Detection and control of lentiviral infections in sheep and goats

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The small ruminant lentiviruses: a bird's-eye view

Infections caused by the small ruminant lentiviruses (SRLV) of sheep (maedi visna virus) and goats (caprine arthritis encephalitis virus) are a serious economical threat to small ruminant farming, particularly in the more intensive settings like dairy farms. Revenue is ultimately negatively influenced by decreased milk production, reduced birth rates and bodyweight and trade limitations [5] [c.f. 12] [21]. Chronically infected animals have a poor body condition and may display progressive dyspnoea, painful arthritis and indurative mastitis, factors that markedly affect animal welfare. However, in well-managed flocks overt disease is hardly seen because affected animals are culled in an early stage of this perniciously progressive disease, hence the only sign is an increased replacement rate. The infection spreads lactogenically from dam to progeny through colostrum and/or milk and horizontally on one hand via the natural close contact between the dam and her progeny [c.f. 12] [28] and on the other hand between flock members via direct and indirect contacts [10] [c.f. 12].

Vertical – prenatal - transmission is generally considered to be of low or no importance. Infections are microscopically characterised by lymphoid infiltrations and inflammatory lesions in the lungs, udder, carpal joints and central nervous system [c.f. 12] [31]. There is no cure for this usually slowly progressive disease.

Identification of infected individuals by laboratory testing and strict farm and animal sanitary management are cornerstones of control programs and the only tools available for reducing the prevalence of infection. Caution with respect to the reliability of laboratory testing for SRLV infections is augmented by the growing knowledge of the RNA viral genomes and their expression products. Immense genetic heterogeneity hampers protein as well as viral genome based tests. Due to sub-optimal laboratory tests that generate false-negative results, an early diagnosis is easily missed and the infection will spread. This reduces the farmers' support for control programs, which was already under pressure because of the costs involved, the low direct market returns, and the 'invisibility' of the long-term returns. Improvement of the diagnostic tools combined with improved test strategies will enable early detection of infection. Moreover, the use of milk samples instead of blood, particularly of bulk milk samples for antibody detection as well as the molecular biological detection of SRLV infections could lead to considerable cost reduction.

The small ruminant lentiviruses, as a group of the genus Lentivirus belonging to the Retroviridae family, will need continuous attention because they are prone to diagnostic test escape as a result of their rapid evolving genomes [c.f. 12] [43] with consequences at the genomic and epitope level [22]. Thus, the use of complementary tests is currently required and molecular-epidemiological surveillance is a prerequisite.

Retroviruses

History and taxonomy

Retroviruses are viruses mainly of vertebrates, but endogenous retrovirus sequences have also been found in genomes of insects [c.f. 12] [44]. Retro (Latin for backwards) refers to the unique property of these RNA viruses to integrate into the host genome as a DNA copy, from which the viral replication proceeds [6]. In the early 1900s, Ellerman and Bang searched for an infectious cause of chicken leukaemia and succeeded in transmitting the disease by cell-free tissue filtrates [18]. Rous identified the viral aetiology of solid tumour formation in chickens by isolating the first retrovirus, Rous Sarcoma Virus [61]. In mammals the first retroviruses discovered were mouse mammary tumour virus (mMTV) and Gross leukaemia virus (GLV) [c.f. 12]. The most prominent representative of the retroviruses, human immunodeficiency virus (HIV) was identified in 1983 [7].

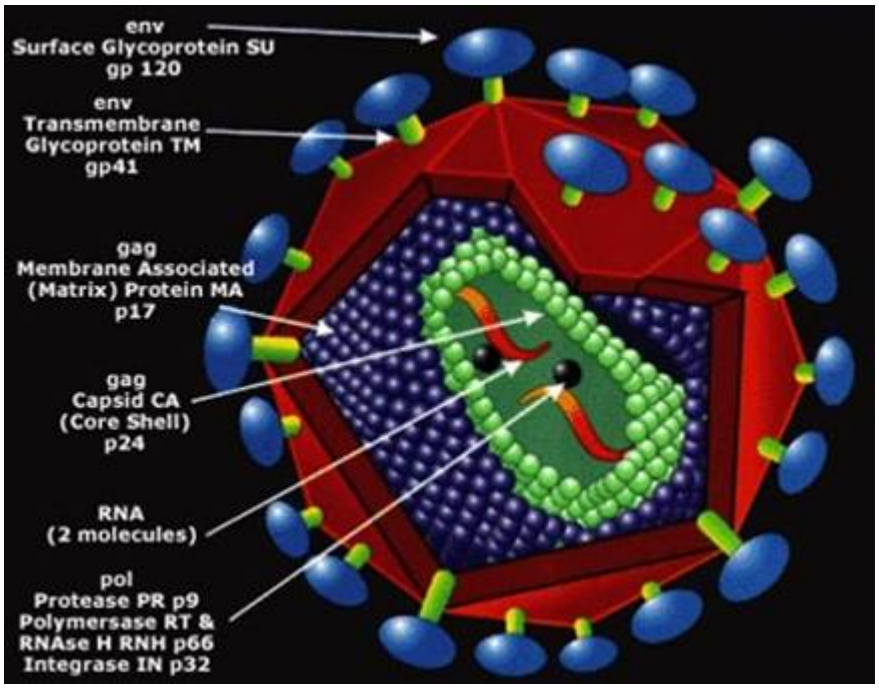

Figure 1. Diagram of a typical lentivirus (www.stanford.edu/.../ret_6_gpedesc.html)

Retroviridae are classified into seven genera (previously divided into three subfamilies) based on amino acid sequences of their reverse transcriptases: Alpha-, Beta-, Gamma-, Delta- and Epsilonretrovirus (the Oncoviruses), Lenti- and Spumavirus [54]. Retroviruses, spherical particles with diameters of 80-100 nm, contain an RNA genome surrounded by a protein capsid and a lipid envelope (Figure 1), and share functional and structural properties. The viral envelope lipid bilayer contains two glycosylated proteins: a surface protein (SU, gp135) and a transmembrane protein (TM, gp46) which is anchored within the matrix protein shell (MA, p17) just underneath the lipid envelope. The outer layer of the capsid (or core) is formed by the capsid protein (CA, p25). The core contains two identical single stranded (+) sense RNA molecules with a 5' cap and a 3' poly-(A) equivalent to mRNA. However, these retroviruses represent the only (+) sense RNA viruses whose genomes have no direct translational function and use the host's mRNA processing machinery for viral RNA synthesis [c.f. 12]. Furthermore, the capsid contains the enzymes reverse transcriptase, integrase and a protease and RNA stabilizing nucleoproteins.

Genomic organization

The RNA genome is composed of terminal non-coding sequences important in replication and internal sequences encoding the viral proteins. The 5' non-coding region includes four sub regions R, U5, PBS and Leader. R, a relative short region containing repeat sequences provides the sequence homology for strand transfer during reverse transcription. U5 is a short unique sequence between R and PBS. PBS (primer binding site) contains 18 nucleotides complementary to the 3' end of a cellular tRNA primer. L (leader) is a signal region for genome packaging [c.f. 12]. The 3' end contains three regions: PPT (polypurine tract, a short 10 base run of A/G), U3 and R. PPT primes the plus-strand DNA synthesis during reverse transcription. U3, a sequence between PP and R contains a signal sequence for transcription of the provirus. The R region is the terminal repeated sequence located on the 3' end of the viral genome.

The internal coding region is generally organised into three (or four) ORFs (open reading frames) called *gag, pol* and *env* where *gag* codes for the polyprotein forming matrix, capsid and nucleoproteins, *pol* for the enzymes protease, reverse transcriptase and integrase and *env* codes for the viral envelope proteins SU and TM. The gag termination sequence may be bypassed leading to the formation of a *gag - pol* precursor polyprotein that is proteolytically processed into the *gag* proteins, reverse transcriptase and integrase. The *env* expression product is a polyprotein to be cleaved into the two viral envelope forming proteins, the outer surface glycoprotein (SU) and the transmembrane glycoprotein (TM). Protease can have its own ORF or may be included in the *gag - pol* genes.

