AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF ANIMAL SCIENCE
LABORATORY OF ANATOMY & PHYSIOLOGY OF FARM ANIMALS

Doctoral (PhD) Thesis

Epizootiology, risk assessment and effects on productivity of ovine progressive pneumonia in intensively reared dairy sheep

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ABSTRACT

Ovine progressive pneumonia (also known as maedi-visna - MV) is a viral, chronic disease of sheep with a long incubation period, caused by small ruminant lentiviruses (SRLV). Chronically infected animals may develop interstitial pneumonia and mastitis, arthritis, encephalitis, and progressive emaciation, leading even to death. Despite the worldwide spreading of SRLV infections, data regarding the significance of transmission routes, the potential associated risk factors, and the effects of SRLV infections on health and productivity in dairy sheep are scarce and mainly derived from cross-sectional sero-epizootiological studies. Also, there is no "gold standard" for the early diagnosis of SRLV infections, and universally applicable diagnostic tools are not available.

Although MV cases have been reported in dairy sheep farms in Greece, the lack of updated epizootiological data does not allow the proposal and implementation of targeted national control programs. Considering this, the overall objectives of the present thesis were: i) to develop and evaluate a diagnostic protocol for the early and accurate diagnosis of SRLV infections; ii) to investigate the epizootiology of SRLV infections, emphasizing the calculation of morbidity frequency measures, the assessment of potential risk factors, and the evaluation of the significance of horizontal and vertical transmission routes; and iii) to quantify the effects of SRLV infections on milk production, health and welfare status in intensively reared dairy sheep.

For this reason, a total of 660 purebred Chios and Lacaune ewes and 195 lambs from four representative intensive dairy farms were included in the study. For the serology-based diagnosis of SRLV infections in the studied farms, an indirect whole virus commercial ELISA test (ELISA, CAEV/MVV Total Ab Test, IDEXX) was utilized and evaluated in serum blood samples from the animals of the study. Also, 13 different sets of primers were used in five nested and three simple conventional PCR protocols in the *pol*, *gag*, *env*, and LTR regions of SRLV genome in DNA samples from the studied animals. Representative PCR products from these protocols were sequenced and used in the subsequent phylogenetic analyses. Pairwise sequence comparisons between the nucleotide sequences from the present study, the available Greek strains and the representative SRLV strains of A, B, C, and E genotypes, and the

construction of the respective phylogenetic trees using the Maximum Likelihood method were performed. The results from the phylogenetic analyses were exploited for the designation and development of a real-time PCR protocol for the early and accurate diagnosis of SRLV infections from the circulating strains. The real-time PCR protocol was evaluated for its specificity after the sequencing of PCR products and for its diagnostic performance with the construction of a standard curve and the calculation of its efficiency and limit of detection (LOD).

Afterwards, a two-year prospective epizootiological study was conducted on the selected ewes and their lambs, which were grouped according to the type of colostrum (unpasteurized and pasteurized) and rearing (natural or artificial). In ewes, blood samplings for serological and molecular diagnosis of SRLV infections were performed twice a year, at premating and pre-lambing. In lambs, four blood samplings were performed during the 1^{st}, 3^{rd}, 8^{th} (at pre-mating), and 13^{th} (at pre-lambing) month of their life. In each sampling occasion, animals were categorized as seropositive and seronegative according to the ELISA results, PCR positive and PCR negative according to the real-time PCR results, and infected and uninfected according to the combination of ELISA and real-time PCR results (in lambs only real-time PCR results were considered for the infection). At the end of the study, animals were categorized according to their temporal serological pattern as constantly seropositive (exclusively seropositive results during the study), constantly seronegative (exclusively seronegative results during the study), seroconverted (seronegative animals at the beginning of the study which converted to seropositive during the study), seroreverted (seropositive animals at the beginning of the study which reverted to seronegative during the study), and animals with an intermittent presence of antibodies (alternating seropositive and seronegative status during the study regardless of their serological status at the beginning of the study). Also, ewes were categorized according to their temporal infection pattern as infected seropositive (tested both PCR positive and constantly seropositive or with an intermittent presence of antibodies or seroconverted until the end of the study), infected seronegative (tested PCR positive and constantly seronegative or seroreverted until the end of the study), and uninfected (tested always both PCR and ELISA negative). Morbidity frequency measures were calculated either based on the ELISA results or the combination of ELISA and real-time PCR results and included point (sero)prevalence, period (sero)prevalence, incidence, and cumulative incidence rates. The potential risk factors associated with SRLV infections in ewes and lambs were evaluated with mixed binary regression models.

Also, milk yield was recorded, and individual milk samples were collected from the studied ewes at the beginning, middle, and end of one milking period. After chemical analysis of milk samples and the measurement of somatic cell counts (SCC), daily milk, fat, protein, lactose, and solids-non-fat yields (DMY, DFY, DPY, DLY, and DSNFY, respectively), as well as the logarithm of SCC were estimated. At the end of the study, the total yields of milk quality traits (TMY, TFY, TPY, TLY, and TSNFY, respectively) were calculated for the first 120 days of the milking period.

During the two-year prospective study, ewes were physically examined, and 17 health and welfare indicators were assessed at the animal level in each sampling occasion. Moreover, blood samples were collected at the last sampling occasion and used for hematological analyses, including 21 parameters of white and red blood cells and platelets. The effects of SRLV infections on milk production and health and welfare status were evaluated with mixed linear regression models and mixed binary regression models, respectively.

The commercial ELISA test provided positive results in all the studied farms, and the sensitivity, specificity, and *k*-value were 82.8%, 93.8%, and 0.620, respectively, using as "gold standard" the positive result in at least one conventional PCR protocol. The phylogenetic analyses of the nucleotide sequences from the study revealed the circulation of viral strains belonging to A and B genotypes in the studied farms. Primers were designed based on conserved regions of the *gag* gene, and a nested SYBR Green real-time PCR protocol was developed for amplifying a 126 bp DNA fragment for the detection of SRLV infections of both genotypes with 99.52% efficiency and LOD 178 viral copies.

A total of 15.2% of the studied ewes were constantly seronegative, 46.2% were constantly seropositive, 20.1% seroconverted, 8.6% seroreverted, and 9.8% presented an intermittent presence of antibodies. Regarding the infection patterns, only a total of 7.1% of the ewes remained uninfected till the end of the study, whereas 76.2% were infected seropositive and 16.7% were infected but seronegative. Point seroprevalence ranged from 57.5% (1^{st} sampling occasion) to 75.4% (4^{th} sampling occasion), whereas prevalence ranged from 70.0% (1^{st} sampling occasion) to 88.3% (5^{th} sampling occasion) in the total of the studied ewes. The overall period seroprevalence, incidence rate, and cumulative incidence were 84.8% (95% CI, 80.9–88.0%), 33.6 new cases per 100 sheep-semesters (95% CI, 27.8–40.3%), and 64.2% (95% CI, 56.8–70.9%) based on the ELISA results. The respective values obtained after the combination of ELISA and PCR results were 90.7% (95% CI, 87.4–93.1%), 40.6 new cases per 100 sheep-semesters (95% CI, 32.6-50.0 new cases per 100 sheep-semesters), and 68.9% (95% CI, 60.2-76.4%).

Also, a one-year increase in age was associated with an increased relative risk for seropositive status (1.78, 95% CI, 1.41-2.25, $p<0.001$), infected status (1.69, 95% CI, 1.25-2.29, $p = 0.001$), constantly seropositive pattern (1.60, 95% CI, 1.35-1.91, $p<0.001$), and infected seropositive pattern (1.31, 95% CI, 1.08-1.60, $p<0.01$). On the other hand, a one-year increase in animal age was associated with a ca. 30% decreased likelihood for constantly seronegative status, infected seronegative ewes, seroconversion, and ewes with an intermittent presence of antibodies. Lacaune ewes were 2.63 times (95% CI, 1.35-5.00, $p<0.01$) more likely to be seropositive during the study, whereas Chios ewes were 4.53 times (95% CI, 1.61-12.76, $p<0.01$) more likely to present an intermittent presence of antibodies. Moreover, ewes were 1.72 times (95% CI, 1.28-2.33, $p<0.001$), 2.94 times (95% CI, 1.82-4.76, $p<0.001$), and 3.23 times (95% CI, 1.85-5.53, $p<0.001$) more likely to be found seropositive, infected, or seroconverted, respectively, at pre-lambing compared to pre-mating sampling occasions.

A total of 78.1% (57/73) and 43.4% (53/122) of the lambs that consumed unpasteurized and pasteurized colostrum, respectively, were infected during the study. Seroreversion and seroconversion incidents occurred until the age of 8 months in more than 90.0% and 70.0% of the seroreverted and seroconverted lambs, respectively. Lambs that consumed unpasteurized colostrum from their dam were 19.29 (95% CI, 2.37-156.85, $p<0.01$) and 6.07 (95% CI, 2.42-15.21, $p<0.001$) times more likely to be found seropositive or infected at the age of 13 months old compared to the lambs that consumed pasteurized colostrum. Also, relative risk for infection during the first 13 months of lambs' life was increased by 2.07 times (95% CI, 1.22-5.88, $p<0.05$) for the lambs from infected dams.

Daily milk and milk quality traits yields (DMY, DFY, DPY, DLY, and DSNFY) were reduced by ca. 15% in the infected seronegative ewes compared to the uninfected ones ($p<0.05$). Also, DFY was reduced by ca. 10% in the infected seronegative ewes compared to the infected seropositive ones ($p<0.05$). Moreover, 120-day milk yield and milk quality traits yields were found to be reduced ca. 20% in the infected seronegative ewes compared to both the uninfected and the infected seropositive ewes ($p<0.05$). The duration of lactation period was 1.25 (95% CI, 1.10-1.45, $p=0.001$) and 1.16 (95% CI, 1.04-1.30, $p<0.01$) times more likely to be shorter in the infected seronegative ewes compared to the uninfected and the infected seropositive ewes, respectively.

Regarding the effects of SRLV infections on health and welfare status, the infected seronegative ewes were 4.17 times (95% CI, 1.25-14.29, $p<0.05$) more likely to develop arthritis at least once during the study compared to the uninfected ewes. Also, the infected

seronegative ewes were 3.03 (95% CI, 1.25-7.14, $p<0.05$) and 2.08 (95% CI, 1.10-3.85, $p<0.05$) times more likely to have swollen supramammary lymph nodes at least once during the study compared to the uninfected and the infected seropositive ewes, respectively. The uninfected ewes presented significantly higher white blood cell, lymphocyte, and monocyte counts compared to the infected ewes ($p<0.05$).

This is the first epizootiological study that prospectively investigated the SRLV prevalence, the risk factors for SRLV infections, and their effects on productivity, health and welfare status after developing and applying a diagnostic protocol for the early and effective diagnosis of SRLV infections with a combination of serological and molecular tests in intensively reared dairy sheep in Greece. The present study confirmed the hypothesis of increased SRLV prevalence in intensively reared dairy sheep in our country and recorded updated epizootiological data for the evidence-based designation of control programs against SRLV infections. Our findings highlighted the weaknesses of cross-sectional sero-epizootiological studies regarding the detection of infections and the investigation of their effects on productivity and health; they also underline the necessity of a combination of serological and molecular tests in control programs to detect seronegative but infected animals that serve as carriers spreading the virus. Based on our results, the administration of pasteurized colostrum in lambs, the serological screening control of replacement animals at the age of 8 months, the serological tests of all adult sheep and the PCR test of seronegative animals at the pre-lambing period, and the removal of infected seronegative animals are proposed as basic management practices to be integrated in SRLV control programs.

Scientific area: Infectious diseases of small ruminants

Keywords: ovine progressive pneumonia, maedi-visna, small ruminant lentiviruses, dairy sheep, longitudinal cohort study, morbidity frequency measures, transmission routes, risk factors, serological diagnosis, molecular diagnosis, effects, milk yield, milk quality traits, health and welfare

Επιζωοτιολογία, εκτίμηση παραγόντων κινδύνου και επιπτώσεις της προϊούσας πνευμονίας στην παραγωγικότητα των εντατικά εκτρεφόμενων προβάτων γαλακτοπαραγωγικής κατεύθυνσης

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ΠΕΡΙΛΗΨΗ

Η προϊούσα πνευμονία, επίσης γνωστή ως maedi-visna, είναι ένα ιογενές, βραδείας εξέλιξης, χρόνιο νόσημα των προβάτων που οδηγεί σε ισόβια λοίμωξη και προκαλείται από τους λεντιιούς των μικρών μηρυκαστικών. Τα χρόνια μολυσμένα ζώα αναπτύσσουν διάμεση πνευμονία, μαστίτιδα, αρθρίτιδα, και εγκεφαλίτιδα και εκδηλώνουν προοδευτική αδυναμία καταλήγοντας έως και το θάνατο. Παρά την παγκόσμια εξάπλωση των λεντιιών των μικρών μηρυκαστικών, τα δεδομένα σχετικά με τη σημασία των οδών μετάδοσης, των δυνητικών παραγόντων κινδύνου, καθώς και των επιπτώσεων των μολύνσεων στην υγεία και την παραγωγικότητα των προβάτων γαλακτοπαραγωγικής κατεύθυνσης είναι ελλιπή και προέρχονται από συγχρονικές οροεπιδημιολογικές μελέτες. Επίσης, δεν υπάρχει παγκοσμίως διαθέσιμη διαγνωστική δοκιμή αναφοράς, ενώ δεν έχει αναπτυχθεί έως σήμερα ένα διαγνωστικό εργαλείο με με καθολική εφαρμογή για την πρώιμη και αξιόπιστη διάγνωση των μολύνσεων από τους λεντιιούς.

Παρότι έχουν αναφερθεί κλινικά περιστατικά προϊούσας πνευμονίας σε εκτροφές προβάτων γαλακτοπαραγωγικής κατεύθυνσης στην Ελλάδα, η έλλειψη επικαιροποιημένων επιζωοτιολογικών δεδομένων δεν επιτρέπει το σχεδιασμό και την εφαρμογή στοχευμένων εθνικών προγραμμάτων ελέγχου. Λαμβάνοντας υπόψιν τα παραπάνω, οι στόχοι της παρούσας διδακτορικής διατριβής ήταν: i) η ανάπτυξη και η αξιολόγηση ενός διαγνωστικού πρωτοκόλλου για την πρώιμη και αξιόπιστη διάγνωση των μολύνσεων από τους λεντιιούς των μικρών μηρυκαστικών με το συνδυασμό ορολογικών και μοριακών δοκιμών, ii) η επιζωοτιολογική διερεύνηση των λεντιιών των μικρών μηρυκαστικών στη χώρα με έμφαση στον υπολογισμό των δεικτών νοσηρότητας, την εκτίμηση της σημασίας των δυνητικών παραγόντων κινδύνου των μολύνσεων, και την αξιολόγηση της σημασίας της κάθετης και της οριζόντιας μετάδοσης των μολύνσεων, και iii) η ποσοτικοποίηση των επιπτώσεων των μολύνσεων στην ποσότητα και την ποιότητα του παραγόμενου γάλακτος, την υγεία και την ευζωία των εντατικά εκτρεφόμενων προβάτων γαλακτοπαραγωγικής κατεύθυνσης.

Συνολικά 660 θηλυκά πρόβατα και 195 αρνιά από τέσσερις αντιπροσωπευτικές εντατικές γαλακτοπαραγωγές εκτροφές καθαρόαιμων προβάτων της φυλής Χίου και Lacaune συμπεριληφθήκαν στην έρευνα. Αρχικά, για την ορολογική διάγνωση των μολύνσεων στις

εκτροφές της μελέτης χρησιμοποιήθηκε και αξιολογήθηκε μία έμμεση εμπορική δοκιμή ELISA ολόκληρου ιού (ELISA, CAEV/MVV Total Ab Test, IDEXX). Επίσης, στα δείγματα DNA των ζώων της έρευνας χρησιμοποιήθηκαν 13 διαφορετικά ζεύγη εκκινητών σε πέντε πρωτόκολλα επάλληλης (nested) και τρία πρωτόκολλα ενός σταδίου απλής PCR (αλυσιδωτής αντίδρασης πολυμεράσης) στη *gag*, *pol*, *env*, και LTR περιοχή του ιικού γονιδιώματος. Αντιπροσωπευτικά PCR προϊόντα των πρωτοκόλλων αυτών αλληλουχήθηκαν και χρησιμοποιήθηκαν σε φυλογενετικές αναλύσεις. Πραγματοποιήθηκαν συγκρίσεις αλληλουχιών κατά ζεύγη ανάμεσα στις αλληλουχίες της παρούσας μελέτης, των στελεχών που έχουν ήδη απομονωθεί στη χώρα, και αντιπροσωπευτικών στελεχών που ανήκουν στους A, B, C, και E γενοτύπους. Στη συνέχεια, κατασκευάστηκαν τα αντίστοιχα φυλογενετικά δέντρα χρησιμοποιώντας τη μέθοδο της Μέγιστης Πιθανοφάνειας (Maximum Likelihood method). Τα αποτελέσματα των φυλογενετικών αναλύσεων χρησιμοποιήθηκαν για το σχεδιασμό και την ανάπτυξη ενός real-time PCR πρωτοκόλλου για την πρώιμη και αξιόπιστη διάγνωση των μολύνσεων από τα διαδεδομένα στελέχη των λεντιιών στις εκτροφές της έρευνας. Το real-time PCR πρωτόκολλο αξιολογήθηκε για την ειδικότητά του μετά από αλληλούχιση των PCR προϊόντων, και για τη διαγνωστική του ικανότητα με την κατασκευή της πρότυπης καμπύλης και τον υπολογισμό της αποτελεσματικότητας και του ελάχιστου ορίου ανίχνευσης.

Στη συνέχεια, πραγματοποιήθηκε μία προοπτική επιζωοτιολογική μελέτη διετούς διάρκειας στα θηλυκά πρόβατα και στα αρνιά τους, τα οποία ομαδοποιήθηκαν ανά εκτροφή σύμφωνα με το είδος πρωτογάλακτος (απαστερίωτο και παστεριωμένο) και το σύστημα γαλουχίας (φυσική ή τεχνητή). Στα θηλυκά πρόβατα οι δειγματοληψίες αίματος για την ορολογική και μοριακή διάγνωση των μολύνσεων από τους λεντιιούς πραγματοποιούνταν δύο φορές το χρόνο, την περίοδο πριν από τις οχείες και πριν από τον τοκετό. Οι αντίστοιχες δειγματοληψίες αίματος στα αρνιά πραγματοποιήθηκαν τέσσερις φορές κατά τη διάρκεια της έρευνας, τον 1^{ο}, τον 3^{ο}, τον 8^{ο} (πριν τις οχείες), και τον 13^{ο} μήνα (πριν τον τοκετό) της ζωής τους.

Σε κάθε δειγματοληψία τα ζώα κατηγοριοποιούνταν ως οροθετικά και οροαρνητικά σύμφωνα με τα αποτελέσματα της ELISA, ως PCR θετικά και αρνητικά σύμφωνα με τα αποτελέσματα του real-time PCR πρωτοκόλλου, και ως μολυσμένα και μη μολυσμένα σύμφωνα με το συνδυασμό των αποτελεσμάτων της ELISA και του real-time PCR πρωτοκόλλου (στα αρνιά μόνο τα αποτελέσματα του real-time PCR πρωτοκόλλου λήφθηκαν υπόψιν για τον προσδιορισμό της μόλυνσης). Στο τέλος της μελέτης τα ζώα κατηγοριοποιήθηκαν σύμφωνα με το ορολογικό τους μοτίβο ως συνεχώς οροθετικά (αποκλειστικά οροθετικά αποτελέσματα

κατά τη διάρκεια της έρευνας), συνεχώς οροαρνητικά (αποκλειστικά οροαρνητικά αποτελέσματα κατά τη διάρκεια της έρευνας), ζώα που έκαναν ορομετατροπή (οροαρνητικά ζώα στην αρχή της έρευνας που μετατράπηκαν σε οροθετικά κατά τη διάρκειά της), ζώα που έκαναν οροαντιστροφή (οροθετικά ζώα στην αρχή της έρευνας που μετατράπηκαν σε οροαρνητικά κατά τη διάρκειά της), και ζώα με διαλείπουσα παρουσία αντισωμάτων (ζώα με εναλασσόμενο οροθετικό και οροαρνητικό αποτέλεσμα κατά τη διάρκεια της έρευνας ανεξαρτήτως του ορολογικού αποτελέσματος στην αρχή της έρευνας). Επίσης, τα θηλυκά πρόβατα κατηγοριοποιήθηκαν σύμφωνα με το μοτίβο μόλυνσης που παρουσίασαν κατά τη διάρκεια της έρευνας ως μολυσμένα οροθετικά (ζώα που βρέθηκαν PCR θετικά και συνεχώς οροθετικά ή με διαλείπουσα παρουσία αντισωμάτων ή παρουσίασαν ορομετατροπή μέχρι το τέλος της έρευνας), μολυσμένα οροαρνητικά (ζώα που βρέθηκαν PCR θετικά και συνεχώς οροαρνητικά ή με οροαντιστροφή μέχρι το τέλος της έρευνας), και μη μολυσμένα (ζώα που βρέθηκαν συνεχώς PCR αρνητικά και οροαρνητικά κατά τη διάρκεια της έρευνας). Οι δείκτες νοσηρότητας υπολογίστηκαν είτε λαμβάνονονας υπόψιν τα αποτελέσματα της ELISA ή το συνδυασμό των αποτελεσμάτων της ELISA και του real-time PCR πρωτοκόλλου και περιελάμβαναν το σημειακό (ορο)επιπολασμό, τον (ορο)επιπολασμό περιόδου, την επίπτωση και την αθροιστική επίπτωση. Η εκτίμηση των δυνητικών παραγόντων κινδύνου των μολύνσεων από τους λεντιιούς στα θηλυκά πρόβατα και τα αρνιά πραγματοποιήθηκε με τη χρήση μικτών μοντέλων διωνυμικής λογιστικής παλινδρόμησης.

Επίσης, πραγματοποιήθηκαν γαλακτομετρήσεις και λήφθηκαν ατομικά δείγματα γάλακτος από τα θηλυκά πρόβατα της έρευνας στην αρχή, τη μέση, και το τέλος της αρμεκτικής περιόδου. Μετά τη χημική ανάλυση των δειγμάτων γάλακτος και την μέτρηση των σωματικών κυττάρων, υπολογίστηκαν η ημερήσια ποσότητα γάλακτος, λίπους, πρωτεϊνών, λακτόζης, και ολικών στερεών άνευ λίπους, καθώς και ο λογάριθμος των σωματικών κυττάρων. Στο τέλος της έρευνας, υπολογίστηκαν η συνολική ποσότητα γάλακτος, λίπους, πρωτεϊνών, λακτόζης, και ολικών στερεών άνευ λίπους για τις πρώτες 120 ήμερες της αρμεκτικής περιόδου.

Κατά τη διάρκεια της διετούς προοπτικής μελέτης, σε κάθε δειγματοληψία τα πρόβατα υποβάλλονταν σε κλινική εξέταση και 17 ατομικοί δείκτες υγείας και ευζωίας αξιολογούνταν. Επιπλέον, στην τελευταία δειγματοληψία της έρευνας πραγματοποιήθηκαν αιματολογικές αναλύσεις στα δείγματα αίματος των ζώων της έρευνας περιλαμβάνοντας 21 παραμέτρους αξιολόγησης χαρακτηριστικών των λευκοκυττάρων, των ερυθρών αιμοσφαιρίων, και των αιμοπεταλίων. Οι επιπτώσεις των μολύνσεων των λεντιιών στη γαλακτοπαραγωγή και στα ποιοτικά χαρακτηριστικά του γάλακτος, καθώς και στην υγεία και

ευζωία των ζώων της έρευνας αξιολογήθηκαν με τη χρήση μικτών μοντέλων γραμμικής και διωνυμικής λογιστικής παλινδρόμησης.

Η εμπορική δοκιμή ELISA ανίχνευσε μολυσμένα ζώα σε όλες τις εκτροφές της έρευνας και η ευαισθησία, η ειδικότητα και η τιμή kappa coefficient (*k*-value) βρέθηκαν 82,8%, 93,8%, και 0,620, αντίστοιχα, χρησιμοποιώντας ως αποτέλεσμα αναφοράς το θετικό αποτέλεσμα σε ένα τουλάχιστον απλό πρωτόκολλο PCR από αυτά που αρχικά χρησιμοποιήθηκαν. Η φυλογενετική ανάλυση που πραγματοποιήθηκε στα στελέχη των εκτροφών που συμμετείχαν στην έρευνα αποκάλυψε τη διάδοση ιικών στελεχών που ανήκουν στους γενοτύπους Α και Β. Οι εκκινητές σχεδιάστηκαν σε συντηρημένες περιοχές του *gag* γονιδίου, και ένα SYBR Green real-time PCR πρωτόκολλο δύο σταδίων που πολλαπλασιάζει ένα τμήμα DNA μεγέθους 126 ζευγών βάσεων αναπτύχθηκε και αξιολογήθηκε για την ανίχνευση των μολύνσεων και των δύο γενοτύπων με 99,52% αποτελεσματικότητα και ελάχιστο όριο ανίχνευσης τα 178 αντίγραφα του ιού.

Συνολικά το 15,2% των θηλυκών προβάτων ήταν συνεχώς οροαρνητικό, το 46,2% συνεχώς οροθετικό, ενώ το 20,1% εμφάνισε ορομετατροπή, το 8,1% οροαντιστροφή, και το 9,8% διαλείπουσα παρουσία αντισωμάτων. Όσον αφορά τα μοτίβα μόλυνσης, μόλις το 7,1% των θηλυκών προβάτων παρέμεινε μη μολυσμένο μέχρι το τέλος της έρευνας, ενώ το 76,2% ήταν μολυσμένο οροθετικό και το 16,7% μολυσμένο οροαρνητικό.

Ο σημειακός οροεπιπολασμός κυμαινόταν από 57,5% (1^{η} δειγματοληψία) έως 75,4% (4^{η} δειγματοληψία), ενώ ο σημειακός επιπολασμός κυμαινόταν από 70,0% (1^{η} δειγματοληψία) έως 88,3% (5^{η} δειγματοληψία). Ο οροεπιπολασμός περιόδου για τη συνολική διάρκεια της έρευνας, η επίπτωση, και η αθροιστική επίπτωση ήταν 84,8% (95% ΔΕ, 80,9–88,0%), 33,6 νέα περιστατικά μολύνσεων ανά 100 προβατο-εξάμηνα (95% ΔΕ, 27,8–40,3%), και 64,2% (95% ΔΕ, 56,8–70,9%) σύμφωνα με τα αποτελέσματα της ELISA. Οι αντίστοιχες τιμές σύμφωνα με το συνδυασμό των αποτελεσμάτων της ELISA και της real-time PCR ήταν 90,7% (95% ΔΕ, 87,4–93,1%), 40,6 νέα περιστατικά μολύνσεων ανά 100 προβατο-εξάμηνα (95% ΔΕ, 32,6-50,0), και 68,9% (95% ΔΕ, 60,2-76,4%).

Σύμφωνα με την εκτίμηση των παραγόντων κινδύνου, η αύξηση της ηλικίας κατά ένα έτος συσχετίστηκε με αύξηση του σχετικού κινδύνου κατά 1,78 φορές για την οροθετικότητα (95% ΔΕ, 1,41-2,25, p<0,001), 1,69 φορές για την μόλυνση (95% ΔΕ, 1,25-2,29, p= 0,001), 1,60 φορές για το συνεχώς οροθετικό μοτίβο (95% ΔΕ, 1,35-1,91, p<0,001), και 1,31 φορές για το μολυσμένο οροθετικό μοτίβο (95% ΔΕ, 1,08-1,60, p<0,01). Από την άλλη πλευρά, η αύξηση της ηλικίας των ζώων κατά ένα έτος συσχετίστηκε με περίπου 30% μειωμένη πιθανότητα για την εμφάνιση συνεχώς οροαρνητικών και μολυσμένων οροαρνητικών ζώων,

καθώς και ζώων που εμφανίζουν ορομετατροπή ή διαλείπουσα παρουσία αντισωμάτων. Τα πρόβατα της φυλής Lacaune ήταν 2,63 φορές (95% ΔΕ, 1,35-5,00, p<0,01) πιο πιθανό να είναι οροθετικά κατά τη διάρκεια της έρευνας, ενώ τα θηλυκά πρόβατα της φυλής Χίου ήταν 4,53 φορές (95% ΔΕ, 1,61-12,76, p<0,01) πιο πιθανό να εμφανίσουν διαλείπουσα παρουσία αντισωμάτων. Επιπλέον, τα θηλυκά πρόβατα ήταν 1,72 φορές (95% ΔΕ, 1,28-2,33, p<0,001), 2,94 φορές (95% ΔΕ, 1,82-4,76, p<0,001), και 3,23 φορές (95% ΔΕ, 1,85-5,53, p<0,001) πιο πιθανό να βρεθούν οροθετικά, μολυσμένα, ή με ορομετατροπή, αντίστοιχα, στη δειγματοληψία πριν από τον τοκετό σε σύγκριση με τη δειγματοληψία πριν από τις οχείες.

Συνολικά 78,1% και 43,4% των αρνιών που κατανάλωσαν απαστερίωτο και παστεριωμένο πρωτόγαλα, αντίστοιχα, βρέθηκαν μολυσμένα κατά τη διάρκεια της έρευνας. Η οροαντιστροφή και ορομετατροπή συνέβη μέχρι την ηλικία των 8 μηνών σε περισσότερα από το 90,0% και το 70,0% των ζώων που εμφάνισαν οροαντιστροφή και ορομετατροπή, αντίστοιχα, κατά τη διάρκεια της έρευνας. Τα αρνιά που κατανάλωσαν απαστερίωτο πρωτόγαλα ήταν 19,29 φορές (95% ΔΕ, 2,37-156,85, p<0,01) και 6,07 φορές (95% ΔΕ, 2,42-15,21, p<0,001) πιο πιθανό να βρεθούν οροθετικά ή μολυσμένα σε ηλικία 13 μηνών συγκριτικά με τα αρνιά που κατανάλωσαν παστεριωμένο πρωτόγαλα. Τέλος, ο σχετικός κίνδυνος για μόλυνση των αρνιών τους πρώτους 13 μήνες βρέθηκε αυξημένος κατά 2,07 φορές (95% ΔΕ, 1,22-5,88, p<0,05) για τα αρνιά που προέρχονταν από μολυσμένες μητέρες.

Οι μέσες ημερήσιες ποσότητες γάλακτος, λίπους, πρωτεϊνών, λακτόζης και ολικών στερεών άνευ λίπους βρέθηκαν στατιστικά σημαντικά μειωμένες κατά περίπου 15,0% στα μολυσμένα οροαρνητικά ζώα σε σύγκριση με τα μη μολυσμένα (p<0,05). Επιπλέον, η μέση ημερήσια ποσότητα λιπαρών βρέθηκε μειωμένη κατά περίπου 10,0% στα μολυσμένα οροαρνητικά ζώα σε σχέση με τα μολυσμένα οροθετικά (p<0,05). Οι απώλειες στις συνολικές ποσότητες γάλακτος, λιπαρών, πρωτεϊνών, λακτόζης και ολικών στερεών άνευ λίπους για τις 120 μέρες αρμεκτικής περιόδου άγγιξαν το 20,0% για τα μολυσμένα οροαρνητικά ζώα σε σχέση τόσο με τα μη μολυσμένα όσο και με τα μολυσμένα οροθετικά ζώα (p<0,05). Τέλος, η διάρκεια της γαλακτικής περιόδου ήταν 1,25 φορές (95% ΔΕ, 1,10-1,45, p= 0,001) και 1,16 (95% ΔΕ, 1,04-1,30, p<0,01) πιο πιθανό να είναι συντομότερη στα μολυσμένα οροαρνητικά ζώα σε σχέση με τα μη μολυσμένα και τα μολυσμένα οροθετικά, αντίστοιχα.

Όσον αφορά τις επιπτώσεις των μολύνσεων των λεντιιών στην υγεία και την ευζωία των ζώων, τα μολυσμένα οροαρνητικά θηλυκά πρόβατα ήταν 4,17 φορές (95% ΔΕ, 1,25-14,29, p<0,05) πιο πιθανό να αναπτύξουν αρθρίτιδα τουλάχιστον μία φορά κατά τη διάρκεια της έρευνας σε σχέση με τα μη μολυσμένα ζώα. Επίσης, τα μολυσμένα οροαρνητικά ζώα ήταν 3,03 φορές (95% ΔΕ, 1,25-7,14, p<0,05) και 2,08 φορές (95% ΔΕ, 1,10-3,85, p<0,05) πιο

πιθανό να εμφανίσουν διογκωμένους οπισθομαστιαίους λεμφαδένες τουλάχιστον μία φορά κατά τη διάρκεια της έρευνας σε σχέση με τα μη μολυσμένα και τα μολυσμένα οροθετικά ζώα, αντίστοιχα. Τα μη μολυσμένα ζώα, επίσης, παρουσίασαν σημαντικά υψηλότερες τιμές λευκοκυττάρων, λεμφοκυττάρων, και μονοκυττάρων συγκριτικά με τα μολυσμένα ($p<0,05$).

Η παρούσα διδακτορική διατριβή αποτελεί την πρώτη προοπτική επιζωοτιολογική μελέτη που διερεύνησε τον επιπολασμό των λεντιιών των μικρών μηρυκαστικών, τους δυνητικούς παράγοντες κινδύνου των μολύσεων από αυτούς, και τις επιπτώσεις αυτών στην παραγωγικότητα, την υγεία και την ευζωία των εντατικά εκτρεφόμενων προβάτων των φυλών Χίου και Lacaune, μέσω της ανάπτυξης και της χρήσης ενός διαγνωστικού πρωτοκόλλου για την πρώιμη και αξιόπιστη διάγνωση των μολύνσεων με το συνδυασμό ορολογικών και μοριακών δοκιμών. Η παρούσα μελέτη επιβεβαίωσε τις ενδείξεις που υπήρχαν σχετικά με τον αυξημένο επιπολασμό των λεντιιών στα εντατικά εκτρεφόμενα πρόβατα γαλακτοπαραγωγικής κατεύθυνσης στη χώρα μας και επικαιροποίησε τα επιζωοτιολογικά δεδομένα, τα οποία μπορούν να χρησιμοποιηθούν για το σχεδιασμό τεκμηριωμένων προγραμμάτων ελέγχου των μολύνσεων. Τα ευρήματα της έρευνας ανέδειξαν τις αδυναμίες των συγχρονικών οροεπιζωοτιολογικών μελετών στην ανίχνευση των μολύνσεων και τη διερεύνηση των επιπτώσεών τους στην παραγωγικότητα και την υγεία των ζώων. Παράλληλα, απέδειξαν την αναγκαιότητα της συνδυαστικής χρήσης των ορολογικών και μοριακών διαγνωστικών δοκιμών στα προγράμματα ελέγχου ώστε να επιτυγχάνεται η ανίχνευση των μολυσμένων ζώων που παραμένουν οροαρνητικά και συνεχίζουν τη διασπορά του ιού. Επίσης, σύμφωνα με τα αποτελέσματα της παρούσας μελέτης, η χορήγηση παστεριωμένου πρωτογάλακτος στα αρνιά, ο ορολογικός έλεγχος των ζώων αντικατάστασης στην ηλικία των 8 μηνών, ο ορολογικός έλεγχος όλων των ενήλικων ζώων και ο μοριακός έλεγχος των οροαρνητικών ζώων την περίοδο πριν τον τοκετό, και η άμεση απομάκρυνση των μολυσμένων οροαρνητικών ζώων προτείνονται ως διαχειριστικά μέτρα στο πλαίσιο εφαρμογής προγραμμάτων ελέγχου των μολύνσεων από τους λεντιιούς των μικρών μηρυκαστικών.

Επιστημονική περιοχή: Λοιμώδη νοσήματα μικρών μηρυκαστικών

Λέξεις-κλειδιά: προϊούσα πνευμονία, maedi-visna, λεντιιοί των μικρών μηρυκαστικών, πρόβατα γαλακτοπαραγωγικής κατεύθυνσης, προοπτική επιζωοτιολογική μελέτη, διαχρονική μελέτη κοορτής, δείκτες νοσηρότητας, οδοί μετάδοσης, παράγοντες κινδύνου, ορολογική διάγνωση, μοριακή διάγνωση, επιπτώσεις, γαλακτοπαραγωγή, ποιοτικά χαρακτηριστικά του γάλακτος, υγεία και ευζωία

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List of publications in peer-reviewed international journals with impact factor

1. **Kalogianni, A.I.**, Bouzalas, I., Bossis, I., Gelasakis, A.I. (2023) 'A Longitudinal Cohort Study of Risk Factors Associated with Small Ruminant Lentivirus Seropositivity in Intensively Reared Dairy Ewes in Greece.' Pathogens, 12(10), 1200. Doi: 10.3390/pathogens12101200.

2. **Kalogianni, A.I.**, Bouzalas, I., Bossis, I., Gelasakis, A.I. (2023) 'Seroepidemiology of Maedi-Visna in Intensively Reared Dairy Sheep: A Two-Year Prospective Study.' Animals, 13:2273. Doi: 10.3390/ani13142273.

3. **Kalogianni, A.I.**, Stavropoulos, I., Chaintoutis, S.C., Bossis, I., Gelasakis, A.I. (2021) 'Serological, Molecular and Culture-Based Diagnosis of Lentiviral Infections in Small Ruminants.' Viruses, 13:1711. Doi:10.3390/ v13091711.

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1. **Kalogianni, A.I.**, Tsigas, N.C., Gelasakis, A.I. 'A comparative study of milk quantity and quality traits between a maedi-visna infected and a maedi-visna free dairy sheep farm.' 10th International Sheep Veterinary Congress, 6-10 March 2023, Seville, Spain. Doi: 10.1016/j.anscip.2023.01.369

2. **Kalogianni, A.I.**, Bouzalas, I., Tsimpouri, E., Bertsias, V., Bossis, I., Gelasakis, A.I. 'Hematological profile of maedi-visna seropositive dairy sheep.' 10th International Sheep Veterinary Congress, 6-10 March 2023, Seville, Spain. Doi: 10.1016/j.anscip.2023.01.136

3. **Kalogianni, A.I.**, Bossis, I., Bouzalas, I., Gelasakis, A.I. 'Diagnostic performance of eight PCR protocols and one indirect ELISA in naturally small ruminant lentiviruses infected ewes in Greece' 9th International Scientific Meeting Days of Veterinary Medicine, 22-25 September 2022, Ohrid, Republic of North Macedonia.

4. **Kalogianni, A.I.**, Bossis, I., Bouzalas, I.G., Gelasakis, A.I. 'Longitudinal changes in the serology of antibodies to naturally maedi-visna infected ewes, and their association with the development of arthritis.' XXI-Middle European Buiatrics Congress-Annual Meeting of ECSRHM, 19-22 May 2022, Statre Jablonki, Poland.

5. **Kalogianni, A.I.**, Tsimpouri, E., Korelidou, V., Ntoulma, V., Bossis, I., Gelasakis, A.I. 'Association between seropositivity for small ruminant lentiviruses and milk production traits in intensively reared dairy sheep.' Virtual Meeting of International Sheep Veterinary Association, 23-25 November 2021.

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List of Abbreviations

MV = Maedi-visna

MVV = Maedi-visna virus

CAE = Caprine arthritis-encephalitis

CAEV = Caprine arthritis-encephalitis virus

SRLV = Small ruminant lentiviruses

WOAH = World Organization for Animal Health

USA = United States of America

FIV = Feline immunodeficiency virus

EIAV = Equine infectious anemia virus

SIV = Simian immunodeficiency virus

HIV = Human immunodeficiency virus

RNA = Ribonucleic acid

RT = Reverse transcriptase

INT = Integrase

PRO = Protease

RNaseH = Ribonuclease H

dUTPase = Deoxyuridine-triphosphatase

DNA = Deoxyribonucleic acid

LTR = Long terminal repeats

PCR = Polymerase chain reaction

TMEM154 = Transmembrane protein 154

DPPA2 = Developmental pluripotency associated 2

DPPA4 = Developmental Pluripotency Associated 4

SYTL3 = Synaptotagmin-Like 3

CCR5 = Chemokine receptor 5

MHC = Major Histocompatability Complex

TLR7 = Toll-like receptor 7

TLR8 = Toll-like receptor 8

TLR9 = Toll-like receptor 9

APOBEC3 = Apolipoprotein B mRNA-editing enzyme

C19orf42 = Chromosome 19 Open Reading Frame 19

TMEM38A = Transmembrane Protein 38A

DLGAP1 = Discs Large (Drosophila) Homolog-Associated Protein 1

TRIM5 = tripartite motif-containing 5

AGID = Agar gel immunodiffusion

ELISA = Enzyme-linked immunosorbent assay

RIPA = Radioimmunoprecipitation

RIA = Radioimmunoassay

WB = Western blot

PBLs = Peripheral blood leukocytes

PBMCs = Peripheral blood mononuclear cells

HMA = Heteroduplex mobility assay

LAMP = Loop-mediated isothermal amplification

RPA-LFD = Recombinase polymerase amplification lateral flow dipstick

POC = Point-of-care

EDTA = Ethylenediaminetetraacetic acid

Ab = Antibodies

OD = Optical density

ACK = Ammonium–chloride–potassium

DNase = Deoxyribonuclease

RNase = Ribonuclease

TAE = Tris-acetate-EDTA

UV = Ultraviolet

NH_4Cl = Ammonium chloride

$NaHCO_3$ = Sodium bicarbonate

k-value = Kappa coefficient value

bp = Base pair

BLAST = Basic Local Alignment Search Tool

T_m = Melting temperature

Ct = Cycle threshold

LOD = Limit of detection

RR = Relative risk

BCS = Body condition score

AIC = Akaike information criterion

DMY = Daily milk yield

DFY = Daily fat yield

DPY = Daily protein yield

DLY = Daily lactose yield

DSNFY = Daily solids-not-fat yield

SCC = Somatic cell counts

LogSCC = Logarithm of somatic cell counts

TMY = Total milk yield

TFY = Total fat yield

TPY = Total protein yield

TLY = Total lactose yield

TSNDFY = Total solids-not-fat yield

AWIN = Animal Welfare Indicators

List of Definitions

- **Sero-epizootiological study:** The epizootiological investigation of SRLV infections based on the detection of SRLV-specific antibodies in the studied animals using ELISA testing.
- **Epizootiological study:** The epizootiological investigation of SRLV infections based on the detection of either SRLV-specific antibodies or viral DNA in the studied animals using ELISA or real-time PCR testing, respectively.
- **Infection:** The detection of either SRLV-antibodies or viral DNA in a ewe using ELISA or real-time PCR testing, respectively. In lambs, infection was considered the detection of viral DNA using real-time PCR testing.
- **Point seroprevalence:** The SRLV infection prevalence calculated based on ELISA results in each sampling occasion.
- **Period seroprevalence:** The SRLV infection prevalence calculated based on the ELISA results during the whole period of the study.
- **Point prevalence:** The SRLV infection prevalence calculated based on the combination of ELISA and real-time PCR results in each sampling occasion. In lambs point prevalence was calculated based exclusively on real-time PCR results.
- **Period prevalence:** The SRLV infection prevalence calculated based on the combination of ELISA and real-time PCR results of ewes during the whole period of the study.
- **Serological status:** The categorization of animals as seropositive or seronegative according to the ELISA results.
- **Serological pattern:** The categorization of animals at the end of the study as constantly seropositive, constantly seronegative, seroconverted, seroreverted, and animals with an intermittent presence of antibodies according to the evolution of their serological status during the study.
- **Constantly seropositive:** Animals with a constantly seropositive status during the study.
- **Constantly seronegative:** Animals with a constantly seronegative status during the study.
- **Seroconverted**: Seronegative animals at the beginning of the study which converted to seropositive during the study.
- **Seroreverted:** Seropositive animals at the beginning of the study which reverted to seronegative during the study.

- **Animals with an intermittent presence of antibodies:** Alternating seropositive and seronegative status during the study regardless of their serological status at the beginning of the study.
- **Seroconversion/seroreversion incident:** The time-point of seroconversion/seroreversion event, namely, the first sampling occasion that the animal was detected seroconverted/seroreverted.
- **PCR status:** PCR positive and PCR negative animal according to the results of the real-time PCR testing.
- **Infection status:** SRLV-infected or SRLV-uninfected ewe according to the combination of ELISA and PCR results; a ewe was defined as infected with a positive ELISA or real-time PCR test and uninfected when both ELISA and real-time PCR test were negative. The infected ewes were further grouped into infected seropositive when both ELISA and real-time PCR results were positive and infected seronegative when only PCR results were positive. In lambs infections status was defined exclusively based on the real-time PCR results (infected and uninfected).
- **Infection pattern:** The categorization of animals at the end of the study as infected seropositive (tested both PCR positive and constantly seropositive or with an intermittent presence of antibodies or seroconverted until the end of the study), as infected seronegative (tested PCR positive and constantly seronegative or seroreverted until the end of the study), or uninfected (tested both PCR and ELISA negative throughout the study).
- **Total milk, fat, protein, lactose, solids-not-fat yields:** The yields of milk, fat, protein, lactose, solids-not-fat yields calculated for the first 120 days of the milking period.

Preface

Dairy sheep farming is the major sector of livestock production in Greece. According to recent data from the Hellenic Statistical Authority (2021), in our country, 7,378,357 sheep are reared in 51,014 farms, producing more than 880,000 tons of milk, which is mainly used for the production of cheeses, yogurt and other dairy products.

Traditionally, dairy sheep were reared under extensive farming systems in Greece, exploiting the diverse, lush pastures for the production of high-quality milk. However, in the last decades, the increasing global demand for Greek dairy products made from ovine milk has led to the intensification of production. This intensification has been followed by various benefits (e.g., increased productivity and profitability, consistent milk production in terms of quantity and quality, integration of modern technologies, and improved biosecurity and hygiene status in farms) and challenges (e.g., emerging health issues, welfare considerations, environmental impacts, etc.).

Infectious diseases are highly rated among the factors deteriorating the sustainability of intensive dairy sheep farms, with ovine progressive pneumonia (also known as maedi-visna) placed at the top of the relevant list. Maedi-visna is a chronic disease of sheep causing severe multi-organ clinical disease, emaciation, and even death of the infected animals, caused by small ruminant lentiviruses (SRLV). Although the disease has been reported since 1915, in the last decades, the international trade of breeding stocks and the close contact of sheep reared under intensive farming systems have favored the global spread of the virus and the constant increase in SRLV infection prevalence. The World Organization for Animal Health (WOAH) has included SRLV in the list of notifiable terrestrial and aquatic animal diseases, and many countries have applied national control programs for the eradication of the disease as there is no treatment or vaccination. However, the early and accurate diagnosis of infections remains a challenging worldwide endeavor, undermining the successful implementation of control programs.

Despite the fact that the impact of the disease on animal productivity, health and welfare has been recognized, it has not been fully elucidated and quantified yet. The long-lasting incubation period of the disease and the lack of a “gold standard” for the accurate diagnosis confound both the epizootiological investigation of the disease and the quantification of its effects in dairy sheep. Also, the vast majority of epizootiological studies are based on cross-sectional observations and recordings, which limit the extraction of safe conclusions regarding chronic infections and diseases.

In Greece, although there is evidence of extensive SRLV spread, relevant epizootiological data are scarce, and the prevalence status in the country is unknown. The lack of updated epizootiological data does not allow the proposal and implementation of targeted national control programs and evidence-based preventive measures. Considering this, the overall objectives of the present thesis were to: i) develop and evaluate a diagnostic protocol for the early and accurate diagnosis of SRLV infections with the combination of serological and molecular tests; ii) investigate the epizootiology of SRLV infections; and iii) quantify the effects of SRLV infections on milk yield and quality traits in representative intensive dairy sheep farms in our country.

In particular, the present study is structured into three Chapters. In the first Chapter, the applied diagnostic protocol is presented, including i) the utilization and evaluation of a commercial ELISA for the serology-based diagnosis of SRLV infections, and ii) the development and evaluation of a molecular diagnostic protocol for the early and accurate diagnosis of circulating SRLV strains in intensively reared purebred Chios and Lacaune sheep.

In the second Chapter, a prospective epizootiological study was conducted in intensively reared ewes and lambs using as diagnostic tools the ELISA test and the real-time PCR protocol described in the first Chapter. The objectives of this study were to: i) calculate morbidity frequency measures for SRLV infections; ii) determine serological patterns; iii) reveal potential risk factors associated with the SRLV infections, the manifestation of specific serological patterns, and the occurrence of seroconversion/seroreversion incidents; and iv) evaluate transmission dynamics and the significance of horizontal and vertical transmission.

The third Chapter, presents a prospective study for the assessment of the effects of SRLV infections on i) milk yield and milk quality traits [fat-, protein-, lactose-, solids-not-fat-yield, somatic cell counts (SCC)], and ii) health and welfare status in dairy ewes.

Part A: Small ruminant lentiviruses-Introduction

Ovine progressive pneumonia (OPP), also known as maedi-visna (MV), is an incurable viral disease of sheep with a very long incubation period that leads to life-long infection (Cutlip *et al.*, 1988; Blacklaws, 2012). It is caused by non-oncogenic exogenous retroviruses, namely, maedi-visna virus (MVV) and caprine arthritis-encephalitis virus (CAEV), both belonging to a subgroup of viruses of the family *Retroviridae* known as small ruminant lentiviruses (SRLV) (Gomez-Lucia, Barquero and Domenech, 2018). Chronically infected sheep may develop pneumonia, mastitis, arthritis, encephalitis, and progressive emaciation, leading even to death (Pépin *et al.*, 1998; Minguijón *et al.*, 2015).

The impact of SRLV infections on farm sustainability is associated with reduced milk production (Leitner *et al.*, 2010; Martínez-Navalón *et al.*, 2013; Echeverría *et al.*, 2020; Juste *et al.*, 2020), impaired lamb growth (Pekelder *et al.*, 1994; Keen *et al.*, 1997; Arsenault *et al.*, 2003; Huttner, Heyne and Heim, 2017), and increased replacement rate due to severe clinical manifestation of the disease or even death of the infected animals (Benavides *et al.*, 2013). Currently, SRLV have a global spread and the World Organization for Animal Health (WOAH) has included them in the list of notifiable terrestrial and aquatic animal diseases, with a significant impact on the international trade of animals and their products (WOAH, 2023). Preventive measures against their transmission between and within the farms proved ineffective to various degrees. This is associated with an evident lack of updated epizootiological data and a "gold standard" assay for the early diagnosis of SRLV infections, which renders the development of efficient control strategies a challenging endeavor.

I. History of small ruminant lentiviruses

Initially, OPP was described in South Africa in 1915 and in Montana, USA, in 1923 (Cutlip *et al.*, 1988; Brodie *et al.*, 1998). Later, OPP was reported in Iceland in 1939, possibly originating from the importation of Karakul sheep from Germany in 1933 (Brodie *et al.*, 1998; Straub, 2004). Two discrete diseases were initially described, namely maedi and visna, from the Icelandic words used for dyspnea and shrinking, respectively. Finally, maedi and visna were attributed to the same infectious viral agent, and the term maedi-visna was established worldwide (Straub, 2004). Maedi-visna virus was the first member of the genus *Lentivirus*, which derived its name from the long latent period and the slow progression of the infections caused by this category of viruses, as lentus means slow in Latin. Eventually, SRLV were transmitted among several countries through the trading of breeding stocks [e.g., Denmark (1968), Canada (1970), Hungary (1972), France (1976), Norway (1979), and

Finland (1994)] (Blacklaws *et al.*, 2004). Nowadays, they have a worldwide spread, with the exception of Iceland, New Zealand, and Australia, which are considered MVV-free (but not CAEV-free) regions (OIE, 2018).

II. Phylogeny of small ruminant lentiviruses

Currently, five genotypes of SRLV, namely A, B, C, D, and E, have been identified in sheep and goats (Shah *et al.*, 2004; Grego *et al.*, 2007). Genotypes A (A1-A22 subtypes) and B (B1-B5 subtypes) have worldwide distribution and consist of MVV and CAEV strains, respectively (Ramírez *et al.*, 2013; Santry *et al.*, 2013; Michiels, Adjadj and De Regge, 2020; Molaee *et al.*, 2020). Genotype C has been isolated in sheep and goats from Norway (Gjerset, Storset and Rimstad, 2006; Gjerset *et al.*, 2009), genotype D in sheep and goats from Spain and Switzerland (Shah *et al.*, 2004; Reina *et al.*, 2006), and genotype E (E1 and E2 subtypes) in goats from Italy (Grego *et al.*, 2007). Nevertheless, SRLV have genetic variants/strains that can infect both sheep and goats (Leroux, Cruz and Mornex, 2010; Blacklaws, 2012) and species-specific categorization of SRLV is not always valid since cross-species transmission of certain genotypes is evident, with the direction of transmission not always apparent (Leroux, Cruz and Mornex, 2010).

III. Immunopathogenesis of maedi-visna

Small ruminant lentiviruses present biological and genetic similarities with other animal and human lentiviruses such as the feline immunodeficiency virus (FIV), the equine infectious anemia virus (EIAV), the bovine immunodeficiency-like virus (BIV), Jembrana Disease Virus (JDV), the simian immunodeficiency virus (SIV), and the human immunodeficiency virus (HIV) (Clements and Zink, 1996; Leroux, Cruz and Mornex, 2010). Also, all the lentiviruses infect cells of the host immune system, causing persistent infection and multi-organ diseases with slow evolution leading even to death (Clements and Zink, 1996; Brodie *et al.*, 1998). In particular, SRLV mainly affect the lungs, the mammary gland, the central nervous system, and the joints, although the underlying mechanisms of pathogenesis have not been fully elucidated (Blacklaws, 2012).

The virion of the SRLV consists of two parts: the external envelope and the internal nucleocapsid core (Figure 1a). The envelope is a phospholipid bilayer containing the glycoproteins gp135SU and gp46TM encoded by the viral gene *env* (Figure 1b). Glycoprotein gp135SU facilitates the entry of the viral genome into the host cell through specific cell receptors, and it is both highly immunogenic and variable among genotypes, whereas gp46TM forms a protein channel in the viral envelope and is presented conserved in

various strains and genotypes (Blacklaws, 2012; Gomez-Lucia, Barquero and Domenech, 2018).

The internal core of the virion is composed of an icosahedral capsid that incorporates the nucleoprotein complex with two linear molecules of RNA and the enzymes reverse transcriptase (RT) and integrase (INT). The protein of the internal core capsid is named p25CA and is encoded by the gene *gag*, which also, encodes for the nucleoprotein p14NC and the matrix protein p16MA. The RT and the INT are encoded by the *pol* gene which also, encodes for protease (PRO), ribonuclease H (RNaseH) and deoxyuridine triphosphatase (dUTPase). The RT enzyme transcribes the viral RNA genome into proviral double-stranded DNA, which enters the host cell nucleus and integrates into the host DNA genome through the action of the INT enzyme. The auxiliary viral genes *tat* and *rev* express proteins that facilitate proviral DNA transcription and mRNA expression/splicing, respectively. The role of the auxiliary gene *vif* is not well understood but could play a role in modulating host innate immune responses and the establishment of infection (Clements and Zink, 1996; Brodie *et al.*, 1998; Gomez-Lucia, Barquero and Domenech, 2018).

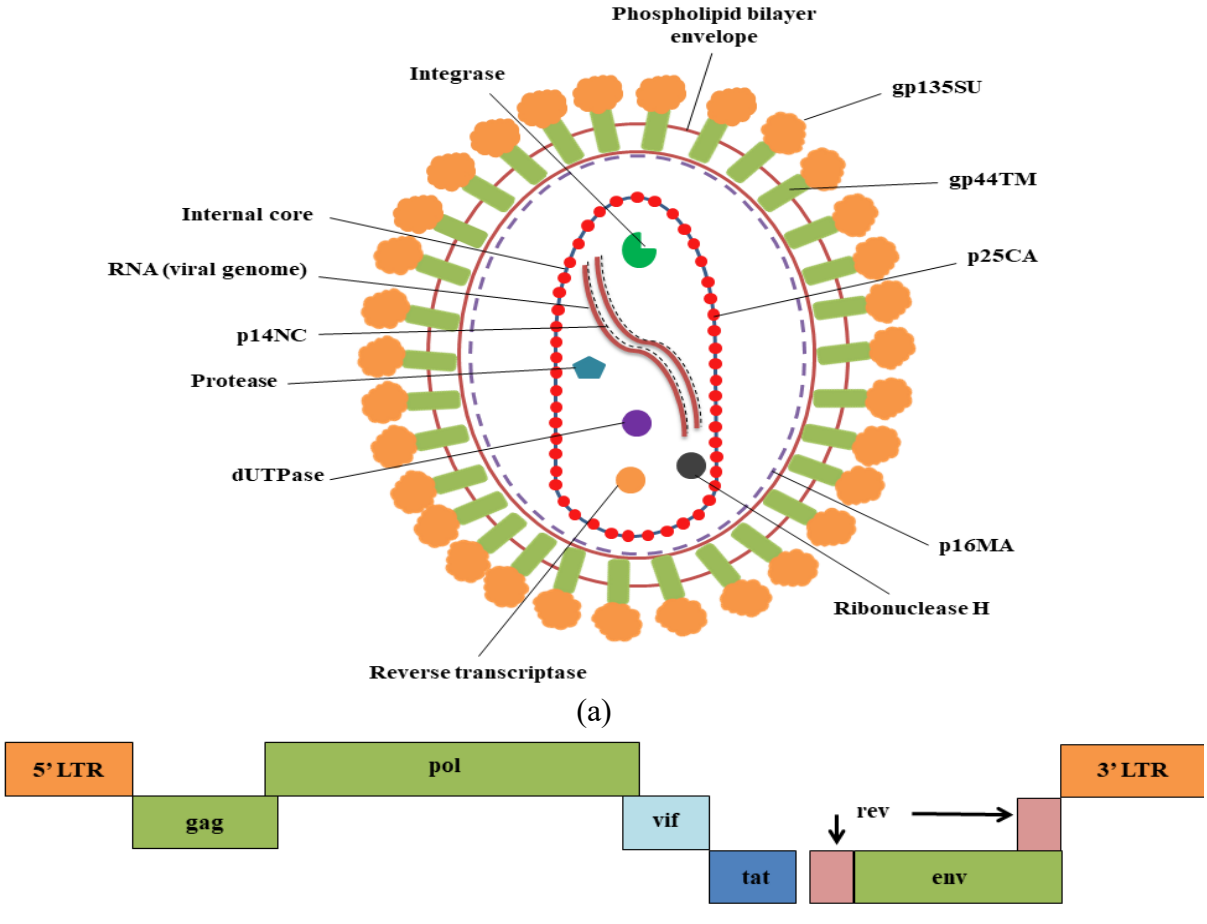


Figure 1. The structure of small ruminant lentivirus particle (a) and genome (b).

Small ruminant lentiviruses show tropism mainly to macrophages and dendritic cells and secondarily to epithelial cells of the mammary gland and endothelial and microglial cells of the central nervous system (Ramírez *et al.*, 2013). The infected dendritic cells transfer the virus to the lymph nodes where the infection of macrophages leads to the systemic infection of the animal (McNeilly *et al.*, 2008). Monocytes are also infected by the virus, but the replication rate is very low until the cells differentiate into macrophages (Blacklaws, 2012; Ramírez *et al.*, 2013). There is controversial evidence suggesting that macrophages can cause life-long infection by entering the bone marrow and infecting progenitor cells of the monocyte lineage (Blacklaws, 2012). The virus enters immature monocytes using cell-specific receptors and spreads in tissues and organs, avoiding immunological response (Ramírez *et al.*, 2013). Recently, a mannose receptor has been identified in sheep as the main viral entry receptor in macrophages and synovial membrane cells (Blacklaws, 2012; Ramírez *et al.*, 2013; Gomez-Lucia, Barquero and Domenech, 2018).

After the integration of the proviral genomic DNA into the cellular DNA, the host develops defense mechanisms to restrict the synthesis of viral RNA (Gomez-Lucia, Barquero and Domenech, 2018). Virus transcription is initiated at the Long Terminal Repeats (LTR), and the process requires the action of viral enzymes expressed by the *pol* gene (Blacklaws, 2012). The binding sites of these enzymes in the nucleotide viral genome are strain-dependent and frequently determine cellular tropism, virulence, and pathogenicity of circulating genotypes (Ramírez *et al.*, 2013).

Small ruminant lentiviruses do not circulate as free virions and do not infect T-cells, which is a major difference from other lentiviruses that cause immunodeficiency (Reina, De Andrés and Amorena, 2013). Nevertheless, they stimulate the production of CD3+ T-cells upon infection; however, the number of T-cells decreases as the infection evolves (Reina, De Andrés and Amorena, 2013). The immune response against SRLV infection is not fully understood, and only indirect evidence exists. This evidence includes the increased presence of T-cells during the early stages of the infection when the number of infected cells and virus replication is low, the greater susceptibility of young animals with an immature immune system, the virus mutations to avoid antibody-mediated immunity, and the necessity of diverting or exhausting the immune response for the progression of the infection (Blacklaws, 2012). Two main theories have emerged in explaining the long-term and persistent infection of SRLV; the first one suggests that the continuous viral antigenic shift in the infected host by mutations of the potential N-linked glycosylation sites (PNG of the viral glycoprotein gp135SU) prevents viral clearance, whereas the second theory is based on evidence showing

isolation of the initial antigenic variants after many years of infection even in the presence of newly emerged mutant variants (Swirski *et al.*, 2009; Arnarson *et al.*, 2017). The latter theory suggests that the virus escapes immune surveillance by modulating the immune response in certain virus reservoir organs, such as the bone marrow and spleen. In these organs, progenitor cells of the monocyte lineage and possibly other hematopoietic stem cells can function as reservoir cells for the virus (Swirski *et al.*, 2009; Arnarson *et al.*, 2017).

IV. Clinical signs and gross pathology of maedi-visna

Small ruminant lentiviruses cause the chronic diseases MV and CAE (Blacklaws, 2012). Chronically infected animals may develop pneumonia (cough and dyspnea), mastitis, arthritis, encephalitis, and progressive emaciation (Minguijón *et al.*, 2015). In the majority of cases, virus replication is slow, and the number of infected blood cells in the circulation is very low (McNeilly *et al.*, 2008). Therefore, the clinical disease is latent or progressive, and in many cases, the clinical signs are not evident or characteristic of the disease at its early stages (Blacklaws, 2012). Immunosuppression of animals due to aging or co-existing diseases and environmental stressors accelerates virus replication, and clinical evidence of the disease becomes apparent. At the flock level, serum detection of antibodies, the severity of clinical disease, and the number of deaths or culled animals, can be affected by management practices and the co-existence of other diseases (Gomez-Lucia, Barquero and Domenech, 2018).

The clinical manifestation of SRLV infection depends on the virus strain, the host immune response, and the host genetic profile regarding resistance or susceptibility to the virus (Reina, De Andrés and Amorena, 2013; Gayo *et al.*, 2018). Lesions of SRLV infection in tissues and organs are caused both by the immune response to the viral antigens and the viral replication itself (Blacklaws, 2012). The cells mainly infected are located at the lungs, the mammary gland, the nervous system, and the joints (Blacklaws, 2012; Minguijón *et al.*, 2015). Pneumonia and mastitis are the predominant clinical manifestations in sheep, and less frequently, lesions such as lymphoid tissue hyperplasia may be apparent in the kidneys, liver, and heart, indicating them as possible target organs (Angelopoulou, Brellou and Vlemmas, 2006; Brellou *et al.*, 2007; Blacklaws, 2012). Multiple-organ infection may be observed in the progression of the disease, but the severity of lesions varies among the affected organs (Minguijón *et al.*, 2015).

Respiratory clinical signs include dyspnea and increased respiratory rate, caused by the characteristic lymphocytic interstitial pneumonia; at necropsy, the lungs appear discolored, enlarged, and diffusely firm with gray spots on the pleural surface, and the mediastinal lymph

nodes are often enlarged (Cutlip *et al.*, 1988; Straub, 2004; Christodoulopoulos, 2006; Minguijón *et al.*, 2015).

Signs from the nervous system include ataxia, paresis, weakness in hind limbs, incoordination, or, in heavier cases, total paralysis due to meningoencephalitis (Straub, 2004; Blacklaws, 2012; Minguijón *et al.*, 2015).

In the mammary gland, SRLV can cause an indurative non-suppurative interstitial mastitis (van der Molen, Vecht and Houwers, 1985; Cutlip *et al.*, 1988; Bolea *et al.*, 2006). The udder is hard but not painful, with decreased milk production mainly noticed the first days postpartum, a situation usually described as "hard udder syndrome" or "hardbag" (Bolea *et al.*, 2006; Minguijón *et al.*, 2015).

Arthritis can also be the outcome of SRLV infection, although it is less common in sheep (Straub, 2004; Christodoulopoulos, 2006; Blacklaws, 2012; Gomez-Lucia, Barquero and Domenech, 2018). The affected joints are usually the carpal and tarsal, but metatarsal, metacarpal, and vertebral joints can also be affected. In advanced arthritis cases, the cartilage is destroyed, and the articular capsule is fibrotic. In the majority of cases, arthritis is progressive, causing lameness and involuntary culling of the animal, whereas less often it may regress (Blacklaws, 2012; Minguijón *et al.*, 2015; Gomez-Lucia, Barquero and Domenech, 2018).

V. Histopathological lesions of maedi-visna

The histopathological analysis of SRLV infected animals reveals characteristic lesions in target organs, which mainly consist of infiltration by mononuclear cells and the formation of lymphoid follicles.

In the lungs, the alveolar walls are thickened due to the interstitial inflammatory infiltration of mononuclear cells and lymphocytes (Georgsson and Pálsson, 1971; Pinczowski *et al.*, 2017). Also, hyperplasia of smooth muscles and peribronchial lymphoid tissue and perivascular fibrosis are observed. In severe cases, total obliteration of alveoli may be noted (Georgsson and Pálsson, 1971; Benavides *et al.*, 2013; Pérez *et al.*, 2015; Pinczowski *et al.*, 2017).

Also, affected joints present infiltration of the synovial membrane and connective tissue by lymphocytes, plasma cells and macrophages which is followed by villous hypertrophy, angiogenesis, and finally fibrosis, mineralization, and necrosis of the synovium and joint capsule (Ravazzolo *et al.*, 2006; Blacklaws, 2012; Pérez *et al.*, 2015; Pinczowski *et al.*, 2017).

In the nervous system, astrocytosis, microgliosis, infiltration of the neuroparenchyma by inflammatory cells, mononuclear perivascular cuffs, and focal secondary demyelination in the brain and the spinal cord are observed (Benavides *et al.*, 2009; Blacklaws, 2012; Ramírez *et al.*, 2012).

In the mammary gland, lymphocytic interstitial infiltration of epithelial cells in gland parenchyma and lymphoid hyperplasia were observed, leading in severe cases to the destruction of the acinar structure and the occurrence of periductal fibrosis (Bolea *et al.*, 2006; Fournier, Campbell and Middleton, 2006; Benavides *et al.*, 2013).

VI. Epizootiology of small ruminant lentiviruses

Currently, SRLV have a worldwide spread, and various prevalence rates have been reported in countries around the world with a developed small ruminant sector (Arsenault *et al.*, 2003; Alba *et al.*, 2008; Hüttner, Seelmann and Feldhusen, 2010; Pérez *et al.*, 2010; Preziuso *et al.*, 2010; Lago *et al.*, 2012; Zhang *et al.*, 2013; Michiels *et al.*, 2018; İnce, 2020; Pazzola *et al.*, 2020). Although the major routes of infection have been identified, there are several factors, either at the animal or at the farm level, that influence the spreading of SRLV within and between the farms and determine the morbidity frequency measures of SRLV infections.

1. Transmission of small ruminant lentiviruses

The mechanisms and significance of horizontal and vertical transmission of SRLV have not yet been fully clarified (Blacklaws *et al.*, 2004). The major routes of transmission have been described; however, their significance and extent remain unclear. This information is critical for the efficient designation of eradication protocols, especially for intensively reared dairy sheep.

The vertical transmission of SRLV refers to the transmission of the virus from the ewe to the lamb during pregnancy (transplacental), at lambing, or during suckling (Peterhans *et al.*, 2004). Results from studies regarding transplacental transmission are controversial; the possibility of horizontal transmission any time post-lambing complicates the assessment of the significance of either transplacental or horizontal transmission, although SRLV have been detected in genital tissues and newborn kids have been found infected (Blacklaws *et al.*, 2004; Peterhans *et al.*, 2004; Broughton-Neiswanger *et al.*, 2010; Cortez-Romero *et al.*, 2011; Araújo *et al.*, 2020). Vertical transmission at lambing refers to the transmission of viruses while the lamb passes through the ewe's genital tract and is exposed to maternal body fluids and blood. The significance of this route of transmission remains unknown and very difficult to be elucidated (Peterhans *et al.*, 2004; Broughton-Neiswanger *et al.*, 2010).

The most significant route of vertical transmission is considered to be the lactogenic, through the ingestion of colostrum and milk from infected dams. Small ruminant lentiviruses show tropism to the epithelial cells of the mammary gland and the resident macrophages, where they can replicate (Bolea *et al.*, 2006). It has been found that isolated lambs fed colostrum or milk from infected ewes seroconverted a few months later, and some of them were diagnosed with clinical disease later in their adult life (Blacklaws *et al.*, 2004). There is evidence that lactogenic transmission is more significant in small ruminants than in primates due to the higher permeability of the digestive tract of small ruminants in the first 24 hours post-lambing, allowing virions and infected cells to be absorbed by the lamb's intestine (Preziuso *et al.*, 2004; Pisoni *et al.*, 2010). However, not all the subgroup variants of SRLV are efficiently transmitted via the lactogenic route, as the envelope varies among the different subgroups, determining some of their physicochemical properties and facilitating or not the lactogenic transmission (Pisoni *et al.*, 2010).

Horizontal transmission of SRLV includes the environmental, mechanical and iatrogenic routes, but mainly refers to transmission through respiratory secretions. Lungs are the main target organs in the respiratory tract. In the lungs, the virus infects monocytes, macrophages, and dendritic cells, and it can be transmitted via secretions containing these cells (Blacklaws *et al.*, 2004; Blacklaws, 2012). In general, the lower respiratory tract constitutes the main route of infection (Blacklaws *et al.*, 2004; McNeilly *et al.*, 2007). This route of transmission is of major importance in intensively reared and permanently housed sheep in sheds with inadequate ventilation and high stocking density (Minguijón *et al.*, 2015), while many researchers claim that airborne transmission could be a more significant route of transmission compared to vertical transmission (Broughton-Neiswanger *et al.*, 2010; Minguijón *et al.*, 2015). For this reason, the segregation of newborn lambs and the separation of uninfected animals from the infected ones are of major importance for the control of SRLV transmission (Blacklaws *et al.*, 2004; Pérez *et al.*, 2013; Villoria *et al.*, 2013). The significance of transmission through contaminated barns, sheds, feeding and water equipment, pastures or reusable veterinary equipment has not yet been fully clarified (Blacklaws *et al.*, 2004; Peterhans *et al.*, 2004). The presence of the virus in the water and air from pens with infected animals indicates that waterborne and airborne transmission on farms cannot be disregarded (Villoria *et al.*, 2013). Infection of dairy sheep via the teat canal during milking has also been reported (Blacklaws *et al.*, 2004).

Sexual transmission is theoretically possible but not yet confirmed. However, there is evidence of virus proliferation in the genitals of infected rams (Cortez-Romero *et al.*, 2013)

and the virus has been found in the semen of rams with leukocytospermia and rams positive for *Brucella ovis* (Preziuso *et al.*, 2003). In another study using real-time PCR (Polymerase chain reaction), proviral DNA of SRLV was found in semen (intermittent shedding) and the genital tract of rams, suggesting possible sexual transmission (Peterson *et al.*, 2008).

2. Risk factors for small ruminant lentiviruses infections

There are several risk factors that influence the transmission of SRLV between and within flocks. These factors determine the likelihood of infection and morbidity frequency measures of the disease. Identification and mitigation of risk factors at the farm level are therefore crucial when establishing a SRLV control or eradication program. Flock size, stocking density, intensification of production (Pérez *et al.*, 2010; Shuaib *et al.*, 2010; Lago *et al.*, 2012; Junkuszew *et al.*, 2016; Michiels *et al.*, 2018), and age distribution (Pérez *et al.*, 2010; Lago *et al.*, 2012; Michiels *et al.*, 2018) affect the likelihood of seropositivity at the flock level, indicating the significant role of horizontal transmission in the epizootiology of the SRLV infections. For example, lower prevalence in extensively reared sheep can be attributed to reduced stocking rates and limited direct contact between animals (Leginagoikoa, *et al.*, 2006a; Leginagoikoa, *et al.*, 2006b), conditions that reduce the exposure to SRLV and the possibility of airborne transmission through respiratory secretions during exhalation, sneezing, and coughing. In flocks where MV co-exists with pulmonary adenomatosis, the transmission is favored by the increased quantities of respiratory secretions produced by the infected sheep (Blacklaws *et al.*, 2004; Pérez *et al.*, 2010). In these cases, late removal of clinical cases and non-isolation of seropositive animals are significant risk factors for the transmission and the increased seroprevalence of the disease.

Inappropriate cleaning and disinfection of milking equipment (Blacklaws *et al.*, 2004; Minguijón *et al.*, 2015), reuse of infected needles and surgical equipment, inadequate hygiene conditions inside the barn, and grazing at common pasturelands are also potential risk factors for the horizontal transmission of SRLV.

Importation of breeding stocks from flocks with unknown SRLV status is associated with increased seroprevalence (Shuaib *et al.*, 2010; Lago *et al.*, 2012). The remarkable absence of certified SRLV-free flocks to produce breeding stocks and the use of seropositive rams for mating or artificial insemination are the main causes. Surprisingly, despite the lactogenic transmission of the virus through colostrum or milk during suckling, a reduced seroprevalence in the replacement stocks has been observed in flocks with an increased suckling period (Pérez *et al.*, 2010). This is possibly the result of a confounding effect of the

farming system; increased weaning age is mainly observed in semi-extensive and extensive systems, where horizontal transmission is limited. On the other hand, early weaning is mainly practiced in intensive systems where virus transmission is facilitated mostly due to the permanent housing, the increased stocking density, and the inappropriate ventilation (Alba *et al.*, 2008; Pérez *et al.*, 2010; Lago *et al.*, 2012). Nevertheless, the use of colostrum or milk from seropositive dams and the natural suckling of newborn animals constitute major risk factors. Also, in mixed-species flocks (sheep and goats), the seroprevalence has been found to be higher, possibly due to cross-species transmission of several SRLV strains (Alba *et al.*, 2008; Lago *et al.*, 2012).

