ASPECTS OF REPRODUCTION and LENTIVIRUS EPIDEMIOLOGY in SMALL RUMINANTS

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ASPECTS OF REPRODUCTION

and

LENTIVIRUS EPIDEMIOLOGY

in

SMALL RUMINANTS

ASPECTEN van REPRODUCTIE en LENTIVIRUS EPIDEMIOLOGIE bij
KLEINE HERKAUWERS
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

Small Ruminants in the Netherlands

Sheep and goats are common farm animals in The Netherlands. In recent years the number of sheep has gradually increased up to 650,000 winter-fed ewes although the number of farms gradually declined [1]. This decline was due to a reduction in the ewe-premium granted by the European Union, declining revenues for wool and meat and EU directives on nitrates aimed to reduce water pollution (manure reduction). Sheep farming is mainly a collateral branch, and nowadays only 18% of the agricultural farms have sheep. The Netherlands has a rich history in sheep farming with a number of local breeds, of which the Texel breed is well known and currently accounts for ¾ of the Dutch sheep population. Historically, several breeds such as the Veluws-, Drents-, and Kempisch heath sheep have adapted to their natural habitat and fulfil a role in nature- and landscape management. Within this context sheep are also used for the maintenance of another typical Dutch habitat, the dikes. They support maintenance of these dikes by grazing and trampling the soil. For commercial farming, composite breeds have been developed characterised by high fertility, easy lambing, good mothering characteristics and relative sober demands. Some of these breeds are kept in substantial flocks with extended in-house periods for lambing (possibly even three times in two years). Commercial sheep farming nowadays aims at the production of lambs for slaughter, wool is of insignificant importance since the price of clipping has overtaken the revenue of the wool. Milk and milk products of sheep are mostly produced on a small local scale. Apart from this commercial farming, many small hobby flocks are present.

Professional dairy goat farming is a relative young agricultural industry in The Netherlands. Traditionally, goats were kept on a small scale to produce milk for personal use. In the 1980's professional dairy goat farms were established in The Netherlands. The milk quotation system in the bovine dairy industry was a major factor for some dairy cattle farms to be transformed into dairy goat farms. In contrast to sheep farming, goat farming is a non-subsidised sector, and therefore highly sensitive to price fluctuations. The goat population has nowadays increased to over 170.000 animals, on approximately 350 professional goat farms. These farms have expanded over the last years and the average number of adult females per farm has increased from approximately 300 to 550, with large farms milking between 1000 and 1500 goats. Because of these large numbers of goats per farm, and the fact that they are predominantly kept indoors, this should be considered an intensive type of production. In addition, many goats are kept for hobby purposes and can for example be found on virtually every children's farm. In fact, in number these pet goats equal those in the professional section.

Reproductive management

Breeding is an important aspect of farming sheep and goats. The vast majority of small ruminants conceive through natural service. However, for genetic improvement supportive reproductive techniques such as artificial insemination (AI) are recommended. In an AI program the selection of males by pedigree, performance and offspring can be rigorous, as only few outstanding males are required to serve a relative large number of females. In addition, the use of frozen-thawed instead of liquid semen enables a wider distribution. AI is primarily used to introduce superior genes into a flock while dissemination of (sexual) transmissible diseases can be prevented. Moreover semen cryopreservation is a suitable way of gene banking for the conservation of rare and endangered breeds.

In the northern hemisphere small ruminants are seasonal breeders and most of the lambs are born in spring. However, out of season breeding enables year round production and results in a more balanced demand for labour. Milk and meat production can be improved both quantitatively and qualitatively by using semen of genetically superior males. AI is usually applied after (hormonal or artificial light induced) synchronisation of the females using frozen-thawed semen produced during the natural breeding season. Another reason to use AI is the limited availability of suitable males. Specifically since government regulations require breeding with rams resistant (homozygous) to the ovine type of Transmittable Spongiform Encephalopathy: Scrapie. By means of AI these -in some breeds rare- genes can more easily be distributed amongst different flocks.

Pregnancy rates after AI following hormonal synchronisation with liquid buck semen are approximately 64%, and 61% when frozen-thawed semen is used [32]. The results may be even poorer when practical experience is limited. Unfortunately, semen quality is still suboptimal with the present semen processing methods both for liquid as well as for frozen/thawed semen. This is even of more importance in The Netherlands where only transvaginal/cervical insemination is applied because trans-abdominal insemination is not allowed for ethical reasons [2]. Fertility is also reduced following an oestrus synchronisation treatment. This treatment includes the application of an intra-vaginal sponge impregnated with progesterone and an intramuscular injection of equine chorionic gonadotrophin and prostaglandins. Non-hormonal induced synchronisation is achieved by keeping the animals under artificial light regimes to simulate the natural breeding season (short days). Single trans-vaginal insemination is performed at a set time after progesterone sponge removal [32]. Following insertion of a speculum, the semen is deposited intra-cervically with an insemination gun; the female's hind legs are lifted of the ground (Fig 1).

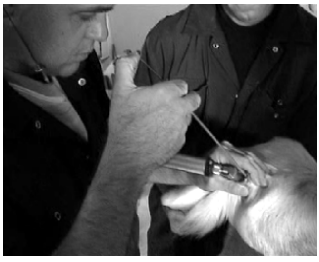

Figure 1: Trans-vaginal artificial insemination (AI) in a goat. The hind side of the doe is lifted; a speculum is inserted into the vagina. The semen is deposited into the cervix with an insemination gun.

The viability of spermatozoa after semen processing and subsequent pregnancy rates after AI are suboptimal and thus should be improved. A key factor in semen processing is the semen extender. The extender which usually contains egg yolk or skimmed milk increases the volume of the insemination dose, protects the spermatozoa against cold-shock and provides them with energy and stabilizes the pH. An important issue is that buck seminal plasma contains compounds that interact with egg yolk and skimmed milk components [32, 40, 43, 44]. If extenders containing skimmed milk and/or egg yolk are used, washing of ejaculates is recommended to prevent or diminish these detrimental chemical reactions [3, 10, 18, 19, 27, 32, 38, 40, 43, 44]. However, as seminal plasma also contains factors favourable for sperm survival, the overall effect of the washing procedure is still under debate and requires further investigation.

With respect to storage of liquid semen, storage temperature and storage period are of significant influence. The currently advised storage temperature is 4°C and semen should be used within 12 hours after collection [17, 24, 32]. However, in case of short-term storage, it remains to be investigated if preservation at ambient (18°C) temperature is favourable for the fertilizing capacity of the spermatozoa. Especially since spermatozoa are notoriously sensitive to temperature changes which may be detrimental for semen quality. Moreover, storage at ambient temperature does not require a refrigerator during transport of semen. Indeed cooling below 15°C is detrimental for fertilizing capacity of spermatozoa in some species e.g. swine. When storage temperature is further decreased as in freeze-thaw procedures, a substantial portion of the spermatozoa do not survive and those surviving have a altered organisation of the plasma membrane [53]. As a consequence pregnancy and lambing or kidding rates are lower when using frozen-thawed semen instead of liquid semen. For freezing a cryoprotectant is required to influence the flux of water and electrolytes during the freeze/thaw process in order to reduce osmotic shrinkage and swelling of cells. Unfortunately, cryoprotectants like glycerol and DMSO despite of the value as cryoprotectant, affect membrane integrity [16, 17] and are metabolically toxic to spermatozoa [53], especially at higher concentrations. The optimal concentrations of these cryoprotectants in the extender are dependent on the freeze/thaw protocol of use both for buck and ram semen.

For the prediction of male fertility, semen evaluation parameters such as the total number of spermatozoa in the ejaculate and the motility and morphology of spermatozoa are often used. Nonetheless, such parameters only have a limited value for predicting the fertilizing capacity of a given ejaculate. Combining a number of parameters to assess semen quality, may improve the reliability of the prediction of fertility as well the applicability of such a semen evaluation procedure for optimising semen handling techniques. Therefore, a comparison of motility scores with flow cytometry assessments of viability and acrosomal integrity of spermatozoa is recommended [20]. In addition, these semen quality assessment

methods as applied in the laboratory, should be correlated with the kidding rate obtained after insemination.

Originally, AI was introduced to prevent the spread of transmissible diseases. Regulations on the intra-community trade of semen foresee in this aspect by requiring strict testing protocols for the donor as well as for the semen. Generally, animals are tested for the absence of antibodies to specific infectious agents. In this way the introduction of most (sexually) transmittable diseases can be prevented. However, a problem exists related to Small Ruminant Lenti Virus (SRLV) transmission via infected semen. In SRLV infected small ruminants the antibody response can be delayed, a phenomenon known as “the serological gap”, and thus serological tests may be negative while the semen donor is actually infected. Subsequently it remains to be investigated whether in these cases and in sero-positive semen donors the virus is indeed excreted in the ejaculate and thus potentially transmitted via AI.

Small Ruminant Lentivirus

Intensive sheep and goat farming evidently can result in increasing health related problems. Infectious diseases nowadays, may spread more vigorous and a small aberration in management can have significant negative effects on production and herd health. For example the SRLV, exemplified by Maedi-Visna Virus (MVV) and Caprine Arthritis and Encephalitis Virus (CAEV), have caused severe outbreaks in herds kept under intensive health control regimes. For infected individuals unfortunately an effective treatment is not available.

“Zwoegerziekte”, the Dutch equivalent of MVV, was first reported in the Netherlands in 1918 on the isle of Texel. In the sixties, research on this disease was initiated at the Netherlands, which resulted in a voluntary accredited SRLV control program in 1982. Initially, export opportunities were the incentive, because exported sheep had to be MVV-free. At present, virtually all breeding flocks are accredited, which is approximately 10% of the total sheep population. According to an estimation of the Animal Health Service 60% of the commercial -lamb producing- flocks and 15-25% of the sheep are infected with MVV [39].

During the expansion of the Dutch professional dairy goat industry in the eighties, goats of high genetic value were imported, mainly from France. At that time, those imported goats were not all officially tested for diseases such as CAEV and Caseous Lymphadenitis (CL). Nowadays, frozen semen is still imported from France and used for AI. Introducing new sires or sperm on a farm bears the risk of simultaneously introducing infectious agents. With gradually increasing herd sizes and particularly further intensification, many goat farmers became aware of the damage CAEV can cause. Meanwhile, CL has been eradicated, but CAEV still is a serious threat to viable dairy goat production.

As result of the SRLV research, which began in Iceland in the 1950's, concepts regarding the epidemiology and the pathogenesis have evolved. Nevertheless, even today, where this research has provided tools and concepts that basically enable the prevention and control of SRLV, unexpected results in disease control programs are incidentally encountered. This indicates that the situation is probably more complicated than the current concepts suggest.
The main reasons for this are; (I) the particular type and manifestation of the virus, (II) the low or late detection of clinical symptoms with hidden economic effects, (III) unpredictable course of lesion development (IV) difficulties in the detection of infected individuals and (V) gaps in the understanding of the transmission.

(I) Type and manifestation of the virus
SRLV are classified as members of the family of Retroviridae. They are non-oncogenic and may cause incurable lethal diseases. SRLV are related to human, simian, bovine and feline immunodeficiency viruses and also to Equine Infectious Anemia virus (EIAV). Retroviridae are enveloped single-stranded RNA viruses that use the enzyme reverse transcriptase in their replication cycle to convert ssRNA in to ssDNA. From the ssDNA, dsDNA is made, which is then integrated into the host chromosome by the viral enzyme integrase. Once integrated into the host genome, the viral dsDNA is referred to as provirus. The provirus remains latent until "triggered" into transcription of mRNA by host cell machinery. The provirus thus serves as a template for the production of mRNA followed by production of ssDNA genomes. These ssDNA genomes serve as the template for the production of ssRNA. Complete virus particles with their ssRNA core and protein coat (virions) are released by budding from the plasma membrane, or by cell lysis. Virions are sensitive to heat, lipid solvents, and detergents but are relatively resistant to ultraviolet light. As reverse transcription is an error-prone process, SRLV's present high genetic heterogeneity [9]. Experimental infection studies indicated that there are biological differences between isolates/strains which are expressed in differences in pathogenicity and tropism [28, 47, 48, 52].

Retroviral genomes consist of two molecules of ssRNA, (+)sense, have 5' cap and 3' poly-(A) (equivalent to mRNA) and three characteristic coding regions, gag, pol, and env (see Fig 2).

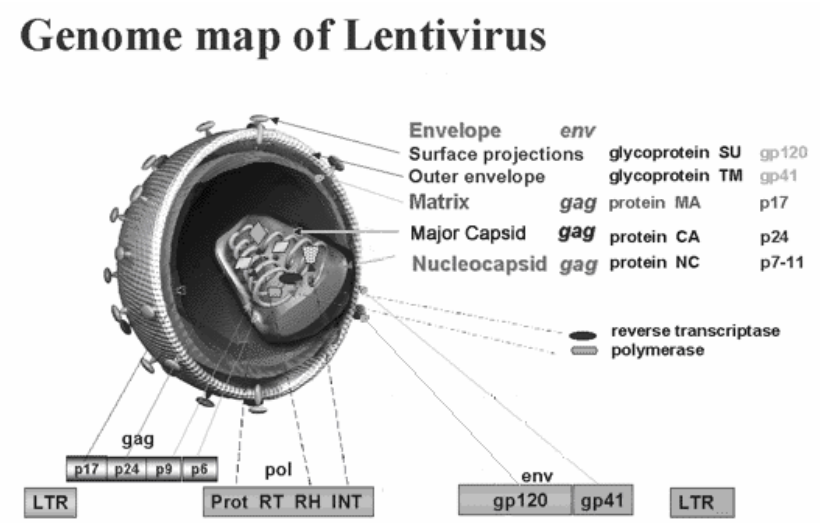

Figure 2: Adapted from C. Büchen-Osmond and J. Whitehead [24]: Genome map of a lentivirus depicting the characteristic coding regions: LTR regions (Long Terminal Repeats); Gag genes (group specific antigen; matrix protein MA p17, major capsid protein CA p24, nucleocapsid proteins NC p6 and NC p9); pol (proteinase Prot, reverse transcriptase RT, RNase-H RH and integrase INT); and env genes (envelope; surface projection glycoprotein SU gp120 and outer envelope transmembrane glycoprotein TM gp41).

A key role for cells of the monocyte / macrophage lineage

The pathogenesis of lentiviral infections of small ruminants is clearly different from that to lentiviral infections in other species such as human, monkey, cat and horse. The most obvious difference is the absence of immunodeficiency, which generally is an important characteristic of lentiviral infection in non-ruminant hosts, except for EIAV. MVV and CAEV apparently do not infect lymphocytes but are restricted to cells of the monocyte and/or macrophage lineage.

Monocytes seem to play an important role in the spread of SRLV throughout the different organs in the infected sheep and goats. Monocyte stem cells may have integrated proviruses that are subsequently passed on to their daughter cells. When provirus-infected monocytes are circulating and arrive in their target-organs they mature to macrophages. Subsequently, virus replication may start. This led to the concept that the monocyte plays the role of “Trojan Horse” by transporting the provirus to various tissues without stimulating the antiviral immune response or serving as a target of an immune response (Fig.3).

Figure 3: Trojan horse. (Part of W. Friedrich: The wooden horse, c.1894.)

Macrophages are present in most tissues of the body. It is still an unanswered question as to why lung, mammary gland, brain and joints are the main organs where lesions develop. Viral proteins or infectious viruses have been detected in macrophages and recovered from lung, bone marrow, mammary gland, lymph node, spleen, synovium, brain, and spinal cord, frequently in association with lymphocytic infiltrates. Viral RNA was also found in epithelium and in a wider range of tissues, with or without SRLV- associated lesions. These findings indicate that the virus can enter a wide variety of cell types. The productive infection, however, is limited to the cells of macrophage lineage, and the cells expressing viral proteins are mainly present in specific tissues that are associated with lentivirus-induced diseases [25].
It remains to be investigated whether the development of lesions in specific target organs, and consequently the increase of clinical symptoms, is related to the duration of infection.
Macrophages have been shown to play a role in the modulation of lung damage such as inflammation, smooth muscle cell proliferation and fibrosis processes through cytokine production, enhanced antigen presentation, fibroblast recruitment and extra cellular matrix protein and fibrin deposition [26].

(II) Clinical evaluation: Low or late detection of clinical symptoms; hidden economic effects

Clinical symptoms following an SRLV infection vary, and usually develop slowly. This may result in initial unawareness of the presence of the infection in a herd or flock. This implies that at the time when clinical problems and downfall in production become obvious, a high percentage of individuals are seropositive (40-50%). The economic effects of Lentivirus infections are often underestimated but solid data are scarce. An average loss of earning capacity of 10 to 20% was calculated for infected sheep flocks as compared to uninfected ones [27]. The overall economic effects are influenced by e.g. environmental factors, husbandry and management systems, individual susceptibility and the relative value of culled individuals compared to that of replacements. Affected animals inevitably loose weight. A typical respiratory symptom in sheep is dyspnoea (zwoegerziekte =laboured breathing) after mild exercise. At a later stage open mouth breathing with a stretched neck can be observed (Fig 4). In dairy goats, a decrease in milk production and lameness due to arthritis are usually perceived. Loss of lambs in the first week of life due to an almost complete lack of milk or a weight shortfall of lambs at weaning due to reduced milk production [28] both as a result from indurative mastitis, are also a frequently encountered problem in herds with MVV infections. In contrast to the procedure on sheep farms, kids in commercial goatherds are routinely separated from the dam shortly after birth and thus early death and retarded growth are not a major problem. SRLV can cause fatal encephalitis in goat kids in the first months of life. Adult small ruminants may also show signs of encephalitis in a less acute form, starting with paresis and paralysis of the hind legs (Visna). On the flock/herd level, the major economic effect of

lentiviral infection is the increased replacement rate. Generally, body weight of affected animals is significantly reduced at slaughter. The carcass may even not be qualified for consumption and therefore represent no value at all. In conclusion, these aberrations cause a significant economic loss.

Figure 4: A flock of Maedi Visna Virus (MVV) infected sheep. One ewe with distinct clinical symptoms: weight loss, dyspnoea, and paresis of the hind legs. The other 4 ewes show no obvious clinical symptoms although they have been infected by MVV for at least 18 months. Two of these are the offspring of the diseased ewe.

(III) Pathological evaluation: slow and un-predictive development of lesions
Post-mortem examination of MVV and CAEV infected individuals shows a variety of alterations in different organs. These alterations can range from mild to severe, and not all target organs are equally affected in an individual animal. The macroscopic lesions are not necessarily specific, but the combination of these multiple lesions are indicative for the post-mortem diagnosis. For confirmation of the diagnosis histological examination is required. Histologically, lymphocytic infiltration with typical foci and fibrosis are the most characteristic changes, particularly in the lung if there is no secondary bacterial infection. Breed-associated and individual differences in susceptibility to the virus and the subsequent development and progression of lesions have been suggested [29], but studies to confirm this are hampered by the fact that lesions develop slow and unpredictably and that experimental infections probably do not mimic the natural infection. To better understand the pathogenic process more should be known of its progressiveness. Unfortunately, the exact timing of the actual transmission of the virus when diagnosed by antibody formation by the host is hampered by the relatively slow and individually variable antibody formation after infection. This also impedes evaluation of longitudinal epidemiological studies, particularly those focusing on the dynamics of the transmission. Theoretically, this can be overcome by using more sensitive virus detection methods, e.g. PCR, instead of antibody detection.

(IV) Laboratory evaluation: Difficulties in detection of infected individuals
Laboratory methods for detecting SRLV infection range from detection of the hosts' specific antibody response, direct detection of viral antigen or nucleic acid (NA) in the host, to culture of the virus.

Detection of the hosts' reaction to the virus
Serology: The conventional method for detection of SRLV-infection is testing for antibodies. Today, the indirect enzyme-linked immuno-sorbent assay (Elisa) that utilises either natural viral of recombinant proteins as antigen is the most widely used. This provides the best compromise between specificity and sensitivity, and economics [30]. It should be noted that both specificity and sensitivity are not 100%, which implies that false-positive (non-specific binding) and false-negative results occur. Moreover, serology has the drawback of the slow and variable antibody formation after infection, which might take several months, the so-called serological gap.

Detection of the virus or its genome.
Virus culture: The classic method of virus detection is the virus culture for which specific fibroblasts are cultured in vitro, subsequently infected and then show typical cytopathic alterations, i.e. multinucleated giant cells. False negative results are to be expected due to specific problems encountered during cell culture. Moreover, it is a very laborious and costly method.
In situ detection: More recent techniques are in situ detection of the virus or its components by means of immunohistochemistry or its DNA through hybridisation with specific nucleic acid probes. Although useful as research tools, these techniques are not suited for routine applications.
Polymerase Chain Reaction (PCR)
The most sensitive method to detect (viral) DNA in substrates is applying the principle of amplification of a specific part of the genome. This assay is extremely sensitive and thus suited to detect the provirus. In theory, PCR would allow viral genome detection immediately after infection and thus close the serology gap. The prerequisite is of course that the genome is present in the sample. Although in theory the PCR is very specific, the heterogeneity of SRLVs is a major hurdle to be overcome. A further restriction is the limited quantity of material that can be tested which may result in reduced sensitivity. So, in case of SRLV-detection, the diagnostic sensitivity basically depends on the presence of cells that contain the provirus, i.e. the monocytes and/or macrophages. Note that the virus itself contains RNA, which is not amplified in this assay and only the proviral state of the SRLV is detected. Monocytes are a small part of the peripheral blood mononuclear cell (PBMC) fraction, which in its turn is part of the white blood cell (WBC) fraction of the blood. Sheep and goat disease handbooks state values for monocytes between 0 and 13 % of the WBC [31], which suggests that they may be absent in a given blood sample, making it unsuited for SRLV-detection. Evidently, this issue requires investigation if blood is to be used for diagnostic PCR-testing. Alternatively, tests in blood should include a check on the presence of monocytes.

(V) Transmission: Gaps in the understanding of the transmission
It is generally accepted that lateral transmission is an important route by which SRLV disseminates. SRLV can spread via droplets or cells discharged from the

respiratory tract. In case of SRLV infection these droplets are likely to contain cells (macrophages, epithelial cells) incorporating virus or even free virus. Lactogenic transmission is another option; free virus and macrophages are present in the mammary gland and the milk. Recent studies have shown that the efficiency of lactogenic transmission is lower than hitherto thought [32, 33]. An epidemiological study demonstrated that the ewe-lamb relationship is an important factor in transmission [34]. The conditions under which the animals are kept are inevitably of paramount importance to lateral transmission, e.g. airborne infections are known to occur particularly indoors. In dairy goats intra-mammary infection might occur through the process of machine milking [35-39]. An alternative indirect route by which the virus might be transmitted via clothes or shoes is highly unlikely as the infectious characteristics of the virus soon fade in such a hostile environment.
In addition, it is plausible that SRLV is transmitted during mating. Is the introduction of SRLV-positive males in a flock a risk because of sexual transmission of the agent or is simply the close contact the cause of infection? Ejaculates from SRLV positive individuals can harbour the virus when these animals are co-infected with *Brucella ovis* [40]. Whether they can be (intermittent) shedders without this additional infection remains to be investigated. Anyway, it is advised to use only proven SRLV-free males for breeding. Nevertheless such SRLV-free individuals can become infected after introduction in a herd if SRLV infected animals are present. Then a male could turn into an efficient transmitter. AI with liquid or frozen-thawed semen from SRLV-free males can be an effective method to exclude sexual transmission, while in the case of infected semen AI turns into an efficient method to disseminate the agent on a wide scale.
Maternal-fetal (trans-placental) transmission of CAEV is also suggested [40-47] and such transmission has been described for lentiviruses other than MVV and CAEV [48, 49]. However, its contribution to the epidemiology of SRLV is generally considered of minor importance [33, 50].

The genetic constitution of the host is considered to influence the susceptibility to a lentiviral infection. A few specific retrospective studies support this concept [29, 34] [51]. For example, the healthy Karakul rams imported into Iceland in 1933 introduced a disastrous epidemic disease in the native sheep population whereas there are records over many years that strongly suggest that the original Karakul population in Germany never showed any symptoms of MVV infection [52, 53]. It is as yet unclear if the suggested breed-associated susceptibility is a true breed characteristic or a matter of the frequency of an individual trait. Most experiments concerning SRLV transmission do not mimic the natural situation, but involve inoculation of a specific dose of an SRLV laboratory strain. Quantitative data on the efficiency of lateral transmission between adult animals under field conditions are lacking. Such data are essential for optimising SRLV-control programs and performing risk calculations in view of preventive measures.