Replication

Retroviruses enter the host cell by attachment of their surface glycoproteins to cellular receptors. The multi-step replication process is initiated by minus-strand DNA synthesis by annealing of the tRNA primer to the primer binding site [c.f. 12] (Figure 2).

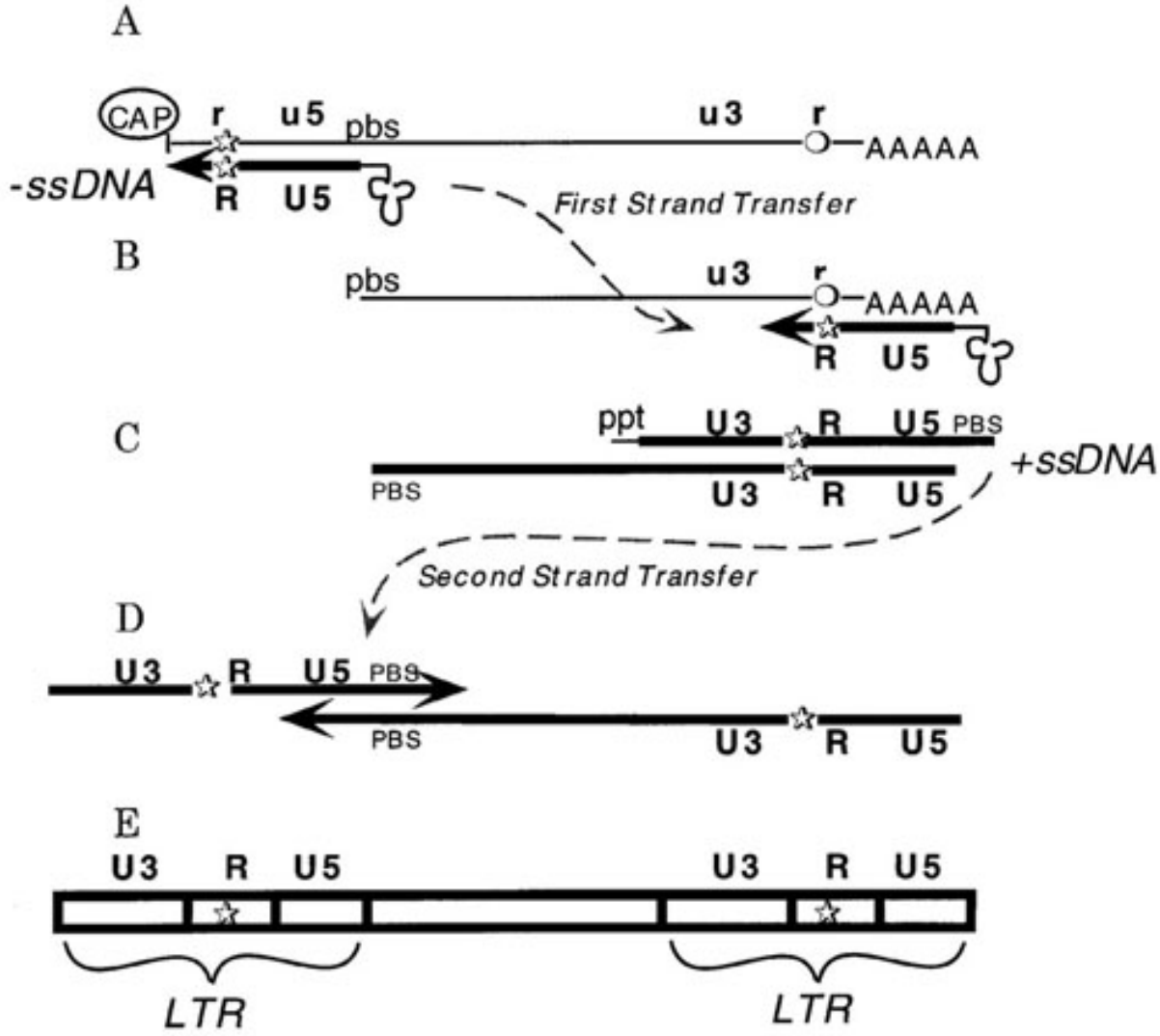

Figure 2. The multi-step replication process of retroviruses resulting into formation of the provirus.

Reverse transcriptase activity elongates the 3' end of the tRNA synthesising DNA complementary to U5 and R. RNAse H activity of reverse transcriptase degrades R and U5 sequences in the viral RNA. Subsequently, the newly formed DNA pairs with the R sequences at the 3' end of the RNA genome forming a DNA/RNA hybrid by reverse transcriptase elongation of the of the negative-strand DNA. The genomic RNA is completely degraded except for the polypurine tract. This sequence is subsequently used as a primer for the synthesis of positive strand DNA. The RNA primer is then degraded by RNAse H and the plus strand DNA primer binding site pairs with its complementary sequence on the negative DNA strand. Reverse transcriptase completes both strands to double-stranded proviral DNA with the schematic structure:

5' - U3 – R - U5 – PBS – Leader – GAG – POL - ENV(PPT) - U3 – R - U5 - 3'

The DNA is translocated into the nucleus and integrated by integrase action as a provirus into the host cell genome. The provirus is transcribed and expressed using the cellular RNA polymerase II. In infected cells three different forms of proviral dsDNA may occur: circular structures containing either one or two LTRs, and the linear form, containing two LTRs. The structures containing two LTRs are infectious [51]. Transcription is regulated by the LTR through interacting with cellular transactivating factors. The viral RNA is translated by cellular ribosomes and proteins and nascent RNA is packaged into new particles that are released from the cell by budding from the plasma membrane or into vacuoles (Figures 3, 4).

