestrijdingsprogramma's.

In **hoofdstuk 4** wordt aangetoond dat in experimentele infecties de mediane tijdsduur tot seroconversie 57 dagen bedraagt en dat het type transmissie de voornaamste factor is die deze tijdsduur beïnvloed. Indien alleen onderzoeken worden beschouwd waarin inoculatie is gebruikt als wijze van transmissie dan bedraagt de mediane tijdsduur tot seroconversie 48 dagen.

Hoofdstuk 5 handelt over de natuurlijke transmissie van BLV en het beschrijft dat de periode rond afkalven een hoog risico periode is en dat zwaar besmette bedrijven een hogere proportie van geïnfecteerd jongvee lijken te hebben. De mate van transmissie werd geschat als relatief hoog en gecombineerd met een lange infectieuze periode resulteert dit in een hoge reproductie ratio (R_0) (8.8).

De basale informatie uit de hoofdstukken 3, 4 en 5 werd gebruikt in studies in de volgende hoofdstukken.

PERSPECTIEF VANUIT DE MODELMATIGE BENADERING

Een belangrijke voorspelling van ons transmissie model is dat, zelfs in relatief kleine gesloten bedrijven de tijdsduur van een BLV uitbraak meerdere decennia kan bedragen (**hoofdstuk 6**). R_0 werd geschat als zijnde 9.5. Een gevoeligheidsanalyse met betrekking tot R_0 toonde aan dat alle maatregelen gericht op de reductie van transmissie potentieel effectief zijn (**hoofdstuk 6**).

Wanneer een infectie met BLV endemisch op een bedrijf aanwezig is, is eradicatie een tijdrovende zaak. Om deze tijdsduur te optimaliseren dient een strategie gevolgd te worden die bestaat uit het tegelijkertijd uitvoeren van meerdere bestrijdingsmaatregelen. Afhankelijk van deze strategie zal het vrijmaken van bedrijven van BLV, en in het bijzonder geldt voor grotere bedrijven, een intensive inspanning vergen en pas effectief zijn op een tijdschaal van meerdere jaren (**hoofdstuk 7**).

DISCUSSIE EN CONCLUSIES

In de algemene discussie worden de resultaten geëvalueerd in het licht van de primaire doelstellingen van dit proefschrift. Modelmatige simulaties geven aan dat na de introductie van één infectieus dier (een vaars of een volwassen koe) in een BLV vrije koppel, de kans

groot is dat er een uitbraak plaats vindt en dat de infectie vervolgens endemisch wordt. Daarom is het voor veehouders die BLV in hun koppel willen uitroeien dan wel hun koppel vrij willen houden van een BLV infectie zeer belangrijk dat zij uiterst voorzichtig zijn wanneer ze nieuwe dieren aan de koppel toevoegen.

Er is aangetoond dat BLV ook is uit te roeien in grotere koppels koeien maar dat dit in vergelijking met kleinere koppels meer inspanningen vergt en langer duurt. Simulaties toonden tevens aan dat de tijdsduur tot uitroeiing niet wordt beïnvloed door de frequentie

van bemonstering (om de 3 of de 6 maanden), hetgeen resulteert in lagere kosten voor de veehouder.

De optimale strategie (combinatie van bestrijdingsmaatregelen, frequentie van bemonstering en type test) om BLV uit te roeien hangt sterk af van de koppelgrootte. Ook een vroege detectie van geïnfecteerde kalveren reduceert de tijdsduur die nodig is voor eradicatie drastisch.

Onze schattingen van eigenschappen (gevoeligheid en specificiteit) van diagnostische testen die routinematig worden gebruikt in de surveillance toonden aan dat deze voldoende zijn om ook gebruikt te worden in een eradicatie programma. Meerdere ELISA testen zijn recentelijk geaccordeerd voor surveillance doeleinden; de zeer voordelige ELISA 108 in melk zou aan dit rijtje toegevoegd kunnen worden.