Problems of the current SRLV control program

The current MVV/CAEV control program in the Netherlands, launched in 1982, aims to acquire and maintain an MVV/CAEV antibody free status at the flock/herd level. For this purpose a representative part of the flock is tested for specific antibodies at regular intervals. Serological tests are unreliable on an individual basis since antibody formation is slow; hence the entire flock/herd or a representative part is tested to assure the uninfected status of the individual. Obviously, the reliability of the "SRLV-free" status of a herd/flock increases with the number of individuals tested and the number of times they were re-tested. The control program requires two successive negative flock tests, regular follow-up tests and the strict application of a of number regulations that aim to prevent re-infection of the flock/herd such as restrictions for the introduction of new animals. Flocks/herds that fulfill these requirements gain the status SRLV-free and individuals sold from these flock/herds obtain an official certificate that is supposed to improve their market value. The sheep breeding societies soon made the program obligatory for their members and thus this part of the sheep industry is virtually free of MVV, albeit every year a small number of flocks loose their status because positives are found at the regular follow-up test. These events, the relatively high costs of the program combined with the low return on investments is a constraint for breeders to continue with the program and the main reason for commercial lamb producers to abstain from participation.

The goat dairy industry on the other hand is very much aware of the economic damage CAE can cause. But due to its specific conditions, efforts to control the infection and to obtain and maintain the CAEV-free status have frequently met with disappointment.

In conclusion, both the sheep and goat industry are waiting for options to control SRLV infections at lower cost, and with fewer restrictive regulations. Better insight into the factors influencing lateral transmission, e.g. housing and host (un-) susceptibility, may provide a first step to this end.

Scope of the thesis

The research described in this thesis aims to improve the reproductive performance of small ruminants, specifically when artificial insemination (AI) is applied. AI is an important technique to enhance the genetic constitution of a population. Unfortunately, the results with AI are poor and thus too expensive. Optimising the semen handling procedures and storage techniques for liquid semen can ameliorate fertility results. For semen evaluation, various techniques were used to assess the changes in the plasma membrane function during semen handling. The study presented in **chapter 2** does focus on: optimising the storage temperature of liquid semen, the effect of glycerol on the quality of spermatozoa during storage, evaluation of the washing procedure for exclusion of seminal plasma and optimising the extender. Data were correlated with fertility rates following AI in goats.

Originally, AI was introduced to prevent the spread of transmissible diseases. However, a problem exists related to SRLV transmission via infected semen. It remains to be investigated whether positive semen donors indeed do excrete the virus in the ejaculate. SRLV infections are common in the Netherlands. Gaps in the knowledge on SRLV transmission hamper the efficiency of such a program. This thesis further investigates ways and efficiency of transmission. For this purpose adequate detection methods are a prerequisite. A PCR test recently developed to identify SRLV strains present in European small ruminants was implemented for blood, tissue and semen evaluation and was utilized in the following chapters and results were compared to those of the standard ELISA test for detection of SRLV specific antibodies.
Both tests are applied in a comparative study on the development and progressiveness of lesions related to a natural MVV infection (**chapter 3**). For this purpose control and infected animals were joined in one flock and the clinical symptoms that developed were evaluated. In addition lesions induced by the infection were investigated post mortem in a time-related histopathological survey. Moreover, to investigate whether a breed specific difference in susceptibility exists, two breeds were studied: a Texel-type sheep and a new composite breed of which field observations suggested a high susceptibility to MVV-infection. As animals were sampled at regular intervals, this study yielded individuals with a known length of naturally acquired infection.

A major problem in detection of SRLV is its masked appearance as proviral DNA incorporated in the host monocytes and/or macrophages. PCR assays detecting the viral genome in blood have a high potential in this respect, but they rely on the availability of monocytes in any given blood sample. The study presented in **chapter 4** investigates -using an accurate flow cytometric evaluation- whether monocytes are indeed available in any blood sample, notwithstanding the 0% value given in the standard value tables.

Differences in susceptibility to acquiring a SRLV-infection have been confirmed in many retrospective studies. However, natural lateral transmission of an infectious agent is effected by many variables, the influence of which cannot be verified retrospectively. The most important variable is probably housing, since lateral (airborne) transmission is expected to increase under these circumstances. Quantification of the horizontal transmission under different husbandry circumstances -indoors versus outdoors-, and the possible difference in breed susceptibility was studied in **chapter 5**. The data will be valuable for risk calculations to improve the strategy in prevention and control programs. Also the performance of the PCR-test itself, in relation to results obtained via detecting antibody concentration in blood samples, is evaluated in this MVV transmission experiment.

The impact of semen in the transmission of SRLV particularly in the perspective of

AI is studied in **chapter 6**. To be able to predict sexual transmittability of SRLV it is first necessary to know whether or not, and if so, when and where, the virus is excreted in the semen. Different fractions of the semen as well as several tissues from the sexual organs from infected rams and bucks were analysed for the presence of proviral DNA using PCR. Moreover, the number of monocytes/macrophages in blood and semen were established to investigate if a positive ejaculate corresponds with a higher number of these cells in the peripheral blood and subsequently the ejaculate.

Finally, the results of the studies described in this thesis are summarized in **chapter 7** and discussed in a broader perspective.

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Chapter 2

Reproductive Management

Theriogenology 67 (2007) 863-871)

Microscopic and flow cytometric semen assessment of Dutch AI-bucks: effect of semen processing procedures and their correlation to fertility.

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Abstract
This study was done to determine the effects of processing techniques on the quality of semen from Dutch AI bucks with the view on improving pregnancy rates after AI with liquid or frozen-thawed semen. Motility of spermatozoa was estimated under a microscope whereas the percentage live spermatozoa and the percentage live spermatozoa with intact acrosomes were determined by means of flow cytometry. Aspects of semen processing that were investigated are storage temperature of liquid semen (i), the effect of glycerol on liquid-stored semen (ii), removal of seminal plasma (iii) and type of extender (iv). The correlation between semen quality and fertility rates in inseminated does was also investigated.
The percentage motile spermatozoa in semen stored in liquid form for 72 hours progressively declined over time, irrespective of whether storage occurred at 4°C or 18°C. The percentage motile spermatozoa in semen stored at 18°C was similar to that in semen stored at 4°C if stored for 24 hours but lower if stored for 48 hours. Goats differ in the sensitivity of their spermatozoa to the deleterious effects of glycerol. Neither the removal of seminal plasma nor the type of extender had any effect on semen quality before freezing but semen frozen in a Tris-citric acid-glucose (TCG) buffer with egg yolk without removal of the seminal plasma had better quality after thawing than semen frozen in another diluent or after removal of seminal plasma.
Remarkably no significant correlation between fertility and membrane integrity of spermatozoa could be found. Thus although integrity assays for spermatozoa are useful to asses resistance to semen handling, the validity of these assays for predicting fertility is questioned.

Keywords: Goat; AI; Flow Cytometry; Diluent

Introduction

Artificial insemination (AI) holds several genetic and economic advantages for goat production. AI is the preferred method of introducing superior genes from individuals free of specific diseases and thereby improving the production of offspring. AI is also useful for breeding goats outside of their natural breeding, which runs from September to February in Western Europe, after oestrus has been induced and synchronised by manipulation of the photoperiod or the use of hormones. Such out of season breeding permits the production of adequate quantities of milk and equalises the need for labour throughout the year. Semen used for out of season breeding can be frozen-thawed or liquid-stored if collected from bucks held under artificial light in order to simulate the short days of the natural breeding season. Although higher pregnancy rates may be obtained with trans-abdominal insemination than with cervical insemination the former method may be prohibited due to ethical considerations [18]**.** Currently, the use of frozen-thawed- or liquid semen in goats in which oestrus was synchronised by means of hormone therapy results in pregnancy rates of approximately 61% or 64%, respectively [15].

The extender used is a key factor in semen processing. Enzymes in the seminal plasma of bucks may interact with specific components of skimmed milk or egg yolk, rendering extenders containing these substances harmful to the bucks' spermatozoa [2, 15, 20, 21]. A practical solution to this is to remove seminal plasma prior to admission of egg yolk or skimmed milk extenders to the spermatozoa. Despite of this possibility, in practise routinely extenders containing skimmed milk or egg yolk are directly added to undiluted semen [2, 5, 14-16, 19]. We may note that seminal plasma also contains factors favourable for sperm survival, it is envisaged that specific inhibitors of the afore-mentioned enzymes could be added to extenders [20, 21].

Storage temperature is another important factor in semen processing. Liquid goat semen destined for use within 12 hours should be stored at 4°C [14]. Significant temperature changes during storage of liquid semen are considered detrimental to semen quality and, under practical conditions, fluctuations in temperature may be easier to avoid at higher storage temperatures than at 4°C.

Glycerol is a cryoprotectant that is added to extenders used for freezing of spermatozoa. Glycerol reduces the viability of spermatozoa from humans and rams [7, 8] but its effects on buck semen are unclear.

Better methods to dilute, wash and store semen may improve the results obtained with AI in goats. Several procedures to process buck semen were therefore evaluated in the present study. The quality of an ejaculate is often expressed as the number, motility and morphology of spermatozoa therein. These indicators of semen quality only have limited value for predicting the potential of a given insemination dose to fertilize the oocytes of a goat and result in a pregnancy. It is therefore important to use adequate evaluation techniques to compare different protocols for the processing of semen [11]**.** The percentage of motile spermatozoa in a sample is most commonly used to evaluate semen quality. Motility is usually

determined subjectively and we therefore decided to compare the percentage of motile spermatozoa with viability and acrosomal integrity as objectively assessed by means of flow cytometry [11].

The aim of the current study was to modify existing protocols in order to improve the preservation of buck semen in liquid or frozen form. More specifically, the aims were to compare the effects of 2 extenders and the removing or not of seminal plasma on the quality of buck semen before and after freezing-thaw process, to compare the effects of 2 storing temperatures on the quality of cooled spermatozoa and, finally, to determine the effect of glycerol on the quality of spermatozoa kept at 18°C. Apart from the effects of the above treatments on semen quality, the correlation between semen quality and fertility of frozen-thawed spermatozoa was determined.

Materials and methods

Extenders

The ejaculates were diluted in a Tris-citric acid-glucose (TCG) buffer or in a glucose solution, both at 37°C. The TCG buffer contained 375 mM tris(hydroxymethyl)aminomethane, 124 mM citric acid, 41 mM glucose, 375 mM trehalose, 0.5 mg/ml dihydrostreptomycin and 0.3 mg/ml benzyl penicillin. The TCG buffer was prepared either with or without 20% (v/v) egg yolk and 4% (v/v) glycerol as described before [1]. The glucose solution buffer contained, 50 mM D-glucose, 0.3 mg/ml benzyl penicillin, 0.5 mg/ml dihydrostreptomycin and was prepared either with or without 100 mg/ml skimmed milk and 4% (v/v) glycerol. All solutions were adjusted to a pH of 7.0 and an osmolarity of 375 mOsm.

Experimental design

Each ejaculate was split into 5 aliquots immediately after collection and initial evaluation. Aliquot 1 was diluted in TCG buffer, centrifuged at 1200 x g for 15 minutes and the supernatant containing the seminal plasma discarded. The dilution and centrifugation of aliquot 1 was repeated and the sediment then diluted in a TCG buffer with egg yolk and glycerol, before it was cooled to 4°C and frozen. Aliquot 2 was diluted in the TCG buffer with egg-yolk and glycerol and thereafter cooled to 4°C and frozen. Aliquot 3 was treated in the same way as aliquot 1, except that a glucose solution was used prior to centrifugation and a glucose solution with skim milk and glycerol to dilute the sediment. Aliquot 4 was diluted in a glucose solution with skimmed milk and glycerol subsequent to which part thereof was cooled to 4°C and frozen and part kept at 18°C for 24 hours (aliquot 4_{18}). The fifth aliquot of each ejaculate was diluted in a glucose solution with skimmed milk and then divided into 2 aliquots of which one was cooled to 4°C and kept at that temperature for 72 hours (aliquot 5_4), whereas the other was cooled to 18°C and kept at that temperature for 72 hours (aliquot 5_{18}). Paragraph 2.1 provides the

composition of each diluent. Figure 1 shows how an ejaculate was split and the aliquots assigned to the different treatments.
Initially, the volume of an ejaculate, as well as the concentration, motility and viability of its spermatozoa were determined. Aliquots 1, 2, 3 and 4 were evaluated for % of motile, % of viable, % live/dead and % of acrosome intact spermatozoa before freezing and after thawing. The motility of aliquot 4_{18} was determined after one and 24 hours whereas the motility of aliquots 5_4 and 5_{18} was determined after 1, 24, 48 and 72 hours. Figure 1 shows which evaluations were performed on each aliquot as well as when the evaluations were done. All experiments were performed twice on two ejaculates that were separately collected at different times in a group of eight bucks.

Semen collection and initial evaluation
Eight Saanen bucks were selected on production and conformation characteristics by the Dutch AI cooperation (Geiten KI Nederland; GKN). The bucks were individually housed in climate-controlled stables (18°C). The photoperiod was 8 hours of light and 16 hours of darkness to simulate short days. Semen was collected by ejaculation into an artificial vagina (37°C) in the presence of a goat in oestrus. Upon collection each ejaculate was weighed to determine its volume. Twenty-five µl of the ejaculates was diluted in 5 ml Tris-buffer (containing 20 mM Tris and 130 mM NaCl; Merck, International BV, Amsterdam, the Netherlands) and mixed on a vortex. The concentration of spermatozoa in the ejaculate was determined by placing 2 ml of the suspension in an ACCUCELL photometer (IVM technologies, L'Aigle, France). Then the total number of cells in the ejaculate was calculated. In order to assess the motility of spermatozoa 10 µl of semen was diluted in 1 ml Tris-buffer, 10 µl of this suspension transferred to a warm (37 °C) glass slide and covered with a glass coverslip (at the same temperature). The slide was then placed on the warm stage of a standard phase contrast microscope (Olympus BX40, Olympus optical Co, Ltd., Japan). Two persons each assessed at least 10 microscope fields at x100 or x200 magnification and, from these assessments, estimated the percentage motile spermatozoa. The viability of the spermatozoa was evaluated on a smear stained with eosine-aniline blue (eosine yellow from Merck Ltd., Darmstadt, Germany and aniline from Gurr BDH Chemicals Ltd., Poole, England), using a bright-field illumination microscope (Olympus BX40, Olympus optical Co, LTD, Japan) at x1000 magnification.

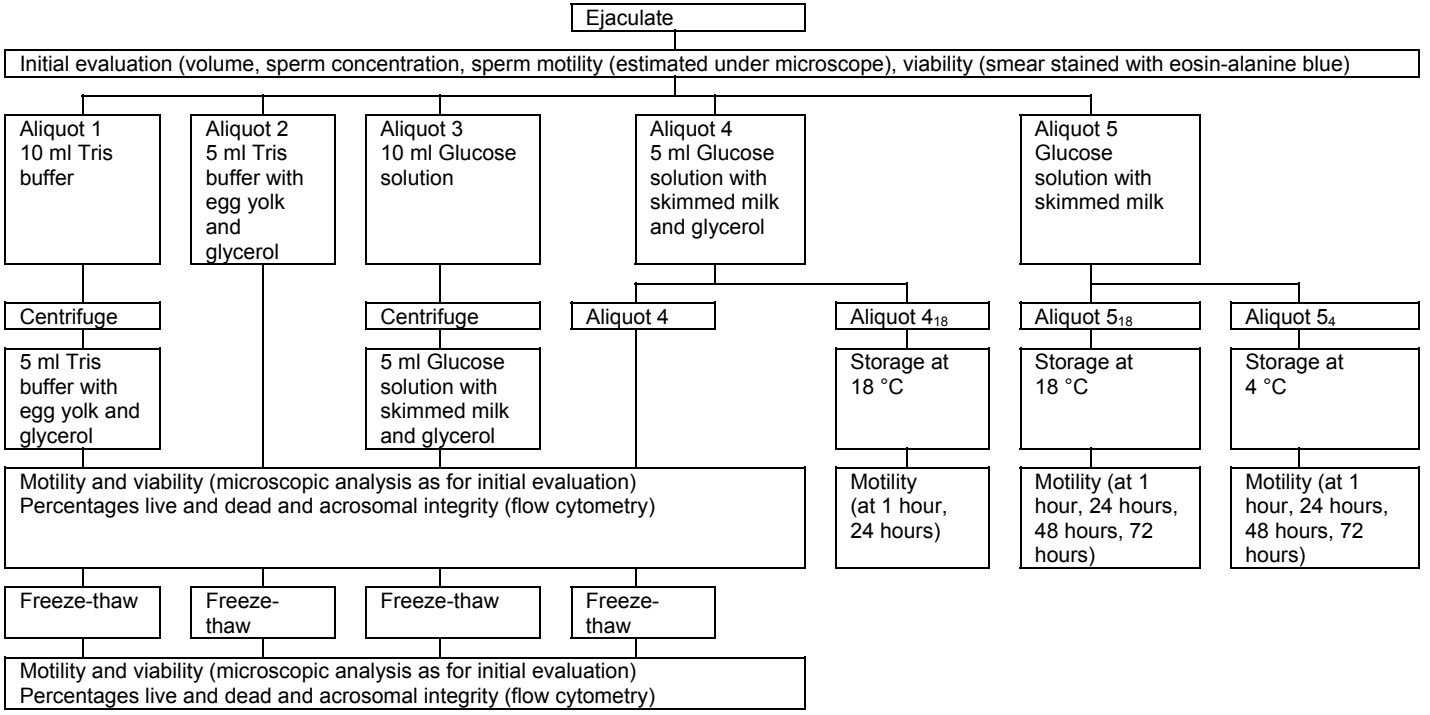

Figure 1. Overview of the experimental design and allocation of semen to the different treatments (split sample, cross-over design). The objectives are to evaluate the influence of seminal plasma, determine the optimal extender in liquid en frozen-thawed semen, to evaluate the effect of the cryoprotectant glycerol, and the survival of spermatozoa in liquid semen at different temperatures over time.

Preparation of samples for storage at 18°C, 4°C or at -196°C
After collection the semen was diluted in the TCG buffer at 37 °C or in the glucose solution, which was also at 37°C, to a final concentration of $400*10^6$ spermatozoa per ml. After dilution the semen was stored in cotton-isolated containers in a Styropor box and subsequently cooled to room temperature over 2 hours. Thereafter, the tubes were either stored in a dry and dark container at 18°C or cooled and equilibrated over a period of 2 hours in a refrigerator at 4 °C.
Aliquots 4_{18} and 5_{18} were stored at 18°C for 24 hours and 72 hours, respectively, whereas aliquot 5_4 was stored at 4°C for 72 hours.

Before cooling and equilibration the spermatozoa destined for cryopreservation (aliquots 1, 2, 3 and 4) was further diluted in their accessory extender to a final concentration of $400*10^6$ spermatozoa/ml. Once cooled and equilibrated at 4°C these aliquots were packaged in pre-printed 0.5 ml French straws, using a machine that automatically fills and seals the straws (IMV, Cedex, France). The straws were then frozen in a Kryo 10 series II freezer (Planer Products Ltd., Sunbury-on-Thames, UK) according to an automated freezing protocol. The cooling rate from 4°C to -8°C was 35°C per minute, and thereafter 30°C per minute to –140°C. Finally; the straws were stored in liquid nitrogen. In each experiment straws were thawed in a water bath at 37 °C for 1 minute.

Flow cytometry analysis
The semen samples were diluted 1:10 in a solution containing phosphate buffered saline (PBS; 1.54mM KH_2PO_4, 155.17mM NaCl, 2.71mM Na_2HPO_4; pH 7.2, Gibco, UK) to which was added 0.1% (w/v) polyvinyl alcohol (PVA; Sigma, ST Louis, USA). In order to distinguish live and dead spermatozoa they were stained with SYBR-14® dye and propidium iodide (PI) that were both present in the LIVE/DEAD staining kit from Molecular Probes, Inc. (Eugene, OR, USA) and used according to the supplier's protocol. For membrane integrity assessments 1 ml of semen samples that were diluted 1:100 in PBS with 0.1% w/v PVA were stained for 10 minutes at 37°C with 50 ng/ml fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; EY Laboratories, Inc., San Mateo, LA, USA) and 0.4 µg/ml PI (Sigma Chemical Company, St. Louis, USA). The cells ($40*10^6$/ml for SYBR-14®/PI and $4*10^6$/ml for FITC-PNA/PI) were then analysed for green and red fluorescence by flow cytometry using a FACScan equipped with an argon laser (488 nm, excitation line, Becton Dickinson, San Jose, CA). Semen specific events were gated for further analysis on the basis of their specific forward and side ways scatter properties [9, 10].
The fluorescence detectors (FL 1 = 500 to 530 nm and FL 3 > 605 nm) were set to asses the amount of the red and green emission of the sperm subpopulations in order to analyse plasma membrane- and acrosome-integrity according to previously described methodology [17]. Liquid semen samples were evaluated within 6-8 hours after collection and within 1 hour post-thawing for frozen samples.

Measurement of fertility and correlation with semen quality

At least 15 ejaculates were obtained from each of 6 bucks, distributed to a minimum of 8 and a maximum of 16 different farms, in order to evaluate fertility. Ejaculates were diluted to a concentration of $400*10^6$ spermatozoa per ml in a glucose solution with skimmed milk (as described above for Aliquot 5), cooled to 4°C and kept at that temperature for a maximum of 8 hours after ejaculation before the semen was used to inseminate does.

The oestrous cycles of 1178 goats (70-260 per buck) were synchronised with progestagen-impregnated intravaginal sponges (Chrono-gest, Intervet, Boxmeer, The Netherlands) for 11 days. On day 9 the does were treated intramuscularly with 400-600 IU of ECG (Folligonan, Intervet, Boxmeer, The Netherlands) and 2 ml of PGF2α. Using 0.5 ml of liquid semen containing $200*10^6$ spermatozoa, an experienced inseminator performed a single intracervical insemination on each doe 41-45 hours after sponge removal [15]. The does' hind legs were lifted off the ground so that the animal was standing on the forelegs. The hind legs were subsequently folded under the body and the body was raised in a near vertical position during insemination.

The techniques described in Sections 2.3 and 2.5 were used to determine the percentages progressively motile, live and live acrosome-intact spermatozoa in the ejaculates. These variables of semen quality were correlated with the kidding rate of does obtained after AI with liquid semen within 8 hours of collection.

Statistical analysis

The parameters of fresh semen quality are presented as means ± SD. The flow cytometric data were analysed with Cell Quest software (Becton Dickinson) and WinMDI (Windows Multiple Document Interface for Flow Cytometry, version 2.8; http://facs.scripps.edu/software.html). Region and quadrant analyses were done on 10,000-gated spermatozoa from each sample. Further analyses of the scores were done with Excel (Microsoft) and SPSS (SPSS Inc., USA; version 10.0). For the analysis of storage temperature, data of 8 bucks were included and a paired sample t-test was used to compare the mean % of motile spermatozoa at different time periods. An one-way ANOVA was used to establish the difference between the two storage temperatures at 24 h, 48 h and 72 h. The effects of storage temperature and glycerol on motility during liquid storage were respectively determined by means of a one-way ANOVA whereas multiple comparisons of means were done with Bonferroni's test. Different combination of effect of removal of seminal plasma in combination with type of extender (n=4) was determined by means of an one-way ANOVA with a Bonferroni post hoc test. Initially, a square root transformation was performed to normalise the data on the percentage of live spermatozoa (SYBR-14® /PI). The separate values for fertility of individual bucks were scored as the number of goats that produced kids divided by the number of goats that received semen from a particular buck through AI. Subsequently the correlation coefficient between fertility and the percentages progressively motile, live and live acrosome-intact spermatozoa were assessed.

Results

Initial evaluation

The volume of the ejaculates was 1.3 ± 0.5 ml, and the concentration of spermatozoa was 6.2 ± 3.9*10^9 per ml. Thus the number of spermatozoa per ejaculate was 7.6 ± 5.4 * 10^9. Ejaculates had 76 ± 11% motile, and 92 ± 3% viable spermatozoa.