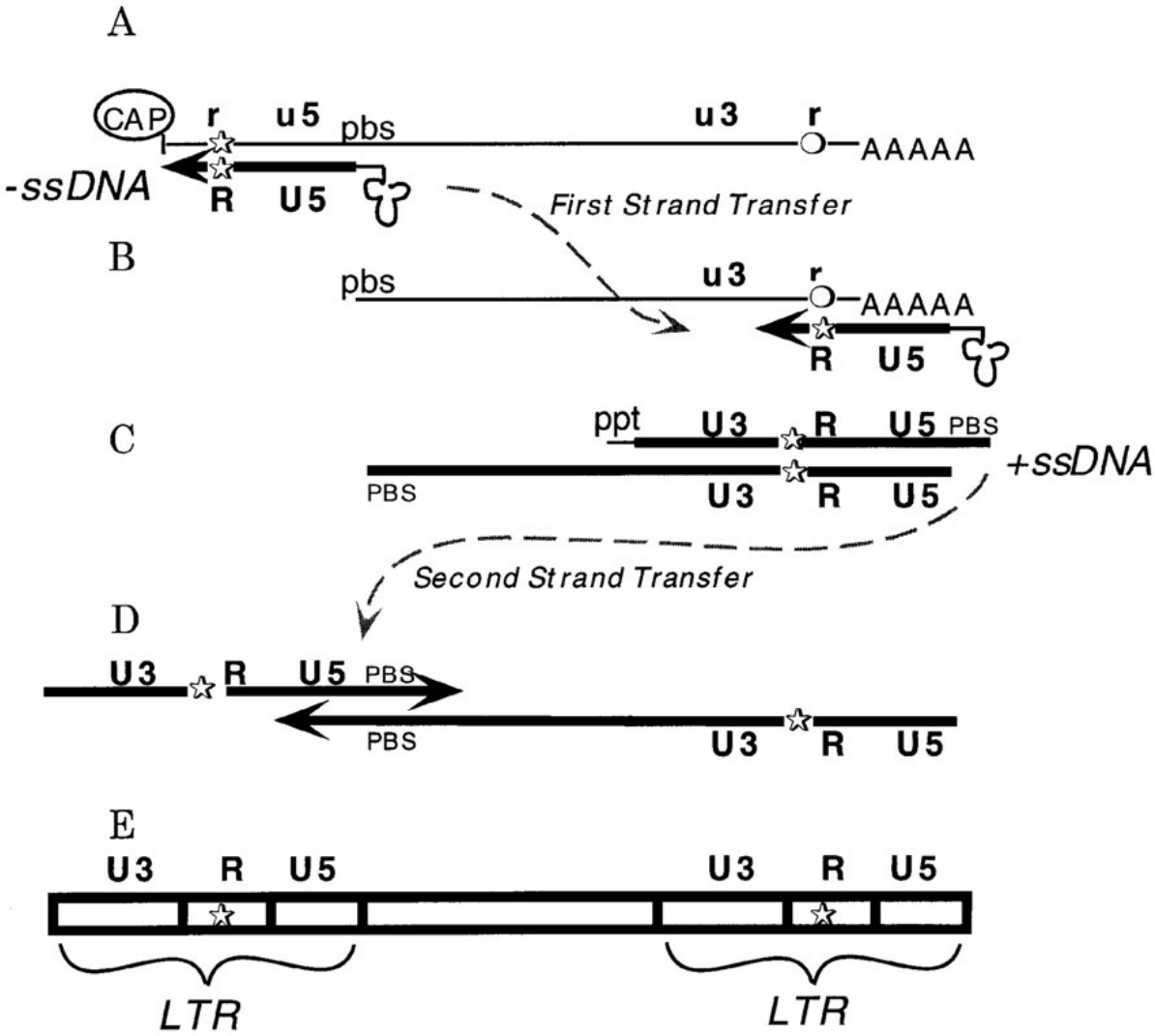

Figure 3. Thin section electron microscopic images of murine leukaemia virus particles budding from infected 3T3 cells. A) A virus particle budding from the plasma membrane. B) Mature (arrows) and immature (arrowheads) particles inside the intracellular endosomes. C) A particle budding into a vacuole [34].

Lentiviruses

Taxonomy

The genus Lentivirus is divided into five groups, based on host specificity: The bovine lentivirus group (bovine immunodeficiency virus, BIV and Jembrana Disease Virus, JDV), the equine group (equine infectious anemia virus, EIAV), the feline group (feline immunodeficiency virus, FIV and puma lentivirus, PLV), the ovine / caprine group (maedi visna virus, MVV and caprine arthritis encephalitis virus, CEAV) and the primate lentivirus group (human immunodeficiency Virus, HIV and simian Immunodeficiency virus, SIV) [c.f. 12]. Another classification of the lentivirus genus is based on viruses that cause immunodeficiency and those that do not. Lenti (Latin for slow) refers to the prolonged time from infection to disease [c.f. 12]. The *in vitro* characteristics of the lentiviruses are different from other retroviruses in that they do not need dividing cells for replication.

Replication

All lentiviruses show similar gene organisation carrying the four genes encoding the structural viral proteins. Replication is strictly controlled as is shown by maedi visna and caprine arthritis encephalitis viruses that infect monocytes while virus replication only significantly takes place when these cells mature to macrophages [20].

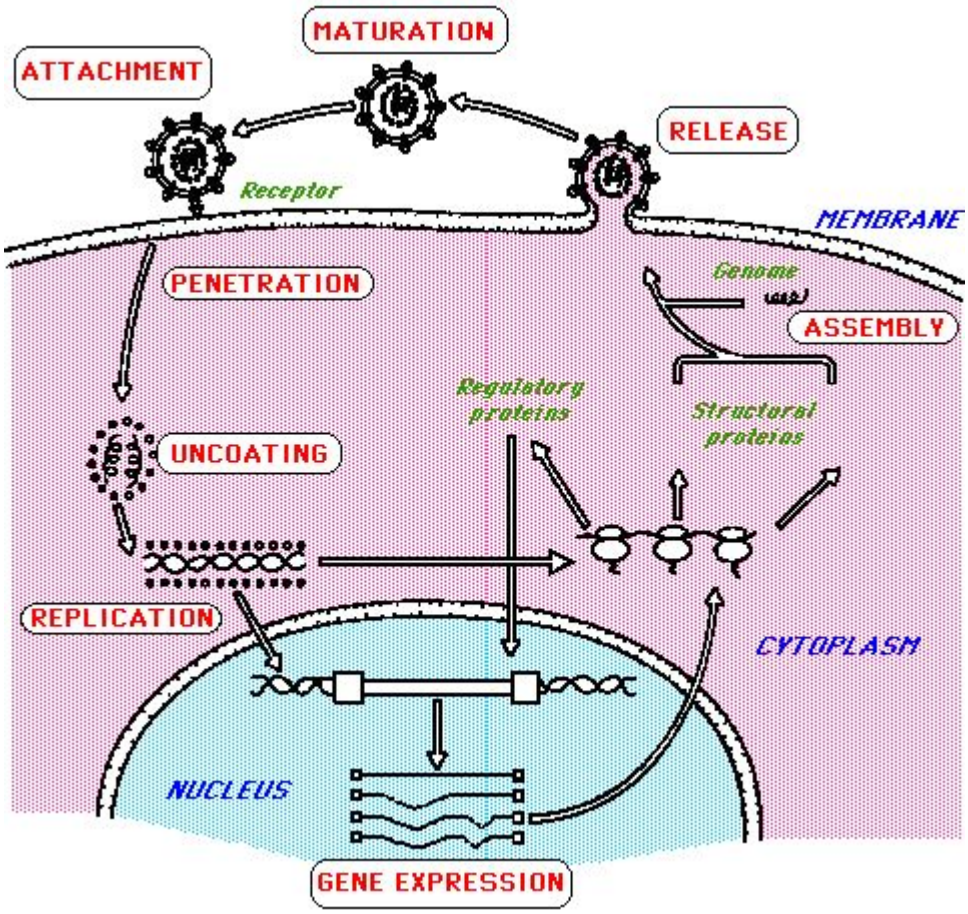

Figure 4. Retroviral replication cycle.

Lentiviruses of sheep and goats: the small ruminant lentiviruses

Historical overview

The first strong indications of an 'ovine progressive pneumonia' were reported in 1862 by Loman, a Dutch veterinarian [41]. In the 20th century the lesions of the infectious disease were described by South African, American, French and Dutch scientists denominating it as Graaff-Reinet disease, Montana disease, la bouhite and zwoegerziekte, respectively [c.f. 12]. In the fifties extensive studies were carried out in Iceland after that apparently uninfected Karakul sheep imported from Germany were recognised as responsible for wide spread dissemination of the infection into the sheep population. Spread was efficient also because of the very long winter housing period. The causative agent of the disease was identified as a virus – the first lentivirus to be isolated - and named after the respiratory and the neurological syndromes shown by affected sheep, in the Icelandic language maedi and visna respectively, hence maedi visna virus, MVV [c.f. 12] [64].

In goats, a disease similar to the ovine progressive pneumonia was described by Indian researchers in 1964 followed by reports from Germany and the USA [c.f. 12] [58]. Based on the symptoms shown by goats, the disease was called caprine arthritis and encephalitis. The causative agent for the disease in goats was identified in 1980 as a lentivirus, the caprine arthritis encephalitis virus, CAEV [c.f. 12].

The sheep and goat lentiviruses are ubiquitous, although MVV, in contrast to CAEV, was never reported in Australia and New Zealand, both major sheep farming countries [c.f. 12]. In Iceland, MVV was eradicated in 1965 by a unique and drastic stamping-out and replacement campaign. During this episode over 100.000 of sheep died of the disease and another 650.000 were killed during the eradication effort [47].

Transmission and epidemiology

MVV and CAEV are closely related but genetically different lentiviruses as was demonstrated by phylogenetic analysis. Since the viruses share many characteristics they are referred to as small ruminant lentiviruses (SRLV) [c.f. 12]. In the USA and Canada, these viruses are still considered as

different entities, Ovine Progressive Pneumonia Virus (OPPV) in sheep and CAEV in goats, although, antigenic cross-reactivity between virion-associated proteins and glycoproteins of the viruses was established rather soon after the identification of CAEV in the USA [c.f. 12]. Natural cross-species transmission was documented so MVV may infect goats and CAEV may infect sheep, the incidence of which appears to depend largely on the management conditions [c.f. 12]. Based on serological, and recently, molecular biological surveys SRLV infections are also manifest in wild small ruminants as moufflons, ibex and chamois but the viruses involved have not been extensively characterised up till now [c.f. 12].

