Diagnostic response to a cross-border challenge for the Swiss caprine arthritis encephalitis virus eradication program

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Summary

Animal trading between countries with different small ruminant lentivirus infectious status is a potential danger for the reintroduction of eradicated genotypes. This was the case in 2017 with the importation of a large flock of seropositive goats into Switzerland. The handling of this case permitted us to test the preventive measures in place. The coordination between the local veterinarian and the cantonal and federal veterinary authorities worked efficiently and rapidly involved the national reference center in the investigations. This case posed a challenge for the reference center and enabled scrutiny of the applied diagnostic tests. ELISA and western blot provided consistent results and pointed to an unusually high infection rate in the flock. This was confirmed by the isolation of several viruses from different organs and cells, demonstrating that the spleen is particularly well suited for isolation of small ruminant lentiviruses. The SU5-ELISA, designed to predict the subtype of the infecting virus, correctly pointed to a B1 subtype as the infectious agent. We confirmed that with this test it is necessary to analyze a representative number of samples from a flock and not just individual sera to obtain reliable results. This analysis permitted us to identify particular amino acid residues in the SU5 peptides that may be crucial in determining the subtype specificity of antibody binding. Different gag-pol and env regions were amplified by PCR using primers designed for this purpose. The phylogenetic analysis revealed a surprisingly high heterogeneity of the sequences, pointing to multiple infections within single animals and the entire flock.

In conclusion, this case showed that the defense of the CAEV negative status of the Swiss goat population with

Kontrolltechnische Herausforderungen und diagnostische Massnahmen zum Erhalt des Caprinen-Arthritis-Enzephalitis-Virus freien Status in der Schweiz

Der Tierhandel mit kleinen Wiederkäuern zwischen Ländern mit unterschiedlichem Lentivirus-Status ist eine potenzielle Gefahr für die Wiedereinführung von ausgerotteten Virus Genotypen. Im Jahre 2017 wurden mehrere Ziegen in die Schweiz eingeführt, die seropositiv auf das Caprine Arthritis-Encephalitis Virus (CAEV) testeten. Dieser Fall erprobte die vorbeugenden Maßnahmen.

Im vorliegenden Fall funktionierte die Koordinierung zwischen dem betreuenden Tierarzt und den kantonalen und bundesstaatlichen Veterinärbehörden effizient und das nationale Referenzzentrum wurde rasch in die Ermittlungen involviert. Die Umsetzung der präventiven Massnahmen stellten das Referenzzentrum vor eine Herausforderung, aber ermöglichte auch die Überprüfung der angewandten Diagnosetests. ELISA und Western Blot auf CAEV lieferten konsistente Ergebnisse und wiesen auf eine ungewöhnlich hohe Infektionsrate in der Herde hin. Dies wurde durch die Isolierung mehrerer Viren aus verschiedenen Organen und Zellen bestätigt. Es zeigte sich, dass die Milz sich besonders gut für die Isolierung von kleinen Wiederkäuer-Lentiviren eignet. Der SU5-ELISA, der zur Vorhersage des Subtyps des infizierenden Virus entworfen wurde, wies korrekterweise auf einen B1-Subtyp als Infektionserreger hin. Es zeigte sich, dass bei diesem Test eine repräsentative Anzahl von Proben aus einer Herde und nicht nur einzelne Seren analysiert werden müssen, um zuverlässige Ergebnisse zu erhalten. Diese Analyse erlaubte es be-

respect to the virulent, prototypic B1 subtype of small ruminant lentiviruses, requires, among other measures, a diagnostic facility capable of performing a thorough analysis of the collected samples.

Keywords: small ruminant lentiviruses, SRLV, CAEV, diagnostic, phylogenetic analysis, outbreak.

stimmte Aminosäurereste in den SU5-Peptiden zu identifizieren, die für die Bestimmung der Subtypspezifität der Antikörperbindung entscheidend sein können. Unter Verwendung von für diesen Zweck entwickelten Primern konnten verschiedene gag-pol- und env-Regionen durch eine PCR amplifiziert. Die phylogenetische Analyse zeigte eine überraschend hohe Heterogenität der Sequenzen, was auf mehrere Infektionen innerhalb einzelner Tiere und der gesamten Herde hinweist.

Zusammenfassend zeigte dieser Fall, dass der Erhalt des CAEV – Negativstatus der Schweizer Ziegenpopulation in Bezug auf den virulenten, prototypischen B1 – Subtyp von kleinen Wiederkäuer – Lentiviren neben anderen Massnahmen eine diagnostische Einrichtung erfordert, die eine gründliche Analyse der Proben ermöglicht.