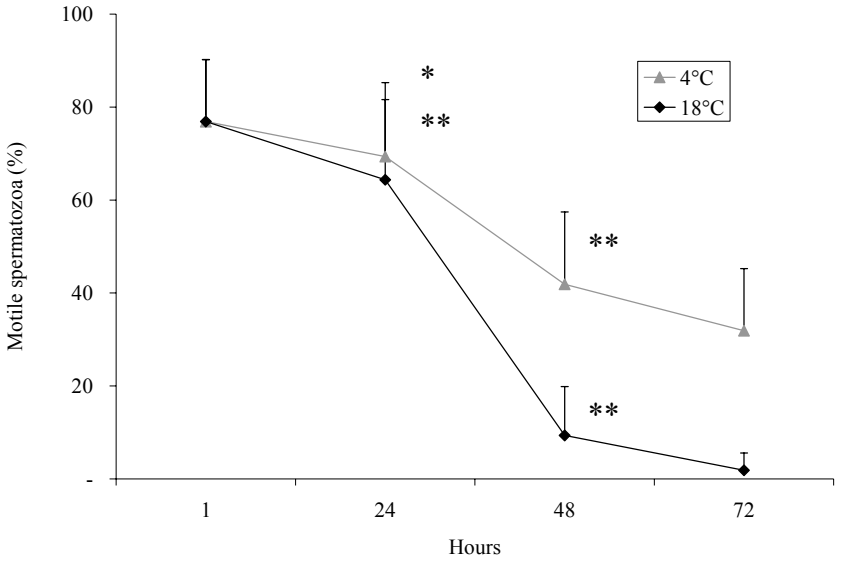

Figure 2. Percentage (mean ± SD) of motile spermatozoa in the ejaculates of 8 bucks stored for up to 72 hours at 18°C and 4°C in a glucose solution containing skimmed milk.

Effects of liquid storage

(i) Storage temperature

Figure 2 shows the effect of storage temperature over time. There was a significant decline in the percentage of motile spermatozoa during the first 24 hours of storage ($p<0.005$ for spermatozoa stored at 18°C and $p<0.01$ for spermatozoa stored at 4°C, $p<0.01$), although there was no significant difference in motility of spermatozoa between the temperature groups ($p>0.05$). Storage from 24 to 48 hours resulted in a prominent decline (18°C $p<0.001$; 4°C $p<0.005$) in the percentage of motile spermatozoa. By 48 hours the percentage motile spermatozoa was significantly lower in the group stored at 18°C than in the group stored at 4°C ($p<0.001$). The percentage of motile spermatozoa further decreased during storage between 48 to 72 hours (18°C $p<0.05$; 4°C $p< 0.05$) but there was

no significant difference in motility of spermatozoa between the temperature groups ($p>0.05$).

(ii) Glycerol in the extender

Glycerol had a negative effect on the motility of spermatozoa over a period of 24 hours and the effect varied among bucks (Fig 3). The spermatozoa of one buck were intolerant towards storage in a glucose solution with skimmed milk and glycerol, as all spermatozoa were immotile within 1 hour. For three other bucks motility of spermatozoa had completely vanished within 24 hours of storage. For the remaining three bucks glycerol did not affect motility of spermatozoa during storage at 18°C (Fig 3).

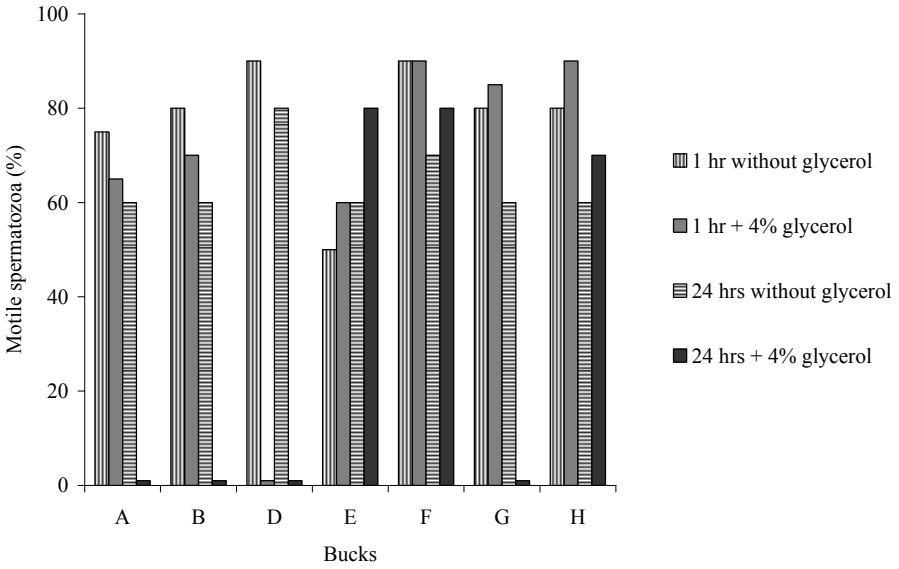

Figure 3: Effect of glycerol on the percentages of motile spermatozoa of 7 bucks (samples were stored for 1 and 24 hours at 18°C in a glucose solution containing skimmed milk with or without 4% glycerol).

(iii) Effects of removal of seminal plasma and (iv) type of extender

Figure 4 shows that the removal of seminal plasma had no effect on the percentage of live spermatozoa before freezing ($p=0.2$, both buffers) or after thawing ($p=0.6$, glucose buffer only). However, in TCG buffer removal of seminal plasma resulted in a significant higher proportion of deteriorated spermatozoa compared to TCG buffer with seminal plasma. Irrespective to the presence of seminal plasma TCG buffer was superior to the Glucose buffer when considering cryoresistance of the spermatozoa. This is best illustrated in figure 4 that shows

that TCG buffer with seminal plasma did only marginally cause cryodamage to the spermatozoa. Furthermore, the survival before freezing was similar in TCG buffer with egg yolk or glucose solution containing skimmed milk (p=0.6). Figure 4 shows that a significantly higher percentage of spermatozoa diluted in TCG buffer with egg yolk without washing survived the freeze-thaw process compared to spermatozoa exposed to the other 3 treatments (p<0.005).

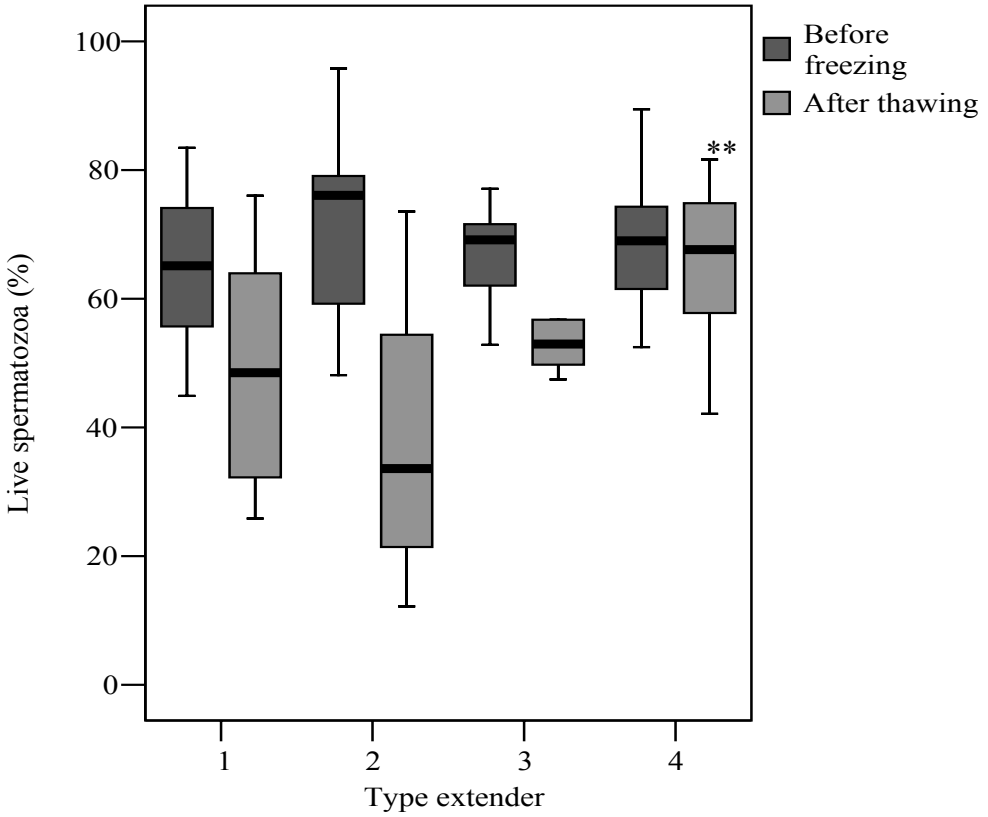

Figure 4: Effects of two diluents (glucose solution with skimmed milk (1 and 2) and TCG with egg yolk (3 and 4) and the removal (1, 3) or not (2, 4) of seminal plasma on the percentage live spermatozoa (flow cytometry after SYBR-14®/PI staining) before freezing or after thawing (horizontal bars indicate the median, shaded blocks the interquartile range that contains 50% of values and the vertical lines the highest and lowest values after exclusion of outliers and extremes).

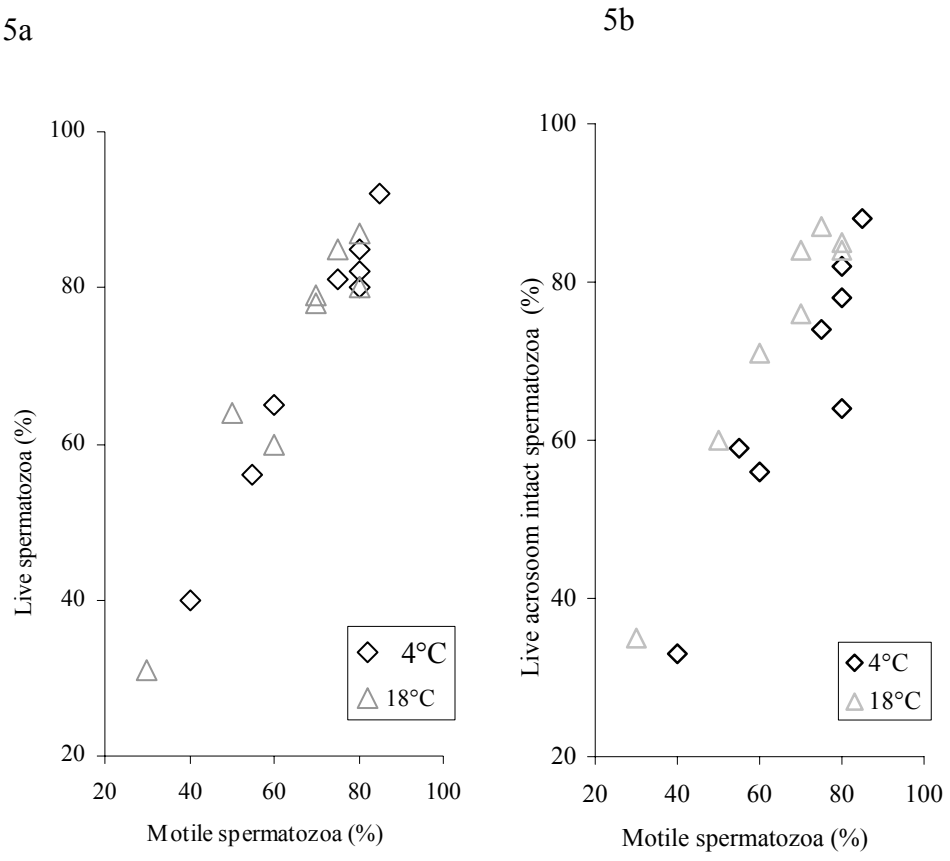

Figure 5. Scatter plots between the percentage of motile spermatozoa as estimated under a microscope and the percentage of live spermatozoa as determined by means of flow cytometry (A) SYBR-14®/PI staining (B) and the percentage of live spermatozoa with intact acrosomes as determined by means of flow cytometry after PNA-FITC-PI staining (spermatozoa from 8 bucks stored for 24 h at 4°C or 18 °C).

Correlation between results of different evaluation methods for buck semen

Figure 5a shows that the proportion of cells with intact membranes correlated with the proportion of motile cells, both, for spermatozoa stored at 18°C and at 4°C (R=0.77 and R=0.98, respectively). Similarly, figure 5b shows that the percentage of motile spermatozoa correlated with the percentage of live spermatozoa with intact acrosomes (R=0.94 for spermatozoa stored at 18°C and R=0.93 for spermatozoa stored at 4°C).

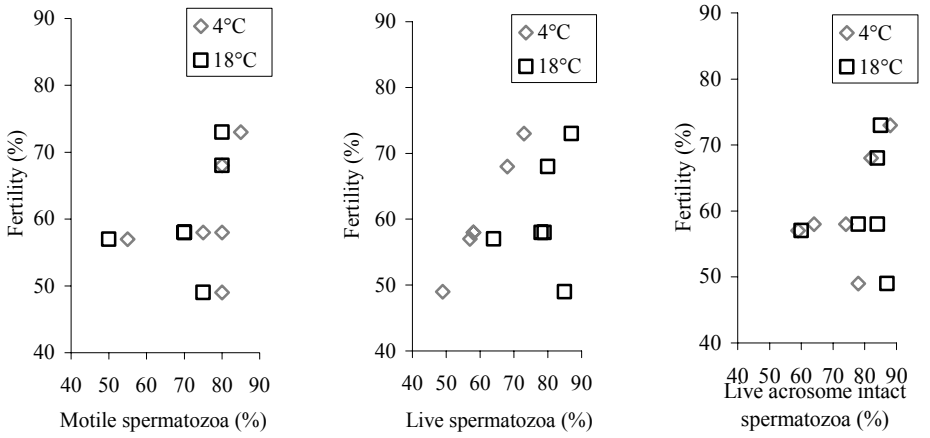

Figure 6. Correlation between fertility (percentage of inseminated does that produced kids) and the (A) percentages of motile spermatozoa estimated under a microscope; (B) live spermatozoa determined by flow cytometry after staining with SYBR-14®/PI; and (C) live spermatozoa with intact acrosomes determined by flow cytometry after staining with PNA-FITC/PI. Sperm from 6 bucks were stored for 24 hours at 4°C or 18 °C.

Correlation between semen quality and fertility

The mean kidding rates after insemination with liquid semen varied from 49% to 73% between the individual bucks. Since 70 up to 208 does were inseminated with semen from one buck, more than one ejaculate had to be used from each buck, with the result that the fertility percentages of a buck varied by 7% to 10% among ejaculates. Kidding rates obtained after AI correlated poorly with the percentage motile cells in the ejaculates used for the inseminations, irrespective of whether motility was assessed after storage at 18°C or at 4°C (R=0.36 for semen from 6 bucks stored at 18°C and R=0.35 for semen from the same 6 bucks stored at 4°C, figure 6a*)*. The percentage of live spermatozoa and the mean kidding rates showed stronger correlations (R=0.44 at 18°C and R=0.27 at 4°C, figure 6b). Figure 6 c shows that the percentage of live spermatozoa with intact acrosomes correlated poorly with fertility (R=0.18 at 18°C and R=0.27 at 4°C).

Discussion

This study shows that storing goat semen for 24 h at 18°C results in similar motility of spermatozoa than storing such semen for 24 h at 4°C. Practically this outcome

implies that liquid semen does not have to be cooled to 4°C, nor stored and transported under refrigerator conditions when used within a day after collection.
Although it is known that glycerol can have a negative effect on semen quality, the number of bucks that reacted negatively on the glycerol was impressive. Glycerol, despite its value as cryoprotectant, is noxious to membrane integrity of spermatozoa of humans and sheep [8, 9], and also metabolically toxic to these cells [25]. Glycerol is the preferred cryoprotectant for goat semen because others are less effective [25]. Glycerol increases osmotic stress and differences among individuals in their sensitivity to glycerol may therefore be due to different abilities to modulate the passage of water and other molecules through cell membranes. Perhaps bucks with a limited resistance to glycerol and/or extender(s) should be eliminated from AI services, since their frozen semen will not result in successful AI.

Even though flow cytometric analysis is considered more accurate to assess semen quality and preservation methods then conventional microscopic evaluation techniques [9, 10, 17], we found a very high correlation between the results of flow cytometric analysis and microscopical assessment of motility (figure 5a and 5b). The percentages of live acrosome intact (FITC-PNA/PI), and live spermatozoa (SYBR-14®/PI) were consistently similar as could be expected. Unlike earlier studies suggesting that semen analysis can partly predict fertility of bucks [5, 19] the current study shows no significant correlations between the percentages of motile spermatozoa and live spermatozoa with intact acrosomes, respectively, and fertility. The correlation between the percentage live spermatozoa and fertility is somewhat stronger and more experiments should be performed to assess the reliability of this outcome. Flow cytometry allows the objective, rapid and simultaneous analysis of a number of properties in a large number of spermatozoa [3], suggesting that the results of flow cytometric analysis may allow the estimation of the fertility of a semen sample. Yet, it has been shown that combining the results of various sperm function tests does not result in more reliable estimation of fertility [3]. Microscopic evaluation of motility is subjective, as it entirely depends on the ability and experience of an operator estimating motility under a phase-contrast microscope. Even so, the microscopic assessment of motility after thawing or immediately prior to insemination is simple, easy and quick and provides the parameter of choice under field conditions to indicate the degree of damage inflicted by cryopreservation. This study shows that motility assessments performed by the experienced operator reliably corresponded to flow cytometric viability assessments. This may implement that the purchase of expensive flow cytometric equipment (for accurate integrity assessments of spermatozoa) is not required to improve goat AI.
The current study shows that the removal of the seminal plasma does not affect the semen quality of liquid-stored or frozen-thawed semen in Dutch dairy goat bucks. Buck seminal plasma contains two compounds that can react with ingredients of diluents. Bulbourethral secreting glycoprotein-60 (BUSgp60) has a triacylglycerol

hydrolase activity that causes deterioration of buck spermatozoa in skimmed milk extender. BUSgp60 causes a decrease in the percentage of motile spermatozoa, a deterioration in the quality of movement, the breakage of acrosomes, and the death of buck spermatozoa [15, 20, 21]. The other compound is egg yolk coagulating enzyme (EYCE) which has phospolipase A2 activity and therefore hydrolyzes egg yolk phosphatidylcholine (PC) into fatty acid and lysophosphatidylcholine (LPC) [2, 5]. LPC acts like a detergent on biomembranes and is therefore toxic to buck spermatozoa. However, the strength of the detergent properties of LPC depend on the amount formed, temperature (enzyme activity and thus LPC formation is temperature dependent), dilution or degree of removal of seminal plasma, season of semen production and breed of fowl providing the egg yolk [15]. In the buck, removal of seminal plasma by washing the spermatozoa immediately after collection increases the percentage of live cells and their motility during storage in egg yolk or milk diluents [4, 22] although this effect does not always occur [6]. The storability of ejaculated and washed semen is less than that of epididymal semen [5, 13, 19], suggesting that seminal plasma contains compounds detrimental to in vitro storage.
The current study shows that washing of semen of Dutch dairy goat bucks does not affect their viability before freezing or after thawing, implying that under field conditions washing is not necessary in The Netherlands. In other breeds washing can be necessarily [12, 23]. Since the type of season affects the composition of seminal plasma this experiment was performed during the natural breeding period in order to obtain optimal results. Trehalose, present in the TCG-buffered extender used in the current study, has been shown to reduce acrosomal detoriation [26]. In the current study the type of extender had no effect on semen quality before freezing because it reduces deterioration of spermatozoa compared to the other extenders used. In contrast, the current study shows that the extender containing TCG and egg yolk is preferred for freezing buck spermatozoa because it reduces live acrosome intact spermatozoa less than the other extenders used. This is partly consistent with the findings of an earlier study [2] in which it was shown that a TCG diluent containing egg yolk reduced deterioration of spermatozoa as assessed with motility characteristics compared to other extenders. In contrast, in the same study an increase was noted of acrosomal damage. Egg yolk has the property to alleviate cold shock by its ability to shield membranes. Although it is well known in farm animals that egg yolk protects sperm cell membranes, egg yolk may also destabilize such membranes, as shown by an increased frequency of acrosomal damage associated with an increase in the concentration of egg yolk [24]. The bucks we used showed large differences in the quality of their semen with respect to motility, percentage of live cells and percentage of intact acrosomes before freezing and after thawing. Although the bucks performed consistently, it is arguable whether or not to draw strong conclusions from mean values.

In conclusion semen analyses are useful to determine cryoresistance, reaction of spermatozoa to extender and effect of removal of seminal plasma, although they

may not predict actual fertilizing potential, but they may predict low fertility and allow exclusion of those samples or even males from an artificial insemination program [11]. It is therefore advisable to test each buck in order to determine the preferred treatment of its semen with respect to resistance to glycerol, extender and removal of seminal plasma. Based on these tests the most preferred storage treatment can be adapted for goat AI.

Acknowledgements
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Chapter 3

SRLV: Clinical and pathological evaluation

In preparation

A semi-quantitative study of the progressiveness of naturally acquired Maedi-Visna in two sheep breeds

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Introduction

Small Ruminant Lentiviruses (SRLV) cause persistent infections in their hosts, which induce typical lymphocytic lesions in several organs. These lesions are generally considered to be slowly, but relentlessly, progressive and thus fatal.

Ever since Maedi Visna (MV) was recognized as a specific infectious disease, differences between breeds of sheep in susceptibility to developing disease have been suggested. For example, the healthy Karakul rams imported into Iceland in 1933 introduced a disastrous epidemic disease in the native sheep population whereas there are records over many years that strongly suggest that the original Karakul population in Germany never showed any symptoms of MVV infection [1, 2]. In addition, breed-associated differences in susceptibility to acquiring the infection have been demonstrated in epidemiological studies and observations [3-5] and chapter 5 of this thesis also deals with this subject.

On the other hand, experimental infection studies indicated that there are differences in pathogenicity and tropism between virus isolates [6-10] and even strains have been described which appear to be non-pathogenic in their original hosts [9, 11]. However, an experimental infection study using different strains in identical twin lambs indicated that particularly host genetic factors determine the pathogenic process [12]. Together, these observations lead to the assumption that lesion development is mainly dependent on the genetic composition of the host.

Experimental studies on the development of lesions are hampered by the facts that lesions develop relatively slowly and that it is uncertain if experimental infections mimic the natural situation. However, naturally acquired infection is unpredictable and the actual moment of infection is difficult to determine, but knowing the actual length of the pathogenic process is necessary for studying its progressiveness.

We recently saw flocks of a particular new composite breed derived from Milksheep-Texel breed crosses with severe losses due to MV and an unusual prominence of the arthritic form hitherto unknown in the Texel-breed sheep, which have been predominant in the Netherlands for decades. It was suggested that this new composite breed was more susceptible to MVV induced disease, i.e. that there was a difference in the progressiveness of the lesions.

To establish a possible host-associated difference in the progressiveness of lesion development a comparative prospective natural transmission study in two flocks of sheep of different genetic constitution, kept under similar conditions, was performed. Sheep were closely monitored for initial MV-infection using Enzyme-Linked Immuno Sorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) in blood to be able to determine the length of the pathogenic process at the time the sheep were subjected to semi-quantitative clinical evaluation and post-mortem examination.

Materials and methods

Experimental design, sheep flocks and sampling

For this natural transmission study two flocks of 30 barren ewes were formed, each consisting of 20 uninfected (certified MVV-free) sheep mixed with 10 naturally infected (ELISA and PCR positive) sheep of the same breed. The average age of the ewes was 4,5 years ranging from 2,5 to 6 years. Flock T consisted of Texel sheep and Flock B of the composite breed drawn from Milksheep-Texel F1's. Both flocks were kept separately on pasture at the Veterinary Faculty's farm and only housed for a two-week period. The ewes were not mated and did not lamb during the study. Early in the study two infected ewes of flock B died of unrelated causes.

Blood samples were taken bimonthly. Blood was collected from the jugular vein with a vacutainer system using either 10 ml plastic serum tubes, or 10 ml glass EDTA-tubes (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK). After 1 year, all MVV positive and part of the MVV negative ewes were euthanized for post-mortem examination. The remaining ELISA- and PCR-negative ewes were put together and blood sampled for another six months. The study was approved by the Institutional Ethics Committee on Animal Experiments (license DEC 0311.0501).

Serology and PCR testing of blood and tissues.

Testing for specific antibodies (ELISA) and proviral MVV DNA in the blood by PCR using LTR-primers was as described in chapter 5 and 6 of this thesis. Whole blood and separated plasma were stored at -20 ^{0}C within 8 hours after collection. Specific antibodies were detected with Elitest-MVV (HYPHEN BioMed, Andrésy, France) according to the manufacturers' instructions. For PCR on blood samples, DNA was extracted from 100 µl whole blood using the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) with its proprietary blood chemistry including the proteinase K treatment. Tissue samples were subjected to the PCR assay after DNA extraction using DNeasy Blood&Tissue Kit (Qiagen GmbH, Hilden, Germany).

Clinical evaluation

At the end of the experimental period an experienced clinician evaluated all ewes according to a preset protocol without knowledge of their status. Manual body condition score (BCS), body weight (BW), age, respiration (frequency, tachypneu, dyspneu, accessory respiration movements, nasal discharge, coughing and lung auscultation), udder (size, constitution, symmetry), joints (lameness, swelling) and the central nervous system (paresis, ataxia) were evaluated. All symptoms were scored on a scale from 0 to 3 (0= no, 1= mild, 2= moderate and 3= severe). The results for lung, udder and joints were grouped. Thereafter the outcomes of these groups (lung, udder and joints) were combined in an overall clinical impression.