Main routes of infection are dam to progeny transmission, i.e. lactogenically via free or cell-associated virus in colostrum and milk, or horizontally by other excretions of the dam during the intimate contact with her progeny [15] [28]. In addition, horizontal transmission between flock members occurs, most probably via the respiratory tract through aerosols [1]. The incidence of this type of transmission is associated with the length and conditions of housing [c.f. 12]. A recent study indicated an important role for infected alveolar macrophages in this respect [c.f. 12]. Intrauterine - vertical - transmission is supposed to be negligible as well is perinatal infection [c.f. 12], other than infected-colostrum or milk feeding. With respect to vertical transmission, there are no reasons to believe that the SRLV genome is present in the germ line of sheep and goats.

Once infection has taken place, a life-long, persistent SRLV infection is established in cells of the lungs, mammary glands, lymph nodes, spleen, joints and the central nervous system. Viral replication is typically associated with the macrophages, eventually giving rise to the development of immune mediated lesions [c.f. 12]. Interestingly, a recent study provided evidence for transmission of OPPV-provirus and antibodies via colostrum/milk to lambs that neither developed a productive infection nor actively produced specific antibodies [25]. This suggests that certain events – probably host determined - are needed to establish infection or to actually prevent it. At least the presented evidence for proviral clearance may imply that the process of lactogenic infection is more complicated than hitherto thought. Obviously, the roles of the infecting virus strain, the neutralizing antibody levels and the antibody dependent cellular cytotoxicity (ADCC) in relation to this proviral clearance need more study, but the concept of transient ‘proviral infection’ in contrast to the generally accepted persistence concept is new and opens alternative views on the efficiency of lactogenic transmission, and possibly on protection against infection.

Recently, evidence was obtained for the presence of viral nucleic acids in seminal plasma and tissues of the genital tract of male small ruminants, suggesting the possibility venereal transmission either through the natural route or via artificial insemination, but its contribution to the epidemiology of SRLV is considered of minor importance [c.f. 12] [50]. The genetic constitution of the host has long been considered to influence the outcome of an SRLV infection [c.f. 12] [33]. The ultimate illustration of this is the importation into Iceland of Karakul sheep from apparently uninfected origin introducing a dramatic epidemic disease in the native sheep population [c.f. 12]. Breed associated susceptibility for disease has been suggested: Texel, Border Leicester and the Finnish Landrace breeds are regarded quite susceptible while Columbia, Rambouillet and Suffolk sheep are regarded rather resistant [c.f. 12] [48]. It has also been suggested that susceptibility is associated with coarse-wool type breeds [c.f. 12]. In goats, the notion of breed associated susceptibility was supported by the linkage between the caprine leukocyte antigen (CLA) system and virus induced arthritis [62]. A recent comparative natural transmission study with a limited number of sheep showed a tendency for association between infection susceptibility and blood PCR consistency suggesting differences in viral load and the progressiveness of the lesions between two breeds [c.f. 12]. Another recent study provided experimental data that support a role of hosts’ genetics in viral replication by demonstrating an association between certain MHC class II alleles and the viral load in blood of naturally infected sheep of different breeds [26]. Other research also showed evidence for the relation between viral load and incidence and severity of lesions [c.f. 12] [27]. Besides the role of host genetic factors in viral replication and as a likely consequence, the development of lesions, there are also studies suggesting that virus strains may differ in pathogenicity or may even be non-pathogenic [3] [c.f. 12]. However, given the established role of the hosts’ genetics, it remains to be seen if strains occur that are non-pathogenic in every host. In this view, it is of course important to question also whether host insusceptibility is strain independent, particularly in view of the genetic dynamics of lentiviruses.

Small ruminant lentiviruses show a tropism for cells of the macrophage lineage. Monocytes have a relatively low susceptibility for infection and show a low level of viral RNA transcription in contrast to mature macrophages where susceptibility to infection increases with maturation and virus replication is amplified [c.f. 12] [20]. The replication *in vivo* is strictly controlled as is shown by the fact that macrophages become infected in specific tissues while *in vitro* the viruses replicate in almost all kinds

of cells, including those of human origin. Because of the broad range of cells of the natural host as well of foreign origin in which they grow, the viruses are categorized as amphotropic. In sheep with advanced disease, a reduction in CD4+ cell number has been observed, similar to that following an HIV infection in man, but without evidence of immunodeficiency [c.f. 12]. Humoral as well as cellular responses may provide protective effects, especially early in infection. However, T-cell proliferation may be related with arthritis and a higher viral load as was described for CAEV infected goats [c.f.12] [65].

The kaleidoscope of diseases

Most SRLV infections are asymptomatic for quite some time after infection. In the earliest stages of disease, sheep may suffer from poor body condition and subsequently slowly develop respiratory signs characterised by progressive dyspnoea. Animals often succumb to secondary bacterial pneumonia. Clinical signs of visna start with subtle neurological symptoms such as trembling of the lips, weakness of the hind limb and loss of condition. Symptoms may progress to ataxia, tremors, paresis, blindness and paraplegia. In sheep as well as in goats, slowly progressive arthritis and indurative mastitis (hard udder) may become manifest, the latter hampering the growth of the lambs because of reduced milk production. A specific manifestation of SRLV infection in goats is encephalomyelitis occurring in young kids. In adult goats the main syndrome is chronic and painful arthritis with synovitis particularly affecting the carpal joints. Early symptoms may include distension of the joint capsule and various degrees of lameness. In association with arthritis and synovitis, chronic progressive pneumonia is occasionally observed. Within-flock-prevalence as expressed by seroprevalence rates ranges up to 60 and 100% in sheep and goat flocks respectively. Morbidity may be high in older animals but as a result of management adaptations, the mortality is usually low in regions where SRLV infections are endemic [c.f. 12] [48]. In areas where the infection is newly introduced, mortality may be considerable since management practices have not yet been adapted.

Diagnosis of small ruminant lentivirus infections

Clinical diagnosis

Sheep may be suspected of SRLV infections in case of wasting with slowly progressive respiratory distress, neurological symptoms, indurative mastitis or arthritis from 2 years of age onwards. Adult goats show symptoms of (poly) arthritis and indurative mastitis, in kids progressive paresis/paralysis should raise suspicion.

Differential diagnosis

The differential diagnosis includes pulmonary adenomatosis (Jaagsiekte), parasitic lung infections and caseous lymphadenitis in animals with respiratory symptoms.

In neurological cases listeriosis, scrapie, rabies, louping ill, parasitic infections of the central nervous system and brain tumours may be considered. The differential diagnosis for SRLV induced arthritis may include traumatic arthritis and infectious arthritis caused by *Mycoplasma* species [c.f. 12].

Laboratory diagnosis

For prevention and control of SRLV infections, early detection is essential. Post-mortem diagnosis is based on the typical, but non-pathognomonic lesions including meningo-leukoencephalitis with secondary demyelinisation in the tissues of the central nervous system, lymphoid hyperplasia and lymphocytic interstitial pneumonia in the lungs and mononuclear infiltration of the periductular stroma obliterating the normal mammary tissue [c.f. 12] [31]. Tools indicated for early detection consist of laboratory methods for the detection of 1) humoral immune responses and 2) (pro-)viral nucleic acid.

1) Methods for antibody detection

A ‘gold standard’ for the detection of SRLV infections does not exist up till now. For antibody detection, the agar gel immunodiffusion test (AGIDT) and enzyme-linked immunosorbent assays (ELISA) are the current internationally prescribed tests [46]. The AGIDT shows limited sensitivity but excellent specificity as compared to radioimmunoassay (RIA) and ELISA. Besides its limited sensitivity, the main drawback of the AGID test concerns its subjective interpretation [c.f. 12]. Initially ELISAs were home-made, but now several kits are commercially available in different formats such as indirect or blocking assays using whole or disrupted virus, recombinant proteins or synthetic peptides for antigens. However, few have been evaluated internationally [c.f. 12]. Pitfalls in serological testing are the relatively long time between infection and antibody production, the so-called serological gap and the possibility of intermittent antibody production. Other false negative results may be due to antigenic

variation between the viral strain(s) used in the test and the actually circulating strains. ELISAs using local strains from a particular area may reduce the risk of misdiagnosis [23]. Tests such as RIA and radioimmunoprecipitation (RIP), applying radiolabels, are of limited use because of restricted availability of suitable laboratory facilities as well as personnel. RIA and RIP share their complexity of methods with the non-radioactive label assay, western blotting (WB) [c.f. 12]. These time consuming methods show a relatively poor reproducibility and repeatability and are not suitable for large scale (automated) testing.