Schlüsselwörter: kleine Wiederkäuer-Lentiviren, SRLV, CAEV, Diagnostik, phylogenetische Analyse, Ausbruch

Introduction

Small ruminant lentiviruses (SRLV) induce chronic debilitating disease in goats and sheep, affecting different organ systems, such as the mammary gland, joints, the lungs and the central nervous system[4,6,17]. SRLV infections have a negative impact on the productivity of the infected animals[3,10,19-21]. In the eighties, Switzerland started an eradication campaign focused exclusively on goats and the caprine arthritis encephalitis virus (CAEV). At that time CAEV was considered a species-specific pathogen of goats. In the following years, this alleged species specificity of CAEV for goats and Visna-maedi virus (VMV) for sheep was disproved by several observations, indicating that these viruses are not distinct species of the genus lentivirus but represent a phylogenetic continuum in the same species[14]. This led to the current SRLV terminology, which as yet has not been adopted by the official taxonomy of the International Committee on Taxonomy of Viruses (ICTV). Currently, SRLV are classified in 5 genotypes (A to E), further subdivided into subtypes, such as A1 to A15, with A1 corresponding to the prototypic VMV strain, B1 to B3, with B1 encompassing the classical CAEV isolates, and E1 to E2[17,30,38]. Genotype E is still considered a species-specific pathogen of goats, while several subtypes of the A and B genotypes have been isolated from goats and sheep[16,27,28]. The demonstrated capacity of SRLV to circumvent the species barrier between goats and sheep represents a potential danger for the Swiss eradication campaign that had not included sheep in the surveillance measures. The current situation in Switzerland can be summarized as follow; the classical CAEV strains (SRLV B1) were successfully eradicated and the Swiss goat population is free of SRLV B1 (communication of the Swiss Federal Food Safety and Veterinary Office, FSVO). In the last 3 years, with the exception of 3 goats (one illegally imported animal, a lost, wandering young animal that joined a flock and an epidemiologically isolated goat), no SRLV B1 seropositive goats have been reported. These three animals were immediately euthanized. Chronic carpitis, the classical hallmark of SRLV B1-induced arthritis, is no longer observed in Swiss goats. In contrast, the general census performed on 85,454 goats between 2011 and 2012 revealed that 0.55% of goats were serologically positive for SRLV A infections[35]. One of the subtypes involved, SRLV A4, was shown to have a marked tropism for the mammary gland and its potential spread in the goat population should be carefully monitored to avoid jeopardizing the successful eradication campaign[8]. The other important threat to the SRLV B1-free status of Switzerland is the introduction of SRLV-infected animals, particularly goats infected with the virulent B1 subtype. To counteract this threat, the national reference center for SRLV is constantly improving its diagnostic tools. As we describe in this publication, this ensured the efficient serological detection of SRLV infected animals in a recently imported flock and the characterization of several SRLV B1 isolates infecting these animals.

Materials and Methods

Animals, animal movements and importation procedures

On the 26th of December 2016, 111 pregnant goats (36 months old) were imported from Belgium to Switzerland, to a newly established goat farm in the Canton of Jura. The health status of the animals was certified

by a TRACES document. In this certificate, the signing official veterinarian declared that to his and the owner knowledge no clinical cases of CAE were observed in the flock for the last 3 years and that the goats did not have contact with infected animals. Alternatively, if clinically affected animals were present in the flock, these goats were euthanized and all the remaining animals tested negative for CAEV in two serological tests performed at least a year after the event.

By the 12th of January 2017, 13 animals were dead from an alleged pregnancy toxemia, at which time a veterinarian visited the premise and examined the imported goats. The veterinarian noticed that about 10% of the goats were coughing and three showed overt symptoms of clinical arthritis. The serological analysis of two of these three goats revealed an SRLV infection. As required by the rules of the Swiss national CAE control program, the flock was placed under quarantine on January 20th 2017 by the veterinary authorities and serum samples taken from all goats. On February 10th 2017, all 98 goats and 97 kids, born after the importation, were slaughtered and the spleen and carpi of two animals showing enlarged carpal joints were sent to the national reference center for SRLV.

Serology

All sera were tested with the CAEV/MVV Total Ab (Idexx Switzerland AG, Liebefeld-Bern, Switzerland), which is the routinely applied screening test used by the national reference center for SRLV and has a reported sensitivity of 98.6% and a specificity of 99.3%[39]. Sera were tested and scored according to the manufacturer's instructions.

Sample to positive (SP) % values were calculated and interpreted as follow:
sera with SP% values below 30 were considered negative, sera with SP% values between 30 and 40 doubtful, and sera with SP% values above 40 were classified as positive. The mean SP% values are reported in the text with the corresponding standard deviations (SD).

A selection of 12 sera were confirmed by western blot (WB) as previously described[37]. We randomly selected 8 sera and added the sera of goats #120, #122, #123 and #155, that were positive in PCR, providing sequence information on the infecting viruses. A weakly positive serum and a negative control serum were applied to permit direct visual comparison of test sera and control sera (Fig. 2).