Post-mortem and histological examination

After euthanasia by intravenous injection of T61 (Intervet, Boxmeer, The Netherlands; 5 ml/50kg BW i.v.), the organs were scored according to a standardised protocol for macroscopic lesions corresponding with MVV infection. Macroscopic and microscopic lesions were scored on a scale from 0 to 3 (0= no, 1= mild, 2= moderate and 3= severe). The lungs were examined for structure, color, consistency, weight and lesions compatible with secondary bacterial infections. The bronchial and mediastinal lymph nodes were comparatively scored for severity of hyperplasia. For the udder the size, weight, consistency and severity of fibrosis were determined. Brain and spinal cord were examined for gross abnormalities. And finally the carpal and tarsal joints were evaluated for external swelling, the synovia was evaluated as to color, amount and viscosity, joint cartilage was judged and bone aberrations were noted.

After macroscopic examination, tissue samples were taken for histological examination. The samples were fixed in 10% formalin, paraffin-embedded and approximately 4 µm sections were cut. Sections were stained with haematoxylin and eosin for routine histopathological evaluation. Lung tissue was scored for the degree of interstitial pneumonia (multi-focal or diffuse), and additional changes of airways, vessels and septa. The udder was scored for the amount of periductal lymphocytes, lymph follicles, fibrosis and alveolar atrophy. In the central nervous system (cerebrum, and spinal cord at three randomly chosen levels) the presence of meningitis, chorioditis, leukoencephalomyelitis and demyelinisation were recorded. The presence and severity of villus hyperplasia and lymphoplasmacellular synovialitis within the joints was also scored.

Statistical analysis

For evaluation, the ewes were divided in three groups; ewes that remained sero- and PCR-negative throughout (non-infected; group 1), ewes that were infected during the course of the study (pathogenic process of less than a year; group 2) and ewes that were already infected at the start of the experiment (pathogenic process lasting longer than one year; group 3). The analyses were performed with the help of Excel (Microsoft) and SPSS (SPSS Inc., USA; version 12.0). Statistical testing was done using the Kruskal-Wallis test (non-parametric test met k-independent samples), Chi^2 and Spearman's Correlation via cross tabs with a significance level of $p<0.05$. To test the influence of the length of the pathogenic process on specific parameters, multinominal regression was used with the histological quantitative scores as dependent, and breed and infection status as independents. There was a trend if $0.05<p<0.10$. In order to have enough frequencies in each group the macroscopic and microscopic scores of 0 (no symptoms) and 1 (mild symptoms) were grouped, and the 2 (moderate symptoms) and 3 (severe symptoms) scores were also grouped. This could be done because none of the symptoms scored are specific for MVV-lesions.

Results

Determination of the length of the pathogenic process

In the ewes that apparently got infected, specific MVV-antibodies appeared virtually together with PCR-positivity. Overall, antibody and viral-DNA detection in blood corresponded well, with the exception of a few specific ewes in flock T in which PCR-positivity was less consistent over time. In flock T only 5 ewes got infected, whereas 9 ewes in flock B acquired infection during the one-year course of the natural exposure.

Since development of the lesions associates with the presence of proviral DNA in macrophages and peripheral blood monocytes [13] the first of the sequentially taken blood samples positive in PCR were considered as the beginning of the pathogenic process, hence its length at the time of post-mortem investigation was known. So, for a total of 14 ewes the length of the pathogenic process was known; the ewes were categorised as having had a pathogenic process of less than a year (group 2), whereas the total of 19 initially infected ewes was categorised as having had a pathogenic process lasting longer than a year (group 3).

Clinical examination

The clinical evaluation revealed alterations in respiration rate, udder-condition and joints. There was a significant increase in severity of deviations scored for the overall clinical impression, irrespective of breed, between the uninfected ewes (group 1) and the recently infected ewes in group 2 ($p<0,001$) and the longer infected ewes in group 3 ($p<0,001$), but there was no difference between the latter groups ($p>0,05$). However, ewes in group 3 yielded higher scores for respiratory symptoms ($p<0,001$) and in overall clinical impression ($p<0,001$) than those in group 2. For joint changes there was no difference. Surprisingly, udders of ewes in group 2 showed more clinical changes than those in group 3. Body weights and BCS were lower in groups 2 and 3 as compared to group 1; this difference was independent of age.

There was a difference between both flocks (Fig 1) regarding the overall clinical impression symptoms, although there is only a statistically significant difference among the uninfected ewes ($p<0,05$) and not in the groups of recent ($p>0,05$) and longer ($p=0,05$) infected individuals. The non-infected ewes (group 1) showed various clinical abnormalities. The scores for respiration and udder changes were significantly lower than in the infected groups 2 and 3 ($p<0,05$).

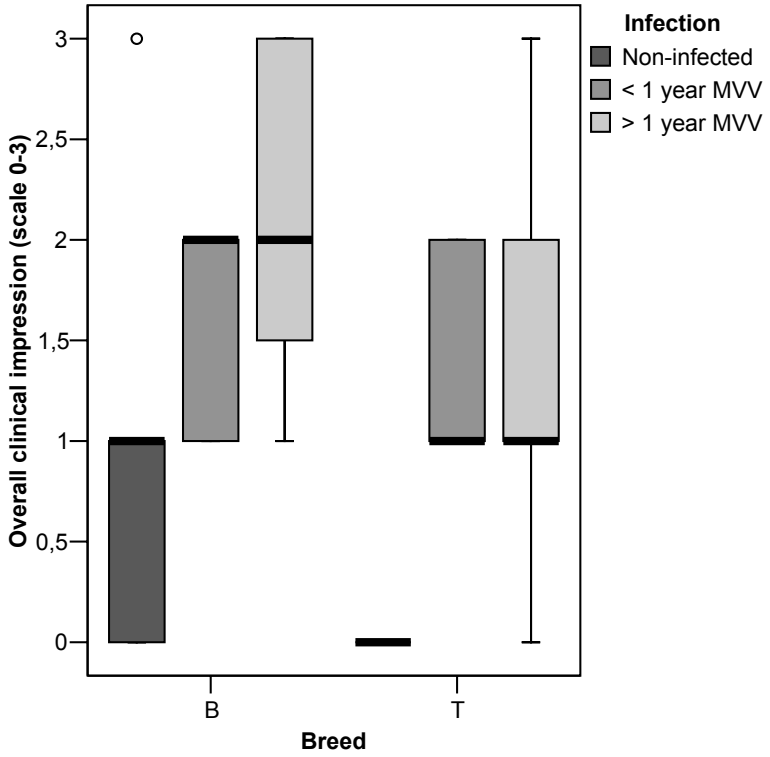


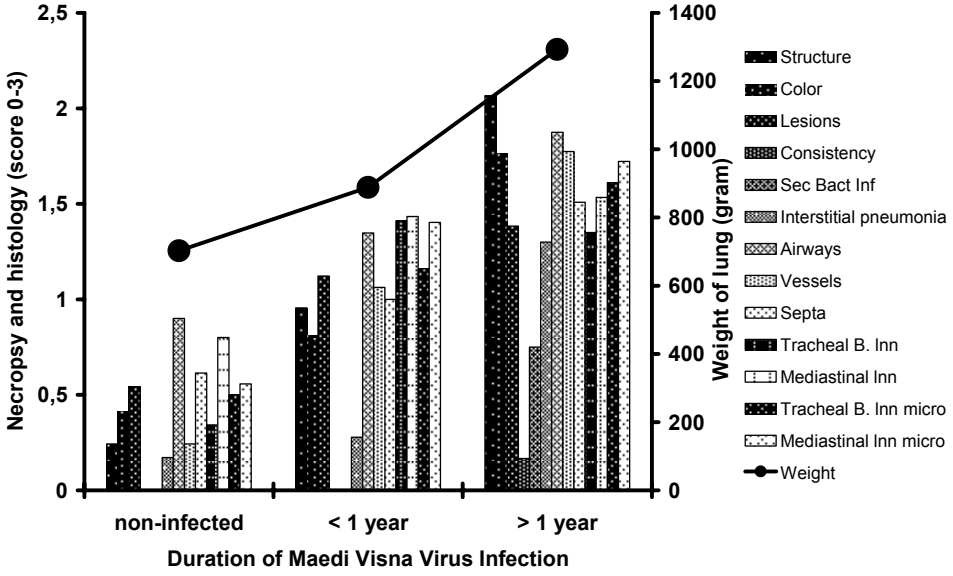
Figure 1: Box plot image of the overall clinical impression of recent (< 1 year) and longer (> 1 year) naturally infected Maedi Visna Virus (MVV) ewes and non-infected flock mates in two different breeds of sheep. (Plots are based on the median and quartiles with interquartile range that contains 50% of values. The whiskers are lines that extend from the box to the highest values, including excluding outliers and extremes.)

Macroscopic evaluation and histological changes

Lungs

At post mortem examination the severity of the gross changes in the lungs clearly correlated with the length of MVV infection (Fig 3a). Overall, the lungs of group 2 and 3 showed an increased firmness and weight. Histologically, it was also clear that the degree of interstitial pneumonia (multi-focal or diffuse), increased with the length of infection (Fig 2a,b). This was also the case for the bronchial and mediastinal lymph nodes in which hyperplasia was more severe in animals that were longer infected.

3a



3b

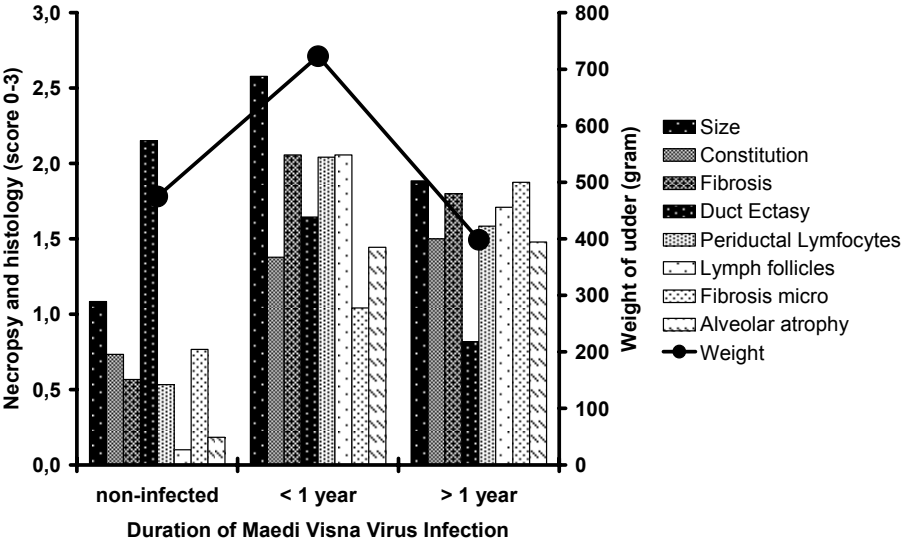


Figure 3a: Necropsy and histological examination of lung and its lymph nodes (3a) and udder (3b) in recent (< 1 year) and chronic (> 1 year) naturally acquired Maedi Visna Virus (MVV) infection in two breeds of sheep and non-infected flock mates. Necropsy and histological symptoms were scored on a scale from 0 to 3 (0= no, 1= mild, 2= moderate and 3= severe).

Mammary glands

In the udders, the macroscopic and microscopic lesions were more severe and weights were higher in group 2 ewes (Fig 3b) then in the other groups. The only increase with the length of the pathogenic process was found for fibrosis (Fig 2c,d). The weights of udders and lungs paralleled the results of the clinical examination and the scores of the histopathology (Fig 3a,b).

CNS

No macroscopic changes were present in the brains and spinal cords of any animal; however, histopathological examination revealed meningitis (3x), leukoencephalo-myelitis (2x) and demyelinisation (4x) in a total of 5 individuals (Fig 2e). These lesions were compatible with Visna.

Joints

Although the macroscopic evaluation of the carpal and tarsal joints revealed external swelling in all groups, no synovial abnormalities, nor changes of joint capsule, cartilage and bone surface were observed. Microscopically, however, villus hyperplasia and lymphoplasmacellular synovialitis were observed (Fig 2f) and their severity correlated with the length of infection, but there was no significant difference between the three groups ($p>0,05$).

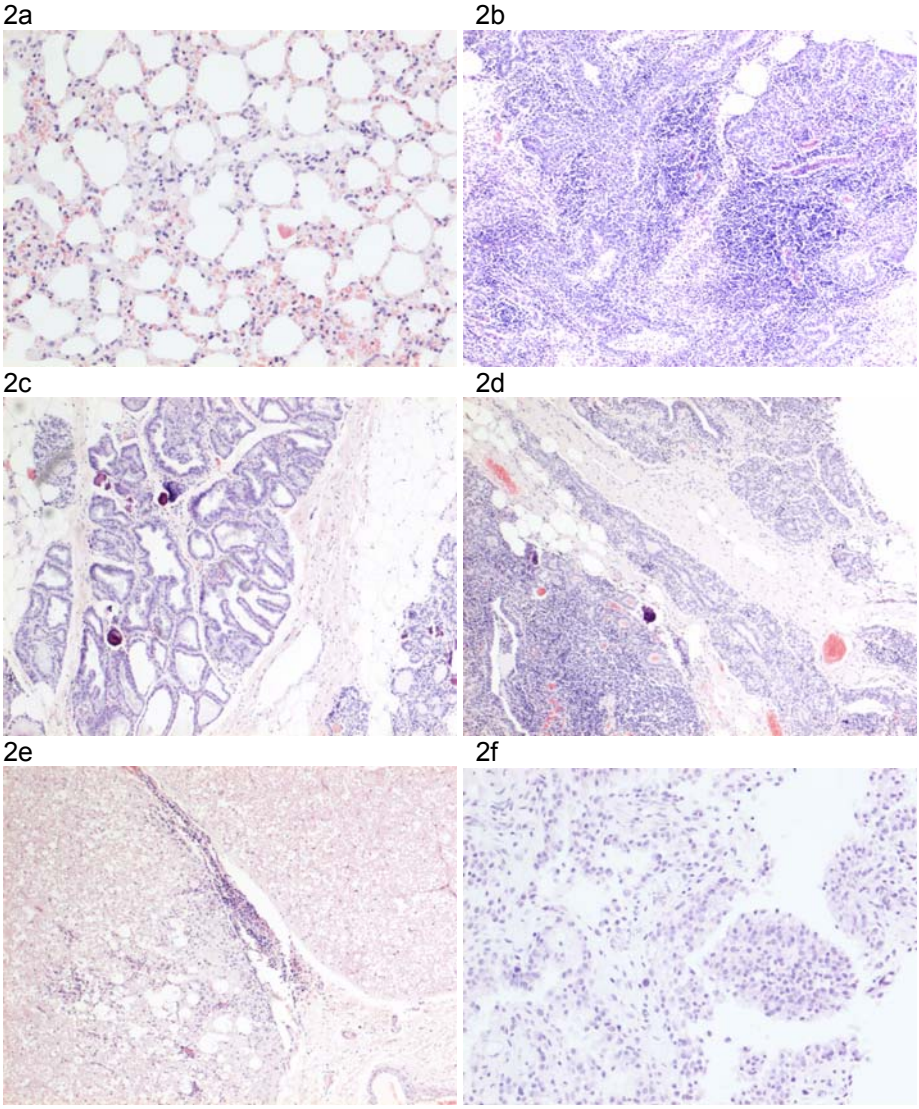


Figure 2: Examples of Histology of haematoxylin and eosin stained sections (± 4 µm) of lung (2a,b), udder (2c,d), spine (2e) and synovialis (2f) of sheep after naturally acquired Maedi Visna Virus infection. Sections showing mild diffuse histiocytic interstitial pneumonia (2a), severe interstitional lymphocytic pneumonia (2b), mild lymphocytic infiltration around individual glands (2c), periglandular lymphocytic infiltration and mild fibrosis (2d), moderate focal axonal sheath swelling with axonal loss and lymphocytic leukomyelitis (2e), and moderate infiltration of hyperplastic villi by lymphocytes and histiocytes(2f).

Flock difference/Breed influence

Figure 4 illustrates that lesions present in the lungs and associated lymph nodes both macroscopically and microscopically (Fig 4a) were more severe in Milksheep-Texel F1's then in Texel sheep. A breed difference between the flocks for deviations in the udder was not apparent (Fig 4b) neither for those in the central nervous system (Fig 4c) and the joints (Fig 4d).

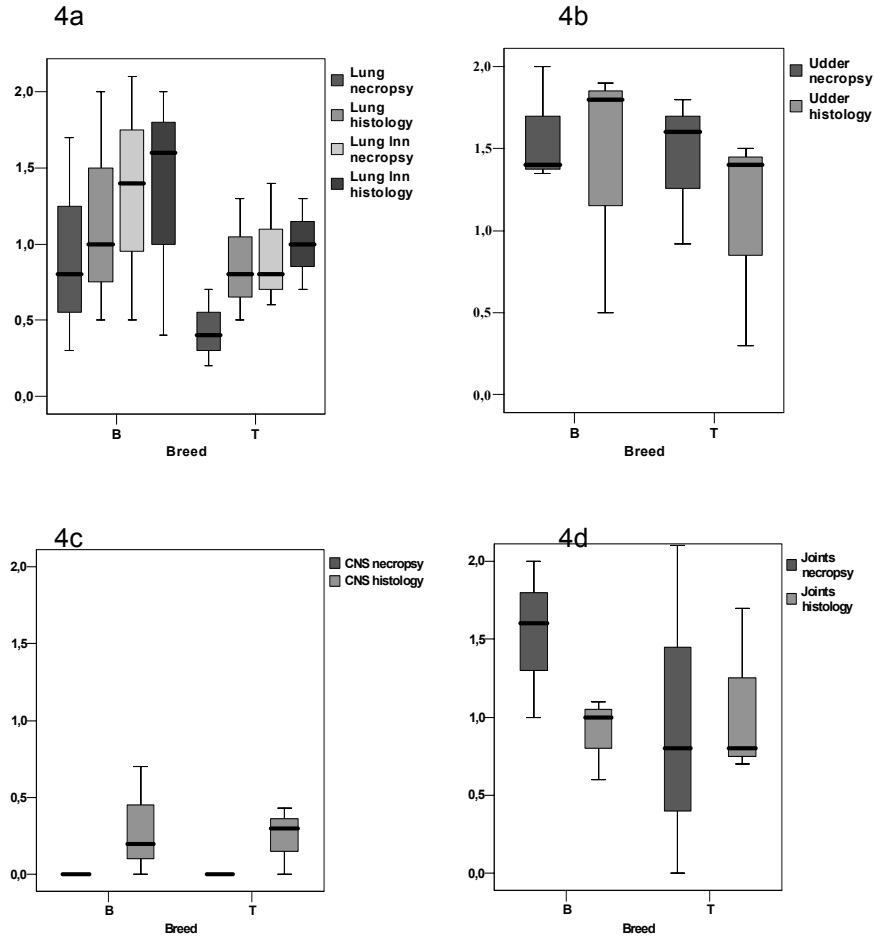


Figure 4a, b, c and d: Necropsy and histological scores (0-3) of the lung and the adherent tracheal bronchial and mediastinal lymph nodes (4a), udder (4b), central nervous system (4c) and joints (4d) in two breeds of sheep with naturally acquired Maedi Visna Virus (MVV) infection (see M&M section for description of scored changes). (Plots are based on the median and quartiles with interquartile range that contains 50% of values. The whiskers are lines that extend from the box to the highest values, excluding outliers and extremes.)

Viral DNA in tissue samples

LTR-PCR was performed on tissue samples from lung, udder and CNS of a limited number of ewes (Table 1). The majority of tissues from infected ewes from both flocks gave a positive result. However, a number of tissues from ewes that remained seronegative and negative in blood-PCR during the study and which were therefore considered appropriate negative controls also gave positive results. As this was unexpected, procedures on these samples were repeated and similar results were obtained. Subsequently, another 5 'negative' ewes from this study were sacrificed and tissues samples were taken with extra precautions to avoid contamination at sampling and further processing. Again, a number of tissues were found positive for SRLV proviral DNA. Also, the application of a different primer set (so-called leader-primers; Brinkhof et al, in preparation) gave positive results.

In order to examine if 'slow conversion' could explain our findings, the serological and blood-PCR monitoring was continued in the remaining 13 negative sheep for six months, but all animals remained negative. Subsequently, an attempt was made to reactivate the supposed latent infection; the sheep were treated 3 times with dexamethason (Voreen, Boehringer Ingelheim BV, Alkmaar, the Netherlands; 15 ml i.m.) on alternating days (license DEC 0311.050). On the last 2 days also a preventive antibiotic treatment (Engemycine 10%, Intervet BV, Boxmeer, The Netherlands, 6 ml i.m.) was provided. Blood samples were taken with intervals increasing from to 2 to 7 days until 5 weeks post treatment. All samples were negative for SRLV-antibodies and proviral DNA.

Table 1: Results of LTR-PCR in tissue samples from the lung, udder and spine of MVV-free negative controls, recent (< 1 year) and longer (> 1 year) MVV-infected ewes, and exposed but consistently sero- and blood-PCR negative ('non-infected') flock mates of two breeds of sheep in different flocks.

Breed / **Tissue**		**Flock T**			**Flock B**		
Infection status	Negative controls (n)	'Non infected' (n)	<1year MVV (n)	>1year MVV (n)	'Non infected' (n)	<1year MVV (n)	>1year MVV (n)
PCR	pos/tot	pos/tot	pos/tot	pos/tot	pos/tot	pos/tot	pos/tot
Lung	**0/4**	**4 / 7**	**2 / 5**	**8 / 10**	**1 / 5**	**7 / 9**	**4 / 6**
Udder	**0/4**	**2 / 7**	**5 / 5**	**10 / 10**	**3 / 5**	**8 / 9**	**6 / 6**
Spine	**0/4**	**4 / 7**	**5 / 5**	**8 / 10**	**4 / 5**	**8 / 9**	**6 / 6**

Discussion

The expected horizontal transmission between adult sheep occurred; a total of 14 ewes (5 in flock T and 9 in flock B) sero-converted during the course of the study. The formation of specific antibodies likely requires a certain level of viral replication and since lesion development associates with local virus replication [13] the moment of seroconversion was considered as the actual start of the pathogenic process. Hence, the length of this process was known in a total of 14 ewes when they were subjected to post-mortem examination. Actually, the synchronicity between the seroconversion and the detection of the viral DNA in the blood of these sheep supported this way of predating the start of the pathogenic process.

The ante mortem clinical evaluation demonstrated that infected animals had higher scores for most criteria, which was as expected. However, there was no clear difference between the recently infected (< 1 year) and the longer infected (> 1 year) groups, which indicate that the actual length of the pathogenic process exerts a limited effect on the clinical presentation. This implies that the progressiveness of the disease is highly variable and that clinical signs may even develop within a period of a few months.

The clinical and histopathological results of the udders were remarkable in that the recently infected ewes showed more severe lesions than the longer infected ones. This demonstrates that the udder lesions are not inevitably progressive, which contrasts with the generally accepted view that MVV-lesions are progressive per definition. Previously, however, a similar observation was made after experimental infections [14] and the results of a field study also suggested this [15], i.e. sero-positive prepubertal lambs had more mammary lymphocytic infiltration than 1.5 year old ewes. The microscopically determined degree of fibrosis was consistent with the length of the pathogenesis, but the degree of alveolar atrophy was not. There was a high prevalence of duct ectasia in these ewes, both negative and positive for MVV; the 3-year old Texel ewes had never lambed and 3 out of 4 showed duct ectasia. Although the numbers are low, this seems to be concurrent with findings in humans where duct ectasia is nonsymptomatic and not associated with the reproductive status. In dogs, duct ectasia is age independent and there may be breed predispositions [16].

The lesions found in the brain and spinal cord -meningitis, leukoencephalomyelitis and demyelinisation- are compatible with Visna [17]. Clinically, none of these sheep showed neurological disorders. This is probably explained by the relative mildness of lesions found. If these lesions were progressive a very unusual number of clinically affected animals were to be expected over time in these sheep. However, Visna (the neurological form of MVV-infection) always has very low occurrence in the field and therefore it seems more likely that CNS lesions are also not necessarily progressive.