2) Methods for (pro-)virus detection

Virus isolation may be performed through co-cultivation of the white blood cell fraction, tissue or body fluid of an infected animal with permissive cells - e.g. sheep plexus choroid cells or goat synovial membrane cells - and subsequent checking for cytopathic effects or for reverse transcriptase activity. These laborious methods may be hampered by the non-permissiveness of cells to particular viral strains. Because of the generally poor growth characteristics of these viruses, tissue culture based methods are not suitable for early detection. Immunohistochemical (IHC) techniques using smears, histological sections or cytospin preparations never became popular for routine screening purposes because of the high costs of these methods and their limited sensitivity [c.f. 12]. Polymerase chain reaction (PCR) based diagnosis may focus on detection of the viral RNA using reverse transcription PCR (RT-PCR) or focus on detection of proviral DNA sequences integrated into the host genome. Because of the immense heterogeneity of the viral RNA genomes, the diagnostic value of these assays very much depends on the design of the oligonucleotides for priming and of the probes for detection. Performing an inventory of local circulating viruses by means of molecular epidemiological methods may create a secure basis for sensitive and reliable detection of viral or proviral nucleotide sequences by means of PCR [24]. The use of real-time PCR (rtPCR) enables quantification of the target under investigation and also limits the risks of amplicon contamination that especially hamper nested PCR methods. An important feature of PCR based methods is the possibility for early diagnosis of SRLV infections by detecting proviral sequences when antibody production is absent, still is at a low level or when maternal antibodies hamper serological detection methods.

Sample suitability

A broad range of samples is basically suitable for serological detection, e.g. blood, serum, plasma, milk, tears and other body fluids. From the cell-fractions in blood and milk and several tissues, viruses can be isolated, which means that PCR assays can also be performed on such samples as well as on histological sections. If samples are to be used for both antibody and proviral DNA detection, ethylenediaminetetraacetic acid (EDTA) -treated blood may represent a useful sample; the plasma can be used for antibody detection and the buffy coat cells for PCR. Milk may be also a suitable sample: the whey can be used for serological methods - except the AGIDT - and the milk cells for PCR. Samples for histology include affected tissues, like lung, brain and udder.

Prevention and Control of SRLV infections at the flock level

Control strategies

To date, no effective vaccines against SRLV infections are available. Moreover, there is no effective treatment for diseased animals. Uninfected populations should be protected from infection by sanitary and regulatory measures. There are basically two options for infected populations: a) adapt animal management practices to reduce economic loss as much as possible, or b) implement control programs based on the identification and removing of infected animals. Timely removal of infected sheep or goats from their flocks combined with strict flock management is a proven method to obtain SRLV-infection-free populations [29]. In severely infected flocks, non-infected animals can be obtained by immediately removing newborn lambs from the maternal environment followed by artificial rearing [28]. Generally, SRLV control programs aim to provide a high level of certainty with respect to the freedom of SRLV of the stock to be sold to participating flocks. Flocks not meeting the requirements of the program loose their so-called certification.

Participation in most control programs usually leads to a certified SRLV-free flock status which enables animal exchange without spread of the virus. Such certified flocks commonly benefit from the absence of the SRLV-associated economic losses and from the extra market value of breeding animals sold. However, many participating farmers feel that the costs are higher than the benefits and that the limitations and obligations of the program are significant. It should be noted however that many of today's sheep farmers have not personally experienced the damage that SRLV eventually brings. Nevertheless, they continue participating because their breeding societies made it mandatory.

On the other hand, many of today's modern goat dairy farmers are perfectly aware of the negative effects of CAEV on their economic performance. Iceland is the only European country that definitively eradicated the disease by an intensive stamping-out campaign and repopulation by introducing SRLV-free animals [47]. Control programs are now conducted in many European countries including the Netherlands - as a pioneer - , Finland, Belgium, Denmark, France, Norway, Sweden and Switzerland, as well as in the USA and Canada.

The Dutch program for SRLV-control

In 1982, the Dutch Animal Health Service launched an SRLV-control program based on the absence of serologically detected infected animals in closed flocks and the implementation of strict regulations to prohibit physical contacts with animals of unknown status. Two consecutive negative flock tests with a six months interval lead to a certified SRLV-free flock status; animals sold from those flocks receive a certificate proving their status. Under this program the status is maintained on the basis of negative flock tests every two years (all animals > one year). Four years later, strategic testing was introduced in which the number of animals to be tested is based on a sample number required to detect a 2 % infection rate with a confidence level of 95%. Upon detection of a positive sample, the flock immediately loses its status. In order to regain it, all seropositive animals, in the case of females including their offspring, should be removed until the entire flock has passed two consecutive negative flock tests with an interval of six months. In other countries, similar approaches are based on this scheme but the number and frequency of retesting to reach the SRLV-free status differ. All these programs are not without problems since the time between infection and seroconversion may vary from weeks to months - the 'serological gap' - while particular animals may never show an immune response [c.f. 12] and seropositive mothers may be tested negative shortly after lambing because of antibodies migrating into the colostrum [c.f. 12]. This basically implies that there is a small risk that certified flocks harbour misdiagnosed animals and that - certified - animals sold from these flocks are infected. A control regime applying serological as well PCR testing may further reduce this risk.

Considerations

The main risks for within flock SRLV transmission are ingestion of infected milk or colostrum and direct or indirect contacts with infected animals. The use of semen from infected males for artificial insemination presents minor risks for transmission of the virus that is of course eliminated by excluding infected semen donors. Also, risk of transmission via embryo transfer is considered negligible, provided the embryos have an intact zona pellucida and are washed according to the International Embryo Transfer Society protocols [c.f. 12]. When infected animals are culled, conservation of genetically valuable offspring may be achieved by caesarean section or separation of the lambs from the infected dams immediately after birth. Newborns must be fed with uninfected colostrum or milk from small ruminants or cattle and/or artificial milk. Farm management should focus on restriction of contacts with other flocks and particularly with flocks of unknown status. Indirect contact with such flocks may e.g. occur via veterinary practitioners, transporting vehicles or breeding males.

The present control programs should be optimised, at least to maintain a population of uninfected breeding animals. SRLV-free flocks will experience higher productivity, breeding sales as well as animal welfare and enhance possibilities for trade. In most countries SRLV control programs are voluntary, but for cross-border movement, most countries apply the restriction that animals should originate from SRLV-free flocks. To ensure and facilitate the continuation of the existing control programs, costs involved must be reduced and, the reliability of negative test results should be improved.

Reliable and cost-efficient laboratory diagnosis

Evaluation of tests for the detection of SRLV antibodies

Serology based methods, especially ELISAs for detection of SRLV antibodies are attractive because of their low costs and a relatively ease of application. Possible drawbacks associated with such methods are the prolonged time between infection and a detectable humoral immune response, the so-called serological gap, or even, the total absence of antibody formation. An additional drawback of serology may be a limited scope for detection of active infections, especially when young animals that were fed with milk or colostrum containing maternally derived anti SRLV antibodies are tested while sensitivity may be hampered by antigenic heterogeneity, i.e. when the protein epitope composition used for the coating of ELISA micro plates does match with locally circulating virus strains.