The same panel of sera was tested with the in-house SU5-ELISA performed as previously described[18]. Serum #185 was no longer available and was randomly substituted by two sera #128 and #195. Sera were incubated on ELISA plates coated with peptides corresponding to the immunodominant SU5 region of the envelope gly-

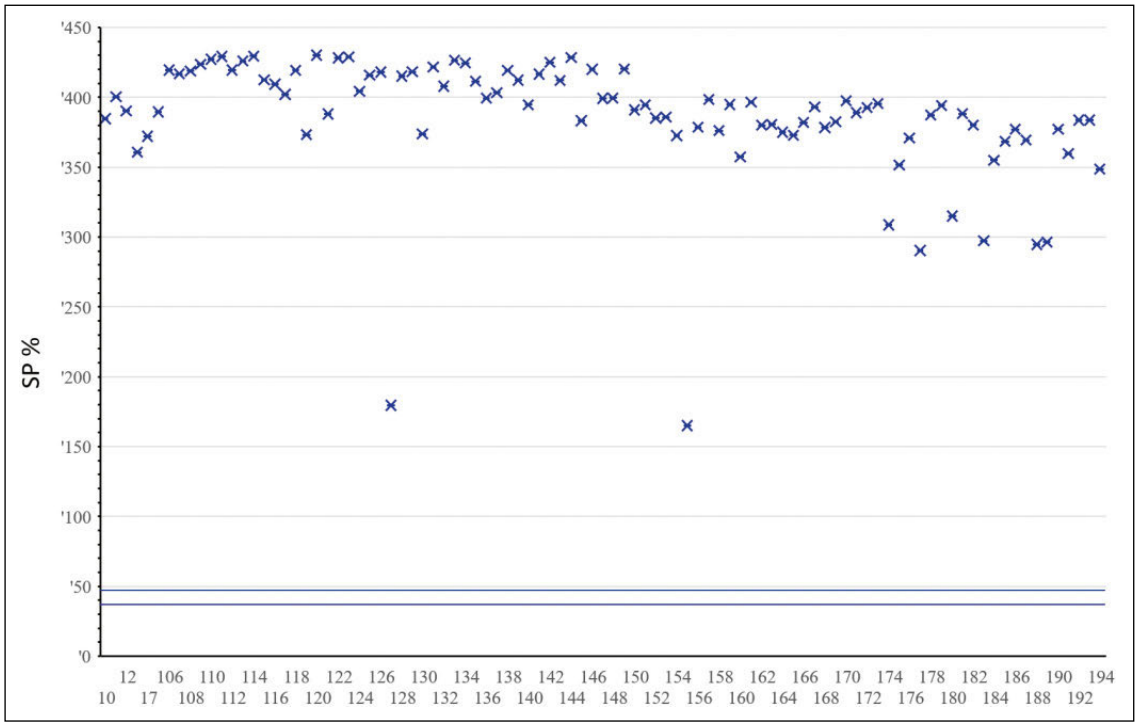

Fig 1: All goats (n=195) were screened using the CAEV/MVV Total Ab ELISA. The calculated sample to positive (SP%) values are shown for every animal to illustrate the unusually high and homogenous results obtained. The upper horizontal blue line (40%) shows the positive cutoff, while the lower line (30%) represent the negative cutoff. Sera reacting between these two cutoffs are considered doubtful.

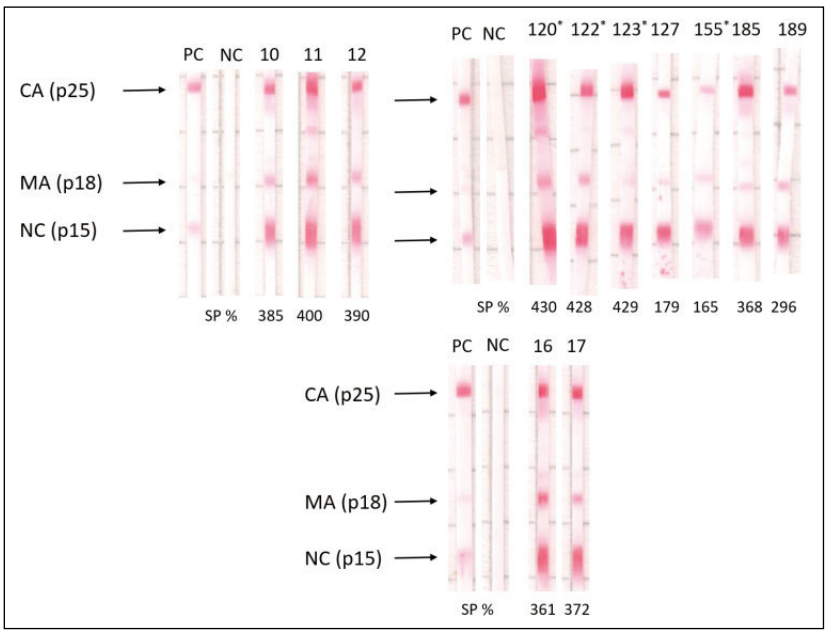

Fig. 2: Goat sera tested in western blot showed strong reactions with the capsid (CA, p25), matrix (MA, p18) and nucleocapsid (NC, p15) antigens that were even stronger than the bands obtained with the positive control serum (PC). The sample to positive (SP%) values calculated for the CAEV/MVV Total ELISA are shown below each strip. Asterisks mark the animals from which sequence information on the infecting virus is available.