Although some of the carpal and/or tarsal joints clinically appeared swollen, which is indicative for MVV-arthritis, the post mortem examination did not confirm this. However, joint lesions of a different nature did occur relatively frequently**.**

Progressiveness
The difference between the two flocks with respect to some of the clinical symptoms, the lesions at necropsy and the histopathology suggests a breed effect, i.e. sheep of flock B developed more severe lesions. This is in line with our expectation. To the authors' knowledge this is the first time that a breed associated difference to developing disease after natural infection is described in a quantitative manner. Several field observations suggested that breed associated differences in susceptibility to developing SRLV induced disease existed, but the decisive comparative study was still lacking. Notably, genetic susceptibility is scarcely studied, but one study found an association between susceptibility to Caprine Arthritis and Encephalitis Virus (CAEV) induced arthritis and certain caprine leukocyte antigens [18].

LTR-PCR on tissue samples.
The results of the viral DNA-detection in tissues substantiate the conclusion that the lesions found, which were actually compatible with those described for MV [17], indeed associated with the local presence of SRLV-proviral-DNA and can therefore be considered as specific.
The consistent finding of SRLV-proviral-DNA in the samples from the ewes that remained sero- and PCR-negative during the study, the non-infected, was completely unexpected. The six months of extra monitoring showed that the 'slow conversion explanation' was most probably invalid. The results of the attempt to re-activate the infection from its suggested latency with the corticosteroid treatment are not entirely conclusive because the expected leucocytosis in the week after the treatment was not observed which indicates that the chosen dosage was possibly insufficient to cause severe immune suppression. To further substantiate our hypothesis that these animals were non-productively infected, 4 sheep from an SRLV-free flock (certified for more that 5 years) were purchased, sacrificed and sampled (license DEC 0311.0501). Their blood samples were negative for antibodies and LTR-PCR negative for proviral DNA. A total of 12 tissues -3 per sheep- were subjected to PCR and all were negative. This outcome substantiates the results with the 'non-'infected', which suggested that non-productive 'infections' occur to a considerable degree in SRLV-exposed populations. These results are in line with an earlier, somewhat disregarded, study showing that only a fraction of the infected sheep in a population are detected with –sensitive- serology [19]. These authors detected virus in the cultured macrophages of a substantial number of exposed seronegative sheep, which suggested that the precursor monocytes contained the provirus. It is likely that in vitro culture of cells changes several biological functions of these cells and thereby did annul the restriction on viral replication. In the present study, however, blood samples remained negative in

PCR suggesting that the monocytes did not contain proviral DNA. However, this contradiction may be explained by a difference in sensitivity between both detection methods; co-culturing intrinsically employs the most extreme form of biological amplification, which in this case most probably exceeds the power of LTR-PCR on blood.
Further tests, e.g. by in situ detection of proviral DNA in tissues, are required to confirm the occurrence of non-productive or latent SRLV-infections.

In conclusion, this study shows that there are individual differences with respect to the progressiveness of the lesions, that severe lesions can develop in a few months, that the progressiveness differs between the breeds examined, but that the lesions in the udder and probably the CNS are not inevitably progressive and may even regress.

Acknowledgements
The authors would like to acknowledge Pim van Dijk, DVM, for clinically examining the sheep, the animal attendants for watching over the flocks as true shepherds, Johan van Amerongen and Louis van den Boom for assisting with the necropsies, Wendy van Hoeyen for meticulously processing and storing the blood samples and Marian Broekhuizen-Stins, Anky Schoormans and Martin van der Wal for assisting with the serology and the PCR.

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Chapter 4

SRLV: Laboratory evaluation

Small Ruminant Research 65 (2006) 136-141

Flowcytometric assessment of circulating peripheral blood monocytes in small ruminants.

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Abstract
The proportions of circulating peripheral blood monocytes were determined in 253 samples from a total of 50 small ruminants (sheep and goats) in various physiological conditions using FITC-labeled anti-CD14 monoclonal antibodies and flowcytometry. Monocytes were detected in all samples, their proportion varied from 0.2 to 35.5% of the peripheral blood mononuclear cell (PBMC) fraction, whereas current reference tables give 0% of the white blood cells as the lower limit. A statistically significant difference was found between sheep and goats and the animals' gender and age exerted an effect; the other potential factor lactation appeared to have no influence.

Keywords: anti-CD14; FACS; flowcytometry; monocytes/macrophages; small ruminants, sheep, goats.

Introduction

Since monocytes can act as the vehiculum of infectious agents they present a potential target for diagnostic approaches. Variation in the number of peripheral monocytes in vivo is likely to occur over time in association with age, gender, reproductive status, and the health-status of the individual [8, 12-15]. Particularly in diagnostics based on amplification of agent-specific DNA/RNA sequences it is important to know the range of variation in the presence of peripheral blood monocytes in order to assess the reliability of negative test results. Current handbooks on goat and sheep diseases quote monocytes as 0 to 4 % and 0 to 13 % of the white blood cell fraction, respectively, suggesting that monocytes can be absent in a given blood sample from sheep or goats [10, 11]. These quotes, however, are mainly based on the differential counting of 100 to 200 white blood cells in stained blood smears, which intrinsically has a low sensitivity. The quantitative variations in the monocyte proportions between individuals and within individuals and the factors that associate with these variations have not yet been studied extensively.

Flowcytometry after labeling the monocytes with a FITC-labeled (fluorescine isothiocyanate) anti-CD14 monoclonal antibody essentially offers a sensitive quantification technique on the basis of a large number of cells [1, 4-7]. CD14 is a myeloid differentiation Ag expressed primarily on peripheral blood monocytes and macrophages. The CD14 antigen is a high affinity receptor for the complex of lipopolysaccharids (LPS) and LPS-Binding protein (LBP) [3].

In this study, we determined the peripheral blood monocyte proportions of the PBMC fractions in groups of sheep and goats of both gender with various ages and physiological conditions as well as the variations in time using a flowcytometric method.

Materials and methods

Animals

White dairy goats (n=24) and sheep from the “Texel” (n=14) and “Zwartbles” (n=12) breeds originating from 5 different herds were used.

First, a single sample study was conducted on these 50 animals. Samples were obtained from 13 males (2 adult bucks and 5 prepubertal bucks, 6 prepubertal rams), 37 females (21 adult goats: 11 lactating, 10 non-lactating, and 16 sheep: 10 lactating, and 6 non-lactating).

Secondly, an additional 28 animals were studied in a longitudinal set-up. This group included 11 female goats (2 prepubertal and 9 adults non-lactating), 3 prepubertal bucks, 11 female sheep (2 prepubertal, and 9 adults of which 4 were lactating) and 3 prepubertal rams. These animals were sampled for 5 successive days and thereafter at days 14, 21 and 28.

Blood was collected from the jugular vein with a vacutainer system using 10 ml glass EDTA-tubes (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK). All samples were processed on the day of collection.

Determination of monocyte proportions.
Peripheral Blood Mononuclear Cells (PBMC's) were collected through density gradient centrifugation; 1250 µl blood was diluted with an equal volume of PBS-0 (Phosphate Buffered Saline, pH 7.2, without Calcium Chloride and Magnesium Chloride; Gibco, UK). Of this dilution, 1250 µl was slowly pipetted upon 750 µl Ficoll (density 1.078, Ficoll 400, Sigma-Aldrich, Steinheim, Germany) in an Eppendorf vial. After centrifugation for 2 min at 10.000 g (Eppendorf 5417R), the interphase was carefully removed and resuspended to a final volume of 2 ml in FACS-buffer (Dulbecco's PBS - 0.0085 M without Ca and Mg, Bio Whittaker, Europe; supplemented with 1% Bovine Serum Albumin and 0.1% $NaNO_3$). Subsequently, the sample was centrifuged again for 2 min at 2600 g (Eppendorf 5417R) and the supernatant discarded. The pellets were resuspended in 100 µl of a 1:50 dilution of anti-CD14-FITC (Clone TÜK4, Mouse IgG2a, Miltenyi Biotec, USA) in FACS-buffer and incubated for 5-10 minutes in the dark at 4°C [2]. The cells were then analyzed for green fluorescence by flow cytometry using a FACScan equipped with an Argon laser (Becton Dickinson, San Jose, CA), and PBS as sheath fluid [4, 7]. Per sample, a total of 10,000 cells of the PBMC fraction were analyzed.

Statistical analysis
The flow cytometric data were analyzed using Cell Quest software (Becton Dickinson, San Jose, CA) and WinMDI (Windows Multiple Document Interface for Flow Cytometry, version 2.8; http://facs.scripps.edu/software.html).
To further analyze the data, two linear mixed-effect models fit by maximum likelihood were designed with the R program (http://www.r-project.org/ version 1.6). Both models used the log-transformation on the data to obtain deviance residuals with a normal distribution. For the single sample study this model is *log (monocytes)~ factor (experiment) + factor (species) + factor (age) + factor (gender) + factor (lactation).* This data set was supplemented with the data from day 2 of the longitudinal study and they were indicated by the factor *(experiment).* In the longitudinal set-up, the model was *log (monocytes) ~ factor (species) + factor (age) + factor (gender)* day.* This is a linear model with random effect for the animals, auto-correlation with one day prior to the sample date and recognition of the different variances between species. Both models are a simplification of an original model in which the factors; monocytes, experiment, species, age, gender, day, lactation and Small Ruminant Lentivirus (SRLV)-infection status were inserted.

Results

Monocytes were demonstrated in all the 253 peripheral blood samples from the 50 individuals tested, proportions ranging from 0.2 to 35.5 % of the PBMC-fraction. Figure 1 depicts the distribution of the samples and shows that the majority of animals have monocyte counts between 2 and 14%. An example of a FACS result is given in Figure 2.

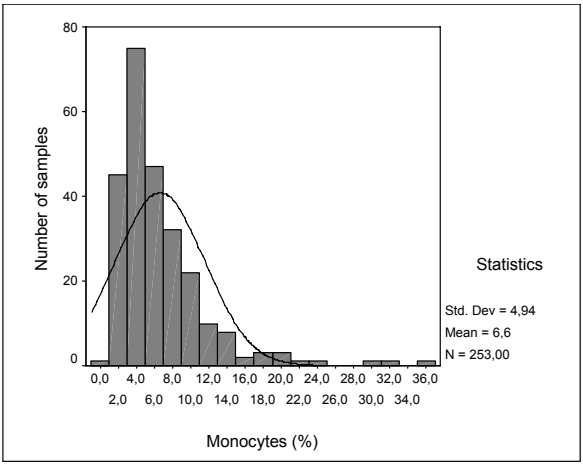

Figure 1: Frequency histogram of samples of CD14-FITC labeled monocytes in peripheral blood (% of PBMC's) as measured by flow cytometry. PBMC's (peripheral blood mononuclear cells) were obtained by density centrifugation in Ficoll (sg 1.078).

In the single sample study, goats and sheep had 4.6 ± 0.4 (mean ± SD) and 4.6 ± 1.2 % monocytes respectively, indicating no differences between these species. In the longitudinal study, however, a significant difference between goats and sheep (**$p < 0.001$) was found (Fig 3).

The longitudinal study also showed significant differences in monocyte proportions over the different sampling dates (**$p < 0.001$), indicating that there is an effect of the specific sampling date on the monocyte counts of the whole group (Fig 3). On account of the standard error at day 1 in goats (2,09) and at day 4 in sheep (2,29) the values are considered different because the ratio of the difference to the standard error is greater than +2.

The single sample study showed no effect of sex whereas the longitudinal study assigned an effect of sex (*$p < 0.05$) (Fig 4a). In both the single sample study (Fig 4b) and the longitudinal study a tendency was found related to age ($p = 0.08$ and $p = 0.06$). In Figures 4a and 4b the extreme value of 31.43% was not depicted. No effect was found of the factor lactation.

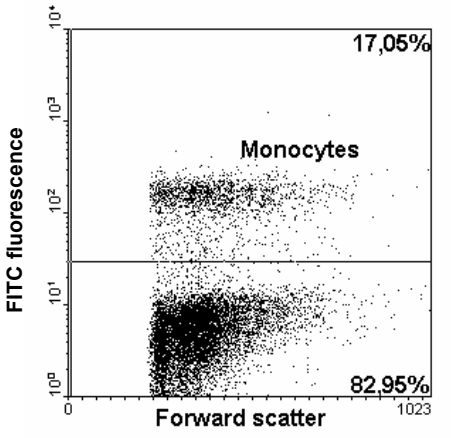

Figure 2: An example of a flowcytometric image of sheep monocytes based on two-dimensional forward-scatter and fluorescence (FL1= 505-605 nm) dot plot properties after CD14-FITC labeling. PBMC (peripheral blood mononuclear cells) were obtained by density centrifugation over Ficoll (sg 1.078). Monocytes present as a specific sub-population (17,05%) of the PBMC's in the upper quadrant of the top panel.

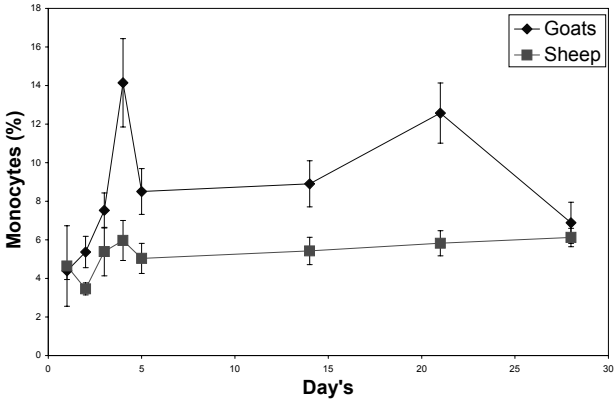

Figure 3: CD14-FITC labeled monocytes in peripheral blood over time (mean ± SD, % of PBMC's) as measured by flow cytometry. PBMC (peripheral blood mononuclear cells) were obtained by density centrifugation in Ficoll (sg 1.078).

Figure 4a

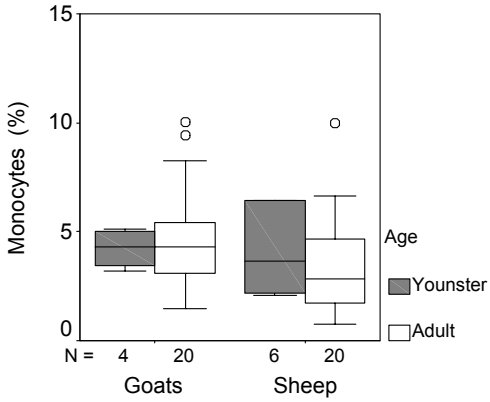

Figure 4b

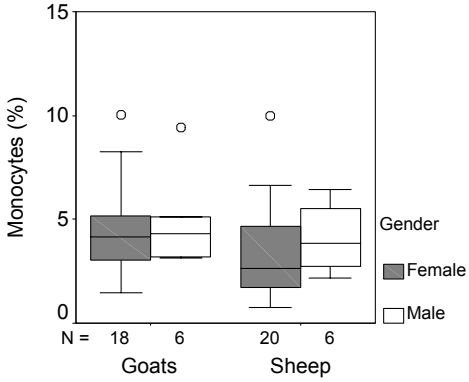

Figure 4a and 4b: Box plots of CD14-FITC labeled monocytes in peripheral blood (% of PBMC's) as measured by flow cytometry, depicting the effect of age (4a) and gender (4b) within the species. (Plots are based on the median and quartiles with interquartile range that contains the 50% of values. The whiskers are lines that extend from the box to the highest and lowest values, excluding outliers, and extremes are not depicted.)

Discussion

Data showed that monocytes were present in all 253 blood samples taken from 50 goats and sheep but that there was a considerable range in proportion, i.e. from 0.2 to 35.5% of the PMBC-fraction which in itself forms about 40 to 70% of the total white blood cell fraction. This is in contrast with the 0% lower limit in the hematological reference tables which indicate that 0-4% of the entire white blood cell fractions in goats and 0-13% in sheep consist of monocytes [10, 11, 14]. Our

quantification method assessed 10,000 cells of the PBMC fraction and this obviously resulted in more sensitive determination. A similar approach in human and bovine blood samples led to similar conclusions [6, 7].
The analysis of the different factors potentially influencing the proportion of monocytes did reveal a species difference as well as an effect according to the moment of sampling. These effects were taken into account for the further analyses. The differences in standard errors at day 1 in goats (2,09) and at day 4 in sheep (2,29) are coursed by large variances (73,38 and 83,52), which in its turn is coursed by outliers (31,43% and 35,5%). In line with similar studies in goats [14] and bovines (unpublished work co-author Koets) we also found an age effect.
Although there were only few males among our animals, the longitudinal study showed an effect of sex. A previous study in goats also found an effect of sex [14].
Our data does not show an effect of lactation on peripheral monocyte proportions, which clearly contrasts with a previous study using flow cytometry, which found significantly lower numbers of CD14+ cells –monocytes- in blood during lactation in ewes, as compared to the dry period [13]. We also determined the SRLV infection status of the animals used in this study by testing them for specific antibodies and by PCR and found 8 of the total of 50 animals SRLV-infected (data not shown). SRLV-infection as such did not appear to have an effect on the monocyte proportions. This is in contrast with another study, which showed reduced proportions of monocytes in the peripheral blood of SRLV-infected (5,98%) compared with healthy control goats (9,92%), also using CD14-FITC and flowcytometry [9]. However, both studies merely determined the presence of the infection, not the presence or stage of any pathogenic process.
Notably, monocytes were detected in all samples irrespective of the factors that appeared to influence their presence.

Monocytes may act as vehiculum for infectious agents like e.g. Mycobacteria and Salmonellae. The SRLV's even integrate their genetic material in the genome of the cells of the macrophage/monocyte lineage. So, in live animals, peripheral monocytes present a convenient substrate for diagnostics of some particular infections. In this view it is important to know that monocytes appear to be present in every blood sample from sheep and goats.

Conclusion

The results of this study refute the notion from the reference tables that blood samples with 0 % monocytes may occur by showing that all 253 samples taken from 50 small ruminants contained monocytes although their proportions in the PBMC fractions ranged considerably. Proportions of monocytes appeared to be influenced by the factors 'species' (sheep or goat) and 'sex' (male/female) and possibly 'age' but not by the factor 'lactation'.

Acknowledgements
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Chapter 5

SRLV: Transmission I

SUBMITTED FOR PUBLICATION

Comparative quantification of the lateral transmission of Maedi Visna virus in two flocks of sheep of different genetic constitution.

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Abstract
A comparative prospective study in two flocks of sheep of different genetic constitution quantified the rate of natural lateral transmission of Maedi Visna virus (MVV). Transmission was monitored by means of detection of specific antibodies and PCR-detection of the viral genome in whole blood. Both tests appeared to be of similar sensitivity in early detection of infection. The recorded rate of transmission at pasture was very low with between 0.12 and 0.19 new infections per infected ewe per year. However, during the short housing period, which simulated the usual indoor lambing season, the transmission rate increased to between 2.27 and 3.4 new infections per infected ewe per year. The differences between the flocks were statistically non-significant. Viral transmission appeared to cease at a certain point in time, which supports the notion that individual differences in susceptibility to lateral infection occur. The results of this study give rise to new views on the prevention, and new options for the control, of MVV-infections.

Key words: Maedi Visna Virus / SRLV / Sheep / lateral transmission / susceptibility

Introduction

Maedi Visna Virus (MVV) of sheep is a single-stranded RNA virus belonging to the group of Small Ruminant Lentiviruses (SRLV's) which causes chronic lymphocytic lesions in multiple organs after relatively long incubation periods. It is transmitted horizontally either by means of ingestion of infected colostrum and milk -lactogenic transmission- or by direct transmission between animals -lateral transmission- [1]. Particularly, the intimate contact between the ewe and her offspring appears to be important [2, 3]. Vertical -prenatal- transmission does occur, but its contribution to the spread of the infection is generally considered limited [1, 4].

Ever since MVV was established as a specific viral disease, a breed associated difference in susceptibility to infection and the subsequent development of disease has been apparent. Probably the most poignant example of this were the healthy Karakul rams imported into Iceland in 1933 which introduced a disastrous disease in the native sheep population [5, 6] whereas there are records over many years that strongly suggest that the original Karakul population in Germany never showed symptoms of disease suggestive of MVV [7]. So far, two studies suggested individual differences in susceptibility to MVV [8, 9] and several studies indicated that certain sheep breeds were less susceptible [3, 10, 11]. The results of these studies cannot be compared because they were influenced by several, typically local, variables like the keeping conditions and the management applied (stocking rate, selection/replacement policy etc) and, particularly, if there were indoor lambing periods. So, comparative studies are required to actually prove the notion of breed susceptibility.

In longitudinal epidemiological studies, particularly those focusing of the dynamics of the transmission, serology as a means to detect infection has the drawback of the relatively slow and individually variable antibody formation after infection, which makes determining the actual point in time of the transmission difficult. It is generally assumed that this can be overcome by using sensitive virus detection methods, e.g. PCR [12].

In order to be able to calculate the risk of transmission under in- and outdoor conditions, e.g. in the framework of prevention and control efforts, basic quantitative data on the transmission rate is required. At the same time this data would give insight in the influence of the conditions on the transmission rate. Such data is currently lacking.

In order to obtain quantitative data on the lateral transmission of MVV and to comparatively study differences between breeds, a prospective study of one year in two cohorts of barren sheep of different genetic constitution on the same farm with a short housing period was performed, using a serological test as well as a PCR-test to detect infection in blood samples taken with short intervals.

Materials and methods

Animals and set-up

Two flocks of 30 ewes each were formed. Flock A consisted of Texel-type sheep and flock B of a relatively new composite breed which was originally drawn from F1 crosses between purebred Texel and purebred Milksheep. The Milksheep breed was almost extinct some four decades ago and genotyping of the current population showed the typical characteristics of an island population, i.e. a high level of inbreeding (personal communication dr J.A. Lenstra). The composite breed drew attention because there were some flocks suffering from MVV-disease to a degree never seen before, i.e. clinical symptoms occurred already in relatively young sheep and particularly arthritis which is very rare in the common Dutch sheep population which mainly consists of Texel-type sheep. The average age of the ewes was 4,5 years ranging from 2,5 to 6 years. Both experimental flocks consisted of 20 MVV-free and 10 infected (seropositive and PCR-positive, see below) barren ewes and were separately kept on pasture on the Veterinary Faculty's farm and were housed separately for 2 weeks to simulate the usual housing period during the lambing season. The stable measured 90 m^2 and had natural ventilation. Hygienic protocols were implemented for all attendants. The ewes did not lamb and were not mated during the study. No concentrates were fed except a handful when luring them into the pens for blood sampling. Early in the study two ewes of Flock B (1 of the infected ewes and one non-infected) died of unrelated causes. The study was approved by the Institutional Ethics Committee on Animal Experiments (license DEC 0311.0501).

Serology and PCR

Every two weeks blood was collected from the jugular vein with a vacutainer system using 10 ml glass EDTA-tubes (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK) . Whole blood and separated plasma were stored at – 20 ^{0}C within 24 hours. Specific antibodies were detected with Elitest-MVV (HYPHEN BioMed, Andrésy, France) according to the manufacturers' instructions. This test was previously described [13] and extensively compared with other tests (Brinkhof et al, submitted for publication**).**

For PCR, DNA was extracted from 100 µl whole blood using the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) with its proprietary blood chemistry including the proteinase K treatment. Extracted whole blood DNA was subjected to real-time PCR using primers directed at the Long Terminal Repeat (LTR) [14]. Amplification was performed with a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using SYBRgreen as reporter agent and melting point analysis. The reaction mix consisted of 10 µl PCR mix (QIAGEN's Quantitect SYBR Green 1), 1 µl of each primer (12.5 pmol/µl), 2 µl sample DNA and 6 µl sterile ultrapure water. PCR procedure started with 15 min at 95°C –hot start- followed by 45 amplification cycles of 20 sec at 65°C, 15 sec at 72°C and 15 sec at 95°C.

Statistical comparison of test results
The results of PCR and Elitest-MVV were compared by a generalised linear mixed model using a binomial error distribution and a logit link function. The result of Elitest-MVV was the dependent variable in the model (1 = positive, 0 = negative) and the result of the PCR (1 = positive, 0 = negative) the explanatory variable. To take into account the interdependence of test results from the same animal, we included animal as a random effect in the model (this interdependence makes comparison by Cohen's Kappa invalid). Moreover, to examine if breed influenced the performance of the tests, we also included the interaction between breed and test result in the model.