Moreover, the genetic resistance/susceptibility against SRLV infections has been investigated in many breeds. Different alleles of the cellular *TMEM154* (*Transmembrane protein 154*) gene have been found to be associated with the occurrence of SRLV infections. Haplotypes carrying nucleotide sequences coding the amino acid glutamate at position 35 are associated with increased susceptibility, whereas haplotypes carrying nucleotide sequences that code lysine at the same position are associated with resistance (Heaton *et al.*, 2012; Leymaster *et al.*, 2013; Alshanbari *et al.*, 2014). Also, the haplotype responsible for the susceptibility seems to be dominant against the "resistant" haplotype (Leymaster *et al.*, 2013). Although there is indication for an association between *TMEM154* mutations and resistance against SRLV infections, there is no proven association for all the haplotypes (Alshanbari *et al.*, 2014). Other genes investigated for their association with virus susceptibility are the *DPPA2* (*Developmental Pluripotency Associated 2*)/*DPPA4* (*Developmental Pluripotency Associated 4*), *SYTL3* (*Synaptotagmin-Like 3*), *CCR5 (Chemokine receptor 5)*, MHC (Major Histocompatability Complex), *TLR7, TLR8, TLR9* (*Toll-like receptors*) genes, and APOBEC3 (Apolipoprotein B mRNA-editing enzyme) proteins (Larruskain and Jugo, 2013; White and Knowles, 2013; Stonos, Wootton and Karrow, 2014), whereas the zinc finger cluster, *C19orf42* (*Chromosome 19 Open Reading Frame 19*)/*TMEM38A* (*Transmembrane Protein 38A*) and *DLGAP1* (*Discs Large (Drosophila) Homolog-Associated Protein 1*) genes have also been proposed for use in genetic selection programs to facilitate the control of the disease (White *et al.*, 2012). The tripartite motif-containing 5 (TRIM5) protein has been studied and has been proven to contribute to the restriction of SRLV (Jauregui *et al.*, 2012).

VII. Impact of small ruminant lentiviruses infections

Currently, the impact of SRLV infection on small ruminants' productivity has not been sufficiently elucidated, as the available studies have produced contradictory results. In

particular, some of the studies have evidenced an adverse effect of SRLV infections on milk yield in dairy sheep (Echeverría *et al.*, 2020; Juste *et al.*, 2020) and goats (Leitner *et al.*, 2010; Martínez-Navalón *et al.*, 2013), whereas other studies concluded that SRLV infections have not a remarkable adverse effect on it in the same species (Nord and Ådnøy, 1997; Legrottaglie *et al.*, 1999; Turin *et al.*, 2005; Kaba *et al.*, 2012; Barquero, Gomez-Lucia, Arjona, Toural, Las Heras, Fernández-Garayzábal, *et al.*, 2013; Pazzola *et al.*, 2020). Moreover, the effects of SRLV infections on milk quality traits in dairy sheep and goats have not been yet sufficiently estimated; many studies claim an adverse impact on fat, protein, and lactose yields in seropositive ewes and goats (Turin *et al.*, 2005; Leitner *et al.*, 2010; Kaba *et al.*, 2012; Martínez-Navalón *et al.*, 2013; Juste *et al.*, 2020), whereas other studies either have not reported any effect of seropositivity on quality traits (Nord and Ådnøy, 1997; Legrottaglie *et al.*, 1999; Barquero *et al.*, 2013c) or have reported a favorable effect on milk fat (Turin et al., 2005; Echeverría et al., 2020). Although SRLV infections are recognized as a major cause of animal culling and death in infected flocks, their effect on the replacement rate has not yet been assessed (Benavides *et al.*, 2013), while according to a study by Leitner *et al.* (2010) the effect of SRLV on the culling rate is not significant.

Furthermore, SRLV infection has been associated with reduced fertility in ewes (Dohoo *et al.*, 1987), reduced birth or weaning weight of lambs (Pekelder *et al.*, 1994; Keen *et al.*, 1997; Arsenault *et al.*, 2003; Huttner, Heyne and Heim, 2017), and increased lamb mortality (Keen *et al.*, 1997; Arsenault *et al.*, 2003). However, in other studies, subclinical SRLV infection in sheep and goats did not have any adverse effect on lamb and goat kids body weight or wool production (Snowder *et al.*, 1990; Nalbert *et al.*, 2019).

The economic impact of SRLV infections in small ruminant farms is relatively important, as they are associated with direct and indirect economic losses undermining the sustainability of sheep farms. In particular, these losses include: i) the increased culling of animals with clinical disease, which results in an increased replacement rate and cost; ii) the reduction in quantity and quality of the produced milk due to the increased incidence of mastitis; iii) the reduction in average daily gain of lambs during suckling due to the reduced milk yield of ewes with mastitis; iv) the use of chemotherapeutic agents to reduce co-infections; and v) the restrictions in trading of breeding stocks and semen. The magnitude of monetary losses is determined by factors related to the clinical signs, the epizootiology, and the control of the disease at the farm level.

VIII. Diagnosis of small ruminant lentiviruses infections

Considering the lack of efficient treatment or vaccination for SRLV infections, early and accurate diagnosis is paramount for the successful implementation of control programs, the eradication of MV and CAE, and the accreditation of SRLV-free regions and farms. Considering that clinical signs of MV or CAE may not be characteristic or apparent early after the infection, the diagnosis of SRLV infections is mainly based on laboratory methods. The detection of SRLV-specific antibodies with serological tests such as agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation (RIPA), radioimmunoassay (RIA), and western blot (WB), or the detection of viral genome with molecular assays (e.g., PCR, real-time PCR) and virus isolation in cell cultures (OIE, 2018) are considered the most reliable methods for the confirmation of the infection.

Lack of a "gold standard" assay for the early diagnosis of SRLV infections has led to various types and combinations of serological and molecular assays being utilized in eradication programs around the world with variable efficacy (Nord, Løken and Orten, 1998; Sihvonen *et al.*, 2000; Peterhans *et al.*, 2004; Modolo *et al.*, 2009; Synge and Ritchie, 2010; Pérez *et al.*, 2010, 2013; Kaba *et al.*, 2011; Polledo *et al.*, 2013; Michiels *et al.*, 2018; Cirone *et al.*, 2019). The limited success of the currently applied programs to control the disease implies that some of the infected animals evade diagnosis, acting as virus reservoirs for the establishment of re-infections. This situation perpetuates the economic impact of SRLV infections, increases the uncertainty and cost of the invested resources for SRLV eradication, and reduces the willingness of farmers to participate in control programs.

Currently, universally applicable diagnostic tools are not available, and the development of highly sensitive and specific diagnostic protocols is urgent; however, it remains a challenging task due to i) the genetic variability of the different strains of SRLV associated with mutations, recombination, and cross-species transmission, and ii) the peculiarities of small ruminants' humoral immune response regarding late seroconversion, as well as intermittent and epitope-specific antibody production.

1. Clinical and histopathologic diagnosis

Clinical evaluation of the suspected SRLV infected animals may confirm the presence of characteristic clinical manifestations of the disease, such as pneumonia, mastitis, arthritis, and encephalitis. However, in many cases, persistently infected animals remain asymptomatic for years or present adjective clinical signs that cannot be clinically evaluated (Blacklaws, 2012;

OIE, 2018). Also, the histopathological examination of tissues and organs for characteristic lesions is feasible at necropsy and can contribute to the confirmation of SRLV presence. Nevertheless, the histopathological examination is not valuable for the early diagnosis of the infected animals, as it is being performed post-mortem.

2. Laboratory diagnosis

2.1 Serological methods

Regarding serological methods, ELISA has been widely exploited in SRLV control programs for the screening of sheep and goat populations in many countries (e.g., Spain, Netherlands, Italy, Switzerland) (Houwers *et al.*, 1987; Mordasini *et al.*, 2006; Schlup *et al.*, 2009; Pérez *et al.*, 2010; Cardinaux *et al.*, 2013; Tavella *et al.*, 2018; De Martin *et al.*, 2019). Viral capsid and matrix proteins (p25CA, p28CA, p14NC, and p16MA), and envelope glycoproteins (gp135SU, gp46TM) coded by the *gag* and *env* genes, respectively, are commonly used as antigens for the detection of SRLV-specific antibodies (De Andrés *et al.*, 2005; Herrmann-Hoesing, 2010; Gomez-Lucia, Barquero and Domenech, 2018). Despite the fact that its performance is not universally constant, ELISA remains a user-friendly, low-cost, semi-quantitative diagnostic test with sufficient repeatability and, in most cases, sensitivity and specificity (De Andrés *et al.*, 2005; Barquero, Domenech and Gomez-Lucia, 2016). Both the commercially available kits (see Table 1) and the in-house assays belong either to the indirect or to the competitive assay type for the detection of circulating antibodies in infected animals. In the indirect ELISA assays, antigens can be the whole virus, recombinant proteins, or synthetic peptides, whereas in the competitive assays, combinations of monoclonal antibodies are utilized for competition with sera antibodies for the coated viral antigens. Although ELISA is the most commonly used diagnostic test, the scarcity of efficient validation protocols using at least one reference standard method (RIPA or WB), according to the guidelines of WOAH (OIE, 2018), constitutes a major flaw in the process of being officially recognized as valid and reliable screening assays.

A considerable advantage of ELISA when compared to other serological methods is its capability to be applied to various biological samples such as blood serum, plasma, and milk (Mazzei *et al.*, 2005; Plaza *et al.*, 2009; Brinkhof *et al.*, 2010; Barquero *et al.*, 2011; Barquero *et al.*, 2013a; Barquero *et al.*, 2013b; Adjadj *et al.*, 2019; Potărniche *et al.*, 2021). Among these samples, milk seems to be the most ambiguous sample matrix given that several factors may adversely affect the reliable diagnosis, such as the progressive reduction of antibodies throughout the lactation, the occurrence of false positive background signals in cases of

mastitis, colostrum, increased milk fat content, or even the specific immune response of the mammary gland depending on the infection stage (Barquero *et al.*, 2013a; Adjadj *et al.*, 2019). Enzyme-linked immunosorbent assays (ELISAs) fluctuate between high sensitivity and low specificity and *vice versa*; for example, the high sensitivity of competitive ELISAs due to the use of undiluted sera is usually combined with low specificity (Herrmann *et al.*, 2003; Herrmann-Hoesing, 2010). In general, the unsatisfactory diagnostic performance of ELISA is mainly attributed to: i) the unfavorable combination of the test's antigen and the infection stage of the tested animal, as the production of antibodies against matrix and capsid proteins (e.g., p25, p28, and p16) during early infection stages precedes the production of other antibodies, while it is almost eliminated at later stages of the infection, where antibodies against gp46 and gp135 prevail (Lacerenza *et al.*, 2006; Sardi *et al.*, 2012; Michiels *et al.*, 2018), ii) the antigenic distance between the viral strain used in the development of the assay and the infecting strain of the examined animals; although SRLV strains are characterized by cross-reactivity (De Andrés *et al.*, 2013; Sanjosé *et al.*, 2015), homologous humoral immune response in strain-specific epitopes reduces dramatically the sensitivity of ELISA test, leading to misdiagnosis (Lacerenza *et al.*, 2006; Reina *et al.*, 2009; Cardinaux *et al.*, 2013; Nogarol *et al.*, 2019), iii) the late seroconversion of animals, the fluctuation of antibody response during animal's life and the alternations between viremia and humoral immune responses (De Andrés *et al.*, 2005; Leginagoikoa *et al.*, 2009; Barquero *et al.*, 2013a; Kalogianni *et al.*, 2020), and iv) the animal species; in goats, a more robust reactivity against transmembrane glycoproteins compared to capsid proteins has been observed (Brinkhof and Van Maanen, 2007; Cardinaux *et al.*, 2013). Therefore, except for the impediments arising from virus nature and immunopathological mechanisms, a critical endeavor for the enhancement of serological diagnostic performance is to enrich the antigenic design of ELISA and improve its negative predictive value. The use of whole virus, incorporation of multiple antigens and synthetic peptide combinations, and genotype-specific immunodominant epitopes have been proposed for the extension of the antigenic spectrum and the amplification of the detection capacity of the assay (Lacerenza *et al.*, 2006; Ramses Reina *et al.*, 2009; Sardi *et al.*, 2012; De Andrés *et al.*, 2013; Sanjosé *et al.*, 2015; Echeverría *et al.*, 2020; Ramírez *et al.*, 2021).

Table 1. Commercially available ELISA kits used for the diagnosis of SRLV infections (Kalogianni *et al.*, 2021).

Commercial Kit Product Name	ELISA Format	Antigen	Sample/ Diagnostic matrix	Se/Sp	Reference Test	Ref
LSIVet™ Ruminant Maedi-Visna/CAEV serum ELISA kit (LSI, Thermo Fisher Scientific, Waltham, MA, USA)	Competitive	gp135 TM protein/A and B genotypes	Serum	90.2%[a]/92.8%[a] 100.0%[b]/85.7%[b]	qPCR	(Michiels *et al.*, 2018)
ID screen® MVV/CAEV indirect (IDvet Innovative Diagnostics, Grabels, France)	Indirect	peptides from the MVV/CAEV, gp135 and p25 proteins/A, B and E genotypes	Serum, plasma and milk	100.0%[a]/97.8%[a] 91.7%-100.0%[b]/97.6-98.9%[b]	qPCR, ELISA[A,B]	(Nowicka *et al.*, 2014; Michiels *et al.*, 2018)
Eradikit™ SRLV screening test (IN3 diagnostic, Italia)	Indirect	*gag* and *env* peptides/A, B and E genotypes	Serum, plasma and milk	96.1%[a]/99.4%[a] 100.0%[b]/94.6%[b]	qPCR	(Michiels *et al.*, 2018)
Elitest MVV/CAEV (Hyphen BioMed, Neuville-sur-Oise, France) or Innotest MVV (Innogenetics, Gent, Belgium)	Indirect	MVV capsid rp25 and gp46 TM protein/EV-1 strain, A genotype	Serum	98.0, 96.9, 97.8, 99.3%[a]/94.7, 99.2, 98.2, 99.4%[a] 95.8%[b]/99.7%[b]	qPCR, Bayesian analysis, AGID and WB	(Saman *et al.*, 1999; Varea *et al.*, 2001; Toft *et al.*, 2007; Michiels *et al.*, 2018)
MVV/CAEV p28 Ab Screening Test (Idexx, Westbrook, ME, USA)	Indirect	peptide of TM protein (*env* gene) and of the recombinant p28 capsid protein/A genotype	Serum and plasma	84.3% [a]/99.6%[a] 91.7%[b]/100.0%[b]	qPCR	(Michiels *et al.*, 2018)
ELISA MAEDI VISNA/CAEV (Institut Pourquier, Montpellier, France)*	Indirect	recombinant p28 *gag* protein and peptide of the *env* protein (gp135)/A genotype	Serum	98%[a]/97.4%[a]	Bayesian analysis	(Toft *et al.*, 2007)
CAEV/MVV Total Ab Test (Idexx, Westbrook, ME, USA) or Checkit CAEV/MVV (Dr. Bommeli AG, Bern, Switzerland)	Indirect	Whole virus/strain OLV, A gentoype	Serum, plasma and milk	98.6%[b]/99.3%[b] 91.4%[c]/98.9%[c]	GAG-GST ELISA**	(Zanoni *et al.*, 1994)
Small Ruminant Lentivirus Antibody Test Kit, cELISA (VMRD, Pullman, WA)	Competitive	SU Antigen of gp135/B genotype	Serum	98.6%[a]/96.9%[a] 100%[b]/96.4%[b]	RIPA	(Herrmann *et al.*, 2003a; Herrmann *et al.*, 2003b)
INgezim Maedi screening™ (Ingenasa, Eurofins Technologies, Spain)	Indirect	synthetic peptides from the *env* protein/ A and B genotypes	Serum	No published data		na
Enferplex Goat/Sheep Multi-Disease 5D (Enfer Scientific, Co. Kildare, Ireland)	Indirect	recombinant p25 core protein, TM1 gp46 synthetic peptide	Serum, plasma and milk	No published data		na

ELISA: Enzyme-linked Immunosorbent Assay ; Se: sensitivity; Sp: specificity; [a]Sensitivity and specificity values for sheep; [b]Sensitivity and specificity values for goats; [c]Sensitivity and specificity values for milk samples; gp: glycoprotein; TM: transmembrane; Ref: reference; na: not available; *before merge of Institute Pourquier by Idexx Laboratories in 2007; **recombinant GAG (group-specific antigens)-GST (glutathione S-transferase) fusion protein expressed in *E. coli*; [A]: Checkit CAEV/MVV monophasic Dr. Bommeli AG, Bern, Switzerland; [B]: ELISA MAEDI VISNA/CAEV Institut Pourquier, Montpellier, France.

Radioimmunoprecipitation, RIA and WB are usually used as "gold standard" methods. Radioimmunoprecipitation and RIA rely on the conformation of antibody-epitope complexes like in the AGID method; however, in these assays, the antigens (RIPA) and the antibodies (RIA) are ^{35}S-labelled, increasing their sensitivity (Reina *et al.*, 2009; Herrmann-Hoesing, 2010). Western Blot uses viral antigens, usually whole virus, which are separated in reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gels, transferred to nitrocellulose membranes, and subsequently incubated with animal sera potentially containing antibodies that recognize and bind to the separated viral antigens (Herrmann-Hoesing, 2010; Barquero, Domenech and Gomez-Lucia, 2016). The denaturing conditions of WB, instead of the native conditions in RIPA and AGID, favor the detection of specific antibodies binding to linear epitopes of CA, MA, and TM proteins (Herrmann-Hoesing, 2010; De Martin *et al.*, 2019). Despite their high sensitivity and specificity, RIPA, RIA, and WB are not suitable for use in large-scale surveillance programs, but they are rather exploited as reference tests since they are costly and time-consuming assays applied in specialized diagnostic laboratories by trained staff (De Andrés *et al.*, 2005; Brinkhof *et al.*, 2010). However, a WB technique (MVV strain ZZV 1050) has been used in the national MV control programs in the Netherlands and Switzerland as a confirmatory method for ELISA positive samples (Houwers *et al.*, 1987; De Martin *et al.*, 2019). Nevertheless, the use of RIPA, RIA, and WB for the validation of new diagnostic tests or for the confirmation of ELISA results should not be considered *a priori* infallible, as both false positive results (due to nonspecific cross-reactivity) and false negative results (due to weak affinity of circulating antibodies with epitopes of viral antigens) have been reported (De Andrés *et al.*, 2005).

2.2 Molecular methods

Proviral DNA of SRLV can be detected in samples of peripheral blood, colostrum and milk, bronchoalveolar fluid and lungs, mammary gland, carpal synovial membranes, brain, and other secondary tissue targets such as bone marrow, spleen, lymph nodes, testicles, ovaries, uterus, heart, kidneys, and liver (Leroux *et al.*, 1997; Extramiana *et al.*, 2002; Angelopoulou, Brellou and Vlemmas, 2006; Brellou *et al.*, 2007; Peterson *et al.*, 2008; Leginagoikoa *et al.*, 2009; Herrmann-Hoesing, 2010; Barquero *et al.*, 2011; Sardi *et al.*, 2012; Barquero *et al.*, 2013a; Pinczowski *et al.*, 2017; Singh *et al.*, 2017; Adjadj *et al.*, 2019; Potărniche *et al.*, 2021). In particular, the LTR of proviral DNA and conserved regions in the *pol*, *gag*, and *env* genes are used as targets for molecular diagnosis (De Andrés *et al.*, 2005; Herrmann-Hoesing, 2010; Gomez-Lucia, Barquero and Domenech, 2018). The presence of SRLV genetic material

has also been reported in air and water samples collected from sheep farms, highlighting the potential for horizontal transmission of SRLV (Villoria *et al.*, 2013). After the development of the first successful PCR protocol applied for the detection of CAEV and MVV (Zanoni, Pauli and Peterhans, 1990), remarkable progress has been made, resulting in more sophisticated and reliable molecular diagnostic protocols. Except for conventional PCR, other PCR techniques have been developed to improve the sensitivity, specificity, and accuracy of molecular diagnostics. Indeed, combinations of PCRs for different genomic regions, multiplex PCRs, (semi-)nested PCRs, and real-time PCRs have been exploited with contradictory results. For the application of PCR, DNA is extracted mainly from peripheral blood leucocytes (PBLs) or mononuclear cells (PBMCs) or milk cells, while DNA extraction from tissues is less frequent for confirmatory purposes. The possibility of detecting viral RNA by applying reverse transcription PCR is low, as circulating cell-free virions are usually non-detectable; however, it has been proven useful for the confirmation of horizontal virus transmission (Leginagoikoa *et al.*, 2009; Herrmann-Hoesing, 2010; Barquero, Domenech and Gomez-Lucia, 2016). On a routine basis, genomic DNA is extracted either by commercial kits or via in-house methods from PBLs or PBMCs, as monocytes/macrophages and dendritic cells are the only cells known to support replication of SRLV. Major determinants for the selection of a DNA extraction protocol are the time required, yield and quality of the extracted DNA (Psifidi, Dovas and Banos, 2010).

The major advantage of PCR technologies compared to serological methods is the early detection of the SRLV infection, preceding the production of antibodies, which may occur much later (Ramírez *et al.*, 2013). Nonetheless, the low viral load of infected animals may hinder the detection of proviral DNA, resulting in false negative results and reduced sensitivity (Reina *et al.*, 2009). A decreased viral load is indicative of a low number of infected monocytes (Zhang *et al.*, 2000; Blacklaws, 2012) or restricted viral replication due to the humoral immune response, which has been hypothesized to act protectively for the infected animals (Blacklaws, 2012; Georgsson *et al.*, 2015). Moreover, the high mutation rate of SRLV due to the low fidelity of the virion's reverse transcriptase and the frequently observed recombination events (Minguijón *et al.*, 2015; Highland, 2017) undermine the diagnostic performance of PCR. To achieve sufficient specificity, the primers have to be designed for conserved regions of the viral genome, avoiding the *env* gene, which is less conserved among genotypes (Zanoni *et al.*, 1992; De Andrés *et al.*, 2005). On the other hand, the problem of virus genetic variability can be addressed by the use of degenerate primers, which expands the detection range and improves the sensitivity of the method (Eltahir *et al.*,

2006; Dolfini *et al.*, 2015; Chassalevris *et al.*, 2020). Although the development of universally applicable PCR assays may be extremely difficult due to the aforementioned obstacles, evidence-based modification of the protocols for the detection of local strains could be a realistic target in the field of SRLV diagnostics. This is a necessary step when planning SRLV surveillance programs, demanding i) sequencing, phylogenetic analyses, and genotyping of the relevant strains; ii) designation of specific and widely applicable primers; and iii) the development of sensitive and specific PCR protocols with the potential and capacity to be applied in a specific geographical region (with available specialized laboratory infrastructure, equipment, and staff).

Heteroduplex mobility assay (HMA) usually follows PCR amplification for the classification of the detected strains in comparison to the reference strains and for the assessment of the homogeneity of strains detected in a region or a flock (Germain and Valas, 2006; Pisoni, Bertoni, *et al.*, 2007; Pisoni, Moroni, *et al.*, 2007; Germain, Croise and Valas, 2008; Olech *et al.*, 2012). It is a qualitative technique and a valuable tool to study the molecular epidemiology of SRLV. In addition, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification lateral flow dipstick (RPA-LFD) techniques have been lately applied with success for CAEV diagnosis (Huang *et al.*, 2012; Balbin *et al.*, 2014; Tu *et al.*, 2017). Although results seem promising when compared to "traditional" serological and molecular techniques, more studies are needed for the validation of the diagnostic performance of these innovative techniques in a wider spectrum of viral strains. Also, newly developed technologies used in HIV diagnosis, such as specific antibody-antigen biomarkers or dried-blood spot testing (Pai *et al.*, 2020) could be exploited in combination with LAMP and RPA-LFD techniques on SRLV diagnosis for the development of *in situ*, rapid, user-friendly, cost-effective, and reliable diagnostic tools. In the future, point-of-care (POC) testing of small ruminant infectious diseases using mobile platform technologies could integrate SRLV diagnostic assays, contributing to the control and elimination of critical epidemic and endemic diseases, including MV and CAE.

2.3 Cell Cultures

Small ruminant lentivirus isolation can be achieved through co-cultures of PBMCs with sheep choroid plexus cells or goat synovial membrane cells (OIE, 2018). The evidence of SRLV infection is co-evaluated by the existence of a cytopathic effect and a positive reverse transcriptase activity assay (Barquero, Domenech and Gomez-Lucia, 2016). However, the expected cytopathic effect, which is the formation of syncytia and/or refractile stellate cells

with dendritic processes, may be difficult to detect for inexperienced staff with limited training in microscopy and cell biology. In addition, strain variability regarding the extent of detectable cytopathic effect cannot be excluded (Reina *et al.*, 2009). It is obvious that cell cultures cannot be routinely used for the diagnosis of SRLV infections given the increased cost, the complexity, the limitations derived from *in vitro* viral replication, and the demands for specialized laboratory and trained personnel. Therefore, cell cultures are mainly applied either for the verification of the results of other molecular diagnostics or for research purposes in the fields of immunopathology, SRLV genetics and molecular epidemiology (Barros, Andrésdóttir and Fevereiro, 2005; Singh, McConnell and Blacklaws, 2006; Cardinaux *et al.*, 2013; Colitti *et al.*, 2019; De Martin *et al.*, 2019).

IX. Prevention and control of small ruminant lentiviruses infections

1. Vaccination

There is neither a treatment nor an effective vaccine against SRLV infections. In the previous years, there have been attempts to develop attenuated and subunit vaccines, but none of them proved effective in preventing viral infections (Cheevers *et al.*, 1994; Pétursson *et al.*, 2005; Torsteinsdóttir *et al.*, 2007; De Andrés *et al.*, 2009)(Cheevers *et al.*, 1994; Pétursson *et al.*, 2005; Torsteinsdóttir *et al.*, 2007; De Andrés *et al.*, 2009). The major obstacles to the development of an effective vaccine include the necessity for the induction of high antibody titers against SRLV, the wide genetic variation of viral strains and their continuous mutations, the increased post-infection immunological reaction, the post-vaccination challenge on the immune system, and the evidence that vaccination could facilitate a possible infection rather than protect against it (Reina, De Andrés and Amorena, 2013).

Current research efforts for the development of an effective vaccine include pseudoviruses/viral particles, recombinant viruses carrying genes from SRLV, and naked plasmids carrying SRLV genes, plus factors enhancing innate immune responses. However, the effectiveness of these alternative strategies has not been sufficiently validated and thus considered inappropriate for commercial use (Reina *et al.*, 2009; Reina, De Andrés and Amorena, 2013).

2. Preventive and eradication measures

After the first eradication program in Iceland, many countries applied their own eradication programs (almost all European countries and Canada) with various results. The major obstacles for the successful implementation of eradication programs are: i) the voluntary participation of the farmers (Peterhans *et al.*, 2004; Pérez *et al.*, 2010); ii) the breed

variability associated with susceptibility and resistance against the disease; iii) the genetic variability of the viral strains and the different epizootiological characteristics of the disease (virulence, transmission, seroconversion, seroprevalence at flock level, etc.); and iv) the heterogeneous farming and herd health management systems. For this reason, the eradication program needs to be adjusted and optimized according to the afore-mentioned factors.

The control programs, when considered on a country-wide scale, should include both species of small ruminants due to the fact that the cross-transmission has been proven and is a significant risk factor in the spread of the virus. Moreover, the existence of reference laboratories for the control of SRLV is of major importance for the surveillance of SRLV at the national level and the coordination of all the efforts for the elimination of the infections (Peterhans *et al.*, 2004).

The preventive measures and management interventions that could aid in controlling or eradicating SRLV should be decided on case-by-case basis and include the following:

1. Frequent blood sampling (annual or biannual based on SRLV prevalence on the farm) from the breeding stocks and serological and molecular testing for the diagnosis of the infected animals.
2. Post-lambing management primarily based on the application of artificial suckling and the use of colostrum and milk substitutes or pasteurized colostrum/milk (56 ^{o}C for 60 min) (Peterhans *et al.*, 2004; Reina *et al.*, 2009; Seyoum *et al.*, 2011; Polledo *et al.*, 2013).
3. Selective culling and replacement or separation of animals with apparent clinical signs and positive laboratory diagnosis regarding the SRLV prevalence rate in the farm (Peterhans *et al.*, 2004; Reina *et al.*, 2009; Pérez *et al.*, 2013).
4. Keeping the replacement animals post-weaning, in a separate place to avoid horizontal transmission of SRLV through contact with adult animals of the remaining flock (Polledo *et al.*, 2013).
5. Should any animal purchase be made, it should be from certified SRLV-free farms. Imported animals need to remain in quarantine until the SRLV status is determined using the most appropriate assays.
6. Regular cleaning and disinfection of facilities and equipment with appropriate disinfectants. The cleaning and disinfection schedule must include the barn (floor, walls, bedding), the milking machine, the feeders, and the waterers.
7. Reduction of stocking density and adequate ventilation.

8. Implementation of general good hygiene practices. Use of disposable needles or sterilization of metal needles before their reuse is necessary. Similarly, medical equipment should be sterilized after use.

9. Seronegative milking ewes should be grouped separately and machine-milked before the seropositive ones.

10. Grazing in communal pastures and sharing of infrastructure and equipment should be avoided when the SRLV status of the flocks is unknown.

11. Rams used either for mating or for semen collection need to be SRLV-free. Currently, attempts are being made to produce SRLV-free breeding stocks via reproductive biotechnologies like artificial insemination and embryo transfer, even from infected males and females, respectively. In the case of embryo transfer, this may be possible via the removal of cumulus oophorus cells (Cortez-Romero *et al.*, 2013).

12. Breeding for resistance could also be considered, but universally accepted resistant genotypes are yet to be found.

Therefore, the implementation of a control program should include the following prerequisites regarding the infrastructure, the equipment, and the consumables:

1. Artificial suckling machine, milk substitute, and isolated area from adult animals for lamb rearing.

2. Machine for pasteurization of colostrum or colostrum substitute.

3. Sufficient area and volume per animal and adequate ventilation (natural or artificial) of the farming facilities.

4. Existence of separate sheds for the appropriate grouping of animals according to their infection status.

5. Existence of a quarantine pen for the imported animals until their testing for SRLV infection.

6. Disinfectants for regular cleaning and disinfection of facilities and equipment.

Part B: Our study

Chapter 1: Development of a diagnostic protocol for the detection of small ruminant lentiviruses infections

I. Objectives

Diagnosis of SRLV infections constitutes the cornerstone for the successful implementation of eradication programs. A "gold standard" test with high values of sensitivity, specificity and accuracy, blindly used in every case does not seem readily feasible when considering the special characteristics of SRLV (i.e., high genetic variability, mechanisms of virus replication, and animal humoral immune response). Nonetheless, the scientific community tries to address these limitations, proposing targeted combinations of diagnostic tools, which are constantly evaluated to reduce the possibility of both newly or persistently infected animals to evade diagnosis (Extramiana *et al.*, 2002; Karanikolaou *et al.*, 2005; Brinkhof *et al.*, 2008; De Regge and Cay, 2013; Michiels *et al.*, 2018; De Martin *et al.*, 2019; Echeverría *et al.*, 2020; Ramírez *et al.*, 2021). Although combination of diagnostics increases cost, time, and effort required, it seems to be inevitable for the early and safe diagnosis in young animals which are likely infected but seronegative. However, in lambs early diagnosis may be limited by interference of maternal antibodies or provirus transmitted during suckling (Herrmann-Hoesing, Palmer and Knowles, 2007). At the same time, genotyping and classification of the circulating SRLV strains in a specific region/breed could permit the targeted application of appropriate serological and molecular tests.

Early and effective diagnosis of SRLV and subsequently the control of MV and CAE are both critical endeavors for countries with a developed small ruminant farming sector. Therefore, linking of the epizootiological characteristics of the disease with the investigation for novel and more efficient diagnostic techniques can ensure an integrated approach for the control of the disease in practice.

The effective control of the diseases can drastically reduce monetary losses associated with the detrimental effects on health, welfare, and productivity of animals, while early diagnosis will facilitate for the first time the large-scale production of certified SRLV-free breeding stocks, enjoying the expected added-value.

The objectives of the Chapter 1 of the present study were: i) the utilization and evaluation of an existed ELISA for serology-based diagnosis of SRLV infections, and ii) the development and evaluation of a molecular diagnostic protocol for the early and accurate diagnosis of

infected animals based on the circulating genotypes in intensively reared purebred Chios and Lacaune sheep in Greece. This diagnostic investigation was prerequisite for the subsequent epizootiological study and the assessment of the effects of SRLV infections on health and productivity in intensively reared dairy sheep in Greece which will be described in the following Chapters.

II. Materials and Methods

1. Animals and blood sample collection

Four intensive dairy sheep farms with purebred Chios (farm A, B, and C) and Lacaune sheep (farm A and farm D), located at different counties of Greece, were selected and enrolled in the study. The farm and animal selection is described in detail in Chapter 2. In brief, from these farms, a total of 660 ewes and 195 lambs were randomly selected and prospectively studied. For each individual studied animal, a serum and a whole blood sample (~9ml each) were collected from jugular vein in clot activator and EDTA-anticoagulated tubes, respectively. Blood samples were transferred under 4 °C in the lab where they were further processed for the serological and molecular analysis. The protocol of this study was approved by the Animal Research Ethics Committee of the Agricultural University of Athens and was in accordance with the national animal welfare regulations.

2. Blood sample processing

2.1 Serum separation

Blood samples collected in clot activator tubes were centrifuged at 3,000 × *g* for 10 minutes. The serum was separated in 2 ml microcentrifuge tubes and was used for ELISA testing.

2.2 Leukocyte pellets and DNA processing

Leukocyte pellets were isolated from whole blood samples. A total of 2 ml blood was mixed with 13 ml of ACK lysis buffer (8.02 g NH_4Cl, 0.84 g $NaHCO_3$, and 0.37 g EDTA per litre, pH 7.2-7.4) in a Falcon™ 15 ml conical centrifuge tube. After 15 minutes of incubation at room temperature, the mixtures were centrifuged at 450 × *g* for 15 minutes. The supernatant was discarded, the leukocyte pellets were diluted in 1 ml ACK lysis buffer and were transferred in 2 ml microcentrifuge tubes. The mixtures were centrifuged at 450 × *g* for 4 minutes and the supernatant was discarded. The leukocyte pellets were resuspended in 1 ml PBS (phosphate buffered saline, pH = 7.4), and were centrifuged at 650 × *g* for 4 minutes. The leukocyte pellets were used for the genomic DNA extraction with a commercial kit (PureLink® Genomic DNA Kit, Life technologies corp.), according to the manufacturer's

instructions. The procedure of DNA extraction was based on the selective binding of DNA to silica-based membrane in the presence of chaotropic salts (ethanol and guanidinium hydrochloride). DNA concentration and purity were measured in a spectrophotometer (Quawell 5000) considering A_{260}/A_{280} and A_{260}/A_{230} ratios. DNA samples were stored at -20 ^{o}C until use.

3. ELISA testing

The serum separated from blood samples was used for the detection of anti-SRLV antibodies with an indirect whole virus (OLV 130/91 strain/A genotype) commercial ELISA test (ELISA, CAEV/MVV Total Ab Test, IDEXX). The analytical sensitivity and specificity values of the ELISA test were 95.5% and 97.2%, respectively when compared to a recombinant GAG (group-specific antigens)-GST (glutathione S-transferase) fusion protein expressed in *E. coli* ELISA (Zanoni *et al.*, 1994). Absorbance values of the ELISA test were measured using a microplate reader (Multiscan FC, Thermoscientific, Singapore) operating at 450 nm. Relative optical density (OD) values of ELISA were calculated using the formula:

$$\text{Relative OD value} = 100 \times (OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control})$$

According to the manufacturer's instruction indicated for sheep sera, samples were considered positive when relative OD values were >60% and suspect when relative OD values were between 50% and 60%. In our study, suspect results were considered as seropositive, and the cut-off value was set at 50%.

4. Assessment of the ELISA and the conventional PCR protocols

For the assessment of the ELISA and the conventional PCR protocols with regard to their diagnostic performance of SRLV infections, a subtotal of 80 adult dairy sheep from the four farms enrolled in the study were randomly selected (20 animals from each farm). Thirteen different set of primers (Table 2) were used in five nested and three simple conventional PCR protocols for the amplification of fragments in the *pol*, *gag*, *env*, and LTR regions of SRLV genome in DNA samples. Cycling conditions (Table 3) and PCR components were optimized according to the relevant literature and the trials conducted in the laboratory. The final PCR reaction volume was 25 µl and contained 2.5 µl DNA sample (100-200 ng), 0.6 µl of 10 µM forward and 0.6 µl of 10 µM reverse primer (240 nM of each primer), 12.5 µl of OneTaq 2X Master Mix (New England, Biolabs Inc., Hitchin, UK), and 8.8 µl DNase-free water. Nested amplifications were carried out with the same components and 1.5 µl from PCR amplicons from the first round. All amplifications were carried out in a thermal cycler (Labcycler,

Sensoquest GmbH, Germany) in duplicate and a negative control reaction with DNase-free water instead of DNA template was used each time to determine any possible nucleic acid contamination. All PCR products were mixed with DNA Gel Loading Dye (Invitrogen, Vilnius, Lithuania) and were electrophoresed in 1.5% agarose gel in 1× RNase-free TAE buffer (Invitrogen, Vilnius, Lithuania) stained with SYBRsafe DNA gel staining (Invitrogen, Carlsbad, USA) and visualized under ultraviolet (UV) light. For the evaluation of the amplicon length a 100bp ladder was used (GeneRuler 100 bp DNA Ladder, Thermoscientific, Vilnius, Lithuania).

Table 2. Primer sets used in conventional PCR for the detection of SRLV infections.

Primer set	Primers Sequence (5'→3')	Targeted Region	Amplicon Size	Strain/ Genotype	Reference
LTR	F: TGACACAGCAAATGTAACCGCAAG R: CCACGTTGGGCGCCAGCTGCGAGA	LTR	291bp	K1514/A	(Sonigo *et al.*, 1985; Rosati, Kwang and Keen, 1995)
LTR nested_1	Round 1 F: ACTGTCAGGRCAGAGAACARATGCC R: CTCTCTTACCTTACTTCAGG	LTR	Round 1: 635bp	EV1/A	(Ryan *et al.*, 2000)
LTR nested_2	Round 2 F: AAGTCATGTAKCAGCTGATGCTT R: 5TTGCACGGAATTAGTAACG		Round 2: 203bp		
Gag	F: TTCCAGCAACTGCAAACAGT R: TCCTTCTGATCCTACATCTC	*gag* gene	600bp	EV1/A K1514/A SA-OMVV/A CAEVCork/B	(Grego *et al.*, 2002)
Gag nested_1	Round 1 F: CAAGCAGCAGGAGGGAGAAGCTG R: TCCTACCCCCATAATTTGATCCAC	*gag* gene	Round 1: 297bp	CAEVCork/B	(Barlough *et al.*, 1994)
Gag nested_2	Round 2 F: GTTCCAGCAACTGCAAACAGTAGCAATG R: ACCTTTCTGCTTCTTCATTTAATTTCCC		Round 2: 185bp		
Pol	F: ATAGTAAATGGCATCAAGATGC R: TCCCGAATTTGTTTCTACCC	*pol* gene	218bp	EV1/A K1514/A SA-OMVV/A CAEVCork/B	(Grego *et al.*, 2002)
Pol nested_1	Round 1 F: ARGGAGGAATMAAGAYICAGGATATCARGG R: CCYGAATWGTTTCTAYCCA	*pol* gene	Round 1: 455bp	all *pol* gene sequences available in GenBank	(Chassalevris *et al.*, 2020)
Pol nested_2	Round 2 F: CAGGGAGGAATMATAGAYGCAGGATAT R: TCATAATGGGTRTARTCYACYTGCCAATG		Round 2: 416bp		

Table 2. Primer sets used in conventional PCR for the detection of SRLV infections. (Continued)

Primer set	Primers Sequence (5'→3′)	Targeted Region	Amplicon Size	Strain/ Genotype	Reference
Gag-pol nested_1	Round 1 F: TGGTGARKCTAGMTAGAGACATG R: CATAGGRGGHGCGGACGGCASCA	*Gag* & *pol* gene	Round 1: 1300bp	EV1/A K1514/A SA-OMVV/A CAEVCork/B	(Grego *et al.*, 2007)
Gag-pol nested_2	Round 2 F: CAAACWGTRGCAATGCAGCATGG R: GCGGACGGCASCACACG		Round 2: 800bp		
Env nested_1	Round 1 F: AGGTAAGTATAAACCCCAGGTAAG R: TTCAGACTTTCTGGAATTATTTCTGCTCC	*env* gene		CAEVCork/B	(Germain and Valas, 2006)
Env nested_2	Round 2 F: TTGCAAAATGGGGATGTCAACC R: GGCATCTTTTCTGTACAGGAGACTGCT		Round 2: 394bp		

PCR: Polymerase chain reaction; SRLV: Small ruminant lentiviruses; F: Forward primer; R: Reverse primer; LTR: long terminal repeat

Table 3. Cycling conditions of conventional PCR protocols.

PCR protocol name	Primer set	Pre-denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
LTR simple	LTR	95°C/5'	94°C/30"	60°C/30"	72°C/40"	72°C/10'	38
LTR nested	LTR nested_1	95°C/5'	94°C/30"	52°C/40"	72°C/40"	72°C/10'	38
	LTR nested_2	95°C/5'	94°C/30"	52°C/30"	72°C/40"	72°C/10'	38
Gag simple	Gag	95°C/5'	94°C/30"	51°C/30"	72°C/40"	72°C/10'	38
Gag nested	Gag nested_1	94°C/5'	94°C/1'	56°C/1'	72°C/45"	72°C/7'	35
	Gag nested_2	94°C/5'	94°C/1'	56°C/1'	72°C/45"	72°C/7'	35
Pol simple	Pol	95°C/5'	94°C/30"	51°C/30"	72 °C/40"	72°C/10'	38
Pol nested	Pol nested_1	95°C/15'	94°C/20"	54°C/30"	72°C/40"	-	32
	Pol nested_2	95°C/2'	94°C/30"	65°C/30"	72°C/40"	-	28
Gag-pol nested	Gag-pol nested_1	94°C/5'	95°C/30"	55°C/30"	72°C/1.5'	72°C/5'	40
	Gag-pol nested_2	94°C/5'	95°C/20"	60°C/30"	72°C/1.5'	72°C/5'	40
Env nested	Env nested_1	95°C/5'	92°C/40"	57°C/50"	72°C/1'	72°C/4'	35
	Env nested_2	95°C/5'	92°C/40"	55°C/50"	75°C/50"	72°C/4'	35

PCR: Polymerase chain reaction

Also, serum samples from the same animals were serologically tested with the above-mentioned commercial ELISA test. Total seroprevalence at the animal level, prevalence based on PCR protocols, and prevalence based on the combination of ELISA and PCR results were estimated. Sensitivity, specificity, and kappa coefficient value (*k*-value) of all PCR protocols were calculated using as "gold standard" a positive result in both ELISA and at least one of the utilized PCR protocols. For the evaluation of the diagnostic performance of ELISA, the positive result in at least one PCR protocol was used as "gold standard".

5. Development of real-time PCR protocol

5.1 Sequencing and phylogenetic analyses

Thirty-seven PCR products from the most efficient conventional PCR protocols were gel-extracted (PureLink™ Quick Gel Extraction Kit, Invitrogen, Vilnius, Lithuania or purified (PureLink™ PCR Purification Kit, Invitrogen, Vilnius, Lithuania) and sequenced in both directions (Sanger dideoxy sequencing) to confirm the amplification of specific products, exclude potential laboratory contamination, and assess the genetic heterogeneity of SRLV strains circulating in our studied farms (Table 4). Sequencing was carried out on an ABI PRISM 3730xl Genetic Analyzer in an external laboratory (Cemia SA, Larissa, Greece). LTR sequences were obtained from samples from farms A, B, C, as LTR simple protocol failed to give any positive result in farm D. On the other hand, *gag* and *gag-pol* sequences were obtained from all the studied farms, whereas *env* sequences were from farms A, C, and D.

Table 4. The sequenced samples from the tested conventional PCR protocols.

Sample name	Farm	PCR protocol	Amplicon length (bp)
31SX LTR	A	LTR simple	291
40SX LTR	A	LTR simple	291
56SX LTR	A	LTR simple	291
35S LTR	B	LTR simple	291
83S LTR	B	LTR simple	291
17FL LTR	C	LTR simple	291
21FL LTR	C	LTR simple	291
9SX *gag*	A	Gag nested	185
16SX *gag*	A	Gag nested	185
17SX gag	A	Gag nested	185
9S *gag*	B	Gag nested	185
11S *gag*	B	Gag nested	185
13S *gag*	B	Gag nested	185
14S *gag*	B	Gag nested	185
10FL *gag*	C	Gag nested	185
14FL *gag*	C	Gag nested	185
18FL *gag*	C	Gag nested	185
3M *gag*	D	Gag nested	185
4M *gag*	D	Gag nested	185
8M *gag*	D	Gag nested	185
9M *gag*	D	Gag nested	185
11SX *env*	A	Env nested	394
16SX *env*	A	Env nested	394
18SX *env*	A	Env nested	394
117FL *env*	C	Env nested	394
8M *env*	D	Env nested	394
16SX *gag-pol*	A	Gag-pol nested	800
40SX *gag-pol*	A	Gag-pol nested	800
56SX *gag-pol*	A	Gag-pol nested	800
11S *gag-pol*	B	Gag-pol nested	800
35S *gag-pol*	B	Gag-pol nested	800
83S *gag-pol*	B	Gag-pol nested	800
10FL *gag-pol*	C	Gag-pol nested	800
21FL *gag-pol*	C	Gag-pol nested	800
42FL *gag-pol*	C	Gag-pol nested	800
8M *gag-pol*	D	Gag-pol nested	800
98M *gag-pol*	D	Gag-pol nested	800

PCR: Polymerase chain reaction; bp; base pair

All nucleotide sequences were edited using the MEGA v.11 software (Tamura, Stecher and Kumar, 2021) and were trimmed according to the obtained chromatographs. The consensus sequences generated were submitted for BLAST (Basic Local Alignment Search Tool) analysis. The nucleotide sequences from LTR, Env, and Gag-pol nested protocol, the sequences most homologues to them, and the representative sequences from SRLV strains of genotypes A, B, C, and E from different geographical regions were aligned by CLUSTAL W (Thompson, Higgins and Gibson, 1994). After the alignment, pairwise genetic distances were

calculated with the *p*-distance model applying the gamma distribution parameter between the nucleotide sequences, the available Greek strains and only the representative SRLV strains of genotype A, B, C, and E (Shah *et al.*, 2004; L'Homme *et al.*, 2011; Santry *et al.*, 2013). Also, three phylogenetic trees (LTR, *env*, and *gag-pol* nucleotide sequences) were constructed using the Maximum Likelihood (ML) method (Huelsenbeck and Crandall, 1997) with the Tamura-Nei gamma distance (Tamura and Nei, 1993) and invariant sites (I) with 100 bootstrap replicates (Felsenstein, 1985).

5.2 Primer design and *in silico* evaluation

Sequences obtained from the Gag-pol nested protocol were used for the designation of primers for the real-time PCR protocol. This selection was based on the fact that i) positive results were produced in all studied farms using the Gag-pol nested protocol, ii) the initial designation of the Gag-pol nested protocol for the detection of both A and B genotype infections, and iii) the ideal size of PCR products (800bp) for the investigation of SRLV genetic heterogeneity. Three datasets were created in MEGA 11 software including the sequences of this study and reference sequences from GenBank; the first one contained 130 *gag-pol* sequences from strains belonging to A, B, C, and E genotypes, the second one contained 81 sequences from strains belonging to A genotype, and the last one contained 50 sequences from strains belonging to B genotype. After alignment, primer designation was based on highly conserved regions between the strains. Melting temperature (Tm), hairpin, and self- or hetero-dimer formation of the designed primers were evaluated with the IDT OligoAnalyzer tool (Owczarzy *et al.*, 2008). Also, primers were tested *in silico* for their specificity with BLAST evaluating their sequence identity with strains of different genotypes.

5.3 Real-time PCR optimization

Three different real-time PCR protocols based on SYBR Green method were developed and evaluated for the detection of SRLV infections of strains belonging to A and B genotypes, and either to A genotype, or to B genotype, exclusively. All amplifications were carried out using extracted DNA from leukocyte pellets with Luna universal qPCR Master Mix (New England, Biolabs Inc., Hitchin, UK) in the same real-time thermal cycler (SaCycler-96, Sacace Biotechnologies, Italy) in duplicate, and non-template negative controls with DNase-free water were tested each time in duplicate to exclude contamination and to assess the primer dimer formation. The conditions of the three real-time PCR protocols (cycling conditions, primer and DNA template concentrations) were optimized after repeated trials with the twelve sequenced samples, which were found positive in the Gag-pol nested protocol and

were used in the phylogenetic analyses. Also, the three PCR protocols were tested as nested using as template the PCR product from the first round of the conventional Gag-pol nested protocol. The conditions for the optimal amplification were determined based on the lowest cycle threshold (Ct) value and the melt curve analysis for each set of primers

5.4 Diagnostic performance of real-time PCR protocols

After the optimization of the three real-time PCR protocols, their specificity was evaluated by gel electrophoresis and nucleotide sequencing of PCR products, as previously described for PCR products from conventional PCR protocols. The efficiency of protocols for the detection of SRLV strains was evaluated using seven consecutive 10-fold dilutions, for the construction of standard curves. In particular, for the detection of specific genotypes, undiluted DNA from sequenced samples was used, whereas, a synthetic double-stranded PCR product, namely gBlock gene fragment (Integrated DNA Technologies, UK) was used to standardize the curve of the real-time PCR protocol for the detection of strains of A and B genotypes. The gBlock gene fragment was resuspended in nuclease-free water to reach a final concentration of 10 ng/μl according to manufacturer's instruction and the number of copies was calculated using the formula below:

$$(C) \times (M) \times (1 \times 10^{-15} \text{ mol/fmol}) \times (\text{Avogadro's number}) = \text{copies}/\mu l$$

where, C = the current concentration of the gBlock gene fragment in ng/μl and, M = the molecular weight in fmol/ng, as provided by Integrated DNA Technologies.

All the dilutions were amplified in three replicates and the standard curves were generated, where Ct value were plotted against the log value of the DNA standard amount. Also, the correlation coefficient (R^2) was estimated, and the efficiency values were calculated as following:

$$\text{Efficiency} = 10^{(-1/s)}-1$$

where, s = the slope of the linear regression line.

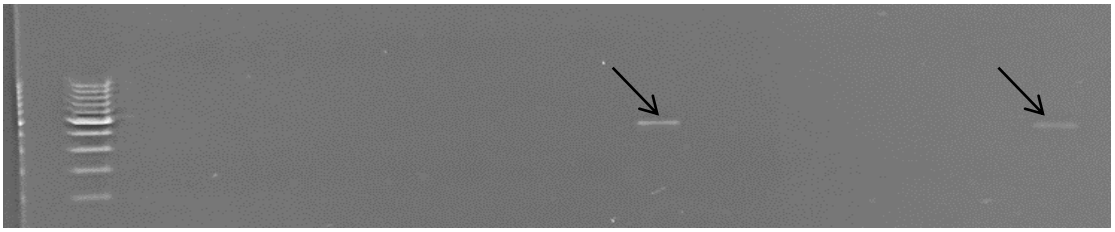
The limit of detection (LOD) was determined by testing 10 replicates of dilutions with 8000, 800, 80, and 8 copies/reaction. Probit analysis was performed in SPSS v26 software, to calculate the LOD with 95% probability.

III. Results

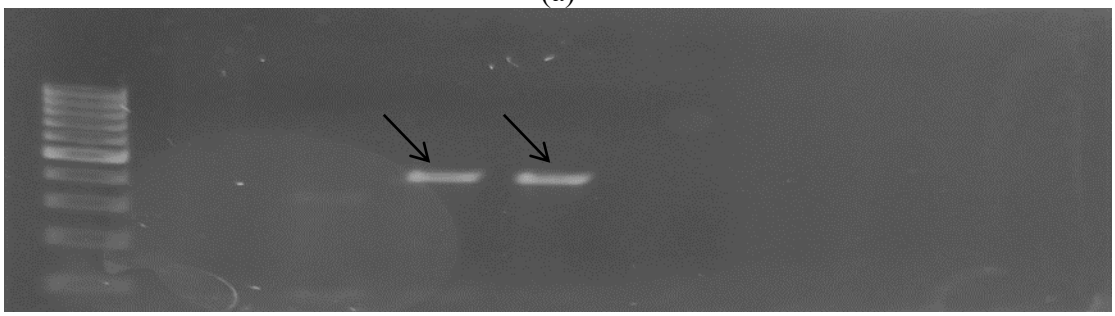
1. Performance of the ELISA and the conventional PCR protocols

The commercial ELISA kit provided positive results in all the studied farms, with a total seroprevalence equal to 67.5% in the studied subpopulation (80 ewes). On the other hand,

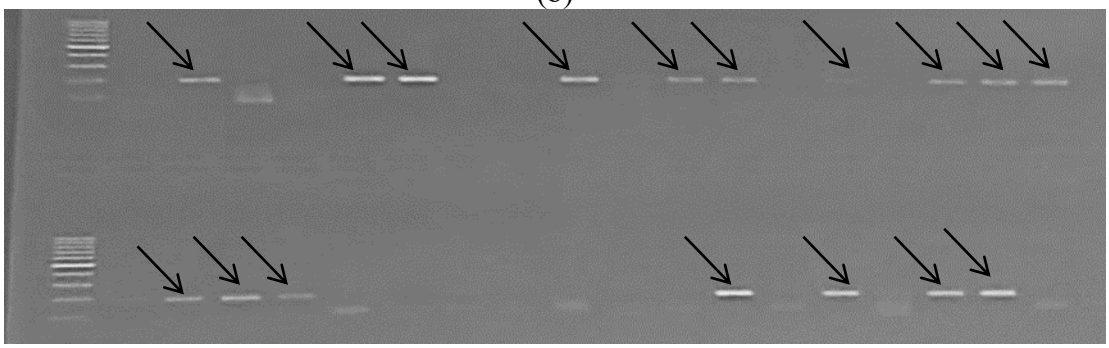
conventional PCR protocols did not give positive results in all farms. Specifically, the Pol simple protocol failed to produce any positive result in any of the studied farm, the LTR simple in farm D, and the LTR nested protocol in farm A. Amplified products from the tested PCR protocols after agarose gel electrophoresis and exposure to UV light are presented in Figures 2a-d.



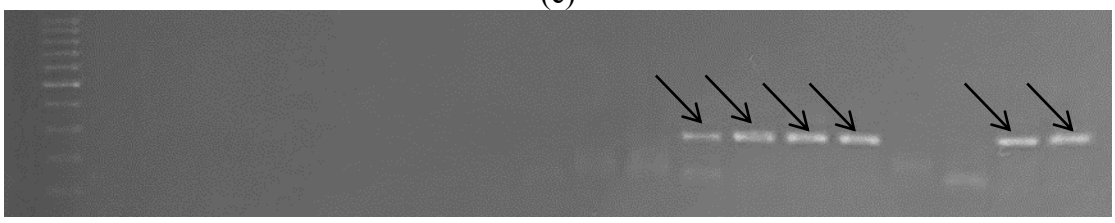
(a)



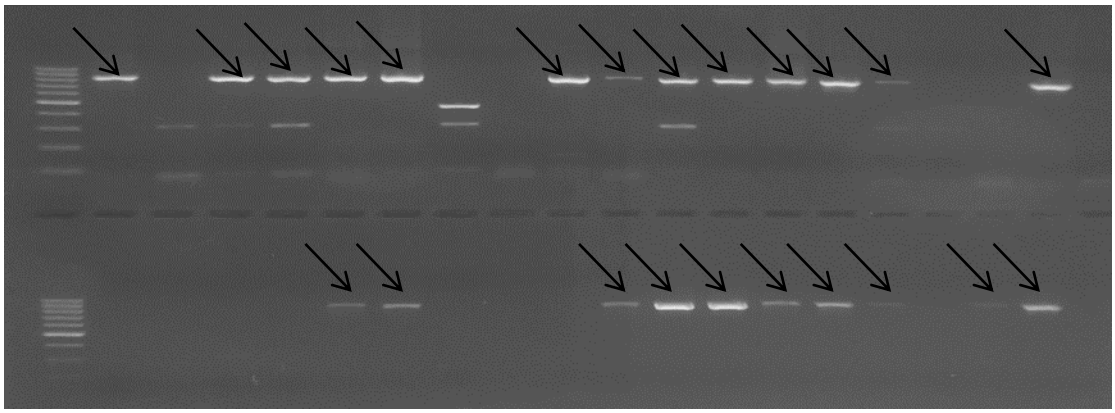
(b)



(c)



(d)



(e)

Figure 2. Agarose gel electrophoresis of a) Pol nested PCR products (416bp); b) Env nested PCR products (394bp); c) Gag nested PCR products (185bp); d) LTR simple PCR products (291bp); e) Gag/pol nested PCR products (800bp) shown with black arrows.

Table 5 summarizes the results from serological analyses and PCR assays. In every case, prevalence of SRLV infections based on a positive output from at least one PCR protocol was higher compared to the seroprevalence. However, except for Gag nested protocol in farm A, PCR protocols did not indicate higher prevalence rates compared to ELISA test when considered separately. Additionally, 11 animals (13.8%) were found positive exclusively with PCR methods, whereas only 1 animal (1.3%) was found positive only in ELISA test. When both the results of ELISA and PCR protocols were considered, overall prevalence increased to 81.3%, while it decreased (66.3%) when positive results in both ELISA and at least one of the utilized PCR protocols were jointly considered to define the infected animals.

Table 5. Results from ELISA and conventional PCR protocols tested in the studied farms.

Method	Farm A	Farm B	Farm C	Farm D	Total
	Frequency (%) of positive animals				
Serology					
ELISA(+)	50.0%	70.0%	85.0%	65.0%	67.5%
Molecular					
LTR	5.0%	50.0%	25.0%	0.0%	20.0%
LTR nested	0.0%	15.0%	5.0%	5.0%	6.3%
Gag	10.0%	10.0%	15.0%	5.0%	10.0%
Gag nested	55.0%	60.0%	40.0%	50.0%	51.3%
Pol	0.0%	0.0%	0.0%	0.0%	0.0%
Pol nested	10.0%	45.0%	10.0%	10.0%	18.8%
Gag-pol nested	50.0%	55.0%	80.0%	50.0%	58.8%
Env nested	35.0%	5.0%	10.0%	30.0%	20.0%
Comparative assessment					
PCR(+) (in at least one protocol)	65.0%	85.0%	90.0%	80.0%	80.0%
ELISA(+) or PCR(+)	70.0%	85.0%	90.0%	80.0%	81.3%
PCR(+)/ELISA(-)	20.0%	15.0%	5.0%	15.0%	13.8%
PCR(-)/ELISA(+)	5.0%	0.0%	0.0%	0.0%	1.3%
PCR(+)/ELISA(+)	45.0%	70.0%	85.0%	65.0%	66.3%

ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; (+): positive result

All protocols, except for the Pol simple protocol which failed to produce any positive result, were evaluated considering as "gold standard" a positive result in both ELISA and at least one of the utilized PCR protocols, and their diagnostic performance is presented in Table 6.

Table 6. Diagnostic performance of the ELISA and PCR protocols.

Method	Sensitivity (%)	Specificity (%)	*k*-value
ELISA	82.8	93.8	0.620
LTR	28.3	96.3	0.027
LTR nested	7.5	96.3	0.576
Gag	11.3	92.6	0.028
Gag nested	66.0	77.8	0.395
Pol*	na	na	na
Pol nested	24.5	92.6	0.127
Gag-pol nested	79.2	81.5	0.576
Env nested	22.6	85.2	0.059
PCR (+) (in at least one protocol)	100.0	59.3	0.658

ELISA: Enzyme-linked Immunosorbent Assay; PCR: Polymerase chain reaction; *k*-value: kappa coefficient value; (+): positive result; *: the diagnostic performance of this protocol was not evaluated as it failed to produce any positive result; na: not applicable

Sensitivity, specificity, and *k*-value for the ELISA test were 82.8%, 93.8% and 0.620, respectively. Sensitivity values for the seven PCR protocols ranged from 7.5% for the nested LTR protocol to 79.2% for the Gag-pol nested protocol, whereas specificity values ranged

from 77.8% for the Gag nested protocol to 85.2% for the Env nested protocols. *K*-value for all PCR protocols ranged from 0.027 for the LTR simple protocol to 0.576 for the Gag-pol and LTR nested protocols. When the results of all PCR protocols were jointly considered, sensitivity, specificity, and k-value were 100.0%, 59.3% and 0.658, respectively.

2. Phylogenetic analyses

2.1 Pairwise sequence comparisons

Pairwise comparisons between LTR sequences and between LTR sequences and the representative SRLV strains of genotype A, B, C, and E (including the strains isolated in Greece and neighbouring countries) are presented in Tables 7 and 8, respectively. The overall pairwise mean distance of LTR nucleotide sequences were 9.9%, ranging from 3.6% (between 35S LTR and 31SX LTR sequences) to 17.6% (between 40SX and 21FL LTR sequences). Also, the intra-farm pairwise comparison was 9.5% for farm A (31SX, 40SX and 56SX sequences), 4.2% for farm B (35S and 83S sequences), and 15.2% for farm C (21FL and 17FL sequences) with an average distance of 9.6% (inter-farm mean distance).

Table 7. Nucleotide sequence diversity (%) between LTR sequences from our study.

Sequence	17FL LTR	21FL LTR	31SX LTR	40SX LTR	56SX LTR	35S LTR
21FL LTR	15.2	-	-	-	-	-
31SX LTR	9.1	13.9	-	-	-	-
40SX LTR	12.7	17.6	12.1	-	-	-
56SX LTR	6.1	12.7	5.5	10.9	-	-
35S LTR	9.1	13.9	3.6	13.5	5.5	-
83S LTR	8.5	13.3	4.2	11.5	4.9	4.2

LTR: Long terminal repeats

The overall mean pairwise distance between LTR sequences and the representative SRLV strains was 39.5%. LTR sequences were similar to isolates from Greece and the neighbouring or nearby countries (Italy, Jordan, and Turkey) belonging to genotype A (mean distance 15.0%). LTR sequences diverged equally (mean distance 62.0%) from all the representative SRLV strains of genotypes A, B, C, and E, except for American OvLv 85/34 strain belonging to genotype A2 (mean distance 24.0%). Also, all LTR sequences presented >60.0% genetic distance from Italian strain genotype B2 (SRLV042). Therefore, these results indicate a higher similarity of LTR sequences with i) A2 strain OvLv 85/34, ii) the other Greek sequences of genotype A, and iii) the sequences of neighboring countries of genotype A.

Table 8. Nucleotide sequence diversity (%) between LTR sequences from our study and representative SRLV strains.

Sequences	17FL LTR	21FL LTR	31SX LTR	40SX LTR	56SX LTR	35S LTR	83S LTR
EV1 (A1/SC)	62.0	61.7	57.8	61.5	62.9	60.6	60.1
K1514 (A1/USA)	63.8	64.1	58.4	62.9	63.4	60.6	60.6
SA-OMVV (A1/SA)	63.8	62.2	57.8	62.4	63.4	60.6	60.6
KV1772 (A1/IC)	63.8	64.1	58.4	62.9	63.4	60.6	60.6
OvLv 85/34 (A2/USA)	22.3	23.7	28.7	23.2	23.7	22.7	23.2
CAEV-Co B1/USA)	61.0	62.2	57.8	60.6	61.5	59.6	60.6
Ov496 (B2/SP)	61.0	62.2	58.4	61.0	61.5	59.6	60.1
Volterra (B3/IT)	61.0	61.7	59.6	59.6	61.5	59.6	60.6
1GA (C/NW)	62.0	62.7	60.8	61.5	62.0	61.5	60.1
Roccaverano (E1/IT)	65.7	65.6	60.8	63.8	64.8	63.8	62.9
MVV-4-lung (A/GR)	12.3	10.6	15.8	14.6	11.3	12.7	11.8
MVV-Gr-LTR58 (A/GR)	12.2	11.5	13.3	12.2	10.8	11.3	10.3
MVV-Gr-LTR63 (A/GR)	13.1	12.4	17.5	17.4	13.6	13.6	13.1
MVV-Gr-LTR80 (A/GR)	13.1	12.9	16.9	14.1	13.1	13.1	13.1
sh301 (A/J)	17.5	9.7	18.3	18.0	13.7	13.3	13.7
sh235 (A/IT)	14.6	8.6	16.3	16.4	12.7	12.2	13.1
TR-2007-Eco2-35 (A/TR)	22.0	23.5	28.8	24.5	25.0	21.5	24.5
SRLV042 (B2/IT)	66.2	65.6	60.8	64.3	65.7	63.4	63.8

LTR: Long terminal repeats; SRLV: Small ruminant lentiviruses; SC: Scotland; USA: United States of America; SA: South Africa; IC: Iceland; SP: Spain; IT: Italy; NW: Norway; GR: Greece; J: Jordan; TR: Turkey.

The mean genetic diversity between *env* sequences was 8.4%. As shown in Table 9, 16SX and 117FL *env* sequences presented great similarity (0.0% genetic nucleotide distance), whereas genetic diversity of 8M with 16SX and 117FL was 12.1%.

Table 9. Nucleotide sequence diversity (%) between *env* sequences from our study.

Sequences	117FL *env*	11SX *env*	16SX *env*	18SX *env*
11SX *env*	9.7	-	-	-
16SX *env*	0.0	9.7	-	-
18SX *env*	6.5	6.8	6.5	-
8M *env*	12.1	10.3	12.1	10.0

All *env* sequences presented higher similarity with strains belonging to B1 and B2 genotypes compared to strains belonging to A, B3, C, and E genotypes (Table 10). Specifically, *env* sequences were most similar to French (Agh536, Cal42140, and Ser2013) and Italian (SRLV001) sequences belonging to B2 genotype with a mean nucleotide divergence 11.6%. Also, mean pairwise genetic distance of *env* sequences from CAEV-Co strain of B1 genotype and from Ov496 strain of B2 genotype was 20.3% and 16.3%, respectively. The respective values for genotypes A, B3, C, and E strains were 64.4%, 29.7%, 31.3%, and 67.8%, respectively.

Table 10. Nucleotide sequence diversity (%) between *env* sequences from our study and representative SRLV strains.

Sequences	117FL *env*	11SX *env*	16SX *env*	18SX *env*	8M *env*
EV1 (A1/SC)	65.8	63.4	65.8	65.5	64.9
K1514 (A1/USA)	66.2	64.7	66.2	66.8	65.0
SA-OMVV (A1/SA)	62.4	60.6	62.4	62.9	60.9
KV1772 (A1/IC)	66.2	64.7	66.2	66.8	65.0
OvLv 85/34 (A2/USA)	64.1	62.4	64.1	64.1	63.2
CAEV-Co (B1/USA)	20.9	20.3	20.9	20.9	18.5
Ov496 (B2/SP)	16.8	17.4	16.8	15.6	15.0
Volterra (B3/IT)	30.3	29.1	30.3	30.6	28.2
1GA (C/NW)	30.8	32.9	30.8	31.7	30.2
Roccaverano (E1/IT)	67.9	67.1	67.9	68.5	67.4
SRLV001 (B2/IT)	12.1	12.1	12.1	12.6	12.9
SRLV042 (B2/IT)	25.6	24.7	25.6	27.6	25.6
Agh536 (B2/FR)	13.5	12.1	13.5	12.1	12.9
Cal42140 (B2/FR)	9.1	10.6	9.1	8.5	8.5
Ser2013 (B2/FR)	11.8	10.0	11.8	9.7	8.2

SRLV: Small ruminant lentiviruses; SC: Scotland; USA: United States of America; SA: South Africa; IC: Iceland; SP: Spain; IT: Italy; NW: Norway; FR: France.

Mean pairwise genetic distance of all *gag-pol* sequences was 12.8% and varied from 0.8% between 8M and 98M sequence to 22.9% between 40SX and 21FL sequences (Table 11). Also, the intra-farm pairwise comparison was 15.4% for farm A (16SX, 56SX and 40SX sequences), 4.2% for farm B (11S, 14S, 35S and 83S sequences), 8.4% for farm C (10FL, 21FL and 42FL sequences), and 0.8% for farm D (8M and 98M sequences) with an average distance of 7.2% (inter-farm mean genetic distance).

Table 11. Nucleotide sequence diversity (%) between *gag-pol* sequences from our study.

Sequences	10FL	21FL	42FL	11S	14S	35S	83S	16SX	56SX	40SX	8M
21FL	10.0	-	-	-	-	-	-	-	-	-	-
42FL	9.7	5.5	-	-	-	-	-	-	-	-	-
11S	4.5	10.6	10.4	-	-	-	-	-	-	-	-
14S	4.4	9.5	9.9	5.1	-	-	-	-	-	-	-
35S	4.5	10.2	9.7	4.5	5.1	-	-	-	-	-	-
83S	4.0	9.7	9.1	2.8	3.8	3.6	-	-	-	-	-
16SX	19.1	22.2	21.8	18.0	18.9	18.9	17.2	-	-	-	-
56SX	5.3	9.7	9.9	5.5	4.6	5.5	4.6	20.6	-	-	-
40SX	20.1	22.9	22.2	19.3	19.7	19.7	18.9	4.4	21.4	-	-
8M	18.8	22.2	20.5	17.8	19.3	18.8	17.4	4.7	19.5	4.9	-
98M	18.8	21.8	20.3	17.6	19.3	18.6	17.2	4.7	19.8	4.9	0.8

The overall mean pairwise genetic distance of *gag-pol* sequences from all the SRLV representative strains and strains from neighbouring countries was 17.3%. As presented in Table 12 *gag-pol* sequences presented the greatest genetic diversity with strains of genotype C (20.9%) and E (27.8%). However, two distinct groups were recognized. One group

including 10FL, 21FL, 42FL, 11S, 14S, 35S, 83S, and 56SX *gag-pol* sequences was most similar (12.6% genetic distance) to the Greek (SRLV-Greece-S1 and SRLV-Greece-S2), the Italian (SRLV038 and It-561), and the Jordan (sh301) sequences belonging to genotype A. On the other hand, the group including 16SX, 40SX, 8M, and 98M sequences was most related (6.2% genetic distance) to Spanish (Ov496) and Italian (SRLV001, SRLV042, lt-Pi1, and lt-007) sequences.

Table 12. Nucleotide sequence diversity (%) between gag-pol sequences from our study and representative SRLV strains.

Sequences	10FL	21FL	42FL	11S	14S	35S	83S	16SX	56SX	40SX	8M	98M
EV1 (A1/SC)	18.9	18.9	18.6	18.0	18.2	19.5	17.2	22.0	19.1	23.5	21.2	21.4
K1514 (A1/USA)	16.3	19.1	18.4	15.0	16.1	17.4	16.1	18.4	17.9	20.1	19.7	20.1
SA-OMVV (A1/SA)	15.0	15.7	15.6	15.3	14.8	15.5	15.2	19.3	15.5	20.6	19.7	19.7
KV1772 (A1/IC)	16.5	19.3	18.2	15.2	16.3	17.6	16.3	18.6	18.1	20.3	19.9	20.3
OvLv 85/34 (A2/USA)	13.8	15.7	15.6	14.4	14.4	14.8	13.4	16.1	15.1	18.6	17.6	18.2
CAEV-Co (B1/USA)	19.3	21.0	19.7	19.3	20.8	18.9	19.1	11.6	21.2	11.7	10.2	10.4
Ov496 (B2/SP)	19.7	23.3	22.4	19.3	19.9	19.9	18.9	7.2	21.0	7.6	6.6	6.8
Volterra (B3/IT)	18.8	21.3	21.7	19.7	19.7	20.7	19.0	16.9	20.3	18.8	16.9	16.9
1GA (C/NW)	22.2	23.4	23.0	23.2	22.8	22.8	21.9	18.1	23.8	18.6	15.6	16.0
Roccaverano (E1/IT)	28.0	29.0	28.1	28.6	27.8	29.4	27.1	26.3	29.2	27.7	26.3	26.7
SRLV-Greece-S1 (A/GR)	9.7	10.2	10.4	9.3	9.3	10.6	8.9	18.9	11.5	20.3	19.5	18.8
SRLV-Greece-S2 (A/GR)	9.7	10.8	10.6	9.3	9.7	10.2	8.5	19.3	11.5	21.2	19.7	18.9
SRLV0 (A19/IT)	13.3	15.5	15.0	14.0	14.0	14.6	14.4	18.8	15.5	20.5	18.8	18.9
It-561 (A/IT)	13.8	16.7	15.2	14.8	15.9	14.4	14.0	21.4	14.7	21.6	19.7	20.5
sh301 (A/J)	12.1	15.3	14.2	12.7	12.7	13.3	11.9	19.7	14.3	20.5	18.2	18.2
sh248 (A22/LB)	18.9	21.4	19.7	20.5	18.9	20.1	20.1	22.3	21.2	22.9	21.4	21.8
SRLV001 (B2/IT)	19.7	22.9	22.0	19.1	20.1	19.7	18.8	6.1	21.0	6.6	5.1	5.1
SRLV042 (B2/IT)	19.3	22.7	21.6	19.5	19.5	19.5	18.2	5.5	21.2	6.6	5.5	5.5
It-Pi1 (B2/IT)	19.3	22.7	21.6	19.5	20.1	19.5	18.6	6.4	20.6	7.2	5.3	5.7
It-007 (B2/IT)	19.9	22.7	22.0	19.7	19.5	19.3	18.6	5.7	21.2	6.4	6.1	6.1

SRLV: Small ruminant lentiviruses; SC: Scotland; USA: United States of America; SA: South Africa; IC: Iceland; SP: Spain; IT: Italy; NW: Norway; GR: Greece; J: Jordan; LB: Lebanon. All sequences in columns are *gag-pol* sequences from our study.

2.2 Phylogenetic trees

All amplified PCR products were assigned as specific SRLV sequences according to the BLAST analysis. The constructed phylogenetic trees with LTR, *env*, and *gag-pol* nucleotide sequences are presented in Figures 3, 4, and 5, respectively. According to the phylogenetic tree, LTR nucleotide sequences (shown with black arrows in Figure 3) were all grouped together in a common branch with the other Greek (DQ480645, DQ084446, DQ084447, and DQ084448) SRLV strains of genotype A, as well those from Italy (KT921316), Jordan (KT921317), and Turkey (GQ862796). However, the bootstrap values reported in this branch are not sufficiently high. Also, 21FL sequence which presented the highest genetic distance from the other sequences from this study was subgrouped with the Italian (KT921316) and Jordan (KT921317) sequences in a separate note, whereas 17FL sequence from the same farm is grouped with all the other LTR sequences of this study. Also, 35S and 31SX LTR sequences which showed the least distance were subgrouped together in the same node with a bootstrap value of 71%.