Five commercially available ELISAs [A–E] were evaluated using sera from sheep and goats with defined SRLV infection statuses. The samples were also tested by AGIDT, which was until recently the method officially prescribed by the Office International des Epizooties (OIE) without, however, defining the antigen to be used. The most commonly used antibody detection test for routine screening is the AGIDT [4]. In the AGIDT for sheep, the test only identifies antibodies against gp135 since precipitation lines are judged for confluence with a monospecific anti-gp135 serum. The AGIDT does not represent a good gold standard neither for sheep nor for goat samples, since the results of many publications show a consistently lower sensitivity (about 70 % relative sensitivity) for the AGIDT as compared to ELISAs. Furthermore, the test is quite laborious and consequently expensive, and in addition it is not suited for automation [4] [c.f. 12]. The specificity of the AGIDT, however, is considered high and therefore the test is often used as a confirmatory test. More sophisticated alternatives for confirmation such as immunoblotting and polymerase chain reaction techniques did not find wide acceptance because of complexity of methods and difficulties regarding standardisation.

ELISAs for detection of SRLV infections can be broadly categorised into indirect ELISAs that use whole virus for coating of the microplates [A, B], competitive ELISAs using monoclonal antibodies [C] and tests that use recombinant core proteins combined with synthetic transmembrane glycoprotein gp46 [D] or recombinant core proteins only [E] for antigens [4]. For the test evaluation, serum samples were collected from certified SRLV-free sheep and goat Dutch flocks, from known infected sheep and goat flocks with clinical and histo-pathologically confirmed cases and from sheep and goat flocks with unknown SRLV status. Furthermore, sera from sheep were obtained from a SRLV free region, Iceland, as well as from experimentally infected sheep and goats that were sequentially sampled (Dr. Christian Vitu, AFFSA, France). For antibody detection in sheep with obvious clinical symptoms, test D was more sensitive than the AGIDT and significantly more sensitive than the other ELISAs. This may be due to the simultaneous detection of antibodies directed against the proteins p25 and gp46 in this test [4] [c.f. 12], whereas in tests C and E only antibodies against p25 are detected. Although antibodies against p25 arise relatively early after infection, these antibodies seem to decline once clinical signs appear. Antibodies to the envelope glycoproteins gp46 and gp135 arise later during infection, but seem to persist also in the clinical phase [4] [11] [32].

The whole virus indirect ELISAs A and B, however, also showed a poor sensitivity for detection of sheep with clinical symptoms of infection but showed a much better sensitivity for detection of infected goats and also for the sample panel originating from clinical cases, reflecting the choice of a CAEV strain for coating of the test microplates [c.f. 12]. Notably, these differences illustrate the antigenic heterogeneity within SRLV strains and its effects on antibody detection. In the category of sheep sera from non-certified flocks the highest seroprevalence was detected with test D. Lower detection limits and earlier seroconversions also sustain a higher analytical sensitivity for test D as compared with tests C and E, and confirm the results presented in another study [11] which demonstrates a higher sensitivity for serodiagnosis of MVV infections for a combined recombinant p25/transmembrane protein gp46 indirect ELISA as compared with a viral core protein p25 based ELISA [E].

In goat flocks where clinical cases were diagnosed, the seroprevalence as measured by tests D, A and B again was relatively high. The relatively poor sensitivity of test C may be explained by the observation that for goats a higher immunoreactivity for the transmembrane glycoprotein than for the core protein has been reported [c.f. 12]. For sheep samples test D showed the lowest detection limit while test E showed better characteristics for goat samples. Test D also detected seroresponses after experimental infection in sheep consistently earlier than the other tests. Antibodies in goat samples are effectively detected by the ELISAs except for test C and the AGIDT which obviously had lower sensitivities. No significant differences were observed between the tests with respect to specificity. Intra- and interplate variances were lowest for test A, but did not show significant differences for the other ELISAs.

In conclusion, the observed sensitivities of the tests were correlated with the host species, underlining the problem of antigenic heterogeneity, and the category of infected animals under investigation while all tests showed similar specificities. Ultimately, the indirect test D of which the solid phase is sensitized with a combination of the core protein p25 of MVV produced in *Escherichia coli* and a peptide derived from the transmembrane protein gp46 had the best overall performance for detecting SRLV infection related antibodies in sheep and goats.

Efficiency and cost-reduction

Costs related with the laboratory diagnosis of SRLV infections in sheep and goats start with the collection of the samples; blood samples are relatively expensive because they require veterinary involvement. Other costs depend for example on prevalence of disease in infected flocks, costs of the

testing kit but also on the hands-on time in the laboratory. It was attempted to reduce the costs of laboratory testing using the relatively inexpensive ELISA technique. Costs related with the use of the technically preferred test were estimated to be still relatively high because of the high dilution rate of serum samples as prescribed for the test and the relatively high costs of the kits. These high serum dilutions, at the same time, did not tolerate pooling of the generally low titre samples by introducing loss of test sensitivity.

Pooling of samples is generally accepted as a cost-effective screening method of samples in human blood banks, provided the seroprevalence is low. Successful pooling strategies without loss of sensitivity and specificity of testing but providing remarkable reduction of the costs of testing were developed for the mass testing of sera for human immunodeficiency virus [c.f. 12] [35] [39] [56] [57]. Also for the mass screening for the serological detection of HTLV types I and II, syphilis and hepatitis B and C infections in man successful pooling strategies were developed and applied [2] [13] [14] [15] [19]. In addition, pooling of samples for the screening of the pathogens *Neisseria gonorrhoe and Chlamydophila trachomatis* was described [36] [37]. Especially the screening in low prevalence populations may imply considerable reduction of the costs of testing as is illustrated by the estimated 75% cost reduction as a result of serum pooling for testing of blood donors for hepatitis C antibodies [38]. Veterinary pooling strategies were reported for the diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* in faecal samples and bovine viral diarrhoea virus (BVDV) antibodies and viral RNA in bulk milk [17] [45].

As mentioned before, a drawback associated with the use of the selected ELISA is the costs per sample associated with the serum pre-dilution. The relatively low antibody titres of SRLV infected sheep and especially goats indicated that pooling of samples using standard test conditions was not feasible. Reports were published [12] on the efforts to design a reliable pooling protocol, hence reducing the costs per sample. Ultimately, testing of serum samples at lower pre-dilutions than advised by the manufacturer of the test was evaluated, resulting in a considerable improvement of the identification of weak positive and doubtful individual samples as compared to testing under standard conditions. At the same time, this modification, turned out to be the key for pooling of sera. At was experienced, traditional pooling of serum samples provoked loss of testing sensitivity because of the higher sample dilutions to be applied. The adaptation of the test enabled the use of pools up to 8 to 10 sera, which as such considerably reduces costs. However, assembling of the pools and retesting of individual samples from positive pools lead to extra costs, which depend on the prevalence of infection in the population under investigation. Thus, the cost and benefit analysis, taking the different cost determining factors into account indicated that the optimal pool size consisted of 5 samples.

In conclusion, testing of pooled samples with the modified protocol proved to be cost-effective and reliable. Cost saving related with the screening of low prevalence sheep and goat flocks for anti SRLV antibodies was estimated to be 40 - 50%. This cost reduction is particularly relevant to the bi-annual SRLV-free-status monitoring of flocks participating in the Dutch national SRLV control program. Further options for cost reduction were explored by evaluating the possibilities of using other samples than serum which was achieved [12] by evaluating the use of the selected ELISA on plasma samples and on individual milk samples and, ultimately, on bulk milk samples. The suitability of plasma as a testing substrate instead of serum for use in the ELISA was considered because EDTA blood samples and thus plasma may be available in the framework of other testing programs. Although plasma samples are not expected to cause technical problems in ELISA assays, this had still to be established. To knowledge, no formal comparison has been published so far. This study found excellent sensitivity, specificity and agreement of test results of plasma samples and corresponding serum samples, demonstrating that plasma can be used instead of serum. However, for plasma as a diagnostic matrix, still blood sample collection by veterinarians is required; various tests could be combined in the same sample, making sample collection more cost-efficient.