Analysis of the transmission
On the basis of the outcome of experimental infection studies [15-17], we assumed for the analysis that antibodies against MVV infection could be detected 4-8 weeks after infection. In addition, we assumed that sheep were infectious immediately after they had been infected and that they remained infectious for the rest of the experiment. Next, if $\lambda(t)$ is taken as the rate of infection for an susceptible individual, then the probability that a randomly assigned susceptible animal that was non-infected at time t has become infected at time $t + dt$ equals

$$\lambda(t)dt + o(dt) \qquad \text{(a)}$$

From eq. (a) can be derived [18] that the probability that a randomly assigned susceptible animal that was non-infected at t1 has become infected at t2 equals

$$1 - \exp(-\int_{t1}^{t2} \lambda(t)dt) \qquad \text{(b)}$$

Based on Eq. (b), a binomial model can be formulated for the number of new infections (C) between t1 and t2 (C(t1,t2)):

$$C(t1,t2) \approx Binom.(S(t1);1 - \exp(-\int_{t1}^{t2} \lambda(t)dt)) \qquad \text{(c)}$$

with S(t1) is the number of susceptible animals at $t1$ and

$$\lambda(t) = \beta \frac{I(t)}{N(t)} \qquad \text{(d)}$$

the rate at which a susceptible individual becomes infected, in which β is the transmission rate parameter (number of new infections caused by one infected sheep per unit of time in a fully susceptible population), I(t) is the number of infected sheep at t and N(t) the total number of sheep at t.
We quantified β by a Generalized Linear Model (GLM), using a binomial error distribution [19]. In this model, the number of new infections in an interval was the dependent variable and breed and housing (outside or inside) were included as explanatory variables. Because of the uncertainty of the time from infection to seroconversion (see above), the housing period was included in the analysis as the

period from four weeks before the housing until four weeks after. The random and systematic components of the model were linked by a complementary log-log function and the average fraction of infected sheep (*I/N*) multiplied by the number of days in the interval was included as an offset variable. As a consequence, the outcome of the model is the number of new infections per infectious sheep per unit of time. The fit of the statistical model was checked by the mean residual deviance and by plotting standardized residuals against predicted values.

Results

Serology and PCR

The results of the serological test and the LTR-PCR are presented in a two by two table (Table I); PCR has fewer positive test results than serology. However, the analysis showed a strong association between both tests. The odd of a seropositive test result giving a positive result in the PCR is 16.9 times (95% CI 6.0-47.9) as high in comparison to the odds of a seropositive test result giving a negative PCR. Although there was a trend for a stronger association between both tests in Flock B, the interaction between breed and test result was not significant ($p = 0.08$).

Serology and LTR-PCR were equally efficient at early detection, i.e. both tests turned positive at virtually the same point in time. However, post conversion, PCR gave a less consistent result over time, particularly in a few specific animals in flock A (not shown).

Two by two table				
	PCR			
		Neg	Pos	Tot
Serology	Neg	167	12	179
	Pos	41	146	187
	Tot	208	158	366

Table I: Two by two table of the results of the serology and the LTR-PCR on whole blood of the sequential samples from 14 sheep that naturally acquired Maedi Visna Virus infection during the sampling period.

Lateral transmission

In Flock B the first new infection was detected in week 18, whereas in Flock A this occurred in week 23. Overall, 9 ewes in Flock B were newly infected during the year, whereas in Flock A only 5 ewes got infected, as determined by serology as well as PCR. Moreover, transmission seemed to extinguish after a certain period of time; 31 weeks in Flock B and 34 weeks in Flock A (Fig 1 and 2).

The transmission modelling showed that the transmission rate was significantly higher (p = 0.0049) during the period of housing, i.e. 3.4 infections per infectious ewe per year in Flock B and 2.27 infections per infected ewe per year in Flock A, as compared to the outdoor period when the transmission rates were 0.19 and 0.12, respectively (Table II). However, although the transmission rate in Flock B, both in- and outdoors, appeared higher than in Flock A, these differences were not statistically significant (p = 0.43).

Flock B

Week / Ewe	0-12	12	15	18	20	21	22	23	24	26	28	30	31	32	35	37	39-49
1	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
2	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
4	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
5	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
7	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
8	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
10-19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	†																
21-29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	†																
Total	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
MVV +	9	9	9	12	12	12	13	13	13	13	14	15	18	18	18	18	18

Figure 1: Lateral transmission pattern of Maedi Visna Virus infection under field conditions during one year, as determined with serology (Elitest-MVV; HYPHEN BioMed, Andrésy, France) in Flock B. The vertical bold lines depict the housing period (week 18 and 19) and an estimated period (8 weeks post infection) for seroconversion related to the housing.

Flock A																	
Week / Ewe	0-12	12	15	18	19	20	21	23	25	28	30	32	34	36	38	40	41-50
1	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
3	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
4	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
5	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
6-20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21-30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Total	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
MVV +	10	10	10	10	10	10	10	11	11	12	13	13	15	15	15	15	15

Figure 2: Lateral transmission pattern of Maedi Visna Virus infection under field conditions during one year, as determined by serology (Elitest-MVV; HYPHEN BioMed, Andrésy, France) in Flock A. The vertical bold lines include the housing period (week 20 and 21) and an estimated period (8 weeks post infection) for seroconversion related to the housing.

Conditions	Breed	New cases per infected animal per year	95% CI
At pasture	Flock a	0,12	(0,015-0,99)
	Flock b	0,19	(0,023-1,50)
Housed	Flock a	2,27	(0,29-18,0)
	Flock b	3,40	(0,43-47,3)

Table II: Number of laterally transmitted Maedi Visna Virus infections per infected ewe per year in two separated flocks on one farm at pasture and during a housing period simulating an indoor lambing season.

Discussion

The methods to detect MVV-infection in this study -serological test and a real-time PCR with LTR-primers in whole blood- gave relatively consistent results. The somewhat limited agreement between both tests is clearly due to a few animals in flock A where PCR-results were less consistent over time. Nevertheless, both methods detected infection at virtually the same point in time which is contrary to our expectation and that of others [12] that PCR would detect earlier. This can be considered as lack of sensitivity, e.g. as a consequence of using whole blood in a plain sample preparation method. However, virtually all the positive DNA preparations sustained 10-fold dilution (results not shown). In addition, other

studies using the same LTR-primers, but also other primers, using separated blood cells and more elaborate preparation methods also showed limited diagnostic sensitivity compared with serology [14, 20]. In this view it is suggested that both serology and LTR-PCR require a certain level of viral replication in the host to become positive which contends that the diagnostic sensitivities of both assays should be in the same range.
In conclusion, the results of the LTR-PCR as applied on whole blood confirmed the results of the serology in this study. The results also demonstrated that most infected animals continuously have provirus carrying monocytes in their circulation, which supports the assumption in the modeling that infected animals are continuously infectious to others.

The calculation of the overall rate of lateral transmission of MVV in the two flocks suggests, but does not prove, a difference in the efficiency of lateral transmission between the breeds. This means that this comparative study did not establish the epidemiological importance of the flocks' genetic constitution, although the observed tendency is in line with the expectation that sheep of Flock B would be more susceptible.
The modeling shows that the lateral spread of the infection was significantly influenced by the short housing period. At pasture, it took an infected ewe in both flocks between 2 and 3 years to infect another one. In conjunction with the average life expectancy of an ewe of four to five years this basically implies that the infection would have faded out if the sheep had not been housed. Note that the sheep were shortly penned for blood sampling every two weeks. However, indoors the efficiency of transmission was totally different; here it took an infected ewe only between 1.2 and 1.9 months to laterally infect another one, which means that the transmission rate was more than tenfold higher during the housing period. It should be noted that the housing conditions were relatively good; a roomy stable with good natural ventilation. Overall, these results suggest that in conditions without housing, MVV-infections will only spread very slowly, if at all, and will probably not reach levels of economic significance, which is in line with recent studies in Spain [20]. However, there is an established epidemiological relationship between the dam and her progeny [3] and if flock management –unknowingly- selects for the progeny of infected dams, MVV-infection rate may increase even without a housing period.
A recent study on lambs born indoors provided data indicating that lactogenic transmission was not as efficient as hitherto contended and consequently that lateral transmission at early age was more important [2]. Results of the current study quantified that lateral transmission between housed sheep can indeed be relatively efficient, which basically corroborates with that outcome. The epidemiological importance of lactogenic transmission is relative to the rate of lateral transmission, which as this study showed, strongly depends on housing. In conclusion, this study quantified that housing is the critical event in the spread of MVV. The generally experienced difficulties with the control of Caprine Arthritis

Encephalitis Virus (CAEV)-infections in modern goat dairies with year-round housing suggest that this conclusion applies for both MVV and CAEV.
An interesting finding was the apparent extinction of lateral transmission at a certain point in time in both flocks. Long standing experience with testing flocks in the field for MVV-infection showed that the initial infection incidences per flock greatly vary, even among flocks of the same breed held under similar conditions and management. Similar observations have been made in other countries. It is suggested that this variation is not reflecting a dynamic process, but a relatively steady situation as the consequence of differences in the overall susceptibility to MVV-infection of the flocks tested. It should be noted that the genetic composition of an established flock only changes slowly in time, but that rams, because of the number of progeny they produce, may have a profound influence in time by introducing susceptibility genes. So, the extinction of transmission documented in this study together with field observations, suggest the existence of individual differences in susceptibility to MVV, which is in line with another recent report [9]. In this view it is hypothesized that the apparent differences between breeds are not breed characteristics, but due to the prevalence of susceptible individuals within the breed. This prevalence is accidental since intentional breeding for resistance has, as yet, not been applied.
Apart from environmental and host factors, SRLV-transmission is probably also influenced by viral factors. In natural transmission studies, however, host and virus cannot be separated and therefore the possible influence of the virus strain cannot be ruled out. So this study employed the natural situation with the viruses that were common to the breeds involved. The fact that sheep seroconverted and became blood-PCR-positive in both flocks and that they had specific lesions at necropsy (to be published elsewhere) proves that the strains involved were at least replicated, which is likely prerequisite for transmission.
In conclusion, this study quantified the lateral transmission of MVV in adult sheep for the first time and demonstrated that housing is a critical event herein. For the prevention and control of MVV this means that management systems without housing run little risk of reaching the infection rates that cause economic damage, which implies that preventative measures should relate to the risk associated with the -mainly geographically determined- management system. This corroborates with the fact that the incidence of MVV in regional sheep populations varies considerably. The recorded cease of transmission in both flocks indicates -genetic- differences in susceptibility to MVV-infection between the individuals in the flock. This theoretically offers the option of controlling MVV in a flock or population simply by decreasing the average susceptibility through structurally selecting the annual replacements from the progeny of the seronegative ewes in an infected flock.

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Chapter 6

SRLV: Transmission II

SUBMITTED FOR PUBLICATION

Presence of pro-lentiviral DNA in male sexual organs and ejaculates of small ruminants

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Abstract
To be able to predict sexual transmitability of small ruminant lenti Viruses (SRLV) it is first necessary to know whether or not, and if so, when and where, the virus is excreted in the semen. This research focussed on the presence of proviral DNA of SRLV in semen and in the male genital tract in small ruminants. After initial results proving the presence of SRLV in serum, in a group of naturally SRLV-infected individuals (13 rams and 4 bucks), the emergence of proviral DNA of SRLV in semen was followed over time using real time polymerase chain reaction (PCR) which was also done for blood samples. These blood samples were also systematically sampled for seroconversion towards SRLV by Enzyme-Linked Immuno Sorbent Assay (ELISA) during the breeding season (August-February). A triple monocyte-macrophage count was performed on both blood and semen using a specific monoclonal antibody and flow cytometry. Epididymal semen and tissue samples of the testis, epididymides, ampullary glands, vesicular glands, prostate glands, and bulbo-urethral glands were tested positive for the presence of proviral DNA. This data indicate that small ruminants show intermittent shedding of SRLV proviral-DNA into epididymal as well as ejaculated semen of rams and bucks. No relation was found between the amount of monocytes and/or macrophages in the blood and the semen and the detection of proviral SRLV in the ejaculates.

Keywords: Small ruminant lentivirus; Maedi Visna Virus; Caprine Arthritis Encephalitis Virus; Sexual Transmission; Semen.

Introduction

Small ruminant lentiviruses (SRLV) such as Maedi Visna Virus (MVV) in sheep and Caprine Arthritis and Encephalitis Virus (CAEV) in goats are single-stranded RNA retroviruses that cause chronic, lymphocytic lesions and inflammation in a variety of organs. The virus spreads mainly through lateral transmission, most probably by droplets or cells discharged from the respiratory tract [1]. In addition, there is lactogenic transmisssion via ingestion of infected colostrum or milk [2-4]. Vertical -prenatal- transmission apparently also occurs, but to a limited extent [5, 6]. It is, however, unclear whether SRLV is sexually transmittable [7, 8]. The aims of this study are to assess the presence of SRLV and its shedding pattern in the male genital tract and in semen of naturally infected small ruminants. To prove that these SRLV are actually sexually transmittable the following steps should be made: semen collection of an infected donor and detection of the causal agent in the ejaculate, in vivo insemination of positive ejaculates in non-infected females, and subsequent symptoms of the infection in those females [9,10]. Thus, the process starts by detection of the virus in the male genital system and in the ejaculate. Indeed, lesions in the testis suggested to be induced by MVV have been observed in 5 out of 7 MVV infected rams [9]. However, the virus was only detected in semen of sheep after super infection with *Brucella ovis,* a bacterium causing epididymitis. [7, 8]. In contrast, presence of CAEV-DNA has been established in the semen of 2 out of 6 experimentally infected bucks without known super infections [10]. Theoretically, SRLV can be present in the sexual organs and in the semen of infected rams and bucks in three forms namely incorporated proviral DNA (in macrophages), vesicles containing the virus and free virus. It is unclear whether infected rams and bucks, if they shed virus in their ejaculate, do so intermittently or continuously. This was shown to be an issue, with *Infectious Bovine Rhinotracheitis* [11].

For diagnostic purposes serological tests are routinely applied to detect SRLV infections. Unfortunately, seroconversion is relatively slow and variable in time and there is no simple relation between the moment of conversion and the actual moment of infection [12, 13]. The method is only reliable at a herd level but not at the individual level and thus a complicating factor for adequate diagnostics that can theoretically be overcome by using more sensitive virus detection methods, such as PCR-tests. Thus, in this study samples from rams and bucks were collected sequentially and tested for SRLV specific antibodies (blood) and for proviral DNA (blood, ejaculate). Furthermore, it was investigated if and where in the male genital tract the (pro-) virus is transferred into the fluids and cells contributing to the ejaculate; after euthanasia, the sexual organs and epididymal and ejaculated semen of the animals were tested for the presence of proviral DNA by PCR. The relation between the number of monocytes-macrophages in blood and ejaculates and the probability of a SRLV positive ejaculate was also evaluated by linking the number of monocytes-macrophages circulating in the peripheral blood just after ejaculation to the number of macrophages present in that ejaculate.

Materials and Methods

Animals and experimental set-up

Thirteen Texel rams and 4 Saanen bucks were repeatedly sampled for blood and semen over a period of 6 months and once again 6 months later at the beginning of the next breeding season. The rams originated from 3 different sources: 10 animals came from a breeding farm having an outbreak of Maedi Visna, 1 was bred from a MVV infected dam and 2 were from unidentified origin. The Saanen bucks came (2 by 2) from 2 different dairy farms with confirmed CAEV infections. The animals were kept indoors in groups together with other individuals originating from the same farms. The housing conditions at the clinic of the Department of Farm Animal Health at the University of Utrecht were well controlled and hygienic. The animals had restricted access to grass silage and concentrates and had water ad lib. Males were trained to ejaculate in an artificial vagina in the presence of an hormonally induced oestrus female. In addition 2 Texel rams and 2 Saanen bucks originating from herds certified to be free of SRLV were used as negative control. These negative control animals were euthanized and sampled directly upon arrival. All animals were tested and proven negative for *Brucella abortus* antibodies (Ceditest® Brucella, Cedi-Diagnostics BV, Lelystad, The Netherlands) and by a serum agglutination test [14].

Blood samples

Blood was collected from the jugular vein with a vacutainer system using either 10 ml plastic serum tubes, or 10 ml glass EDTA-tubes (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK). Samples were processed within 4 hours after collection. For PCR the blood samples from the EDTA-tubes were centrifuged at 1100xg for 25 min at room temperature. Thereafter, the buffy-coat was obtained and diluted in 2 ml of Phosphate Buffered Saline (PBS; 1.54mM KH_2PO_4, 155.17mM NaCl, 2.71mM Na_2HPO_4; pH 7.2, Gibco, UK), subsequently layered upon 4 ml Ficoll (Ficoll 400; density 1.078, Sigma-Aldrich, Steinheim, Germany) and centrifuged for 25 min at 800g at 4°C. The cells at the interphase, the peripheral blood mononuclear cells (PBMC), were recovered, washed twice by re-suspension in 14 ml PBS and subsequent centrifugation at 600g for 10 min.

Semen and tissue samples

Ejaculated semen samples could be collected from 11 adult Texel rams, naturally infected with MVV and 2 adult Saanen bucks, naturally infected with CAEV. Epididymal semen and tissue samples of the testis, epididymis, ampullary glands, vesicular glands, prostate glands, bulbo-urethral glands were collected at the end of the experiment, from all individuals following euthanasia by intra-venous injection of 150-200 mg per kg body weight. Pentobarbital Natrium (Euthesate, Ceva Sante Animale, Naaldwijk, The Netherlands). From the tissue samples taken at necropsy small pieces (approximately 20 mg) were prepared for further testing.

Semen washing
For semen washing a double Percoll gradient was prepared. Percoll (100%, isotonic, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was first pH and osmotically stabilized with 11,8 ml of medium A (92,4 mM NaCl, 3,1 mM KCl, 24 mM $NaHCO_3$, 0,31 mM Na_2HPO_4, 22 mM lactate, 100 mM HEPES, 1,5 mM; pH 7.4) and diluted to an end concentration of 35% or 80% with incomplete medium D (92 mM NaCl, 3,11 mM KCl, 24 mM $NaHCO_3$, 0,31 mM Na_2HPO_4, 22 mM lactate, 100 mM HEPES, 1,5 mM $MgCl_2$, 25 nM phenol red which was tested for endotoxins; pH 7.3, 285 mOsmol). Gradients were prepared immediately before use by placing 2 ml of the 85% Percoll into a 15 ml centrifuge tube and overlaid with 4 ml 35% Percoll. The semen (ejaculated and epididymal) was diluted 1:1 (v:v) in PBS and gently pipetted on top of the double Percoll gradient and centrifuged (10 min at 300 g and thereafter 20 min at 750 g). Four different fractions were recovered; on top the seminal plasma (a), the fraction between the seminal plasma and the 35% Percoll (containing cytoplasmic droplets and macrophages) (b), underneath the 35% Percoll (aggregated- and parts of spermatozoa as well as cells originating from the sexual organs) (c), and finally under the 80% Percoll (the life intact spermatozoa) (d). The cell fractions (b, c, and d) were washed twice in 14 ml of PBS by centrifugation at 600g for 10 min.

ELISA
Serum from blood samples derived from the serum tubes was used for serological testing. Elitest-MVV (HYPHEN BioMed, Andrésy, France) was performed according to the manufacturer's instructions. This test uses synthetic recombinant peptides derived from MVV envelope protein and from Gag proteins.

PCR
For the PCR test on blood, samples from the EDTA-tubes were prepared as described above. In addition, semen fractions a, b and c were tested as well as tissue samples from the genital organs. DNA-extraction (from blood, semen and tissue) was performed with the QIAamp®DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to manufacturers instructions. For real-time PCR a LightCycler 1.5 instrument (Roche, Diagnostics GmbH, Mannheim, Germany) and primers for the Long Terminal Repeat (LTR) sequences of the genome of SRLV were used [15]. The mix contained 10 µl PCR mix (QIAGEN's Quantitect SYBR Green I PCR kit), 2 µl primer mix (0.5 µM of each primer, 2.5 mM $MgCl_2$), 5 µl of the extracted DNA (150 – 250 ng) and 3 µl sterile water. The procedure started with a period of 15 min at 95°C followed by 45 cycles of 15 s 95°C, 20 s 65°C, 20 s min 72°C. Amplicon detection was based on measuring fluorescence caused by the intercalating compound SYBR Green I combined with melting point analysis.

Monocyte counting by flow cytometry
The pellets containing the monocytes and/or macrophages from semen and blood samples were re-suspended in 100 µl of a 1:50 dilution of anti-CD14-FITC (Clone TÜK4, Mouse IgG2a, Miltenyi Biotec, Auburn, California, USA) in FACS-buffer (Dulbecco's Phosphate Buffered Saline (D-PBS) 137 mM NaCl, 6.4 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM NaH_2PO_4 without $CaCl_2$ and $MgCl_2$, Bio Whittaker, Walkersville, Maryland, USA supplemented with 1% w/v Bovine Serum Albumin and 11.7 mM $NaNO_3$ and incubated for 5-10 minutes in the dark at 4°C [16]. The cells were then analyzed for the presence or absence of green fluorescence by flow cytometry using a FACScan equipped with an Argon laser, (488 nm, excitation line, Becton Dickinson, San Jose, CA), and PBS as sheath fluid [17, 18]. The fluorescence emission detectors (FL 1 = 500 to 530 nm and FL 3 > 605 nm) were set to asses the amount of green emission of the monocytes/macrophage subpopulations according to previously described methodology [19]. Per sample, 10,000 monocyte and/or macrophage specific evens were analyzed. This experiment was repeated three times at a month interval within the natural breeding season (September to February in the Netherlands).

Statistical analysis
The results were analysed with Microsoft® Excel spreadsheet software (Microsoft Corporation, Redmond, Washington, USA) and SPSS (SPSS Inc., Chicago, Illinois, USA; version 10.0.). The PCR results from blood samples were compared to those from semen in a two-by-two table and chi-squares were calculated.
ELISA (serology) and PCR were compared in a two-by-two table and a chi-square test. Also a kappa measurement of agreement (Cohen's kappa coefficient) was determined.
The flow cytometric data were analyzed using Cell Quest software (Becton Dickinson, San Jose, CA) and WinMDI (Windows Multiple Document Interface for Flow Cytometry, version 2.8; http://facs.scripps.edu/software.html). To determine if there was a correlation between cell counts and PCR positive results a general linear model (design: Intercept+animal+date+infection+date*infection) and a test of between-subjects effects was performed. Since the cell counts in semen were not normally distributed a log transformation was performed on these data.

Results

SRLV proviral DNA in blood
Twelve rams and 3 bucks originating from infected herds tested positive for presence of SRLV antibodies (serology) or proviral DNA (PCR) by analysis of blood samples at the start of the experiment. One ram and 1 buck tested negative in both assays at the start. In addition, the negative control males tested negative in both tests.

Of the 11 rams and 2 bucks that tested positive in both assays at the start of the experiment 10 rams and 2 bucks continuously remained positive. Whereas only 1 ram (2) and 1 buck (1) tested negative by serological examination and intermittently positive on the PCR test in blood, and another ram (12) tested intermittently positive at the serological and PCR tests, but was positive for both tests at the end of experiment. Three rams (1, 3 and 6) and 2 bucks (2 and 3) were sacrificed during the experimental period.

SRLV proviral DNA in ejaculated and epididymal semen

Three fractions collected with the discontinuous Percoll gradient were tested for the presence of proviral DNA by PCR at three subsequent moments in time. The seminal plasma fraction tested consistently negative. The fraction containing aggregated- and parts of spermatozoa as well as cells originating from the sexual organs gave a positive test result only once. The fraction containing cytoplasmic droplets and macrophages gave positive results in 55% of the samples. With respect to PCR testing for SRLV proviral DNA in semen it is noted that none of the animals had detectable SRLV specific DNA in their ejaculate at the start of the experiment, even though SRLV specific antibodies were present in 11 rams and 2 bucks. After two months the first ram (7), and two weeks later another ram (2), showed shedding of the proviral DNA in semen. One week later 4 rams (4, 7 again, 9 and 10) and 1 buck (1) showed intermittent PCR positive ejaculates (shedding). In total 12 rams and 3 bucks were intermittently shedding proviral SRLV DNA in the semen during the sampling period. Notably, it was not always possible to collect semen; some animals refused to ejaculate in the artificial vagina. These individuals were sacrificed before the end of the experiment. Of the remaining animals only 2 rams remained SRLV proviral DNA shedders into semen (1 was intermittently negative) and in the other 4 rams and 1 buck no SRLV proviral DNA was detected in the semen in the last two months. Samples from negative control bucks and rams remained negative throughout the experiment.

Epididymal semen, collected after euthanasia, was PCR-positive for 6 rams and 3 bucks and negative for 5 rams and 1 buck. In summary, this study demonstrated using PCR analysis that SRLV proviral DNA is present in the cytoplasmic droplets and macrophages fraction of semen of rams and bucks that were tested SRLV-positive by serological and PCR tests. However, only part of the SRLV seropositive small ruminants showed (intermittent) shedding of SRLV proviral DNA into semen and then only a few month after infection.