	SU5-peptide				
goat #	A4	A3	A1	B1	B2
pos	100	100	100	100	100
10	249	349	400	700	278
11	166	285	551	698	233
12	126	240	292	509	36
16	14	547	277	625	138
17	135	0	19	477	141
120	43	96	88	337	81
122	2	0	10	335	0
123	305	428	280	354	232
127	34	110	65	338	96
128	108	346	240	367	289
155	13	132	93	192	61
189	52	111	102	273	79
195	31	173	128	320	81

Fig. 3: Thirteen goat sera were tested with the in-house SU5-ELISA and the results expressed as SP% values compared to the positive control serum. SU5 peptides encompassing the immunodominant and variable region of the SRLV subtypes A4, A3, A1, B1 and B2 were used. Results were visualized using a color scale implemented in Excel (Microsoft Office 2018). Sera such as those of goats #10, #123, #128 and, partly, #155 showed a broad reactivity with SU5 peptides of different subtypes and do not permit us to define the infecting virus; however, the general picture clearly points to the B1 subtype as the infecting genotype.

coprotein (Env) of SRLV subtype A1, A3, A4, B1 and B2 (amino acid sequences are shown in Fig. 4). Sample to positive (SP) % values were calculated, setting an arbitrary cutoff value for seropositivity at 50%.

PCR

Primer positions are related to the prototypic sequence CAEV-CO, GenBank: NC_001463.

gag-pol

The gag-pol fragments were amplified using the QIAGEN OneStep RT-PCR Kit according to the manufacturer's instructions and using a nested strategy based on the following primers.

The first amplification was performed with the primer pair

Gag-Pol EF: 5'-GCCATGATGCCTGGAAATAGAGC-3' (position 1112-1134)

Gag-Pol ER: 5'-TCTGCGGGTGTAGTTCAAATCCTA-3' (position 2843-2866).

RT-PCR conditions were set as follows: RT-Step: 50 °C, 30 min, Polymerase activation: 95 °C, 15min followed by 35 cycles subdivided as follow: 94 °C, 30 sec at 59 °C, 1 min at 72 °C, 1 min and a final extension at 72 °C for 10 min.

Five µl of the amplified product were used for the second round of amplification using the following primers:

gag-pol IF: 5'-GCCATGATGCCTGGAAATAGAGC-3' (position 1112-1134)

gag-pol IR: 5'-TCTGCGGGTGTAGTTCAAATCCTA-3' (position 2843-2866)

PCR conditions were set as follows: Polymerase activation: 95 °C, 15 min followed by 35 cycles subdivided as follow: 94 °C, 30 sec. at 45 °C, 1 min at 72 °C, 1 min and a final extension at 72 °C for 10 min.

env SU4-5

The SU4-5 encoding region of env was amplified using the following primers

EdM-SU4-5 F 5'-ATAACAGTAGGAAATGGAACVATAAC-3' (7326-7351)

EdM-SU4-5 R 5'-GCHGCAGTTGCATTAGCAAG-3' (8022-8041)

RT-Step: 50 °C, 30 min, Polymerase activation: 95 °C, 15 min followed by 35 cycles subdivided as follows: 94 °C, 30 sec at 57 °C, 1 min at 72 °C, 1 min and a final extension at 72 °C for 10 min.

Virus Isolation

Virus was isolated from peripheral blood mononuclear cells, spleen and the carpal joints of different animals. With the exception of the synovial membrane cells isolated from the joints of two selected animals, all other virus isolation attempts were performed by co-cultivating the isolated cells with primary goat synovial mem-