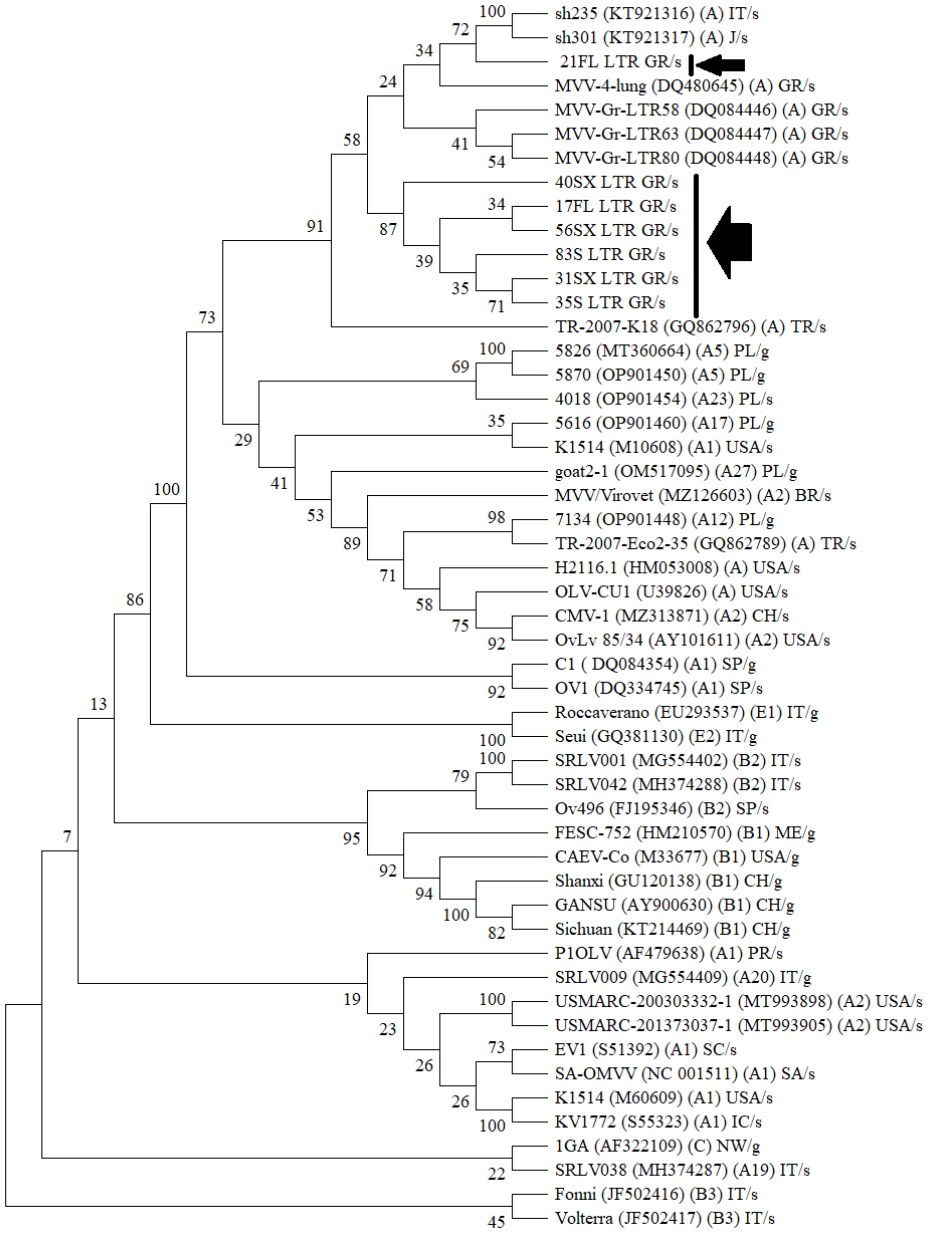


Figure 3. Phylogenetic tree indicates the relationship of LTR nucleotide sequences (~300bp) of this thesis with the available database (Genbank) SRLV strains originating from different geographical areas. Sequences from the present study are shown with black arrows. Database derived sequences are denoted with their strain/isolate name, GenBank accession number and genotype in parenthesis, the abbreviation of the country and the host (IT: Italy; J: Jordan; GR: Greece; TR: Turkey; PL: Poland; USA: United States of America; BR: Brazil; CH: China; SP: Spain; ME: Mexico; PR: Portugal; SC: Scotland; SA: South-Africa; IC: Iceland; NW: Norway; s: sheep; g: goat). This unrooted tree was inferred in MEGA11 by using the Maximum Likelihood method and Tamura-Nei model, as described in Materials and Methods chapter. Bootstrap values are based on 100 repetitions and are shown at the nodes. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 1.6864)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 3.73 sites). This analysis involved 50 nucleotide sequences.

Env sequences (shown with black arrows in Figure 4) were all grouped together with B2 Italian (MG554402) and French (AY842738, AY842746, and AY842750) SRLV strains with a bootstrap value of 90%. However, the bootstrap values of the nodes in this cluster were not quite high.

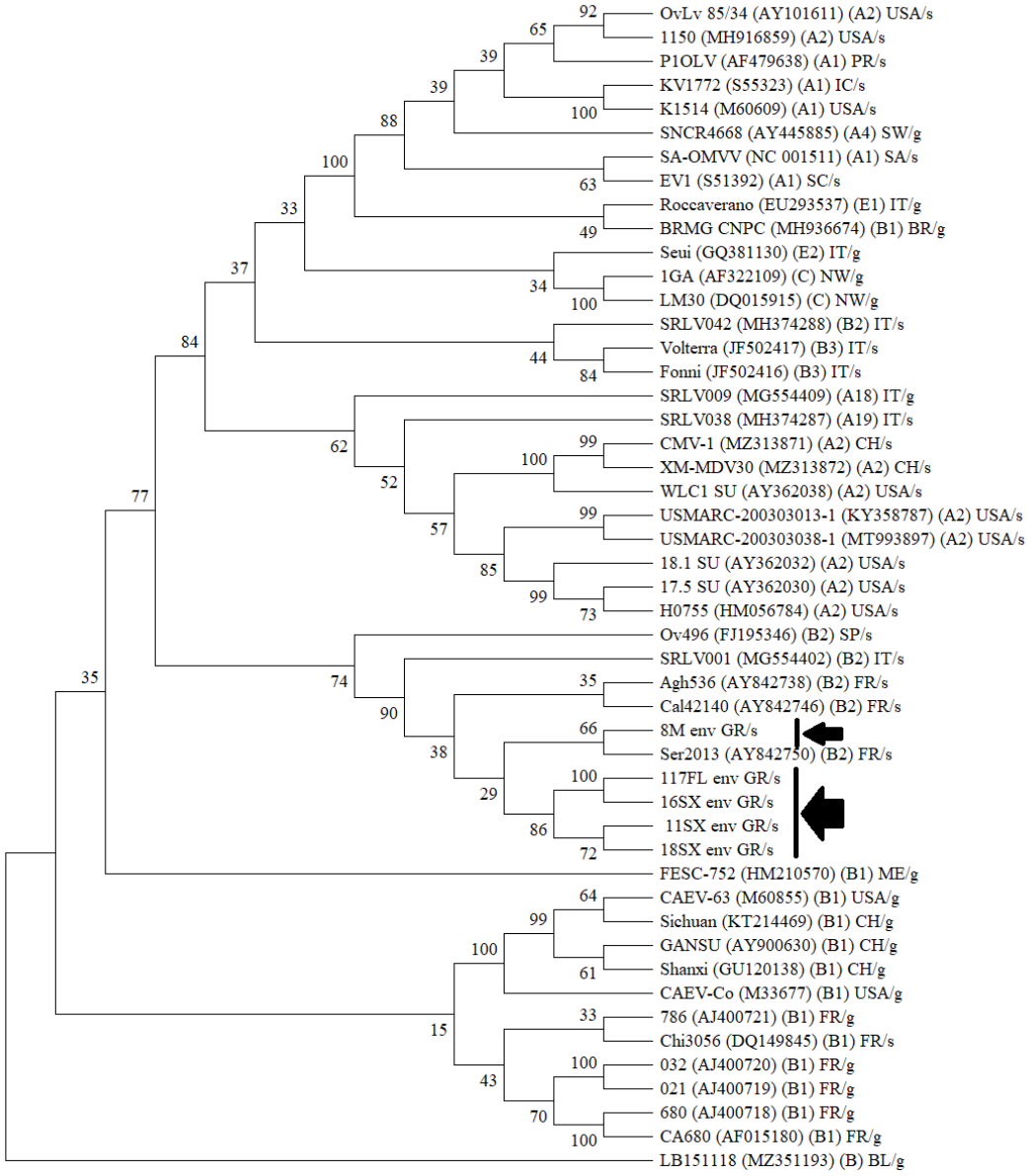


Figure 4. Phylogenetic tree indicates the relationship of *env* nucleotide sequences (~400bp) of this thesis with the available database (Genbank) SRLV strains originating from different geographical areas. Sequences from the present study are shown with black arrows. Database derived sequences are denoted with their strain/isolate name, GenBank accession number and genotype in parenthesis, the abbreviation of the country and the host (FR: France; BL: Belgium; USA: United States of America; CH: China; ME: Mexico; SP: Spain; IT: Italy; NW: Norway; BR: Brazil; SC: Scotland; SA: South-Africa; IC: Iceland; SW: Switzerland; PR: Portugal; sheep; g: goat). This unrooted tree was inferred in MEGA11 by using the Maximum Likelihood method and Tamura-Nei model, as described in Materials and Methods chapter. Bootstrap values are based on 100 repetitions and are shown at the nodes. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 1.1889)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 1.79% sites). This analysis involved 49 nucleotide sequences.

Gag-pol sequences (shown with black arrows in Figure 5) were clustered in two groups in the phylogenetic tree. The first group included 8M, 98M, 16SX, and 40SX *gag-pol* sequences and was strongly associated with Italian (MG554402, EU010126, AY265456, and MH374288) B2 genotype SRLV strains with a bootstrap value of 98%. The other group included 10FL, 21FL, 42FL, 11S, 14S, 35S, 83S, and 56SX *gag-pol* sequences and clustered with other Greek (AY530289 and AY530290) A genotype SRLV strains with a bootstrap value of 91%. This clustering is in consistency with the genetic distance of the sequences presented in pairwise sequence comparison in Table 12. The *gag-pol* sequences reported in this study were deposited in GenBank with accession numbers OR283217 to OR283228.

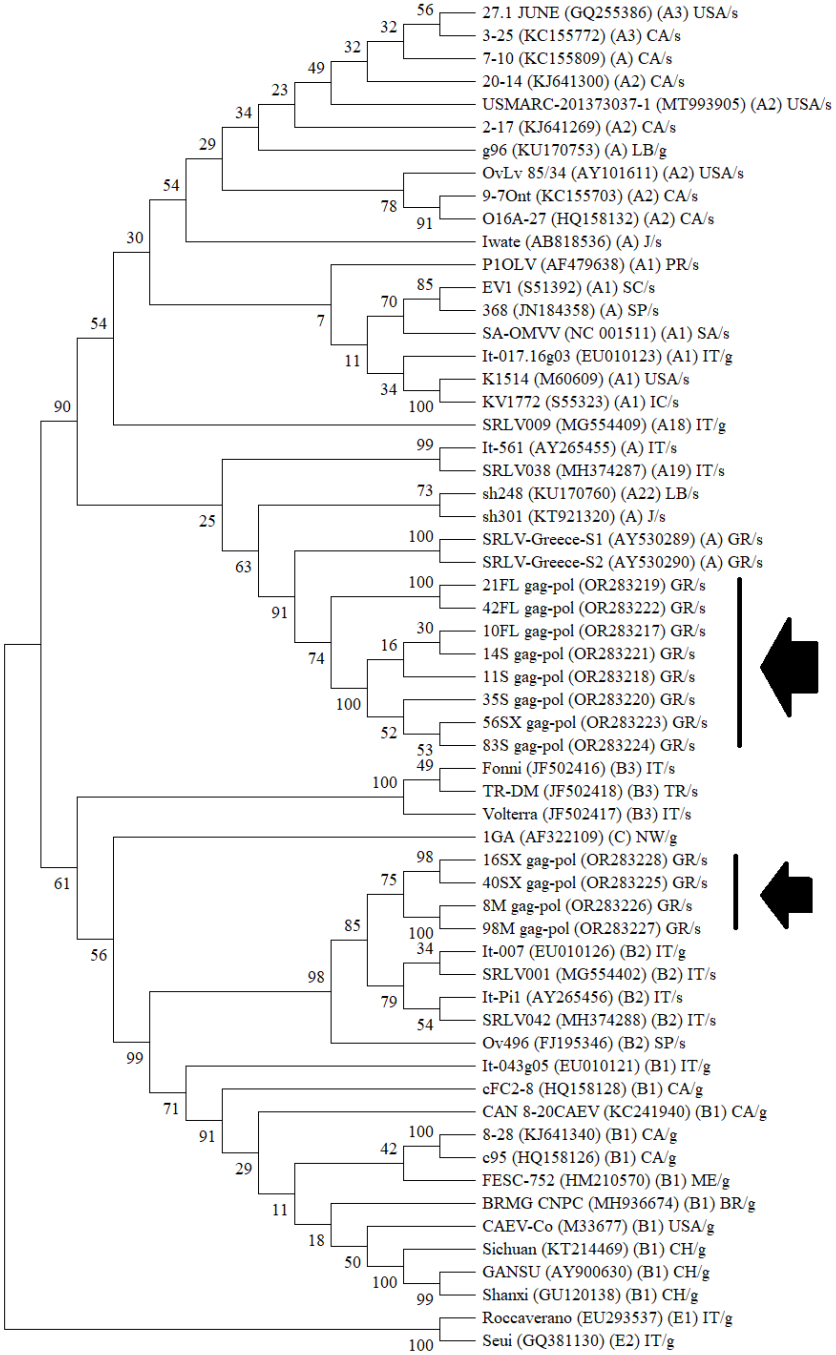


Figure 5. Phylogenetic tree indicates the relationship of *gag-pol* nucleotide sequences (~800bp) of this thesis with the available database (Genbank) SRLV strains originating from different geographical areas. Sequences from the present study are shown with black arrows. Database derived sequences are denoted with their strain/isolate name, GenBank accession number and genotype in parenthesis, the abbreviation of the country and the host (CH: China; USA: United States of America; BR: Brazil; CA: Canada; ME: Mexico; IT: Italy; SP: Spain; NW: Norway; TR: Turkey; LB: Lebanon; J: Jordan; GR: Greece; PR: Portugal; SC: Scotland; SA: South-Africa; IC: Iceland; s: sheep; g: goat). This unrooted tree was inferred in MEGA11 by using the Maximum Likelihood method and Tamura-Nei model, as described in Materials and Methods chapter. Bootstrap values are based on 100 repetitions and are shown at the nodes. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 0.6901)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 19.62% sites). All SRLV sequences characterized in this study are available under accession number (GenBank: OR283217 to OR283228).

3. Development of three real-time PCR protocols

3.1 Primer selection

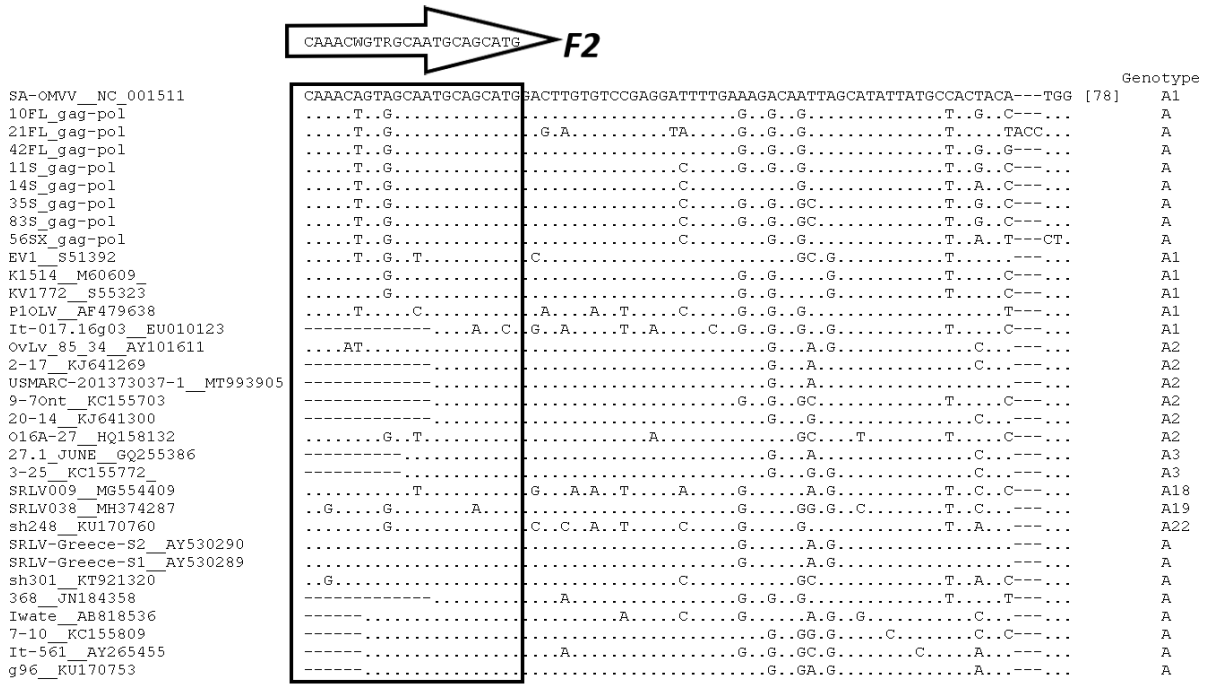
Phylogenetic analysis of *gag-pol* sequences was considered as the most appropriate strategy for the development of real-time PCR protocol for the detection of SRLV infections in the studied farms. Gag-pol nested protocol was efficient in all the studied farms presenting in total the best diagnostic performance compared to the other protocols. Also, phylogenetic tree of *gag-pol* sequences was the most comprehensive compared to *env* and LTR trees, revealing the circulation of strains belonging to A and B2 genotype in the studied farms. The dataset of sequence alignments revealed a deleted region in *gag-pol* sequences and reference strains belonging to genotype A in positions 201-206 compared to sequences and strains belonging to genotype B. Based on this, three different sets of degenerate primers were designed in conserved regions of strains belonging to i) all genotypes, ii) only A genotype, and iii) only B genotype (F2-R, F2-Ra, F2-Rb, respectively). Degenerate primers were selected to match the maximum number of sequences in the three datasets. The forward primer of the conventional Gag-pol nested_2 set (Grego *et al.*, 2007) was maintained in the three new sets of primers (F2), except for the last nucleotide that was discarded for the optimization of the primer sets. The specificity of reverse primers was tested *in silico* with BLAST, and the primer Ra showed sequence identity only towards strains of genotype A, the primer Rb showed identity only towards strains of genotype B, whereas the primer R showed identity to all genotypes. The sequences of the three sets of primers, their $T_{m,}$ and their positions are summarized in Table 13.

Table 13. Primer sets of new real-time PCR protocols for the detection of SRLV infections in the studied farms.

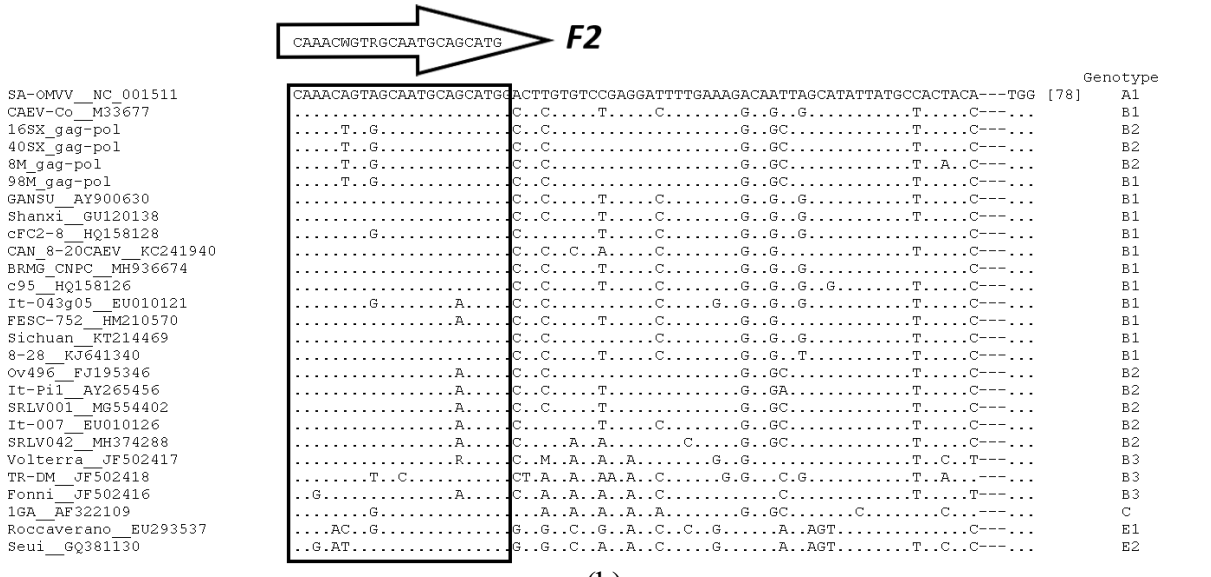
Protocol	Primers Sequence (5'→3')	T_m (°C)	Positions in genome	Targeted Region	Amplicon Size (bp)
F2-R	F2: AAACWGTRGCAATGCAGCATG	64.0	1018-1039*[1] 1010-1031*[2]	*gag* gene of all genotypes	125
	R: GCYCTRTTYCCWGGCATCAT	63.3	1123-1142*[1] 1115-1134*[2]		
F2-Ra	F2: AAACWGTRGCAATGCAGCATG	64.0	1018-1039*[1] 1010-1031*[2]	*gag* gene of A genotype	209
	Ra: GTAAGGACRTTTGGCCCYG	62.6	1208-1226*[1]		
F2-Rb	F2: AAACWGTRGCAATGCAGCATG	64.0	1018-1039*[1] 1010-1031*[2]	*gag* gene of B genotype	214
	Rb: TTAAYCCTCCTCCTRCYKGAG	62.5	1203-1223*[2]		

PCR: Polymerase chain reaction; SRLV: Small ruminant lentiviruses; bp: base pair; T_m: primer melting temperature; F2: forward primer; R, Ra, Rb: reverse primer; *[1] Numbering according to nucleotide sequence of reference SRLV strain SA-OMMV (GenBank accession number: NC_001511); *[2] Numbering according to nucleotide sequence of reference SRLV strain CAEV-Co (GenBank accession number: M33677)

Schematic representation of the newly designed primers and the respective sequence alignments are shown in Figures 6, 7, 8 and 9.

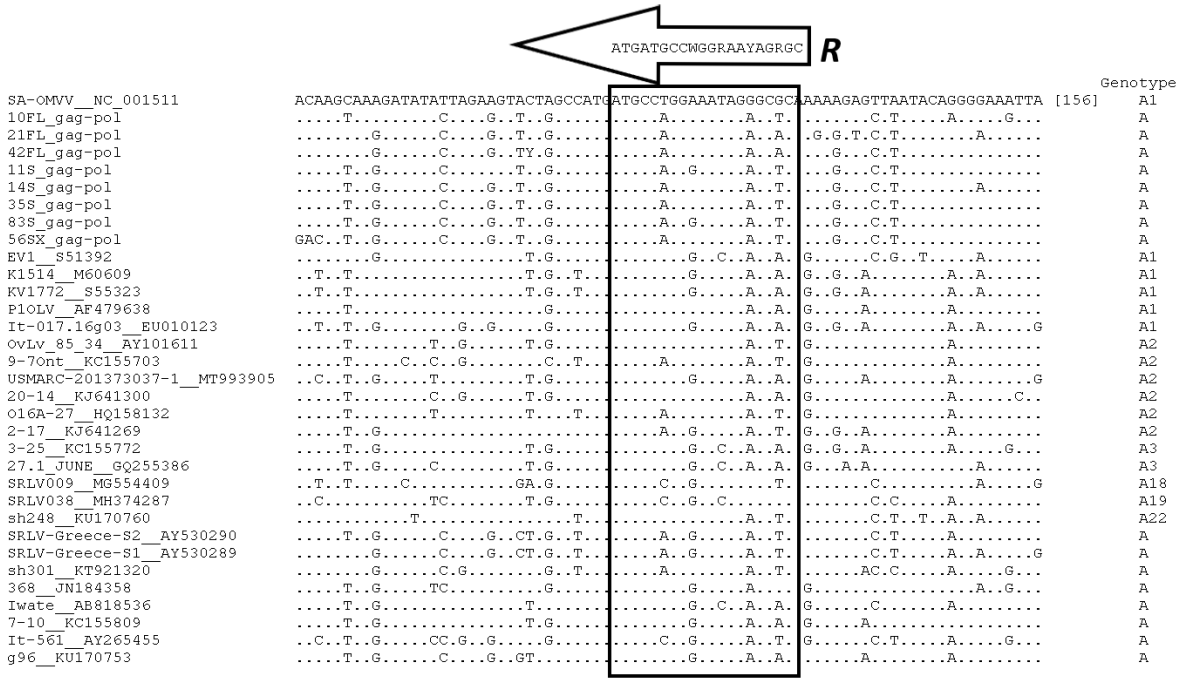


(a)

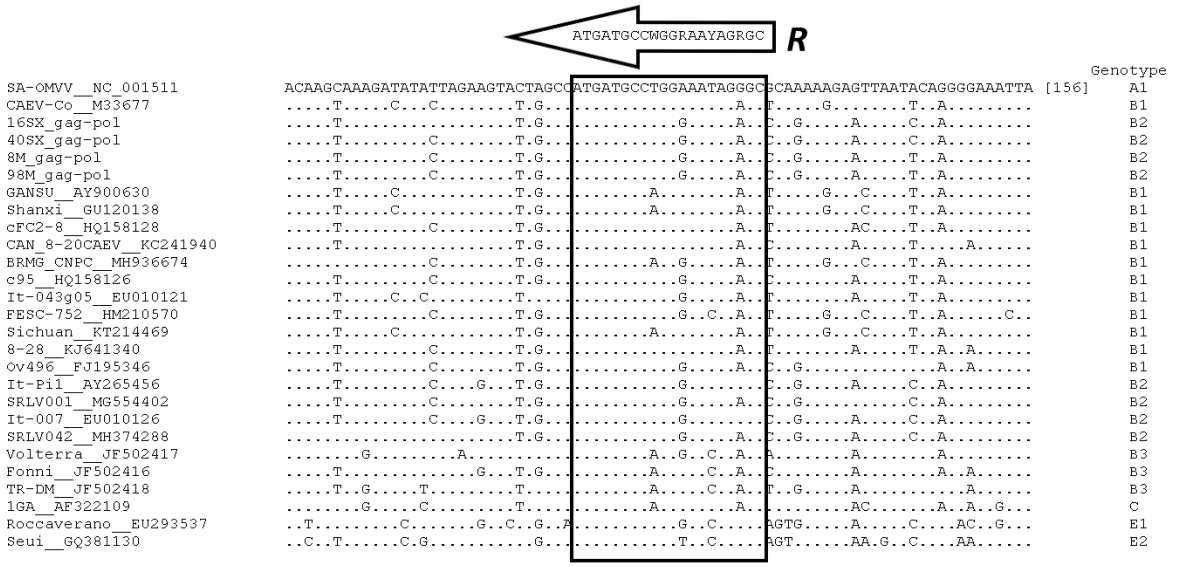


(b)

Figure 6. Amino acid sequence alignment of *gag-pol* sequences and selected reference SRLV strains from GenBank belonging to genotype A (a), and genotype B, C, and E (b). Dots indicate identity with SA-OMVV strain of genotype A, whereas dashes indicate deletions. The region of common forward primer (F2) for the three real-time PCR protocols is boxed [positions 1018-1039 in SA-OMVV strain (acc. NC_001511)]. Above the sequence alignment the sequence of degenerate F2 primer is indicated into the black arrow. W:A/T; R: A/G.



(a)



(b)

Figure 7. Amino acid sequence alignment of *gag-pol* sequences and selected reference SRLV strains from GenBank belonging to genotype A (a), and genotype B, C, and E (b). Dots indicate identity with SA-OMVV strain of genotype A, whereas dashes indicate deletions. The region of reverse primer R is boxed [positions 1123-1142 in SA-OMVV strain (acc. NC_001511) and 1115-1134 in CAEV-Co strain (acc. M33677)]. Above the sequence alignment the reverse complement sequence of degenerate R primer is indicated into the black arrow. W:A/T; Y: C/T.

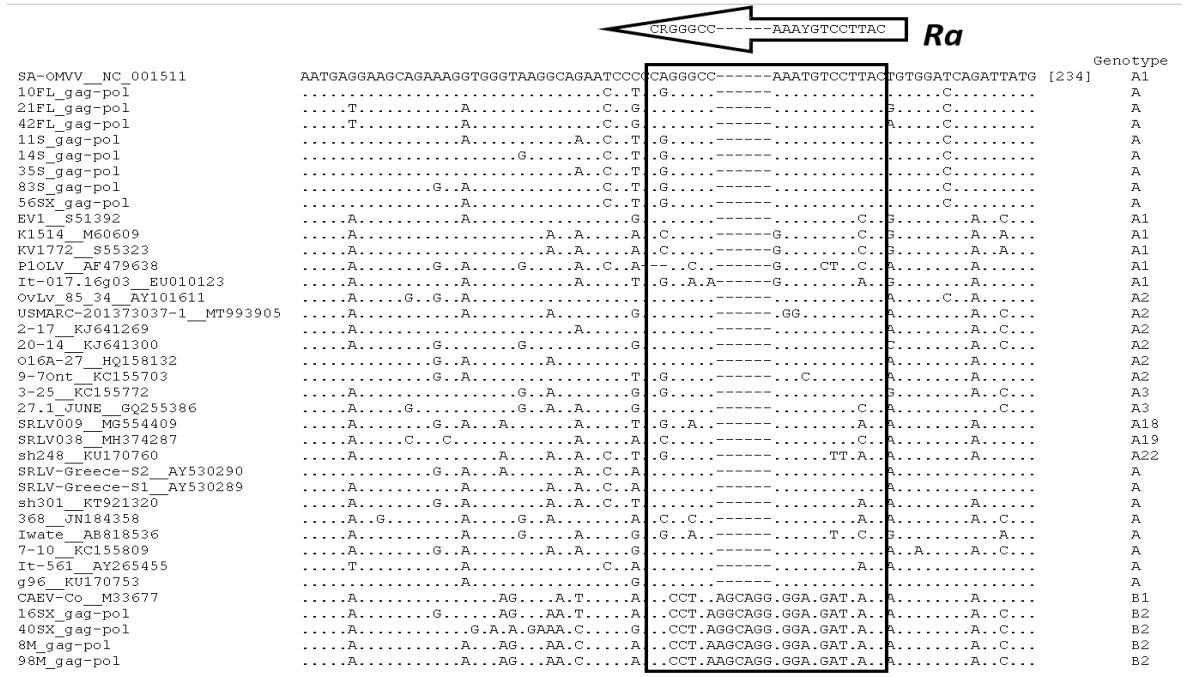


Figure 8. Amino acid sequence alignment of *gag-pol* sequences and selected reference SRLV strains from GenBank belonging to genotype A, B, C, and E. Dots indicate identity with SA-OMVV strain of genotype A, whereas dashes indicate deletions. The region of reverse primer Ra is boxed [positions 1208-1226 in SA-OMVV strain (acc. NC_001511)]. Above the sequence alignment the reverse complement sequence of degenerate Ra primer is indicated into the black arrow. R: A/G; Y: C/T.

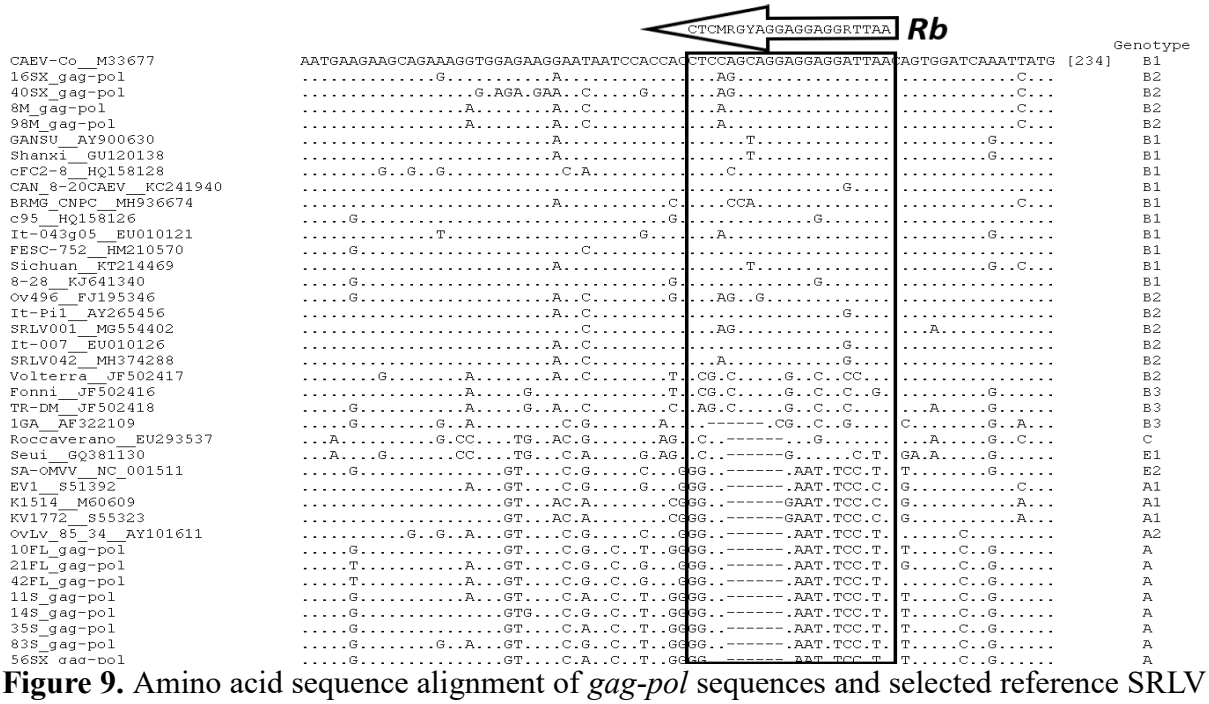


Figure 9. Amino acid sequence alignment of *gag-pol* sequences and selected reference SRLV strains from GenBank belonging to genotype A, B, C, and E. Dots indicate identity with CAEV-Co strain of genotype B, whereas dashes indicate deletions. The region of reverse primer Rb is boxed [positions 1203-1223 in CAEV-Co strain (acc. M33677)]. Above the sequence alignment the reverse complement sequence of degenerate Rb primer is indicated into the black arrow. M: A/C; R:A/G; Y: C/T.

3.2 Real-time PCR setup

After the optimization of the three real-time PCR assays, the final real-time PCR reaction volume was 20 µl and contained 0.5 µl PCR product from the first round of the conventional Gag-pol nested protocol, 0.5 µl of 10 µM forward and 0.5 µl of 10 µM reverse primer (200 nM of each primer), 10 µl Luna universal qPCR Master Mix (New England, Biolabs Inc., Hitchin, UK), and 8.5 µl DNase-free water. The first round of PCR included 30 cycles and same cycling conditions as previously described. Cycling conditions for the three real-time PCR protocols included initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 15s and 59 °C for 30s. After the amplification cycles, melting temperature (T_m) analysis from 50 ^oC to 95 ^oC at 0.5 ^oC increments was conducted to determine T_m of specific real-time PCR products.

4. Diagnostic performance of real-time PCR protocols

PCR products from the three PCR protocols without the melting curve phase were gel electrophoresed (Figure 10) and the bands were gel extracted and sequenced. The nucleotide sequencing confirmed the amplification of specific PCR products in F2-R and F2-Rb protocols, whereas non-specific products were amplified with F2-Ra protocol.

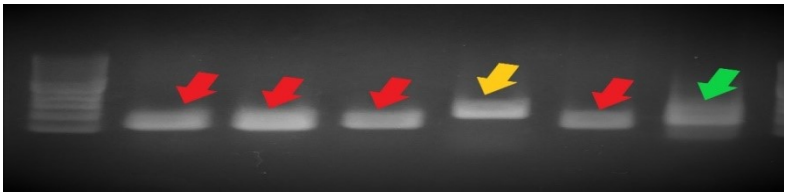
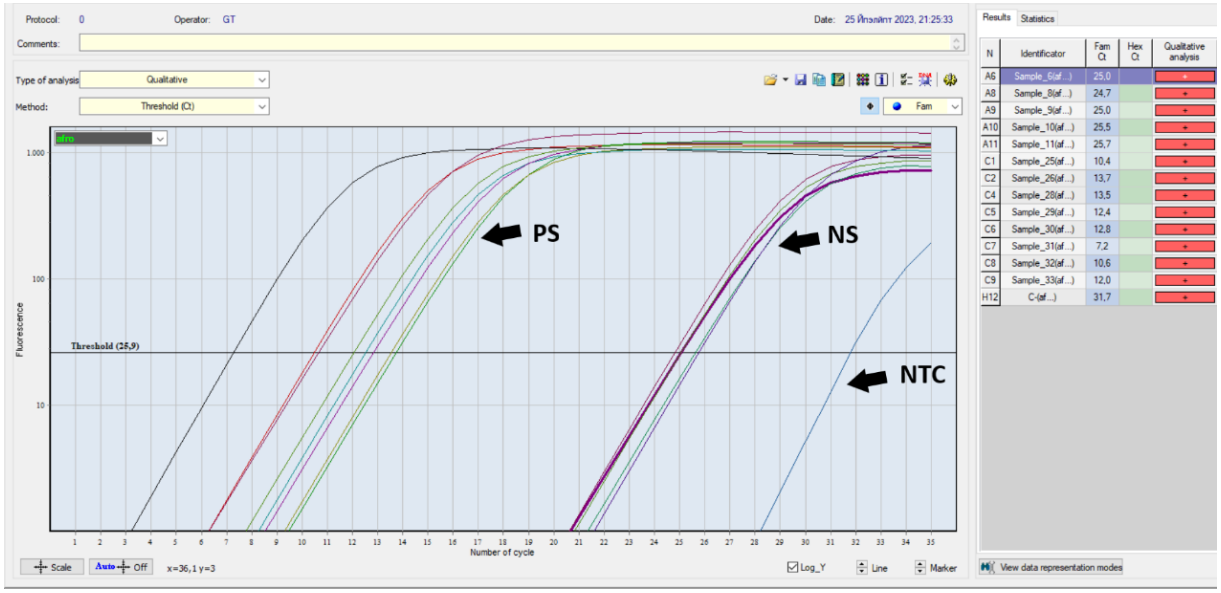
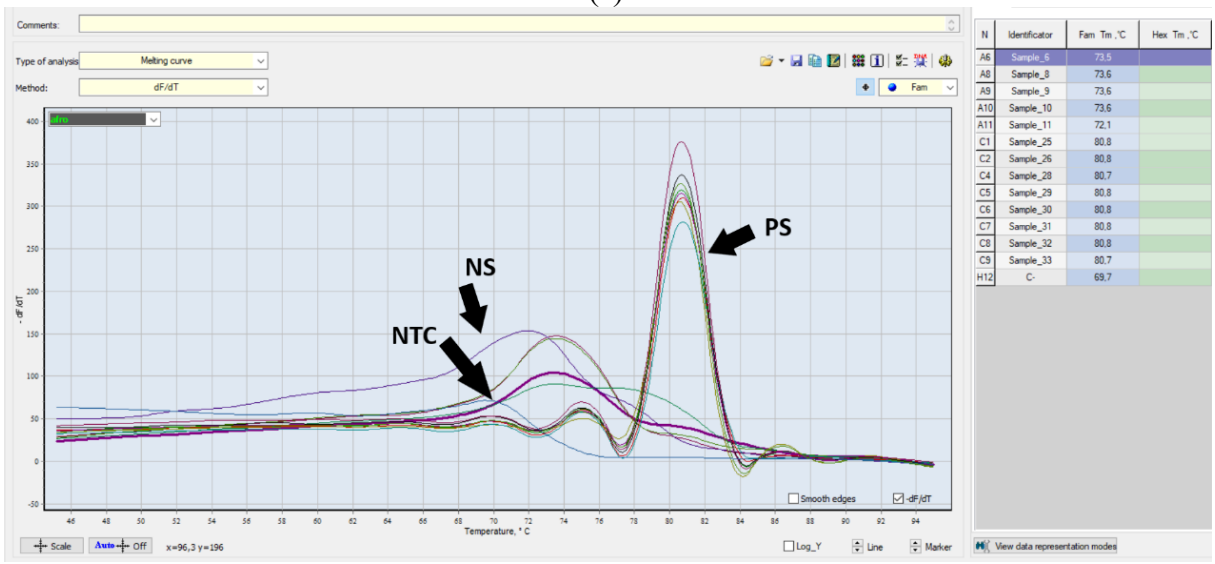


Figure 10. Agarose gel electrophoresis of F2-R PCR products (126bp, shown with red arrows), F2-Rb PCR product (214 bp, shown with yellow arrow), and F2-Ra PCR product (209bp, shown with green arrow).

In Figures 11 and 12 Ct curves and melting curves of positive and negative samples, and non-template controls are shown for F2-R and F2-Rb protocols, respectively.

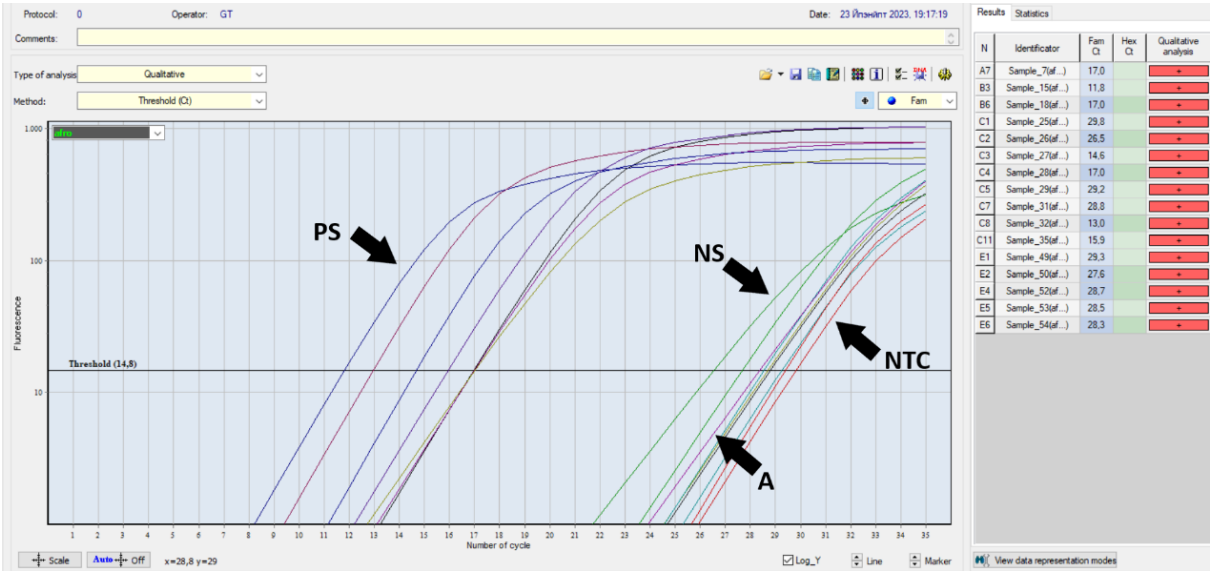


(a)

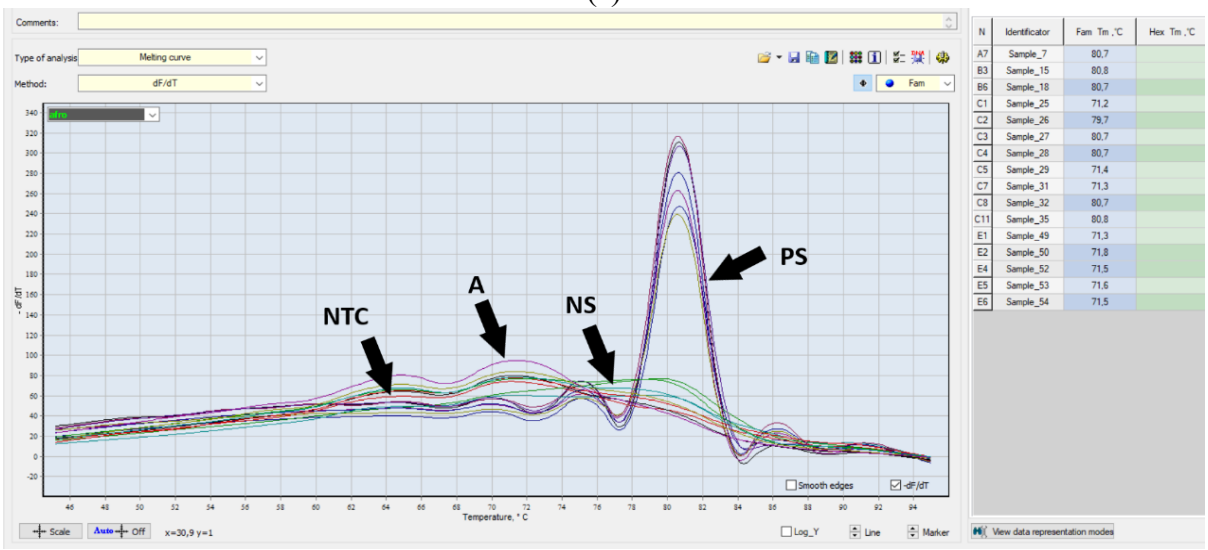


(b)

Figure 11. Cycle threshold (Ct) curves (a) and melting curves (b) of F2-R protocol in positive samples (PS), negative samples (NS), and non-template control (NTC). Positive samples produced a single peak melting curve (80.4-80.8 ^{o}C).



(a)

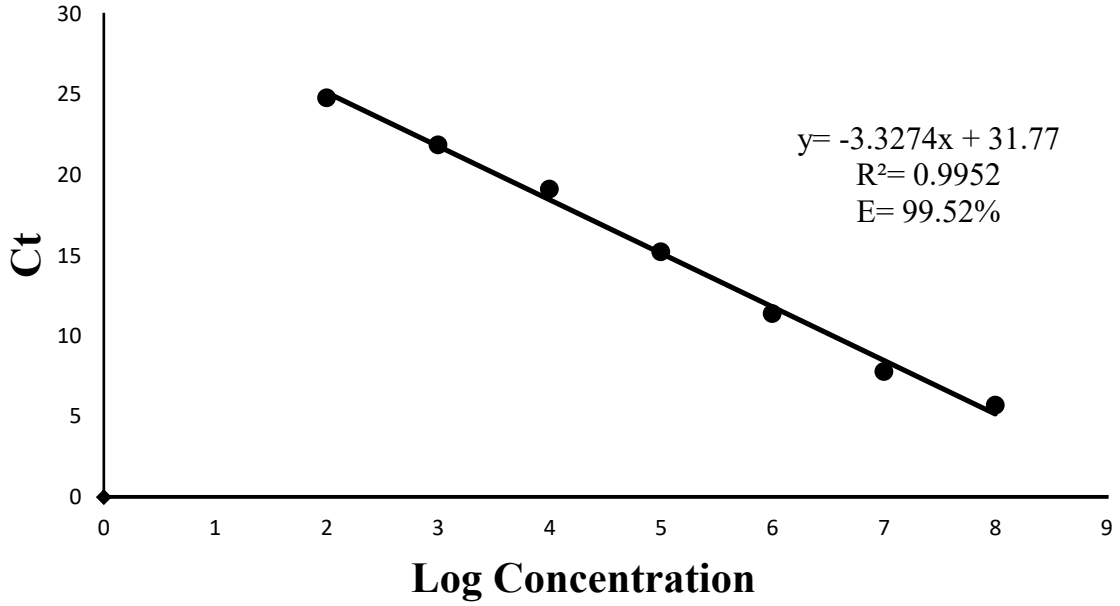


(b)

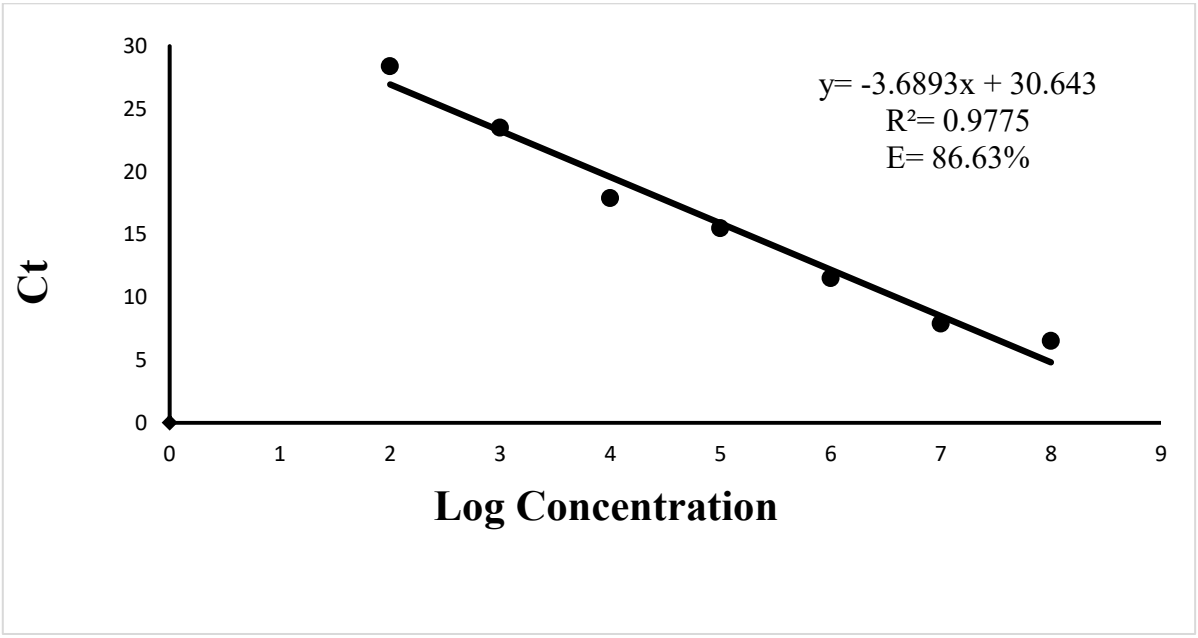
Figure 12. Cycle threshold (Ct) curves (a) and melting curves (b) of F2-Rb protocol in positive samples (PS) of genotype B, positive samples of genotype A (A), negative samples (NS), and non-template control (NTC). Positive samples of genotype A produced a single peak melting curve (80.7-80.9 ^{o}C).

As shown in Figures 11 and 12, tested samples can be considered as positive or negative after evaluating both the Ct value and the melting curve. In particular, a sample was considered positive when Ct value was lower than 20 and the melting curve presented a single peak at temperature 80.4-80.8 ^{o}C and 80.7-80.9 ^{o}C in protocol F2-R and F2-Rb, respectively. Non-template controls and negative controls produced primer dimers with low Tm (~70 ^{o}C) which are not confused with the Tm of the specific real-time PCR products. Also, sequences of genotype A did not produce positive results when tested with the protocol specific for the strains of genotype B.

The efficiency and R^2 of F2-R and F2-Rb real-time PCR assays were 99.52% and 0.9952, and 86.63% and 0.9775, respectively, as shown in the standard curves generated with the serial 10-fold dilutions (Figures 13a and 13b).



(a)



(b)

Figure 13. Standard curves of the F2-R (a) and F2-Rb (b) real-time PCR assays. Ct (cycle threshold) values of serial dilutions were plotted against the log concentration of gBlock gene fragment (a) and DNA sample (b). Regression equations with the coefficient of correlation (R^2) and efficiency of the reaction (E) are also shown. The dots in the regression line represent the result of a triplicate amplification of each serial dilution.

The LOD was calculated for the F2-R assay amplifying ten replicates of dilutions of gBlock gene fragment with known copies. The probit analysis revealed that the assay was able to detect 178 copies.

IV. Discussion

The commercial indirect ELISA used in the present thesis detected SRLV infected animals in all the studied farms, despite the genetic variability of the circulating strains between and within the farms. This is possibly attributed to the presence of whole virus as antigen, which increases the detection spectrum of specific antibodies compared to ELISA based on capsid or transmembrane peptides (De Andrés *et al.*, 2013). At the same time, the use of whole virus-based ELISA possibly permitted the detection of infected animals regardless of the infection stage and the presence of antibodies, either against gp135 in the case of chronically infected animals or p28/p25 capsid antigens in the case of currently infected animals (Singh, McConnell and Blacklaws, 2006). The sensitivity and specificity values of the ELISA test were 82.8% and 93.8%, respectively, and are lower compared to the results from a previous study evaluating its diagnostic performance in sheep (98.6% and 100%, respectively) (De Andrés *et al.*, 2005). Also, the concordance of ELISA with the results obtained from all PCR (*k*-value = 0.620) protocols was substantial according to the approach of Landis and Koch (Landis and Koch, 1977). However, in this study, the combination of several different conventional PCR results as a reference method rather than serological methods decreased the detection of infected animals from the ELISA test, as many infected animals might have not seroconverted yet.

The molecular investigation of SRLV infections in this study confirmed the fact that the diagnostic performance of PCR protocols is variable and is undermined by the mismatching of primers at the binding sites due to the genetic variability of the circulating viral strains. In this study, seven conventional PCR protocols with target sequences in highly (LTR, *gag* gene, *pol* gene) or less (*env* gene) conserved genomic regions (Zanoni *et al.*, 1992; De Andrés *et al.*, 2005) were evaluated for their diagnostic performance. Their sensitivity values ranged from 7.5% for the nested LTR protocol to 79.2% for the Gag-pol nested protocol, indicating the gag/pol region as more conserved and appropriate for the designation of primers. On the other hand, the specificity values, which ranged from 77.8 to 85.2%, were attributed to the "gold standard" used in the study (as reference infected animals were considered the animals positive in both ELISA and at least one PCR protocol). The concordance of PCR protocols with reference results was found to be moderate for LTR nested and Gag-pol nested protocol,

fair for gag nested protocol, and poor for all the other PCR protocols. The evaluation of the diagnostic performance of PCR protocols for SRLV infections is extremely difficult to be objective, as the lack of a "gold standard" or universally applicable molecular protocols leads to the categorization of detected animals with the new PCR protocol as false positives. In this case, the real specificity and concordance values can be evaluated only after the sequencing of all the ambiguous PCR products. In our study, all 37 sequenced PCR products were SRLV-specific, proving the high specificity of molecular tests.

Also, the poor diagnostic performance of some conventional PCR protocols in the studied farms resulted from the mismatch between the circulating strains and the detection spectrum of primers. For example, the LTR protocol, which was designed in the LTR region of the K1514 strain of genotype A (Sonigo *et al.*, 1985), did not produce any positive results in farm D, where the circulation of strains belonging to the B genotype was shown after the phylogenetic analyses of *gag-pol* and *env* sequences. On the other hand, the Gag nested protocol presented high sensitivity and specificity values (66.0% and 77.8%, respectively) compared to other protocols and produced positive results in all farms, although it was designed using the CAEV-Co strain of genotype B (Barlough *et al.*, 1994). The most possible explanation is that primers for this protocol were designed in highly conserved regions of the *gag* gene, as the phylogenetic tree of these sequences produced two separate clusters: one cluster was mostly associated with strains of genotype A and the other with strains of genotype B. However, the highest diagnostic performance was recorded for the Gag-pol nested protocol which was designed using strains of both A and B genotypes (Grego *et al.*, 2007), confirming the significance of primer designation for the efficient molecular diagnosis of infected animals in different regions. On the other hand, the very low diagnostic performance of Gag and Pol protocols (Grego *et al.*, 2002), despite their primer designation using strains of different genotypes, may be attributed to the production of weak signals, due to the low viral load, which is enhanced in the cases of nested protocols.

Phylogenetic analyses revealed the circulation of SRLV strains belonging to genotypes A and B (subtype B2) in the studied farms. Although phylogenetic data regarding the circulating strains in our country are limited, previous studies have also reported the existence of strains belonging to genotypes A (Angelopoulou *et al.*, 2005, 2006; Chassalevris *et al.*, 2020) and B (Chassalevris *et al.*, 2020). Specifically, regarding genotype A, the most homologous strains to the sequences from this study were previously isolated in Greece (Angelopoulou *et al.*, 2005), in Italy, and in neighbouring or nearby countries of the Middle East (Turkey, Italy, Lebanon, and Jordan). On the other hand, the most homologous strains to the B2 sequences

from this study were isolated in Italy, France, and Spain (Grego *et al.*, 2005, 2007; Germain and Valas, 2006; Glaria *et al.*, 2009). These findings are in consistency with previous phylogenetic studies indicating the Middle East as the ancestral region of the A genotype and Central Europe of B genotype (Molaee *et al.*, 2020; Carrozza *et al.*, 2023).

Although genotype A includes MVV-like strains, whereas genotype B includes CAEV-like strains, cross-species transmission of strains of these two genotypes between sheep and goats has been previously reported (Leroux, Cruz and Mornex, 2010; Da Cruz *et al.*, 2013; Michiels, Adjadj and De Regge, 2020). In particular, subtype B2, which was found in our study, has been reported in sheep in many studies in the past (Shah *et al.*, 2004; Grego *et al.*, 2005, 2007; Glaria *et al.*, 2009). The combination of the results obtained from all the constructed phylogenetic trees indicates the circulation of strains of both A and B2 genotypes in farms A and C, whereas in farms B and D all the sequences were associated with strains of genotypes A and B2, respectively. This is in consistency with the results from mean pairwise genetic distances between the sequences; all sequences originating from the same farm presented genetic heterogeneity <15.0%, which is considered the limit for the categorization of sequences in the same subtype (Shah *et al.*, 2004), except for LTR sequences in farm C (15.2%), and the *gag-pol* sequences in farm A (15.4%). The clustering of a sample (40SX) from farm A with the strains of genotype A in the case of LTR phylogenetic analyses and with strains of subtype B2 in the case of *gag-pol* phylogenetic analyses may be attributed either to co-infection with A and B2 genotypes (Pisoni, Bertoni, *et al.*, 2007; Ramírez *et al.*, 2012; Fras *et al.*, 2013) or to the circulation of a recombinant strain due to the animal co-infection with multiple strains (Ramírez *et al.*, 2011; L'Homme *et al.*, 2015; Olech and Kuźmak, 2021). Based on the results from the phylogenetic analyses, three real-time PCR protocols were designed and evaluated for their efficiency (the universal protocol, and protocols for A and B genotypes). The limited specificity of the protocol for the detection of strains belonging to the A genotype may be associated with weaknesses in primers' designation, leading to matching with non-specific regions in the DNA samples. The fact that all primers were blasted and checked for their specificity during their designation highlights the complexity of the development of efficient molecular diagnostic protocols for SRLV infections.

Also, despite the designation of primers based on the sequencing of field samples, a first round of PCR was necessary to increase the sensitivity of the assay. Nested PCR protocols reduce non-specific binding in the case of samples rich in genomic DNA and enhance the sensitivity and specificity of the assay.

Currently, only a TaqMan real-time PCR protocol in the *pol* region has been previously developed in our county for the detection of SRLV infections belonging to A and B genotypes (Chassalevris *et al.*, 2020). Similarly, in the present study, the real-time PCR protocols developed were evaluated in field samples belonging to A and B genotypes from both indigenous Chios and imported Lacaune sheep reared in four intensive farms, demonstrating high efficiency and R^2 values. Also, considering the economic aspect, these real-time PCR protocols are based on the SYBR Green method, and the primers do not include costly modifications, significantly reducing the cost of the reaction and permitting its use as a routine molecular diagnostic test. Also, the degeneracy of the primers could be easily modified in the future to improve the diagnostic performance of the protocol, considering the emergence of new strains in the country. Moreover, the F2-R protocol was initially designed for the detection of SRLV strains of all genotypes, and its future evaluation with strains belonging to genotypes C and E could confirm its universal application for the detection of infections from these genotypes.

Taking into account all the above, the newly developed nested SYBR Green real-time PCR protocol F2-R presented high diagnostic performance for the detection of SRLV infections from strains of A and B genotypes and was considered appropriate for use in the subsequent prospective epizootiological study. Also, beyond the present study, this real-time PCR protocol could be exploited as a valuable diagnostic molecular tool for the early and accurate diagnosis of SRLV infections in our country. In any case, further evaluation of its diagnostic performance in more field samples belonging to various genotypes of SRLV is needed.

Chapter 2: Prospective cohort study and risk assessment for small ruminant lentiviruses infections in intensively reared dairy ewes and their lambs

I. Objectives

Formal data regarding SRLV prevalence in European countries are insufficient and derive mainly from limited voluntary epizootiological studies conducted in specific regions and breeds, including animals reared under dissimilar farming systems, rather than from systematically applied national surveillance programs. However, these studies may not be representative of the current situation. Indeed, the inclusion of MV and CAE in the list of notifiable terrestrial and aquatic animal diseases by the WOAH and the subsequent limitations in the trading of breeding stocks discourage sheep breeders from voluntarily investigating the presence of the disease on their farm.

Currently, investigation of SRLV prevalence and associated risk factors is based on cross-sectional sero-epizootiological studies. According to these studies, seroprevalence values vary among countries, and several potential risk factors have been recognized to be associated with SRLV seropositivity at the animal level (e.g., age, breed, sex) and the farm level (e.g., intensive farming system, increased flock size, unfavorable housing conditions, breeding stocks trade etc.) (Arsenault *et al.*, 2003; Shuaib *et al.*, 2010; Hüttner, Seelmann and Feldhusen, 2010; Pérez *et al.*, 2010; Lago *et al.*, 2012; Kaba *et al.*, 2013; Norouzi *et al.*, 2015; Junkuszew *et al.*, 2016; Alves *et al.*, 2017; Michiels *et al.*, 2018; Pavlak *et al.*, 2022). However, results from this type of study are not always reliable due to the underdiagnosis of infected animals, as some of them may remain constantly seronegative from months to years after the infection, demonstrate fluctuating antibody titers, or even serorevert (Minguijón *et al.*, 2015). Moreover, in cross-sectional sero-epizootiological studies, the major drawback is the imperfect diagnostic performance of the applied diagnostic tools, which can lead to the misclassification of animals based on a single observation of their serological status. Underdiagnosis of infected animals in these studies undermines the successful implementation of control programs and hampers the eradication of SRLV. On the contrary, prospective epizootiological studies, which are based on both serological and molecular diagnostic tools and follow the studied animals over time, limit the effects of imperfect diagnosis and allow the extraction of safer conclusions regarding the epidemiology and risk factors of chronic diseases (Lau, Gange and Moore, 2007; Song and Chung, 2010).

In Greece, the increasing demand for high-yielding breeding stocks has lately led to a remarkable increase in the importation of breeding stocks from European countries and/or

from local intensive farms with unknown or, in some cases, high SRLV prevalence rates. Despite this fact and the evidence of extensive virus spread, currently, the epizootiological data regarding SRLV infections in the country is scarce (Ploumi *et al.*, 2001; Karanikolaou *et al.*, 2005) and the role of potential risk factors has not been sufficiently documented yet. The scarcity of epizootiological data on SRLV does not allow the designation of evidence-based control programs, which should be precisely adapted per region and farming system according to the morbidity frequency measures and the potential risk factors (genetic predisposition of breeds, management, and productive orientation).

The objectives of this epizootiological study were to: i) calculate morbidity frequency measures; ii) determine serological patterns; iii) prospectively study the potential risk factors associated with animal seropositivity and infection, the manifestation of specific serological patterns, and the occurrence of a seroconversion or a seroreversion incident; and iv) evaluate the significance of horizontal and vertical transmission of SRLV in intensive dairy sheep farms in Greece.

II. Materials and Methods

1. Farm and animal selection

The selected farms that were used in the development of the diagnostic protocol described in Chapter 1 were also enrolled in the prospective epizootiological study. In detail, a total of ten intensive, zero-grazing dairy sheep farms were initially surveyed during on-site visits and interviews with the farmers, using a structured questionnaire (Appendix A) to collect data regarding the farms' characteristics and management practices. Among them, four intensive dairy sheep farms with purebred Chios (farms A, B, and C) and Lacaune sheep (farms A and D), located at different counties in Greece (Figure 14), were selected and enrolled in the study on the basis of i) being representative of the intensive system (Gelasakis *et al.*, 2012), ii) applying similar management schemes (Table 14), iii) being recently found to be seropositive in SRLV, and iv) fulfilling the terms of participation and collaboration during the whole duration of the study.

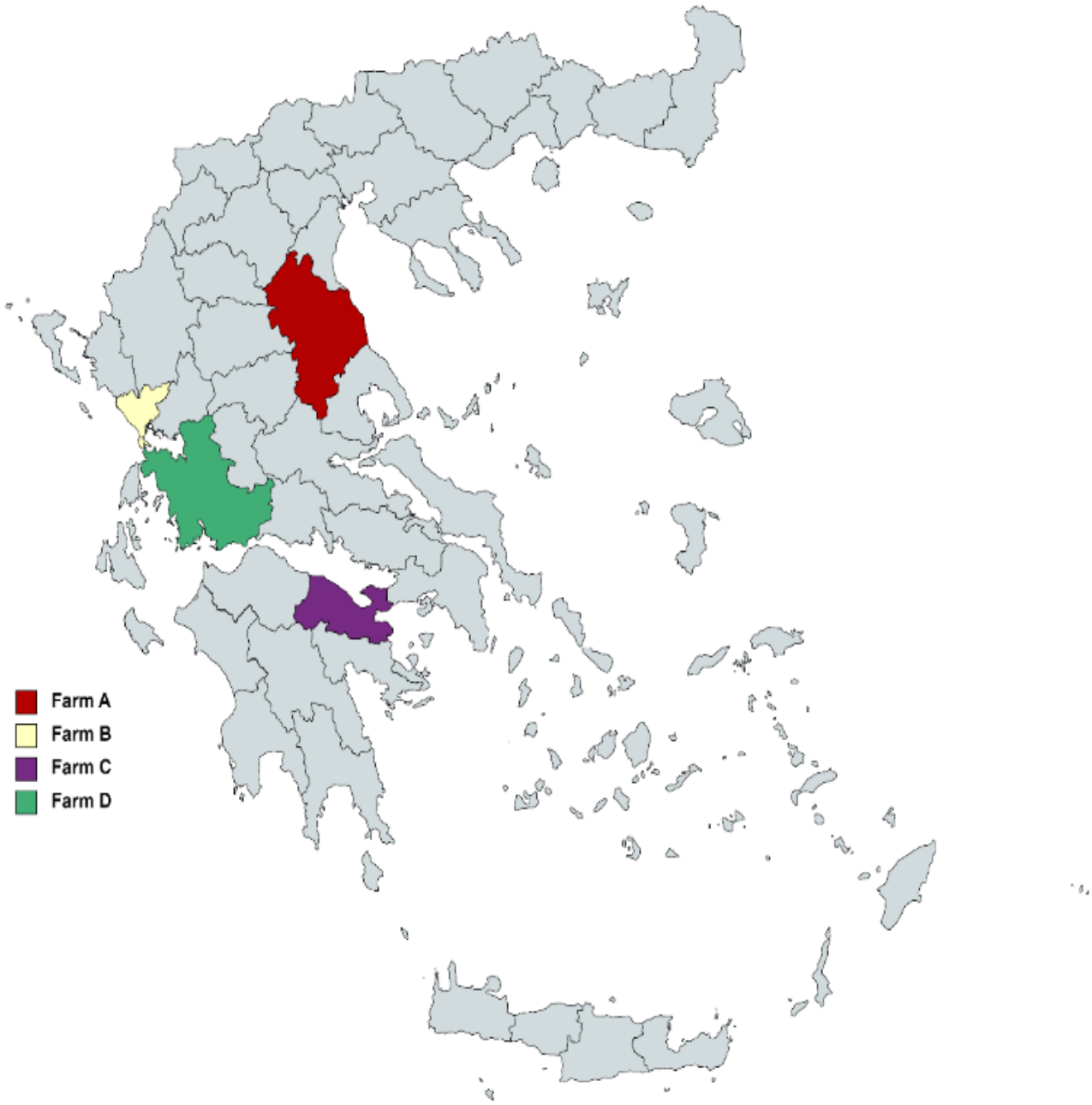


Figure 14. Geographical distribution of the studied farms.

Table 14. Farm characteristics and herd management practices in the studied farms.

	Farm A	**Farm B**	**Farm C**	**Farm D**
Location	Larissa	Preveza	Korinthos	Aetolia-Acarnania
Breed	Chios/Lacaune	Chios	Chios	Lacaune
Years of operation	7	7	4	9
Animals per employee (n)	118	100	224	115
Total animal number (n)	241	208	925	230
Milking ewes (n)	156	160	615	148
Replacement rate (%)	30	25	40	25
Shed area/ewe (m^2)	1.42	2.70	1.53	1.50
Shed volume/ewe (m^3)	7.10	13.50	12.24	5.20
Type of ventilation	Natural	Natural	Natural and mechanical	Natural and mechanical
Ventilation conditions	Poor	Good	Very good	Medium
Type of bedding	Straw	Straw	Straw	Straw
Frequency of manure removal (times/year)	1	3	6	1
Feeder space/sheep (cm)	20.7	31.3	35.9	34.4
Exercise paddock	No	Yes	No	Yes
Method of mating	Natural/groups	Natural/single sire groups	Natural/groups	Natural/single sire groups
Ewes:rams ratio	20	20	24	24
Milk yield/ewe/lactation 210 days (kg)	300	400	250	450
Prolificacy (lambs/ewe)	1.4	2	1.9	1.6
Method of lamb rearing	Natural	Artificial	Artificial	Artificial
Colostrum	Unpasteurized ewe colostrum	Unpasteurized ewe colostrum	Pasteurized ewe colostrum	Pasteurized ewe colostrum
Weaning age (days)	40	45	50	35
Method/frequency of milking (times/day)	Mechanical/2	Mechanical/3-2	Mechanical/3-2	Mechanical/2
Vaccinations—treatments:				
Enterotoxemia	✓	✓	✓	✓
Pasteurellosis	–	–	✓	–
Contagious agalactia	✓	✓	✓	✓
Enzootic abortion	✓	✓	✓	✓
Anthelmintic treatment	✓	✓	✓	✓
Dry-off treatment	–	✓	✓	✓
Health issues:				
Mastitis	<10%	5%	10%	<10%
Abortions	<5%	<5%	5%	<5%
Lameness	<5%	<5%	<5%	5%
Pregnancy toxaemia	<5%	<5%	<5%	<5%
Confirmed maedi-visna clinical cases	No	No	No	No

2. The epizootiological study design in ewes

From the selected farms, a total of 660 ewes (6 months to 7 years old) were randomly selected and included in the study, which initiated 3–4 weeks pre-mating. In particular, 93 ewes from farm A, 187 ewes from farm B, 200 ewes from farm C, and 180 ewes from farm D were enrolled in the study. The selected ewes were separately penned within each farm and prospectively studied for two consecutive years, from May 2020 to July 2022. Samplings were performed semiannually for two consecutive years, in specific physiological stages across the production life cycle of sheep, namely 3–4 weeks pre-mating and 2–4 weeks pre-lambing, and for each individual animal, a serum and a whole blood sample were collected for the ELISA and real-time PCR testing as previously described in Chapter 1. In each sampling occasion ear tag, breed, age, and body condition score (BCS, 1–5, 1=emaciated, 5=obese with 0.25 increments) (Russel, Doney and Gunn, 1969) were recorded. Also, ewes were physically examined, and 17 health and welfare indicators were assessed at the animal level. The recorded health and welfare indicators and the effects of SRLV infections on their occurrence are presented in detail in Chapter 3.

3. The epizootiological study design in lambs

A total of 195 lambs from the studied ewes (20, 53, 37, and 85, from farms A, B, C, and D, respectively) were prospectively studied from their birth until their first parturition. As shown in Table 14, farm A applied natural suckling with unpasteurized colostrum, farm B applied artificial rearing with unpasteurized colostrum, whereas farms B and C applied artificial rearing with pasteurized colostrum. Colostrum was pasteurized by heating at 56 ˚C for 60 min. The studied lambs on all farms were kept separately until the age of 8-9 months, when they were mixed with adult ewes and rams during mating. Lambs from the studied ewes were sampled four times during the study: the first month of their life during suckling or artificial rearing, the third month of their life (post-weaning), at pre-mating (8 months old), and at pre-lambing (13 months old). For each individual animal, a serum and a whole blood sample were collected for the ELISA and real-time PCR testing as previously described in Chapter 1. Also, the serological and infection status of the dams were recorded at pre-lambing for 169 out of the 195 studied lambs.

4. ELISA and real-time PCR-based diagnosis of small ruminant lentiviruses infections

All serum samples were analyzed as previously described in Chapter 1 for the detection of anti-SRLV antibodies with an indirect whole-virus commercial ELISA test. Also, all DNA samples extracted from whole blood samples, as described in Chapter 1, were tested with the

newly developed real-time PCR protocol F2-R for the detection of SRLV infections. In particular, a sample was considered positive when the Ct value was lower than 20 and the melting curve presented a single peak at a temperature 80.4-80.8 ^{o}C. In total, 3,301 ELISA and 2,487 real-time PCR tests were performed during the study.

5. Statistical analyses and epizootiological assessment in ewes

From the initially enrolled ewes, only the records of the ones with at least four consecutive samplings (full set of measurements) were retained and used for the statistical analyses. This resulted in a total of 407 ewes (234 Chios: 25, 143, and 66 from farms A, B, and C, respectively, and 173 Lacaune: 32 and 141 from farms A and D, respectively) with a full set of measurements. At the beginning of the study, ewes were categorized into five age classes as follows: 1 ($x \leq 1$), 2 ($1 < x \leq 2$), 3 ($2 < x \leq 3$), 4 ($3 < x \leq 4$), and 5 ($x > 4$).

5.1 Categorization of ewes according to their serological, PCR, and infection status

In each sampling occasion, ewes were categorized according to the ELISA and real-time PCR results as follows:

-seropositive and seronegative according to the ELISA results (serological status),

-PCR positive and PCR negative according to the real-time PCR results (PCR status),

-infected and uninfected according to the combination of ELISA and PCR results (infection status); an animal was defined as infected with a positive ELISA or real-time PCR test and uninfected when both ELISA and real-time PCR tests were negative. Also, the infected ewes were further grouped into infected seropositive when both ELISA and real-time PCR tests were positive and infected seronegative when only PCR test was positive.