Date	28/07/03	29/08/03	17/09/03	23/09/03	29/09/03	30/09/03	09/10/03	13/10/03		16/10/03	21/10/03	16/11/03	25/11/03		22/12/03		21/01/04		18/02/04		
Animal	B	**A**	**B**	**C**	**C**	**B**	**C**	**A**	**B**	C	**C**	**B**	**A**	**C**	**B**	**C**	**B**	**C**	**A**	**B**	**C**
Ram 1	+	+	+	0	0	+	0	+	+	0	0	+	+	0	0	†	0	0	0	0	0
Ram 2	+	-	+	-	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-
Ram 3	+	+	+	-	-	+	0	+	+	0	0	+	+	0	0	†	0	0	0	0	0
Ram 4	+	+	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	-	+	+	-
Ram 5	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+
Ram 6	+	+	+	0	0	+	0	+	+	0	0	+	+	0	0	†	0	0	0	0	0
Ram 7	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+
Ram 8	+	+	+	-	-	+	-	+	+	-	-	+	+	+	+	-	+	-	+	+	-
Ram 9	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	-
Ram 10	+	+	+	-	-	+	-	+	+	+	0	+	+	+	+	-	+	-	+	+	-
Ram 11	+	+	+	-	-	+	-	+	+	-	+	+	+	-	+	+	+	-	+	+	-
Ram 12	+	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-
Ram 13	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-
Buck 1	+	-	+	-	-	-	-	-	+	+	+	-	-	+	-	-	+	-	-	-	-
Buck 2	+	+	+	0	0	+	0	+	0	0	0	+	+	0	0	†	0	0	0	0	0
Buck 3 1289	+	+	+	0	0	+	0	+	0	0	0	+	+	0	0	†	0	0	0	0	0
Buck 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1: Small ruminant lentivirus infection detected by the presence (+) or absence (-) of antibodies (ELISA, columns A), or proviral DNA (PCR, columns B) in blood and in semen (columns C). 0= Not tested †= Deceased.

Serology/PCR
There was a significant agreement in the results obtained via the ELISA and the PCR test, measured by the chi-square test (p= 0,024 with a confidence interval of 95%). Also the Cohen's kappa coefficient was 0 indicating that both methods have a perfect agreement in blood. However, the PCR tests on blood and the PCR tests on semen were incongruent (p=0,976 within a confidence interval of 95%). This indicates that an individual with PCR positive blood did not automatically have a PCR positive ejaculate.

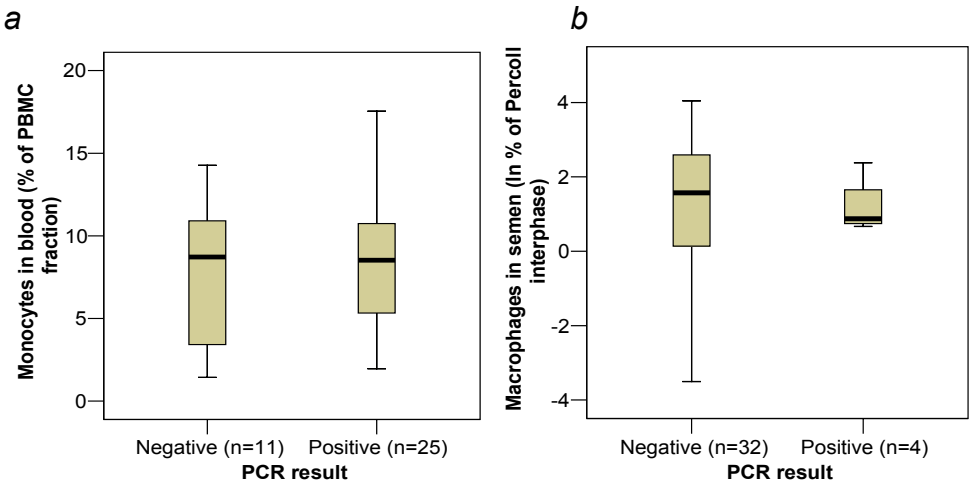
Tissue samples
Table 2 shows the prevalence of SRLV in tissues from the genital tract and epididymal semen compared to that in blood and ejaculates as tested by PCR. Nine epididymal semen samples were positive. From the tissues 12 testis-, 8 epididymis-, 8 ampullary glands-, 11 vesicular glands, 9 prostate- and 9 bulbo-urethral glands samples were positive for proviral SRLV DNA. Some of the tissues of one SRLV negative but exposed ram (13) and one similar buck (4) were also PCR-positive, although none of their seminal fluids were. All samples from the control animals (2 rams and 2 bucks) from SRLV free flocks were negative. Two rams died prematurely due to unrelated causes.

Animal	Blood	**Ejaculated Semen**	**Epididymal Semen**	**Testis**	**Epididymis**	**Ampullary gland**	**Vesicular gland**	**Prostate gland**	**Bulbourethral gland**
Ram 1	+	0	-	+	+	+	+	0	-
Ram 2	+	+	+	+	-	+	+	+	+
Ram 3	+	-	0	0	0	0	0	0	0
Ram 4	+	+	+	+	+	+	+	+	+
Ram 5	+	+	0	0	0	0	0	0	0
Ram 6	+	0	+	+	+	-	-	0	-
Ram 7	+	+	+	-	-	-	-	-	+
Ram 8	+	+	-	+	+	-	-	+	+
Ram 9	+	+	+	+	-	-	+	-	-
Ram 10	+	+	-	-	+	-	+	+	-
Ram 11	+	+	+	+	-	-	+	+	-
Ram 12	+	+	-	-	+	+	+	+	+
Ram 13	-	-	-	+	-	+	+	+	+
Ram 14	-	0	-	-	-	-	-	-	-
Ram 15	-	0	-	-	-	-	-	-	-
Buck 1	+	+	+	+	+	+	-	+	+
Buck 2	+	0	+	+	-	+	+	0	+
Buck 3	+	0	+	+	+	-	+	0	+
Buck 4	-	-	-	+	-	+	+	+	-
Buck 5	-	0	-	-	-	-	-	-	-
Buck 6	-	0	-	-	-	-	-	-	-

Table 2: Presence (+) or absence (-) of Small ruminant lentivirus proviral DNA as demonstrated by PCR in tissues from the genital tract, in epididymal semen, in blood and in ejaculates. 0= Not tested

Monocytes/ macrophages

There was no correlation between monocyte and/or macrophage counts in blood and semen and PCR positive results in blood and semen (Figure 1). In blood cell counts, monocytes and/or macrophages are represented as percentage of the PBMC fraction. In semen, macrophages counts are represented as % of the fraction containing cytoplasmic droplets and macrophages. Also there were no between-subjects effects for individual animals, date and infection.



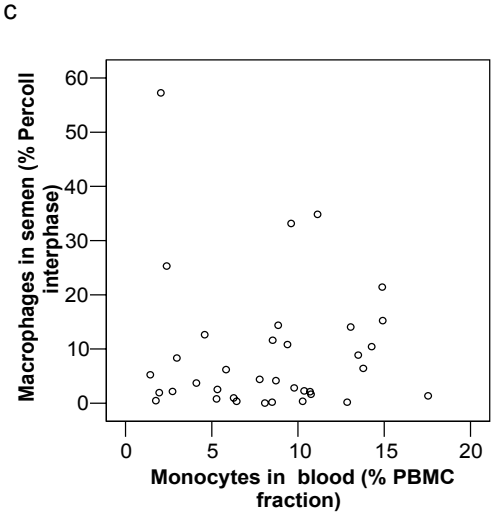


Figure 1a: The percentage of monocytes of total events of the PBMC fraction from the peripheral blood of male small ruminants in relation to presence (positive) or absence (negative) of proviral SRLV DNA (PCR) in blood. Figure 1b: The percentage of macrophages of the total events from the 35%/70% Percoll gradient interphase fraction in ejaculates (n=36) of small ruminants in relation to presence (positive) or absence (negative) of proviral SRLV DNA (PCR) in the ejaculate. (Horizontal bars indicate the median, shaded blocks the interquartile range that contains 50% of values and the vertical lines the highest and lowest values after exclusion of outliers and extremes.) Figure 1c: Scatterplot of monocytes (% of total events of the PBMC fraction) in peripheral blood versus macrophages (% of total events of the 35%/70% Percoll gradients interphase fraction) in semen of male small ruminants of SRLV infected and control animals.

Discussion

This study demonstrated that proviral SRLV DNA is shed into the semen of many rams and all bucks that were positive for SRLV antibodies (ELISA) and for proviral DNA in the blood (PCR). Not all rams did shed proviral DNA into semen. Those that started shedding of proviral DNA into semen did so after a lag period of a few months after the start of the experiment. SRLV-DNA shedding in the ejaculates was intermittent. If present, the proviral DNA was predominantly detected in the fraction of semen containing cytoplasmic droplets and macrophages. The proviral DNA of SRL was also found in the fraction with aggregated and fragmented spermatozoa. Samples taken from the sexual organs of euthanized positive animals often also contained the proviral DNA of SRLV. This indicates that the sexual organs may directly contribute to shedding of the proviral SRLV DNA in the ejaculated semen, which implies that SRLV transmission via semen is possible. Furthermore, the results demonstrate that in line with the observed intermittent shedding patterns, a single PCR-negative semen sample cannot be used as a diagnostic tool to predict that subsequent ejaculates will be free of SRLV DNA. PCR on blood samples appears to have a better predictive value in this respect. Hence, for control programs, the PCR testing of blood samples is recommended to ensure that the animals are SRLV proviral DNA free prior to breeding or collection of semen for artificial insemination.

The SRLV detection methods in this study, serological and PCR tests, gave consistent results, except for ram 2 and buck 1. These individuals were consistently serologically negative, but blood-PCR positive. They obviously did not produce antibodies against both antigens applied by the test used (Elitest-MVV) during the observation of 9 months. Similar observations using others tests have been made before [20] and are henceforth expected to occur [21]. The results of these two animals underline the added value of blood-PCR in detecting infected animals. Nevertheless, there was a high level of agreement at the sample level between the serological and PCR tests. Notably, both methods detected infection at virtually the same moment in time. Also, once animals were diagnosed as infected, both tests remained positive in the consecutive samples of all except the two above-mentioned individuals and in semen samples. It could be argued that PCR tests require a certain level of viral replication in the host for proviral DNA to be present in a specific blood sample, which contends that the diagnostic sensitivities of serological and PCR assays should be in the same range.

The route of shedding SRLV from the blood circulation into the semen is supposed to be mediated through infected macrophages. All blood PCR positive animals were positive in one or more reproductive tissues and fluids. In bucks proviral DNA of SRLV was only present in the testis and vesicular glands as well as in ejaculated and epididymal semen. For rams the proviral DNA was detected in all tissues that were assessed. In contrast to what was expected some of the reproductive tissues of both exposed but consistently blood- and semen-PCR negative and seronegative males (ram and buck) were also PCR positive, whereas the negative

control animals were completely negative. To our knowledge there is one study that also reports on this discrepancy [12]. All cases proved to be individual animals that had been in contact with SRLV positive herd members. It can be hypothesized that these individuals were infected but that the viral replication was strongly restricted by host factors hence no antibodies were induced and blood- and semen-PCR remained negative.
All of the rams and bucks that were SRLV positive in the blood also showed the presence of proviral SRLV DNA in reproductive tissues and fluids. All positive bucks showed SRLV provirus in ejaculated and epididymal semen and in the testis and bulbo-urethral glands. In conclusion, all small ruminant males that tested positive (Elisa, PCR) in the blood showed SRLV-DNA in the reproductive tissues and fluids and should therefore be considered as potential shedders of SRLV.
There was no correlation between the amount of monocytes and/or macrophages in the blood and the semen, which is basically not surprising because the influx of macrophages is governed by typically local processes, which have little effect on the general monocyte content of the blood. Such local processes in the accessory sex glands, where proviral DNA was detected in this study, could result in direct shedding of virus into the seminal plasma. The absence of a correlation between the number of these cells and presence of proviral DNA in this study does not corroborate with a previous hypothesis contending that other infections, eg *Brucella ovis*, cause influx of macrophages which are –accidentally- carrying proviral DNA [8]. Moreover, it is inconsistent with findings in man where polynuclear cells in semen are a risk factor for seminal HIV-1 excretion and blood viral load was the only predictive factor for the intermittent shedding of HIV-1 in semen over time. The males in the present study were free of *B. ovis* infection and as far as could be ascertained had not been suffering from other infections affecting the sexual organs.
Notably, it is currently not known if the local production of -infectious- virus can be predicted on the basis of the local presence of proviral DNA, let alone if there is a quantitative connection. However, cells infected with SRLV DNA have been isolated from male sexual organs, which demonstrates that semen can be potentially infectious, indeed.

Conclusions

Shedding of proviral SRLV DNA in semen has been proven for 9 out of 12 seropositive males, rams and bucks. Most males shed intermittently. Also a large proportion of the tissue samples from the male genital tract tested positive for SRLV-DNA. These findings support the possibility that SRLV are sexually transmittable and that shedding in semen can be accomplished by alternative routes than the postulated macrophage dependent delivery.
PCR proved to be useful in detecting proviral DNA in blood as well as in semen. To determine if an individual semen donor is infected with SRLV, blood-PCR was shown to be an essential adjunct to antibody detection. Infected males should not

be used as semen donors. Blood, not semen, should be sampled since intermittent SRLV DNA shedding has only been determined in animals with specific antibodies or proviral DNA in the peripheral circulation. The hypothesis that shedding of virus is related to a high number of susceptive cells in the blood and semen is not supported by our data.

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Chapter 7

Summarising discussion

In commercial small ruminant farming the aims to optimise production and to lower costs have resulted in increased flock/herd sizes and a considerable intensification. Genetic improvement is a well-established method to improve production. Contiguously, many small ruminants are accommodated in small hobby flocks, which for their genetic diversity also rely in introduction of new genes. Genetic improvement is generally obtained by the introduction of superior males. Although the vast majority of small ruminants successfully conceive through natural service, assisted reproductive techniques are increasingly in demand. Oestrus synchronisation and/or artificial insemination (AI) are methods of choice. The benefits of AI are: more strict selection of the males as one male can serve more females, a wider distribution of (valuable or rare) genes, out of season breeding, prevention of spread of (sexually) transmissible diseases, and conservation of biodiversity through gene banking. Since reproductive performance after AI and oestrus synchronisation is suboptimal both in sheep and goats, more research was performed to optimise this technique.
For disease control as part of a breeding program, AI seems to be a less hazardous then the introduction of new males. Although AI was introduced to prevent the spread of transmissible diseases, a problem exists related to Small Ruminant Lentivirus (SRLV) transmission via infected semen, as shedding of virus might go undetected in apparently healthy animals. SRLV are common infections in the Netherlands. The voluntary SRLV accredited disease control program is quite effective in the control and eradication, but incidentally unexpected results are encountered e.g. when SRLV specific antibodies are detected in an animal of a herd, certified as “SRLV free” or when outbreaks occur. The main reasons for this are; (I) the particular type and manifestation of the virus (II) the low or late detection of clinical symptoms with hidden economic effects, (III) unpredictable course of lesion development (IV) difficulties in the detection of infected individuals and (V) gaps in the understanding of the transmission. These issues are addressed in this thesis.

Reproductive management
One of the aims of this thesis was to strengthen the small ruminant industry by improving AI results, aiming at better reproductive performance and optimal out of season breeding. Semen quality is of major importance in this respect. The study presented in **chapter 2** focused on: optimisation of the storage temperature of liquid semen, the effect of glycerol on the quality of spermatozoa during storage, evaluation of the washing procedure for exclusion of seminal plasma and optimisation of the extender. Furthermore, these data were correlated with fertility rates following AI.
To study semen quality and preservation methods microscopical- as well as flow cytometric techniques were applied in **chapter 2**. Flow cytometric analysis is considered more accurate then conventional microscopic evaluation [1-3]. However, we found a very high correlation between the results of flow cytometric analysis and the more practical microscopic methods when motility assessments

are performed by an experienced operator. On the one hand this implies that the purchase of expensive flow cytometric equipment (for accurate integrity assessments of spermatozoa) is not required to improve semen quality evaluation. While on the other hand microscopic assessment of motility after thawing or immediately prior to insemination is simple, easy and quick and appeared to provide the parameter of choice under field conditions to evaluate the degree of damage inflicted by cryopreservation.
The flow cytometric assessments of viability and acrosomal integrity of spermatozoa was used to study semen storage and handling procedures. Storage of liquid and frozen-thawed semen begins with cooling to ambient temperatures followed by gradual cooling to 4°C. The currently advised storage temperature is 4°C and semen should be used within 12 hours after collection [4]. However, in case of short-term storage preservation at ambient (18°C) temperature might be favourable for the fertilising capacity of the spermatozoa. The results in chapter 2 indicated that storing goat semen for 24 h at 18°C resulted in similar motility of spermatozoa as storing such semen for 24 h at 4°C. The practical implication of this outcome is that under field conditions, liquid semen does not have to be cooled to 4°C, nor stored and transported under refrigerator conditions when used within a day after collection.

For freezing a cryoprotectant is required in order to reduce osmotic shrinkage and swelling of cells. Unfortunately, cryoprotectants affect membrane integrity [1, 5], and are metabolically toxic to spermatozoa [6]. Glycerol is the preferred cryoprotectant for goat semen because others are less effective [6], nevertheless a relatively high percentage of Dutch AI-bucks produced semen that reacted negatively to the glycerol as morphologically evaluated. Individual differences in glycerol-sensitivity may be due to different osmotic, and semi-permeable properties of sperm membranes. Bucks that were tested with a limited sperm cell resistance to glycerol and/or extender(s) should be eliminated from AI services, since their frozen semen will not result in successful AI.

In contradiction to earlier studies [7, 8] the work in chapter 2 showed no significant correlations between the percentages of functional spermatozoa and fertility. (Fertility is represented as number of goats that kidded divided by the number of goats that were inseminated with semen from a specific buck.) At least 15 ejaculates were obtained from each buck, distributed to a minimum of 8 and a maximum of 16 different farms, and 70 to 260 inseminations per buck were performed in order to evaluate fertility. Although the number of inseminations is high according to Dutch standards it could be argued that more experiments should be performed to assess the reliability of this outcome.

The composition of the semen extender -a key factor in semen processing- is important because buck seminal plasma contains factors that may negatively influence spermatozoa and obnoxiously interact with the egg yolk present in the

freeze-thawing buffer [9]. Removal of seminal plasma prevents or diminish this detrimental influences [9]. In chapter 2 it is shown that washing of semen of the bucks used in the study does not affect their viability both before and after thawing, implying that under field conditions washing is not necessary. Although the preservability of ejaculated and washed semen is less than that of epididymal semen [8, 10] -suggesting that seminal plasma contains compounds detrimental to in vitro storage- seminal plasma also contains factors favourable for sperm survival [11, 12]. The finding might be influenced by breed or even individual genetic composition since in other breeds washing can be necessarily [13, 14]. Moreover, the season affects the composition of seminal plasma hence further research might primarily focus on seasonal influences.

From the studies described in this thesis it was concluded that Tris–citric acid glucose buffer and egg yolk containing extender is preferred for freezing buck spermatozoa because it best preserves spermatozoa integrity when compared with other extenders containing glucose, egg yolk and or skimmed milk. Egg yolk has the property to alleviate cold shock by its ability to shield membranes. Although it is well known that egg yolk protects sperm cell membranes of farm animals, egg yolk may also destabilize such membranes, as shown by an increased frequency of acrosomal damage associated with an increase in the concentration of egg yolk [4]. Indeed an increase in acrosomal damage with even the best extender was still noted. The bucks used in this thesis showed large differences in the quality of their semen with respect to motility, percentage of live cells and percentage of intact acrosomes before freezing and after thawing. It is therefore advisable to test each buck in order to determine the preferred treatment of its semen with respect to resistance to glycerol, extender and removal of seminal plasma. Based on these tests the most preferred semen storage method should be adjusted to the individual buck.

SRLV

The attempt to control and eradicate SRLV has only partly been successful. The voluntary SRLV accredited disease control program has been quite successful in the sheep breeding sector, since most breeding flocks became certified MVV-free, partly as a result of the decision of the breeding societies to make the program obligatory. Breeders of predominantly Texel sheep were interested to join the voluntary program, mostly because of export opportunities.

However, today's breeders find the program costly and cumbersome and see no obvious return on their investment. In addition, every year a small number of flocks loose their certificate as a result of the finding of positives at a follow-up test. Actually, the self-imposed obligation to take part in the program is currently subject of intense debate in most breeding societies. Unfortunately the interest in eradication did not attain the commercial lamb producing sector and many (small flock) hobby farmers. Notably, for the commercial sector certification as such is

useless because their output is for slaughter, so their interest lies in simple low cost measures that keep the rate of infection low. This is in contrast with hobby farmers were costs are not a major concern. These, in general, small flock farmers are not aware of the possibilities or simply not interested. The only exceptions are farmers whose animals attend shows where SRLV certification is mandatory. In the dairy goat sector, the situation is different since the intensive management conditions -year around housing and machine milking- appear to facilitate lateral transmission and hamper effective control.
The above-mentioned issues have been reported in chapter 1. The effectiveness of the control program is influenced by the low or late detection of clinical symptoms (II) that cause significant (hidden) economic effects and the unpredictable course of lesion development (III), which are further investigated in chapter 3. To be able to quantify clinical symptoms ante- and post-mortem, as well as predict the course of lesion development, it is essential to know the length of infection. Therefore a longitudinal transmission experiment in sheep, naturally exposed to MVV was performed. To comparatively study a possible breed effect, the experiment was performed in duplicate with different breeds. This chapter gives the results of the clinical evaluation and the post-mortem examination of these sheep. The severity of the clinical effects and of the macroscopic and microscopic lesions were not clearly associated with the length of the infection, which means that the progression of the lesions is not a simple function of time. The lymphocytic lesions in the mammary glands even showed regression. The microscopically determined degree of fibrosis was progressive consistent with the duration of infection, but the degree of alveolar atrophy remained constant after an initial increase.
The lesions observed in the brain and spinal cord specifically signs of meningitis, leukoencephalomyelitis and demyelinisation were indicative for Visna, the neurological form of MVV. This was remarkable since clinical Visna is rarely seen in the Netherlands. Clinically none of the individuals with one or more of these lesions showed neurological disorders. This could be explained by the relative mildness of the lesions found and by the fact that the ewes were probably not infected long enough in order to show clinical symptoms. Moreover this could lead to the tentative conclusion that these CNS lesions are also not always progressive. Swelling of the carpal and/or tarsal joints seemed indicative for MVV infection, although at more detailed examination only a slight but not significant progressive lymphoplasmacellular synovialitis was found. Arthritis is not a common feature of MVV infection, although carpal swelling has been reported in ewes from the synthetic breed. In the ewes sacrificed in these experiments lesions in the joints were not prominent, neither macroscopically nor microscopically. Future research should determine whether arthritis could be considered as a breed related symptom of SRLV.

Difficulties in detection of SRLV infected individuals (IV) are still a major concern. Antibody detection is routinely used for this purpose, but has the drawback of the relatively slow and unpredictable formation of antibodies, which results in infected

animals going undetected. Proper initial phase detection of the manifestation of the virus is important. PCR assays detecting the viral genome, specifically in the monocytes and/or macrophages, have potential in this respect, but they rely on the availability of these cells in any given sample. Detection of proviral SRLV DNA is dependent on the presence and amount of provirus containing cells. Monocytes are the target cells because they specifically carry the integrated SRLV-provirus. Current handbooks on goat and sheep diseases quote monocytes as 0–4 and 0–13% of the white blood cell fraction, respectively, suggesting that monocytes can be absent in a given blood sample from sheep or goats [15, 16]. These quotes, however, are mainly based on the differential counting of 100–200 white blood cells in stained blood smears, which intrinsically has a low sensitivity. Immune fluorescence and detection by flow cytometry is a method with a very high sensitivity as well as specificity and this method is applied in chapter 4 where the availability of monocytes in sheep and goat blood is investigated. Since the quantitative variations in the monocyte proportions between individuals and within individuals and the factors that associate with these variations have not yet been studied extensively, the aim was to establish these individual variations and to see if blood samples without monocytes do occur. The study showed that monocytes were indeed available in all blood samples (253) taken from 26 sheep and 24 goats (adults and prepubertals, lactating and non-lactating, male and female, and SRLV-infected or not) taken over a period of time. However, there was a considerable range in the proportion of monocytes, i.e. from 0.2 to 35.5% in the PMBC-fraction, which in itself forms about 40 to 70% of the total white blood cell fraction. The proportions appeared to be influenced by the factors ‘species’ and ‘sex’ and possibly ‘age’ but not by the factor ‘lactation’. SRLV-infection as such did not appear to have an effect on the proportion of monocytes in the PBMC fractions. These conclusions are crucial for identification of SRLV-infection through proviral DNA detection, as the target is incorporated in the monocyte genome. Thus, in theory, detection does not only rely on the number of monocytes in a sample, but on the actual number of monocytes with incorporated SRLV-DNA.