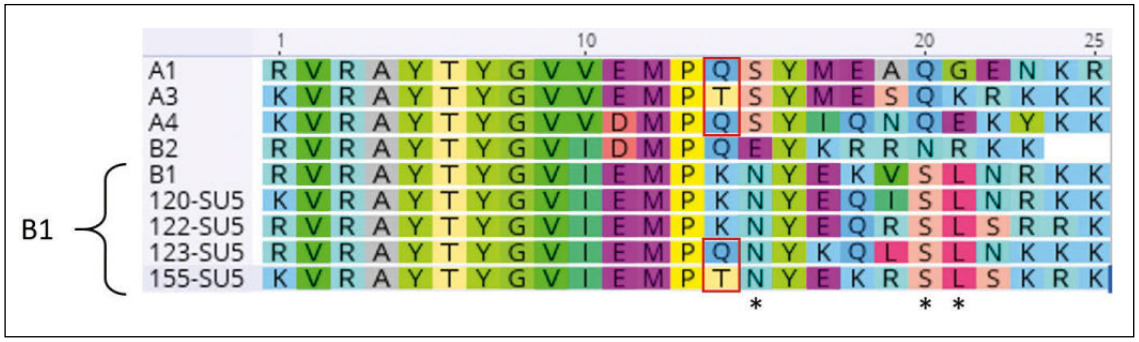

Fig. 4: An alignment of amino acid sequences of the SU5 peptides routinely used in our SU5-ELISA and those deduced from sequencing the corresponding region of the viruses isolated from four goats of the imported flock are shown. All peptides are identical between position 2 and 9. Considering few conservative mutations, the region from position 1 to the proline at position 13 can be considered constant. In contrast, the carboxy-terminal region of these peptides is highly variable and confers the subtype specificity of this immunodominant region. The B1 SU5 sequence is boxed in red and the amino acid positions shared between the B1 peptide and the deduced SU5 sequences of the viruses infecting the four goats are marked with asterisks. The Q at position 14 shared between the SU5 sequences of the virus infecting goat #123 and the A1 and A4 SU5 peptides and the T at the same position shared between the virus infecting goat #155 and the A3 SU5 peptide are boxed in red.

brane (GSM) cells obtained from a certified SRLV-negative goat.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from 5ml EDTA-anticoagulated blood. Blood samples were diluted 1:1 in Ca2+ and Mg2+ -free phosphate-buffered saline, pH 7.3, supplemented with 0.3 mM EDTA (PBS/EDTA) and gently overlaid on 4 ml of Biocoll (density 1.077) separation solution (Biochrom Gmbh, Merck, Berlin, Germany) in a 15 ml conical tube (Sarstedt, Nümbrecht, Germany). The prepared tubes were centrifuged 25 min at 800 × g at RT, after which plasma was partially discarded and the PBMC band collected and transferred to a 50 ml conical tube (Greiner Bio-One Gmbh, Frickenhausen, Germany), adding 10 ml of ice-cold PBS/EDTA. The cells were centrifuged at 350 × g for 10 min at 4 °C, resuspended in 30 ml ice-cold PBS-EDTA, and pelleted again at 350 × g for 10 min at 4 °C. Finally, PBMC were resuspended in tissue culture medium Dulbecco's MEM (Seraglob, Bioswisstec AG, Schaffhausen, Switzerland), supplemented with 10% FCS (Life Technologies Europe, Zug, Switzerland) Amphotericin B (Biochrom, Berlin, Germany) Penicillin-Streptomycin (Biochrom, Berlin, Germany) and added to a 25 cm^2 tissue culture flask seeded with subconfluent GSM cells.

Spleen cells

The spleen was thoroughly disinfected with 70% ethanol and the splenic capsule removed. Small slices of splenic tissues were cut with a sterile scalpel and digested in 100 ml of Trypsin (1:250, 0.25% w/v in PBS, Biochrom, Berlin, Germany) at RT with constant stirring for 15 min, after which Trypsin solution was discarded and replaced with fresh, pre-warmed Trypsin (1:250), continuing the incubation at 37 °C with constant stirring for an additional 30 min. Finally, cells were filtered through a sterile gauze and collected in a conical tube on ice. Cells were washed twice with Dulbecco's MEM supplemented with 10% FCS (Life Technologies Europe, Zug, Switzerland), penicillin-streptomycin (10'000 U/ml, 10'000 mg/ml, respectively, Biochrom, Berlin, Germany), 0.5% neomycin-bacitracin (1mg/ml, 50 IU/ml, respectively, Biochrom, Berlin, Germany) and 1% Amphotericin B (250 mg/ml, Biochrom, Berlin, Germany). Between every washing step, cells were centrifuged at 300 × g for 10 min at 4 °C. Finally, the cells were resuspended in tissue culture medium and seeded in 75 cm^2 tissue culture flasks. Four days later 1.6×10^6 indicator GSM cells were added and the co-cultures monitored for the appearance of SRLV-induced cytopathic effects such as syncytia.

Goat synovial membrane cells

The front legs of the slaughtered animals were severed through the radius, about 20 cm above the antebrachiocarpal joint and immediately chilled on ice. The skin was removed and the exposed parts thoroughly disinfected with 70% ethanol before proceeding with the opening of the antebrachiocarpal joint. After cutting and fixing the superficial tendons with forceps, the antebrachiocarpal joint was exposed by cutting a half-moon incision from one side to the other of the joint. The open joint was accessible with a curette and the synovial membrane cells were gently scratched from the walls of the joint and transferred to a 6-well plate containing Dulbecco's MEM supplemented with 10% FCS (Life Technologies Europe, Zug, Switzerland) 1% penicillin-streptomycin solution (10'000 U/ml, 10'000 µg/ml, Biochrom, Berlin, Germany), 0.5% neomycin-bacitracin (1mg/ml, 50 IU/ml, Biochrom, Berlin, Germany)

and 1% Amphotericin B (250 µg/ml, Biochrom, Berlin, Germany). The same procedure was used to collect the synovial membrane cells from the middle-carpal and the carpometacarpal joints. The next day the cells were washed with pre-warmed tissue culture medium, before adding fresh medium. The operation was repeated 3 days later and after reaching confluence the cells of every well were harvested and transferred to a 75 cm^2 tissue culture flask and monitored for the appearance of SRLV-induced syncytia.