RESUMEN

El virus de la leucosis bovina (VLB) es un retrovirus que produce linfoma (leucosis) y otros desórdenes en bovinos. La leucosis bovina ocasiona grandes pérdidas económicas en el sector ganadero de numerosos países del mundo. En explotaciones ganaderas infectadas con VLB las pérdidas económicas se relacionan con muerte prematura de animales, decomisos en frigoríficos, mayores costos por atención veterinaria, pero también se ve reflejada como consecuencia de limitaciones en las exportaciones de reproductores en pie. Bajo condiciones naturales han sido reportados como infectados, solamente los bovinos (*Bos taurus*), zebuinos (*Bos indicus*), búfalos y capibaras. Sin embargo, bajo condiciones experimentales la transmisión en ovejas ha sido verificada.

La leucosis bovina se reportó por primera vez en Argentina en el año 1968. Recientemente, en un estudio de prevalencia que se llevó a cabo a lo largo del país, se demostró que la prevalencia serológica a nivel individual es del 33%, mientras que la prevalencia a nivel predial alcanza el 84%. Luego de estratificar por niveles de prevalencia serológica individual, el 16% de los predios mostraron resultados negativos, aproximadamente un 20% de los predios mostraron prevalencias menores al 15%, un 17% de los predios mostaron prevalencias que variaban entre el 15 y 30%, y casi el 50% mostraron valores por encima del 30%. A pesar de que existen diferencias en prevalencias entre las distintas areas geográficas del país, se puede concluir que si tomamos como base la proporción de predios infectados, la mayor parte de las regiones están afectadas en forma excesiva.

El área bajo estudio (Cuenca Santafecina Central) es la región que cuenta con la prevalencia más baja de Brucelosis (Res. SENASA 150/2002, Res. SENASA 497/2002) y Tuberculosis (Res. 115/99, SENASA) del país, con aproximadamente un 45% y 30% de los predios certificados como libres. Dado que estas importantes enfermedades se encuentran bajo control, las asociaciones de productores y el servicio de salud animal provincial están interesados en comenzar con un programa piloto de control obligatorio que reemplace al programa nacional voluntario (RES. 337/94, SENASA), vigente en este momento. Dado que la enfermedad se presenta más frequentemente en explotaciones tamberas que explotaciones de cría o invernada, el principal objectivo de esta tesis fue proveer un marco analítico de refererencia que sirva para la cuantificación de diversos aspectos que se relacionan con la epidemilogía y control de VLB en explotaciones tamberas de la República Argentina. Para tal fin, es esencial la comprensión y cuantificación de la transmisión del VLB en el ganado lechero, para diseñar estrategias de control efectivas y en general para la prevención de la enfermedad.

EPIDEMIOLOGÍA MOLECULAR DEL VIRUS DE LA LEUCOSIS BOVINA

En el **Capítulo 2** se describe la presencia en predios lecheros de la Cuenca central Santafecina, de 2 genogrupos de aislados del VLB – Australiano y Argentino. El análisis filogenético muestra claramente que los aislados del genogrupo argentino representan un genogrupo independiente y homogéneo en comparación con los restantes genogrupos. Se ha podido demostrar una respuesta serológica en la mayor parte de los animales pero algunos animals, aunque se mantienen infectados persistentemente, no mostraron un nivel detectable de respuesta serológica (ver también Capítulo 5).

La menor tasa de sustituciones no-sinónimas en comparación con las sustituciones sinónimas que encontramos en el estudio, afirman la hipótesis de que ocurre una selección

purificante de los genes *env*, *gag* y *pol* y varios subunidades de ellos, de tal manera que ocurre la evolución molecular, pero bajo limitaciones funcionales. Pareciera que – al menos para los aislados argentinos - las variaciones genómicas del dominio transmembrana hidrofóbico (THR) localizado en el gen *env,* son el resultado de las interacciones entre agente-hospedador y la presión de selección es influenciada por el hospedador. Por lo tanto, este dominio representa un buen candidato para establecer futuros estudios funcionales.