5.2 Categorization of ewes according to their serological and infection pattern

At the end of the study, ewes were categorized according to their temporal serological pattern as follows:

-constantly seropositive (constantly seropositive results during the study),

-constantly seronegative (constantly seronegative results during the study),

-seroconverted (seronegative ewes at the beginning of the study converted to seropositive during the study),

-seroreverted (seropositive ewes at the beginning of the study reverted to seronegative during the study), and

-ewes with an intermittent presence of antibodies (alternating seropositive and seronegative status during the study, regardless of their serological status at the beginning of the study).

The seroconversion/seroreversion incident was defined as the time-point of the seroconversion/seroreversion event, namely, the first sampling occasion that the animal was detected as seroconverted/seroreverted.

Also, at the end of the study, the ewes were categorized according to their temporal infection pattern as follows:

-infected seropositive (tested both PCR positive and constantly seropositive, or with an intermittent presence of antibodies, or seroconverted until the end of the study),

-infected seronegative (tested PCR positive and constantly seronegative, or seroreverted until the end of the study),

-uninfected (tested always both PCR and ELISA negative).

5.3 Comparative assessment of the ELISA and the real-time PCR protocols

The comparison between ELISA and real-time PCR results included a total of 1,907 samples. The concordance between ELISA and real-time PCR results was estimated considering the samples that tested either positive or negative in both assays. Also, *k*-value was calculated and classified as poor (<0.00), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), or almost perfect (0.81–1.00) (Landis and Koch, 1977) .

5.4 Morbidity frequency measures

Morbidity frequency measures included (sero)prevalence (point and period), incidence rate, and cumulative incidence for SRLV infections and were calculated considering only the ELISA results and the combination of ELISA and real-time PCR results. The following formulas were used for the calculation of morbidity frequency measures:

$$\text{Point (sero)prevalence}_n = \frac{\text{positive ewes within n sampling occasion}}{\text{total ewes population within n sampling occasion}}$$

$$\text{Period (sero)prevalence} = \frac{\text{new and pre-existing cases of positive ewes during the study}}{\text{total ewes population during the study}}$$

$$\text{Incidence rate} = \frac{\text{new cases of positive ewes during the study}}{\text{sum of healthy sheep-semesters during the study}}$$

$$\text{Cumulative incidence} = \frac{\text{new cases of positive ewes during the study}}{\text{ewes at risk at the beginning of the study (negative ewes)}}$$

For the calculation of the above-mentioned measures, the following assumptions were followed: i) as a new case was defined the sheep found seropositive for the first time when only ELISA results were considered, and once it was found infected in the case of the combination of ELISA and real-time PCR results; ii) ewes at risk were the seronegative ewes

at the beginning of the study when only ELISA results were considered and the uninfected ewes in the case of the combination of ELISA and real-time PCR results; and iii) for the calculation of the incidence rate, sheep-semester was defined as the unit of the time–animal component. Each seronegative or uninfected ewe contributed to the healthy sheep-semesters until being found positive; once found positive, it did not contribute any healthy sheep-semesters in the study, even if seroreversion occurred or a PCR negative result was recorded.

Prevalence rates and 95% confidence intervals were calculated with R package *epi.prev* using the Blaker method and adjusting for the analytical sensitivity and specificity values of the ELISA test (Zanoni *et al.*, 1994). The respective incidence values were calculated with R package *epi.conf* using the Byar method for incidence rates and the Wilson method for cumulative incidence rates.

5.5 Risk assessment analysis

Descriptive statistics (frequencies and mean values) for various serological and infection status or patterns, seroconversion and seroreversion incidents, age, and BCS were calculated using SPPS v.26.

Multivariable adjusted relative risks (RRs) for i) seropositive, PCR positive, and infected status, ii) seroconversion and seroreversion incidents, and iii) seropositive status in ewes with intermittent presence of antibodies, mixed binary logistic models with repeated measures were generated in SPPS v.26. In these models, breed (2 levels, Chios and Lacaune), sampling occasion (2 levels, pre-mating and pre-lambing), and year of the study (3 levels, 1^{st}, 2^{nd}, 3^{rd}; in the third year only the pre-mating period was considered) were used as fixed effects, age and BCS as covariates, and the random effects of farm and animal were also considered. The animals that were found infected on the first sampling occasion were excluded from the statistical analysis for the RR for PCR positive status for the first time in seroconverted animals.

Also, adjusted RRs for serological and infection patterns were estimated using the breed (2 levels, Chios and Lacaune), the age of the ewes at the beginning of the study (covariate) as fixed effects, and the farm as a random effect in mixed binary logistic models.

In all models, scaled identity was selected as the most appropriate covariance structure according to Akaike's information criterion (AIC).

6. Statistical analyses and epizootiological assessment in lambs

6.1 Categorization of lambs according to their serological and infection status

In each sampling occasion, lambs were categorized as seropositive and seronegative according to the ELISA results (serological status) and as infected or uninfected (infection status) according to the real-time PCR results.

6.2 Categorization of lambs according to their serological pattern

At the end of the study, the lambs were categorized according to their temporal serological pattern as previously described for the ewes (constantly seropositive, constantly seronegative, seroconverted, seroreverted, and lambs with an intermittent presence of antibodies).

6.3 Morbidity frequency measures

Point seroprevalence and prevalence were calculated for each sampling occasion, considering only the ELISA results and the real-time PCR results, respectively.

6.4 Risk assessment analysis

Descriptive statistics (frequencies and mean values) and multivariable adjusted RRs were calculated using SPPS v.26. Multivariable adjusted RRs for i) seropositive status at the age of 13 months old and ii) infected status at the age of 1, 3, 8, and 13 months old were calculated with mixed binary logistic regression models using the breed (2 levels, Chios and Lacaune), the infection status of the dam (2 levels, uninfected and infected), and the type of the colostrum (2 levels, unpasteurized and pasteurized) as fixed effects and the farm as a random effect. In all models, scaled identity was selected as the most appropriate covariance structure according to Akaike's information criterion (AIC).

III. Results

1. Epizootiology of small ruminant lentiviruses infections in ewes

1.1 Serological and infection patterns

A total of 15.2% (62/407) of the studied ewes were constantly seronegative, 46.2% (188/407) were constantly seropositive, 20.1% (82/407) seroconverted, 8.6% (35/407) seroreverted, and 9.8% (40/407) presented an intermittent presence of antibodies. The frequencies of serological patterns per breed are presented in Figure 15. Similar percentages of constantly seronegative and constantly seropositive ewes were observed in both breeds. However, the Lacaune breed demonstrated a higher percentage of seroconverted ewes, and the Chios breed demonstrated higher percentages of seroreverted ewes and ewes with an intermittent presence

of antibodies. Frequencies of serological patterns per farm and breed are presented in Table S1 in Appendix B.

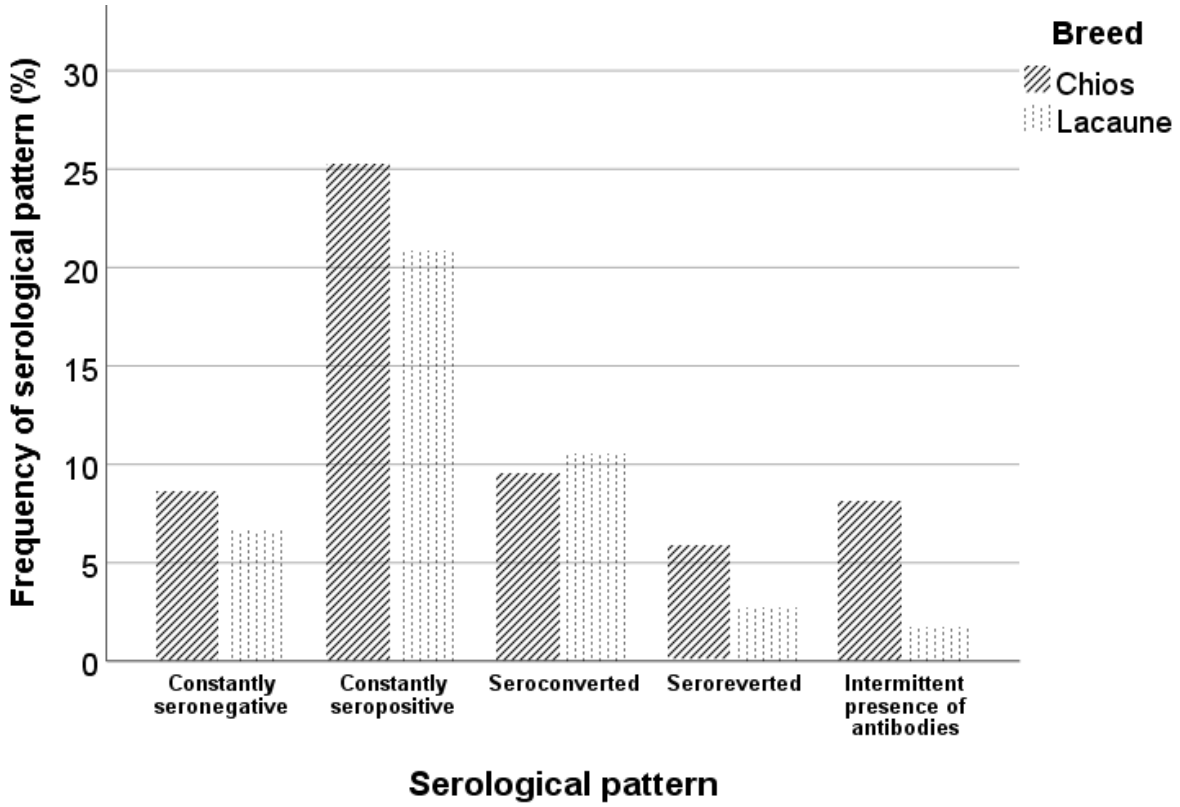


Figure 15. The frequencies of serological patterns in Chios and Lacaune breed.

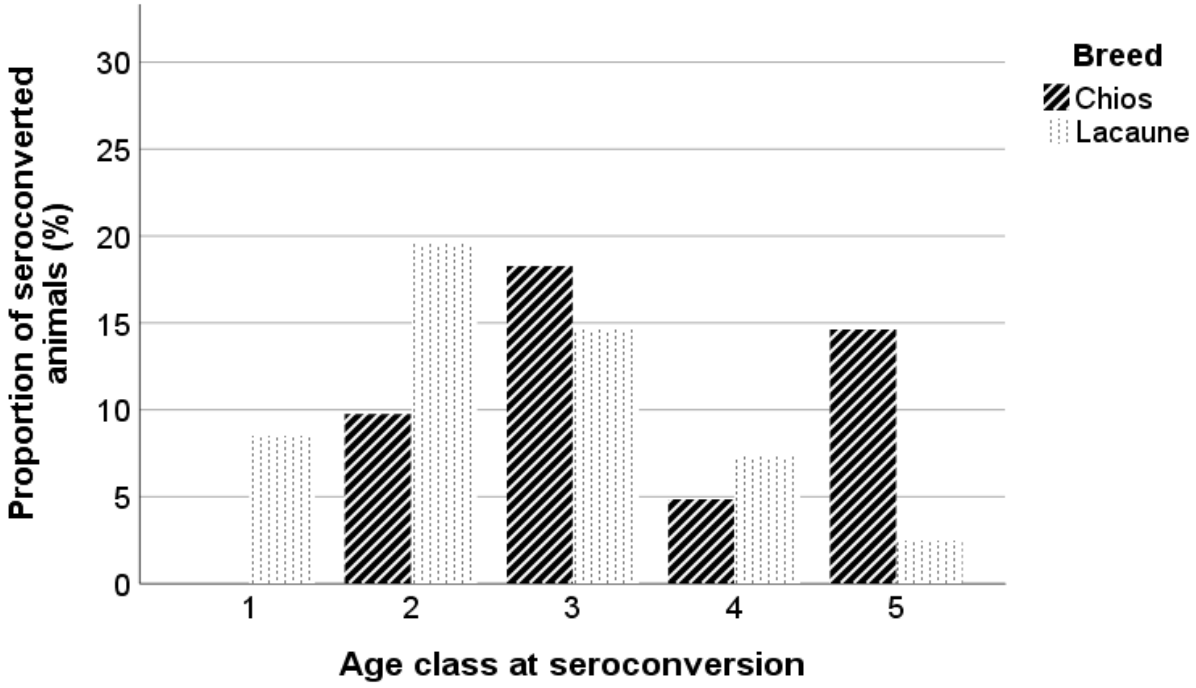
The age of the studied ewes at the beginning of the study ranged from 6 months to 7 years, with a mean value equal to 2.4 ± 1.46 years. The mean ages of Chios and Lacaune ewes were 2.7 ± 1.55 and 1.8 ± 1.16 years, respectively. The mean ages of ewes per serological pattern when introduced in the study are presented in Table 15.

Table 15. Mean age of ewes when introduced in the study per serological pattern.

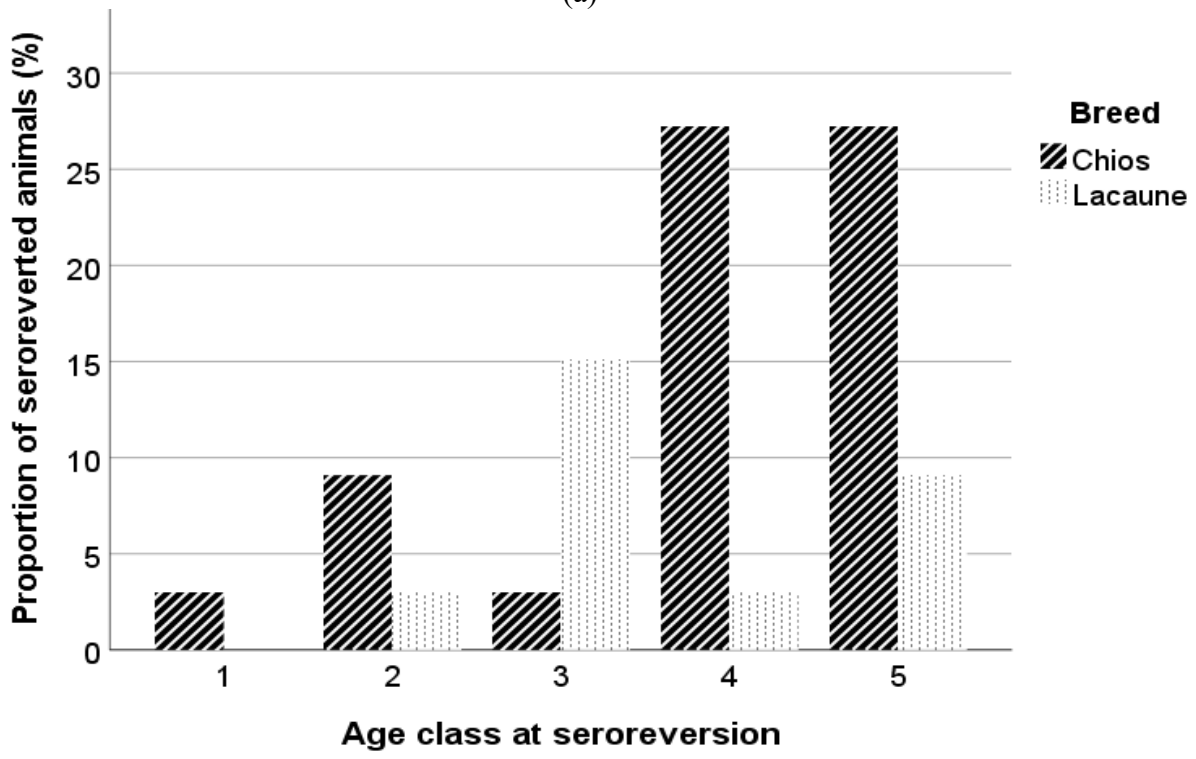
Serological pattern	Mean age ± SD (in years)
Constantly seronegative	1.9 ± 1.18
Constantly seropositive	2.7 ± 1.62
Seroconverted	2.0 ± 1.28 / 2.9 ± 1.38* / 2.7 ± 1.38**
Seroreverted	2.2 ± 1.27 / 3.7 ± 1.47***
Intermittent presence of antibodies	2.1 ± 1.17

*at the seroconversion incident; **when found PCR positive; ***at the seroreversion incident

Among seroconverted ewes, in 8.5%, 29.3%, 32.9%, 12.2%, and 17.1%, of the cases, seroconversion occurred at the first, second, third, fourth, and greater than fourth year of age, while 70.0% of the seroreverted ewes were more than three years old at the seroreversion incident. In Figures 16a and 16b, the age class at the seroconversion and the seroreversion incident in Chios and Lacaune breeds are presented.



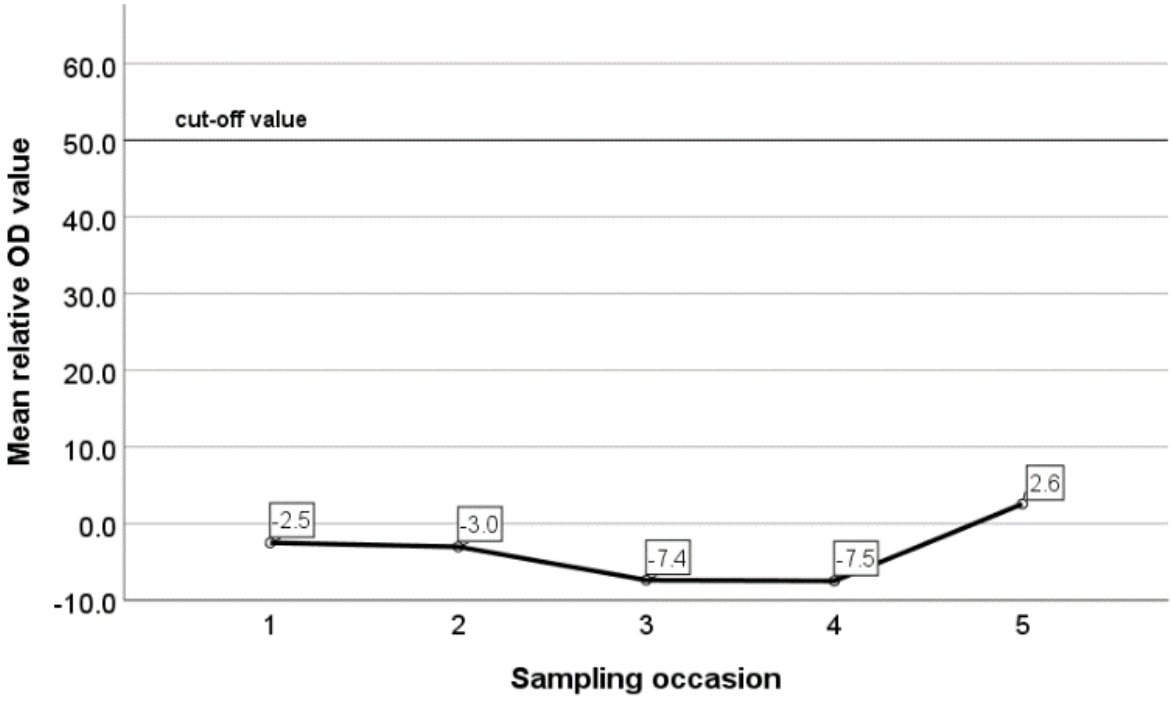
(a)



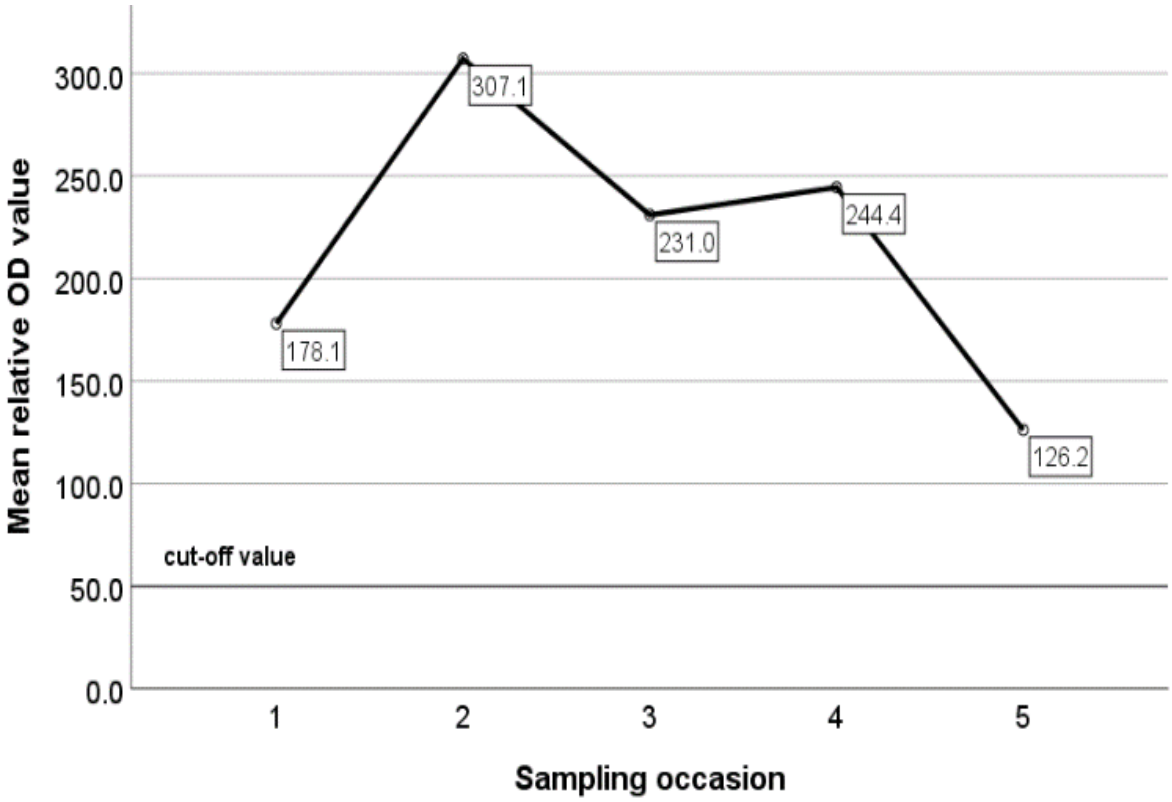
(b)

Figure 16. Age class of Chios and Lacaune ewes at the seroconversion (a) and the seroreversion (b) incident. Age is presented in age classes, 1 ($x \leq 1$), 2 ($1 < x \leq 2$), 3 ($2 < x \leq 3$), 4 ($3 < x \leq 4$), and 5 ($x > 4$).

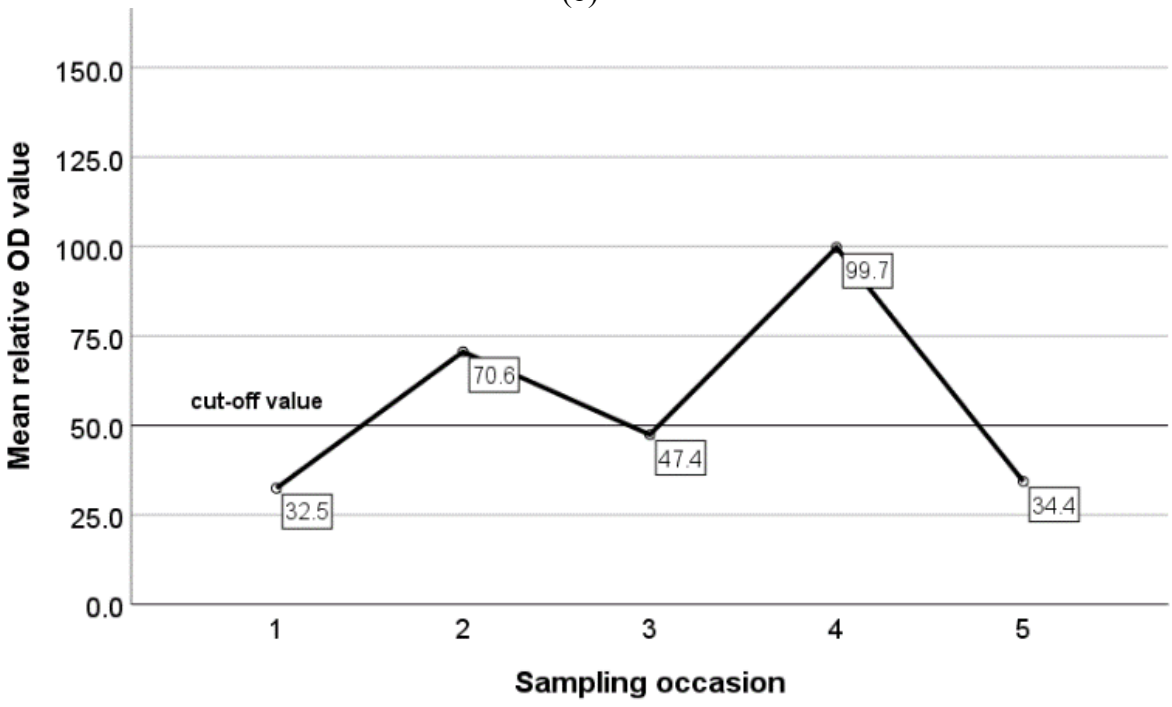
The mean relative OD values of ELISA for constantly seronegative, constantly seropositive, and ewes with an intermittent presence of antibodies were −4.0, 224.1, and 58.2, respectively. In constantly seronegative ewes, the mean relative OD values remained very low and did not present any remarkable variation during the study (Figure 17a), whereas in constantly seropositive ewes and in ewes with an intermittent presence of antibodies, the mean relative OD values increased pre-lambing and decreased pre-mating (Figures 17b and 17c). In seroconverted and seroreverted ewes, and in ewes with an intermittent presence of antibodies, the mean relative OD values were 205.5, 126.6, and 110.6 for the seropositive status and −0.71, 29.4, and 19.0 for the seronegative status, respectively. In Figures 18a and 18b, the mean relative OD values of seroconverted and seroreverted ewes are presented before and after seroconversion/seroreversion incident.



(a)

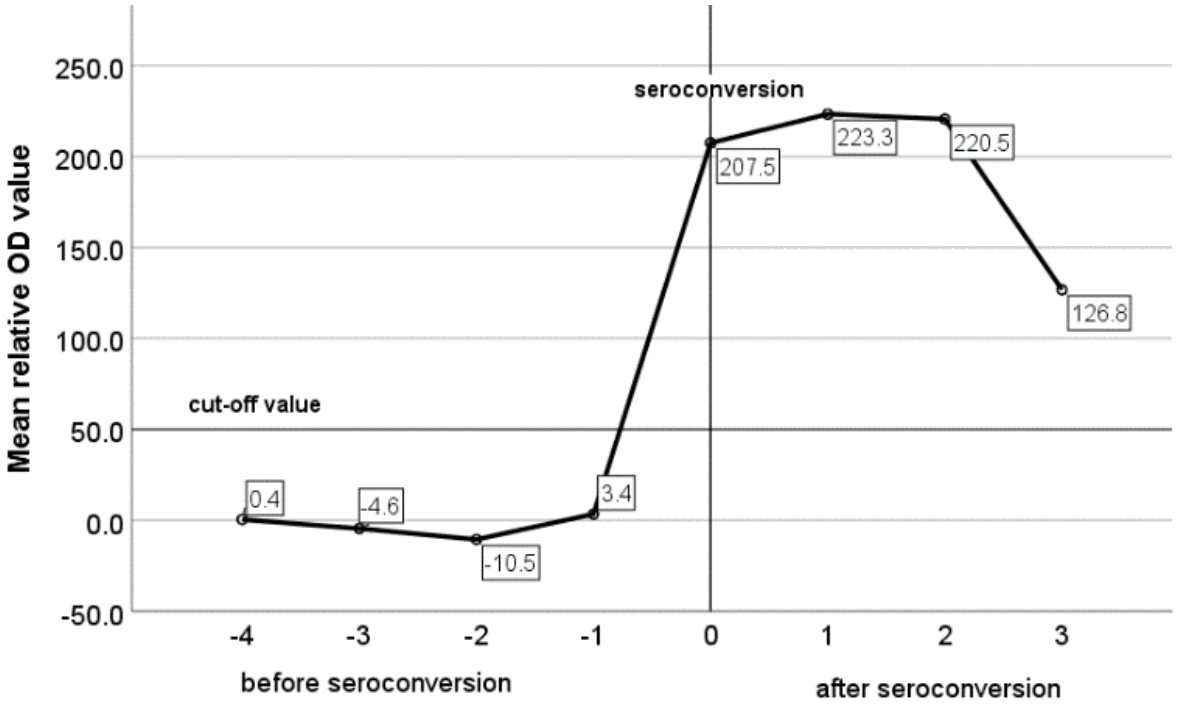


(b)

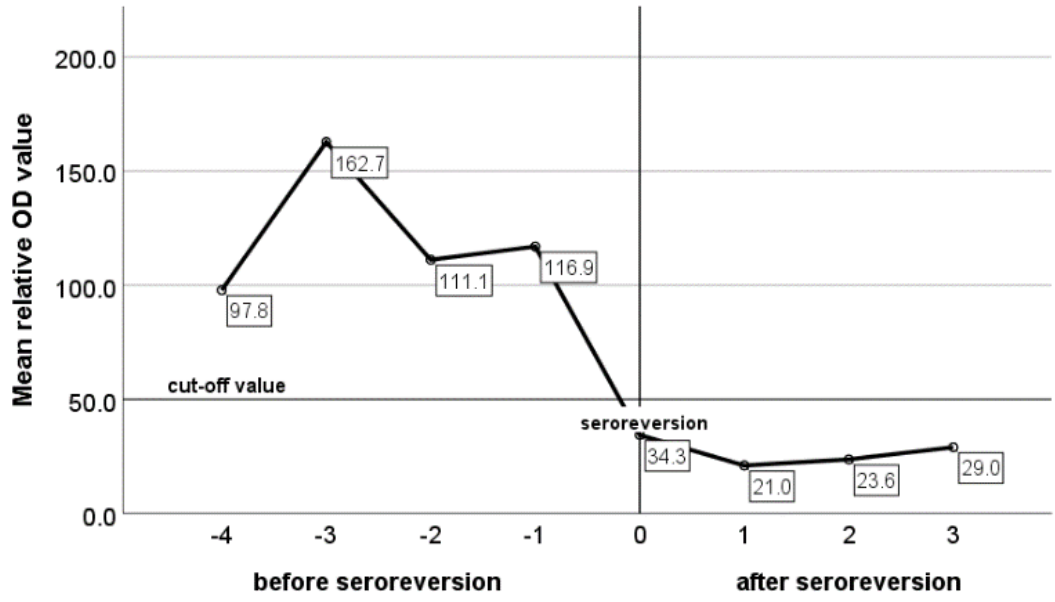


(c)

Figure 17. Mean relative optical density (OD) values of ELISA in constantly seronegative ewes (a), constantly seropositive ewes (b), and ewes with an intermittent presence of antibodies (c) during the study.



(a)



(b)

Figure 18. Mean relative OD values of ELISA in seroconverted (a) and seroreverted (b) ewes before and after the seroconversion/seroreversion incident.

Regarding the infection patterns, only a subtotal of 7.1% (29/407) of the studied ewes were uninfected, whereas 76.2% (310/407) were infected seropositive and 16.7% (68/407) were infected seronegative. The frequencies of infection patterns per breed are presented in Figure 19. The frequency of infected seropositive ewes was similar in both breeds, whereas the percentage of uninfected ewes was higher in the Lacaune breed. On the other hand, the Chios breed demonstrated a higher percentage of infected seronegative ewes. The frequencies of

infection patterns per farm and breed are presented in Table S2 in Appendix B. The mean ages of uninfected, infected seropositive, and infected seronegative ewes when introduced in the study were 1.7 ± 0.96, 2.5 ± 1.52, and 2.3 ± 1.35, respectively.

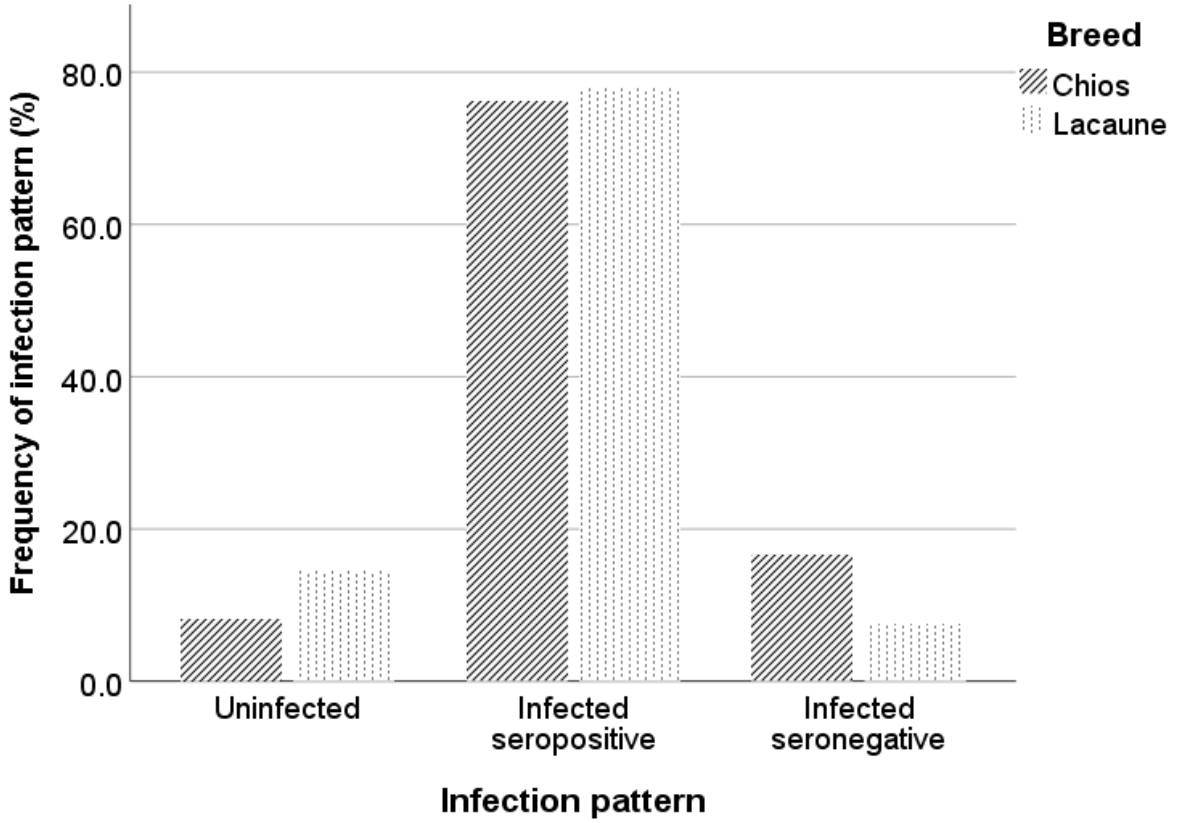


Figure 19. Infection patterns in Chios and Lacaune breeds.

The mean Ct values of real-time PCR for uninfected, infected seropositive, and infected seronegative ewes were 26.1, 14.9, and 13.0, respectively. The infected seropositive ewes had higher Ct values compared to the infected seronegative ewes in each sampling occasion during the study (Figure 20).

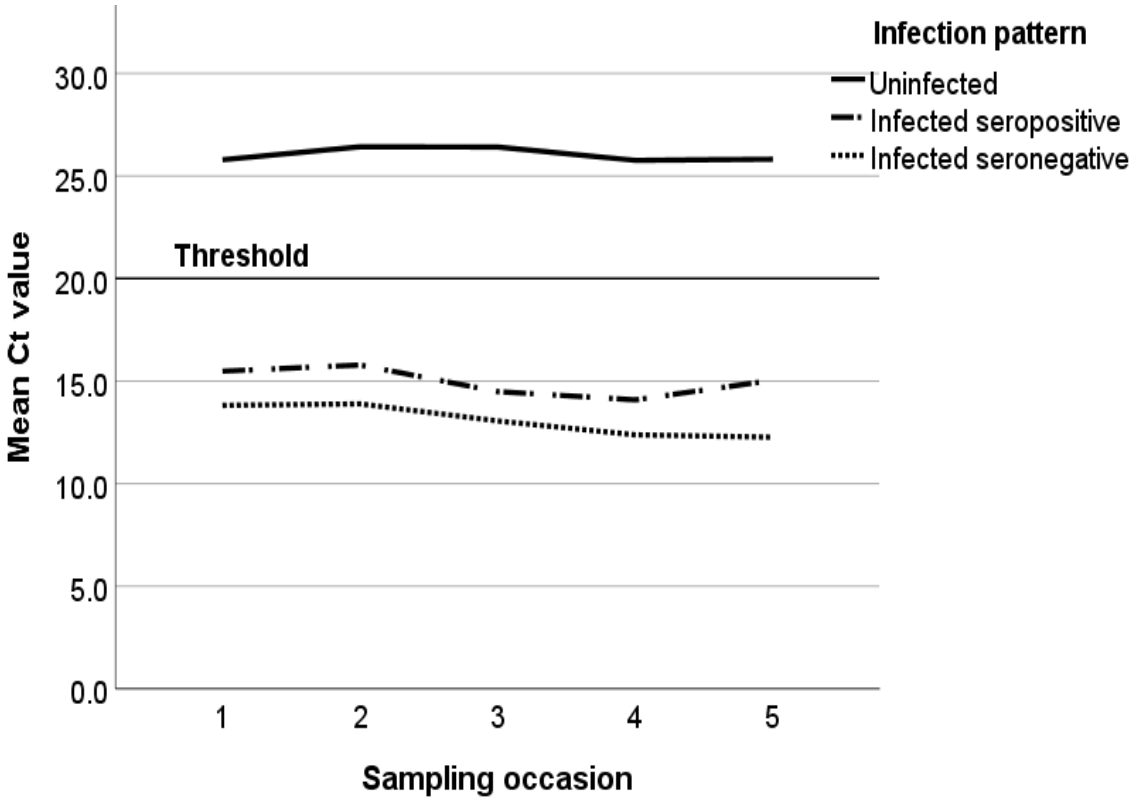


Figure 20. Mean Cycle threshold (Ct) values of real-time PCR testing for uninfected, infected seropositive and infected seronegative animals during the study.

Also, the mean Ct values of real-time PCR for constantly seropositive ewes, seroreverted ewes, and ewes with an intermittent presence of antibodies were 15.4, 13.2, and 15.0, respectively. In seroconverted (after the first PCR positive result) and seroreverted ewes, and ewes with an intermittent presence of antibodies, the mean Ct values were 14.7, 13.5, and 12.8 for the seropositive status and 14.1, 12.8, and 16.6 for the seronegative status, respectively. The mean Ct values for constantly seropositive ewes remained stable during the study (Figure 21).

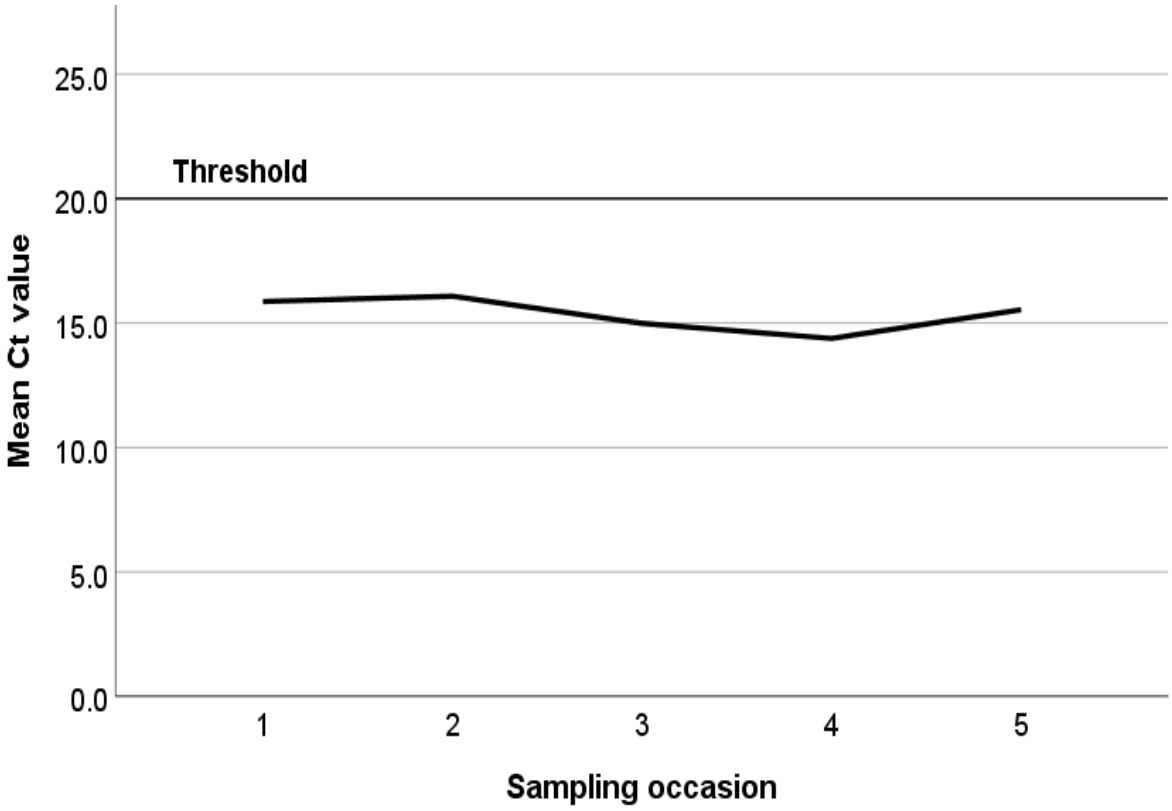
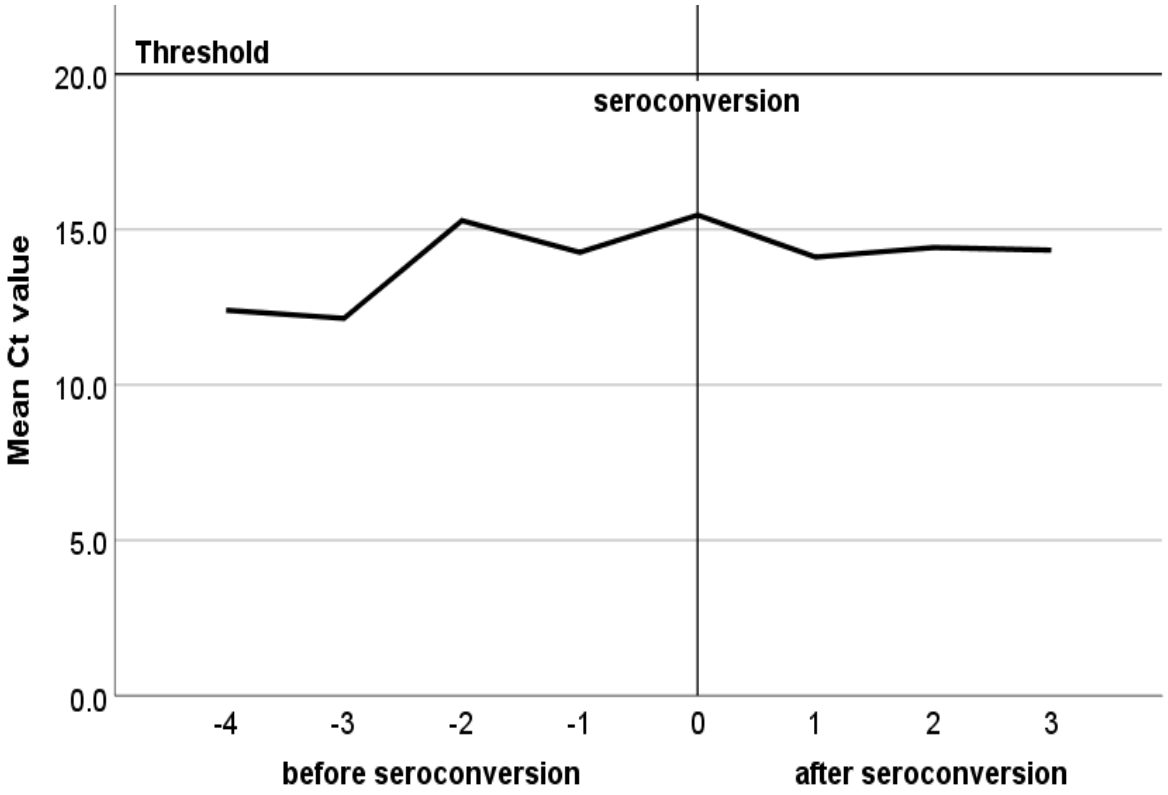
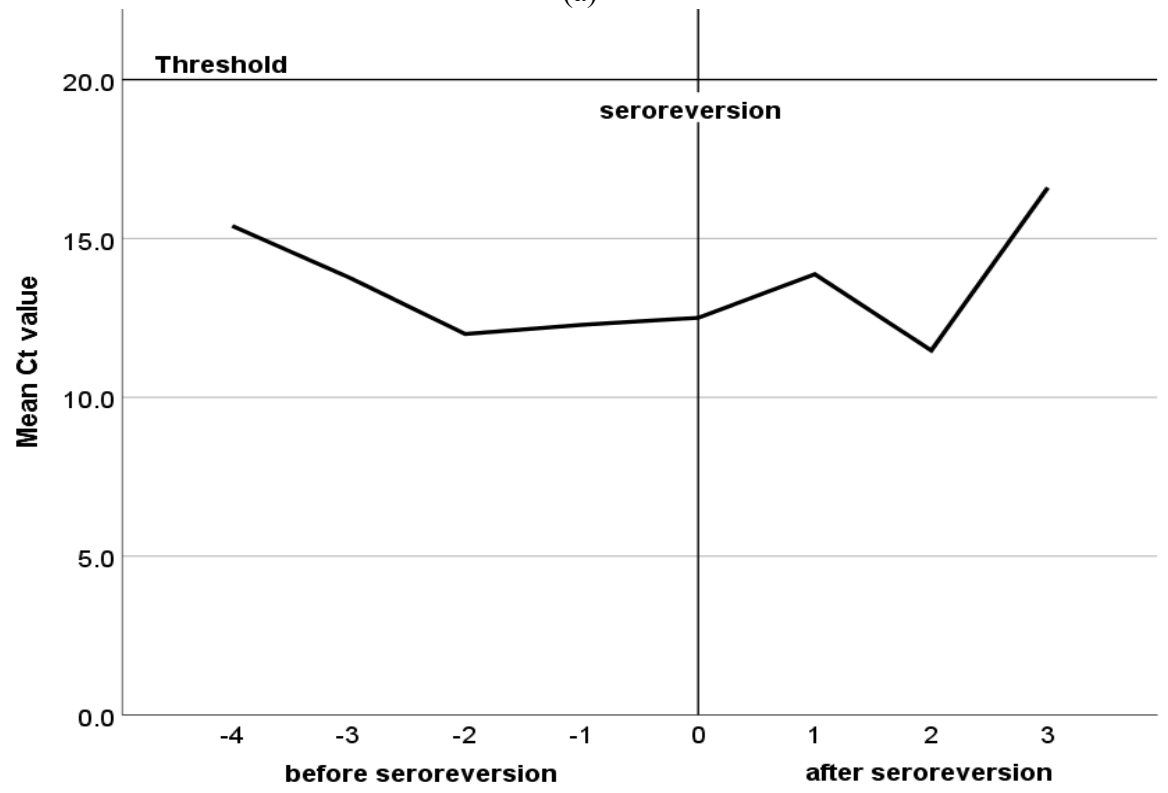


Figure 21. Mean Cycle threshold (Ct) values of real-time PCR testing for constantly seropositive ewes.

The mean Ct values for seroconverted (after the first PCR positive result) and seroreverted ewes before and after the seroconversion/seroreversion incident are presented in Figures 22a and 22b. Seroconverted ewes presented the lowest Ct values in the first sampling occasion after the infection and then the Ct values remained almost stable regardless of the seroconversion incident. The Ct values of seroreverted ewes fluctuated, and the highest values were reported on the most distant sampling occasions from the seroreversion point.



(a)



(b)

Figure 22. Mean Cycle threshold (Ct) values of real-time PCR for seroconverted and seroreverted ewes before and after the seroconversion/seroreversion incident.

1.2 Comparative assessment of the ELISA and PCR protocols

The concordance between ELISA and PCR results in the total of studied ewes during the study was found moderate (*k*-value = 0.477, p<0.001), as 1,486 out of 1,907 samples (77.9%) tested either positive or negative in both assays. The ELISA test detected 91.2% of PCR positive, whereas the real-time PCR protocol detected 97.1% of seropositive animals. *K*-values and concordance between ELISA and PCR results are summarized per age class, farm, breed, and serological pattern in Table 16. The lowest *k*-values and concordance values were recorded in age class 5, in farm C, and in Chios ewes, while in the case of serological patterns, the respective lowest values were estimated for seroreverted ewes and for ewes with an intermittent presence of antibodies. On the other hand, the highest *k*-values and concordance values were recorded in age class 1, in Lacaune ewes, and in seroconverted ewes. In the case of farms, the highest *k*-value was observed in farm D, whereas farm A presented the highest concordance value.

Table 16. *K*-values and concordance between ELISA and PCR results per age class, farm, breed, and serological pattern.

	***k*-value**	**Concordance (%)**
Age class		
1	0.630	81.6 (115/141)
2	0.543	78.1 (313/401)
3	0.498	79.6 (414/520)
4	0.435	77.2 (287/372)
5	0.254	75.5 (357/473)
Farm		
A	0.557	85.3 (233/273)
B	0.480	76.7 (517/674)
C	0.132	64.3 (189/294)
D	0.595	82.1 (547/666)
Breed		
Chios	0.391	74.5 (810/1087)
Lacaune	0.596	82.4 (676/820)
Serological pattern		
Constantly seronegative	na	74.5 (217/291)
Constantly seropositive	na	86.0 (752/874)
Seroconverted	0.563	79.6 (309/388)
Seroreverted	0.001	60.2 (100/166)
Intermittent presence of antibodies	0.204	57.4 (108/188)

ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; *k*-value: kappa coefficient value; age is presented in age classes, 1 ($x \leq 1$), 2 ($1 < x \leq 2$), 3 ($2 < x \leq 3$), 4 ($3 < x \leq 4$), and 5 ($x > 4$); na: the calculation of *k*-value is not available in these cases due to constantly negative or positive ELISA result.

In particular, 345/407 ewes (84.8%) were found seropositive at least once, while 369/407 ewes (90.7%) had a positive PCR result at least once during the study. The PCR results per serological pattern are presented in Table 17.

Table 17. Real-time PCR results per serological pattern.

Serological pattern	PCR (+)*	PCR (-)**
Constantly seronegative	61.3% (38/62)	38.7% (24/62)
Constantly seropositive	97.8% (182/188)	3.2% (6/188)
Seroconverted	97.6% (80/82)	2.4% (2/82)
Seroreverted	97.1 (34/35)	2.9% (1/35)
Intermittent presence of antibodies	97.5% (39/40)	2.5% (1/40)

PCR: Polymerase chain reaction: PCR (+): PCR positive result; PCR (-): PCR negative result *PCR positive result in at least one sampling occasion; **PCR negative result in all sampling occasions.

1.3 Morbidity frequency measures

1.3.1 Point seroprevalence and prevalence

Point seroprevalence ranged from 57.5% (first sampling occasion) to 75.4% (fourth sampling occasion), whereas prevalence ranged from 70.0% (first sampling occasion) to 88.3% (fifth sampling occasion). The point seroprevalence and prevalence for each sampling occasion are presented in Figure 23. Point seroprevalence increased until the fourth sampling and then decreased, whereas point prevalence increased until the last sampling occasion.

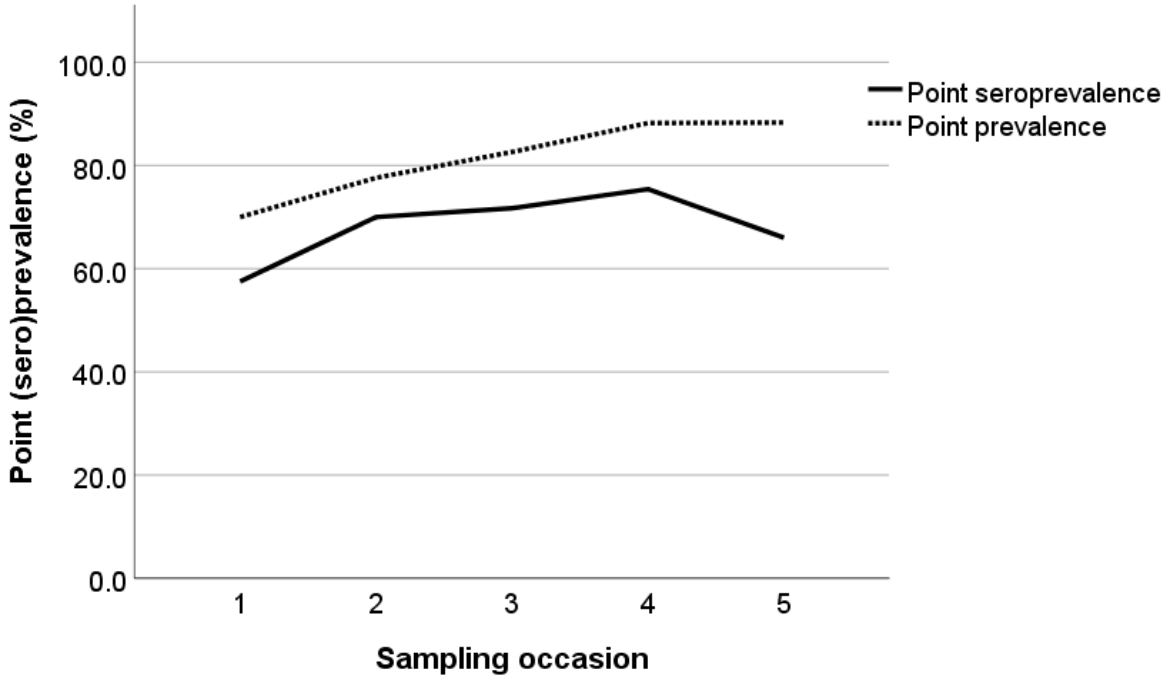
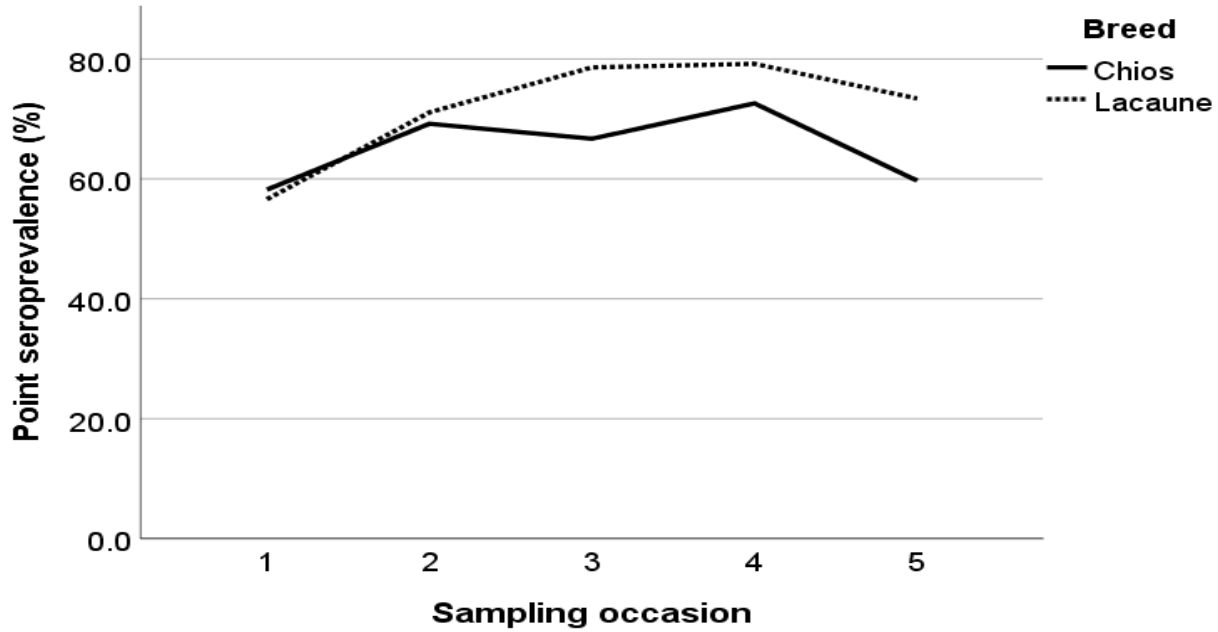


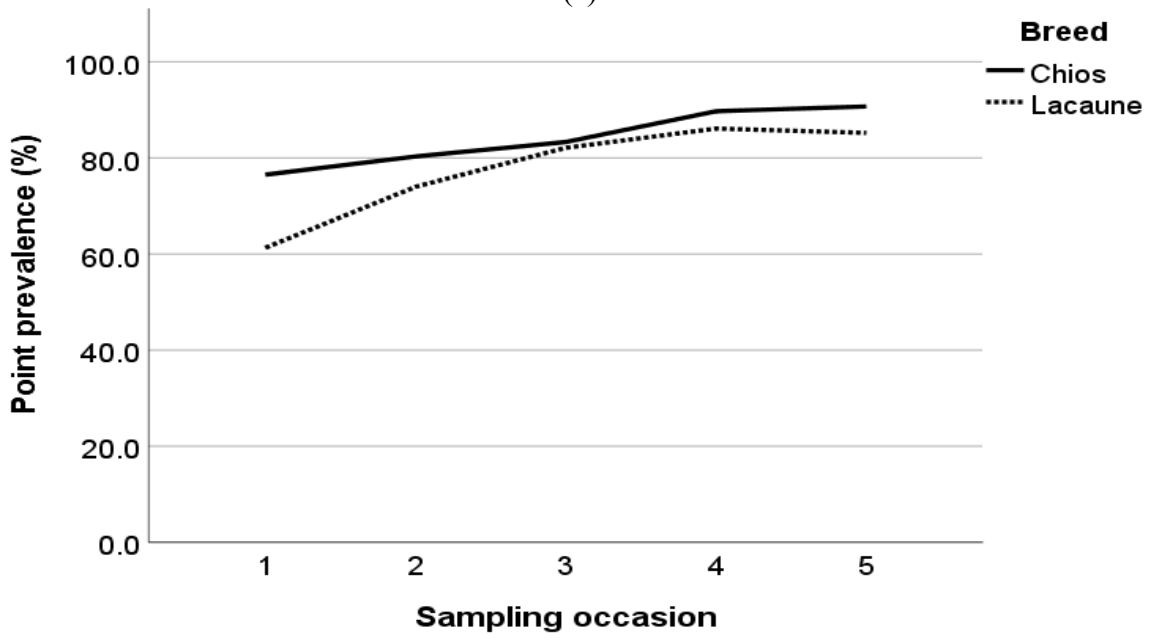
Figure 23. Point seroprevalence and prevalence in ewes in each sampling occasion during the study.

Point seroprevalence and prevalence per farm are presented in Figures S1a, S1b, S1c, and S1d in Appendix B.

Point seroprevalence and prevalence in Chios and Lacaune ewes are presented in Figures 24a and 24b, respectively. Except for the first sampling occasion, Lacaune ewes had higher seroprevalence rates compared to Chios ewes, whereas Chios ewes presented higher prevalence rates in all sampling occasions. In Chios ewes, seroprevalence fluctuated during the study, whereas prevalence was continuously increasing. On the other hand, in Lacaune ewes, both point seroprevalence and prevalence increased till the fourth sampling occasion and decreased in the last one.



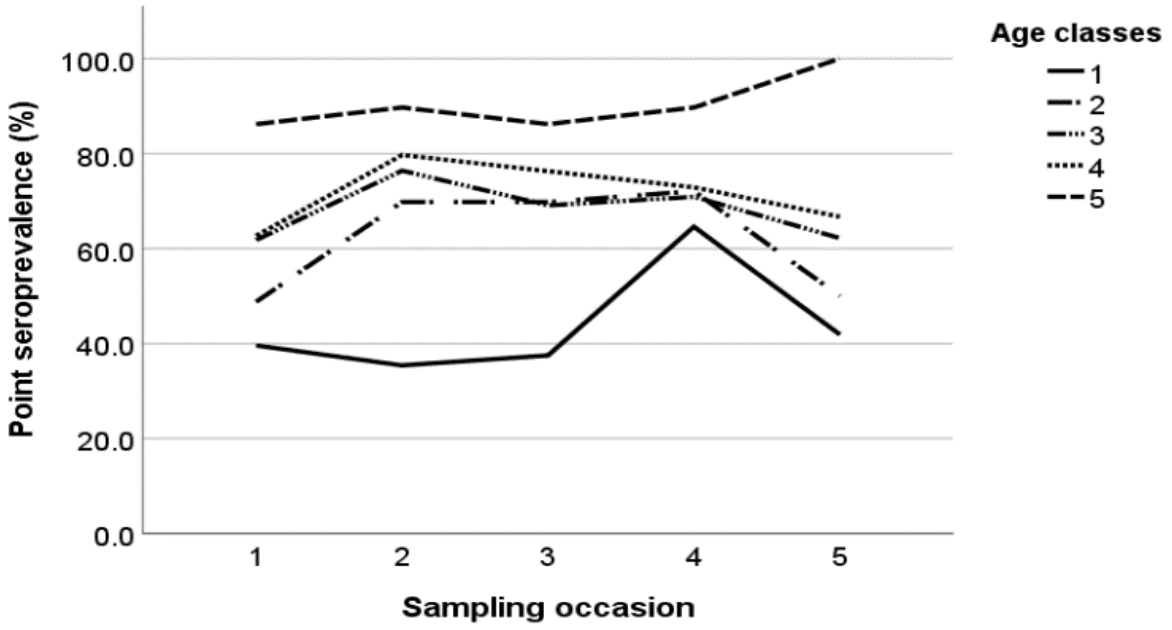
(a)



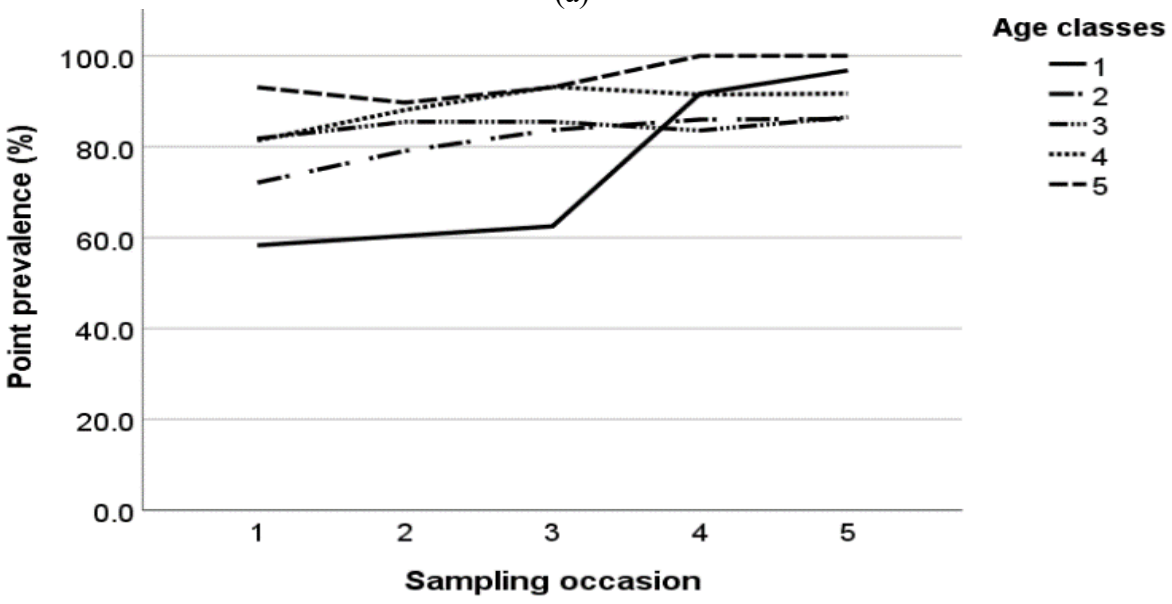
(b)

Figure 24. Point seroprevalence (a) and prevalence (b) in Chios and Lacaune ewes in each sampling occasion during the study.

The point seroprevalence at the beginning of the study was 49.1%, 47.6%, 61.3%, 63.5%, and 82.5% in the age classes 1 ($x \leq 1$), 2 ($1 < x \leq 2$), 3 ($2 < x \leq 3$), 4 ($3 < x \leq 4$), and 5 ($x > 4$), respectively. The respective values for the point prevalence at the beginning of the study were 57.8%, 61.9%, 77.4%, 79.7%, and 87.5%. The point seroprevalence and prevalence during the study per age class and breed are presented in Figures 25 and 26, respectively. The point seroprevalence and the prevalence at the beginning of the study per age class and farm are presented in Appendix B (Figures S2a and S2b, respectively).

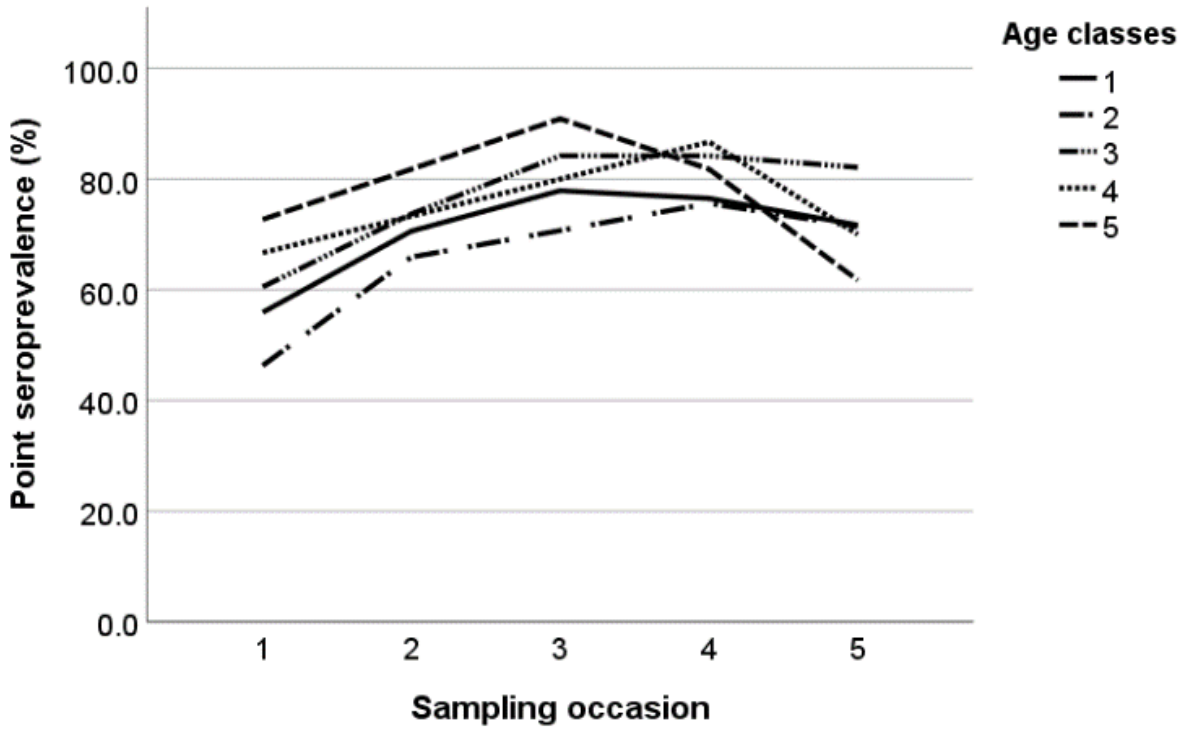


(a)

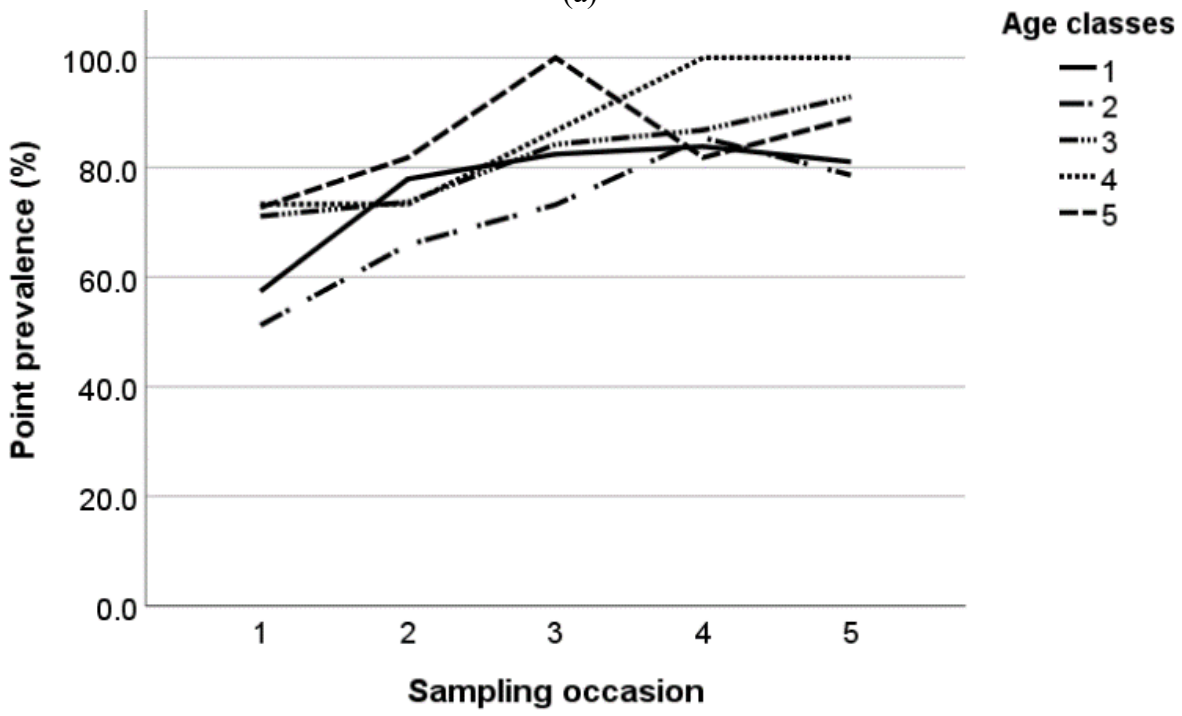


(b)

Figure 25. Point seroprevalence (a) and prevalence (b) in each sampling occasion during the study per age class in Chios ewes; the five age classes are: 1 ($x \leq 1$), 2 ($1 < x \leq 2$), 3 ($2 < x \leq 3$), 4 ($3 < x \leq 4$), and 5 ($x > 4$).



(a)



(b)

Figure 26. Point seroprevalence (a) and prevalence (b) in each sampling occasion during the study per age class in Lacaune ewes; the five age classes are: 1 ($x \leq 1$), 2 ($1 < x \leq 2$), 3 ($2 < x \leq 3$), 4 ($3 < x \leq 4$), and 5 ($x > 4$).

1.3.2 Period seroprevalence and prevalence, incidence rate, and cumulative incidence

The overall period seroprevalence, incidence rate, and cumulative incidence were 84.8% (95% CI, 80.9–88.0%), 33.6 new cases per 100 sheep-semesters (95% CI, 27.8–40.3%), and 64.2% (95% CI, 56.8–70.9%) based on ELISA results. The respective values obtained after the combination of ELISA and PCR results were 90.7% (95% CI, 87.4–93.1%), 40.6 new cases per 100 sheep-semesters (95% CI, 32.6-50.0 new cases per 100 sheep-semesters), and 68.9% (95% CI, 60.2-76.4%). Morbidity frequency measures for Chios and Lacaune ewes for the first year and the whole duration of the study are presented in Tables 18 and 19, respectively.

Table 18. Period prevalence, incidence rate, and cumulative incidence (95% CI) for Chios ewes.

Morbidity Frequency Measure	ELISA		ELISA and PCR	
	12 months	24 months	12 months	24 months
Period prevalence (%)	73.1 (66.9–78.5)	84.6 (79.4–88.8)	87.2 (82.3-90.9)	94.4 (90.7-96.7)
Incidence rate (new cases per 100 sheep-semesters)	37.8 (27.9–50.1)	32.8 (25.4–41.7)	35.2 (23.3-51.1)	37.6 (27.0-51.1)
Cumulative incidence (%)	45.9 (36.4–55.8)	64.3 (54.4–73.1)	45.5 (33.0-58.5)	74.5 (61.1-84.5)

CI: confidence interval.

Table 19. Period prevalence, incidence rate, and cumulative incidence (95% CI) for Lacaune ewes.

Morbidity Frequency Measure	ELISA		ELISA and PCR	
	12 months	24 months	12 months	24 months
Period prevalence (%)	81.5 (75.0–86.9)	84.4 (78.2–89.2)	83.8 (77.6–88.6)	90.8 (85.5-94.2)
Incidence rate (new cases per 100 sheep-semesters)	51.2 (37.4–68.5)	34.8 (26.0–45.7)	56.5 (40.8–76.4)	48.1 (36.2-62.7)
Cumulative incidence (%)	56.0 (44.7–66.7)	64.0 (52.7–73.9)	58.2 (46.3–69.3)	76.1 (64.7-84.7)

CI: confidence interval.

In Figures S3, S4, and S5 in Appendix B morbidity frequency measures are presented for each farm according to ELISA results and the combination of ELISA and PCR results.

1.4 Risk assessment analysis

1.4.1 Adjusted relative risks for the seropositive status

Table 20 summarizes the adjusted RR from the repeated measures binary models for i) the seropositive status during the study and ii) the seropositive status in ewes with an intermittent presence of antibodies. A one-year increase in age was associated with 1.78 times (95% CI, 1.41-2.25, p<0.001) increased RR of seropositive status. Also, Lacaune ewes were 2.63 times (95% CI, 1.35-5.00, p<0.01) more likely to be seropositive during the study. Moreover, the RR of seropositive status was increased by 1.72 times (95% CI, 1.28-2.33, p<0.001) at pre-lambing compared to pre-mating, while seropositive status exclusively in ewes with an intermittent presence of antibodies was also increased by 2.78 times (95% CI, 1.48-5.00, p<0.01) during pre-lambing. Finally, the RR of seropositive status in the studied ewes increased during the second year of the study compared to the third year (p<0.001), while ewes with an intermittent presence of antibodies were more likely to be seropositive during the first and second year of the study compared to the third one (p<0.05).

Table 20. Adjusted relative risks for seropositive status during the study and seropositive status in ewes with an intermittent presence of antibodies.