Sequence analysis

Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method implemented in Geneious version 10.2.6.[11] with the Tamura-Nei gamma distance[33]. The statistical confidence of the topologies was assessed with 1000 bootstrap replicates[9].

Protein alignments were generated using Geneious version 10.2.6.[11].

Results

Serology:

ELISA

Ninety-five sera were tested with the routinely used CAEV/MVV Total Ab Screening Test and all showed SP% values well above the positive cutoff of 40. The mean SP% value calculated was unusually high at 386 ± 44, with a range between 165 and 430. As shown in Fig. 1, all but two animals (goats # 127: SP% 179 and #155: SP% 165) showed SP% values above 290 (SP% value of serum #177).

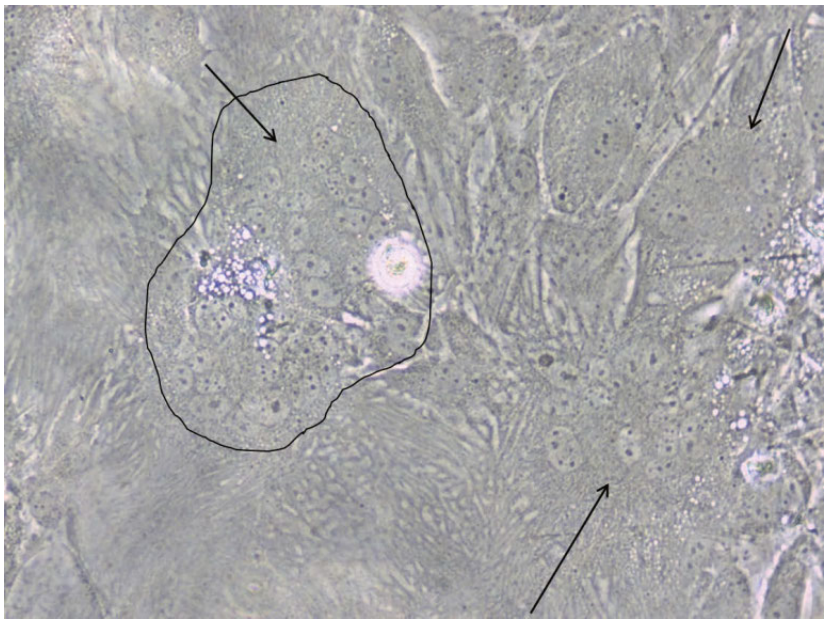

Fig. 5: Numerous syncytia were observed 10 days after starting the co-culture between isolated PBMC and the indicator GSM cells. The largest syncytium is highlighted with a black line and the black arrows point to the several multinucleated cells.

Western blot

Twelve selected sera were tested in WB and showed robust reactions to the capsid (p25), matrix (p18) and nucleocapsid (p15) proteins (Fig. 2). The intensity of the capsid and nucleocapsid bands were quite similar for all sera, while the matrix band showed intensities that were more variable. No obvious association between the SP% values and the intensity of the bands in WB was observed.

SU5-ELISA

In order to predict the genotype of the infecting SRLV, 13 sera were tested with an in-house SU5-ELISA. The results shown in Fig. 3 illustrate the strength and weaknesses of this test when applied to a series of sera from the same flock. While the results of individual serum may provide ambiguous results, e.g. goats #10, #123, #128 did not point to a particular genotype, the analysis of several individuals of the same flock allowed accurate prediction of the subtype of the infecting virus, in this case SRLV B1. As described in the sequencing paragraph, we also determined the deduced SU5 amino acid sequences of the SRLV infecting 4 of the SU5-ELISA-tested goats (Fig. 4). The most variable region of SU5 encompasses the amino acid positions 14 to 25 and determines the genotype specificity of this serological test. The serum of goat #123, infected with a B1 virus, showed a broad reactivity to all SU5 peptides tested. Similarly, the serum of goat #155 reacted with the SU5-B1 peptide corresponding to the infecting genotype and showed a relatively high SP% values with the A3 SU5 peptide. Position 14 of the deduced amino acid sequence of the SU5 region of the B1 viruses infecting these two goats showed two mutations, corresponding to the amino acids found in the A1 and A4 viruses (K to Q) for goat #123, or in the A3 (K to T) SU5 sequence for goat #155 (Fig. 4). Both mutations substitute a positively charged K with two amino acids (Q or T) with uncharged polar side chains, potentially explaining the broader A-B reactivity of these two sera (Fig. 3).