EVALUACIÓN CUANTITATIVA DE LAS PRUEBAS DIAGNÓSTICAS, ESTIMACIÓN DEL TIEMPO QUE TRANSCURRE ENTRE LA INFECCIÓN Y LA SEROCONVERSIÓN Y DE LA TRANSMISSION NATURAL.

Para poder desarrollar modelos de simulación es necesario contar con información relacionada con muchos parámetros de entrada. En el **Capítulo 3** se evaluó el ELISA 108 (que puede utilizarse tanto en muestras de suero o leche) y los resultados demostraron que esta prueba diagnóstica posee una alta sensibilidad (más del 97%) y especificidad diagnóstica (más del 96%) por lo que puede ser incorporado como prueba tamiz o confirmatoria en los programas de monitoreo o control.

El **Capítulo 4** demuestra que cuando se consideran todos los tipos de infecciones experimentales, la mediana del tiempo que transcurre entre la infección y la seroconversión es de 57 días y que los principales factores que influyen sobre este intervalo es el tipo de transmisión experimental utilizado. Cuando se considera sólo infecciones experimentales producidas por inoculación, la mediana del intervalo se estimó en 48 días.

El **Capítulo 5** se relaciona con la transmisión natural del VLB y el estudio demuestra que el período alrededor del parto es un importante período de riesgo de transmissión y además demuestra que lo predios utilizados en el estudio que se encontraban excesivamente infectados poseían también una alta proporción de animales infectados jóvenes. El parámetro que define la tasa de infección se estimó en un valor relativamente alto y combinado con un largo período de infecciosidad, resultando en una tasa de reproducción básica (R_0) de 8.8. La información básica obtenida en los capítulos 3 al 5 fue usada en los siguientes capítulos.

PREDICCIONES OBTENIDAS POR MODELAJE

Una predicción importante de nuestro modelo de transmisión es que aún en predios cerrados que cuenten con pocos animales, una vez introducido el virus, la escala de tiempo que mide el brote de VLB puede extenderse a varias décadas. (**Capítulo 6)** y la R_0 se estimó en 9.5. El análisis de sensibilidad de los distintos factores sobre R_0 demostró que todas las medidas tendientes a reducir en forma directa la tasa de transmisión son potencialmente las más efectivas para ser utilizadas como medidas operacionales de control (**Capítulo 6).**

La escala de tiempo que mide la erradicación intra-predial del VLB en explotaciones lecheras puede ser muy larga una vez que la infección se convirtió en endémica. Para optimizar el tiempo requerido para alcanzar la erradicación del virus, es necesario adoptar una estrategia que incluya simultáneamente varias medidas de control. Dependiendo de la estrategia elegida, la erradicación intra-predial del VLB requiere intensivos esfuerzos, especialmente en predios que cuenten un gran número de animales, y sólo es efectiva luego de varios años de implementación (**Capítulo 7)**.

DISCUSIÓN Y CONCLUSIONES

En la discusión general los resultados de la tesis fueron discutidos en relación a los objectivos iniciales. Las simulaciones demostraron que luego de la introducción de un animal infectado (ya sea una vaca adulta o una vaquillona) en una población totalmente susceptible existe una alta probabilidad de que ocurra un brote de la enfermedad que en la mayoría de los casos terminará estableciéndose en forma endémica. Por lo tanto, si un ganadero desea erradicar VLB de su predio o mantenerse libre de la enfermedad, debe mantenerse extremadadmente cauteloso con las adiciones de animales nuevos en su predio. Hemos demostrado que aunque es posible erradicar VLB en predios que cuenten con un gran número de animales, se necesita más tiempo para alcanzar la erradicación y se demanda más esfuerzos, comparado con predios que cuenten con menos animales. Las simulaciones demostraron además que el tiempo necesario para alcanzar la erradicación no se ve afectado significantemente si la frecuencia de muestreo que se lleva a cabo para la comprobación de infección, extiende de una frecuencia de 3 á 6 meses, lo que resulta en un costo menor para el ganadero. La decisión de adoptar una estrategia específica de erradicación del VLB (vale decir una combinación de medidas de control, frecuencia de muestreo y prueba diagnóstica) que optimice las chances de alcanzar la erradicación y el tiempo esperado de erradicación, son en gran medida influenciados por el número de animals con que cuenta el predio. Además, el tiempo necesario para alcanzar la erradicación se vé drásticamente reducido cuando se incluye a los terneros en el monitoreo.