Dependent variable	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
SS during the study	Age	*	0.579	1.78	1.41-2.25	<0.001
	Breed	Chios	-0.964	0.38	0.20-0.74	<0.01
		Lacaune		Ref		
	BCS	*	0.142	1.15	0.46-2.92	ns
	Year of the study	1	0.560	1.75	0.81-3.77	ns
		2	1.032	2.81	1.64-4.80	<0.001
		3		Ref		
	Production stage	Pre-mating	-0.552	0.58	0.43-0.78	<0.001
		Pre-lambing		Ref		
SS in ewes with an intermittent presence of antibodies	Age	*	-0.134	0.87	0.70-1.09	ns
	Breed	Chios	-0.101	0.90	0.40-2.02	ns
		Lacaune		Ref		
	BCS	*	-0.630	0.53	0.10-2.88	ns
	Year of the study	1	1.383	3.99	1.19-13.33	<0.05
		2	1.701	5.48	1.35-22.24	<0.05
		3		Ref		
	Production stage	Pre-mating	-1.011	0.36	0.20-0.68	0.001
		Pre-lambing		Ref		

SS: Seropositive status; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: Body condition score;*Continuous variable; Ref: Reference category; ns: not statistically significant

1.4.2 Adjusted relative risks for the seroconversion and seroreversion incidents

Adjusted RRs for the seroconversion and the seroreversion incidents are summarized in Table 21. Ewes were 3.23 times (95% CI, 1.85-5.53, p<0.001) more likely to be seroconverted at

pre-lambing compared to pre-mating sampling occasions. Also, the year of the study was associated with seroreversion incidents; ewes were 33.3 times (95% CI, 7.69-100.00, p<0.001) and 20.0 times (95% CI, 3.85-100.00, p<0.001) less likely to serorevert the first and the second year of the study, respectively, compared to the last one.

Table 21. Adjusted relative risks for the seroconversion and seroreversion incidents.

Dependent variable	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
Seroconversion incident	Age	*	-0.135	0.87	0.74-1.03	ns
	Breed	Chios	-0.296	0.74	0.49-1.14	ns
		Lacaune		Ref		
	BCS	*	0.299	1.35	0.48-3.78	ns
	Year of the study	1	0.260	1.30	0.47-3.61	ns
		2	0.082	1.09	0.36-3.30	ns
		3		Ref		
	Production stage	Pre-mating	-1.162	0.31	0.18-0.54	<0.001
		Pre-lambing		Ref		
Seroreversion incident	Age	*	-0.077	0.93	0.68-1.26	ns
	Breed	Chios	0.730	2.08	0.82-5.26	ns
		Lacaune		Ref		
	BCS	*	-0.711	0.49	0.03-7.12	ns
	Year of the study	1	-3.569	0.03	0.01-0.13	<0.001
		2	-2.941	0.05	0.01-0.26	<0.001
		3		Ref		
	Production stage	Pre-mating	-0.937	0.39	0.12-1.33	ns
		Pre-lambing		Ref		

β: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: Body condition score; *Continuous variable; Ref: Reference category; ns: not statistically significant

1.4.3 Adjusted relative risks for serological patterns

Adjusted RRs for the observed serological patterns, as estimated by the binary models, are presented in Table 22. A one-year increase in age was associated with an increased RR of an animal being constantly seropositive by 1.60 times (95% CI, 1.35-1.91, p<0.001). On the other hand, age was negatively associated with the occurrence of the constantly seronegative and the seroconverted patterns; a one-year increase in animal age, was associated with 32% (95% CI, 3-67%, p<0.05) and 28% (95% CI, 3-59%, p<0.05) decreased likelihood of an animal being constantly seronegative or seroconverted during the study, respectively. Moreover, intermittent presence of antibodies was 4.53 times (95% CI, 1.61-12.76, p<0.01) more likely to occur in Chios ewes, whereas a one-year increase in age was associated with a decreased RR of the intermittent presence of antibodies by 32% (95% CI,1-72%, p<0.05).

Table 22. Adjusted relative risks for serological patterns.

Dependent variable	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
Constantly seropositive	Age	*	0.473	1.60	1.35-1.91	<0.001
	Breed	Chios	0.236	1.27	0.52-3.10	ns
		Lacaune		Ref		
Constantly seronegative	Age	*	-0.272	0.76	0.60-0.97	<0.05
	Breed	Chios	-0.253	0.78	0.28-2.19	ns
		Lacaune		Ref		
Seroconverted	Age	*	-0.252	0.78	0.63-0.97	<0.05
	Breed	Chios	-0.372	0.69	0.30-1.57	ns
		Lacaune		Ref		
Seroreverted	Age	*	-0.173	0.84	0.64-1.11	ns
	Breed	Chios	0.469	1.60	0.55-4.63	ns
		Lacaune		Ref		
Intermittent presence of antibodies	Age	*	-0.279	0.76	0.58-0.99	<0.05
	Breed	Chios	1.510	4.53	1.61-12.76	<0.01
		Lacaune		Ref		

β: Coefficient; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not statistically significant

1.4.4 Adjusted relative risks for the PCR positive status

Adjusted RRs for PCR positive status i) for the total of the studied ewes; ii) exclusively for seroreverted ewes; and iii) for ewes with an intermittent presence of antibodies; and iv) for PCR positive status for the first time in seroconverted ewes are summarized in Table 23.

Breed was recognized as a risk factor for PCR positive status; RRs for Chios ewes were 1.99 (95% CI, 1.19-3.34, p<0.01) and 8.00 (95% CI, 1.47-43.66, p<0.05) times higher during the study and among ewes with an intermittent presence of antibodies, respectively, when compared to Lacaune ewes. Moreover, a one-unit decrease in BCS was associated with an increased RR by 20.00 (95% CI, 2.50-100.00, p<0.01) times in ewes with an intermittent presence of antibodies.

Also, the year of the study was associated with PCR positive status. In particular, RR for PCR positive status was 5.56 (95% CI, 3.23-9.10, p<0.001) and 1.52 (95% CI, 1.01-2.27, p<0.05) times lower in the first and second year, respectively, compared to the third one, and RR for PCR positive status of ewes with an intermittent presence of antibodies during the study was 9.09 (95% CI, 1.45-50.00, p<0.05) and 5.26 (95% CI, 1.16-25.00, p<0.05) times lower in the first and second year, respectively, compared to the third one. In seroconverted ewes, RR was increased by 6.86 (95% CI, 1.49-31.61, p<0.05) times in the second year compared to the third one.

Table 23. Adjusted relative risks for PCR positive status for the total of the studied ewes, seroreverted ewes, ewes with an intermittent presence of antibodies, and seroconverted ewes.

Dependent variable	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
PCR + (total of ewes)	Age	*	0.135	1.15	0.96-1.37	ns
	Breed	Chios	0.690	1.99	1.19-3.34	<0.01
		Lacaune		Ref		
	BCS	*	-0.411	0.66	0.36-1.21	ns
	Year of the study	1	-1.701	0.18	0.11-0.31	<0.001
		2	-0.421	0.66	0.44-0.99	<0.05
		3		Ref		
	Production stage	Pre-mating	-0.126	0.89	0.69-1.12	ns
		Pre-lambing		Ref		
PCR + of seroreverted ewes	Age	*	-0.099	0.91	0.50-1.66	ns
	Breed	Chios	1.589	4.90	0.76-31.45	ns
		Lacaune		Ref		
	BCS	*	-0.414	0.66	0.02-18.89	ns
	Year of the study	1	-1.103	0.33	0.04-3.10	ns
		2	0.347	1.42	0.15-13.16	ns
		3		Ref		
	Production stage	Pre-mating	-0.565	0.57	0.16-1.97	ns
		Pre-lambing		Ref		
PCR + of ewes with an intermittent presence of antibodies during the study	Age	*	0.148	1.16	0.64-2.01	ns
	Breed	Chios	2.080	8.00	1.47-43.66	<0.05
		Lacaune		Ref		
	BCS	*	-3.084	0.05	0.01-0.40	<0.01
	Year of the study	1	-2.188	0.11	0.02-0.69	<0.05
		2	-1.654	0.19	0.04-0.86	<0.05
		3		Ref		
	Production stage	Pre-mating	-0.277	0.76	0.35-1.63	ns
		Pre-lambing		Ref		
PCR + in seroconverted ewes[†]	Age	*	-0.025	0.98	0.76-1.26	ns
	Breed	Chios	-0.282	0.75	0.4-1.42	ns
		Lacaune		Ref		
	BCS	*	-0.261	0.77	0.19-3.11	ns
	Year of the study	1	1.308	3.70	0.71-19.21	ns
		2	1.925	6.86	1.49-31.61	<0.05
		3		Ref		
	Production stage	Pre-mating	-0.295	0.36	0.74-1.40	ns
		Pre-lambing		Ref		

β: Coefficient; PCR +: PCR positive status; $CI_{95\%}$: 95% confidence interval; BCS: Body condition score; [†]PCR positive result for the first time; *Continuous variable; Ref: Reference category; ns: not statistically significant

1.4.5 Adjusted relative risks for the infected status

Adjusted RRs for the infected status during the study for the studied animals are presented in Table 24. Age was significantly associated with infected status; in particular, RR for infected status increased with age by 1.69 times (95% CI, 1.25-2.29, p = 0.001). Also, ewes were 2.94 times (95% CI, 1.82-4.76, p<0.001) more likely to be found infected at the pre-lambing

compared to the pre-mating sampling occasion. Year of the study was also associated with infected status; infections were 20.0 (95% CI, 6.25-50.0, p<0.001) and 3.3 (95% CI, 1.45-7.69, p<0.01) times less likely in the first and second year, respectively, compared to the third one.

Table 24. Adjusted relative risks for infected status during the study.

Dependent variable	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
Infected status	Age	*	0.527	1.69	1.25-2.29	0.001
	Breed	Chios	0.459	1.58	0.68-3.68	ns
		Lacaune		Ref		
	BCS	*	-1.052	0.349	0.09-1.36	ns
	Year of the study	1	-3.014	0.05	0.02-0.16	<0.001
		2	-1.204	0.30	0.13-0.69	<0.01
		3		Ref		
	Production stage	Pre-mating	-1.081	0.34	0.21-0.55	<0.001
		Pre-lambing		Ref		

β: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: Body condition score; *Continuous variable; Ref: Reference category; ns: not statistically significant

1.4.5 Adjusted relative risks for infection patterns

Adjusted RRs for infection patterns are summarized in Table 25. A one-year increase in animal age was associated with an increased RR of an animal being infected seropositive by 1.31 times (95% CI, 1.08-1.60, p<0.01) during the study and a decreased RR of an animal being infected seronegative by 1.30 times (95% CI, 1.03-1.61, p<0.05). On the other hand, breed was not significantly associated with any of the infection patterns.

Table 25. Adjusted relative risks for infection patterns.

Dependent variable	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
Infected seronegative	Age	*	-0.257	0.77	0.62-0.97	<0.05
	Breed	Chios	-0.039	0.96	0.34-2.75	ns
		Lacaune		Ref		
Infected seropositive	Age	*	0.273	1.31	1.08-1.60	<0.01
	Breed	Chios	-0.018	0.98	0.43-2.27	ns
		Lacaune		Ref		
Uninfected	Age	*	-0.225	0.80	0.56-1.14	ns
	Breed	Chios	-0.316	0.73	0.05-11.86	ns
		Lacaune		Ref		

β: Coefficient; $CI_{95\%}$: 95% confidence interval;*Continuous variable; Ref: Reference category; ns: not statistically significant

2. Epizootiology of small ruminant lentiviruses infections in lambs

2.1 Morbidity frequency measures

2.1.1 Point seroprevalence

Point seroprevalence in lambs was 48.2%, 34.6%, 27.7%, and 31.5% at the ages of 1, 3, 8, and 13 months old, respectively. At the beginning of the study, seroprevalence was 55.0% (11/20) in farm A, 50.9% (27/53) in farm B, 35.1% (13/37) in farm C, and 50.6% (43/85) in farm D in 1-month-old lambs. The evolution of seroprevalence in the studied lambs is presented in Figure 27 for each farm.

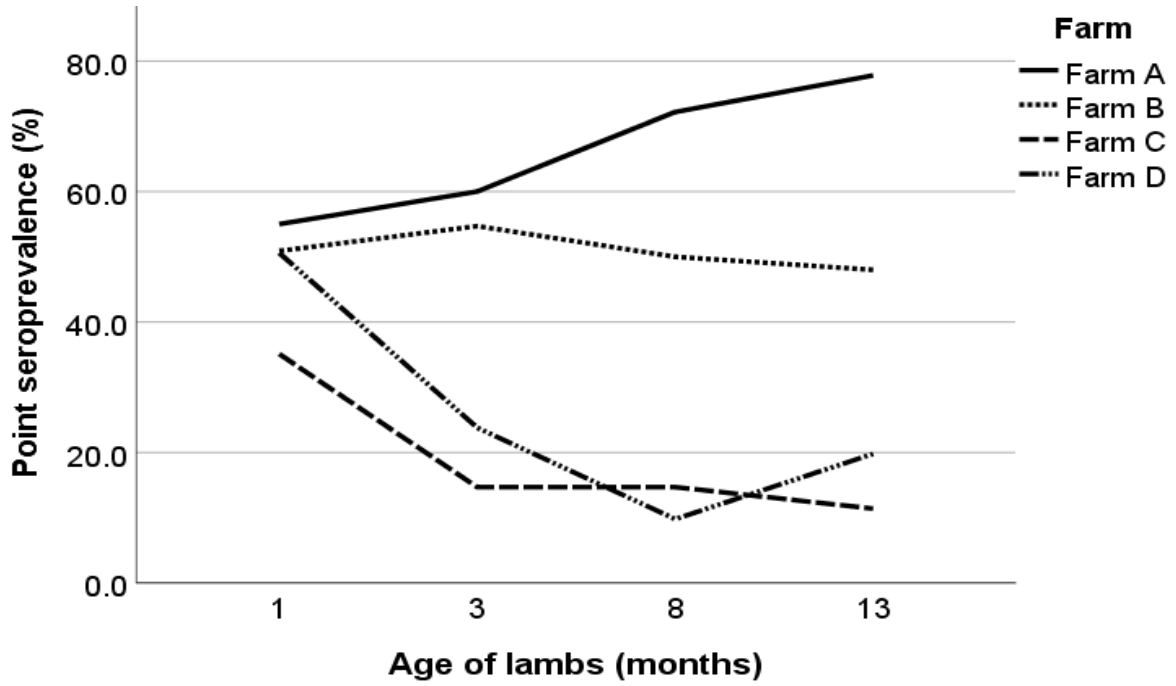


Figure 27. Point seroprevalence in lambs in each farm and sampling occasion during the study.

As shown in Figure 28, seroprevalence in lambs fed unpasteurized colostrum observed to be almost stable during the study (52.1%-56.2%). On the other hand, seroprevalence in lambs fed pasteurized colostrum was 45.9% (56/122) in the first sampling occasion, decreased till the third sampling (10.7%; 13/122) and then increased in the last sampling occasion (16.4%; 20/122).

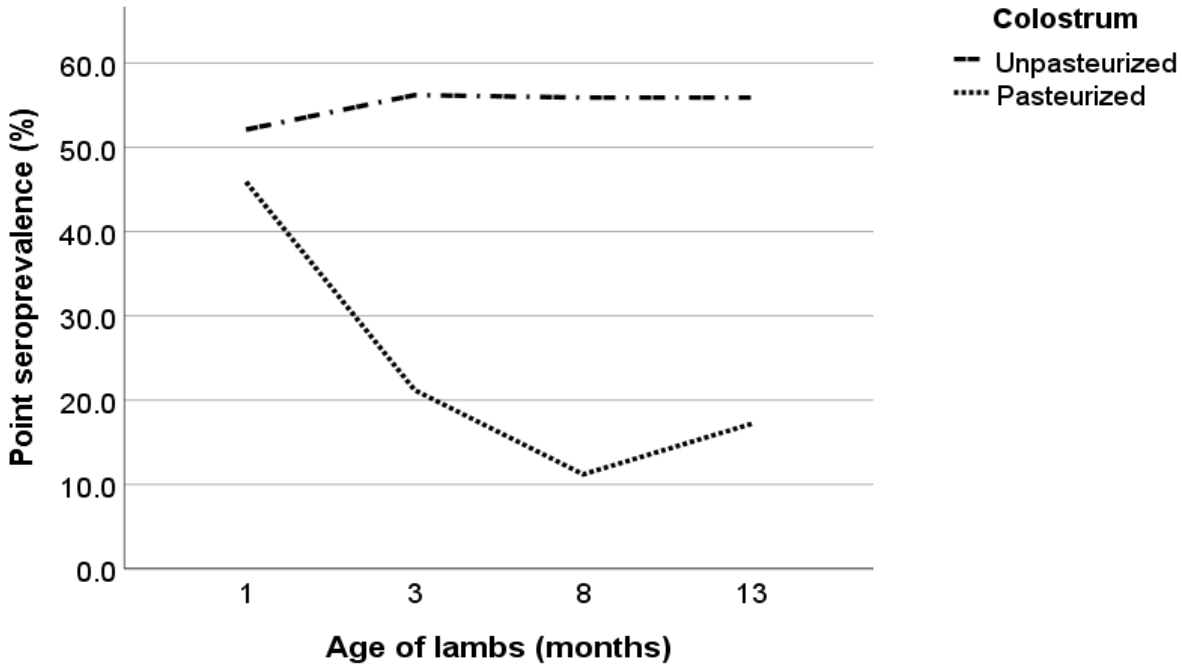
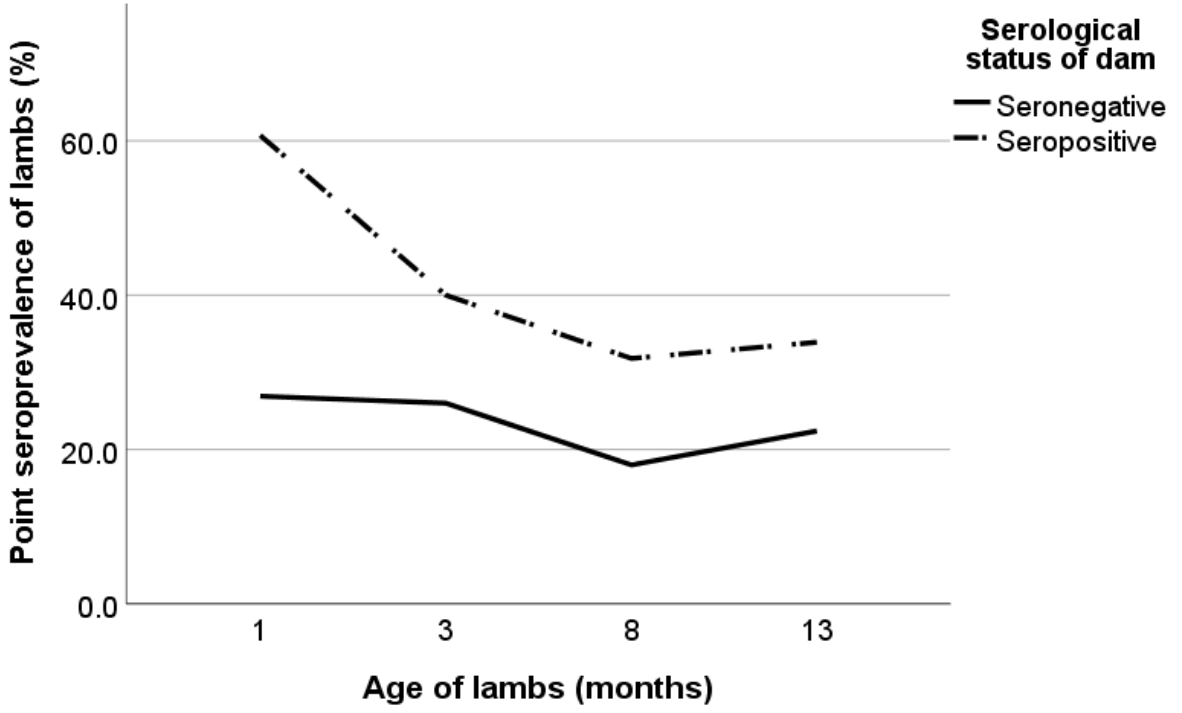
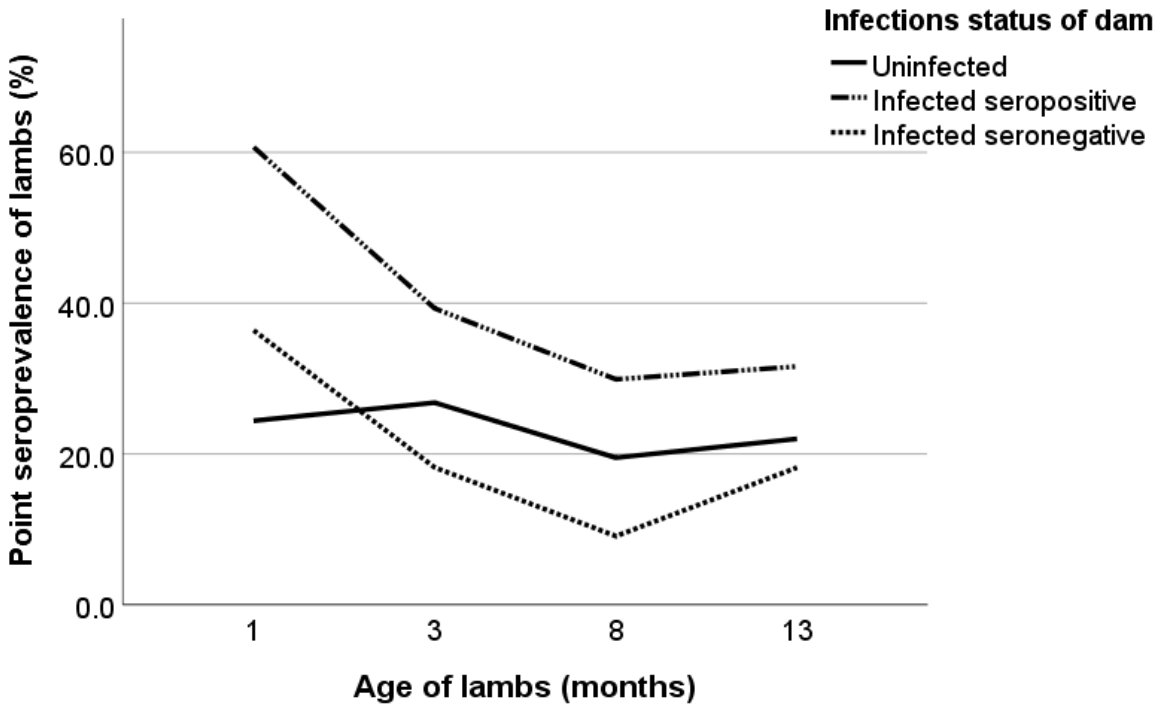


Figure 28. Point seroprevalence in each sampling occasion during the study in lambs fed unpasteurized and pasteurized colostrum.

The serological and infection status of the dams were recorded at pre-lambing for 169 out of the 195 studied lambs; 30.8% (52/169) was seronegative, whereas 24.3% (41/169) was uninfected, 69.2% (117/169) was infected seropositive, and 6.5% (11/169) was infected seronegative. The evolution of point seroprevalence in lambs with regard to the serological and infection status of their dam is presented in Figures 29a and 29b, respectively.



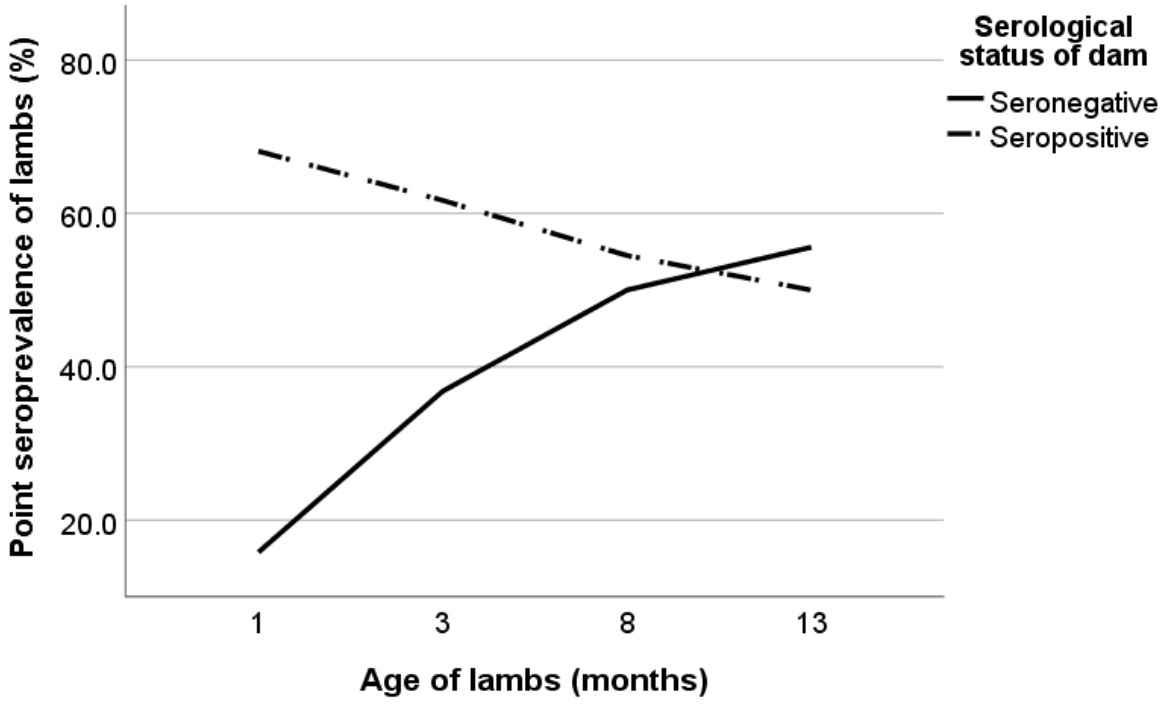
(a)



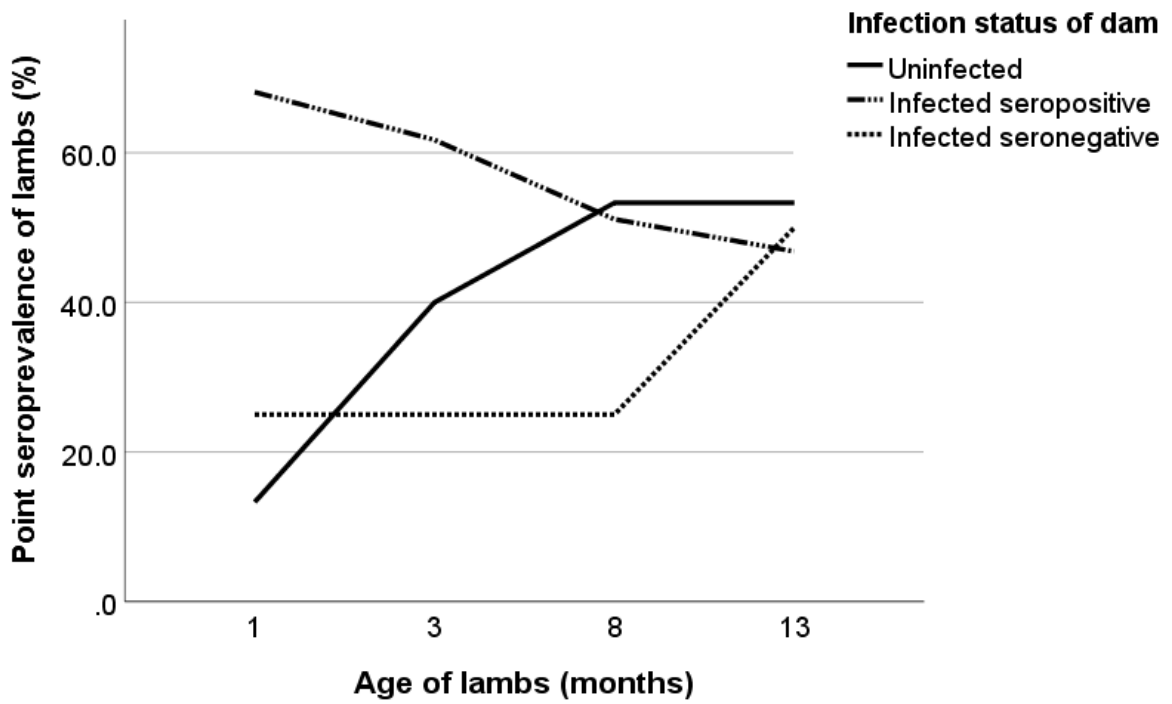
(b)

Figure 29. Point seroprevalence of lambs in each sampling occasion during the study in association to the serological (a) and the infection (b) status of their dams at pre-lambing.

The evolution of seroprevalence regarding the serological and infection status of dams only for farms A and B, where the lambs consumed unpasteurized colostrum from their dams is presented in Figures 30a and 30b.



(a)



(b)

Figure 30. Point seroprevalence of lambs from farms A and B in each sampling occasion during the study in association to the serological (a) and the infection (b) status of their dams at pre-lambing.

2.1.2 Point prevalence

During the study (13 months), 56.4% of lambs (110/195) were infected. In particular, 12.3% (24/195), 21.5% (42/195), 13.3% (26/195), and 9.2% (18/195) were found infected at the ages of 1, 3, 8, and 13 months, respectively. The evolution of point prevalence per farm is presented in Figure 31. Farms A and B, as well as farms C and D, demonstrated similar point prevalence patterns in lambs during the first 13 months of their life.

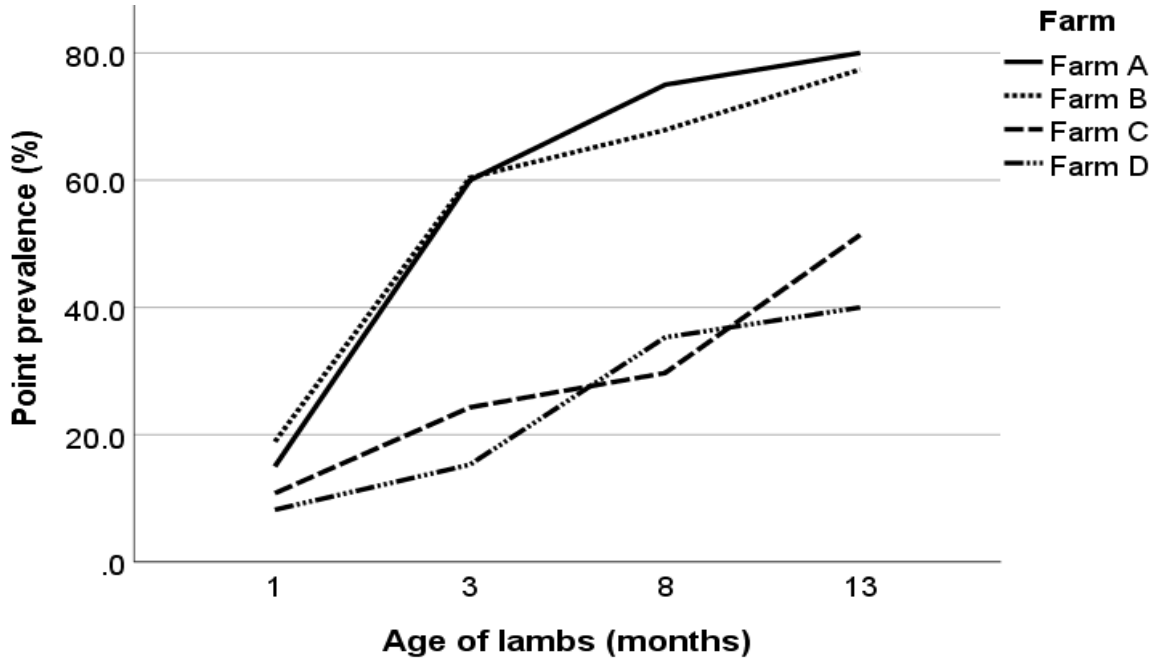


Figure 31. Point prevalence in lambs in each farm and sampling occasion during the study.

A total of 78.1% (57/73) and 43.4% (53/122) of the lambs that consumed unpasteurized and pasteurized colostrum, respectively, were infected during the study. The evolution of point prevalence in lambs according to the type of colostrum is presented in Figure 32. A total of 14 out of 73 (19.2%) lambs that consumed unpasteurized colostrum were found infected in the first sampling occasion (1 month), whereas only 11 out of 122 (9.0%) lambs that consumed pasteurized colostrum were infected at the 1st month of their life. The point prevalence in lambs that consumed unpasteurized colostrum dramatically increased in the second sampling occasion and was gradually increasing thereafter until the end of the study. The prevalence in lambs that consumed pasteurized colostrum increased during the study but much lower compared to the lambs that consumed unpasteurized colostrum.

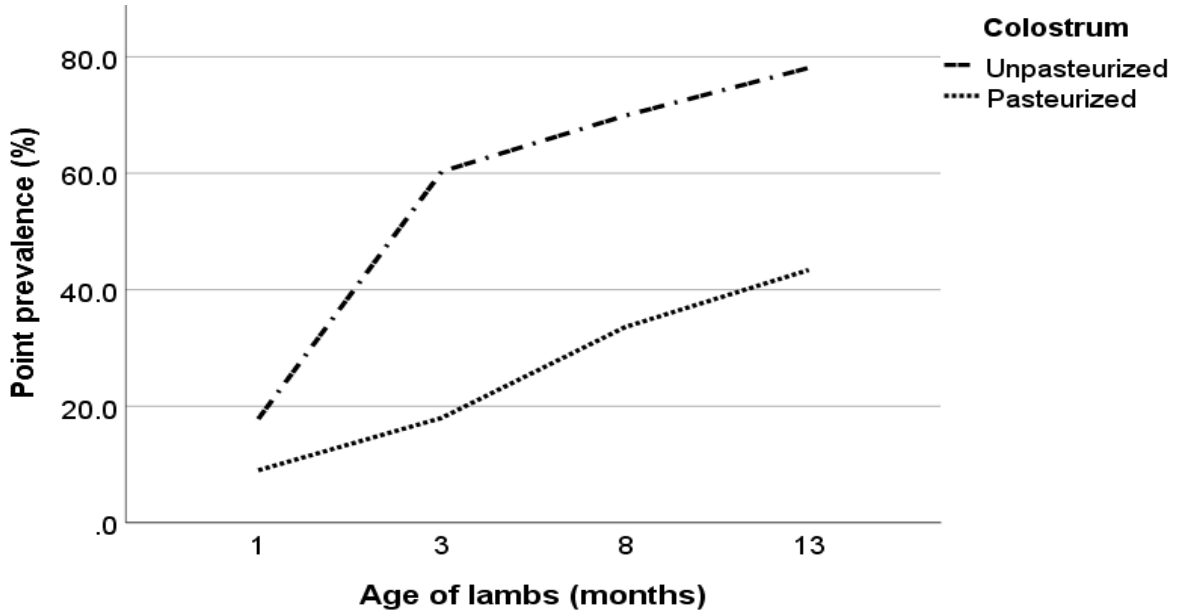
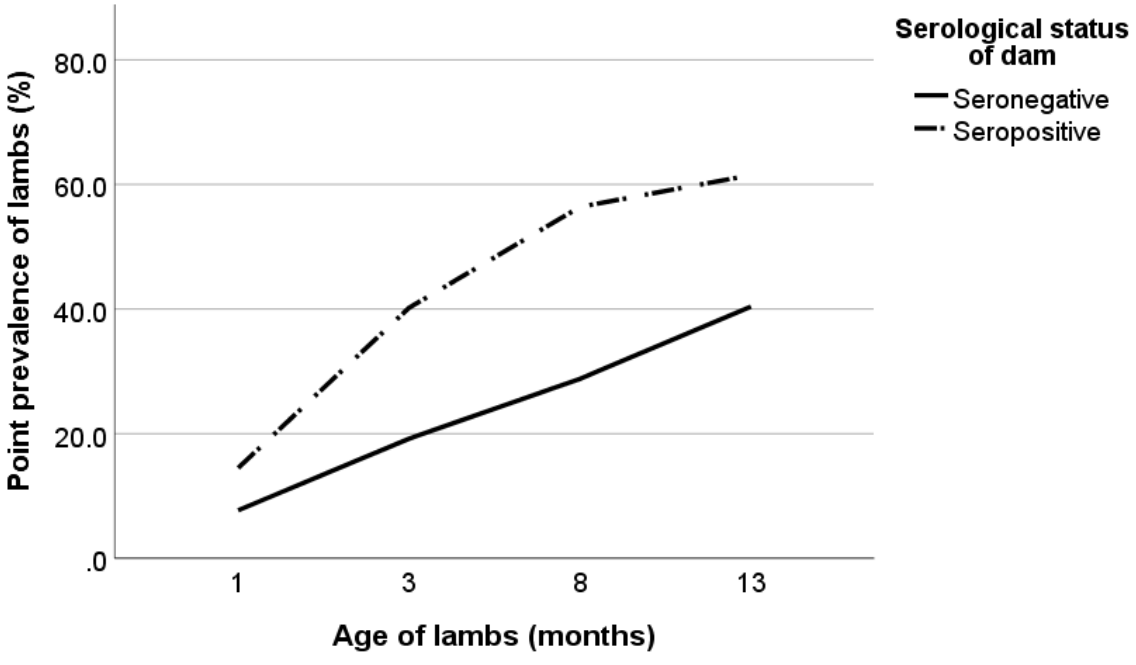
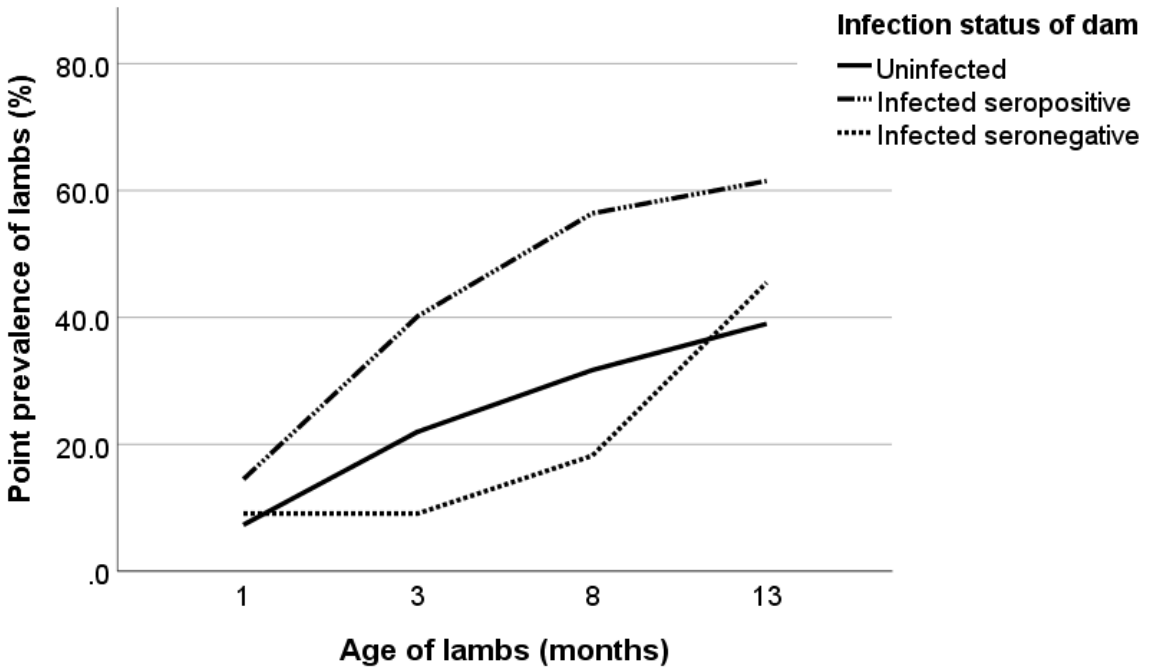


Figure 32. Point prevalence in lambs that consumed unpasteurized and pasteurized colostrum in each sampling occasion during the study.

A total of 39.0% (16/41) of lambs from uninfected dams were found infected during the study. Also, 61.5% (72/117) and 45.5% (5/11) of lambs from infected seropositive and infected seronegative dams, respectively, were infected during the first 13 months of their life. The evolution of point prevalence in lambs considering the serological and infection status of their dams is presented in Figures 33a and 33b. The prevalence in lambs from seropositive dams was constantly higher compared to the prevalence in lambs from seronegative dams.



(a)



(b)

Figure 33. Point prevalence in lambs in each sampling occasion during the study in association to the serological (a) and infection (b) status of their dams at pre-lambing.

2.2 Serological patterns

All serological patterns were observed in each farm except for farm C, where constantly seropositive lambs were not recorded (Figure 34). In farm D, the percentage of constantly seropositive lambs was low (3.5%; 3/85), whereas in farms A and B, it was higher (35.0%; 7/20 and 18.9%; 10/53, respectively). Moreover, farms C and D demonstrated a higher percentage of constantly seronegative lambs (51.4%; 19/37 and 38.8%; 33/85, respectively), while in farms A and B the respective values were 20.0% (4/20) and 18.9% (10/53), respectively. Seroconverted lambs were increased in farms A and B (30.0%; 6/20 and 22.6%; 12/53, respectively), compared to farms C and D (8.1%; 3/37 and 9.4%; 8/85, respectively). On the other hand, seroreverted animals were increased in farms C and D (32.4%; 12/37 and 40.0%; 34/85, respectively) compared to farms A and B (5.0%; 1/20 and 26.4%; 14/53, respectively). The percentage of animals with an intermittent presence of antibodies was similar in all farms.

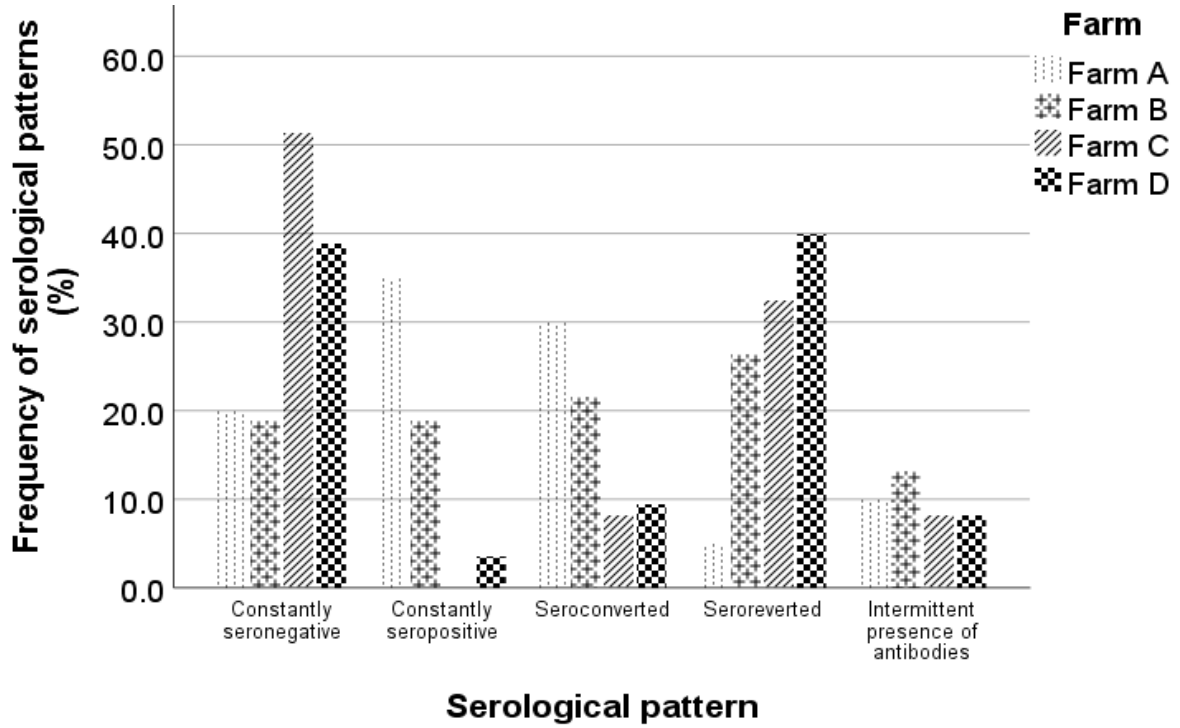


Figure 34. The frequencies of serological patterns in lambs on each farm.

More than 50.0% (15/29) of the seroconverted lambs were found seroconverted at the age of 3 months old (2nd sampling occasion), whereas 20.7% (6/29) were found seroconverted at the age of 8 months old (3rd sampling occasion), and 27.6% (8/29) at the age of 13 months old (4th sampling occasion). Regarding seroreverted animals, 62.3% (38/61), 29.5% (18/61), and 8.2% (5/61) were found seroreverted at the ages of 3, 8, and 13 months. Also, 63.2% (12/19) of lambs with an intermittent presence of antibodies were found to be seropositive at the beginning of the study, seronegative in the next sampling occasion, and again seropositive till the end of the study. The rest 7 animals demonstrated a random pattern regarding the presence of antibodies.

Lambs fed pasteurized colostrum demonstrated higher percentages of constantly seronegative pattern and seroreversion incidents, whereas percentages of constantly seropositive status and seroconversion incidents were increased in lambs fed unpasteurized colostrum (Figure 35).

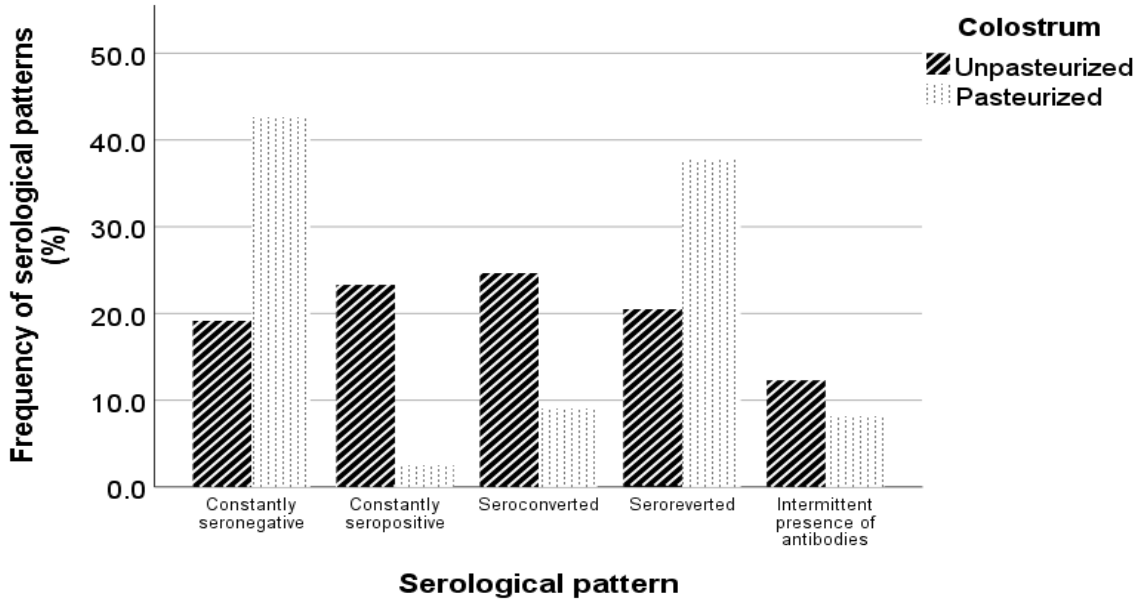
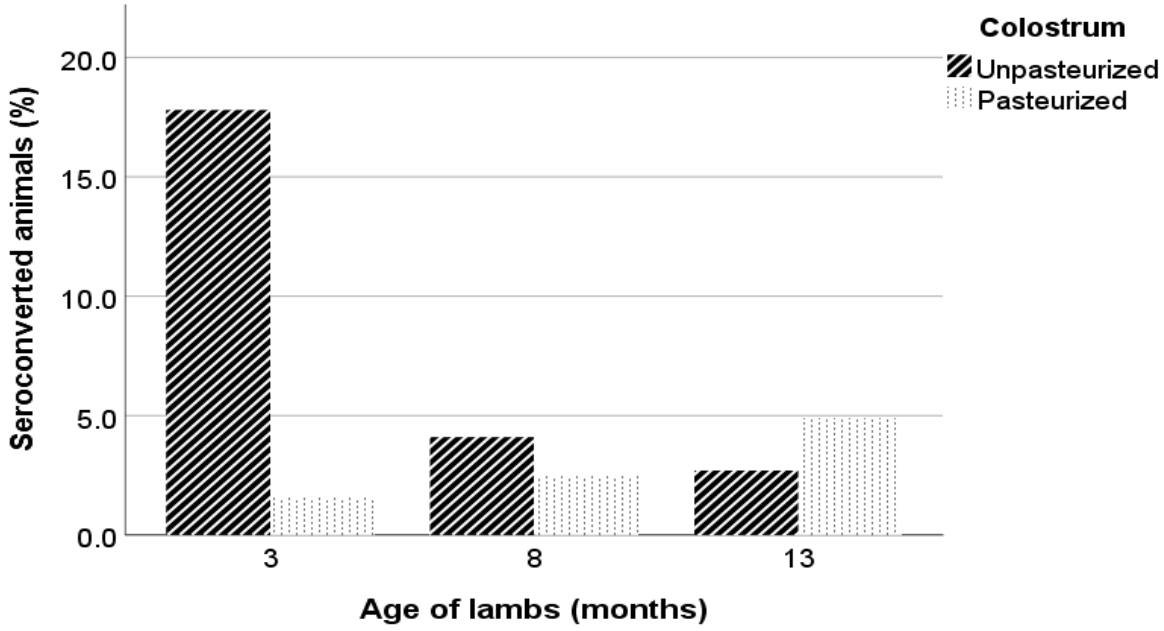
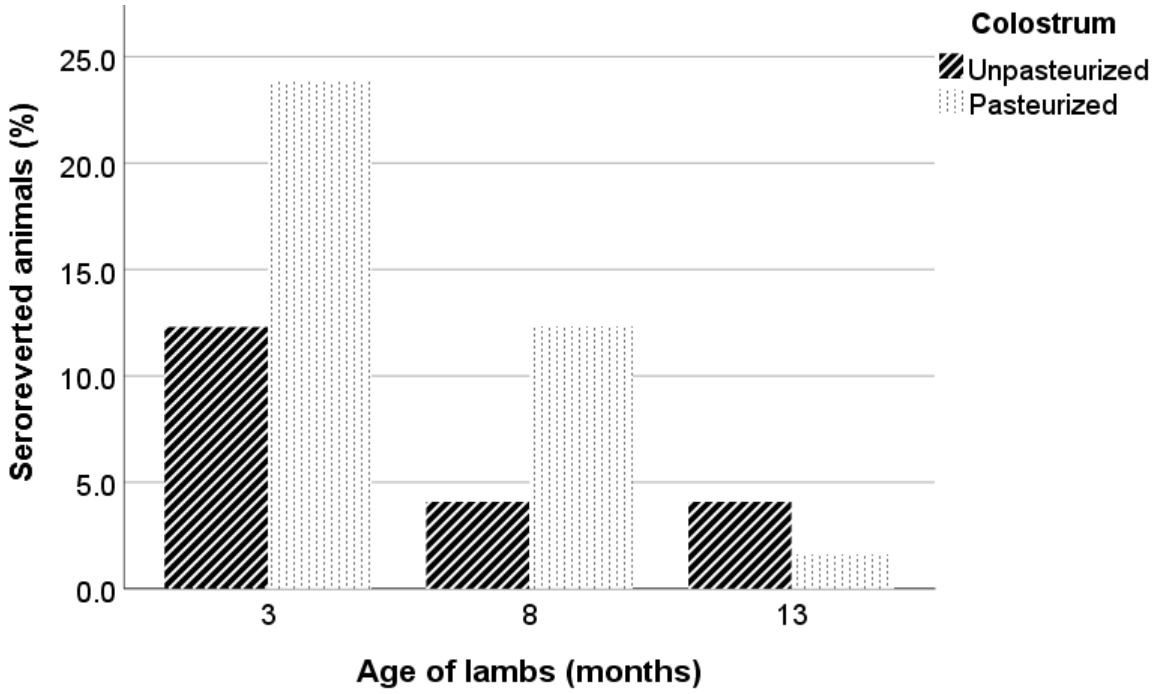


Figure 35. The frequencies of serological patterns of lambs fed unpasteurized and pasteurized colostrum.

Also, as shown in Figure 36a, 17.8% (13/73) of lambs fed unpasteurized colostrum seroconverted within the first 3 months of age, whereas only 1.6% (2/122) of lambs fed pasteurized colostrum seroconverted until this age. Regarding seroreversion (Figure 36b), 12.3% (9/73) of lambs fed unpasteurized colostrum and 23.8% (29/122) of lambs fed pasteurized colostrum seroreverted within the first 3 months. Also, all the uninfected lambs that presented antibodies in the first sampling occasion, seroreverted until the age of 8 months, whereas seroconversion incidents occurred until the age of 8 months in 72.4% (21/29) of the seroconverted lambs.



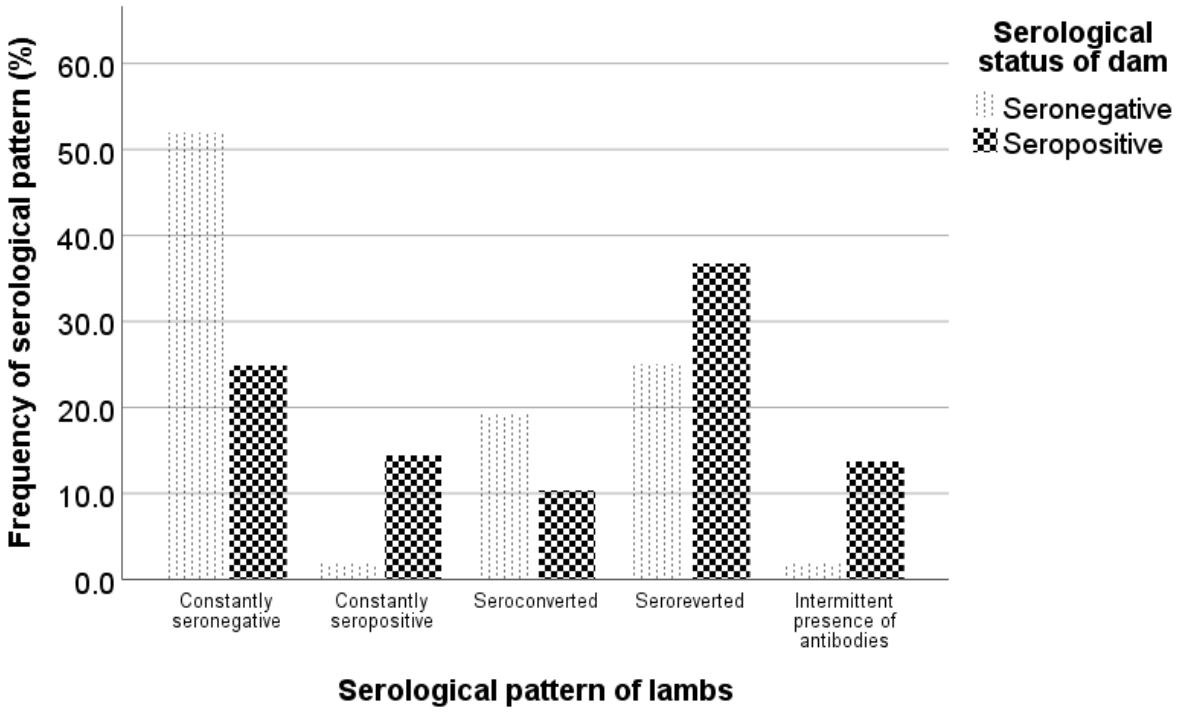
(a)



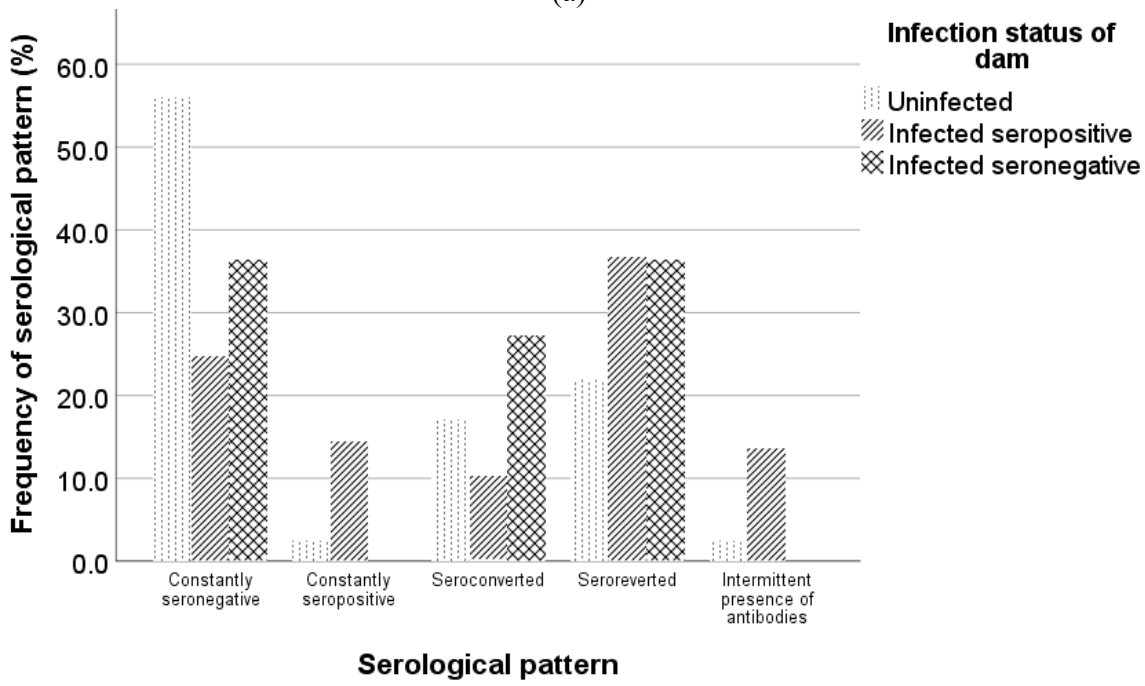
(b)

Figure 36. Percentages of seroconverted (a) and seroreverted (b) lambs in each sampling occasion during the study for the two types of colostrum.

More than 50.0% (27/52) of lambs from seronegative dams were constantly seronegative, whereas only 1.9% (1/52) were constantly seropositive during the study (Figure 37a). On the other hand, seropositive dams demonstrated a higher percentage of seroreverted lambs (36.8%; 43/117) and lambs with an intermittent presence of antibodies (13.7%; 16/117). When the infection status of dams was considered, uninfected dams demonstrated the highest percentage of constantly seronegative lambs (56.1%; 23/41), whereas only 2.4% (1/41) were constantly seropositive (Figure 37b). None of the constantly seropositive lambs and lambs with an intermittent presence of antibodies originated from infected seronegative dams; on the contrary, these dams presented the highest percentage of seroconverted lambs. Infected dams (seropositive or seronegative) demonstrated a similar percentage of seroreverted lambs, whereas infected seropositive dams demonstrated the highest percentage of lambs with an intermittent presence of antibodies.



(a)



(b)

Figure 37. The frequencies of serological patterns of lambs in association to the serological (a) and the infection (b) status of dams at pre-lambing.

During the study, 36.4% (24/66), 95.0% (19/20), 96.6% (28/29), 41.0% (25/61), and 84.2% (16/19) of constantly seronegative, constantly seropositive, seroconverted, seroreverted lambs and lambs with an intermittent presence of antibodies, respectively, were found to be infected. The evolution of prevalence during the study in lambs of different serological

patterns is presented in Figure 38. Constantly seronegative lambs demonstrated the lowest point prevalence rates during the study, whereas the highest point prevalence rates were observed in constantly seropositive lambs, with the exception of the last sampling occasion where seroconverted animals demonstrated a higher prevalence rate (96.6%; 28/29). Seroreverted lambs demonstrated a similar prevalence pattern to the constantly seronegative lambs, with a gradual increase in prevalence. On the other hand, the prevalence of seroconverted lambs and lambs with an intermittent presence of antibodies increased sharply during the study, following a similar trend. At the end of the study, three lambs with an intermittent presence of antibodies, one seroconverted lamb, and one constantly seropositive lamb were found to be PCR negative.

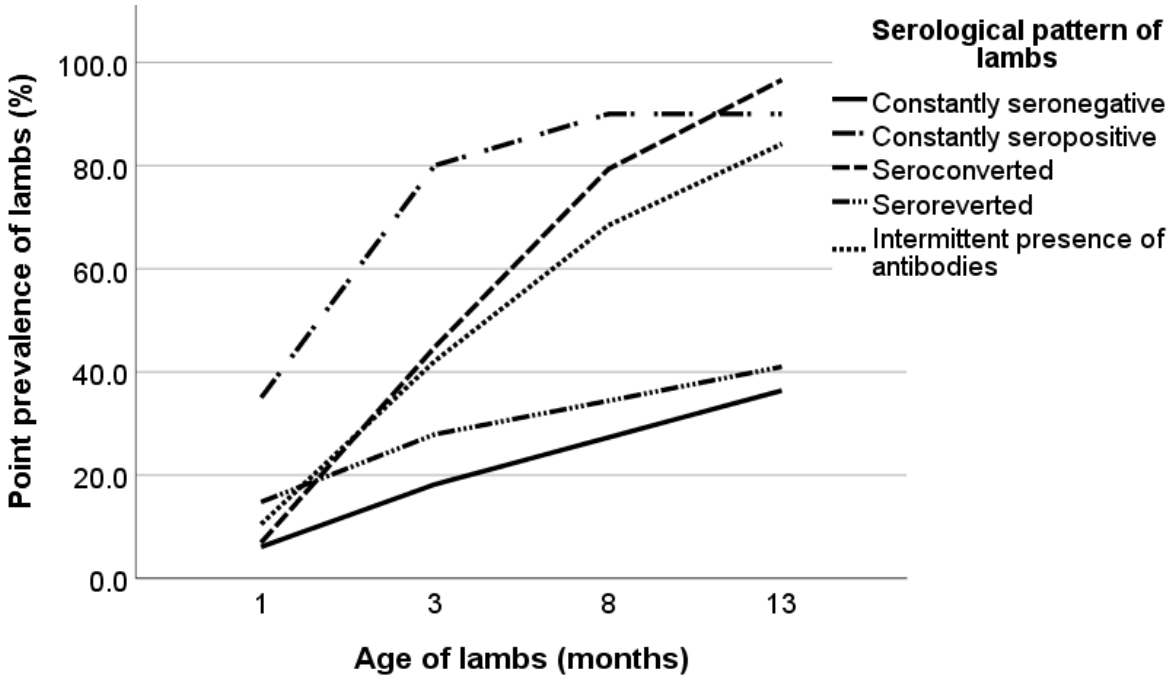


Figure 38. Point prevalence in lambs in each sampling occasion during the study in association to their serological pattern.

2.3 Risk assessment analysis

2.3.1 Adjusted relative risks for the seropositive status

Adjusted RRs for the seropositive status at the age of 13 months are presented in Table 26. Lambs that consumed unpasteurized colostrum from their dam were 19.29 (95% CI, 2.37-156.85, $p<0.01$) times more likely to be found seropositive at the age of 13 months old compared to the lambs that consumed pasteurized colostrum.

Table 26. Adjusted relative risks for seropositive status in lambs at the age of 13 months.

Dependent variable	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
SS at the age of 13 months	Breed	Chios	-1.664	0.19	0.02-1.59	ns
		Lacaune		Ref		
	Infection status of dam	Uninfected	-0.737	0.48	0.19-1.24	ns
		Infected		Ref		
	Colostrum	Unpasteurized	2.960	19.29	2.37-156.85	<0.01
		Pasteurized		Ref		

SS: Seropositive status; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; Ref: Reference category; ns: not statistically significant

2.3.2 Adjusted relative risks for the infected status

Adjusted RRs for the infected status of lambs at the ages of 1, 3, 8, and 13 months old are presented in Table 27. The type of colostrum was statistically significant in any case; lambs that consumed unpasteurized colostrum from their dam were 3.43 (95% CI, 1.01-11.60, p<0.05), 6.39 (95% CI, 2.38-17.16, p<0.001), 8.00 (95%CI, 3.04-21.02, p<0.001) and 6.07 (95%CI, 2.42-15.21, p<0.001) times more likely to be found infected at the ages of 1, 3, 8, and 13 months old, respectively, compared to the lambs that consumed pasteurized colostrum. Also, RR for infected status during the first 8 and 13 months of lambs' life was increased by 2.94 (95%CI, 1.30-6.67, p = 0.01) and 2.07 times (95% CI, 1.22-5.88, p<0.05), respectively, for the lambs from infected dams.

Table 27. Adjusted relative risks for infected status in lambs at the age of 1, 3, 8, and 13 months old.

Infected status	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
1 month old	Breed	Chios	-0.373	0.69	0.21-2.32	ns
		Lacaune		Ref		
	Infection status of dam	Uninfected	-0.662	0.52	0.15-1.82	ns
		Infected		Ref		
	Colostrum	Unpasteurized	1.232	3.43	1.01-11.60	<0.05
		Pasteurized		Ref		
3 months old	Breed	Chios	0.265	1.30	0.49-3.49	ns
		Lacaune		Ref		
	Infection status of dam	Uninfected	-0.873	0.42	0.17-1.05	ns
		Infected		Ref		
	Colostrum	Unpasteurized	1.854	6.39	2.38-17.16	<0.001
		Pasteurized		Ref		
8 months old	Breed	Chios	-0.703	0.50	0.19-1.27	ns
		Lacaune		Ref		
	Infection status of dam	Uninfected	-1.088	0.34	0.15-0.77	0.01
		Infected		Ref		
	Colostrum	Unpasteurized	2.079	8.00	3.04-21.02	<0.001
		Pasteurized		Ref		

β: Coefficient; $CI_{95\%}$: 95% confidence interval; Ref: Reference category; ns: not statistically significant

Table 27. Adjusted relative risks for infected status in lambs at the age of 1, 3, 8, and 13 months old. (Continued)

Infected status	Risk factor	Categories	*β*	Relative risk	$CI_{95\%}$	*P*
13 months old	Breed	Chios	-0.115	0.89	0.37-2.14	ns
		Lacaune			Ref	
	Infection status of dam	Uninfected	-0.997	0.37	0.17-0.82	<0.05
		Infected			Ref	
	Colostrum	Unpasteurized	1.803	6.07	2.42-15.21	<0.001
		Pasteurized			Ref	

β: Coefficient; $CI_{95\%}$: 95% confidence interval; Ref: Reference category; ns: not statistically significant

IV. Discussion

1. Epizootiological study in ewes

This is the first prospective epizootiological study of SRLV infections in intensively reared dairy sheep flocks regarding the most popular and productive indigenous (Chios) and foreign (Lacaune) dairy sheep breeds in Greece, with the combined use of ELISA and PCR assays. Also, it is the first time that seroreversion incidents and cases of an intermittent presence of antibodies are systematically recorded and further assessed in combination with molecular testing in SRLV naturally infected ewes, supporting the need for the introduction of serological and infection patterns for the classification of animals rather than the current typical classification into seropositive and seronegative.

Our study demonstrated that the exclusive use of ELISA for the diagnosis of SRLV infections can lead to underdiagnosis, raising concerns about the validity of SRLV prevalence rates estimated by the most recent cross-sectional sero-epizootiological studies in other countries. For this reason, we selected to estimate both seroprevalence based exclusively on ELISA results and on the combination of ELISA and PCR results and comparatively assess them. In any case, the findings confirm the hypothesis of an increased SRLV infection rate in intensively reared dairy sheep flocks in Greece. This is in accordance with the results from other Mediterranean countries with a developed dairy sheep sector (e.g., Spain, Italy, Turkey, etc.) (Pérez *et al.*, 2010; Albayrak *et al.*, 2012; Lago *et al.*, 2012; Pazzola *et al.*, 2020). Before this study, limited epizootiological data on SRLV infections was available in our country through one cross-sectional sero-epizootiological study, which included 143 sheep from six infected flocks and aimed at the serological and molecular detection of SRLV infections (Karanikolaou *et al.*, 2005), and one prospective sero-epizootiological study (>25 years ago) in which 378 Chios ewes originating from one experimental flock (Animal Research Institute, ELGO-DIMITRA) were followed across one lactation to assess the effect of SRLV seropositivity on the milk yield (Ploumi *et al.*, 2001). The seroprevalence rates in the

aforementioned studies were 65.0% and 47.0%, respectively, and in both cases, they were similar to our results (57.7%–75.4%).

The point seroprevalence found in our study is higher compared to the seroprevalence documented in other countries. In particular, low seroprevalence rates were found in Poland (5.4–14.9% in 6,470 ewes from 98 flocks) (Junkuszew *et al.*, 2016), Croatia (10.0% in 460 sheep from 17 farms) (Pavlak *et al.*, 2022), Belgium (9.0% in 555 sheep from 87 farms) (Michiels *et al.*, 2018), and Turkey (15.3% in Istanbul and 5.7% in Afyonkarahisar in 542 sheep from 4 flocks and 248 sheep from 22 flocks, respectively) (Preziuso *et al.*, 2010; İnce, 2020). Moreover, in Japan, a survey in 267 adult sheep from 14 sheep flocks using both AGID and ELISA tests revealed only three seropositive animals, indicating a limited spread of MVV in the country (Giangaspero *et al.*, 2011). Medium prevalence rates were observed in Germany (28.8% in 2229 sheep from 41 farms) (Hüttner, Seelmann and Feldhusen, 2010), Canada (32.0% in 1954 sheep from 29 farms) (Arsenault *et al.*, 2003), and Iran (34.5% in 220 sheep from 30 flocks) (Norouzi *et al.*, 2015), while in Spain seroprevalence rates ranged from medium to high among the studied regions [24.8%, 52.83%, and 54.4% in three studies in Spain including 15,155 sheep from 78 flocks (Lago *et al.*, 2012), 274,048 sheep from 554 flocks (Pérez *et al.*, 2010), and 5,120 sheep from 239 flocks (Alba *et al.*, 2008)], and in China ranged from 4.6–50.0% in a study involving 672 sheep from 24 flocks (Zhang *et al.*, 2013). Nevertheless, direct comparisons between the seroprevalence values in the aforementioned studies and the seroprevalence found in our study are rather arbitrary, as in the vast majority of those studies, animals were tested once (cross-sectional studies) and various farms (of unknown SRLV infection status), breeds, and production systems were considered. On the other hand, in our study, farms were selected using specific criteria, namely: i) the seropositive status at the farm level, which was necessary for the assessment of the morbidity frequency measures during the study and at predetermined stages of the production cycle; and ii) zero-grazing, intensive management, where animals are more exposed to SRLV infections due to closer contact and extensive horizontal transmission of the MVV (Leginagoikoa *et al.*, 2006a; Leginagoikoa *et al.*, 2010; Pérez *et al.*, 2010; Lago *et al.*, 2012; Barquero *et al.*, 2013c). Moreover, the increased seroprevalence found in the present study could be partially associated with the type of ELISA used for testing; it was a whole-virus ELISA with the capacity to increase the detection spectrum of specific antibodies and, subsequently, the sensitivity and the overall performance of serological testing.

Longitudinal studies investigating the morbidity frequency measures of SRLV are limited, and the estimation of these measures in these studies is mainly based on the results

exclusively from serological testing (Berriatua *et al.*, 2003; Leginagoikoa *et al.*, 2010); thereby, benchmarking our results with similar studies to comparatively assess and update the current situation regarding SRLV infections on various occasions is partially feasible. The prevalence and incidence rates observed in our study are in agreement with those found in a prospective serological study on intensively reared Assaf sheep (Leginagoikoa *et al.*, 2010). On the contrary, the overall cumulative incidence rate found herein was higher (64.2%) compared to the respective estimates by studies on semi-intensively reared Latxa breed flocks (19.6% and 27.0%) (Berriatua *et al.*, 2003; Leginagoikoa *et al.*, 2010).

In our study, the actual prevalence increased compared to the seroprevalence, with the differences between the two values being higher in Chios compared to Lacaune ewes, thus, confirming the significant role of the late seroconversion and the intermittent presence of antibodies mainly in the Chios breed. Point prevalence was ca. 10% higher than seroprevalence, and ca. 35.0% of constantly seronegative animals were tested PCR positive at least once during the first year of the study. This is consistent with previous studies, indicating late seroconversion or no seroconversion incidents after the infection (Barquero *et al.*, 2011; De Regge and Cay, 2013; Barquero *et al*., 2013a; Dolfini *et al.*, 2015; Chassalevris *et al.*, 2020). The continuous increase in point prevalence indicates the significant role of horizontal transmission in the spreading of SRLV infections within the herd. On the other hand, the decreased seroprevalence in the last sampling occasion in all age classes, in combination with the constant increase in the point prevalence, could be explained by the seroreversion of some animals. Moreover, the stabilization of seroprevalence rates in animals older than three years old could be associated with the fact that there is a dynamic balance between seroconverted and seroreverted animals after that age; indeed, in most cases, seroconversion occurred before the age of three, whereas seroconversion and seroreversion incidents were about the same in older animals. Nevertheless, period prevalence increased by ca. 15.0% in the first year of the study and further increased by ca. 5% in the second year, suggesting that SRLV prevalence gradually reaches a plateau, with ca. 7.0% of animals remaining uninfected (negative results in both ELISA and PCR during the study). The presence of uninfected animals in farms with high SRLV prevalence is attributed either to the young age of some of the animals or to a potential underlying genetic resistance against the infection (Molaee, Eltanany and Lühken, 2018; Tumino *et al.*, 2022).

Moreover, this is the first time different serological patterns, as determined by the seroconversion and seroreversion incidents, are defined and described in SRLV naturally infected sheep under field conditions in a large-scale study. Based on our findings, 9.8%

(40/407) of animals demonstrated an intermittent presence of antibodies, and 8.6% (35/407) seroreverted at some point. The presence of these serological patterns is not likely to result from a poor diagnostic performance of the ELISA test; in fact, all serological analyses were performed by the same trained veterinarian in a single laboratory, using the same equipment and protocols, shortly after the blood samplings to avoid inconsistencies and minimize the possibilities of diagnostic errors and the misclassification of animals. Moreover, the proportion of seroreverted animals and animals with an intermittent presence was too high to be attributed to false negative results. The latter is also supported by the fact that more than 50.0% of the animals in the aforementioned categories demonstrated specific serological patterns during the study, which were not characterized by a single seroconversion or seroreversion event. In fact, the majority of seroreverted animals were found to be seronegative more than once after consecutive positive results, while most of the animals with an intermittent presence of antibodies had an alternating serological status between sampling occasions. Furthermore, only one animal with an intermittent presence of antibodies and one seroreverted animal were constantly negative in PCR testing.