Costs of any monitoring program can be significantly reduced using individual milk samples instead of serum [8] [40] [66]. In this study, a good to excellent diagnostic performance was found in terms of agreement, correlation, sensitivity and specificity for milk samples from goats and sheep as compared with their corresponding serum samples. The results show that milk represents a reliable replacement for serum, or plasma, when using the ELISA for SRLV-antibody detection. Testing of milk will save the cost of veterinary involvement in the sampling. For the small ruminant dairy industry, the use of bulk milk as a testing substrate would offer a very substantial cost reduction in SRLV-infection monitoring, if sufficiently sensitive. The results of this study show high specificity, in that SRLV-free flocks were all found antibody negative in bulk milk. Sensitivity could not be evaluated since the infection rates of the non-free flocks were unknown. In a more theoretical approach, using dilution series in negative milk of pooled, ELISA positive milk samples (n = 36) and tested according to instructions of the manufacturer,

a putative detection limit of 1.5 - 3% within-herd prevalence was obtained and when these tested these series without pre-dilution, a putative detection limit of < 1% within-herd prevalence was found. Thus, the results obtained so far are encouraging enough for further elaboration of the use of bulk milk for the detection of SRLV-antibodies. Because of the variable constitution of bulk milk due to variation in antibody levels, volume of milk contributed, and the dilution characteristics of individual antibodies for each animal contributing to the bulk milk, more work needs to be done, i.e. determining the cut-off for undiluted milk and establishing the detection sensitivity related with the within-herd prevalence and individual antibody titres. It is supposed that because of the low costs related with bulk milk investigation for SRLV-antibodies a higher frequency of testing is feasible that may compensate for variations in the composition of the bulk milk, at the same time providing early detection of infection which is a corner stone of effective control of infection.

Early detection of infection by PCR

Development of real-time PCR for provirus detection

Antibody detection for the laboratory diagnosis of SRLV infections implicates misdiagnosing of infected animals because of 1) detecting maternally antibodies, 2) detecting no antibodies because of the prolonged time between infection and the appearance of humoral immune responses to infection or the complete absence of response to infection and, additionally, by the simple fact that 3) every biological detection test has limitations with respect to sensitivity and specificity. Nevertheless, antibody detection methods are widely used in diagnostic laboratory settings because of the relatively low costs and practicability [12].

In an effort to be able to identify false positive as well as false negative results of antibody testing, the development of molecular biological detection of proviral DNA sequences integrated into the genomes of SRLV infected sheep and goats by means of real-time polymerase chain reaction (PCR) was described in detail [12]. In view of the notorious heterogeneity of the lentiviral genomes, the main obstacle for successful test development is the design of oligonucleotide primers that are not hindered by this phenomenon. In the course of a European project (CRAFT 70536) it became clear that this was not an easy challenge. Initially, a published traditional PCR test [c.f. 12] was adapted to the real-time PCR format using SYBR Green I for melting point based detection. Real-time formats, especially those based on SYBR Green I, are highly practical and cost efficient in comparison to other PCR formats. Since this assay showed a limited sensitivity particular for goat samples, probably because of its design for detection of sheep circular LTR sequences, additionally an alternative primer set was designed based on evaluation of proviral SRLV sequences from sheep and goats available in GenBank as a source of bio information. Hereto, the focus was on sheep as well as goat long terminal repeat and leader SRLV proviral DNA sequences. The resulting primer 1 was designed very similar to the viral genomic primer binding site (PBS). This highly conserved retroviral sequence, consisting of 18 nucleotides, essential for reverse transcription of the viral genome, was chosen despite of its sub-optimal thermodynamics. Primer 2 was designed to anneal with sequences in the conserved early gag region. Together, these primers form the basis of the leader-gag assay.

Sample preparation is crucial in PCR practise. Comprehensive studies in the framework of the mentioned European project afore had resulted in a high yield, albeit laborious, peripheral blood mononuclear cells (PBMC) collection and extraction protocol. A previous study, also in the framework of this project, had shown that PBMC are present in any blood sample from sheep and goats, contrary to the reference tables that suggested that they might be absent [49].

Based on the negative outcome of the 189 samples from several SRLV free flocks it was concluded that both assays are highly specific. With respect to sensitivity, the leader-gag assay appeared to be rather efficient in detecting proviral nucleic acid sequences in seropositive animals. Antibody negative animals from infected flocks were also found positive in both assays but to a larger extent using the leader-gag test. Particularly the samples positive in both assays were not likely to be false-positives but most probably accounted for infected animals in which specific antibodies could not (yet) be detected. Several other studies using PCR for SRLV related nucleic acid detection reported seronegative animals that were found positive in PCR tests [c.f. 12] [59] [67]. In addition, the results from the series of sequential samples from sheep with naturally acquired infections [49] also clearly demonstrated this tendency. In four out of nine sheep, the leader-gag assay detected infections weeks to months before antibodies were detected.

These results, together with results obtained with samples from other countries strongly suggested that especially the leader-gag primers are directed to a highly conserved region of the proviral DNA. Results of an Italian molecular epidemiological study [52] indicating that their lentivirus strains are

closely related to the prototype strain CAEV-CO that was isolated in the US three decades ago also support to the conserved nature of the sequences chosen for leader-gag priming. In addition, studies on the human immunodeficiency virus (HIV) indicated that the most conserved part of its lentiviral genome is not found in one of the open reading frames, but in the 5' untranslated leader region [9] [c.f. 12].

The poor sensitivity of the LTR assay, especially for goat samples may be explained a) by the design of the favourably evaluated ELISA, biasing for selecting animals mainly reactive to genotype A, the heterogeneous MVV type and b) by the fact that the LTR PCR primers were designed mainly based on sheep circular proviral DNA sequences [60]. As may be concluded from transmission experiments [49], the sensitivity of the LTR assay is comparable to that of antibody detection indicating that these tests are functional in the case of productive infections. In this situation, because of efficient activation of the immune system, antibody production proceeds rapidly. The leader-gag assay, however, showed clearly better performance than the LTR test with respect to the results of the transmission experiment and the identification of samples of infected goats. No clear differences were noticed with respect to the overall score of samples from the different geographical areas, although in Norway predominantly phylogenetic group C sequences were found [c.f. 12] and in Spain, the more sheep and goat SRLV prototypes related, groups A or D are predominant [c.f. 12]. However, the number of samples per country investigated in these studies is rather small, prohibiting firm conclusions. The performance of the ELISA used in this study needs further evaluation because of the observed relatively low identification rate of infected goats as indicated by the results of the leader-gag PCR.

In conclusion, the leader-gag real-time PCR presents a valuable additional test to determine the SRLV infection status of individual seronegative or inconclusive animals and is a valuable addition to the existing diagnostic tools. Use of PCR as a supplement to serology, so limiting diagnostic test escape, will improve the efficiency of the detection of SRLV infected animals, thus improving the efficacy of control programs. The diagnostic performance of the two newly developed real-time PCRs was also evaluated in WBC fractions and individual milk samples in comparison with the ELISA results of the corresponding serum samples as the gold standard [12]. This implies that truly infected but seronegative animals are regarded as uninfected and PCR-positive results as false-positive, leading to poor specificity values for PCR. From the SRLV certified flocks, a subset of 50 milk samples was analysed in both PCRs: for the goat samples specificity was 94 and 88% for the LTR and the leader-gag PCR, respectively, and for the sheep samples specificity was 99 and 89% for the LTR and the leader-gag PCR, respectively. As indicated [12], specificity of both PCRs for EDTA-blood samples of sheep and goats was 100%. The initially rather crude sample preparation of the individual milk samples tested in this study may explain these false-positive results, since for the bulk samples from certified flocks the specificity was 100%, using a slightly adapted sample preparation protocol.

Both infected flocks had a high seroprevalence. In the goat flock (99% seroprevalence) relative sensitivities of both PCRs were clearly higher in individual milk samples than in blood samples. In the infected sheep flock (66% seroprevalence) sensitivities of both PCRs were higher than for goats, predominantly in individual milk samples. Since the leader-gag PCR showed a specificity of 100% in bulk milk samples from certified sheep flocks, it is tempting to conclude that the leader-gag PCR detected as infected an additional 5 out of 30 seronegative samples in the infected sheep flock under investigation, showing its additional detection value, probably for recent infections. As was shown [12], antibody negative animals from infected flocks were detected by both PCR assays but to a larger extent using the leader-gag assay. In conclusion, the performance of the PCR assays was rather disappointing in this study, especially for the goat individual samples from infected flocks. This may be a reflection of the obvious strain differences of the circulating viruses and stresses for phylogenetic research which will provide the basis for more reliable application of PCR technology in this field by reducing diagnostic test escape. Specificity data indicate detection of animals by PCR in the certified SRLV-negative flocks. The question remains whether this concerns false positive detection but incidentally these results were confirmed by serology using plasma, which may be tendentious for the presence of infected animals.