Virus isolation from different cells and organs:

Virus isolation attempts from the PBMC of four goats and from the carpal joints and spleen of two additional animals were successful, suggesting that the viral load in these animals was particularly high. As shown in Fig. 5, GSM cells incubated with the PBMC of an infected animal showed prominent syncytia, characteristic of SRLV infections in general and the subtype B1 in particular. Virus isolation was particularly rapid and efficient for spleen cells co-cultivated with GSM cells.

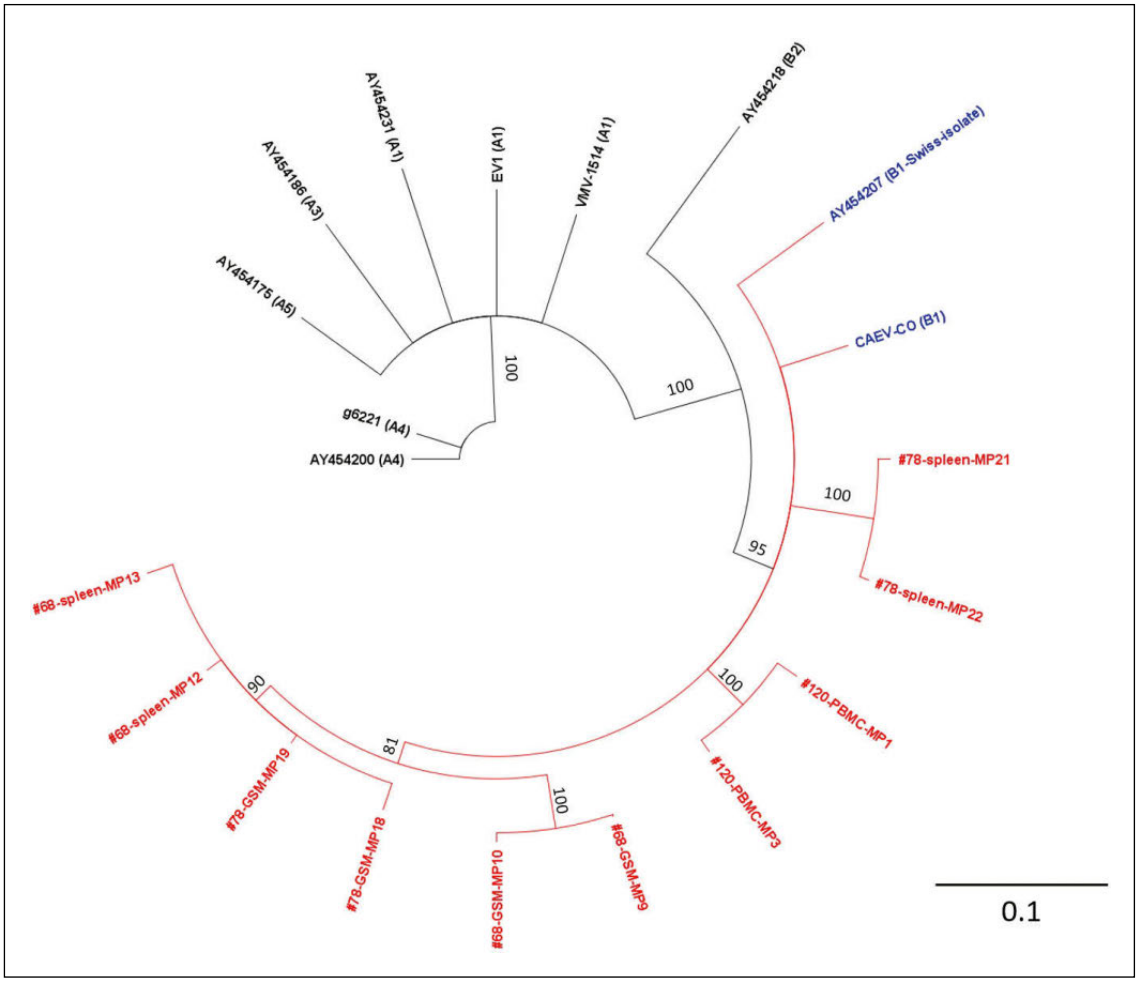

Fig. 6: The figure shows an unrooted phylogenetic tree based on the pol-gag sequences obtained from viruses isolated from the imported goats (red), the prototypic CAEV-CO SRLV B1 sequence (blue), a previously described Swiss SRLV B1 isolate (blue) and several Swiss SRLV A4, A3, A5, A1 sequences (black) and the prototypic EV1 and VMV-1415 SRLV A1 sequences (black). Sequences obtained from the spleen or GSM cells of goats #68 and #78 segregated on separated branches with high bootstrap values, suggesting that these animals were infected with multiple viruses, colonizing different compartments.