Seroreversion reactions have been previously reported in studies with a limited number of animals associated with the transient presence of maternal antibodies (lambs and kids), the experimental infection of animals with inadequate immune response (goats) (De Andrés *et al.*, 2005), and advanced MV clinically manifested cases (Mekibib *et al.*, 2018); however, the mechanism behind this serological reaction has not been elucidated. Seroreversion has also been described in HIV infected adults and children following antiretroviral therapy after the acute infection phase (Jurriaans *et al.*, 2004; Kassutto, Johnston and Rosenberg, 2005; Amor *et al.*, 2006; Hare *et al.*, 2006; Eberle *et al.*, 2010; De Souza *et al.*, 2016) as well as in end-stage HIV patients (Gutiérrez *et al.*, 1994); in the first case, it derives from the long-lasting viral suppression and the subsequent restricted production of antibodies, whereas, in the second case, the most possible explanation is the loss of antibodies against capsid proteins. In the present study, the first explanation could be reasonable as animals were infected and maintained the seropositive status for a long period before the seroreversion incident; moreover, the mean relative OD values of seroreverted animals and animals with an intermittent presence of antibodies in a seronegative status remained relatively high and closer to the threshold of the ELISA test (28.91 and 18.50, respectively), compared to the constantly seronegative animals (−1.45). These values could be used as evidence of seroreversion rather than a seronegative status, which is further confirmed by the molecular testing of these animals, where only 2 out of 35 seroreverted animals were found PCR

negative at the seroreversion incident, one animal was found continuously PCR negative during the study, and the rest of the seroreverted animals were found PCR positive either continuously or intermittently during the study. Also, the mean Ct values of seroreverted animals remained almost stable after the seroreversion incident, indicating that the loss of antibodies may not be associated with a respective viral suppression.

The mean relative OD values of animals with an intermittent presence of antibodies were below the threshold at pre-mating and were above it at pre-lambing sampling occasions, suggesting an underlying regulatory humoral immune response mechanism associated with late pregnancy. The results from molecular investigation of these animals confirmed the intermittent presence of the virus within detectable limits in 22 out of 40 animals, whereas all the other animals were tested constantly positive (except for one that remained constantly negative). Also, the mean Ct values of these animals were slightly higher in the seronegative status compared to the respective values in the seropositive status (16.6 and 12.8, respectively), suggesting an association of serorevesion with the decrease of viral load in blood circulation.

The concordance between ELISA and PCR was found to be moderate (77.9%) confirming the results by Chassalevris *et al.*, (2020), who compared a semi-nested real-time PCR with a commercial indirect ELISA. During our study, PCR positive results were found in 97.1% of animals found seropositive at least once, with only 6 constantly seropositive animals, 2 seroconverted, 1 seroreverted and 1 ewe with an intermittent presence of antibodies remaining PCR negative across the study. This could be attributed to the low circulating viral load at the post seroconversion phase, which remains under the LOD of the PCR assay (De Andrés *et al.*, 2005), or to the reduced sensitivity of the applied real-time PCR for the specific strain of these infected animals. However, the reduced sensitivity of the applied PCR assay cannot be a possible explanation in our study, as the development and evaluation of the applied real-time PCR protocol were based on the phylogenetic analyses of the circulating strains in the studied farms, and the number of seropositive animals that tested PCR negative was small. Also, ca. 50% of constantly seronegative animals were found PCR positive, which has been previously described and attributed to either the late seroconversion or the occurrence of viral latency in monocyte and myeloid stem cells (De Andrés *et al.*, 2005; Blacklaws, 2012; Cardinaux *et al.*, 2013; Ramírez *et al.*, 2013). Nevertheless, not all of the seroconverted animals were detected to be PCR positive at an earlier stage, while the mean period of immune response estimated in our study was about 3 months.

PCR positive animals that were found seronegative at least once during the study underpin the possibility of misdiagnosis in cross-sectional sero-epizootiological study designs. This could at least partially explain the failure of current ELISA-based control programs to eradicate SRLV in specific regions, highlighting the significance of consecutive screening controls in infected flocks and/or the combination of serological and molecular testing.

Also, it is the first time that risk factors for SRLV infections are prospectively evaluated in intensively reared dairy sheep farms in Greece, while their effects on seropositive and infected status, on the occurrence of different serological and infection patterns, and on seroconversion and seroreversion incidents are assessed.

Among the studied risk factors, increased age has been extensively assessed in several cross-sectional studies, and its significance for seropositive status has been sufficiently documented (Arsenault *et al.*, 2003; Leginagoikoa *et al.*, 2006b; Lago *et al.*, 2012; Norouzi *et al.*, 2015). This association has been attributed to the late seroconversion of infected animals (De Andrés *et al.*, 2005), the establishment of latent infection for a long period after the initial infection (Blacklaws, 2012), and the increased risk of older animals for infection due to a longer exposure to the virus compared to the younger animals (Lago *et al.*, 2012). In our study, it was the first time that age was evaluated as a potential risk factor for i) both the seropositive status, the PCR positive status, and the infected status; ii) the manifestation of specific serological and infection patterns; and iii) the seroconversion/seroreversion incidents under a prospective study design. Our results confirmed that the RR for the seropositive and infected status and the constantly seropositive pattern were increasing with age. On the other hand, age was negatively associated with the constantly seronegative pattern and the intermittent presence of antibodies, as well as the seroconversion incident. The negative association between age and seroconversion incidents could be explained by the fact that older animals had already seroconverted before the initiation of the study, remaining constantly seropositive during it.

Other studies have also suggested a breed-related susceptibility, especially for the purebred animals compared to the cross-bred ones (Hüttner, Seelmann and Feldhusen, 2010; Barquero, *et al.*, 2013c; Pavlak *et al.*, 2022). In our study, breed was recognized as a risk factor for the seropositive status and the PCR positive status, but not for the infected status. Lacaune ewes were more likely to be found seropositive, whereas Chios ewes were more likely to be found PCR positive. Another remarkable finding of this study, which could also provide an explanation of the above-mentioned results, is the increased likelihood of an intermittent presence of antibodies in Chios ewes. Although this specific serological pattern had been

reported in the past (De Andrés *et al.*, 2005), it had not been further investigated or linked to specific animal characteristics. The increased frequency of the intermittent presence of antibodies pattern in the Chios breed could be, at least partially, attributed to a breed-specific immune response to SRLV infections, as the diagnostic performance of the applied ELISA had been evaluated and found to be adequate before the initiation of the study. The RRs of seropositive and infected statuses in both breeds need to be further investigated on more farms. In our study, the confounding effect of the farm cannot be excluded; however the inclusion of the farm as a random factor in the statistical model diminishes this effect. Based on the current study design and the fact that the breed was not found to be significantly associated with the PCR positive status, it was not possible to imply a potential genetic susceptibility/resistance to SRLV infection for the studied breeds.

To our knowledge, for the first time, the production stage is evaluated as a potential risk factor with regard to the seropositive and infected status. In our study, the sampling occasions were predetermined twice during the production cycle, namely 3-4 weeks before the onset of the mating season and 2-4 weeks before the lambing season. It was found that the seropositive and infected status, the seropositive status in animals with an intermittent presence of antibodies, and the seroconversion incidents were increased at pre-lambing. Contrarily, PCR positive status was not associated with the production stage. Therefore, the increased seropositivity of ewes at pre-lambing could be attributed to the increased antibody titers during the last stage of gestation. However, this finding is not consistent with a previous study in goats, where a drop in the antibody titers against SRLV was observed in seropositive animals during the last month of gestation (Czopowicz *et al.*, 2017). In general, a decline in blood serum IgG antibodies is known to naturally occur during the last month of gestation in sheep, attributed to the transferring of IgG antibodies to the colostrum and the suppressed immunological response (Beasley, Kahn and Windon, 2010; Herr, Bostedt and Failing, 2011; Chniter *et al.*, 2016; Walraph *et al.*, 2018). However, this is not the first time that a viral disease has been linked to an increased antibody titer during the last stage of gestation. For example, in a study on Bovine Viral Diarrhea virus in cattle, total IgG and IgG1 antibodies were reduced, while IgG2 antibodies were increased at that stage (Bachofen *et al.*, 2013). Of course, our findings are not directly comparable to the aforementioned ones due to the different species, pathogen, and immunological response (IgG1 instead of IgG2 antibodies) (Singh *et al.*, 2006). Consecutive measurement of anti-SRLV specific total IgG for a long period pre- and post-lambing could elucidate this serological reaction and its association with the periparturient period. Based on our findings, the increased likelihood of seropositive

status at pre-partum should be considered when designing control programs to avoid underdiagnosis of infected but seronegative animals that could serve as reservoirs of the virus, resulting in the gradual re-emergence of high prevalence rates.

Seroreversion incidents increased during the third year of the study compared to the first year, while seroreverted animals in our study belonged mainly to age class 4 ($3 < x \leq 4$) and their mean age at the seroreversion incident was 5.2 ± 0.64. These findings could indicate a modified humoral immune response that results in seroreversion in animals that have been infected and have been seropositive for a long time, as previously described in HIV patients, after a long period of clinical disease and immunosuppression (Gutiérrez *et al.*, 1994)

Poor BCS has been previously reported in seropositive animals (Junkuszew *et al.*, 2016). Chronic incurable disease and progressive weakness caused by SRLV infections is observed in some of the animals with clinical signs. In our study, decreased BCS was not associated with seropositive status. However, it was lower in PCR positive animals with an intermittent presence of antibodies. It could be hypothesized that the intermittent presence of antibodies coincides with early clinical signs of the disease, which include body weight loss. On the other hand, a reverse mechanism could not be excluded; the loss of antibodies could result in the virus circulation within detectable limits and in BCS deterioration. The elucidation of this underlying mechanism demands another study design with more frequent serological and molecular monitoring of animals with an intermittent presence of antibodies. Considering that none of the studied farms had a history of clinical cases of MV and animals were reared under intensive farming conditions with satisfying preventive herd health management (antiparasitic treatments and vaccinations) and nutrition according to their demands (production stage, lactation stage and milk production), it is possible that the association between BCS and infection/serological status or patterns was not evidenced due to the high health and management status.

2. Epizootiological study in lambs

SRLV seropositivity and infections in Chios and Lacaune lambs, as well as potential risk factors, are prospectively studied for the first time. According to the available literature, although lactogenic transmission is a major route of SRLV spreading within the flock (Blacklaws *et al.*, 2004), relevant epizootiological studies for the investigation of seropositivity and infection status of lambs are scarce (Álvarez *et al.*, 2005, 2006; Araújo *et al.*, 2020).

In our study, lambs were not grouped within the same farm according to different types of colostrum, the infection status of dams, or the rearing method; instead, the lambs in each farm were all reared under the system already applied. This allowed the assessment of vertical transmission dynamics per farm and under real-world conditions, but did not allow the assessment of natural versus artificial suckling as a control measure.

Seroprevalence at the age of 1 month was higher in lambs originating from seropositive and infected ewes compared to seronegative and uninfected ones. Considering that bulk pasteurized colostrum was administered to lambs on farms C and D, the seroprevalence rates in lambs for this comparison were calculated only for farms A and B, where lambs were fed unpasteurized colostrum derived from their dams. The presence of high seroprevalence rates in lambs originating from seropositive ewes is expected and has been previously reported (Álvarez *et al.*, 2005), due to the presence of maternal antibodies that pass through the digestive track in the lambs' blood circulation after colostrum consumption.

At the age of three months, the seroprevalence in lambs originating from seropositive ewes was substantially decreased, as many lambs lost their maternal antibodies. According to the available literature, this is the first time that evidence of the duration of maternal immunity against SRLV in lambs has been presented. Seroreversion and seroconversion incidents occurred until the age of 8 months in more than 90.0% and 70.0% of the seroreverted and seroconverted lambs, respectively. Hence, the age of 8 months (which coincides with the pre-mating period in most of the farms) could be proposed as the most appropriate time to apply an early screening program based on ELISA testing while minimizing the possibility of false positive results due to the presence of maternal antibodies. Nevertheless, ca. 40.0% of lambs that were infected during the first 3 months of their life did not seroconvert until the age of 13 months, a percentage that is higher than the respective (19.0%) reported by Álvarez *et al.*, (2006) in Latxa lambs. This finding highlights the necessity of either repeating ELISA testing at the age of 13 months or applying a combination of ELISA and PCR testing for the detection of SRLV infections in yearlings.

The fact that only a few lambs fed pasteurized colostrum were found infected in the first month of their life (9.0%) confirms the efficiency of feeding pasteurized colostrum as a preventive measure. Assuming that the administration of pasteurized colostrum was performed in newborn lambs immediately after their birth and no physical contact was permitted with their dam, the 5 lambs originated from infected ewes that were found infected at the age of 1 month, were either infected transplacentally, at lambing from maternal body fluids and blood, or horizontally from other infected lambs during rearing. This finding is

consistent with the occurrence of SRLV infections in newborn lambs reported in previous studies, where the vertical transmission was assessed before suckling or in lambs fed pasteurized or substitute colostrum (Blacklaws *et al.*, 2004). The significance of transplacental transmission cannot be assessed with certainty under the present study design, as the possibility of infection from the unnoticed consumption of infected colostrum and the horizontal transmission during the first month between lambs reared in the same shed cannot be excluded. In any case, it is concluded that the maternal transmission of SRLV accounts for less than 10.0% of the total SRLV prevalence in newborn lambs.

On the other hand, in lambs that naturally suckled colostrum from their dams, the SRLV prevalence was ca. 20.0% in both farms A and B, despite the fact that in farm A the lambs were naturally reared, whereas in farm B the lambs were isolated from their dams and artificially reared after the consumption of colostrum from their dams. The prevalence values estimated in lambs in the present study are similar to the values previously reported by Álvarez *et al.*, (2006); even though they were obtained under dissimilar study designs (in the latter study, lambs were grouped and reared depending on the infection status of their dam, the type of colostrum and mode of feeding, and the method of rearing).

The administration of either colostrum from uninfected ewes or pasteurized and substitute colostrum has already been suggested as a management intervention and has been successfully implemented in combination with other preventive measures for the control or eradication of SRLV infections in infected flocks (Peterhans *et al.*, 2004; Ramsés Reina *et al.*, 2009; Polledo *et al.*, 2013). In our study, the significant role of colostrum on SRLV transmission was confirmed; SRLV prevalence in lambs that consumed colostrum from their dams, regardless of the subsequent method of rearing (natural or artificial), was almost double compared to the lambs that consumed pasteurized colostrum. Also, the latter lambs were less likely to be found seropositive or infected until the age of 13 months. Therefore, disruption of the lactogenic transmission in intensive farms with high SRLV prevalence rates is of paramount importance given that the presence of infected newborn lambs due to transplacental transmission or transmission at birth cannot be avoided.

Herein, the presence of infected lambs due to maternal transmission resulted in a gradual increase of SRLV infections until the age of 8 months in the lambs fed pasteurized colostrum. Contrarily, in farms where lambs consumed unpasteurized colostrum, the infection rate was higher due to both maternal and lactogenic transmission, which facilitated the rapid virus spread during the following months. The continuous increase in infection rates in lambs fed pasteurized colostrum is possibly associated with the horizontal transmission between the

lambs. Horizontal transmission was even more extensive at the age of 8-9 months, when all the studied lambs were mixed with adult male and female animals at mating.

As expected, the infection status of dams was found to be significantly associated with the infection of lambs until the age of 8 and 13 months; lambs from uninfected ewes were less likely to be found infected. This is expected, as in that case lactogenic transmission does not occur, while potential genetic resistance could also be assumed for these lambs. To confirm this hypothesis, candidate genes associated with susceptibility or resistance against SRLV infections have to be sought and assessed.

3. Control of small ruminant lentiviruses infections in Greece

In Greece, dairy sheep farming is the major sector of livestock production, and the wide spreading of infectious diseases in high-yielding purebred dairy sheep could result in its substantial deterioration. Considering the findings from the present study and the undeniable significance of SRLV infections, direct measures are imperative to reduce SRLV dispersion and restrict their subsequent effects.

Several control programs have been implemented worldwide at the country level (Houwers *et al.*, 1987; Sihvonen *et al.*, 2000; Kampen *et al.*, 2008; Tavella *et al.*, 2018; De Martin *et al.*, 2019) or at the farm level (Williams-Fulton and Simard, 1989; Pérez *et al.*, 2010, 2013; Seyoum *et al.*, 2011; Polledo *et al.*, 2013) with various results. Although different management practices were implemented and evaluated, the diagnostic protocol for the detection of infected animals was based exclusively on serological methods, leaving the infected seronegative animals undiagnosed and retarding the eradication of the infections.

The designation and implementation of successful control programs against SRLV infections need to be based on epizootiological data and adjusted according to the applied farming systems and the potential risk factors at the animal and farm levels.

Considering that SRLV infections cause a chronic disease, prospective studies can lead to safer conclusions compared to cross-sectional ones as regards relevant epizootiological indices. Nonetheless, our study was limited to four intensive dairy sheep farms with high SRLV prevalence rates and investigated specific risk factors at the animal level. The investigation of SRLV epizootiology in more animals of various breeds reared under dissimilar farming systems at farms with different prevalence rates and the evaluation of more risk factors either at the animal or at the farm level (environmental factors, housing conditions, management practices, etc.) could enrich and extend the findings of this study regarding the proposal of an integrated control program for our country. Nevertheless,

considering the current situation with the complete lack of epizootiological data regarding SRLV infections and the inexistence of applicable control programs in our country, the results from our epizootiological study can form a stepping stone for the development of an evidence-based control program for dairy sheep in our country, exploiting the diagnostic protocol proposed herein.

To implement such a program, an initial serological screening is imperative for the classification of the flocks according to their seroprevalence rates as highly (>50.0%), medium (26.0-50.0%), and low (1.0-25.0%) infected or SRLV-free flocks (<1.0%) (Peterhans *et al.*, 2004; Reina *et al.*, 2009; Pérez *et al.*, 2010). After this initial screening, the management practices and steps for the reduction and the medium-term elimination of SRLV infections are determined, as described in Figure 39. In every case, the sufficient diagnostic performance of the applied ELISA and PCR protocols used in the control program should be ensured for the circulating strains.

As shown in Figure 39, culling of positive animals and replacement with breeding stocks from SRLV-free flocks could be a sustainable option only in areas where the seroprevalence is very low and SRLV-free flocks are available. Otherwise, there is a serious threat of significant monetary losses and the restriction of genetic resources, which may undermine the sustainability of the farms, particularly in areas with a developed dairy sheep farming industry (Reina *et al.*, 2009). Therefore, the eradication of SRLV infections in flocks with low seroprevalence could be less time-consuming compared to flocks with medium and high prevalence rates. Also, regardless of the observed prevalence rate, the implementation of general good hygiene practices, the reduction of stocking density, the improvement of ventilation, and the importation of breeding stocks only after serological and molecular testing should be considered standard preventive measures. Also, the above-mentioned practices limit stress factors and the presence of co-infections, which could further deteriorate the effects of SRLV infections on the health and productivity of dairy sheep. Last but not least, all efforts for the eradication of SRLV infections should be coordinated under a national control program and reference laboratories responsible for the surveillance of the infections.

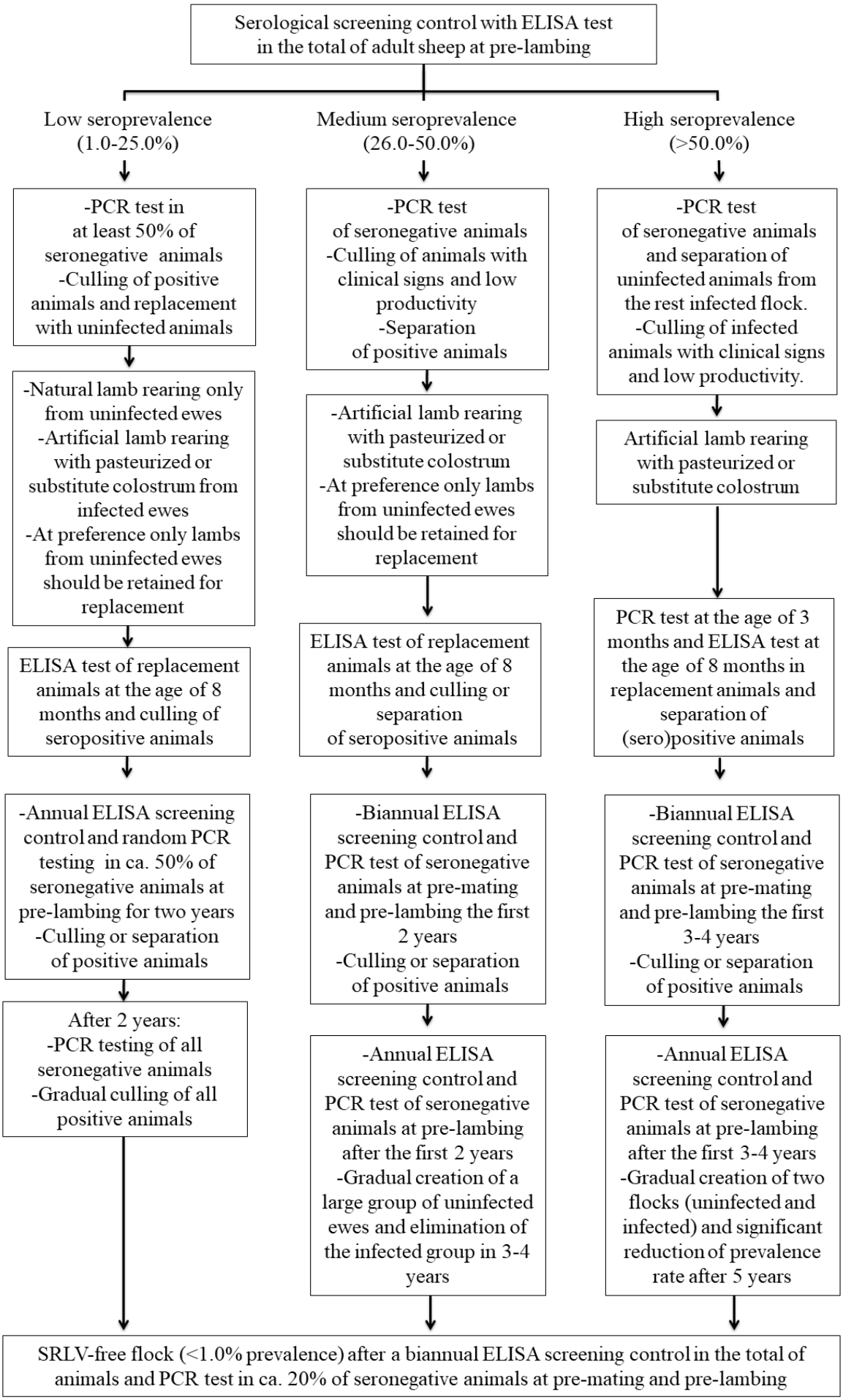


Figure 39. Flow chart describing the management practices for the control of small ruminant lentiviruses infection in intensive dairy sheep farms

Chapter 3: The effects of small ruminant lentiviruses infections on productivity and health and welfare status of intensively reared dairy sheep

I. Objectives

Although it is evident that clinical manifestation of SRLV infection leads to impaired health and welfare status and reduced productivity in infected animals, the severity of clinical signs varies. The occurrence of subclinical SRLV infections and the multivariability of the factors determining animals' productivity (farming system, breed, age, farm management practices, etc.) do not allow a universal quantification of the effects of SRLV infections on animal productivity and farm sustainability. Also, a crucial factor hindering the assessment and quantification of SRLV effects on animal health and productivity is the reliable detection of infected animals. In previous studies, the diagnosis of infected animals was performed only with serological testing, mainly with ELISA, except for one study where serological and molecular testing were combined (Echeverría *et al.*, 2020). Although ELISA is widely used for routine screening control as it is considered highly sensitive compared to previous serological tests such as AGID, its utilization can lead to the misclassification of many infected animals as seronegative. Delayed seroconversion has been previously reported and proved in the present study, while SRLV infected animals may never seroconvert, may serorevert, or may demonstrate an intermittent presence of antibodies, as presented in Chapter 2 of the dissertation. In these cases, the infected animals may evade serological diagnosis, masking the effects of SRLV infections on health and productivity.

In our country, although dairy small ruminant farming is well-developed, the effects of SRLV infections have not been studied and quantified, except for one serological study that was conducted over 25 years ago in an experimental dairy sheep setting, where seropositive animals were removed from the farm leading to biased results (Ploumi *et al.*, 2001). Although SRLV infections and clinical disease thereof have been previously reported in Greece (Karanikolaou *et al.*, 2005; Angelopoulou, Brellou and Vlemmas, 2006; Brellou *et al.*, 2007; Giadinis *et al.*, 2015), a detailed recording of clinical signs, directly or indirectly associated with SRLV infections, has not been attempted in infected flocks.

Considering the existing gaps in assessing and quantifying the effects of SRLV infections on milk production, health and welfare traits, a prospective cohort study was conducted to evaluate them, utilizing a diagnostic protocol combining ELISA and PCR testing. In particular, the objectives of the present study were to evaluate and quantify the effects of

SRLV infections on i) milk yield and milk quality traits [fat, protein, lactose, and solids-not-fat yield, and SCC)], and ii) health and welfare status in intensively reared dairy sheep.

II. Materials and Methods

1. Animal population and study design

A total of 527 milking ewes (1 to ≥ 7 years old) from the initially enrolled ones described in Chapter 2 were involved in the study at post-weaning (60 days post-partum). These ewes were prospectively studied bimonthly for 4 months (3 sampling occasions) for one milking period to assess the effects of SRLV infections on milk yield and quality traits. The percentages of ewes at the 1^{st}, 2^{nd}, 3^{rd}, or $\geq 4^{th}$ lactation were 26.6% (140/527), 22.0% (116/527), 20.3% (107/527), and 31.1% (164/527), respectively.

For the assessment of the impact of SRLV infections on the health and welfare status of animals, all of the initially enrolled ewes described in Chapter 2 were prospectively studied for two consecutive years.

2. Milk samplings and analyses

In each sampling occasion, milk yield was recorded, and milk samples were collected from each ewe to perform physicochemical analyses and estimate SCC. The milk yield recording and milk sampling were performed using ICAR (International Committee of Animal Recording)-approved equipment (Waikato Milkmeter, InterAg, Hamilton, New Zealand) and protocols during the morning milking. One composite milk sample (ca. 70 ml) was collected from the milkmeter's sampler at the end of the milking. After the collection of milk samples, sodium azide tablets (Supelco®, Merck Milipore, Burlington, MA, USA) were added, and milk samples were transferred under 4 °C in the lab to be analyzed within 24 h. Milk samples were analyzed for fat, protein, lactose, solids-not-fat, and SCC (Lactoscan Combo, Milktronic Ltd). In each sampling occasion, ear tag, breed, and body condition score (BCS) were recorded.

3. Blood samplings and recordings of health and welfare indicators

All ewes were blood sampled, and serum and whole blood samples were used for ELISA and real-time PCR testing, respectively, for the detection of SRLV infections semiannually, at pre-mating and pre-lambing, for two consecutive years, as detailed in Chapter 2. In each sampling occasion, ewes were physically examined using a modified version of the AWIN (Animal Welfare Indicators) protocol, and 17 health and welfare indicators were assessed at the animal level. Recordings included BCS (1-5, 1 = emaciated, 5 = obese with 0.25

increments) (Russel, Doney and Gunn, 1969), the occurrence of foot-related lameness, arthritis, respiratory disease (cough and abnormal respiratory sound), ocular and nasal discharge, body abscesses, mastitis, udder lesions and deformities (skin lesions, abscess, wart-like lesions, mammary cyst, fibrosis and asymmetry) (0 = absence, 1 = presence), the size of supramammary lymph nodes (1-5), and the wool quality (0 = good quality, 1 = poor quality). Also, ear tag, breed, and age were recorded.

Moreover, blood samples from 284 ewes of the study were collected at the last sampling occasion and used for hematological analyses (Mindray BC-30Vet), which included: white blood cell count (WBC), granulocyte count (GC), lymphocyte count (LC), monocyte count (MC), percentages of granulocytes (GP), lymphocytes (LP), and monocytes (MP), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean values of corpuscular volume (MCV), corpuscular hemoglobin (MCH), corpuscular hemoglobin concentration (MCHC), red cell distribution width coefficient of variation (RDW-CV) and standard deviation (RDW-SD), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), platelet large cell count (P-LCC), and platelet large cell ratio (P-LCR).

4. Statistical analyses

4.1 Milk production traits

4.1.1 Calculation of daily and total milk production

The final data set included 1,378 recordings of milk yield and quality traits. For the statistical analyses, SCC were log transformed (LogSCC), while daily milk, fat, protein, lactose, and solids-not-fat yields (DMY, DFY, DPY, DLY, and DSNFY, respectively) were calculated using the morning milking records and after adjusting following the ICAR recommendations (ICAR, 2018). Afterwards, the total milk, fat, protein, lactose, and solids-non-fat yields (TMY, TFY, TPY, TLY, and TSNFY, respectively) were calculated for the first 120 days of the milking period using the Fleischmann method and the ICAR instructions (ICAR, 2018).

4.1.2 Categorization of milking ewes according to their serological and infection pattern

Based on the last year's ELISA results at pre-mating (the last third of the previous milking period), at pre-lambing (before the beginning of the studied milking period), and again at pre-mating (the last third of the studied milking period) sampling occasions, the animals were categorized as constantly seropositive, constantly seronegative, seroconverted, seroreverted, and with an intermittent presence of antibodies during the last year.

Similarly, according to the combination of ELISA and PCR results, animals were categorized as uninfected (both ELISA and PCR negative), infected seropositive (both ELISA and PCR positive), and infected seronegative (only PCR positive) during the same period.

4.1.3 The effects of small ruminant lentiviruses infections on milk yield and quality traits

Descriptive statistics (mean ± SD) were calculated for milk yield and milk quality traits. The following mixed linear regression model was built in SPPS v.26 for the assessment of the effects of serological and infection patterns on DMY, DFY, DPY, DLY, DSNFY, and LogSCC:

$$Y_{ijklm} = \mu + P_i + S_j + B_k + a_1 \times age + a_2 \times BCS + E_l + F_m + \delta_{lj} + e_{ijklm}$$

where Y_{ijklm} = dependent variables (DMY, DFY, DPY, DLY, DSNFY, and LogSCC); μ = intercept; P_i = fixed effect of either the serological pattern (i = 5 levels; constantly seronegative, constantly seropositive, seroconverted, seroreverted, and intermittent presence of antibodies) or the infection pattern (i = 3 levels; uninfected, infected seropositive, and infected seronegative); S_j = fixed effect of the sampling occasion (j = 3 levels; 1^{st} to 3^{rd} sampling occasion); B_k = fixed effect of the breed (k = 2 levels, Chios and Lacaune breed); a_1 = fixed effect of the regression coefficient of age; a_2 = fixed effect of the regression coefficient of BCS; E_l = random variation of the l^{th} ewe; F_m = random variation of the m^{th} farm; δ_{lj} = repeated variation of the l^{th} ewe in the j^{th} sampling occasion; and e_{ijklm} = residual error.

A linear regression model was also built for the assessment of the effects of the serological and infection patterns on the 120-day milk, fat, protein, lactose, and solids-not-fat yields, as described in the following model:

$$Y_{ijk} = \mu + P_i + B_j + a_1 \times age + F_k + e_{ijk}$$

where Y_{ijk} = dependent variables (TMY, TFY, TPY, TLY, and TSNFY); μ= intercept; P_i = fixed effect of either the serological pattern (i = 5 levels; constantly seronegative, constantly seropositive, seroconverted, seroreverted, and with an intermittent presence of antibodies) or the infection pattern (i = 3 levels; uninfected, infected seropositive, and infected seronegative); B_j = fixed effect of the breed (j = 2 levels, Chios and Lacaune breed); a_1 = fixed effect of the regression coefficient of age; F_k = random variation of the k^{th} farm; and e_{ijk} = residual error.

The first-order autoregressive covariance structure was selected as the most appropriate for all the models based on Akaike's information criterion (AIC) value. The assumptions of normal distribution, homoscedasticity, and linearity for the models were checked using the scatterplot of standardized predicted values against the standardized residuals and the probability-probability and quantile-quantile plots of standardized residuals.

To assess the effects of the serological or infection patterns (5 levels and 3 levels, respectively, as previously described), adjusted for the fixed effects of breed (2 levels, Chios and Lacaune), and age (covariate), and the random effect of farm, on the lactation persistency (binary outcome, 0 = lactation period ≥ 7 months, 1 = lactation period < 7 months) a mixed binary logistic regression model was built. In this model, scaled identity was selected as the most appropriate covariance structure.

4.2 Health and welfare status

4.2.1 Categorization of ewes according to their serological and infection status and pattern

In the statistical analyses for the assessment of the effect of SRLV infections on health status only the 407 ewes described in Chapter 2 were considered and were categorized according to their serological and infection statuses and patterns as previously described.

4.2.2 The effects of small ruminant lentiviruses infections on health and welfare status

Descriptive statistics (frequencies) of health disorders and welfare issues were calculated, and the effects of SRLV infections on their occurrence were assessed using mixed binary logistic models. Mixed binary logistic models with repeated measures were built for the assessment of the occurrence of health disorders and welfare issues during the study. In these models, serological or infection status [2 levels (seronegative or seropositive) and 3 levels (uninfected, infected seropositive, or infected seronegative, respectively)], breed (2 levels, Chios and Lacaune), sampling occasion (2 levels, pre-mating and pre-lambing), and year of the study (3 levels, 1^{st}, 2^{nd}, 3^{rd}; in the third year only pre-mating period was considered) were used as fixed effects, age and BCS as covariates, while the random effects of farm and animal were also considered.

For the assessment of the occurrence of health disorders and welfare at least once during the study period, mixed binary logistic models were used with serological or infection pattern [5 levels (constantly seronegative, constantly seropositive, seroconverted, seroreverted, and with an intermittent presence of antibodies), and 3 levels (uninfected, infected seropositive or

infected seronegative), respectively], breed (2 levels; Chios and Lacaune), and age (covariate) being forced into them as fixed effects, and farm as a random effect. Scaled identity was selected as the most appropriate covariance structure according to Akaike's information criterion (AIC).

Moreover, another set of mixed linear regression models was used for the assessment of SRLV infections on hematological parameters, with either the serological or infection animal status at the specific sampling occasion [2 levels (seronegative or seropositive) and 3 levels (uninfected, infected seropositive or infected seronegative) respectively] or the serological and infection animal pattern during the study [5 levels (constantly seronegative, constantly seropositive, seroconverted, seroreverted, and with an intermittent presence of antibodies), and 3 levels (uninfected, infected seropositive or infected seronegative), respectively], breed (2 levels; Chios and Lacaune), and age (covariate) being used as fixed effects, and farm as random effect. The first-order autoregressive covariance structure was selected as the most appropriate one for all the mixed linear regression models based on Akaike's information criterion (AIC) value.

III. Results

1. The effects of small ruminant lentiviruses infections on productivity of milking ewes

1.1 Descriptive statistics of daily milk yield and quality traits

A total of 527 ewes (329 Chios and 198 Lacaune ewes) were recorded for their milk yield and sampled in the first sampling occasion, whereas 387 (229 Chios and 155 Lacaune ewes) had a full set of recordings and analyses during the studied period. The mean age (± SD) of ewes was 3.0 ± 1.5 years and the mean value (± SD) of BCS during the whole lactation period was 2.8 ± 0.21, ranging between 2.7 ± 0.17 in the first sampling occasion and 2.9 ± 0.21 in the third sampling occasion. A total of 22.4% (118/527) ewes were constantly seronegative, 53.5% (282/527) constantly seropositive, 17.6% (93/527) seroconverted, 4.7% (25/527) seroreverted, and 1.7% (9/527) presented an intermittent presence of antibodies. Regarding their infection pattern, 14.8% (78/527) of ewes were uninfected, 73.1% (385/527) were infected seropositive, and 12.1% (64/527) were infected seronegative.

The overall mean values (± SD) of DMY, DFY, DPY, DLY, DSNFY, and logSCC during the lactation period were 2.0 ± 0.99 kg, 115.6 ± 50.86 g, 106.0 ± 49.99 g, 100.6 ± 47.43 g, 224.1 ± 105.64 g, and 5.4 ± 0.60 cells/ml, respectively. The mean values of DMY, DFY, DPY, DLY, DSNFY, and logSCC for the serological patterns during the study are summarized in Tables 28-33. The respective values for the infection patterns are shown in Figures 40a-f.

Table 28. Mean values (SD) of daily milk yield during the study in different serological patterns.

Serological Pattern	1st sampling occasion	2nd sampling occasion	3rd sampling occasion	Overall
	Mean value (kg/ewe)			
Constantly seronegative	2.4 (1.18)	1.9 (0.73)	1.5 (0.66)	2.0 (0.99)
Constantly seropositive	2.6 (1.12)	1.9 (0.77)	1.4 (0.59)	2.0 (1.00)
Seroconverted	2.7 (0.93)	2.0 (0.77)	1.4 (0.61)	2.1 (0.96)
Seroreverted	2.0 (1.12)	1.5 (0.68)	1.3 (0.53)	1.6 (0.90)
Intermittent presence of antibodies	2.3 (1.11)	1.8 (0.67)	1.2 (0.66)	1.8 (0.94)

SD: Standard deviation

Table 29. Mean values (SD) of daily fat yield during the study in different serological patterns.

Serological Pattern	1st sampling occasion	2nd sampling occasion	3rd sampling occasion	Overall
	Mean value (g/ewe)			
Constantly seronegative	120.5 (62.46)	119.7 (43.88)	97.5 (37.11)	114.1 (51.53)
Constantly seropositive	128.0 (55.22)	127.1 (48.46)	91.3 (36.82)	117.2 (50.99)
Seroconverted	136.0 (46.19)	131.2 (51.05)	89.0 (38.19)	120.9 (49.99)
Seroreverted	93.1 (52.75)	87.8 (39.46)	83.5 (30.81)	88.8 (42.87)
Intermittent presence of antibodies	99.4 (33.91)	136.3 (48.62)	74.4 (41.80)	104.4 (47.04)

SD: Standard deviation

Table 30. Mean values (SD) of daily protein yield during the study in different serological patterns.

Serological Pattern	1st sampling occasion	2nd sampling occasion	3rd sampling occasion	Overall
	Mean value (g/ewe)			
Constantly seronegative	122.2 (60.16)	101.6 (38.33)	80.8 (37.03)	104.2 (50.76)
Constantly seropositive	134.7 (55.06)	102.9 (38.02)	75.8 (31.22)	107.5 (50.04)
Seroconverted	141.3 (46.46)	105.6 (39.60)	75.4 (35.62)	110.1 (49.00)
Seroreverted	105.3 (58.57)	78.8 (34.15)	65.9 (28.81)	85.8 (46.76)
Intermittent presence of antibodies	118.5 (53.36)	92.9 (33.02)	67.9 (37.72)	95.3 (47.01)

SD: Standard deviation

Table 31. Mean values (SD) of daily lactose yield during the study in different serological patterns.

Serological Pattern	1st sampling occasion	2nd sampling occasion	3rd sampling occasion	Overall
	Mean value (g/ewe)			
Constantly seronegative	115.9 (57.08)	96.3 (36.36)	76.6 (35.13)	98.9 (48.11)
Constantly seropositive	127.7 (52.31)	97.6 (36.05)	71.9 (29.62)	101.9 (47.50)
Seroconverted	134.0 (44.07)	100.1 (37.57)	71.5 (33.80)	104.4 (46.49)
Seroreverted	99.9 (55.60)	74.7 (32.39)	62.5 (27.12)	81.4 (44.38)
Intermittent presence of antibodies	112.5 (52.51)	88.2 (31.29)	64.5 (35.81)	90.4 (44.59)

SD: Standard deviation

Table 32. Mean values (SD) of daily solids-not-fat yield during the study in different serological patterns.

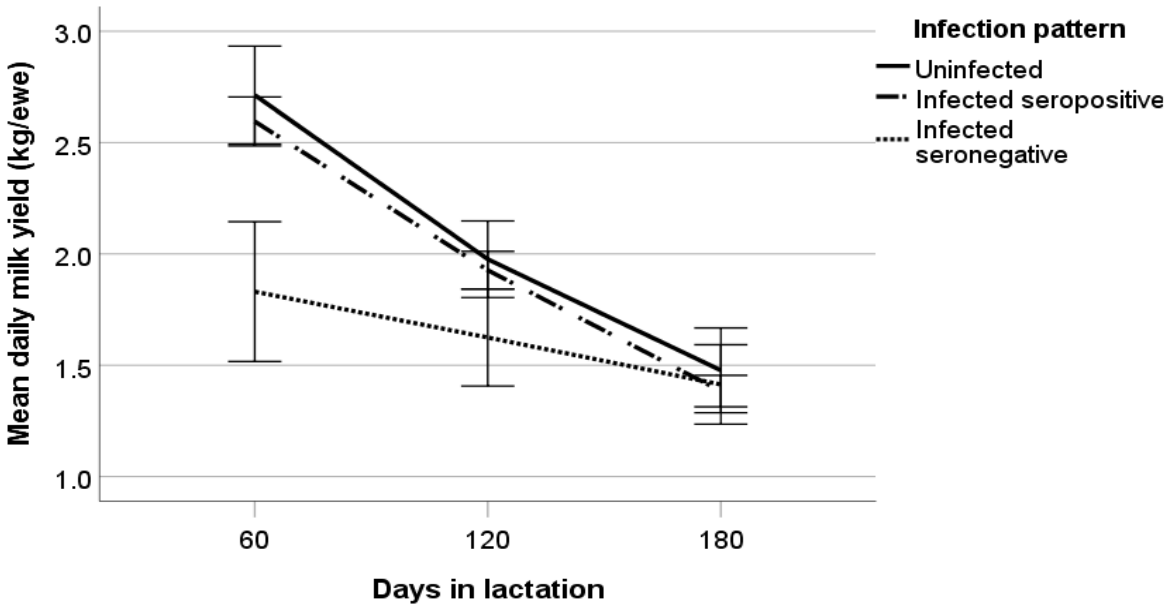
Serological Pattern	1st sampling occasion	2nd sampling occasion	3rd sampling occasion	Overall
	Mean value (g/ewe)			
Constantly seronegative	258.5 (127.12)	215.4 (80.73)	170.6 (78.20)	220.6 (107.23)
Constantly seropositive	284.9 (116.29)	217.5 (80.11)	160.0 (65.91)	227.2 (105.75)
Seroconverted	298.4 (98.16)	222.8 (83.62)	159.1 (75.26)	232.4 (103.52)
Seroreverted	222.5 (123.74)	166.3 (72.09)	139.2 (60.88)	181.3 (98.79)
Intermittent presence of antibodies	250.4 (116.97)	196.1 (69.67)	143.5 (79.68)	201.1 (99.31)

SD: Standard deviation

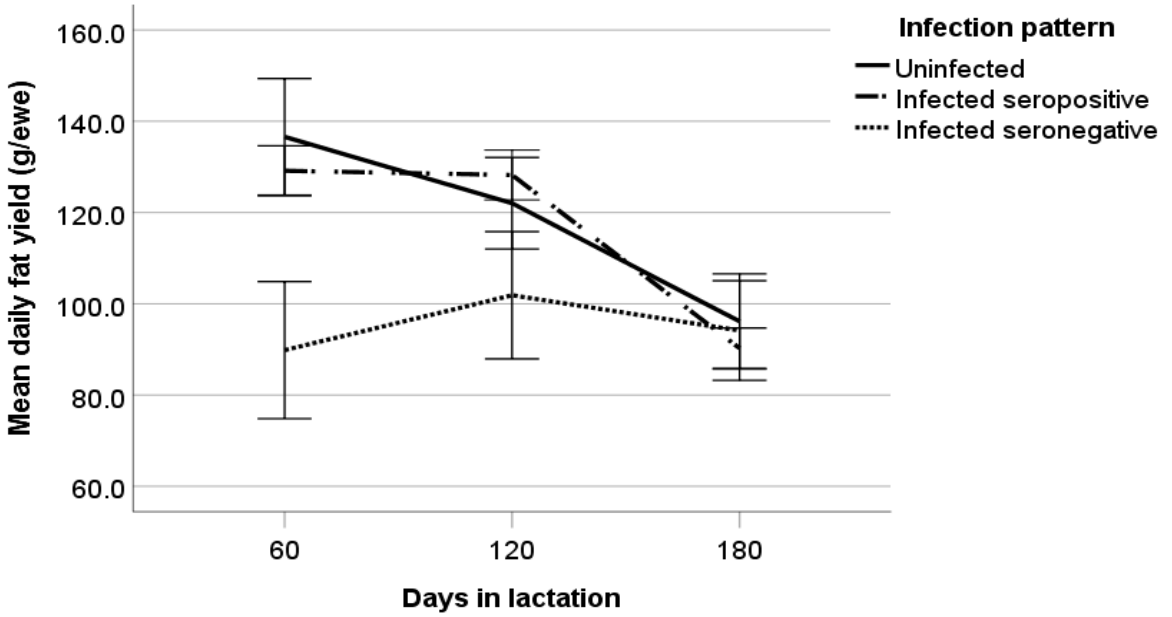
Table 33. Mean values (SD) of daily logarithm of SCC during the study in different serological patterns.

Serological Pattern	1st sampling occasion	2nd sampling occasion	3rd sampling occasion	Overall
	Mean value (cells/ml)			
Constantly seronegative	5.3 (0.65)	5.5 (0.58)	5.6 (0.54)	5.5 (0.60)
Constantly seropositive	5.3 (0.61)	5.5 (0.58)	5.6 (0.56)	5.4 (0.60)
Seroconverted	5.2 (0.61)	5.5 (0.54)	5.5 (0.66)	5.4 (0.62)
Seroreverted	5.1 (0.64)	5.5 (0.44)	5.4 (0.52)	5.3 (0.56)
Intermittent presence of antibodies	5.4 (0.53)	5.2 (0.44)	5.4 (0.28)	5.3 (0.42)

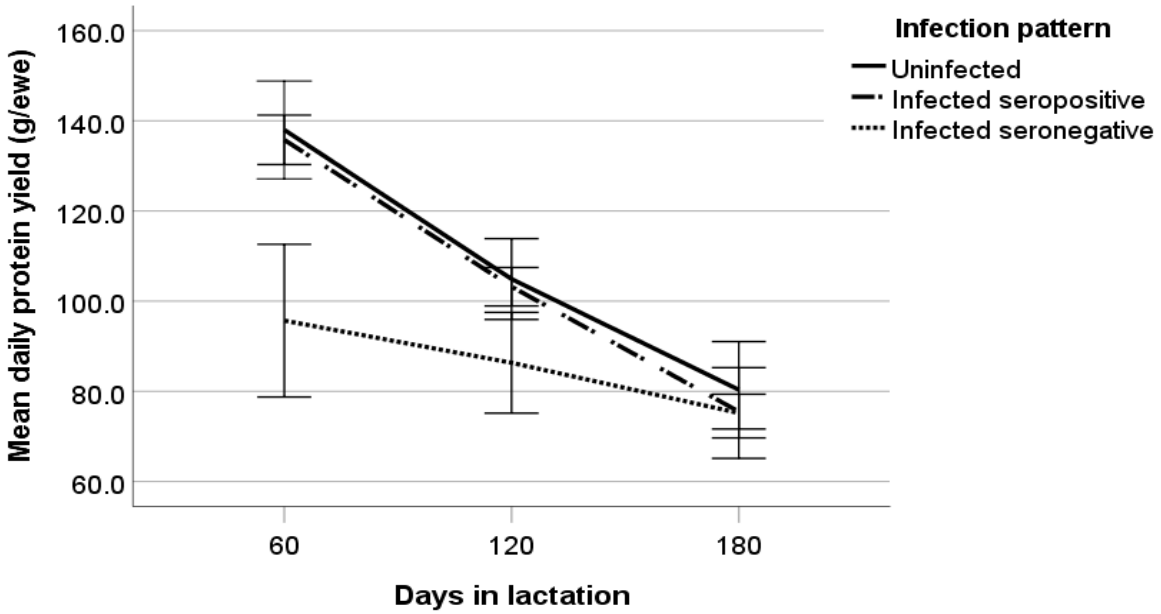
SD: Standard deviation


Infection pattern
Uninfected
Infected seropositive
Infected seronegative
Mean daily milk yield (kg/ewe)
3.0
2.5
2.0
1.5
1.0
60
120
180
Days in lactation

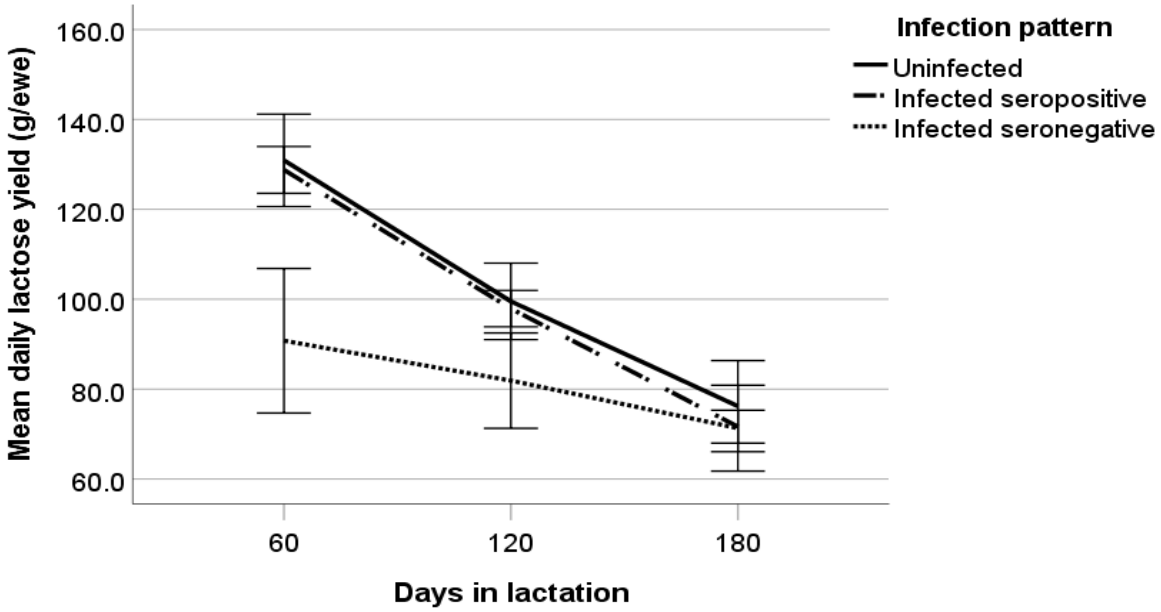
(a)


Infection pattern
Uninfected
Infected seropositive
Infected seronegative
Mean daily fat yield (g/ewe)
160.0
140.0
120.0
100.0
80.0
60.0
60
120
180
Days in lactation

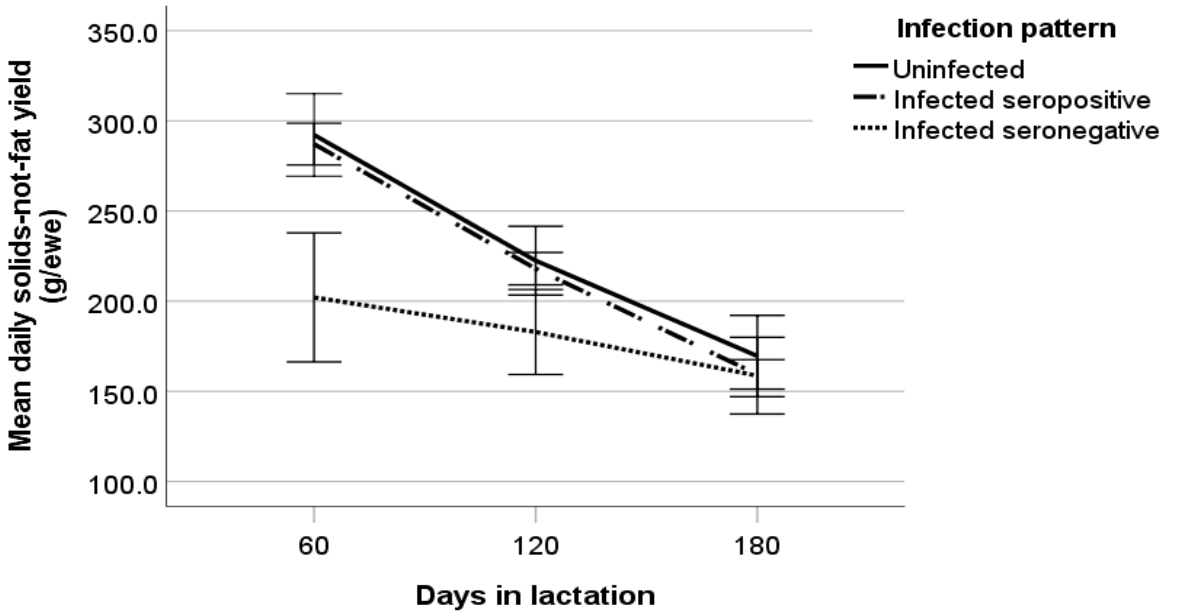
(b)


Infection pattern
Uninfected
Infected seropositive
Infected seronegative
Mean daily protein yield (g/ewe)
160.0
140.0
120.0
100.0
80.0
60.0
60
120
180
Days in lactation

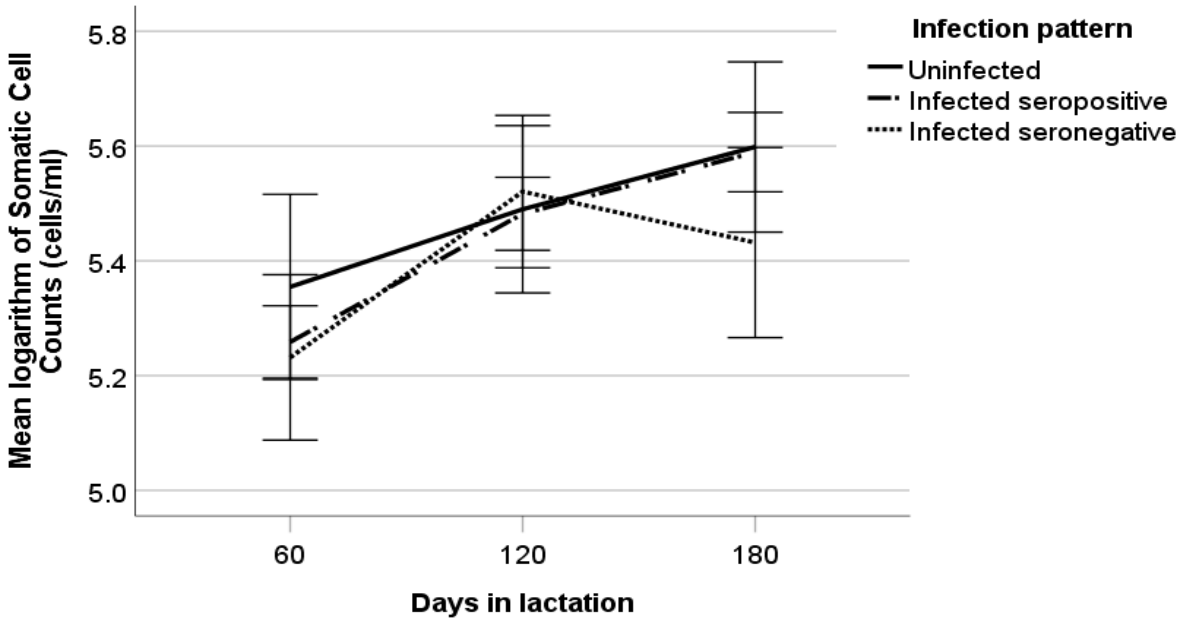
(c)


Infection pattern
Uninfected
Infected seropositive
Infected seronegative
Mean daily lactose yield (g/ewe)
160.0
140.0
120.0
100.0
80.0
60.0
60
120
180
Days in lactation

(d)



(e)



(f)

Figure 40. Mean values of (a) daily milk yield; (b) daily fat yield; (c) daily protein yield; (d) daily lactose yield; (e) daily solids-not-fat yield; (f) logarithm of somatic cell counts for i) uninfected ewes, ii) infected seropositive ewes, and iii) infected seronegative ewes during the study.

1.2 The effects of serological pattern on daily milk yield and milk quality traits

The overall fixed effect of the serological pattern was not statistically significant in any case. However, the pairwise comparisons between the serological patterns revealed statistically significant differences regarding the DFY. Namely, the constantly seronegative, the constantly seropositive, and the seroconverted ewes had higher DFY compared to the seroreverted ones (15.50 g, 95% CI 0.15-30.85 g, $p<0.05$, 15.29 g, 95% CI 0.60-29.98 g, $p<0.05$, and 19.00 g, 95% CI 3.81-34.20 g, $p<0.05$, respectively). Chios breed, sampling

occasion, and BCS were negatively associated with daily milk yield and all the milk quality traits ($p<0.001$, in all cases), except for the LogSCC, where the Chios breed and the sampling occasion were positively associated ($p<0.001$ in both cases), whereas the age was negatively associated ($p<0.05$) with them. Also, age was positively associated with the DFY, DPY, DLY, and DSNFY ($p<0.05$ in all cases). Tables S3-S8 in Appendix C summarize the effects of serological pattern, breed, sampling occasion, age, and BCS on DMY, DFY, DPY, DLY, DSNFY, and LogSCC and pairwise comparisons between the serological patterns as derived from the regression models.

1.3 The effects of infection patterns on daily milk yield and milk quality traits

Although the overall effects of the infection patterns were not statistically significant in any case, the pairwise comparisons between the infection patterns revealed many statistically significant differences for the studied milk yield and quality traits. Namely, the uninfected ewes had higher DMY (ca. 300 g, 95% CI 30-560 g, $p<0.05$), DPY (14.58 g, 95% CI 0.34-28.83 g, $p<0.05$), DLY (13.84 g, 95% CI 0.33-27.34 g, $p<0.05$), and DSNFY (31.35 g, 95% CI 1.14-61.57 g, $p<0.05$) compared to the infected seronegative ones. Also, infected seronegative ewes had lower DFY compared to both uninfected and infected seropositive ewes (15.29 g, 95% CI 2.73-27.85 g, $p<0.05$ and 11.88 g, 95% CI 0.13-23.62 g, $p<0.05$, respectively). Chios breed, sampling occasion, and BCS were negatively associated with the DMY and all the milk quality traits ($p<0.001$ in all cases), except for the LogSCC. In the latter case, Chios breed and sampling occasion were positively associated ($p<0.001$ in both cases), whereas age was negatively associated ($p<0.05$) with LogSCC. Also, age was positively associated with DFY, DPY, DLY, and DSNFY ($p<0.05$). Tables S9-S14 in Appendix C summarize the effects of infection pattern, breed, sampling occasion, age, and BCS on daily milk yield and milk quality traits.

1.4 Descriptive statistics of total milk yield and quality traits

The mean values of TMY, TFY, TPY, TLY, and TSNFY are summarized in Table 34. Seroconverted and uninfected ewes presented the highest values of total milk yield and milk quality traits, whereas seroreverted and infected seronegative ewes presented the lowest ones.

Table 34. Mean values (SD) of total milk, fat, protein, lactose, solids-not-fat yields estimated for the first 120 days of the milking period in different serological and infection patterns.

	TMY	TFY	TPY	TLY	TSNFY
Serological pattern			Mean value (kg/ewe)		
Constantly seronegative	193.4 (110.94)	11.3 (6.49)	10.0 (5.80)	9.6 (5.45)	21.1 (12.25)
Constantly seropositive	203.9 (108.53)	12.1 (6.42)	10.7 (5.63)	10.2 (5.34)	22.7 (11.84)
Seroconverted	225.6 (93.62)	13.5 (5.71)	11.7 (4.93)	11.2 (4.66)	24.6 (10.45)
Seroreverted	163.4 (97.25)	9.1 (5.30)	8.6 (5.08)	8.2 (4.82)	17.7 (11.06)
Intermittent presence of antibodies	196.2 (105.32)	12.0 (5.88)	10.1 (5.38)	9.6 (5.10)	21.3 (11.36)
Infection pattern					
Uninfected	228.8 (90.24)	13.3 (5.26)	11.8 (4.72)	11.3 (4.39)	24.9 (9.99)
Infected seropositive	208.8 (105.32)	12.4 (6.26)	10.9 (5.48)	10.4 (5.19)	23.1 (11.52)
Infected seronegative	140.0 (110.12)	8.2 (6.42)	7.4 (5.82)	7.1 (5.52)	15.3 (12.35)

SD: Standard deviation; TMY, TFY, TPY, TLY, TSNFY: total milk, fat, protein, lactose, and solids-not-fat yields, respectively for the first 120 days of milking period

1.5 The effects of serological patterns on total milk yield and milk quality traits

The overall effect of the serological patterns was significant only in the case of TMY, whereas the pairwise comparisons between the serological patterns were significant in many cases. In particular, the seroconverted ewes yielded 32.21 kg more milk (95% CI, 5.86-58.55 kg, $p<0.05$), 1.93 kg more fat (95% CI, 0.31-3.54 kg, $p<0.05$), 1.61 kg more protein (95% CI, 0.24-2.99 kg, $p<0.05$), 1.51 kg more lactose (95% CI, 0.21-2.82 kg, $p<0.05$), and 3.34 kg more solids-not-fat (95% CI, 0.43-6.25 kg, $p<0.05$) compared to constantly seronegative during the first 120 days of the milking period. Also, seroconverted ewes yielded 32.14 kg more milk (95% CI, 9.22-55.06 kg, $p<0.01$), 1.86 kg more fat (95% CI, 0.45-3.37 kg, $p<0.05$), 1.55 kg more protein (95% CI, 0.36-2.75 kg, $p<0.05$), 1.56 kg more lactose (95% CI, 0.43-2.69 kg, $p<0.01$), and 2.30kg more solids-not-fat (95% CI, 0.47-5.53 kg, $p<0.05$) compared to constantly seropositive ewes, and ca. 3.0 kg (95% CI 0.10-5.39 kg, $p<0.05$) more fat compared to the seroreverted ewes at the same period. Age was positively associated with milk yield and all milk quality traits ($p<0.05$), whereas the Chios ewes had significantly lower fat yield ($p<0.01$). Tables S15-S19 in Appendix C summarize the effects of serological pattern, breed, and age on TMY, TFY, TPY, TLY, and TSNFY for the first 120 days of the milking period.

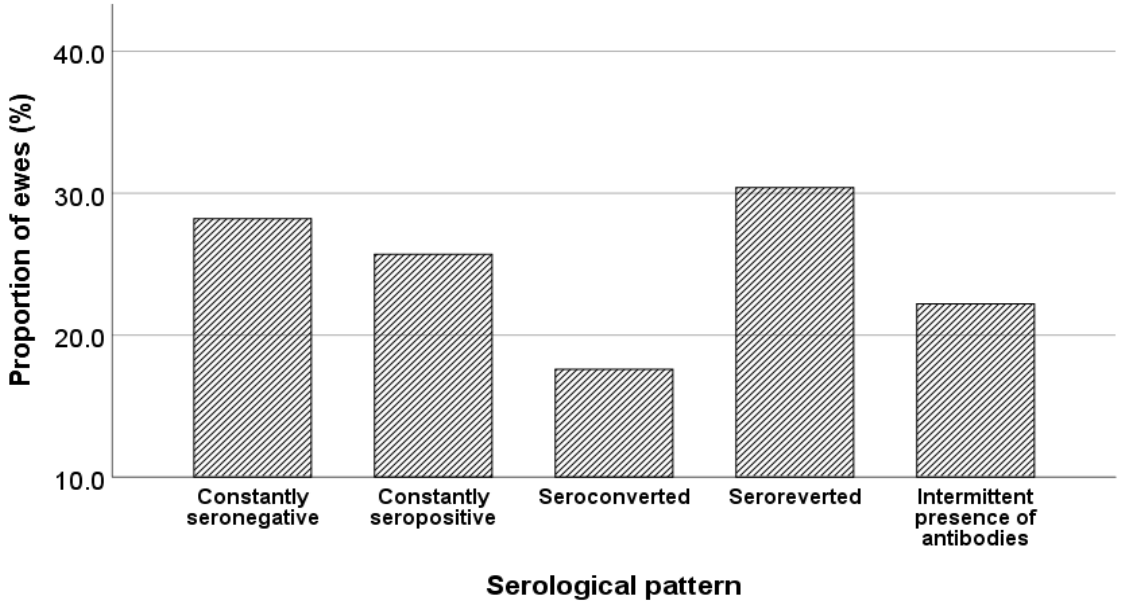
1.6 The effects of infection patterns on total milk yield and milk quality traits

The overall fixed effect of the infection pattern was significant in all cases ($p<0.05$); in particular, infected seronegative ewes yielded ca. 43 kg (95% CI, 11.02-75.85 kg, $p<0.01$) and 35 kg (95% CI, 8.68-61.21, $p<0.01$) less milk, ca. 2.7 kg (95% CI, 0.72-4.70 kg, $p<0.01$) and 2.2 kg (95% CI, 0.63-3.38 kg, $p<0.01$) less fat, ca. 2.0 kg (95% CI, 0.30-3.68 kg, $p<0.05$) and 1.7 kg (95% CI, 0.29-3.03 kg, $p<0.05$) less protein, ca. 2.0 kg (95% CI, 0.36-3.56 kg, $p<0.05$) and 1.6 kg (95% CI, 0.26-2.86 kg, $p<0.05$) less lactose, and ca. 5.0 kg (95% CI, 0.90-8.05 kg, $p<0.05$) and 4.0 kg (95% CI, 0.96-6.76 kg, $p<0.01$) less solids-not-fat yield compared to the uninfected ewes and the infected seropositive ewes, respectively. The age was positively associated with milk yield and all the milk quality traits ($p<0.05$), whereas Chios ewes produced 3.92 kg less TFY (95% CI, 1.44-6.41 kg, $p<0.01$) compared to Lacaune ewes. Tables S20-S24 in Appendix C summarize the effects of infection pattern, breed, and age on TMY, TFY, TPY, TLY, and TSNFY for the first 120 days of the milking period.

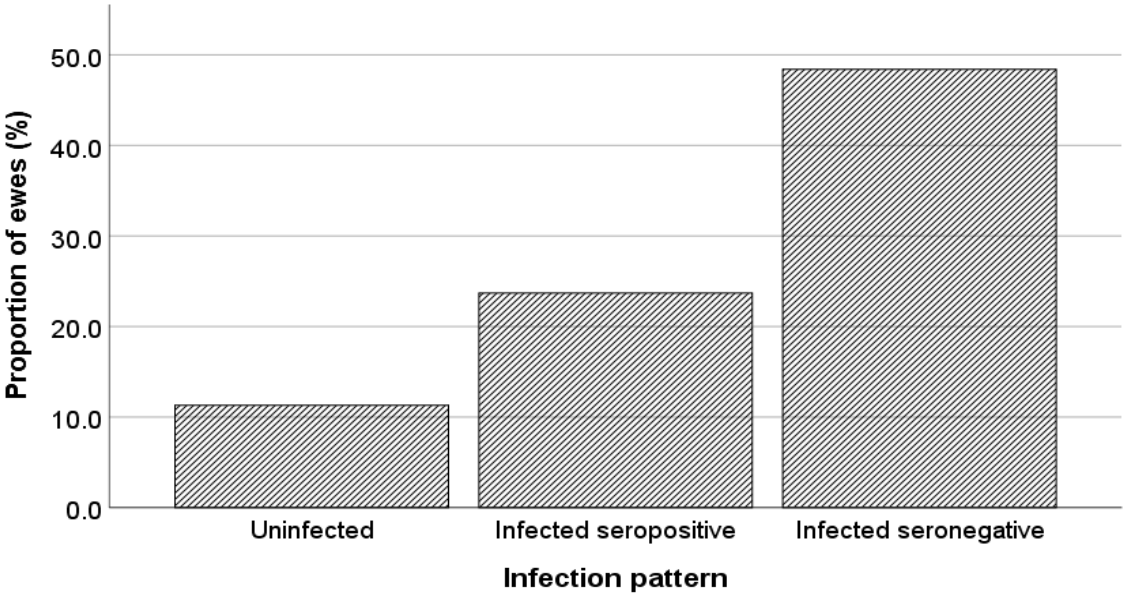
1.7 The effects of serological and infection patterns on the duration of lactation period

A total of 124 ewes (25.0% of the total studied ewes) presented a lactation period of <7 months. The proportion of the ewes that presented a lactation period <7 months is presented

in Figures 41a and 41b per serological and infection pattern, respectively. Seroreverted and infected seronegative animals presented the highest proportions (30.4% and 48.4%, respectively). On the other hand, seroconverted and uninfected animals demonstrated the lowest proportions (17.6% and 11.3%, respectively).



(a)



(b)

Figure 41. Proportion of ewes that presented shorter lactation period per serological (a) and infection (b) pattern.

Constantly seronegative and constantly seropositive animals were 1.09 (95% CI, 1.02-1.27, $p<0.05$) and 1.15 (95% CI, 1.04-1.26, $p<0.01$) times more likely to have a short lactation period compared to the seroconverted ones. Also, infected seronegative ewes were 1.25 (95%

CI, 1.10-1.45, p=0.001) and 1.16 (95% CI, 1.04-1.30, p<0.01) times more likely to present shorter lactation period compared to the uninfected and infected seropositive ewes. The odds ratios of serological and infection patterns regarding the occurrence of short lactation period (<7 months) are presented in Tables S25 and S26 in Appendix C, respectively.

2. The effects of small ruminant lentiviruses infections on the health and welfare status of dairy ewes

2.1 Descriptive statistics of health and welfare issues

During the study, lameness, arthritis in at least one limb, respiratory disease, nasal discharge, poor wool quality, and body abscesses were recorded at least once in 3.7% (15/407), 23.8% (97/407), 3.4% (14/407), 15.2% (62/407), 7.1% (29/407), and 8.4% (34/407) of the studied ewes, respectively. Also, regarding udder disorders, mastitis, abscess, skin lesions, wart-like lesions, mammary cysts, fibrosis, asymmetry (mild to intense), and swollen supramammary lymph nodes were recorded at least once in 3.2% (13/407), 31.2% (127/407), 27.0% (110/407), 6.6% (27/407), 19.7% (80/407), 20.9% (85/407), 47.7% (194/407), and 44.5% (181/407) of the studied ewes, respectively. The frequencies of health and udder disorders in ewes of different serological and infection patterns are summarized in Tables 35 and 36, respectively (occurrence of the health disorder at least once during the study period).

Constantly seropositive ewes demonstrated the highest frequency of mammary cysts (20.7%), body abscesses (10.1%), and wart-like lesions (8.0%), whereas seroconverted ewes demonstrated the highest frequency of poor wool quality (8.5%) and mastitis (4.9%). On the other hand, seroreverted ewes presented the highest frequency of swollen supramammary lymph nodes (68.6%), arthritis (45.7%), udder skin lesions (37.1%), udder fibrosis (25.7%), and respiratory disease (5.7%), whereas ewes with an intermittent presence of antibodies had the highest frequency of udder asymmetry (57.5%), udder abscesses (35.0%), nasal discharge (20.0%), and lameness (7.5%). Regarding infection pattern, infected seropositive ewes presented the highest frequency of udder abscesses (31.8%), lameness (4.5%), respiratory disease (3.9%), and mastitis (3.9%), whereas infected seronegative ewes demonstrated the highest frequency of swollen supramammary lymph nodes (59.6%), udder asymmetry (48.1%), arthritis (40.4%), udder skin lesions (36.5%), udder fibrosis (28.8%), mammary cysts (25.0%), nasal discharge (21.2%), and poor wool quality (9.6%). On the other hand, uninfected ewes presented the highest frequency of body abscesses (9.1%).

Table 35. The mean value of body condition score (±SD) and frequencies of health disorders in ewes of different serological and infection patterns during the study.

Serological pattern	BCS	Lameness	Arthritis	Respiratory Disease	Nasal Discharge	Poor wool quality	Body abscesses
Constantly seronegative	2.9(±0.23)	0.0%	14.5% (9/62)	0.0%	19.4% (12/62)	3.2%(2/62)	9.7% (6/62)
Constantly seropositive	2.9(±0.24)	4.8% (9/188)	21.8% (41/188)	3.7% (7/188)	13.3% (25/188)	7.4% (14/188)	10.1% (19/188)
Seroconverted	2.9(±0.23)	2.4% (2/82)	15.9% (13/82)	3.7% (3/82)	14.6% (12/82)	8.5% (7/82)	4.9% (4/82)
Seroreverted	2.9(±0.21)	2.9% (1/35)	45.7% (16/35)	5.7% (2/35)	14.3% (5/35)	4.4% (4/35)	8.6% (3/35)
Intermittent presence of antibodies	2.9(±0.22)	7.5% (3/40)	45.0% (18/40)	5.0% (2/40)	20.0% (8/40)	5.0% (2/40)	5.0% (2/40)
Infection pattern							
Uninfected	3.0(±0.20)	0.0%	9.1% (4/44)	0.0%	13.6% (6/44)	2.3% (1/44)	9.1% (4/44)
Infected seropositive	3.0(±0.25)	4.5% (14/311)	23.2% (72/311)	3.9% (12/311)	14.5% (45/31)	7.4% (23/311)	8.4% (26/311)
Infected seronegative	3.0(±0.21)	1.9% (1/52)	40.4% (21/52)	3.8% (2/52)	21.2% (11/52)	9.6% (5/52)	7.7% (4/52)

SD: standard deviation; BCS: body condition score

Table 36. The frequencies of udder disorders in ewes of different serological and infection patterns during the study.

Serological pattern	Mastitis	Abscesses	Skin lesions	Wart-like lesions	Mammary cyst	Fibrosis	Asymmetry	Swollen supramammary lymph nodes
Constantly seronegative	1.6% (1/62)	30.6% (19/62)	24.2% (15/62)	3.2% (2/62)	9.7% (6/62)	16.1% (10/62)	45.2% (28/62)	33.9% (21/62)
Constantly seropositive	3.7% (7/188)	33.0% (62/188)	29.8% (56/188)	8.0% (15/188)	20.7% (39/188)	25.5% (48/188)	47.3% (89/188)	46.0% (88/188)
Seroconverted	4.9% (4/82)	28.0% (23/82)	17.1% (14/82)	7.3% (6/82)	12.2% (10/82)	12.2% (10/82)	43.9% (36/82)	36.6% (30/82)
Seroreverted	0.0%	25.7% (9/35)	37.1% (13/35)	2.9% (1/35)	20.0% (7/35)	25.7% (9/35)	51.4% (18/35)	68.6% (24/35)
Intermittent presence of antibodies	2.5% (1/40)	35.0 (14/40)	30.0% (12/40)	7.5% (3/40)	15.0% (6/40)	20.0% (8/40)	57.5% (23/40)	45.0% (18/40)
Infection pattern								
Uninfected	2.3% (1/44)	27.3% (12/44)	18.2% (8/44)	2.3% (1/44)	6.8% (3/44)	9.1% (4/44)	47.7% (21/44)	29.5% (13/44)
Infected seropositive	3.9% (12/311)	31.8% (99/311)	26.7% (83/311)	7.7% (24/311)	20.6% (64/311)	21.2% (66/311)	47.6% (148/311)	44.1% (137/311)
Infected seronegative	0.0%	30.8% (16/52)	36.5% (19/52)	3.8% (2/52)	25.0% (13/52)	28.8% (15/52)	48.1% (25/52)	59.6% (31/52)

The mean values of BCS in constantly seronegative and constantly seropositive ewes are presented in Figure 42. In constantly seronegative ewes, BCS was reducing until the third sampling occasion and then increasing, whereas in constantly seropositive ewes, BCS was reducing until the fourth sampling occasion and increasing in the last one. In seroconverted ewes, BCS was almost constant at the sampling occasions until the seroconversion incident and decreased at the first sampling occasion after it, whereas in seroreverted ewes, BCS decreased at the sampling occasion of the seroreversion incident and increased in the next one (Figures 43a and 43b).