The overall results [12] show that, apart from potential cost reductions through using milk samples and, ultimately, bulk milk samples for antibody testing, infection detection sensitivity may be further improved by also applying the leader-gag assay. This is particularly interesting if the monitoring for infection is based upon more frequent bulk milk testing instead of the present testing with two year intervals. Notably, the conditions on an average modern dairy farm favour rapid spread of infection, which makes the timely detection of infection even more significant.

SRLV control in practice: application of ELISA and PCR

Rapid elimination of SRLV infection from sheep flock using a tailor-made approach

SRLV eradication and control in the Netherlands is only partly successful because of the voluntary nature of the disease control program. Since sheep breeding societies made the program mandatory, however, most breeding flocks became certified MVV-free. Initially Texel sheep breeders were interested in joining the voluntary program because of the associated export possibilities. Unfortunately, the commercial lamb producers were hardly interested in SRLV free production flocks although the benefits of an adjusted control program in this sector were clear from the beginning [30] [55]. In hindsight, this is explained by the fact that lamb production in the Netherlands is a side activity for most farmers which requires little input and involves little professionalism. On the other hand, it could also be argued that the breeding sector generally did not offer the kind of production ewe the lamb producer wanted.

Reports on the combined use of antibody and proviral DNA detection in an effort to eliminate the infection after an outbreak in a breeding sheep flock were presented [12]. Application of ELISA and PCR tests combined with relatively short sampling intervals should lead to the earliest possible detection of infected ewes and lambs. Strict flock management in view of the testing results also reduced the options for lateral transmission as much as possible. The results show that this tailor-made approach controlled the ongoing infection relatively quickly. This case report demonstrates that even under conditions which obviously favour spread of infection, application of the most sensitive detection method, antibody and proviral DNA detection, and strict adherence to the advised measures related to the test results may control inadvertent outbreaks efficiently. Actually, SRLV was eradicated after two samplings with two to three months intervals enabling this flock to regain its SRLV-free status in the quickest possible way.

The added value of proviral DNA detection by applying the highly specific leader-gag PCR was obvious: 9 samples were positive for proviral DNA while antibody negative, whereas a total of 46 samples was seropositive. This meant that 9 lambs/sheep were identified as infected and culled earlier than if serology had been used alone. On the other hand, applying PCR also saved lambs that would have been culled on the basis of serology alone. It is likely that these PCR-negative lambs were only seropositive because of maternal antibodies and were not infected. With respect to test performance, it is interesting to note that the leader-gag PCR is relatively sensitive compared to serology; 41 of 46 samples (89%) were seropositive which is in line with the study described [12] and considerably better than other PCR-based assays as presented so far [4].

Retrospective considerations

The aspects of the control of SRLV infections, analyzed and summarized with emphasis on the timeframe from the starting of the Dutch control program up to recently The Dutch national voluntary control program for maedi visna virus infections was initiated in 1982 is described [12]. At that time, about 80 percent of the flocks were infected with an average within-herd prevalence of 30 percent [63]. The program was conducted by six regional Animal Health Services in collaboration with the Central Veterinary Institute in Lelystad. After passing two consecutive laboratory tests for SRLV antibodies successfully, flocks acquired the SRLV-free status which was controlled yearly by testing all animals older than 12 months. Important part of the control program consisted of strict farm management which focused on proper animal identification and the prevention of contacts with animals of uncontrolled origin. For antibody testing, an indirect ELISA was used in combination with an AGIDT [c.f. 12]. The quality of antibody testing was improved significantly by the introduction in 1984 of a blocking ELISA using monoclonal antibodies. Doubtful or positive results of this screening test were confirmed using AGIDT or western blotting [c.f. 12].

Houwers and co-workers [30] evaluated the first four years of the voluntary control program. Participation among breeding flocks was about 70 percent (1711 flocks). More than 70 percent acquired the SRLV-free status at the end of the 4 year period by repopulation (36 percent), testing and culling (20 percent) and artificial rearing (15 percent). About 20 percent of the participation breeding flocks entered the program without measures because no antibody positive animals were detected. Since it became apparent that the aetiology of caprine arthritis encephalitis virus and maedi visna virus infections in goats and sheep, respectively, is very similar, the participation of the rapid developing dairy goat industry in the control program is substantial and growing. In the period up to 2000 the program was adapted for reduction of costs by controlling SRLV-free flocks biannually and the introduction of the requirement of only one successful test after loosing the SRLV-free status for re-certification.

In this period, technical problems hampered the quality of the laboratory testing for SRLV antibodies significantly. The blocking ELISA turned out to be no longer available and therefore the Animal Health Services for the northern, the western and the central parts of the Netherlands located in Drachten and Gouda cooperated with the Central Veterinary Institute in Lelystad on the development of an alternative blocking assay and the development of an indirect ELISA. The attempts to develop monoclonal antibodies for use in a new blocking test were not fruitful so for antibody testing the newly developed indirect ELISA was used in combination with western blotting and AGIDT testing for confirmation. Antibody testing was performed in Gouda and Drachten. However, quality of testing was disputable and very expensive because of the labour-intensive procedure, factors urging for changes also because of the absence of standardised and harmonised data management systems combined with reorganisation of the structure of the Animal Health Service resulting in one organisation. Therefore, reliable retrospective analysis over this 'black' period was difficult. Only some descriptive data could be retrieved from the archives of the former Animal Health Service in the North of the Netherlands over the period 1982 - 1994.

The period 2000 – 2008 is characterised by 1) risk factor analysis related with re-infections in SRLV-free flocks based on a case-control study and 2) the improvement of the tools for the laboratory diagnosis of SRLV infections in sheep and goats. Significant risk factors identified are mainly related with management, concerning age of weaning and colostrum feeding. A trend was observed towards less space per sheep and longer housing periods as possible risks, factors favouring horizontal, and mainly aerogenic transmission.

Suggestions for future research

1. Efficiency: bulk milk based SRLV control

Further research is needed with respect to the relationship of within-flock prevalence of infection with the detection limits of antibody based as well as PCR based testing of bulk milk samples. Early detection in SRLV monitoring programs is of crucial importance, particularly in view of the relatively slow response of the hosts' immune system by the formation of antibodies. A future experimental SRLV-monitoring program based on high-frequency sampling should include polymerase chain reaction testing to assess its additional value. The enormous cost-reduction that will be achieved with bulk milk testing in stead of (bi-) annual individual testing is expected to compensate for the extra costs of including PCR testing.

2. Molecular epidemiology

Genetic analysis of in the Netherlands circulating small ruminant lentiviruses will contribute to the understanding of the genetic, protein and antigenic make-up and their relations with the outcome of pathogenesis, epidemiology and phylogenetic relationships. Additionally, polymerase chain reaction as a tool for the reliable diagnosis of SRLV infections needs in view of the appropriate design of oligonucleotide concepts for priming of the polymerase chain reaction, the continuing monitoring of viral genomes originating from local and international sources or outbreaks because of molecular variations of the genomes due to interspecies spread of virus strains combined with the imperfect action of the RNA dependent RNA polymerases for copying of the RNA viral genomes. Also, molecular epidemiological data may be useful with respect to the working decisions with respect to control in case of SRLV outbreaks.

3. Lactogenic transmission

Further research into the immunological and viral determinants that play a role in transient presence of proviral DNA sequences after lactogenic transmission may enlighten the protective factors in breast milk that are critical to thwarting lactogenic SRLV transmission, possibly as a model for lactogenic HIV transmission.

4. Fat-lamb production

Initiation of research into the economical effects of SRLV infections for fat-lamb producers.

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