The development of a golden standard for the detection of SRLV is necessary to determine and define the “health status” of an animal [17]. The ultimate test would have a 100% specificity for identification of the presence of the infectious agent in the individual. Since such a test is not (yet) available a combination of tests is used to approach this standard. Presently two types of test are used in control programs, Enzyme Linked Immuno-sorbent Assay (ELISA) variants and agar gel immune precipitation tests (AGIDT). A reliable field test should be easy to handle, efficient, robust and cheap. Of course the sensitivity and the specificity should be high. The production of antigen needed for the test should be relatively easy, fast, stable and efficient. Because of the individual variation in recognition of antigens and the antigenic variation between SRLV-strains, a wide antigenic window is necessary if not essential for a reliable test. To improve reliability, ideally two tests should be

used for testing under field circumstances. Both the ELISA and the PCR tests were used in this thesis to provide a more reliable assessment of the presence of SRLV.

In this thesis two methods to detect SRLV-infection, the serological test and a real-time PCR with LTR-primers were compared in **chapter 3, 5** and **6**. The serological test and the real-time PCR, gave relatively consistent results. In chapter 6 two individuals gave seronegative and PCR positive results and thus represent an example of the serological gap: a lag time between infection and seroconversion. Still there was a high level of agreement at the sample level between the ELISA and PCR results in chapter 6 and a somewhat limited agreement in chapter 3 due to a few animals of the Texel breed where PCR-results were less consistent over time. Nevertheless, both methods detected infection at virtually the same point in time which is contrary to what was expected also by others [18]; due to a supposed delay in seroconversion, the PCR test was supposed to be the first in detecting infected animals. This finding may indicate that seroconversion is relatively rapid or simultaneous with the detected presence of proviral SRLV DNA. However, virtually all positive DNA preparations sustained 10-fold dilution. This suggested that, for both assays, a positive test result requires a certain level of viral replication in the host, which contends that the diagnostic sensitivities of both assays should be in the same range.

The gaps in the understanding of transmission (V) are of major importance in dealing with SRLV infections and hamper control programs. Identifying conditions which facilitate transmission, and quantification of the risk involved are important epidemiological issues. Differences in susceptibility to acquiring SRLV-infection have been suggested in several retrospective studies. Hence a longitudinal study of horizontal transmission of naturally acquired SRLV infection was performed in two flocks of different breeds of sheep (Texel and a synthetic breed). This comparative study described in **chapter 5** did not establish the epidemiological importance of the hosts' genetic constitution. The results suggested, but did not prove, a difference in the efficiency of lateral transmission between the breeds. In contrast, the differences in some of the clinical symptoms and in lesions at necropsy and histopathology between the two herds do indicate a genetic influence (chapter 3).
An interesting finding was the apparent extinction of lateral transmission at a certain point in time in both flocks. This suggests that there was a difference in susceptibility to acquiring the SRLV-infection between the individuals of the flocks. Long standing experience with testing flocks in the field for MVV-infection showed that the incidence of infection greatly varied per flock and even among flocks of the same breed held under similar conditions and management. It is suggested that this variation is not reflecting a dynamic process, but a relatively steady situation as the consequence of differences in the individual susceptibility to MVV-infection of the flocks tested. It should be noted that the genetic composition of an established flock only changes slowly in time, but that rams, because of the number of progeny

they produce, may have a profound influence by introducing susceptibility genes. It is hypothesized that the apparent differences between breeds in susceptibility to MVV are not breed characteristics, but are due to the prevalence of susceptible individuals within the breed –this prevalence is accidental since intentional breeding for resistance has, as yet, not been applied. The significance of specific virus strains for inducing clinical symptoms is still under debate [19-21] although a recent publications indicates that this is of minor importance [22].

This study unequivocally demonstrated the epidemiological importance of housing; indoors the lateral transmission rate was over 10 times higher than at pasture. The quantitative data demonstrates that housing is essential to get relevant transmission in a flock. At pasture, it took an infected ewe in both flocks between 2 and 3 years to infect another one. This basically implies that the conditions at pasture did not facilitate lateral transmission. This observation may, by itself, explain why SRLV has not spread in certain extensively kept populations notwithstanding the evident exposure. Together with the ewe's limited lifespan these results suggest that lateral transmission of MVV at pasture is of limited epidemiological importance. However, indoors this was totally different; here it took an infected ewe only between 1,2 to 1,9 months to laterally infect another one, which means that the transmission rate was increased more than tenfold. The observed extinction of transmission, however, suggests individual differences in susceptibility since –at least apparently- no new infections occurred. Overall, these results indicate that without housing, MVV-infections will only spread very slowly, if at all, and will probably not reach levels of economic significance, which is in line with recent studies in Spain [23, 24]. However, there is an established epidemiological relationship between the dam and her progeny [25] and if flock management -unknowingly- selects for the progeny of infected dams, MVV-infection rate may increase even without a housing period. The data is valuable for risk calculations concerning prevention and control. Moreover, this data can be used to evaluate testing intervals at the flock level.

Gaps in the knowledge of (sexual) transmission and genetic susceptibility of SRLV could explain unexpected results that are incidentally encountered in the current disease control programs. Although AI was originally introduced to prevent the spread of transmissible diseases, transmission of SRLV via infected semen cannot be excluded because infected donors may not be identified due to a delayed antibody response. The study presented in **chapter 6** investigates if proviral DNA is excreted into the semen, and if so, in what semen fraction it resides. In addition, several male sexual organs were examined for the presence of proviral DNA. Most animals in the study produced ejaculates containing proviral DNA, specifically in the cellular fraction. These results imply that SRLV transmission via semen is possible. In addition, most tissues tested were also positive. Remarkably, provirus detection did not associate with the presence of monocytes/macrophages in the blood and the semen. Although infected macrophages probably shed the virus into

the semen, there seems to be no relation between the amount of monocytes and/or macrophages in the blood and the semen and between the number of these cells and presence of SRLV. SRLV could also be transferred into the semen by epididymal (epithelial) cells [26]. However, all sero/PCR positive animals showed SRLV provirus in ejaculated and epididymal semen, in the testis and the bulbourethral gland. Thus it can be assumed that SRLV can follow a number of routes to enter the ejaculated semen. In conclusion, these results indicate that semen is a potential vehicle for SRLV. Although the infectivity of semen remains to be established, it is clear that semen donors should be free of SRLV.

Future of SRLV control

Today, virtually all breeding flocks participate in the voluntary, certification program, which includes approximately 10% of the total sheep population. According to an estimation of the Animal Health Service 60% of the commercial -lamb producing- flocks and 15-25% of the sheep are infected with MVV [27]. The main question regarding SRLV control is what the incentives and goals should be. This can vary from eradication of the virus to maintaining the incidence of infection at a low level, e.g. by certain measures in the management

If the goal is to eradicate the virus the following measures should be applied:

- Total replacement of flock, or alternatively
- Repeated testing and culling of positives and their progeny

In general, there are many ways to control the infection and keep the incidence below levels of economic significance. Given the shortcomings of the detection of infected animals and the voluntary nature of control efforts, it is clear that regional or national eradication is not a realistic option. Incentives to control the infection should be taken at the flock/herd level. The method of control should be designed on the basis of the production goal of the flock/herd (breeding, lamb production, milk) and its local conditions (e.g. stocking rate, housing period). To lower disease incidence a system of “control by management” with following recommendations can be applied:

- Buying replacements from SRLV-free certified flocks
- Isolation of kids at birth/ artificial rearing (goat dairy)
- Selection for unsusceptibility (this thesis), e.g. by specifically recruiting the replacements among the progeny of sero-negative ewes/does in the flock/herd; this also reduces the contribution of ewe-lamb transmission
- Refrain from or restrict housing period (this thesis)
- Combinations of the above

Notably, “control by management” does only to a limited extent rely on blood sampling and testing. However, the efficacy of the ‘genetic route’ remains to be proven in the field.

Are the above-mentioned goals feasible? The decision as to whether or not to participate in a certified disease control program is foremost a concern of the farmer and e.g. depend on the availability of financial and management resources. In addition, market limitations for infected animals also play an important role. Even so, the success depends on region or country versus flock (sero-) prevalence as well as the availability of certified negative individuals for substitution.
The current disease control program has disadvantages such as; the costs, loss of the certificate in case of infection which may occur unexpectedly and the restricting rules & regulations. The costs will inevitable be considered high. Once a seropositive animal has been identified, the costs to regain the certificate include elaborate retesting. Being certified does not absolutely guarantee that the herd is free of virus. It means that the chance that seropositives are present is minimal. In conclusion, the rules and regulations are strict, and not designed for a “tailor-made” approach.

Positive PCR results in the tissues of uninfected ewes
The observation in chapter 3 (and chapter 6) of PCR-positive tissues in sheep (flock mates of infected animals) that were consistently antibody and PCR negative in blood samples sequentially taken over long periods of time was unexpected. The possibility that these results were false positive has been examined. However, the results were confirmed and further substantiated by the negative results with the tissues from the four certified MVV-free sheep. Obviously, these findings need further confirmation e.g. by in situ detection techniques on the positive tissues as earlier demonstrated [28].
Presence of proviral DNA in tissues of “non-infected” ewes (chapter 3), flock mates of SRLV-positive animals, opens the discussion regarding non-productive infection. This is supported by the relatively high degree of macroscopically respectively microscopically identified lesions in these individuals. In order to determine whether immune suppression could lead to (re-) activation of the virus, a follow-up experiment was performed. Experimental immune suppression by corticosteroid treatment in these ewes did however, not result in (re-) activation of (in-active) provirus.
If viral replication is restricted in a certain host this animal will probably not shed the virus and thus not be infective to others. Virus-host combinations that do become productive due to an as yet unknown mechanism and that live among susceptible hosts may give rise to an -unexplained- outbreak of infection. In this context, it is also possible, and maybe even likely, that the restriction on viral replication has a quantitative nature: hosts with little restriction sero-convert and develop lesions rapidly, while those with moderate restriction are slow to convert and develop lesions and those with total restriction are truly non-productively infected.

Important causes for failures in the eradication of SRLV could be explained by the findings in this thesis. Moreover, the specific strain of the virus might play a role in the low or late detection of clinical symptoms. Difficulties in the detection of infected

animals could be due to alternative manifestation of the virus. The significance of the seronegative carriers has been underlined by the experiments performed in the scope of this thesis.

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Samenvatting

Dit proefschrift behandeld aspecten van de voortplanting, in het bijzonder het optimalisering van bokkensperma bewerking en opslag, alsmede aspecten van Small Ruminant Lentivirus (SRLV) epidemiologie.

Aangezien de resultaten van kunstmatige inseminatie (KI) na oestrussynchronisatie binnen Nederland tegenvallen, zijn verschillende facetten van de bewerking en opslag van bokkensperma nader onderzocht in **hoofdstuk 2**. Er is specifiek gekeken naar: de optimale opslagtemperatuur voor vers sperma, het effect van glycerol op de kwaliteit van de spermatozoön tijdens de opslag, de wasprocedure waarbij seminaal plasma wordt verwijderd en de optimalisatie van de spermaverdunner. Tevens is de kwaliteit van sperma gekoppeld aan vruchtbaarheidresultaten na KI. Zowel microscopie als flow cytometrie zijn gebruikt om de kwaliteit van spermatozoön te bepalen, waarbij er een hoge correlatie is gevonden tussen deze twee methoden. De kwaliteit van vers sperma, beoordeeld aan de hand van bewegelijkheid, was tijdens 24 uur opslag bij 18°C en 4°C gelijk. Glycerol is een cryoprotectant welke veelvuldig gebruikt wordt bij het invriezen van (bokken)sperma. Verschillende Nederlandse KI-bokken produceren sperma waarvan de kwaliteit (snel) achteruit gaat in aanwezigheid van glycerol. De samenstelling van de spermaverdunner is cruciaal bij de verwerking van bokkensperma aangezien er in het seminaal plasma factoren zitten die een reactie aan kunnen gaan met componenten (eigeel of ondermelk) uit deze verdunner. In theorie zou het wassen van het ejaculaat, en daarmee het verwijderen van het seminale plasma, deze effecten kunnen minimaliseren. Uit dit onderzoek blijkt echter dat de kwaliteit zowel voor invriezen als na ontdooien niet beïnvloed wordt door de aan/ of afwezigheid van seminaal plasma. Tevens is gebleken dat een Tris buffer met eigeel verdunner de beste resultaten geeft bij invriezen.
Het percentage functionele (levende, acrosoom intacte) spermatozoön bleek in deze studie niet gerelateerd te kunnen worden aan vruchtbaarheidcijfers. De belangrijkste conclusie is dat bokkensperma bewerking en opslag geoptimaliseerd kunnen worden. Bovendien bestaan er grote verschillen in kwaliteit, bewerk- en bewaarbaarheid van de ejaculaten van de verschillende bokken. De vruchtbaarheidsresultaten met KI kunnen dus verbeterd worden als de sperma bewerking en opslag wordt afgestemd op de eigenschappen van het ejaculaat van het individuele dier.

In het kader van dierziektebestrijding is KI een veilig alternatief voor de introductie van nieuwe rammen en bokken. Helaas bestaat er ten aanzien van SRLV geen zekerheid aangezien infecties niet altijd (direct) herkenbaar zijn en ogenschijnlijk gezonde dieren wellicht virus uitscheiden in het ejaculaat. Bovendien komen SRLV infecties -Maedi Visna (MV) of Zwoegerziekte bij schapen en Caprine Artritis en Encephalitis (CAE) bij geiten- veel voor in Nederland. Ondanks dat het vrijwillige bestrijdingsprogramma behoorlijk effectief is, worden er incidenteel toch nog onverklaarbare uitbraken binnen gecertificeerd vrije koppels waargenomen. In dit proefschrift worden verschillende verklarende aspecten van dit fenomeen belicht.

Om te beginnen worden de variërende klinische symptomen veelal pas laat ontdekt en in verband gebracht met een mogelijke SRLV-infectie. De klinische symptomen kunnen zowel wat betreft lokalisatie als in ernst variëren. Door dit sluipende verloop worden de economische gevolgen pas inzichtelijk als reeds een aanzienlijk deel van de koppel geïnfecteerd is. Deze aspecten worden nader onderzocht in **hoofdstuk 3**. In dit hoofdstuk wordt een longitudinaal MV transmissie experiment beschreven binnen twee koppels schapen van verschillende genetische achtergrond. Deze dieren zijn zowel op klinische symptomen als pathologische verschijnselen en histologisch veranderingen gescoord. De ernst van de klinische symptomen en de macroscopische en microscopische laesies was niet duidelijk gerelateerd aan de lengte van infectie. De histologische bevindingen in het uier duiden voor wat betreft de specifieke lymfocytaire laesies zelf op een vermindering naarmate de infectieduur toenam. Een andere opvallende bevinding is dat bij verschillende schapen microscopische veranderingen in het centraal zenuw stelsel gevonden zijn die duiden op Visna, de neurologische vorm van zwoegerziekte, terwijl er geen neurologische symptomen waargenomen waren. Alhoewel zwelling van de carpale en tarsale gewrichten indicatief is voor een SRLV-infectie, werden er geen significante verschillen waargenomen tussen niet, recent (< 1jaar) en langdurig (>1 jaar) geïnfecteerde individuen.

Problemen bij de bestrijding van SRLV worden mede veroorzaakt door complicaties bij de detectie van geïnfecteerde individuen. De routinematig gebruikte serologie heeft als nadeel dat de SRLV antilichaam productie relatief langzaam en onvoorspelbaar is. Hierdoor kunnen geïnfecteerde dieren onopgemerkt blijven. PCR testen, die het virale DNA herkennen, kunnen ingezet worden om dit probleem te omzeilen. In het geval van SRLV's zit het (pro)virale DNA ingebouwd in het gastheer DNA van specifieke witte bloedcellen, de monocyten en/of macrofagen. Indien de PCR-testen ingezet worden is het van belang om te weten of in elk willekeurig (bloed-)monster van elk willekeurig individu deze specifieke, mogelijk geïnfecteerde cellen aanwezig zijn. Om antwoord te kunnen geven op deze vraag is in **hoofdstuk 4** beschreven hoe middels immuunfluorescentie en met behulp van flow cytometrie het percentage monocyten in bloedmonsters van kleine herkauwers bepaald is. In alle onderzochte monsters zijn monocyten aangetroffen waarbij het opviel dat er een zeer grote variatie te zien was. Deze variatie bleek beïnvloed te kunnen worden door diersoort-, sekse- en mogelijk zelfs leeftijdsverschillen, maar niet door al of niet melk geven of geïnfecteerd zijn met SRLV.
De serologische test en de PCR test zijn in de **hoofdstukken 3**, **5** en **6** uitvoerig met elkaar vergeleken en gaven relatief constante uitslagen.

Er is nog geen volledige opheldering over het verloop van de transmissie van SRLV's. Verschillende epidemiologische parameters zoals de identificatie van predisponerende factoren en het kwantificeren van transmissie onder verschillende omstandigheden zijn nog onvoldoende in kaart gebracht. In **hoofdstuk 5** wordt

onderzoek beschreven naar een mogelijke genetisch bepaalde gevoeligheid en de het verschil in transmissiesnelheid onder weide en stalomstandigheden. Een rasgebonden gevoeligheid is niet met zekerheid vastgesteld, terwijl er wel duidelijke individuele verschillen te zien zijn. Op opvallende bevinding is dat de verspreiding van de infectie op een bepaald moment lijkt te stoppen, er worden dan geen dieren meer geïnfecteerd ondanks dat ze nog steeds in dezelfde koppel en onder de zelfde omstandigheden gehouden worden. In de studie beschreven in hoofdstuk 5 komt heel duidelijk naar voren dat de omstandigheden waaronder de dieren gehouden worden, in de wei of op stal, van invloed is op de snelheid waarmee de infectie zich binnen de koppel verspreid. Op stal is de transmissiesnelheid 10x zo hoog als in de wei.

Een ander epidemiologische factor die van belang is bij de verspreiding van SRLV's is de vraag of we al dan niet met een seksueel overdraagbare aandoening te maken hebben. In **hoofdstuk 6** wordt een proef beschreven waarbij gekeken is naar het voorkomen van (pro)viraal DNA in de ejaculaten en accessoire geslachtsklieren van SRLV geïnfecteerde rammen en bokken. De meeste individuen produceerde (intermitterend) positieve ejaculaten. Ook de meeste weefselmonsters van de accessoire geslachtsklieren testen positief voor (pro-)viraal SRLV DNA. In tegenstelling tot de verwachting was er geen correlatie tussen het percentage monocyten in het bloed en/of macrofagen in de ejaculaat en een positief of negatief testresultaat.

De belangrijkste conclusies wat betreft de SRLV epidemiologie zijn: Om de besmetting met deze langzaam progressief verlopende ziekte reeds in een vroeg stadium aan te tonen prevaleren virusdetecterende testen boven testen die de immuunreactie van het dier aangeven. De efficiëntie van de overdracht van lentivirussen verschilt naar gelang de omstandigheden waaronder de dieren gehouden worden. Bovendien is seksuele overdraagbaarheid zeer waarschijnlijk. Daarnaast verlopen lentivirus infecties niet altijd progressief en bovendien verschilt de mate van aantasting tussen individuen. Al met al kan economische en fysieke schade veroorzaakt door lentivirus-infecties bij schapen en geiten beperkt worden door eenvoudige maatregelen waarbij de risico's voor overdracht geminimaliseerd worden en besmetting snel herkend wordt. De belangrijkste aanbevelingen zijn dan ook dat om lentivirus-infecties efficiënt aan te kunnen pakken een duidelijk doel gesteld moet worden, met daaropvolgend een op maat gemaakt plan van aanpak op bedrijfsniveau.

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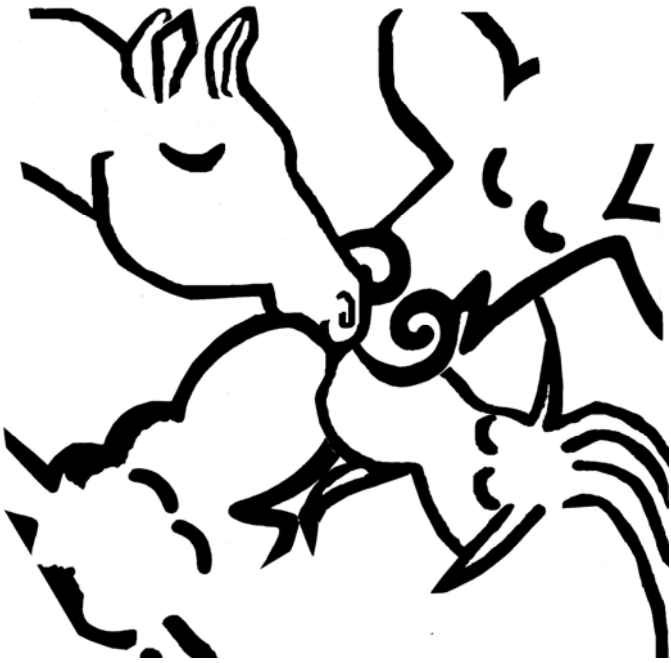

Curriculum vitae

Karianne Peterson werd geboren op 23 maart 1971 te Rotterdam. In 1988 behaalde ze het HAVO diploma aan het Montessori Lyceum en vervolgens in 1990 het VWO diploma aan de Openbare Scholengemeenschap Libanon, beide te Rotterdam. Vervolgens is ze gestart aan de lerarenopleiding, richting Gezondheidskunde, aan de Hogeschool van Utrecht, welke studie i.v.m. inloten voor de studie Diergeneeskunde niet is voortgezet. I.v.m. een deficiënt vakkenpakket is een schaduwplaats aangevraagd en dat jaar (1991) heeft de auteur als dierenartsassistente gewerkt bij dierenarts Wim van Dijk in Krimpen a/d IJssel. Na het behalen van het Deelcertificaat Natuurkunde aan het James Boswell Instituut is ze in 1992 gestart met de studie Diergeneeskunde aan de Faculteit Diergeneeskunde te Utrecht. Gedurende de studie zijn verschillende nevenactiviteiten ontplooid, waaronder aansluiting bij de Utrechtsche Vrouwelijke Studenten Vereniging/ Nieuwe Vereniging Vrouwelijke Studenten te Utrecht, de Vrouwelijke Studenten Rijvereniging Kimmarone (1992-1993 bestuurslid, 1993-1994 praeses), het Vrouwelijk Veterinair Dispuut Mastitis (1994-1995 bestuurslid, 1995-1996 praeses tijdens het 3de lustrum), “veulenbrigadier” bij de afdeling Inwendige Ziekte (1996-1998) en de Kascommissie van de Utrechtse Studenten Rijvereniging de Solleysel (1997-1999). Daarnaast heeft de auteur zich ook ingezet voor de organisatie van het Concours Hippique International Official (CHIO) Rotterdam (Stafofficier Hospitality services), en als commissielid met de portefeuille PR/Marketing van het Jaarlijks Internationaal Diergeneeskundig Congres Voorjaarsdagen (2000-2005 o.a. tijdens het WSAVA/FECAVA World Congress in 2000 en het FECAVA-FEEVA congres in 2005).

Het Dierenartsexamen, met differentiatie Landbouwhuisdieren werd in 2000 behaald. De afstudeerscriptie “Tegenvallers bij de georganiseerde zwoegerziektebestrijding in Nederland” i.s.m. de Gezondheidsdienst voor Dieren te Drachten, sloot wat onderwerp betreft mooi aan op het vervolgens gestarte AIO traject bij de afdeling Voortplanting van het Departement Gezondheidszorg Landbouwhuisdieren aan de Veterinaire Faculteit te Utrecht. Aldaar heeft onderzoek plaatsgevonden op het gebied van de voortplanting bij kleine herkauwers en de Small Ruminant Lentivirussen onder leiding van promotor prof. dr. Ben Colenbrander en copromotoren dr. Dirk Houwers en dr. Bart Gadella. In het kader van het promotietraject is er in verschillende EU-projecten geparticipeerd. Tijdens de aanstelling heeft de auteur zich specifiek ingezet om het onderwijs op het gebied van de kleine herkauwers te ontwikkelen en geven binnen verschillende curricula. Sinds september 2006 is de auteur werkzaam als junior docent bij de afdeling Klinische Pathofysiologie van het Departement Gezondheidszorg Landbouwhuisdieren. Medio 2007 vertrekt het gezin Lievaart-Peterson naar Wagga Wagga, New South Wales, Australië om de carrière voort te zetten aan de Veterinary Faculty van de Charles Sturt University.

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