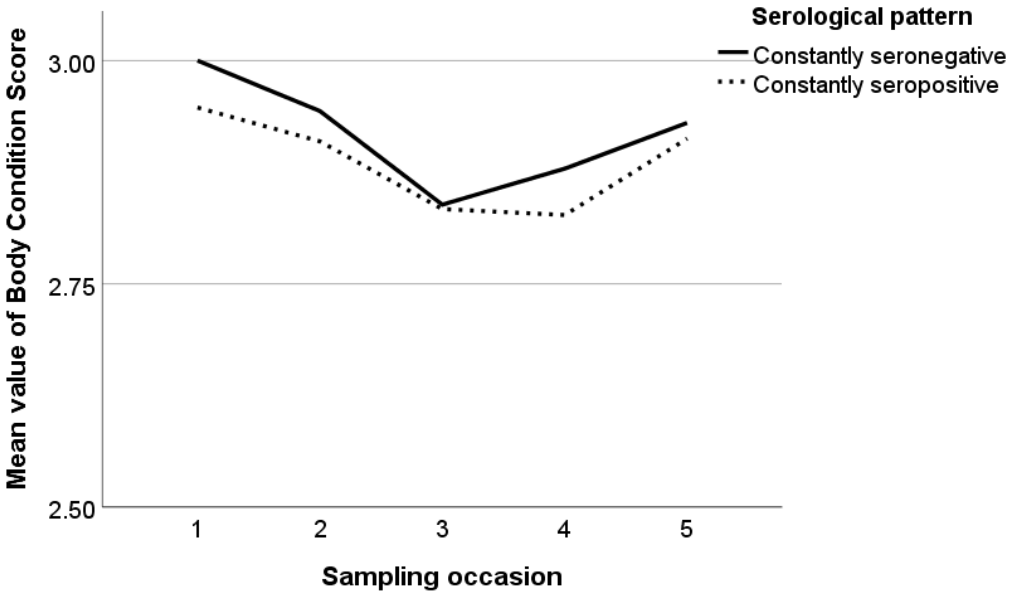
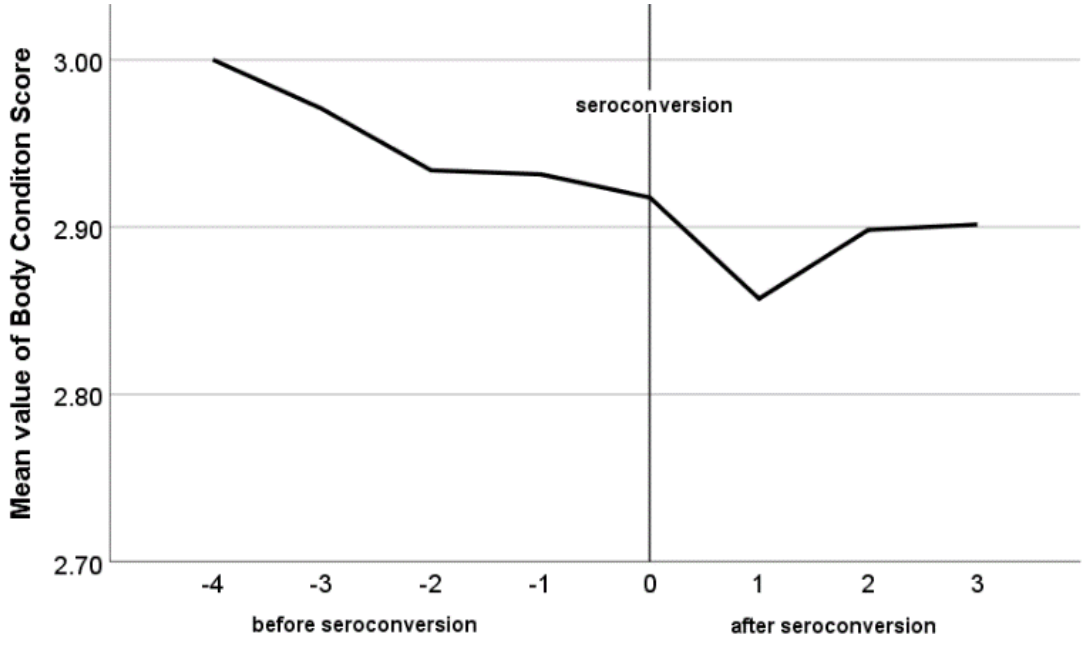
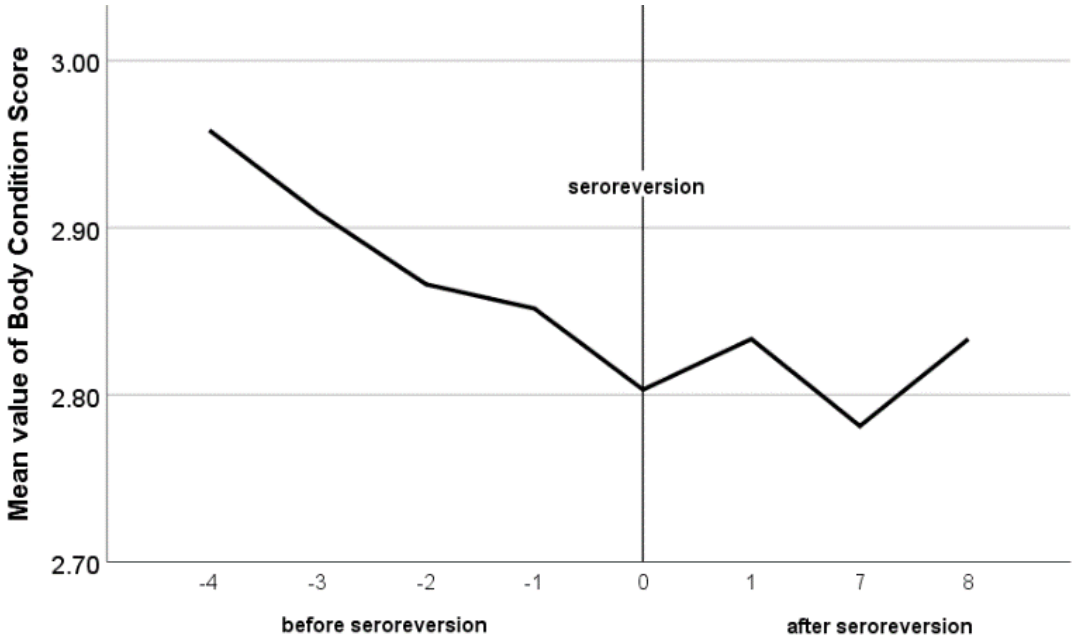


Figure 42. Mean values of body condition score during the study in constantly seronegative and constantly seropositive ewes.



(a)



(b)

Figure 43. Mean values of body condition score before and after the seroconversion (a) and the seroreversion (b) incident.

2.2 The effects of serological status on health and welfare issues

The effect of serological status of ewes was statistically significant only on the occurrence of nasal discharge. Seropositive animals were 2.44 times more likely (95% CI, 1.06-5.56, $p<0.05$) to have nasal discharge during the study compared to the seronegative ones (Table S27 Appendix C).

2.3 The effects of infection status on health and welfare issues

Infected seronegative animals were 14.29 times (95% CI, 1.52-100.00, $p<0.05$) more likely to be lame compared to infected seropositive ewes. Also, infected seropositive and seronegative ewes were ca. 33 and ca. 50 times more likely to have mastitis during the study compared to uninfected ones (95% CI, 25.00-100.00, $p<0.01$, and 95% CI, 20.00-100.00 $p<0.05$, respectively). Moreover, udder skin lesions were more commonly observed in infected ewes; infected seropositive ewes were 3.03 times (95% CI, 1.45-6.25, $p<0.01$), while infected seronegative ewes were 2.63 times (95% CI, 1.06-6.67, $p<0.05$) more likely to develop udder skin lesions compared to the uninfected ones. In addition, wart-like lesions were 20.00 times (95% CI, 1.52-100.00, $p<0.05$) more likely to occur in infected seronegative ewes compared to the uninfected ones. The statistically significant effects of infection status on the occurrence of health and welfare issues derived from repeated measures mixed binary logistic models are presented in Tables S28-S31 in Appendix C.

2.4 The effects of serological patterns on health and welfare issues

Seroreverted ewes were 4.55 (95% CI, 1.67-12.5, $p<0.01$), 2.78 (95% CI, 1.23-6.25, $p<0.05$), and 4.35 (95% CI, 1.67-11.11, $p<0.01$) times more likely to develop arthritis at least once during the study compared to constantly seronegative, constantly seropositive, and seroconverted ewes, respectively. Also, ewes with an intermittent presence of antibodies were ca. 3.0 times more likely to present arthritis compared to constantly seronegative (95% CI, 1.22-9.09, $p<0.05$) and seroconverted ewes (95% CI, 1.22-12.5, $p<0.05$). Moreover, seroreverted ewes were 4.55 (95% CI, 1.82-11.12, $p<0.01$), 3.23 (95% CI, 1.45-7.14, $p<0.01$), 3.84 (95% CI, 1.62-9.09, $p<0.01$), and 3.42 (95% CI, 1.27-9.22, $p<0.05$) times more likely to have swollen supramammary lymph nodes at least once during the study compared to constantly seronegative, constantly seropositive, seroconverted, and ewes with an intermittent presence of antibodies, respectively. The statistically significant effects of serological patterns on the occurrence of health and welfare issues at least once during the study, as derived from the respective mixed binary logistic models, are presented in Tables S32 and S33 in Appendix C.

2.5 The effects of infection patterns on health and welfare issues

Infected seronegative ewes were 4.17 times (95% CI, 1.25-14.29, $p<0.05$) more likely to develop arthritis at least once during the study compared to uninfected ewes. Also, infected seronegative ewes were 3.03 (95% CI, 1.25-7.14, $p<0.05$) and 2.08 (95% CI, 1.10-3.85, $p<0.05$) times more likely to have swollen supramammary lymph nodes at least once during the study compared to uninfected and infected seropositive ewes, respectively. The statistically significant effects of infection patterns on the occurrence of health and welfare issues at least once during the study, as derived from the respective mixed binary logistic models are presented in Tables S34 and S35 in Appendix C.

2.6 Descriptive statistics of hematological parameters

A total of 96 ewes were found seronegative and 188 seropositive, according to the ELISA results. The combination of ELISA and PCR results revealed a total of 34 ewes as uninfected, 188 ewes as infected seropositive, and 62 ewes as infected seronegative. The mean values (± SD) of hematological parameters of white blood cells, red blood cells, and platelets are presented in Tables 37, 38, and 39, respectively, for ewes with different serological and infection statuses. Based on their serological pattern, a total of 43 ewes were constantly seronegative, whereas a total of 121 ewes were constantly seropositive. Also, 60 ewes seroconverted during the study and 27 ewes seroreverted, whereas a total of 29 ewes

presented an intermittent presence of antibodies. The combination of ELISA and PCR results revealed a total of 28 ewes as uninfected, 210 ewes as infected seropositive, and a total of 42 ewes as infected seronegative. The mean values (± SD) of hematological parameters regarding white blood cells, red blood cells, and platelets are presented in Tables 40, 41, and 42, respectively, for ewes with different serological and infection patterns.

Table 37. Mean values (SD) of hematological parameters regarding white blood cells in ewes with different serological and infection status.

	Mean value (SD)						
Serological Status	**WBC (10^3/µl)**	**GC (10^3/µl)**	**LC (10^3/µl)**	**MC (10^3/µl)**	**GP (%)**	**LP (%)**	**MP (%)**
Seronegative	7.8 (2.71)	3.5 (1.51)	3.8 (1.46)	0.5 (0.19)	44.7 (9.51)	49.1 (9.42)	6.2 (1.14)
Seropositive	8.1 (2.49)	3.6 (1.45)	3.9 (1.43)	0.5 (0.19)	44.7 (8.93)	49.0 (8.85)	6.2 (1.24)
Infection Status							
Uninfected	9.3 (2.61)	4.1 (1.29)	4.6 (1.69)	0.6 (0.19)	44.1 (8.64)	49.5 (8.75)	6.4 (1.22)
Infected seropositive	8.1 (2.49)	3.6 (1.45)	3.9 (1.43)	0.5 (0.19)	44.7 (8.93)	49.0 (8.85)	6.2 (1.24)
Infected seronegative	7.0 (2.40)	3.2 (1.55)	3.3 (1.08)	0.4 (0.15)	45.0 (10.00)	48.9 (9.83)	6.1 (1.09)

SD: Standard deviation; WBC: white blood cell count; CG: granulocyte count; LC: lymphocyte count; MC: monocyte count; GP: percentage of granulocytes, LP: percentage of lymphocytes; MP: percentage of monocytes; Reference ranges: WBC: 5.1-15.8 10^3/µl, GC: 1.3-7.6 10^3/µl, LC: 2.0-7.8 10^3/µl, MC: 0.0-1.3 10^3/µl, GP: 21.5-68.0%, LP: 28.0-71.5%, MP: 0.0-9.5%

Table 38. Mean values (SD) of hematological parameters regarding red blood cells in ewes with different serological and infection status.

	Mean value (SD)							
Serological Status	**RBC (10^6/µl)**	**HGB (g/dl)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dl)**	**RDW-CV (%)**	**RDW-SD (fL)**
Seronegative	8.6 (1.30)	9.8 (1.46)	30.3 (4.63)	35.5 (3.44)	11.4 (0.92)	32.3 (1.96)	17.0 (2.46)	23.5 (4.91)
Seropositive	8.4 (1.36)	9.6 (1.37)	29.9 (4.74)	35.7 (3.15)	11.5 (1.12)	32.4 (2.43)	16.7 (2.07)	23.5 (4.37)
Infection Status								
Uninfected	8.5 (0.94)	9.7 (1.28)	29.7 (4.53)	34.7 (2.52)	11.4 (0.73)	32.7 (2.39)	16.4 (1.23)	22.2 (2.65)
Infected seropositive	8.4 (1.36)	9.6 (1.37)	29.9 (4.74)	35.7 (3.15)	11.5 (1.12)	32.4 (2.43)	16.7 (2.07)	23.5 (4.37)
Infected seronegative	8.6 (1.46)	9.8 (1.56)	30.6 (4.69)	35.9 (3.81)	11.5 (1.02)	32.1 (1.67)	17.3 (2.88)	24.2 (5.69)

SD: Standard deviation; RBC: red blood cell count; HGB: hemoglobin concentration; HCT: hematocrit; MCV: mean value of corpuscular volume; MCH: mean value of corpuscular hemoglobin; MCHC: corpuscular hemoglobin concentration; RDW-CV: red cell distribution width coefficient of variation; RDW-SD: red cell distribution standard deviation; fL: femtoliter; Pg: petagrams; Reference ranges: RBC:6.5-15.2 10^6/µl, HGB: 6.8-14.5 g/dl, HCT: 20.0-42.5%, MCV: 25.0-41.0 fL, MCH: 8.0-12.3 pg, MCHC: 29.0-37.0 g/dl, RDW-CV: 14.5-26.2%, RDW-SD: 17.0-32.0 fL

Table 39. Mean values (SD) of hematological parameters regarding platelets in ewes with different serological and infection status.

	Mean value (SD)					
Serological Status	**PLT (10^9/l)**	**MPV (fL)**	**PDW**	**PCT (ml/l)**	**P-LCC (10^9/l)**	**P-LCR (%)**
Seronegative	278.0 (158.97)	6.1 (0.51)	15.0 (0.40)	1.7 (0.94)	130.8 (72.51)	48.6 (8.01)
Seropositive	248.9 (143.14)	6.1 (0.46)	15.2 (0.40)	1.5 (0.85)	118.0 (68.82)	48.6 (7.05)
Infection Status						
Uninfected	268.0 (122.54)	6.0 (0.43)	15.1 (0.32)	1.6 (0.75)	124.9 (60.14)	47.3 (6.90)
Infected seropositive	248.9 (143.14)	6.1 (0.46)	15.2 (0.40)	1.5 (0.85)	118.0 (68.82)	48.6 (7.05)
Infected seronegative	283.5 (176.48)	6.1 (0.55)	14.9 (0.43)	1.7 (1.04)	134.1 (78.8)	49.3 (8.52)

SD: Standard deviation; PLT: platelet count; MPV: mean platelet volume; PDW: platelet distribution width; PCT: plateletcrit: P-LCC: platelet large cell count; P-LCR: platelet large cell ratio; fL: femtoliter; Reference ranges: PLT: 200.0-800.0 10^9/l, MPV: 3.5-6.8 fL, PDW: 12.0-17.5, PCT: 1.0-4.2 ml/l, P-LCC: 30.0-260.0 10^9/l, P-LCR: 12.6-60.0%

Table 40. Mean values (SD) of hematological parameters regarding white blood cells in ewes with different serological and infection patterns during the 24 months.

	Mean value (SD)						
Serological Pattern	**WBC (10^3/µl)**	**GC (10^3/µl)**	**LC (10^3/µl)**	**MC (10^3/µl)**	**GP (%)**	**LP (%)**	**MP (%)**
Constantly seronegative	8.7 (2.67)	3.8 (1.48)	4.3 (1.46)	0.5 (0.19)	44.0 (8.04)	49.6 (7.95)	6.3 (1.23)
Constantly seropositive	8.1 (2.49)	3.6 (1.46)	4.0 (1.42)	0.5 (0.19)	44.8 (8.82)	49.1 (8.72)	6.2 (1.30)
Seroconverted	8.0 (2.55)	3.6 (1.43)	3.9 (1.48)	0.5 (0.19)	44.7 (9.27)	49.0 (9.25)	6.3 (1.14)
Seroreverted	6.9 (2.38)	3.2 (1.48)	3.3 (1.21)	0.4 (0.15)	44.8 (11.26)	49.1 (11.19)	6.1 (1.07)
Intermittent presence of antibodies	7.5 (2.71)	3.5 (1.55)	3.5 (1.45)	0.5 (0.18)	45.9 (9.82)	48.0 (9.69)	6.1 (1.05)
Infection Pattern							
Uninfected	9.1 (2.43)	4.1 (1.23)	4.5 (1.47)	0.6 (1.86)	44.4 (7.67)	49.1 (7.73)	6.5 (1.32)
Infected seropositive	8.0 (2.53)	3.6 (1.46)	3.9 (1.44)	0.5 (0.19)	44.9 (9.06)	48.9 (8.97)	6.2 (1.22)
Infected seronegative	7.2 (2.60)	3.3 (1.60)	3.5 (1.29)	0.4 (0.16)	44.3 (10.40)	49.6 (10.24)	6.1 (1.05)

SD: Standard deviation; WBC: white blood cell count; CG: granulocyte count; LC: lymphocyte count; MC: monocyte count; GP: percentage of granulocytes, LP: percentage of lymphocytes; MP: percentage of monocytes; Reference ranges: WBC: 5.1-15.8 10^3/µl, GC: 1.3-7.6 10^3/µl, LC: 2.0-7.8 10^3/µl, MC: 0.0-1.3 10^3/µl, GP: 21.5-68.0%, LP: 28.0-71.5%, MP: 0.0-9.5%

Table 41. Mean values (SD) of hematological parameters regarding red blood cells in ewes with different serological and infection patterns during the 24 months.

	Mean value (SD)							
Serological Pattern	**RBC (10^6/µl)**	**HGB (g/dl)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dl)**	**RDW-CV (%)**	**RDW-SD (fL)**
Constantly seronegative	8.6 (0.98)	9.9 (1.27)	30.4 (4.35)	35.2 (2.32)	11.5 (0.74)	32.4 (1.85)	16.8 (2.61)	23.3 (4.98)
Constantly seropositive	8.5 (1.30)	9.8 (1.34)	30.4 (4.62)	35.8 (3.15)	11.5 (1.07)	32.2 (2.41)	16.8 (2.28)	23.9 (4.93)
Seroconverted	8.2 (1.49)	9.3 (1.38)	28.8 (4.96)	35.5 (3.21)	11.5 (1.21)	32.6 (2.37)	16.6 (1.60)	22.8 (2.92)
Seroreverted	8.9 (1.42)	9.9 (1.61)	31.2 (5.21)	35.0 (2.58)	11.1 (0.78)	32.3 (2.05)	16.4 (1.06)	22.2 (2.12)
Intermittent presence of antibodies	8.3 (1.48)	9.5 (1.57)	29.3 (4.20)	36.1 (5.06)	11.6 (1.24)	32.4 (2.38)	17.5 (2.99)	24.8 (6.17)
Infection Pattern								
Uninfected	8.5 (0.96)	9.7 (1.29)	29.8 (4.63)	34.8 (2.43)	11.4 (0.76)	32.5 (2.02)	16.4 (1.20)	22.4 (2.41)
Infected seropositive	8.4 (1.38)	9.6 (1.40)	29.8 (4.70)	35.8 (3.47)	11.5 (1.13)	32.4 (2.39)	16.8 (2.24)	23.7 (4.68)
Infected seronegative	8.9 (1.28)	10.0 (1.47)	31.3 (4.66)	35.3 (2.40)	11.3 (0.79)	32.2 (1.86)	16.9 (2.59)	23.2 (4.96)

SD: Standard deviation; RBC: red blood cell count; HGB: hemoglobin concentration; HCT: hematocrit; MCV: mean value of corpuscular volume; MCH: mean value of corpuscular hemoglobin; MCHC: corpuscular hemoglobin concentration; RDW-CV: red cell distribution width coefficient of variation; RDW-SD: red cell distribution standard deviation; fL: femtoliter; Pg: petagrams; Reference ranges: RBC:6.5-15.2 10^6/µl, HGB: 6.8-14.5 g/dl, HCT: 20.0-42.5%, MCV: 25.0-41.0 fL, MCH: 8.0-12.3 pg, MCHC: 29.0-37.0 g/dl, RDW-CV: 14.5-26.2%, RDW-SD: 17.0-32.0 fL

Table 42. Mean values (SD) of hematological parameters regarding platelets in ewes with different serological and infection patterns during the 24 months.

	Mean value (SD)					
Serological Pattern	**PLT (10^9/L)**	**MPV (fL)**	**PDW**	**PCT (mL/L)**	**P-LCC (10^9/L)**	**P-LCR (%)**
Constantly seronegative	291.8 (153.53)	6.1 (0.59)	15.0 (0.42)	1.8 (0.91)	136.1 (70.94)	47.8 (8.76)
Constantly seropositive	246.8 (142.54)	6.1 (0.47)	15.2 (0.40)	1.5 (0.87)	119.3 (66.62)	49.2 (6.75)
Seroconverted	252.9 (145.27)	6.0 (0.46)	15.1 (0.39)	1.5 (0.83)	115.6 (59.04)	47.5 (7.69)
Seroreverted	292.3 (183.60)	6.0 (0.41)	14.9 (0.30)	1.8 (1.11)	138.7 (86.35)	48.6 (6.84)
Intermittent presence of antibodies	241.9 (140.04)	6.1 (0.44)	15.0 (0.43)	1.5 (0.80)	114.6 (58.43)	49.8 (7.57)
Infection Pattern						
Uninfected	266.8 (121.92)	6.1 (0.41)	15.1 (0.33)	1.6 (0.76)	126.0 (61.59)	47.7 (6.56)
Infected seropositive	247.9 (142.35)	6.1 (0.46)	15.1 (0.40)	1.5 (0.84)	117.5 (63.02)	48.8 (7.16)
Infected seronegative	308.8 (187.11)	6.1 (0.60)	14.9 (0.40)	1.9 (1.10)	144.4 (85.15)	48.4 (8.94)

SD: Standard deviation; PLT: platelet count; MPV: mean platelet volume; PDW: platelet distribution width; PCT: plateletcrit: P-LCC: platelet large cell count; P-LCR: platelet large cell ratio; fL: femtoliter; Reference ranges: PLT: 200.0-800.0 10^9/L, MPV: 3.5-6.8 fL, PDW: 12.0-17.5, PCT: 1.0-4.2 mL/L, P-LCC: 30.0-260.0 10^9/L, P-LCR: 12.6-60.0%

2.7 The effects of serological status on hematological parameters

Statistically significant effects of serological status on hematological parameters were not observed.

2.8 The effects of infection status on hematological parameters

Infection status had a significant effect on WBC, GC, LC, and MC. Namely, the uninfected ewes had significantly higher WBC, LC, and MC compared to the infected seropositive ($p<0.05$) and infected seronegative ones ($p<0.01$). Also, uninfected ewes demonstrated significantly higher GC compared to the infected seronegative ones ($p<0.05$). Moreover, the infected seropositive ewes had significantly higher WBC, LC, and MC ($p<0.05$) compared to the infected seronegative ones. The statistically significant effects of infection status on hematological parameters, as derived from the respective mixed linear logistic models are presented, in Tables S36-S39 in Appendix C.

2.9 The effects of serological patterns on hematological parameters

The seroreverted ewes had significantly lower WBC and LC compared to the constantly seronegative ones ($p<0.05$ in both cases). Also, the seroreverted ewes had significantly lower RDW-SD compared to the constantly seropositive and ewes with an intermittent presence of antibodies ($p<0.05$ in both cases) and lower PDW compared to the constantly seropositive ones ($p<0.05$), whereas they also had higher RBC and HGB compared to the seroconverted ones ($p<0.05$). Ewes with an intermittent presence of antibodies had lower PLT and P-LCC compared to the constantly seronegative ones ($p<0.05$ in both cases). The statistically significant effects of serological pattern on hematological parameters, as derived from the respective mixed linear logistic models, are presented in Tables S40-S47 in Appendix C.

2.10 The effects of infection patterns on hematological parameters

The infection pattern had a statistically significant effect on MC, HGB, and PDW. Namely, the uninfected ewes had significantly higher MC compared to the infected seropositive or seronegative ewes ($p<0.05$ in both cases). Also, the infected seronegative ewes had significantly higher HGB but lower PDW compared to the infected seropositive ewes ($p<0.05$ in both cases). The statistically significant effects of infection pattern on hematological parameters, as derived from the respective mixed linear logistic models, are presented in Tables S48-S50 in Appendix C.

IV. Discussion

It is the first prospective cohort study for the assessment of the effects of SRLV infections on the productivity and health of intensively reared dairy sheep in Greece. Also, the classification of animals according to their serological and infection pattern permitted further investigation of the effects, indicating the significance of the accurate diagnosis and prospective study of SRLV infections.

Currently, the effect of SRLV infections on milk yield and quality traits remains unclear, as the results of previous studies are contradictory; milk, fat, protein, and lactose yields were found reduced in SRLV seropositive ewes and goats (Turin *et al.*, 2005; Leitner *et al.*, 2010; Kaba *et al.*, 2012; Martínez-Navalón *et al.*, 2013; Juste *et al.*, 2020), whereas in other studies no effect of seropositivity was reported on milk yield (Nord and Ådnøy, 1997; Legrottaglie *et al.*, 1999; Turin *et al.*, 2005; Kaba *et al.*, 2012; Barquero *et al.*, 2013c) and quality traits (Nord and Ådnøy, 1997; Legrottaglie *et al.*, 1999; Barquero *et al.*, 2013c), or a favorable effect was reported on milk fat (Turin *et al.*, 2005; Echeverría *et al.*, 2020). In the present study, the serological pattern had a significant effect on daily fat yield and on 120-day milk, fat, protein, lactose, and solids-not-fat yields. The constantly seronegative, the constantly seropositive, and the seroconverted ewes had ca. 14% significantly higher daily fat yield compared to the seroreverted ones. Also, the seroconverted ewes presented higher 120-day milk, fat, protein, lactose, and solids-not-fat yields compared to the constantly seronegative and seropositive ewes ($p<0.05$). Moreover, 120-day milk fat yield was found to be higher in the seroconverted compared to the seroreverted ewes ($p<0.05$).

On the other hand, the infection pattern had a significant effect on both daily and 120-day milk, fat, protein, lactose, and solids-not-fat yields. The DMY and milk quality traits were reduced by ca. 15% in the infected seronegative ewes compared to the uninfected ones. Also, the DFY was reduced by ca. 10% in infected seronegative ewes compared to infected seropositive ones. The 120-day milk yield and milk quality traits were found to be reduced ca. 20% in the infected seronegative ewes compared to both the uninfected and the infected seropositive ewes. The findings of this study are partially in consistency with a previous study where milk yield and protein content were reduced, but fat content was increased in infected animals diagnosed by both ELISA and PCR (Echeverría *et al.*, 2020). The inconsistency regarding the effect of SRLV infection on milk fat between the previous and the present study may be attributed to the statistical analyses used in the first one, which were

limited to Mann-Whitney's comparisons of contents compared to the estimation of the effect on milk component yields with the mixed linear regression models used in the present study.

Also, in our study, no effect of serological and infection pattern on the SCC was reported, in consistency with previous studies (Legrottaglie *et al.*, 1999; Leitner *et al.*, 2010; Kaba *et al.*, 2012). However, an increase in SCC in seropositive (Nord and Ådnøy, 1997; Turin *et al.*, 2005) or infected (Echeverría *et al.*, 2020) goats and ewes has been previously reported. Although the limits of SCC in sheep milk have not yet been clarified, the increase of SCC has been associated with the presence of clinical or subclinical mastitis (Paape *et al.*, 2007; Gelasakis *et al.*, 2015; Albenzio *et al.*, 2019). However, in our study, the overall mean value of SCC was observed to be low (<300.000 cells/ml) indicating an enhanced udder health status of the studied ewes, possibly due to artificial lamb rearing, machine milking, and implementation of general hygiene practices. On the other hand, the presence of mastitis, swollen supramammary lymph nodes, and reduced milk yield in infected animals could be indicators of mammary inflammation, which could lead to an increase in SCC. However, in our study, the SRLV infections may not cause an intense concentration of leukocytes in the mammary parenchyma to significantly increase the SCC in the milk of infected animals. Moreover, a shorter duration of lactation period was recorded in constantly seronegative and constantly seropositive animals compared to the seroconverted ones, and in infected seronegative ewes compared to the uninfected and the infected seropositive ones. Reduced duration of lactation period has, also, been observed in seropositive goats (Martínez-Navalón *et al.*, 2013), whereas in other studies no effect on lactation duration was reported (Barquero *et al.*, 2013c; Juste *et al.*, 2020).

In the present study, the assessment of the effects of SRLV infections on milk yield and quality traits after the classification of the animals according to their serological or infection pattern revealed an adverse impact of the infection in cases of a lack of antibodies in infected animals (lack of seroconversion or loss of the produced antibodies). This may explain the current ambiguity regarding the effect of SRLV on the productivity of dairy sheep and goats; the seroreverted animals and the infected animals, which never seroconvert, evade the serological diagnosis and are classified with the uninfected animals, confounding the results. Hence, a highly sensitive and accurate diagnostic method is an essential prerequisite for the investigation of the effects of SRLV infections on animal productivity. This is further highlighted in the present study by the low performance of constantly seronegative animals compared to the seroconverted ones in cases of 120-day milk, fat, protein, lactose, and solids-not-fat yields and the duration of lactation period, whereas uninfected animals did not present

lower performance compared to the infected ones in any case. It is obvious that the infected animals that never seroconverted were classified as constantly seronegative during the study, significantly reducing the mean values of milk yield and quality traits in this category. On the other hand, the constantly seropositive ewes presented lower day milk, fat, protein, lactose, and solids-not-fat yields compared to the seroconverted ones, indicating that the chronic and persistent SRLV infection may lead to inflammatory udder lesions in some animals, reducing their performance compared to the animals more recently infected and seroconverted.

The protective role of neutralizing antibodies produced after SRLV infection has not been fully elucidated, though it has been hypothesized (Singh *et al.*, 2006; Torsteinsdóttir *et al.*, 2007; Blacklaws, 2012). The adverse impact of SRLV on the productivity of infected seronegative animals in the present study may confirm the protective role of neutralizing antibodies against virus replication. This neutralizing effect may inhibit the rapid evolution of the disease and the development of lymphocytic inflammatory lesions in the mammary gland, which leads to a reduction in milk production. On the other hand, the possibility of animals with impaired health status that cannot trigger an effective immunological response against the virus and present low productivity cannot be excluded, though it was rather than possible in the studied farms since the farming conditions, the nutrition, and the preventive veterinary measures ensured a high animal health status with a low prevalence of other infectious (e.g., bacterial mastitis, foot-rot, enzootic pneumonia) or metabolic (e.g., pregnancy toxemia, ruminal acidosis) diseases.

Although SRLV clinical cases have been reported not only worldwide but also in our country (van der Molen, Vecht and Houwers, 1985; Karanikolaou *et al.*, 2005; Angelopoulou, Brellou and Vlemmas, 2006; Fournier, Campbell and Middleton, 2006; Benavides *et al.*, 2007, 2009; Brellou *et al.*, 2007; Giadinis *et al.*, 2015; Borquez Cuevas *et al.*, 2021), the prevalence of clinical signs of the disease or other health disorders in infected flocks has not been adequately investigated. In the studied farms, the typical clinical manifestations of SRLV infections recorded during the study were the arthritis in at least one limb, the cough and dyspnea (respiratory disease), and the “hard udder” syndrome (interstitial mastitis), whereas any animal with neurological signs was reported during the study or the last years in these farms. An interesting finding of the study was that the 8 animals that manifested the “hard udder” syndrome were all SRLV infected and removed from the farms during the study, as they manifested it post-lambing, accompanied by very low milk production. Therefore, due to their early removal from the study, these 8 animals did not have milk recordings and they were not retained in the final database for the subsequent statistical analyses.

SRLV seropositivity and infection in the present study were associated with the occurrence of many health disorders. Namely, the seropositive animals were more likely to present nasal discharge, whereas seroreverted animals were associated with the occurrence of arthritis and swollen supramammary lymph nodes. Also, the manifestation of arthritis during the study was associated with the ewes with an intermittent presence of antibodies. Moreover, the infection was associated with the occurrence of mastitis and udder skin lesions, whereas infected seronegative ewes were more likely to present arthritis, lameness, wart-like lesions, and swollen supramammary lymph nodes. These findings indicate a possible association between the lack of antibodies (seroreversion and lack of seroconversion) and clinical signs associated with the organ targets of SRLV infections (joints and udder). This, in combination with the above-mentioned results from reduced milk production in the infected seronegative animals, reinforces the diagnostic value of the combination of serological and molecular investigation. Also, considering these results, the hypothesis of the protective role of neutralizing antibodies against the virus replication (Georgsson *et al.*, 2015) needs to be further investigated.

The presence of nasal discharge in seropositive animals is not a typical symptom, as the pneumonia in MV cases is dry (Straub, 2004), and no association with respiratory disease (cough and dyspnea) was identified in our studied cases. However, the association between internal parasitism and SRLV infection has been previously reported (Hüttner, Seelmann and Feldhusen, 2010; Mekibib *et al.*, 2018), explaining possibly the presence of nasal discharge in seropositive animals in our study. The co-existence of parasites and SRLV infection creates a vicious circle of stress factors for the immune system of animals, which can lead to the manifestation of signs of both diseases. Also, the increased manifestation of wart-like lesions in infected seronegative animals, which are probably caused by the papilloma virus, could be associated with the decreased immune response of these animals.

Although SRLV tropism is related to monocytes, macrophages, and dendritic cells, and infection of myeloid stem cells has been suggested (Blacklaws, 2012), scientific evidence to support hematological changes in infected sheep is limited (Lipecka *et al.*, 2010; Serkal Gazyagci, 2011). In the present study, the infected ewes were associated with lower white blood cell, lymphocyte, granulocyte, and monocyte counts, whereas the infected seronegative animals had significantly higher hemoglobin concentration but lower platelet distribution width, white blood cell, lymphocyte, and monocyte counts compared to the infected seropositive ewes. These differences in hematological parameters are consistent with previous studies where a reduced white blood cell count was reported in lambs from

seropositive ewes (Lipecka *et al.*, 2010) and increased hemoglobin was associated with SRLV infection in adult sheep (Serkal Gazyagci, 2011). Hematological disorders such as anemia, lymphopenia, neutropenia, leukopenia, and thrombocytopenia have been previously described in HIV patients before antiretroviral treatment (Saif, 2001; Vishnu and Aboulafia, 2015; Damtie *et al.*, 2021), mainly due to the infection of bone marrow mesenchymal stem cells. Although the lack of breed- and age-specific reference values of hematological parameters in dairy sheep does not permit the extraction of safe conclusions about the manifestation of similar hematological abnormalities in SRLV infected ewes, the statistically significant differences in mean values of hematological parameters indicate a similar mechanism of infection of progenitor and stem cells in bone marrow, leading not only to persistent infection of the animal via the continual production of infected cells but also to blood cell abnormalities. Also, the results from our study support a more intense leukopenia in infected seronegative animals compared to the seroconverted animals, indicating a more severe immunosuppression in these animals. Nevertheless, the existence of a reverse mechanism (the lack of seroconversion or the loss of antibodies in already immunosuppressed ewes due to co-infections or stressed status) explaining these hematological abnormalities cannot be excluded under the present study design and the measurement of hematological parameters only in the last sampling occasion.

In the present study, infected seronegative animals were associated with health and hematological disorders and low productivity. Considering these findings, the hematological analyses could be performed in combination with the diagnosis of SRLV infections for the selective removal of animals with white blood cell abnormalities. However, further large-scale prospective studies including animals with different infection patterns could elucidate the potential association of SRLV infection with hematological disorders and reinforce the diagnostic value of hematological analyses in SRLV infections.

Chapter 4: Conclusions

It is the first time that a holistic approach to diagnosis and epizootiological investigation for the assessment of risk factors and the effects of SRLV infections on productivity, health, and welfare traits was attempted in intensively reared dairy sheep in Greece.

A novel diagnostic protocol, based on both serological and molecular assays, was developed and evaluated for the early and efficient diagnosis of the circulating SRLV strains. The nested real-time PCR protocol that was developed, evaluated, and applied in the present study demonstrated high diagnostic performance and could be further exploited as an affordable routine molecular test for the diagnosis of SRLV infections in the country.

The prospective study design of the epizootiological investigation revealed the presence of different serological and infection patterns in infected animals and highlighted the increased misdiagnosis of SRLV infections in cross-sectional sero-epizootiological studies. The high values of morbidity frequency measures in the studied farms confirmed the hypothesis of increased SRLV prevalence and the significance of horizontal transmission in intensively reared purebred Chios and Lacaune ewes in Greece. Risk assessment analysis confirmed the increased age as a risk factor for SRLV infections and revealed a breed-related effect on the occurrence of different serological patterns. The pre-lambing period was also associated with seropositivity, indicating that this period is more appropriate for serological screening control.

The epizootiological study in lambs underpinned the significance of the lactogenic transmission route in lambs and the necessity for the administration of pasteurized colostrum in combination with artificial rearing. Also, the molecular control of lambs at the age of 3 months was evaluated as appropriate for early interruption of horizontal virus spreading, whereas the serological screening control of replacement animals could be performed at the age of 8 months.

The prospective study design and the classification of animals according to their infection pattern permitted the quantification of the effects of SRLV infections on productivity and health. Milk production losses reached up to ca. 20% regarding milk, fat, protein, lactose, and solids-not-fat yields in infected animals that did not seroconvert or serorevert during the study. Similarly, health disorders including lameness, arthritis, mastitis, swollen supramammary lymph nodes, udder skin and wart-like lesions, and white blood cel parameters were more frequent in these animals.

The present study recorded updated epizootiological data that could be exploited in the evidence-based designation of control programs against SRLV in intensive dairy farms in

Greece for the mitigation of production losses, the improvement of animal health and welfare status, and the enhancement of farms' sustainability. Further large-scale epizootiological studies including animals reared under various farming systems and prevalence rates could enrich and extend the knowledge regarding the epizootiology, the early and accurate diagnosis, the transmission dynamics, and the impact of SRLV infections in our country. At the same time, the genetic resistance/susceptibility to the SRLV infection and its association with production traits should be further investigated and assessed for use in genetic selection programs, and basic research should be extended, including cell cultures for the isolation of the circulating viral strains, the further phylogenetic analysis and characterization, and the development of highly sensitive and specific serological and molecular assays with universal applicability in the country.

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Appendix A: The questionnaire used for the collection of data regarding the farms' characteristics and management practices during on-site visits.

ΔΕΛΤΙΟ ΕΡΕΥΝΑΣ ΕΚΤΡΟΦΗΣ*

Ημερομηνία: ..../…./……..

ΣΤΟΙΧΕΙΑ ΕΚΤΡΟΦΗΣ

Ονοματεπώνυμο ιδιοκτήτη	
Ηλικία ιδιοκτήτη	
Περιοχή εκτροφής	
Κύρια δραστηριότητα	
Χρόνια άσκησης επαγγέλματος	
Σύστημα εκτροφής	
Μέλη οικογένειας που εργάζονται	
Ξένοι εργάτες	

ΚΤΙΡΙΑΚΕΣ ΕΓΚΑΤΑΣΤΑΣΕΙΣ

Κτίριο	Αριθμός	Διαστάσεις
Κυρίως στάβλος		
Βοηθητικός στάβλος		
Αποθήκη χονδροειδών ζωοτροφών		
Αποθήκη συμπυκνωμένων ζωοτροφών		
Παρασκευαστήριο ζωοτροφών		
Κατάλυμα προσωπικού-γραφείο		

ΜΗΧΑΝΙΚΟΣ ΕΞΟΠΛΙΣΜΟΣ

Μηχάνημα	Αριθμός	Χαρακτηριστικά
Σφυρόμυλος κυλινδρόμυλος		
Αναμικτήρας ζωοτροφών		
Συσκευή τεχνητής γαλουχίας		
Σύστημα παγίδευσης		
Άλλος εξοπλισμός		
Αρμεχτικό συγκρότημα		

ΖΩΙΚΟ ΚΕΦΑΛΑΙΟ - ΑΠΟΔΟΣΕΙΣ

Ομάδα	Αριθμός
Πρώιμες προβατίνες αρμεγόμενες	
Όψιμες προβατίνες αρμεγόμενες	
Πρώιμες αρνάδες αντικατάστασης	
Όψιμες αρνάδες αντικατάστασης	
Ενήλικα κριάρια	
Αρσενικά ζυγούρια αντικατάστασης	
Αρσενικά αρνιά αντικατάστασης	
Στέρφα	

Φυλή/ές προβάτων: .....……
Άλλα παραγωγικά ζώα (πρόβατα/αγελάδες κ.α.): □ ……
Άλλα ζώα (σκύλοι/γάτες): □ ….....
Ρυθμός αντικατάστασης κριών:…….
Αρνάδες αντικατάστασης: ……..……

ΠΑΡΑΓΩΓΙΚΑ ΧΑΡΑΚΤΗΡΙΣΤΙΚΑ

Χαρακτηριστικό	Επίδοση
Συνολική ετήσια γαλακτοπαραγωγή ποιμνίου	
Αριθμός αρνιών που γεννήθηκαν σε ένα έτος	
Γαλακτοπαραγωγή/ προβατίνα/ γαλακτική περίοδο	
Μέση πολυδυμία	
Αρνιά που πωλήθηκαν για σφαγή	
Μέσο βάρος σφάγιου αρνιών	
Προβατίνες που πωλήθηκαν για σφαγή	
Μέσο βάρος σφάγιου προβατίνων	
Κριάρια που πωλήθηκαν για σφαγή	
Μέσο βάρος σφάγιου κριαριών	

ΠΟΙΟΤΙΚΑ ΧΑΡΑΚΤΗΡΙΣΤΙΚΑ ΓΑΛΑΤΟΣ:

Στάδιο γαλακτικής περιόδου	Λίπη	Πρωτεΐνες	Σωματικά κύτταρα	Μικροβιακό φορτίο
Αρχή				
Μέση				
Τέλος				

ΤΙΜΕΣ ΠΡΟΪΟΝΤΩΝ (/χλγ):

Γάλα	
Σφάγιο αρνιού	
Σφάγιο προβατίνας	
Σφάγιο κριαριού	
Μαλλί	
Κοπριά	
Δέρμα	

ΖΩΟΤΡΟΦΕΣ

ΙΔΙΟΠΑΡΑΓΟΜΕΝΕΣ ΖΩΟΤΡΟΦΕΣ:

Για Βόσκηση		
ΕΙΔΟΣ	ΕΚΤΑΣΗ	Βοσκή (Άσκηση) - Πρόγραμμα
		.....
		.....
		.....

Για Συγκομιδή					
Χονδροειδείς			Συμπυκνωμένες		
Είδος	Έκταση	Ποσότητα	Είδος	Έκταση	Ποσότητα
Σανός μηδικής			Καλαμπόκι		
Lollium			Κριθάρι		
Κτηνοτροφικό μπιζέλι			Σιτάρι		
Κτηνοτροφικό κουκί			Βρώμη		
Άλλο:			Άλλο:		

ΑΓΟΡΑΖΟΜΕΝΕΣ ΖΩΟΤΡΟΦΕΣ:

Χονδροειδείς			Συμπυκνωμένες		
Είδος	Ποσότητα	Τιμή	Είδος	Ποσότητα	Τιμή
Σανός μηδικής			Καλαμπόκι		
Άχυρο			Κριθάρι		
Ενσ. μηδικής			Πίτυρα		
Ενσ. αραβοσίτ			Σόγια		
Άλλο σανό			Βαμβακόπιτα		
			Βαμβακόσπορος		
			Ηλιόπιτα		
			Ζαχαρόπιτα		

Ισσοροπιστής: Ναι □ Όχι □ Ποσότητα
Αναλύσεις ζωοτροφών: Ναι □ Όχι □
Αγορά συμπυκνωμένων ζωοτροφών: Σταδιακή □ Μαζική □
Υπεύθυνος καταρτισμού σιτηρεσίων: .....
Προέλευση ύδατος: Δίκτυο κοινότητας □ Γεώτρηση □

ΓΑΛΟΥΧΙΑ ΑΡΝΙΩΝ
Φυσική γαλουχία**:** Ναι □ Όχι □
Τύπος φυσικής γαλουχίας: Ελεύθερη □ Περιορισμένη □
Διάρκεια γαλουχίας:.....εβδομάδες
Άρμεγμα κατά την περίοδο της γαλουχίας: .. Ναι □ Όχι □

Τεχνητή γαλουχία: Ναι □ Όχι □
ΧΟΡΗΓΗΣΗ-ΛΗΨΗ ΠΡΩΤΟΓΑΛΑΤΟΣ: Ημέρες ..... Γεύματα ..... Ποσότητα.....
Σκέυη: Μπουκάλι με θηλή □ Κουβάς με θηλή □ Αυτόματη συσκευή □
Γεύματα: Αριθμός/ημέρα..... Ποσότητα/ γεύμα:.....
Διάρκεια γαλουχίας: .....

Παράθεση:
1) Νερού: Ημέρα .....
2) Μίγματος συμπ. ζωοτροφών: Εβδομάδα ..... Είδος .....…….....
3) Χονδροειδών ζωοτροφών: Εβδομάδα ..... Είδος ..……

ΣΙΤΗΡΕΣΙΑ
ΟΜΑΔΑ Α: Αρμεγόμενες προβατίνες

	$1^{ος}$ μήνας	$2^{ος}$ μήνας	$3^{ος}$ μήνας	$4^{ος}$ μήνας	$5^{ος}$ μήνας	$6^{ος}$ μήνας	$7^{ος}$ μήνας	$8^{ος}$ μήνας
Φύραμα								
Σανό								
Άχυρο								
Ώρες βόσκησης								

Παρατηρήσεις : .....

ΟΜΑΔΑ Β: Προβατίνες στην ξηρά περίοδο

	Ξηρά περίοδος	Ένα μήνα πριν τον τοκετό
Φύραμα		
Σανό		
Άχυρο		
Ώρες βόσκησης		

Παρατηρήσεις : .....

ΟΜΑΔΑ Γ: Αρνάδες – Ζυγούρες

	$2^{ος}$ μήνας	$3^{ος}$ μήνας	$4^{ος}$ μήνας	$5^{ος}$ μήνας	$6^{ος}$ μήνας	$7^{ος}$ μήνας	$8^{ος}$ μήνας	$9^{ος}$ μήνας	$10^{ος}$ μήνας
Φύραμα									
Σανό									
Άχυρο									
Ώρες βόσκησης									

Παρατηρήσεις : .....

ΟΜΑΔΑ Δ: Κριάρια

	Συντήρησης	Προετοιμασίας	Οχείες
Φύραμα			
Σανό			
Άχυρο			
Ώρες βόσκησης			

Παρατηρήσεις : .....

Σύνθεση φυραμάτων:

	Ομάδες			
Ζωοτροφές	Αρμεγόμενες	Ξηρά περίοδος	Αρνάδες-ζυγούρες	Κριάρια
Καλαμπόκι				
Κριθάρι				
Σιτάρι				
Βρώμη				
Πίτυρα σιταριού				
Σογιάλευρο				
Βαμβακόπιτα				
Βαμβακόσπορος				
Πούλπα ζαχαροτ.				
Μηδικάλευρο				

Ώρες βόσκησης και σύνθεση βοσκότοπου

Μήνας	1ος	2ος	3ος	4ος	5ος	6ος	7ος	8ος	9ος	10ος	11ος	12ος
Ώρες βόσκησης												
Απόσταση που διανύουν ημερησίως												

ΑΝΑΠΑΡΑΓΩΓΗ

Μέθοδος γονιμοποίησης: Τεχνητή σπερματέγχυση □ Οχεία □
Συγχρονισμός οίστρων: Ναι □ (.....%. Ομάδες……..…….……) Όχι □
Προέλευση κριών: Μονάδα □ Αγορά □ Αναλογία …../….. Επίδραση αρσενικού □

ΣΥΣΤΗΜΑ ΟΧΕΙΩΝ :

Α. Ελεύθερες οχείες στην τύχη	Β. Προκαθορισμένες οχείες κατά ομάδες	Γ. Τελείως ελεγχόμενες οχείες

ΑΝΑΠΑΡΑΓΩΓΙΚΗ ΠΕΡΙΟΔΟΣ:

	Έναρξη αναπαραγωγικής περιόδου (μήνας)	Λήξη αναπαραγωγικής περιόδου (μήνας)
Πρώιμα		
Όψιμα		

Ηλικία στην έναρξη του αναπαραγωγικού τους βίου:
Ζυγούρια:.....
Κριάρια:…….....

Συστηματική τήρηση αναπαραγωγικών στοιχείων: Ναι □ Όχι □
Αποβολές το τελευταίο έτος:……
Θνησιγενή:.....

ΣΤΑΒΛΙΣΜΟΣ

Υποομάδες:.....
Μέγεθος υποομάδων:.....……
Συστέγαση:……..

Ταΐστρες – ποτίστρες:
Εξωτερικές □ Εσωτερικές □
Στεγασμένες □ Ακάλυπτες □
Απόσταση από χώρο ανάπαυσης:……..
Ποτίστρα: Σκαφοειδής με φλοτέρ □ Σκαφοειδής χωρίς φλοτέρ □ Άλλο □
Εκμηχάνιση τροφοδοσίας: Ναι □ Όχι □
Κτίριο Κτίσμα: Ανοικτό □ Κλειστό □
Δάπεδο: Χωμάτινο □ Τσιμεντένιο □ Σχαρωτό □
Διαστρωμάτωση δαπέδου: .....
Στρωμνή: Ναι □ (Είδος: ..... Επαρκής □ Ανεπαρκής □) Όχι □
Συχνότητα ανανέωσης στρωμνής: .....

Προαύλιο: Ναι □ Όχι □
Δάπεδο προαυλίου: Χωμάτινο □ Τσιμεντένιο □
Επιφάνεια κτιρίου: Επαρκής □ Ανεπαρκής □
Όγκος κτιρίου: Επαρκής □ Ανεπαρκής □
Αερισμός κτιρίου: Καλός □ Μέτριος □ Κακός □
Τύπος αερισμού: Φυσικός □ Τεχνητός □
Υλικό οροφής: Λαμαρίνα □ Κεραμίδι □ Πλάκα □ Ψευδοροφή □

ΑΡΜΕΓΜΑ
1.Με τα χέρια
Αριθμός αρμεχτών:.....
Αρμέγματα ανά ημέρα:.....
Διάρκεια αρμέγματος:.....
Πρόβατα ανά ώρα:…….
Σκεύος: Πλαστικό □ Μεταλλικό □ Ανοξείδωτο □
Παγολεκάνη: Ναι □ (Στο στάβλο □ Στο σπίτι □) Όχι □
Συχνότητα παραλαβής του γάλακτος: .....

2.Με μηχανή
Κατασκευαστής:.....
Παλαιότητα:.....
Αριθμός αρμεχτών:.....
Αριθμός αρμεκτικών μονάδων: .....
Παλμοί:…….....
Ύψος κενού:.....……
Αρμέγματα ανά ημέρα:.....
Διάρκεια αρμέγματος:.....
Παγολεκάνη: Ναι □ Όχι □ Χωρητικότητα (λίτρα) .....
Συχνότητα καθαρισμού αρμεκτικού συγκροτήματος: .....
Συχνότητα αλλαγής θηλάστρων:…….
Συχνότητα συντήρησης:...……
Εφεδρική πηγή ενέργειας: Ναι □ Όχι □
Συχνότητα παραλαβής του γάλακτος: .....

Εργασίες αρμέγματος
Έλεγχος γάλακτος: Ναι □ (μέθοδος.....) Όχι □
Έλεγχος μαστού (μαστίτιδα): Ναι □ Όχι □
Χορήγηση τροφής: Ναι □ Όχι □ Ποσότητα : .....
Απάρμεγμα: Ναι □ Όχι □
Εμβάπτιση θηλών σε αντισηπτικό: Ναι □ Όχι □

ΓΕΝΙΚΕΣ ΠΡΑΚΤΙΚΕΣ
Περιποίηση ομφάλιου λώρου: Βράχυνση □ Δέσιμο □ Απολύμανση □
Κόψιμο ψευδοθηλών: Ναι □ (Ηλικία.....) Όχι □
Περιποίηση χηλών: Ναι □ Όχι □
Στείρευση γαλακτοπαραγωγής: Μείωση τροφής..... Μείωση νερού.....
Διακοπή αρμέγματος: Απότομη □ Σταδιακή □

ΠΡΟΛΗΠΤΙΚΗ ΚΤΗΝΙΑΤΡΙΚΗ

Συστηματική προληπτική χορήγηση στα αρνιά:
Βιταμίνες: .....
Ανθελμινθικά: .....
Άλλο : .....

Συστηματική προληπτική χορήγηση στις προβατίνες:
Βιταμίνες : .....
Ανθελμινθικά : .....
Άλλο : .....

ΕΜΒΟΛΙΑΣΜΟΙ

Εμβόλιο/προληπτική αγωγή	Εφαρμογή	Λεπτομέρειες
Εντεροτοξιναιμία		
Λοιμώδης αγαλαξία		
Ενζωοτική αποβολή		
Μαστίτιδες		
Λοιμώδες έκθυμα		
Άλλο εμβόλιο		
Ενδομαστικά ξηράς περιόδου		

ΚΤΗΝΙΑΤΡΙΚΑ

ΑΡΝΙΑ

Πάθηση	Ηλικία	Νοσηρότητα	Θνησιμότητα
Διάρροια			
Πνευμονία			

Προβλήματα υγείας το τελευταίο έτος (αριθμός ζώων):
Αποβολές……
Μαστίτιδα……
Λοιμώδης αγαλαξία…….
Ποδοδερματίτιδα……..
Προϊούσα πνευμονία……..……...
Άλλο……

Εφαρμογή θεραπείας: Κτηνοτρόφος □ Υπάλληλος □
Αναρρωτήριο: Ναι □ Όχι □
Τήρηση αρχείου θεραπείας: Ναι □ Όχι □
Σήμανση ζώων στα οποία χορηγήθηκαν αντιβιοτικά: Ναι □ Όχι □

* Gelasakis A.I. (2011) 'Investigation of the relationship between lameness, milk production and rearing methods of Chios dairy sheep.', Doctoral dissertation. Aristotle University of Thessaloniki.

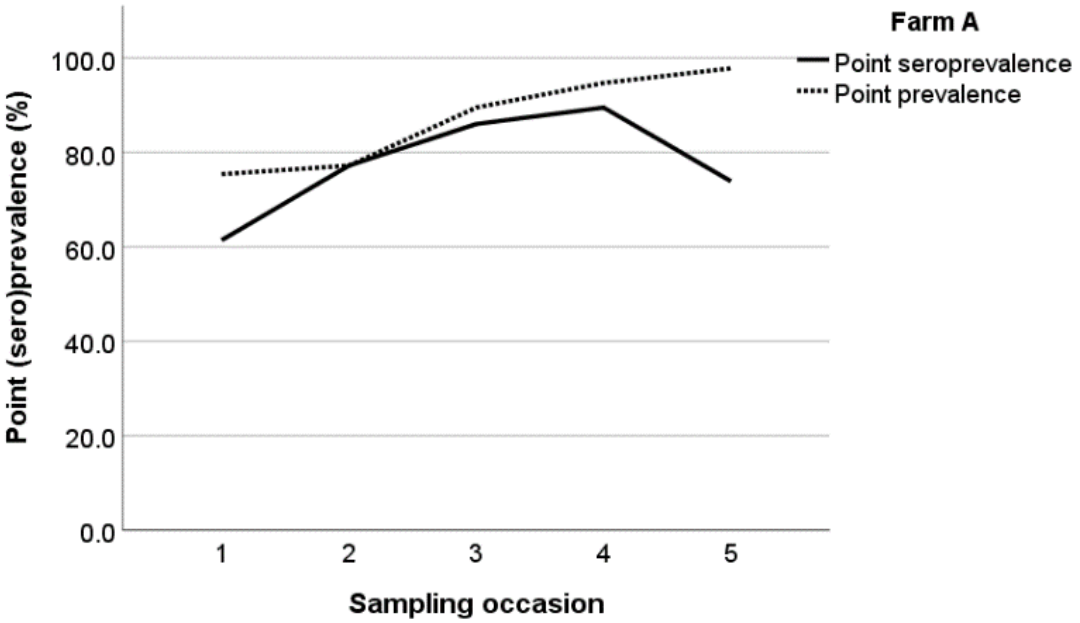
Appendix B: Tables and figures with epizootiological results for the studied farms.

Table S1. The frequencies of serological patterns per farm and per breed.

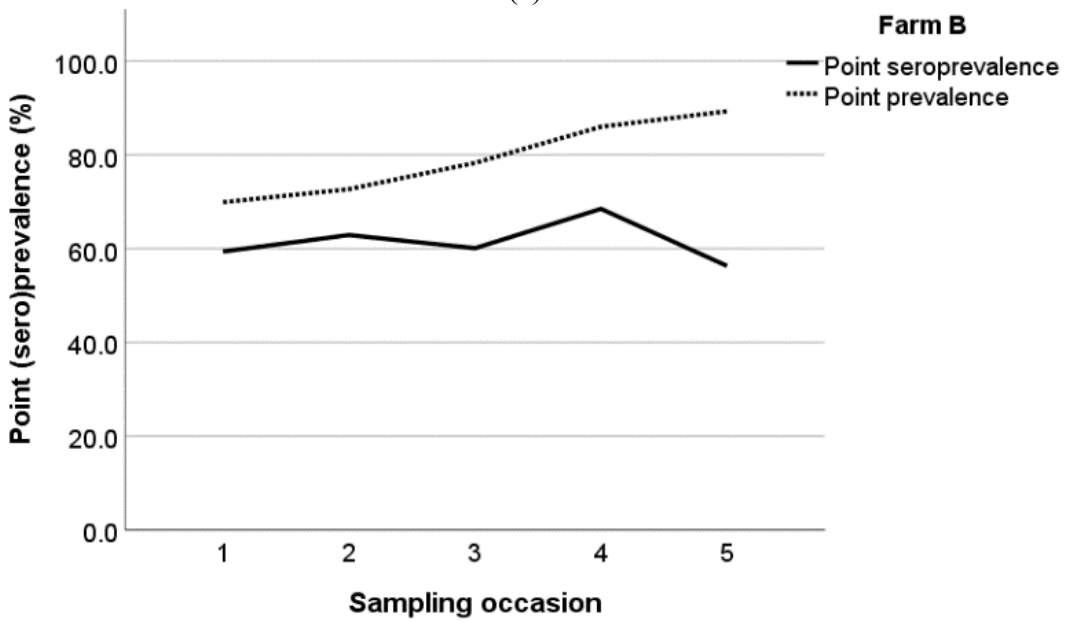
Serological pattern	Chios			Lacaune		
	Farm A	Farm B	Farm C	Farm A	Farm D	All Farms
Constantly seronegative	0.0% (0/25)	19.6% (28/143)	10.6% (7/66)	12.5% (4/32)	16.3% (23/141)	15.2% (62/407)
Constantly seropositive	64.0% (16/25)	42.7% (61/143)	39.4% (26/66)	37.5% (12/32)	51.8% (73/141)	46.2% (188/407)
Seroconverted	20.0% (5/25)	14.0% (20/143)	21.2% (14/66)	31.3% (10/32)	23.4% (33/141)	20.1% (82/407)
Seroreverted	12.0% (3/25)	11.2% (16/143)	7.6% (5/66)	12.5% (4/32)	5.0% (7/141)	8.6% (35/407)
Intermittent presence of antibodies	4.0% (1/25)	12.6% (18/143)	21.2% (14/66)	6.3% (2/32)	3.5% (5/141)	9.8% (40/407)

Table S2. The frequencies of infection patterns per farm and breed.

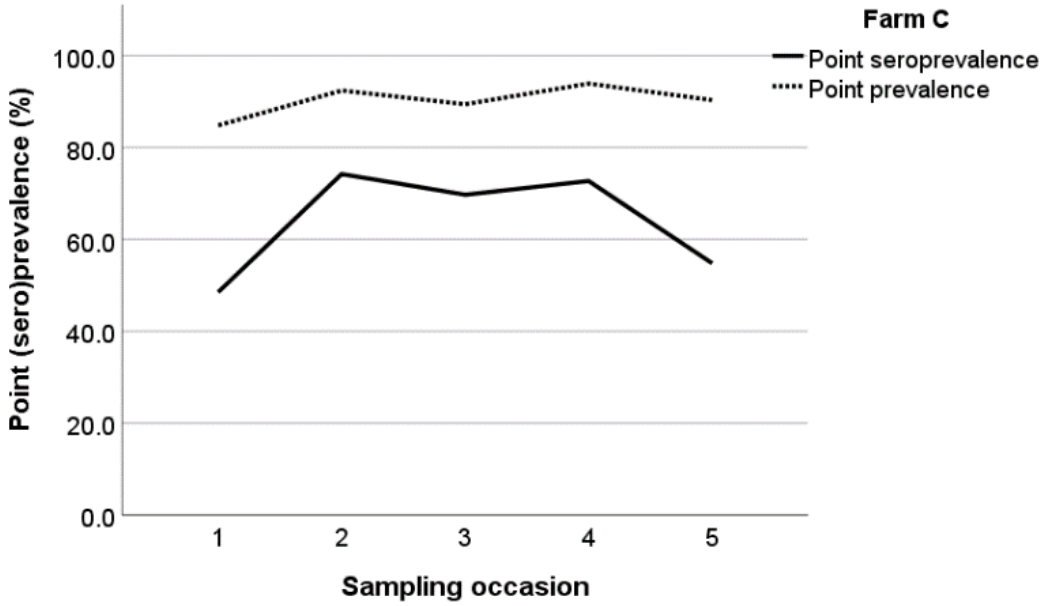
Infection Pattern	Chios			Lacaune		
	Farm A	Farm B	Farm C	Farm A	Farm D	All Farms
Uninfected	0.0% (0/25)	8.4% (12/143)	1.5% (1/66)	0.0% (0/32)	11.3% (16/141)	7.1% (29/407)
Infected seropositive	88.0% (22/25)	69.2% (99/143)	81.8% (54/66)	75.0% (24/32)	78.7% (111/141)	76.2% (310/407)
Infected seronegative	12.0% (3/25)	22.4% (32/143)	16.7% (11/66)	25.0% (8/32)	9.9% (14/141)	16.7% (68/407)


Farm A
Point seroprevalence
Point prevalence
Point (sero)prevalence (%)
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
5
Sampling occasion

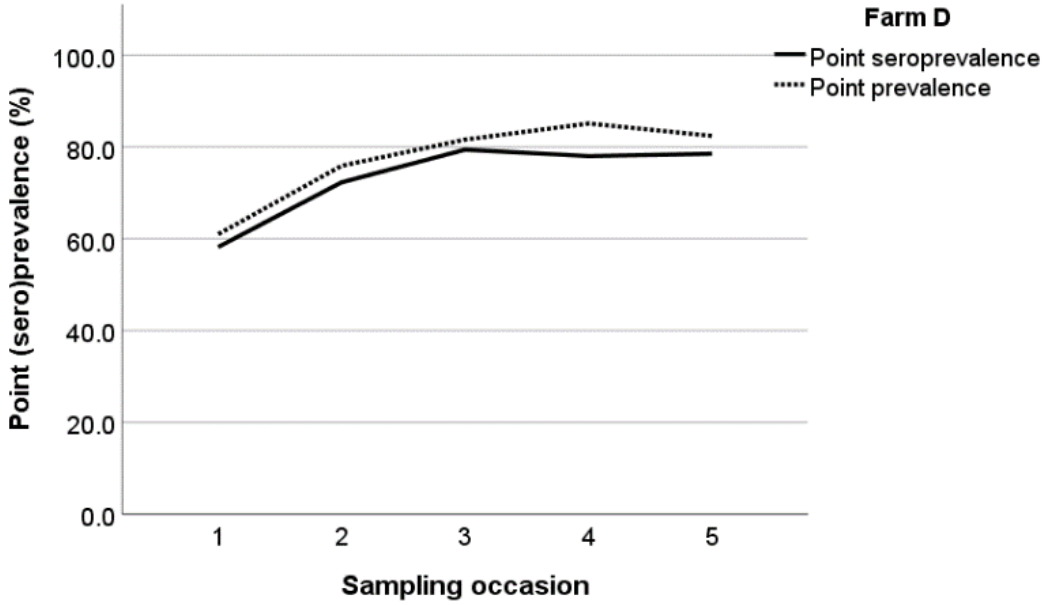
(a)


Farm B
Point seroprevalence
Point prevalence
Point (sero)prevalence (%)
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
5
Sampling occasion

(b)

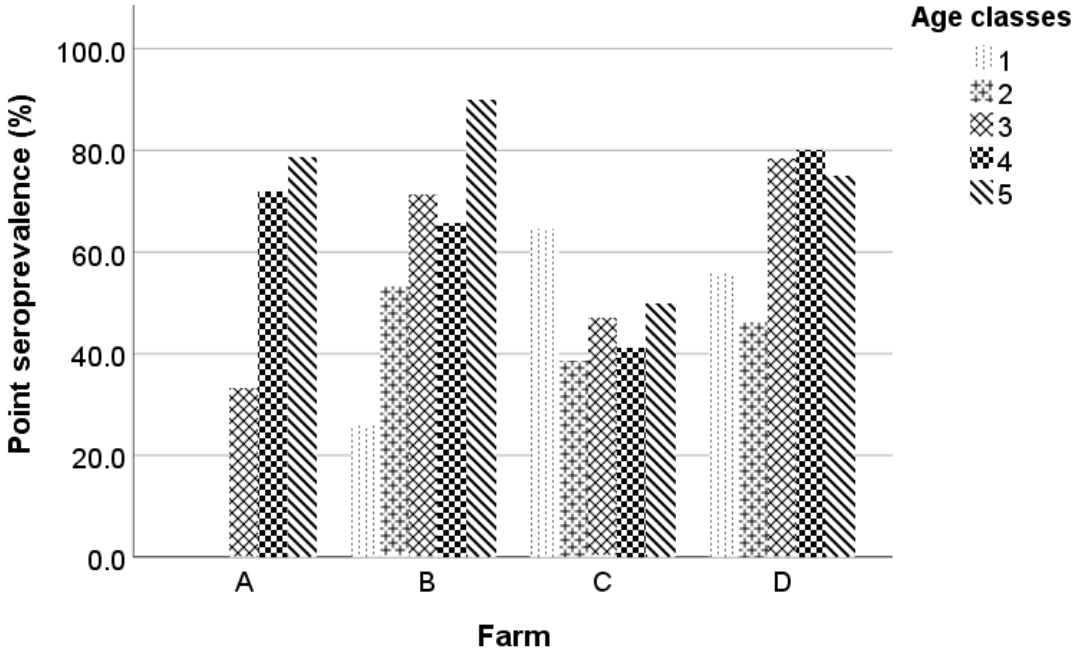


(c)

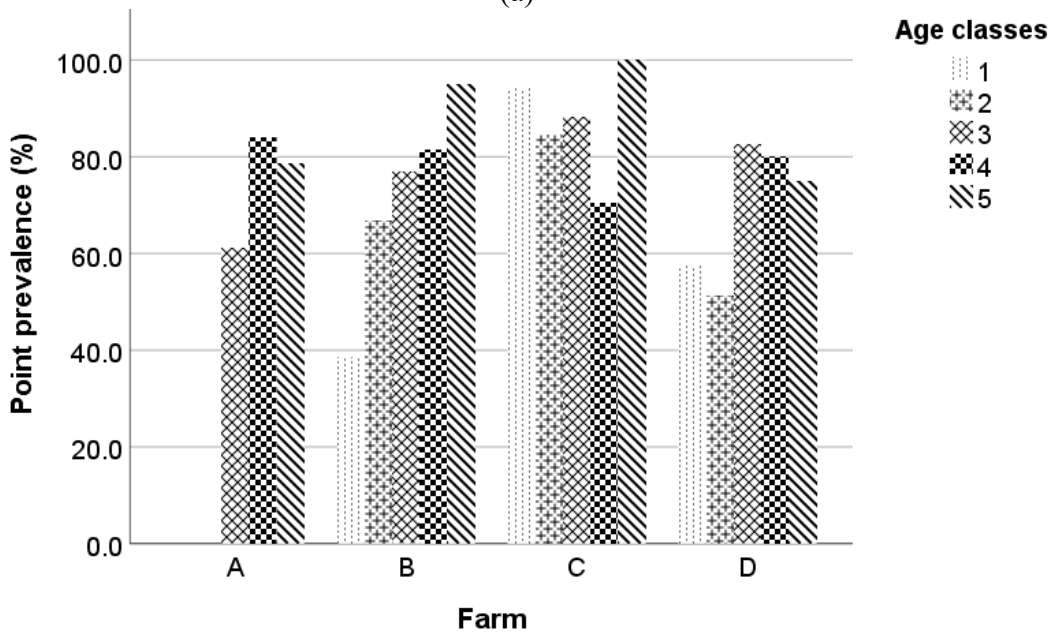


(d)

Figure S1. Point seroprevalence and prevalence in ewes from farms A (a), B (b), C (c), and D (d) in each sampling occasion during the study.

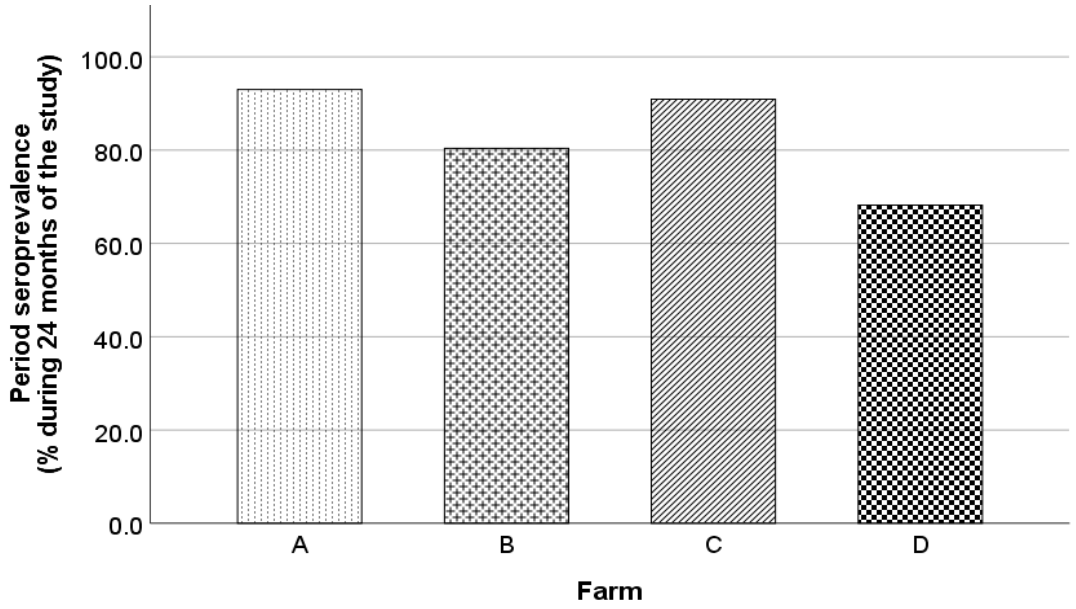


(a)

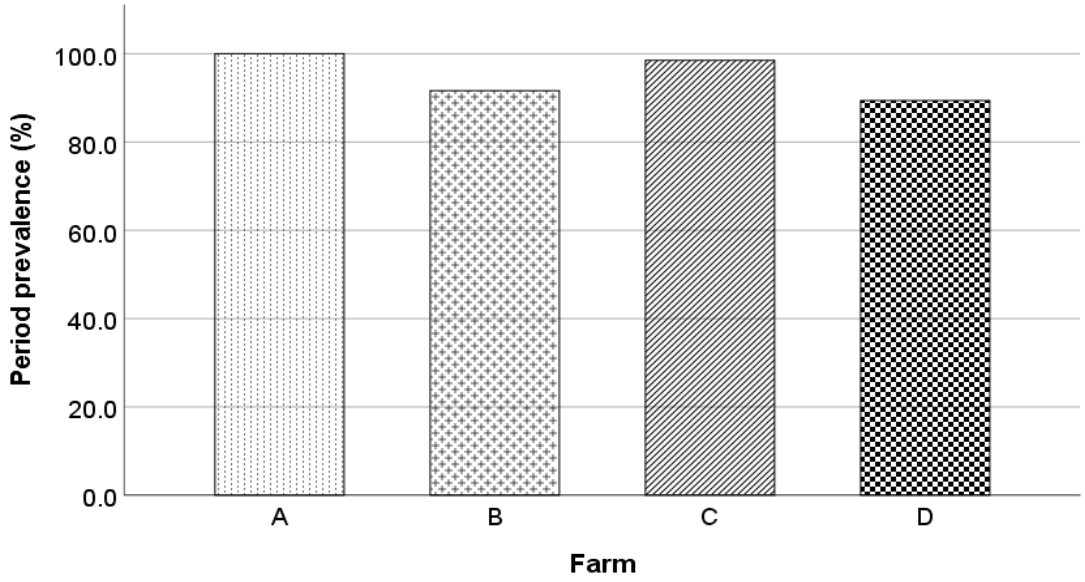


(b)

Figure S2. Seroprevalence (a) and prevalence (b) at the beginning of the study per age class and farm; the five age classes are: 1 ($x \leq 1$), 2 ($1 < x \leq 2$), 3 ($2 < x \leq 3$), 4 ($3 < x \leq 4$), and 5 ($x > 4$).