Nuestras estimaciones de las propiedades específicas (sensibilidad y especificidad) de las pruebas diagnósticas comúmmente utilizadas o disponibles en Argentina, demostraron que son lo suficientemente altas para ser utilizadas en programas de erradicación, cuando diferentes estrategias fueron simuladas utilizando diferentes combinaciones de valores. Aunque varios tipos de ELISAS han sido recientemente aprobados oficialmente para su uso en Argentina, el programa actual todavía no contempla el uso del ELISA 108 en muestras de leche, el cual podría ser utilizado ventajosamente en el monitoreo de la enfermedad.

ACKNOWLEDGEMENTS

These lyrics describe all my feelings at once (including the memory of those that are not with us anymore):

'Hush me love now don't you cry, everything will be all right,
Close your eyes and drift in dream, rest in peaceful sleep.
If there is one thing I hope I showed you: just give love to all.
Oh my love… in my arms tight, every day you give me life.
As I drift off to your world will rest in peaceful sleep.
I know there is one thing that you showed me: just give love to all,
Let's give love to all.'

CURRICULLUM VITAE

Gustavo Enrique Monti was born in Rafaela (Argentina), on 28th October of 1962. He completed high school in 1980 at the institute Escuela Nacional de Comercio de Rafaela with the degree of commercial appraiser. In 1981, he started the career of Veterinary Medicine for one semester. Afterwards, he fulfilled the military service in the Argentine Navy, at board of the A.R.A. Crucero 'General Belgrano' that which was torpedoed and it sunk in the Atlantic Ocean during Malvina's war in May 1982. Gustavo Monti received honors and a distinctiona as war veteran. Nevertheless, he continued and got his Veterinarian degree in 1989, at the former Faculty of Agronomy and Veterinary of Esperanza (currently Faculty of Veterinary Sciences of Esperanza (FCV) at the Littoral University (UNL) (Argentina). During 1989 he worked as assistant in a Science and Technology Project of the CONICET. From 1990 to 1994 he worked as Veterinarian and Farmer's adviser, at the Farmer's Technical Assistance Department of MANFREY (Farmers cooperative of trade and dairy industry) and as Veterinarian and zone manager, for the company 'La Ramada S.A'. In 1995 he enrolled at the Master of Science program at Free University of Berlin (Germany) and in July 1996 he got the degree of Master of Science in Tropical Veterinary Epidemiology, with mention 'Distinction' in his thesis. In 1996 he got a position as Assistant Professor, at the Department of Epidemiology and Department of Biostatistics, FCV, UNL. He was lecturer in the Master of Science Program at the Faculty that started in 1997, and he lead several courses related with epidemiology. During that time he participated in several research projects, congresses and workshops, he published several papers, he gave courses to veterinarians and farmers, and he provided advisory to several dairies. In 1999, he started a Phd research at Quantitative Veterinary Epidemiology group at Wageningen University and part of the research was carried out at the Federal Research Centre for Virus Diseases of Animals, Institute of Epidemiological Diagnostic; Wusterhausen, Germany under the supervision of Dr. Dagmar Beier. From February 2004 to June 2005, he worked as Senior Researcher at NRS (Royal Dutch Cattle Syndicate) CR-Delta B.V Holding. From August 2005, he has been appointed as Associated Professor at the Institute of Preventive Medicine in the Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile.