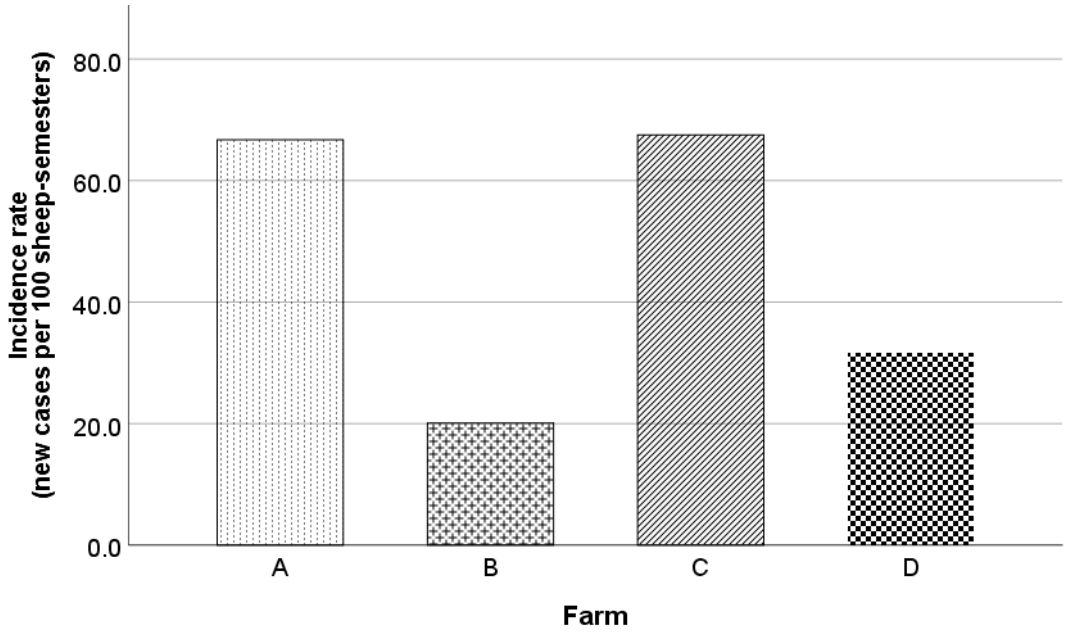


(a)

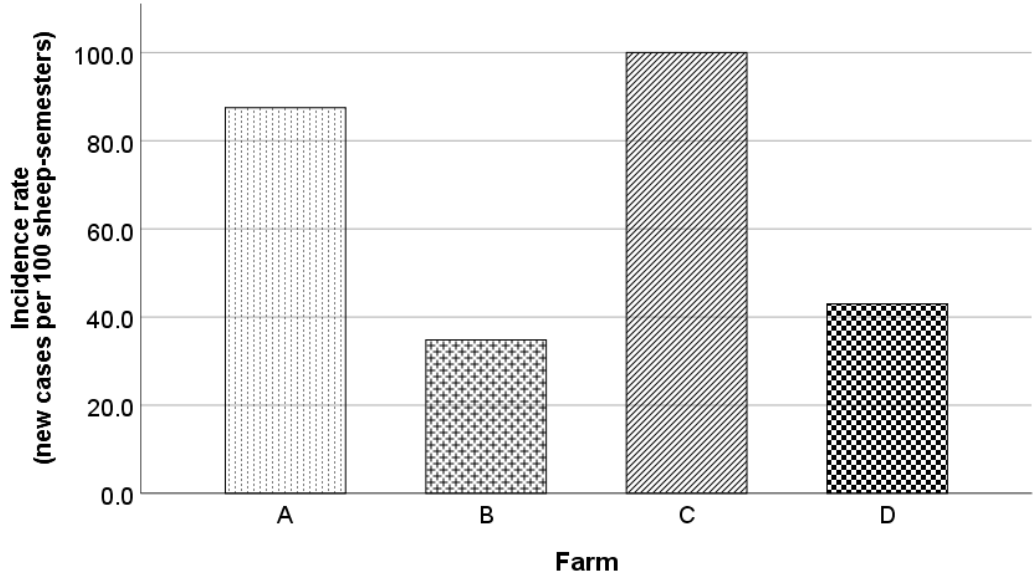


(b)

Figure S3. Period seroprevalence (a) and prevalence (b) for each farm during the study.

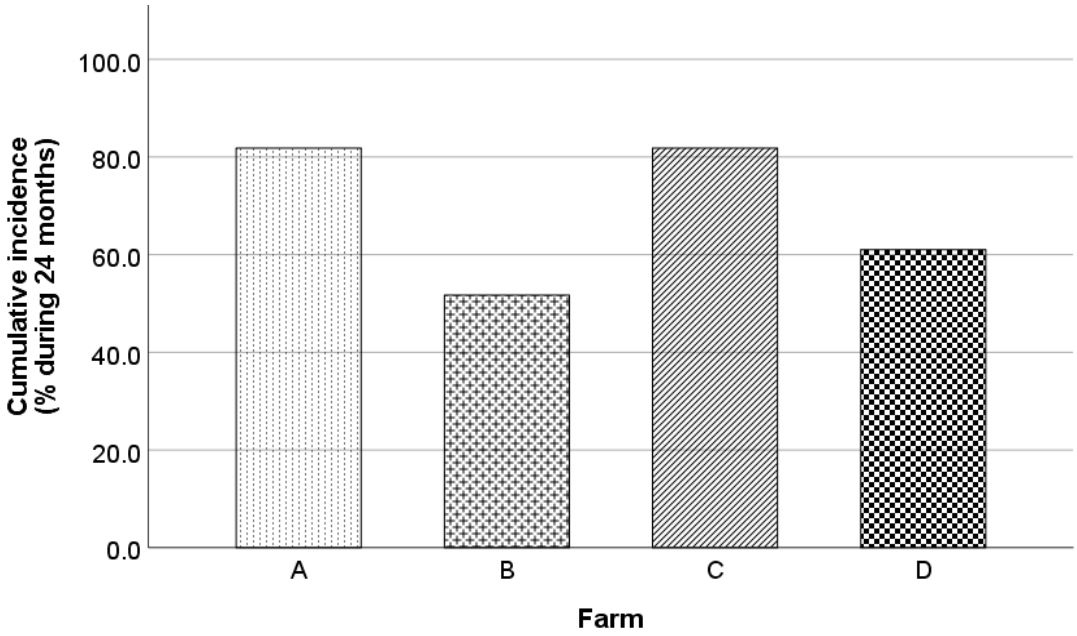


(a)

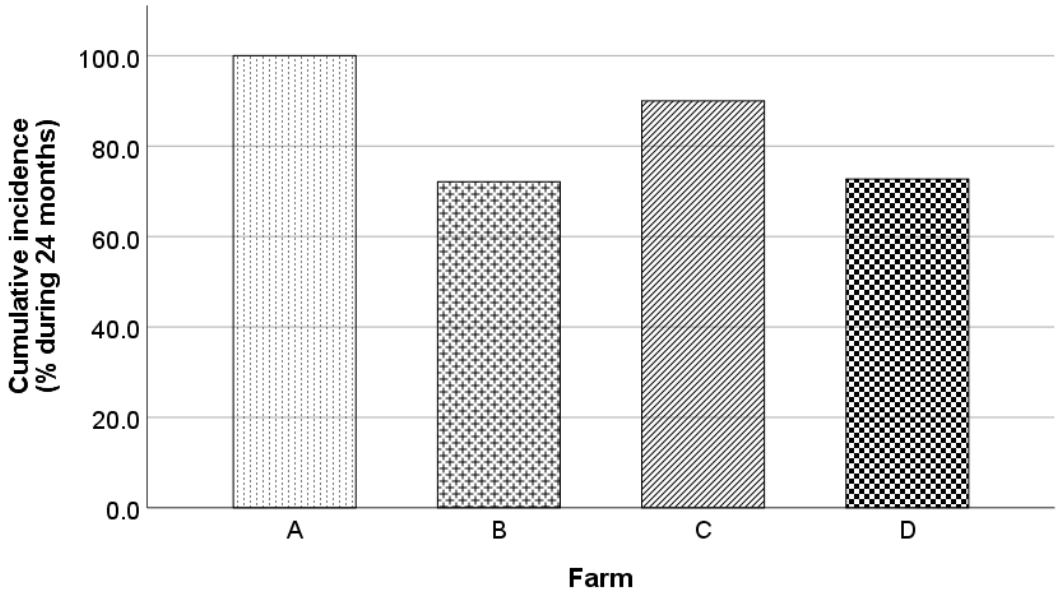


(b)

Figure S4. Incidence rate according to ELISA results (a) and the combination of ELISA and PCR results (b) for each farm during the study.



(a)



(b)

Figure S5. Cumulative incidence according to ELISA results (a) and the combination of ELISA and PCR results (b) for each farm during the study.

Appendix C: Statistical tables for the effects of small ruminant lentiviruses infections on productivity and health and welfare status.

Table S3. The effects of serological pattern, breed, sampling occasion, age, and BCS on daily milk yield (kg/ewe) during the study and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	**Upper Bound**
Breed	Chios	-0.73	0.07	<0.001	-0.87	-0.58
	Lacaune			Ref		
Sampling occasion	1	1.27	0.425	<0.001	1.19	1.36
	2	0.50	0.029	<0.001	0.45	0.56
	3			Ref		
Age	*	0.03	0.025	ns	-0.02	0.08
BCS	*	-0.41	0.109	<0.001	-0.63	-0.20
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.01	0.092	ns	-0.19	0.17
Constantly seronegative-Seroconverted		-0.11	0.099	ns	-0.30	0.09
Constantly seronegative-Seroreverted		0.18	0.183	ns	-0.18	0.54
Constantly seronegative-Intermittent presence of antibodies		-0.02	0.231	ns	-0.48	0.43
Constantly seropositive-Seroconverted		-0.10	0.082	ns	-0.26	0.06
Constantly seropositive-Seroreverted		0.18	0.176	ns	-0.16	0.53
Constantly seropositive-Intermittent presence of antibodies		-0.01	0.223	ns	-0.45	0.42
Seroconverted-Seroreverted		0.28	0.180	ns	-0.07	0.64
Seroconverted-Intermittent presence of antibodies		0.09	0.230	ns	-0.36	0.54
Seroreverted-Intermittent presence of antibodies		-0.20	0.274	ns	-0.74	0.34

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S4. The effects of serological pattern, breed, sampling occasion, age, and BCS on daily fat yield (g/ewe) during the study and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-38.50	3.429	<0.001	-45.23	-31.77
	Lacaune			Ref		
Sampling occasion	1	35.05	2.728	<0.001	29.69	40.40
	2	31.05	2.239	<0.001	26.66	35.44
	3			Ref		
Age	*	2.36	1.128	<0.05	0.14	4.57
BCS	*	-31.13	6.158	<0.001	-43.21	-19.05
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		0.20	4.343	ns	- 8.32	8.73
Constantly seronegative-Seroconverted		-3.51	4.737	ns	-12.80	5.79
Constantly seronegative-Seroreverted		15.50	7.825	<0.05	0.15	30.85
Constantly seronegative-Intermittent presence of antibodies		5.93	9.154	ns	-12.03	23.89
Constantly seropositive-Seroconverted		-3.71	3.883	ns	-11.33	3.91
Constantly seropositive-Seroreverted		15.29	7.489	<0.05	0.60	29.98
Constantly seropositive-Intermittent presence of antibodies		5.73	8.698	ns	-11.34	22.79
Seroconverted-Seroreverted		19.00	7.746	<0.05	3.81	34.20
Seroconverted-Intermittent presence of antibodies		9.44	9.053	ns	-8.32	27.20
Seroreverted-Intermittent presence of antibodies		-9.57	10.913	ns	-30.98	11.84

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S5. The effects of serological pattern, breed, sampling occasion, age, and BCS on daily protein yield (g/ewe) during the study and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-38.54	3.608	<0.001	-45.62	-31.47
	Lacaune			Ref		
Sampling occasion	1	61.41	2.196	<0.001	57.10	65.72
	2	25.01	1.525	<0.001	22.02	28.00
	3			Ref		
Age	*	2.70	1.219	<0.05	0.31	5.09
BCS	*	-26.61	5.646	<0.001	-37.69	-15.53
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-1.81	4.645	ns	-10.92	7.31
Constantly seronegative-Seroconverted		-6.76	4.962	ns	-16.50	2.97
Constantly seronegative-Seroreverted		7.75	9.242	ns	-10.38	25.88
Constantly seronegative-Intermittent presence of antibodies		0.30	11.495	ns	-22.25	22.85
Constantly seropositive-Seroconverted		-4.96	4.076	ns	-12.95	3.04
Constantly seropositive-Seroreverted		9.56	8.903	ns	-7.91	27.02
Constantly seropositive-Intermittent presence of antibodies		2.11	11.059	ns	-19.59	23.80
Seroconverted-Seroreverted		14.51	9.033	ns	-3.21	32.24
Seroconverted-Intermittent presence of antibodies		7.06	11.385	ns	-15.27	29.40
Seroreverted-Intermittent presence of antibodies		-7.45	13.653	ns	-34.24	19.33

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S6. The effects of serological pattern, breed, sampling occasion, age, and BCS on daily lactose yield (g/ewe) during the study and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-36.58	3.422	<0.001	-43.29	-29.86
	Lacaune			Ref		
Sampling occasion	1	58.24	2.083	<0.001	54.16	62.33
	2	23.72	1.446	<0.001	20.88	26.55
	3			Ref		
Age	*	2.55	1.159	<0.05	0.28	4.83
BCS	*	-25.13	5.369	<0.001	-35.66	-14.60
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-1.71	4.407	ns	-10.36	6.93
Constantly seronegative-Seroconverted		-6.42	4.708	ns	-15.65	2.82
Constantly seronegative-Seroreverted		7.34	8.771	ns	-9.87	24.55
Constantly seronegative-Intermittent presence of antibodies		0.26	10.903	ns	-21.13	21.65
Constantly seropositive-Seroconverted		-4.70	3.868	ns	-12.29	2.88
Constantly seropositive-Seroreverted		9.05	8.450	ns	-7.53	25.63
Constantly seropositive-Intermittent presence of antibodies		1.97	10.490	ns	-18.61	22.55
Seroconverted-Seroreverted		13.76	8.573	ns	-3.06	30.57
Seroconverted-Intermittent presence of antibodies		6.68	10.799	ns	-14.51	27.86
Seroreverted-Intermittent presence of antibodies		-7.08	12.953	ns	-32.49	18.33

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S7. The effects of serological pattern, breed, sampling occasion. age, and BCS on daily solids-not-fat yield (g/ewe) during the study and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-80.76	7.636	<0.001	-95.74	-65.78
	Lacaune			Ref		
Sampling occasion	1	130.03	4.634	<0.001	120.94	139.12
	2	53.04	3.217	<0.001	46.73	59.36
	3			Ref		
Age	*	5.56	2.585	<0.05	0.49	10.64
BCS	*	-56.26	11.902	<0.001	-79.60	-32.91
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-3.94	9.856	ns	-23.27	15.40
Constantly seronegative-Seroconverted		-13.96	10.516	ns	-34.59	6.67
Constantly seronegative-Seroreverted		16.94	19.575	ns	-21.46	55.35
Constantly seronegative-Intermittent presence of antibodies		1.08	24.378	ns	-46.75	48.91
Constantly seropositive-Seroconverted		-10.02	8.624	ns	-26.94	6.90
Constantly seropositive-Seroreverted		20.88	18.848	ns	-16.10	57.86
Constantly seropositive-Intermittent presence of antibodies		5.02	23.448	ns	-40.98	51.01
Seroconverted-Seroreverted		30.90	19.117	ns	-6.60	68.41
Seroconverted-Intermittent presence of antibodies		15.04	24.134	ns	-32.31	62.39
Seroreverted-Intermittent presence of antibodies		-15.86	28.929	ns	-72.62	40.89

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S8. The effects of serological pattern, breed, sampling occasion, age, and BCS on the logarithm of somatic cell counts (cells/mL) during the study and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.16	0.044	<0.001	0.07	0.24
	Lacaune			Ref		
Sampling occasion	1	-0.32	0.037	<0.001	-0.39	-0.25
	2	-0.08	0.033	<0.05	-0.14	-0.01
	3			Ref		
Age	*	-0.04	0.015	<0.05	-0.06	-0.01
BCS	*	0.06	0.084	ns	-0.10	0.22
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.02	0.053	ns	-0.13	0.08
Constantly seronegative-Seroconverted		0.07	0.065	ns	-0.06	0.20
Constantly seronegative-Seroreverted		0.18	0.106	ns	-0.03	0.39
Constantly seronegative-Intermittent presence of antibodies		0.13	0.160	ns	-0.19	0.44
Constantly seropositive-Seroconverted		0.09	0.057	ns	-0.02	0.20
Constantly seropositive-Seroreverted		0.20	0.102	ns	0.00	0.40
Constantly seropositive-Intermittent presence of antibodies		0.15	0.157	ns	-0.16	0.46
Seroconverted-Seroreverted		0.11	0.109	ns	-0.11	0.32
Seroconverted-Intermittent presence of antibodies		0.06	0.162	ns	-0.26	0.38
Seroreverted-Intermittent presence of antibodies		-0.05	0.182	ns	-0.41	0.31

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S9. The effects of infection pattern, breed, sampling occasion, age, and BCS on daily milk yield (kg/ewe) during the study and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-0.70	0.071	<0.001	-0.84	-0.56
	Lacaune			Ref		
Sampling occasion	1	1.26	0.043	<0.001	1.18	1.35
	2	0.50	0.029	<0.001	0.44	0.56
	3			Ref		
Age	*	0.03	0.024	ns	-0.02	0.07
BCS	*	-0.43	0.108	<0.01	-0.64	-0.22
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.06	0.082	ns	-0.01	0.22
Uninfected - Infected seronegative		0.30	0.134	<0.05	0.03	0.56
Infected seropositive - Infected seronegative		0.23	0.127	ns	-0.02	0.48

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S10. The effects of infection pattern, breed, sampling occasion, age, and BCS on daily fat yield (g/ewe) during the study and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-37.72	3.352	<0.001	-44.30	-31.15
	Lacaune			Ref		
Sampling occasion	1	34.82	2.736	<0.001	29.45	40.19
	2	30.83	2.251	<0.001	26.41	35.24
	3			Ref		
Age	*	2.19	1.113	<0.05	0.01	4.38
BCS	*	-31.59	6.141	<0.001	-43.64	-19.55
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		3.42	4.011	ns	-4.45	11.29
Uninfected - Infected seronegative		15.29	6.401	<0.05	2.73	27.85
Infected seropositive - Infected seronegative		11.88	5.986	<0.05	0.13	23.62

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S11. The effects of infection pattern, breed, sampling occasion, age, and BCS on daily protein yield (g/ewe) during the study and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-37.33	3.505	<0.001	-44.20	-30.45
	Lacaune			Ref		
Sampling occasion	1	60.98	2.204	<0.001	56.66	65.31
	2	24.76	1.538	<0.001	21.75	27.78
	3			Ref		
Age	*	2.41	1.171	<0.05	0.11	4.71
BCS	*	-27.44	5.621	<0.001	-38.46	-16.41
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		1.94	4.207	ns	-6.31	10.19
Uninfected - Infected seronegative		14.58	7.262	<0.05	0.34	28.83
Infected seropositive - Infected seronegative		12.64	6.666	ns	-0.04	25.72

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S12. The effects of infection pattern, breed, sampling occasion age, and BCS on daily lactose yield (g/ewe) during the study and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-35.42	3.325	<0.001	-41.95	-28.90
	Lacaune			Ref		
Sampling occasion	1	57.83	2.090	<0.001	53.73	61.94
	2	23.48	1.458	<0.001	20.63	26.34
	3			Ref		
Age	*	2.28	1.114	<0.05	0.09	4.46
BCS	*	-25.91	5.345	<0.001	-36.40	-15.43
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		1.85	3.99	ns	-5.98	9.67
Uninfected - Infected seronegative		13.84	6.89	<0.05	0.33	27.34
Infected seropositive - Infected seronegative		11.99	6.32	ns	-0.42	24.40

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S13. The effects of infection pattern, breed, sampling occasion, age, and BCS on daily solids-not-fat yield (g/ewe) during the study and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-78.17	7.418	<0.001	-92.73	-63.62
	Lacaune			Ref		
Sampling occasion	1	129.12	4.650	<0.001	119.99	138.24
	2	52.52	3.243	<0.001	46.16	58.88
	3			Ref		
Age	*	4.98	2.484	<0.05	0.10	9.85
BCS	*	-58.01	11.848	<0.001	-81.26	-34.77
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		4.28	8.938	ns	-13.26	21.81
Uninfected - Infected seronegative		31.35	15.402	<0.05	1.14	61.57
Infected seropositive - Infected seronegative		27.07	14.117	ns	-0.62	54.77

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S14. The effects of infection pattern, breed, sampling occasion, age, and BCS on the logarithm of somatic cell counts (cells/ml) during the study and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.16	0.045	<0.001	0.07	0.25
	Lacaune			Ref		
Sampling occasion	1	-0.32	0.037	<0.001	-0.39	-0.24
	2	-0.08	0.033	<0.05	-0.14	-0.01
	3			Ref		
Age	*	-0.03	0.015	<0.05	-0.06	-0.01
BCS	*	0.06	0.084	ns	-0.10	0.23
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.04	0.058	ns	-0.08	0.15
Uninfected - Infected seronegative		0.15	0.078	ns	-0.01	0.30
Infected seropositive - Infected seronegative		0.11	0.066	ns	-0.02	0.24

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S15. The effects of serological pattern, breed, and age on milk yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-40.02	22.665	ns	-84.55	4.52
	Lacaune			Ref		
Age	*	7.66	3.031	<0.05	1.71	13.62
Serological Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.07	10.654	ns	-21.00	20.87
Constantly seronegative-Seroconverted		-32.21	13.407	<0.05	-58.55	-5.86
Constantly seronegative-Seroreverted		7.69	21.103	ns	-33.77	49.16
Constantly seronegative-Intermittent presence of antibodies		-39.56	31.972	ns	-102.38	23.26
Constantly seropositive-Seroconverted		-32.14	11.665	<0.01	-55.06	-9.22
Constantly seropositive-Seroreverted		7.76	20.392	ns	-32.31	47.83
Constantly seropositive-Intermittent presence of antibodies		-39.49	31.262	ns	-100.92	21.93
Seroconverted-Seroreverted		39.90	21.895	0.06	-3.12	82.92
Seroconverted-Intermittent presence of antibodies		-7.35	32.328	ns	-70.87	56.17
Seroreverted-Intermittent presence of antibodies		-47.25	36.329	ns	-118.64	24.13

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S16. The effects of serological pattern, breed, and age on fat yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-3.83	1.30	<0.01	-6.38	-1.27
	Lacaune			Ref		
Age	*	0.54	0.186	<0.01	0.17	0.90
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.06	0.654	ns	-1.35	1.22
Constantly seronegative-Seroconverted		-1.93	0.824	<0.05	-3.54	-0.31
Constantly seronegative-Seroreverted		0.82	1.297	ns	-1.73	3.37
Constantly seronegative-Intermittent presence of antibodies		-2.21	1.965	ns	-6.08	1.65
Constantly seropositive-Seroconverted		-1.86	0.717	<0.05	-3.27	-0.45
Constantly seropositive-Seroreverted		0.88	1.253	ns	-1.58	3.34
Constantly seropositive-Intermittent presence of antibodies		-2.15	1.921	ns	-5.92	1.62
Seroconverted-Seroreverted		2.74	1.345	<0.05	0.10	5.39
Seroconverted-Intermittent presence of antibodies		-0.29	1.987	ns	-4.19	3.61
Seroreverted-Intermittent presence of antibodies		-3.03	2.232	ns	-7.42	1.36

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S17. The effects of serological pattern, breed, and age on protein yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-1.97	1.186	ns	-4.30	0.36
	Lacaune			Ref		
Age	*	0.41	0.158	<0.01	0.10	0.72
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.06	0.556	ns	-1.15	1.03
Constantly seronegative-Seroconverted		-1.61	0.699	<0.05	-2.99	-0.24
Constantly seronegative-Seroreverted		0.21	1.100	ns	-1.95	2.38
Constantly seronegative-Intermittent presence of antibodies		-2.03	1.667	ns	-5.31	1.24
Constantly seropositive-Seroconverted		-1.55	0.608	<0.05	-2.75	-0.36
Constantly seropositive-Seroreverted		0.27	1.063	ns	-1.82	2.36
Constantly seropositive-Intermittent presence of antibodies		-1.98	1.630	ns	-5.18	1.23
Seroconverted-Seroreverted		1.83	1.142	ns	-0.42	4.07
Seroconverted-Intermittent presence of antibodies		-0.42	1.686	ns	-3.73	2.89
Seroreverted-Intermittent presence of antibodies		-2.25	1.894	ns	-5.97	1.476

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S18. The effects of serological pattern, breed, and age on lactose yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-1.82	1.123	ns	-4.02	0.39
	Lacaune			Ref		
Age	*	0.37	0.150	<0.05	0.08	0.67
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		0.05	0.527	ns	-0.99	1.08
Constantly seronegative-Seroconverted		-1.51	0.663	<0.05	-2.82	-0.21
Constantly seronegative-Seroreverted		0.35	1.043	ns	-1.70	2.40
Constantly seronegative-Intermittent presence of antibodies		-1.82	1.580	ns	-4.92	1.29
Constantly seropositive-Seroconverted		-1.56	0.576	<0.01	-2.69	-0.43
Constantly seropositive-Seroreverted		0.31	1.008	ns	-1.68	2.29
Constantly seropositive-Intermittent presence of antibodies		-1.87	1.545	ns	-4.90	1.17
Seroconverted-Seroreverted		1.87	1.082	ns	-0.26	3.99
Seroconverted-Intermittent presence of antibodies		-0.30	1.598	ns	-3.44	2.84
Seroreverted-Intermittent presence of antibodies		-2.17	1.795	ns	-5.70	1.36

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S19. The effects of serological pattern, breed, and age on solids-not-fat yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-3.57	2.517	ns	-8.52	1.37
	Lacaune			Ref		
Age	*	0.81	0.335	<0.05	0.15	1.46
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.34	1.177	ns	-2.65	1.97
Constantly seronegative-Seroconverted		-3.34	1.481	<0.05	-6.25	-0.43
Constantly seronegative-Seroreverted		0.84	2.331	ns	-3.74	5.42
Constantly seronegative-Intermittent presence of antibodies		-4.31	3.531	ns	-11.24	2.63
Constantly seropositive-Seroconverted		-2.30	1.288	<0.05	-5.53	-0.47
Constantly seropositive-Seroreverted		1.18	2.252	ns	-3.25	5.60
Constantly seropositive-Intermittent presence of antibodies		-3.97	3.453	ns	-10.75	2.82
Seroconverted-Seroreverted		4.18	2.418	ns	-0.58	8.93
Seroconverted-Intermittent presence of antibodies		-0.97	3.571	ns	-7.98	6.05
Seroreverted-Intermittent presence of antibodies		-5.14	4.012	ns	-13.03	2.74

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S20. The effects of infection pattern, breed, and age on milk yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-42.82	22.364	ns	-86.76	1.13
	Lacaune			Ref		
Age	*	6.09	2.992	<0.05	0.21	11.96
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		8.49	12.007	ns	-15.11	32.08
Uninfected - Infected seronegative		43.43	16.497	<0.01	11.02	75.85
Infected seropositive - Infected seronegative		34.94	13.367	<0.01	8.68	61.21

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S21. The effects of infection pattern, breed, and age on fat yield (kg/ewe) for the first 120 days of the milking period and the pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-3.92	1.263	<0.01	-6.41	-1.44
	Lacaune			Ref		
Age	*	0.44	0.183	<0.05	0.08	0.81
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.46	0.737	ns	-0.99	1.91
Uninfected - Infected seronegative		2.71	1.013	<0.01	0.72	4.70
Infected seropositive - Infected seronegative		2.24	0.820	<0.01	0.63	3.85

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S22. The effects of infection pattern, breed, and age on protein yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-2.13	1.17	ns	-4.43	0.17
	Lacaune			Ref		
Age	*	0.34	0.156	<0.05	0.03	0.64
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.33	0.626	ns	-0.90	1.56
Uninfected - Infected seronegative		1.99	0.861	<0.05	0.30	3.68
Infected seropositive - Infected seronegative		1.66	0.697	<0.05	0.29	3.03

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S23. The effects of infection pattern, breed, and age lactose yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-1.98	1.109	ns	-4.15	0.20
	Lacaune			Ref		
Age	*	0.30	0.148	<0.05	0.01	0.59
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.40	0.594	ns	-0.77	1.57
Uninfected - Infected seronegative		1.96	0.816	<0.05	0.36	3.56
Infected seropositive - Infected seronegative		1.56	0.661	<0.05	0.26	2.86

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S24. The effects of infection pattern, breed, and age on solids-not-fat yield (kg/ewe) for the first 120 days of the milking period and pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-3.86	2.484	ns	-8.74	1.02
	Lacaune			Ref		
Age	*	0.66	0.330	<0.05	0.01	1.31
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.61	1.324	ns	-1.99	3.21
Uninfected - Infected seronegative		4.47	1.819	<0.05	0.90	8.05
Infected seropositive - Infected seronegative		3.86	1.474	<0.01	0.96	6.76

Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S25. Odds ratios of serological pattern, breed, and age regarding the occurrence of short lactation period (<7 months) and the pairwise comparisons between the serological patterns.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.101	1.11	ns	0.64	1.57
	Lacaune			Ref		
Age	*	-0.027	0.97	<0.05	0.95	1.00
Serological Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.013	0.98	ns	0.90	1.08
Constantly seronegative-Seroconverted		0.125	1.09	<0.05	1.02	1.27
Constantly seronegative-Seroreverted		0.006	1.01	ns	0.84	1.20
Constantly seronegative-Intermittent presence of antibodies		0.248	1.28	ns	0.98	1.67
Constantly seropositive-Seroconverted		0.138	1.15	<0.01	1.04	1.26
Constantly seropositive-Seroreverted		0.019	1.02	ns	0.86	1.21
Constantly seropositive-Intermittent presence of antibodies		0.260	1.30	ns	1.00	1.68
Seroconverted-Seroreverted		-0.119	0.89	ns	0.74	1.07
Seroconverted-Intermittent presence of antibodies		0.123	1.13	ns	0.86	1.48
Seroreverted-Intermittent presence of antibodies		0.242	1.27	ns	0.94	1.72

Cat: Category of the independent variable; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S26. Odds ratios of infection pattern, breed, and age regarding the occurrence of short lactation period (<7 months) and the pairwise comparisons between the infection patterns.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.086	1.09	ns	0.95	1.24
	Lacaune			Ref		
Age	*	-0.019	0.98	ns	0.96	1.01
Infection Pattern	-	-	-	<0.01	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		-0.074	0.93	ns	0.84	1.03
Uninfected - Infected seronegative		-0.229	0.80	0.001	0.69	0.91
Infected seropositive - Infected seronegative		-0.155	0.86	<0.01	0.77	0.96

Cat: Category of the independent variable; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S27. Odds ratio of serological status, breed, age, production stage and year of the study regarding the occurrence of nasal discharge during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	1.421	4.14	<0.001	1.97	8.72
	Lacaune			Ref		
Age	*	0.003	1.00	ns	0.80	1.26
BCS	*	2.453	11.62	ns	2.63	51.38
Production stage	Pre-mating	0.701	2.02	<0.05	1.16	3.49
	Pre-lambing			Ref		
Year of the study	1	-0.421	0.66	ns	0.21	2.03
	2	0.670	1.95	ns	0.77	4.95
	3			Ref		
Serological Status	Seronegative	-0.885	0.41	<0.05	0.18	0.94
	Seropositive			Ref		

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S28. Odds ratio of infection status, breed, age, production stage and year of the study regarding the occurrence of lameness during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-1.408	0.25	ns	0.05	1.20
	Lacaune			Ref		
Age	*	0.170	1.19	ns	0.76	1.85
BCS	*	-0.034	0.97	ns	0.03	37.70
Production stage	Pre-mating	1.289	3.63	ns	0.80	15.21
	Pre-lambing			Ref		
Year of the study	1	3.396	29.86	<0.05	2.25	396.93
	2	3.355	28.65	<0.05	1.81	454.75
	3			Ref		
Infection Status	-	-	-	-	ns	-
Pairwise comparisons						
Uninfected - Infected seropositive		-0.144	0.87	ns	0.04	18.85
Uninfected - Infected seronegative		-2.806	0.06	ns	0.00	2.41
Infected seropositive - Infected seronegative		-2.662	0.07	<0.05	0.01	0.66

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S29. Odds ratio of infection status, breed, age, production stage and year of the study regarding the occurrence of mastitis during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-0.284	0.75	ns	0.17	3.43
	Lacaune			Ref		
Age	*	0.258	1.30	ns	0.83	2.02
BCS	*	-3.689	0.03	<0.001	0.01	0.06
Production stage	Pre-mating	0.319	1.38	ns	0.94	2.01
	Pre- lambing			Ref		
Year of the study	1	-0.305	0.74	ns	0.27	2.00
	2	-0.102	0.90	ns	0.51	1.60
	3			Ref		
Infection Status	-	-	-	<0.001	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		-5.085	0.03	<0.001	0.01	0.04
Uninfected - Infected seronegative		-4.986	0.02	<0.001	0.01	0.05
Infected seropositive - Infected seronegative		0.099	1.10	ns	0.91	1.99

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S30. Odds ratio of infection status, breed, age, production stage and year of the study regarding the occurrence of udder skin lesions during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	2.737	15.44	<0.001	7.17	33.23
	Lacaune			Ref		
Age	*	0.430	1.54	<0.001	1.30	1.81
BCS	*	-1.930	0.15	0.001	0.05	0.46
Production stage	Pre-mating	1.792	6.00	<0.001	3.52	10.22
	Pre-lambing			Ref		
Year of the study	1	1.187	3.28	<0.01	1.48	7.26
	2	2.230	9.30	<0.001	4.53	19.11
	3			Ref		
Infection Status	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		-1.116	0.33	<0.01	0.16	0.69
Uninfected - Infected seronegative		-0.969	0.38	<0.05	0.15	0.94
Infected seropositive - Infected seronegative		0.148	0.86	ns	0.57	2.36

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S31. Odds ratios of infection status, breed, age, production stage and year of the study regarding the occurrence of wart-like lesions during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.332	1.39	ns	0.36	5.45
	Lacaune			Ref		
Age	*	0.781	2.18	<0.01	1.54	3.09
BCS	*	-0.857	0.42	ns	0.03	5.40
Production stage	Pre-mating	0.186	1.20	ns	0.44	3.29
	Pre-lambing			Ref		
Year of the study	1	-0.809	0.45	ns	0.07	3.07
	2	-0.083	0.92	ns	0.18	4.75
	3			Ref		
Infection Status	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		-2.400	0.09	ns	0.01	1.25
Uninfected - Infected seronegative		-3.057	0.05	<0.05	0.01	0.66
Infected seropositive - Infected seronegative		-0.657	0.52	ns	0.06	4.74

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; BCS: body condition score; *Continuous variable; Ref: Reference category; ns: not significant

Table S32. Odds ratios of serological patterns, breed and age regarding the occurrence of arthritis during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-0.555	0.57	ns	0.18	1.82
	Lacaune			Ref		
Age	*	0.084	1.09	ns	0.90	1.30
Serological Pattern	-	-	-	<0.01	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.503	0.60	ns	0.26	1.41
Constantly seronegative-Seroconverted		-0.064	0.94	ns	0.35	2.50
Constantly seronegative-Seroreverted		-1.534	0.22	<0.01	0.08	0.61
Constantly seronegative-Intermittent presence of antibodies		-1.192	0.30	<0.05	0.11	0.82
Constantly seropositive-Seroconverted		0.439	0.64	ns	0.73	3.28
Constantly seropositive-Seroreverted		-1.031	0.36	<0.05	0.16	0.81
Constantly seropositive-Intermittent presence of antibodies		-0.689	0.50	ns	0.23	1.11
Seroconverted-Seroreverted		-1.470	0.23	<0.01	0.09	0.60
Seroconverted-Intermittent presence of antibodies		-1.127	0.32	<0.05	0.08	0.82
Seroreverted-Intermittent presence of antibodies		0.343	0.71	ns	0.53	3.78

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S33. Odds ratio of serological patterns, breed and age regarding the occurrence of swollen supramammary lymph nodes during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.532	1.70	ns	0.67	4.34
	Lacaune			Ref		
Age	*	0.358	1.43	<0.001	1.20	1.70
Serological Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.329	0.72	ns	0.38	1.40
Constantly seronegative-Seroconverted		-0.161	0.85	ns	0.41	1.76
Constantly seronegative-Seroreverted		-1.515	0.22	0.001	0.09	0.55
Constantly seronegative-Intermittent presence of antibodies		-0.286	0.75	ns	0.32	1.77
Constantly seropositive-Seroconverted		0.168	1.18	ns	0.67	2.09
Constantly seropositive-Seroreverted		-1.186	0.31	<0.01	0.14	0.69
Constantly seropositive-Intermittent presence of antibodies		0.044	1.04	ns	0.50	2.19
Seroconverted-Seroreverted		-1.354	0.26	<0.01	0.11	0.62
Seroconverted-Intermittent presence of antibodies		-0.124	1.13	ns	0.39	2.00
Seroreverted-Intermittent presence of antibodies		1.230	3.42	<0.05	1.27	9.22

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S34. Odds ratio of infection patterns, breed and age regarding the occurrence of of arthritis during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-0.472	0.62	ns	0.20	1.94
	Lacaune			Ref		
Age	*	0.060	1.06	ns	0.89	1.27
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		-0.814	0.44	ns	0.15	1.35
Uninfected - Infected seronegative		-1.440	0.24	<0.05	0.07	0.80
Infected seropositive - Infected seronegative		-0.626	0.53	ns	0.28	1.03

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S35. Odds ratio of infection patterns, breed and age regarding the occurrence of swollen supramammary lymph nodes during the study.

Independent variables	Cat.	*β*	Odds ratio	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.529	1.70	ns	0.68	4.22
	Lacaune			Ref		
Age	*	0.356	1.43	<0.001	1.21	1.69
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		-0.374	0.69	ns	0.34	1.40
Uninfected - Infected seronegative		-1.102	0.33	<0.05	0.14	0.80
Infected seropositive - Infected seronegative		-0.728	0.48	<0.05	0.26	0.91

Cat: Category of the independent variable; ; *β*: Coefficient; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S36. The effects of infection status, breed, age, and BCS on white blood cell count (10^3/µl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$ Lower Bound	$CI_{95\%}$ Upper Bound
Breed	Chios	0.25	0.553	ns	-0.84	1.34
	Lacaune			Ref		
Age	*	-0.28	0.121	<0.05	-0.52	-0.05
BCS	*	-0.11	0.537	ns	-1.17	0.94
Infection Status	-	-	-	<0.01	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.89	0.429	<0.05	0.05	1.74
Uninfected - Infected seronegative		1.72	0.505	0.001	0.73	2.72
Infected seropositive - Infected seronegative		0.83	0.358	<0.05	0.13	1.53

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S37. The effects of infection status, breed, age, and BCS on granulocyte count (10^3/µl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$ Lower Bound	$CI_{95\%}$ Upper Bound
Breed	Chios	0.34	0.339	ns	-0.33	1.00
	Lacaune			Ref		
Age	*	-0.13	0.071	ns	-0.27	0.01
BCS	*	-0.73	0.311	<0.05	-1.340	-0.12
Infection Status	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.28	0.248	ns	-0.21	0.77
Uninfected - Infected seronegative		0.66	0.292	<0.05	0.08	1.23
Infected seropositive - Infected seronegative		0.38	0.207	ns	-0.03	0.78

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S38. The effects of infection status, breed, age, and BCS on lymphocyte count (10^3/µl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$ Lower Bound	$CI_{95\%}$ Upper Bound
Breed	Chios	-0.19	0.300	ns	-0.78	0.40
	Lacaune			Ref		
Age	*	-0.14	0.068	<0.05	-0.27	-0.01
BCS	*	0.63	0.307	<0.05	0.02	1.23
Infection Status	-	-	-	<0.01	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.54	0.245	<0.05	0.06	1.02
Uninfected - Infected seronegative		0.95	0.288	0.001	0.38	1.51
Infected seropositive - Infected seronegative		0.41	0.204	<0.05	0.01	0.81

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S39. The effects of infection status, breed, age, and BCS on monocyte count (10^3/µl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$ Lower Bound	$CI_{95\%}$ Upper Bound
Breed	Chios	0.08	0.046	ns	-0.01	0.17
	Lacaune			Ref		
Age	*	-0.02	0.009	<0.05	-0.04	-0.01
BCS	*	-0.01	0.039	ns	-0.09	0.07
Infection Status	-	-	-	<0.01	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.07	0.031	<0.05	0.01	0.13
Uninfected - Infected seronegative		0.13	0.037	0.001	0.05	0.20
Infected seropositive - Infected seronegative		0.06	0.026	<0.05	0.01	0.11

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S40. The effects of serological pattern, breed, age, and BCS on white blood cell count (10^3/µl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.36	0.582	ns	-0.78	1.51
	Lacaune			Ref		
Age	*	-0.26	0.126	<0.05	-0.50	-0.01
BCS	*	-0.09	0.546	ns	-1.16	0.99
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		0.45	0.421	ns	-0.38	1.28
Constantly seronegative-Seroconverted		0.66	0.465	ns	-0.26	1.57
Constantly seronegative-Seroreverted		1.33	0.574	<0.05	0.21	2.46
Constantly seronegative-Intermittent presence of antibodies		0.85	0.560	ns	-0.26	1.95
Constantly seropositive-Seroconverted		0.21	0.377	ns	-0.54	0.95
Constantly seropositive-Seroreverted		0.88	0.508	ns	-0.12	1.88
Constantly seropositive-Intermittent presence of antibodies		0.40	0.496	ns	-0.58	1.37
Seroconverted-Seroreverted		0.68	0.541	ns	-0.39	1.74
Seroconverted-Intermittent presence of antibodies		0.19	0.530	ns	-0.85	1.23
Seroreverted-Intermittent presence of antibodies		-0.49	0.620	ns	-1.71	0.73

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S41. The effects of serological pattern, breed, age, and BCS on lymphocyte count (10^3/μl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.01	0.341	ns	-0.66	0.68
	Lacaune			Ref		
Age	*	-0.12	0.071	ns	-0.26	0.02
BCS	*	0.64	0.311	<0.05	0.03	1.26
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		0.30	0.240	ns	-0.17	0.78
Constantly seronegative-Seroconverted		0.38	0.265	ns	-0.14	0.90
Constantly seronegative-Seroreverted		0.72	0.327	<0.05	0.08	1.36
Constantly seronegative-Intermittent presence of antibodies		0.49	0.319	ns	-0.14	1.12
Constantly seropositive-Seroconverted		0.07	0.215	ns	-0.35	0.50
Constantly seropositive-Seroreverted		0.42	0.289	ns	-0.15	0.98
Constantly seropositive-Intermittent presence of antibodies		0.19	0.283	ns	-0.37	0.74
Seroconverted-Seroreverted		0.34	0.308	ns	-0.27	0.95
Seroconverted-Intermittent presence of antibodies		0.11	0.302	ns	-0.48	0.71
Seroreverted-Intermittent presence of antibodies		-0.23	0.353	ns	-0.93	0.47

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S42. The effects of serological pattern, breed, age, and BCS on red blood cell count (10^6/µl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$ Lower Bound	$CI_{95\%}$ Upper Bound
Breed	Chios	-0.08	0.300	ns	-0.67	0.51
	Lacaune			Ref		
Age	*	-0.01	0.068	ns	-0.14	0.12
BCS	*	1.10	0.295	<0.001	0.52	1.68
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		0.04	0.228	ns	-0.41	0.48
Constantly seronegative-Seroconverted		0.37	0.251	ns	-0.13	0.86
Constantly seronegative-Seroreverted		-0.28	0.310	ns	-0.89	0.33
Constantly seronegative-Intermittent presence of antibodies		0.38	0.302	ns	-0.22	0.97
Constantly seropositive-Seroconverted		0.33	0.203	ns	-0.07	0.73
Constantly seropositive-Seroreverted		-0.31	0.274	ns	-0.85	0.23
Constantly seropositive-Intermittent presence of antibodies		0.34	0.268	ns	-0.19	0.87
Seroconverted-Seroreverted		-0.65	0.292	<0.05	-1.22	-0.07
Seroconverted-Intermittent presence of antibodies		0.01	0.286	ns	-0.56	0.57
Seroreverted-Intermittent presence of antibodies		0.65	0.335	ns	-0.01	1.31

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S43. The effects of serological pattern, breed, age, and BCS on hemoglobin concentration (g/dl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.34	0.293	ns	-0.23	0.92
	Lacaune			Ref		
Age	*	0.05	0.067	ns	-0.08	0.18
BCS	*	2.03	0.294	<0.001	1.45	2.61
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		0.10	0.227	ns	-0.35	0.54
Constantly seronegative-Seroconverted		0.48	0.251	ns	-0.02	0.97
Constantly seronegative-Seroreverted		-0.15	0.309	ns	-0.76	0.46
Constantly seronegative-Intermittent presence of antibodies		0.37	0.302	ns	-0.22	0.97
Constantly seropositive-Seroconverted		0.38	0.203	ns	-0.02	0.78
Constantly seropositive-Seroreverted		-0.25	0.273	ns	-0.78	0.29
Constantly seropositive-Intermittent presence of antibodies		0.28	0.267	ns	-0.25	0.80
Seroconverted-Seroreverted		-0.63	0.291	<0.05	-1.20	-0.05
Seroconverted-Intermittent presence of antibodies		-0.10	0.286	ns	-0.67	0.46
Seroreverted-Intermittent presence of antibodies		0.52	0.334	ns	-0.14	1.18

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S44. The effects of serological pattern, breed, age, and BCS on red cell distribution standard deviation (fL).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	1.79	0.638	<0.01	0.54	3.05
	Lacaune			Ref		
Age	*	-0.16	0.220	ns	-0.59	0.28
BCS	*	0.54	1.045	ns	-1.52	2.60
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.83	0.811	ns	-2.43	0.77
Constantly seronegative-Seroconverted		0.32	0.896	ns	-1.44	2.09
Constantly seronegative-Seroreverted		1.20	1.106	ns	-0.98	3.37
Constantly seronegative-Intermittent presence of antibodies		-1.23	1.082	ns	-3.36	0.90
Constantly seropositive-Seroconverted		1.15	0.721	ns	-0.27	2.57
Constantly seropositive-Seroreverted		2.03	0.972	<0.05	0.11	3.94
Constantly seropositive-Intermittent presence of antibodies		-0.40	0.953	ns	-2.27	1.48
Seroconverted-Seroreverted		0.87	1.044	ns	-1.18	2.93
Seroconverted-Intermittent presence of antibodies		-1.55	1.024	ns	-3.56	0.47
Seroreverted-Intermittent presence of antibodies		-2.42	1.198	<0.05	-4.78	-0.06

fL: femtoliter; BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S45. The effects of serological pattern, breed, age, and BCS on platelet distribution width.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.12	0.106	ns	-0.08	0.33
	Lacaune			Ref		
Age	*	0.04	0.021	<0.05	0.00	0.08
BCS	*	0.06	0.090	ns	-0.12	0.23
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		-0.09	0.069	ns	-0.22	0.05
Constantly seronegative-Seroconverted		-0.05	0.076	ns	-0.20	0.10
Constantly seronegative-Seroreverted		0.12	0.094	ns	-0.06	0.31
Constantly seronegative-Intermittent presence of antibodies		-0.04	0.092	ns	-0.22	0.14
Constantly seropositive-Seroconverted		0.04	0.062	ns	-0.09	0.16
Constantly seropositive-Seroreverted		0.21	0.083	<0.05	0.05	0.37
Constantly seropositive-Intermittent presence of antibodies		0.04	0.082	ns	-0.12	0.21
Seroconverted-Seroreverted		0.17	0.089	ns	0.00	0.35
Seroconverted-Intermittent presence of antibodies		0.01	0.087	ns	-0.16	0.18
Seroreverted-Intermittent presence of antibodies		-0.17	0.102	ns	-0.37	0.03

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S46. The effects of serological pattern, breed, age, and BCS on platelecrit (ml/l).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	-0.38	0.226	ns	-0.82	0.07
	Lacaune			Ref		
Age	*	-0.12	0.046	<0.01	-0.22	-0.03
BCS	*	-0.31	0.198	ns	-0.70	0.08
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		0.14	0.153	ns	-0.162	0.44
Constantly seronegative-Seroconverted		0.31	0.169	ns	-0.025	0.64
Constantly seronegative-Seroreverted		0.09	0.208	ns	-0.321	0.50
Constantly seronegative-Intermittent presence of antibodies		0.41	0.203	<0.05	0.010	0.81
Constantly seropositive-Seroconverted		0.17	0.137	ns	-0.102	0.44
Constantly seropositive-Seroreverted		-0.05	0.184	ns	-0.414	0.31
Constantly seropositive-Intermittent presence of antibodies		0.27	0.181	ns	-0.085	0.63
Seroconverted-Seroreverted		-0.22	0.196	ns	-0.605	0.17
Seroconverted-Intermittent presence of antibodies		0.10	0.192	ns	-0.276	0.48
Seroreverted-Intermittent presence of antibodies		0.32	0.225	ns	-0.121	0.77

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S47. The effects of serological pattern, breed, age, and BCS on platelet large cell count (10^9/l).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$ Lower Bound	$CI_{95\%}$ Upper Bound
Breed	Chios	-20.37	17.223	ns	-54.28	13.53
	Lacaune			Ref		
Age	*	-8.92	3.462	0.01	-15.74	-2.11
BCS	*	-15.42	15.003	ns	-44.96	14.12
Serological Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Constantly seronegative-Constantly seropositive		8.86	11.581	ns	-13.95	31.65
Constantly seronegative-Seroconverted		23.63	12.773	ns	-1.52	48.77
Constantly seronegative-Seroreverted		5.44	15.736	ns	-25.54	36.42
Constantly seronegative-Intermittent presence of antibodies		31.45	15.361	<0.05	1.21	61.69
Constantly seropositive-Seroconverted		14.77	10.368	ns	-5.64	35.18
Constantly seropositive-Seroreverted		-3.42	13.934	ns	-30.85	24.01
Constantly seropositive-Intermittent presence of antibodies		22.60	13.660	ns	-4.30	49.49
Seroconverted-Seroreverted		-18.19	14.838	ns	-47.40	11.02
Seroconverted-Intermittent presence of antibodies		7.83	14.547	ns	-20.81	36.46
Seroreverted-Intermittent presence of antibodies		26.01	17.018	ns	-7.49	59.52

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S48. The effects of infection pattern, breed, age, and BCS on monocyte count (10^3/µl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.08	0.047	ns	-0.01	0.18
	Lacaune			Ref		
Age	*	-0.02	0.009	ns	-0.03	0.01
BCS	*	-0.01	0.039	ns	-0.08	0.08
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.07	0.034	<0.05	0.01	0.14
Uninfected - Infected seronegative		0.10	0.042	<0.05	0.02	0.18
Infected seropositive - Infected seronegative		0.03	0.029	ns	-0.03	0.09

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S49. The effects of infection patterns, breed, age, and BCS on hemoglobin concentration (g/dl).

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.39	0.290	ns	-0.18	0.96
	Lacaune			Ref		
Age	*	0.09	0.064	ns	-0.04	0.22
BCS	*	2.08	0.293	<0.001	1.50	2.66
Infection Pattern	-	-	-	ns	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		0.11	0.254	ns	-0.39	0.61
Uninfected - Infected seronegative		-0.35	0.311	ns	-0.96	0.27
Infected seropositive - Infected seronegative		-0.46	0.216	<0.05	-0.89	-0.03

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Table S50. The effects of infection patterns, breed, age, and BCS on platelet distribution width.

Independent variables	Cat.	*β*	SE	*P*	$CI_{95\%}$	
					Lower Bound	Upper Bound
Breed	Chios	0.12	0.105	ns	-0.09	0.33
	Lacaune			Ref		
Age	*	0.04	0.020	<0.05	0.01	0.08
BCS	*	0.06	0.089	ns	-0.11	0.24
Infection Pattern	-	-	-	<0.05	-	-
Pairwise comparisons						
Uninfected - Infected seropositive		-0.04	0.077	ns	-0.19	0.11
Uninfected - Infected seronegative		0.13	0.095	ns	-0.06	0.32
Infected seropositive - Infected seronegative		0.17	0.066	<0.05	0.04	0.30

BCS: body condition score; Cat: Category of the independent variable; *β*: Coefficient; SE: Standard error; $CI_{95\%}$: 95% confidence interval; *Continuous variable; Ref: Reference category; ns: not significant

Appendix D: Photos from the field and the laboratory research.

Photo 1. The studied animals on farm D.

Photo 2. The studied animals on farm C.

Photo 3. The studied animals on farm B.

Photo 4. Physical examination of the studied animals on farm A.

Photo 5. The shed for artificial lamb rearing on farm C.

Photo 6. The milk machine for artificially lamb rearing on farm C.

Photo 7. Milk measurement and milk sampling on farm C.

Photo 8. The shed for artificial lamb rearing on farm B.

Photo 9. Equipment used for the pasteurization of bulk milk colostrum on farm C.

Photo 10. Individual colostrum pasteurization from ewes with known infection status on farm D.

Photo 11. Physical examination of the studied animals on Farm B.

Photo 12. Milk measurement and milk sampling on farm B.

(a)

(b)

Photo 13. Blood sampling (a) and blood samples stored and transferred under refrigeration (b) on farm A.

Photo 14. Chemical milk analysis in milk samples from the studied animals.

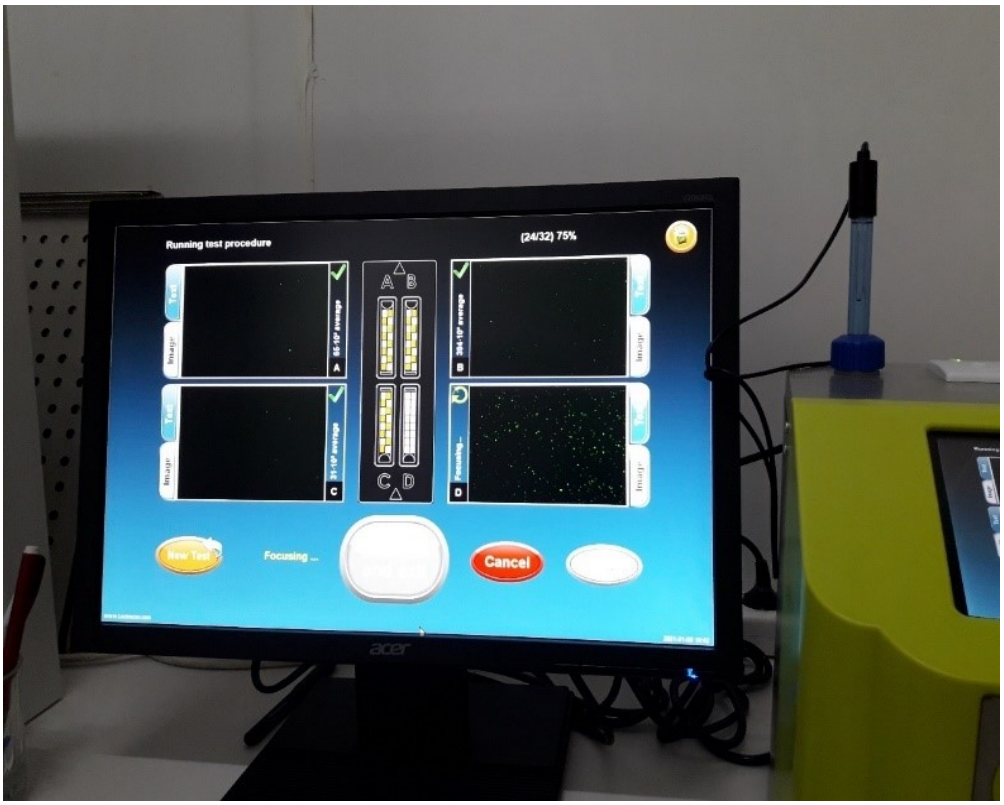


Photo 15. Measurement of somatic cell counts in milk samples from the studied animals.

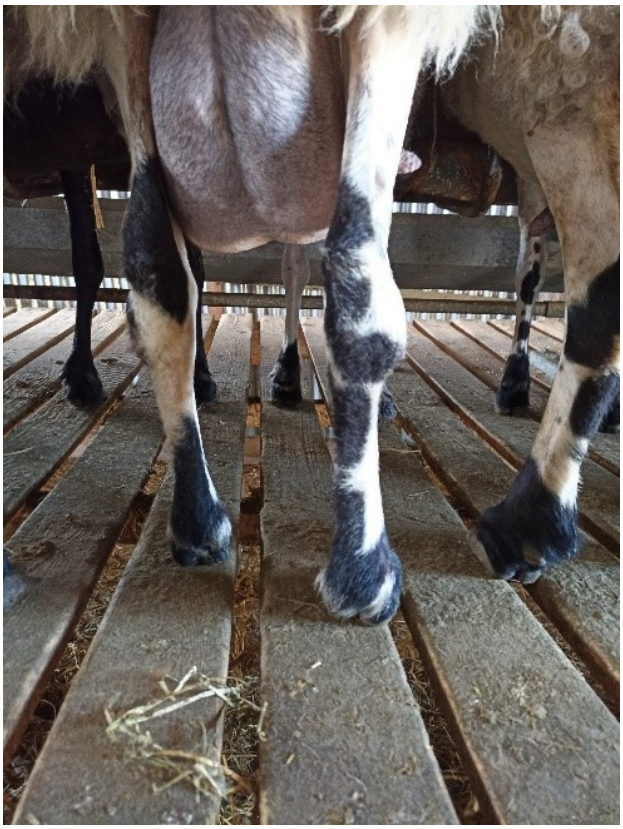


Photo 16. Occurrence of arthritis in a SRLV infected ewe of the study.

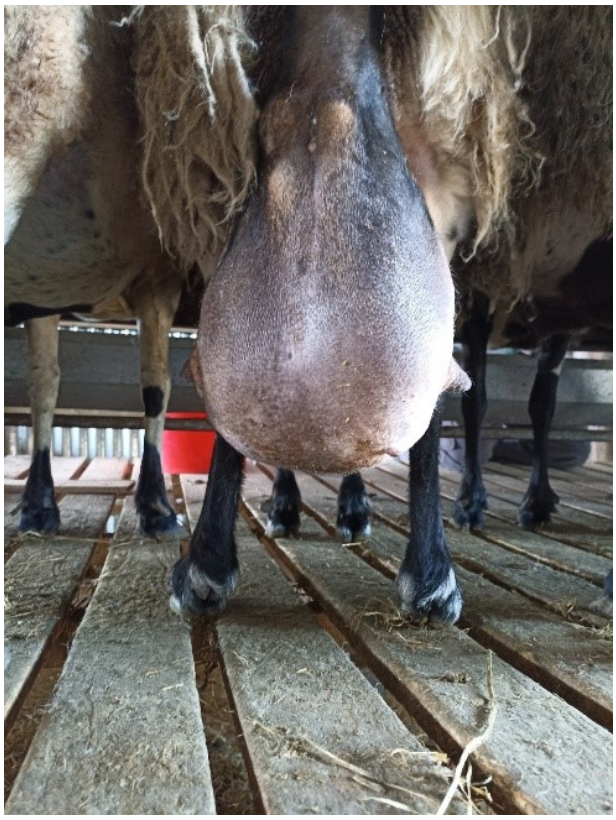


Photo 17. The “hard udder” syndrome in a SRLV infected ewe of the study.

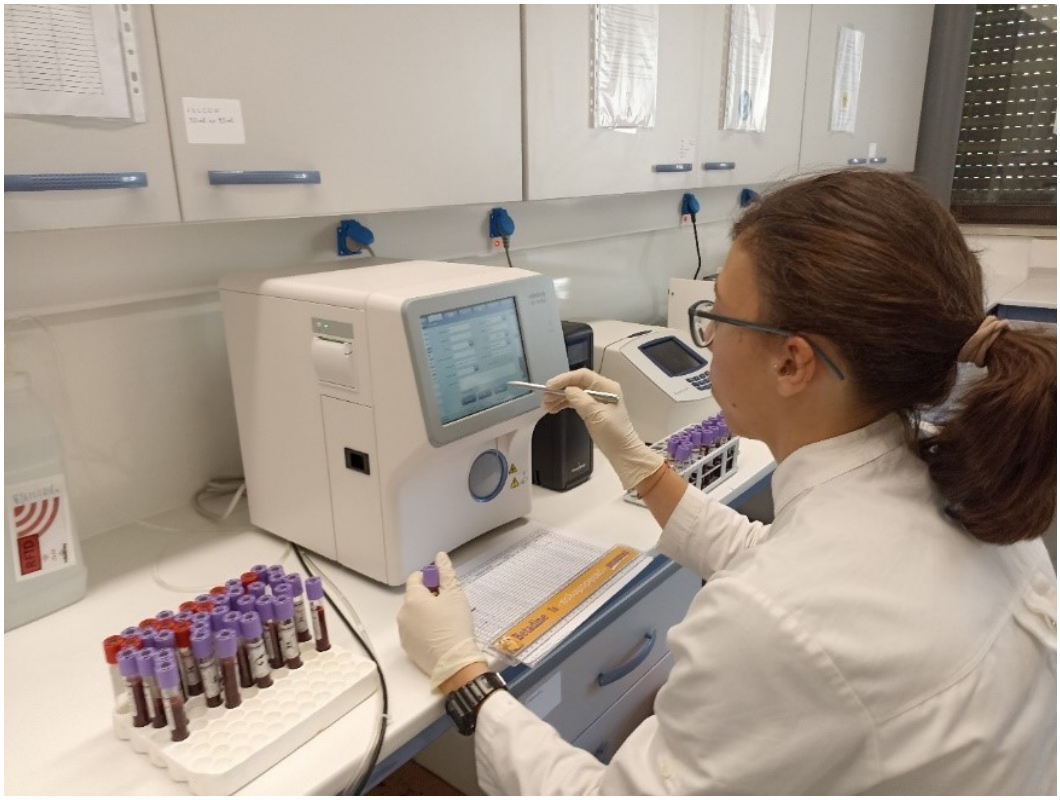


Photo 18. Hematological analysis in blood samples from the studied animals.

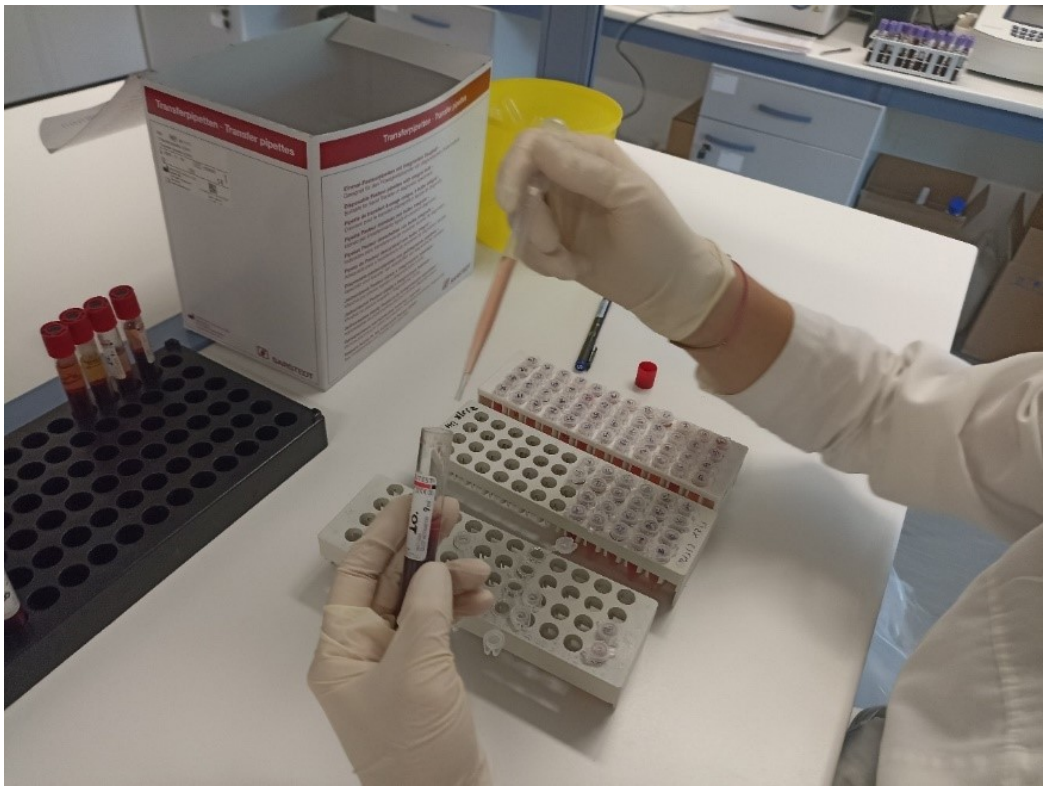


Photo 19. Serum extraction for ELISA testing in blood samples.

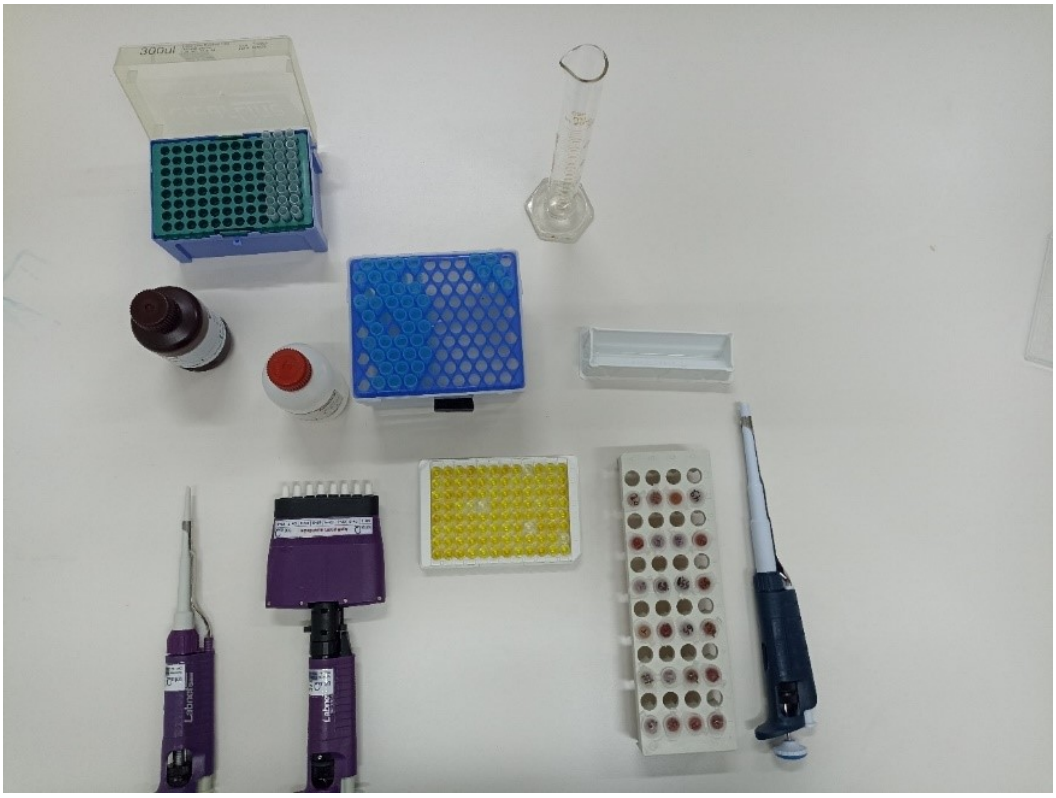
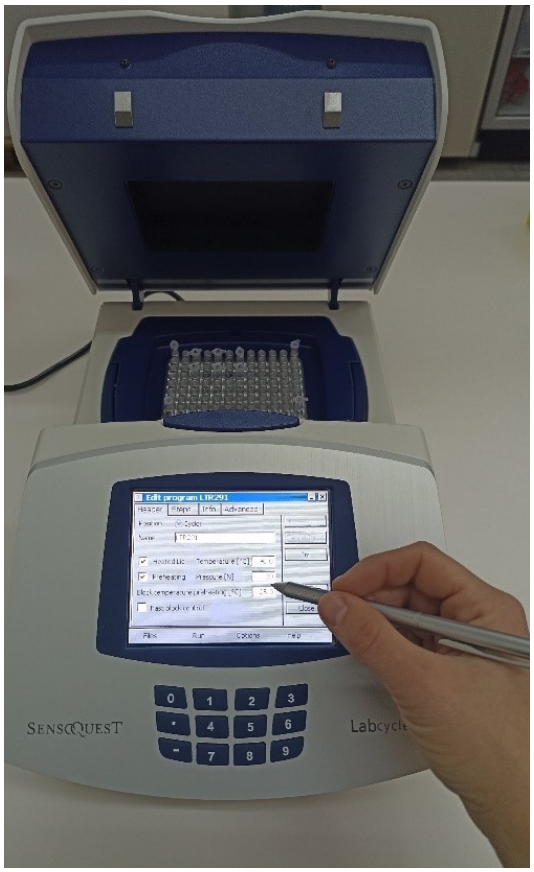
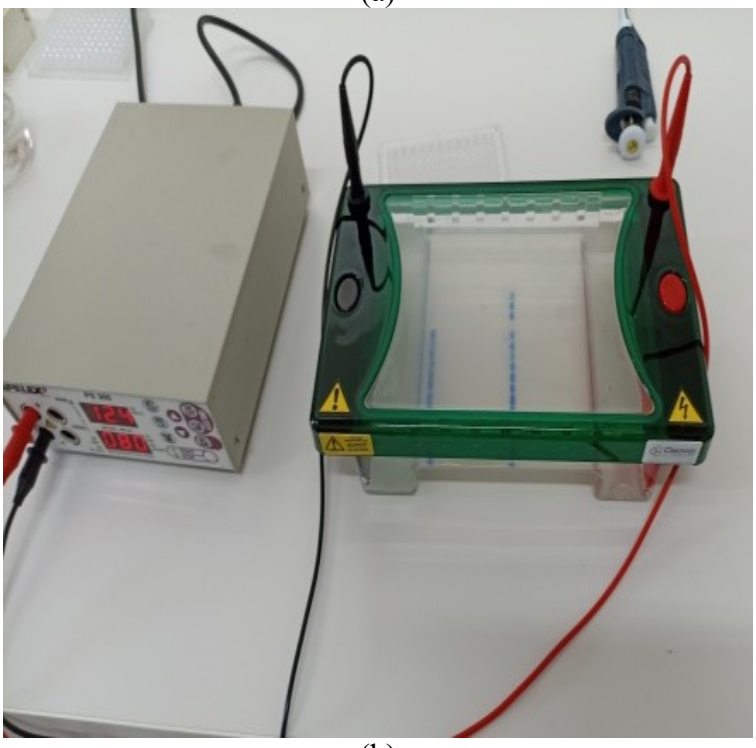


Photo 20. Serological diagnosis with ELISA test in serum samples from the studied animals.



(a)



(b)

Photo 21. Molecular diagnosis of the studied animals with conventional PCR assays (a) and gel electropheresis of PCR products (b).

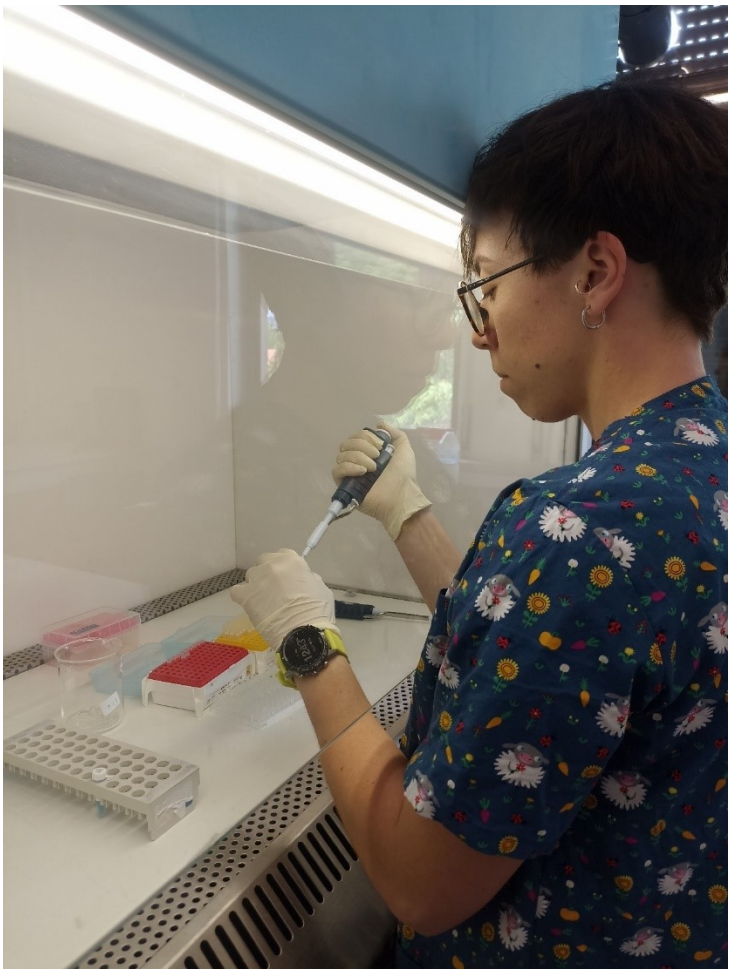


Photo 22. The real-time PCR workstation for the processing and preparation of real-time PCR reactions in the DNA samples from the studied animals.