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OTHER

CONTINUING EDUCATION FOR FARMERS
1. Monti, GE. How to control Bovine Leukemia. Supercampo. 2000.

TRAINING AND SUPERVISION PLAN

EDUCATION AND TRAINING

THE BASIC PACKAGE	year	credit points
WIAS Common Course	1999	2.0
Course on philosophy of science and ethics	2000	1.0

SCIENTIFIC EXPOSURE

INTERNATIONAL CONFERENCES

	year	credit points
9th International Society of Veterinay Epidemiology and Economics Symposium (ISVEE), Breckenridge, United States of America	2000	1.0
Society of Veterinary Economics and Preventive Medicine Conference (SVEPM), Noordwijkerhout, The Netherlands	2001	0.5
X International Symposium of Veterinary Laboratory Diagnosticians (WAVLD), Salsomaggiore, Italy	2001	0.8
IX Argentine Congress of Microbiology (AAM), Buenos Aires, Argentina	2001	0.4
Society of Veterinary Economics and Preventive Medicine Conference (SVEPM), Cambridge, United Kingdom	2002	0.5
XXII World Buiatrics Congress (WAB), Hannover, Germany	2002	1.0

SEMINARS AND WORKSHOPS

	year	credit points
The Placenta, WIAS	2000	0.5
Advanced Methods for Test Validation and Interpretation in Veterinary Medicine, Free University of Berlin-University of California-Davis, Berlin, Germany	2000	0.6
Mathematical Epidemiology of Infectious Diseases. Utrecht University, The Netherlands	2002	0.6

PRESENTATIONS

POSTERS

	year	credit points
Society of Veterinary Economics and Preventive Medicine Conference (SVEPM), Noordwijkerhout, The Netherlands	2001	0.5
IX Argentine Congress of Microbiology. Buenos Aires, Argentina	2001	0.5
XXII World Buiatrics Congress. Germany	2002	0.5
SVEPM Conference.Cambridge, United Kingdom	2002	0.5

ORAL PRESENTATIONS

	year	credit points
X International Symposium of Veterinary Laboratory Diagnosticians, Salsomaggiore, Italy	2001	0.5
XXII World Buiatrics Congress. World Association for Buiatrics (WAB), Hannover, Germany	2002	0.5
PhD Retreat "Gateway to the future". Nunspeet, The Netherlands	2002	0.5

	year	credit points
WIAS Science Day 2003	2003	0.5

IN-DEPTH STUDIES

DISCIPLINARY AND INTERDISCIPLINARY COURSES

Disease Modeling. University of Utrecht, The Netherlands	1999	0.8
Conducting On-Farm Surveillance and Epidemiological Studies at the National Level, United States of America	2000	0.3
Biology underpinning Animal Sciences: Broaden your Horizon. WIAS course	2001	0.8

ADVANCED STATISTICS COURSES

Analysis of High-Dimensionality Epidemiological Data, Denmark	1999	1.0
Mixed Models for Continuous and Discrete Data in Veterinary Epidemiology, United States of America	2000	0.8
WIAS Advanced Statistics Course: Design of Animal experiments	2000	0.6

UNDERGRADUATE COURSES

System Analysis, Simulation and System management. WUR	2002	4.0

PROFESSIONAL SKILLS SUPPORT COURSES

WIAS Course Techniques for Scientific Writing	2002	0.8
Career Orientation	2003	1.0
Time Planning & Project Management	2004	1.0

RESEARCH SKILLS TRAINING

Preparing own PhD research proposal	1999	4.0
External training period in Germany (6 months; Federal Research Centre for Virus Diseases of Animals. Institute of Epidemiological Diagnostics. Wusterhausen, Germany)	2000	1.0

TUTORSHIP

Klinkert, Heelen (2.5 months, In Argentina)	2001	1.0

Education and Training Total   **30.0**