RT-PCR, PCR:

We collected the supernatants of GSM-PBMC co-cultures obtained from the first four goats tested and analyzed those samples by RT-PCR. We amplified, cloned and sequenced several amplicons derived from the genomic regions encoding for a portion of gag-pol and for the highly variable SU4-5 regions of the envelope glycoprotein. Similarly, we generated several gag-pol and SU4-5 sequences from the supernatants of the spleen and carpal joints cells obtained from the sacrificed animals, or from DNA isolated from these cells at the end of the experiments.

Sequence analysis:

The gag-pol and the env sequences encoding for the SU4-5 epitopes confirmed the serological results obtained in SU5-ELISA pointing to an SRLV B1 infection. As shown in Fig. 6 for gag-pol, the infecting viruses were quite variable. Remarkably, the viruses found in the spleen and the carpi of both, goat #68 and goat #78, segregated on different branches with high bootstrap values, indicating that different viruses colonized these distinct organs. An alignment of the SU4 sequences, encompassing a previously characterized neutralizing epitope of VMV, demonstrated the striking variability of the infecting viruses. These sequences showed several mutations, insertions, and deletions around the mapped neutralizing epitope (data not shown).

As mentioned above, the deduced amino acid sequences of the SU5 peptides used in our diagnostic SU5-ELISA were determined, providing a potential explanation for the differences in the SU5 peptide specificity of the sera tested (see SU5-ELISA and Fig. 4).

Discussion

Animal trading is a well-recognized risk for spreading infectious diseases, such as SRLV and can be particularly problematic when trading between countries with a different infectious status for a particular disease[1]. In fact, molecular evidence points to Switzerland as a historically important source of internationally circulating SRLV B1[30]. Noteworthy, is the importation of SRLV-infected sheep to Iceland in 1933 and can be considered the triggering event and starting point for lentivirus research[22,31].

The case described in this manuscript is a typical example of problems arising from the importation of animals from outside the country and shows the critical points that should be addressed to avoid jeopardizing the SRLV B1 status of Switzerland. The eradication of this particular virulent subtype of SRLV is the result of a long ongoing CAEV control program that demands a continuous serological monitoring of resident and imported goats in Switzerland. This is not the case for the European Union where SRLV eradication plans are implemented on a compulsory basis only in particular regions[34], or are established as voluntary programs. In this context, the SRLV-TRACES certification accompanying the imported flock cannot match the requirements of the Swiss eradication campaign. As mentioned in the materials and methods section, this certificate is based on a simple declaration by the signing official veterinarian and the flock owner that the animals were free of clinical SRLV-induced pathologies in the three years prior to selling the animals. This is certainly insufficient and may pave the way to potential fraudulent abuses, as might be the case for the described flock. Indeed, the discovery of clinically affected animals a few days after the importation, the unusually high SP% values observed (Fig. 2), the successful virus isolation from all samples analyzed and the phylogenetic complexity of the obtained sequences (Figs. 6), all point to an extremely high infection pressure in the flock and a very high viral load in the clinically affected animals[25,26]. The seroprevalence in this flock was much higher than the average prevalence reported for CAEV-infected flocks in Belgium, pointing to an unusual high infection rate in these animals[15].

The immediate recognition of a potential SRLV infection by the veterinarian in charge after observing animals with clinical carpitis in the flock indicates that in spite of SRLV cases being eliminated in Switzerland for many years, disease awareness has not been reduced in the profession. We may speculate that the importation of clinically inapparent, infected animals, which represent the vast majority (>70%) of SRLV infected goats, would have been much more difficult to detect, emphasizing the importance of performing timely serological tests on imported animals. The prompt reaction of the veterinary authorities imposing a quarantine on the flock and the rapid involvement of the national reference center for SRLV permitted quick isolation and characterization of the infecting virus, leading to the elimination of the entire flock and stopping the potential spread of the infection.

This case was also a perfect opportunity to test the strengths and weaknesses of the virological and serological tests used by the national reference center. The CAEV/MVV Total Ab ELISA that previously showed some limitations in detecting animals infected with SRLV A4 viruses was extremely efficient in detecting goats infected with different SRLV B1 strains[7]. The homogeneous, very high SP% values observed in this flock were striking. As mentioned above, we interpret this observation as strong evidence for the presence of a very high infectious pressure in this flock. Western blot is considered the gold standard in SRLV serology and is routinely applied by the reference center to confirm doubtful and positive ELISA results[37]. On previous occasions, this test failed to detect animals infected with SRLV A4 strains, revealing some unexpected limitations[7]. This was not the case for the serological screening of this SRLV B1-infected flock in which the applied WB confirmed its reliability and showed a very solid performance. This suggests that in spite of its costs and cumbersomeness, WB remains a very consistent test, indispensable for the confirmation of serological results, obtained from animals infected with virulent SRLV B1 isolates[37].

Particularly interesting was the performance of the in-house SU5-ELISA, a test based on synthetic peptides encompassing the variable and immunodominant SU5 region of Env, that correctly predicted an SRLV B1 infection in these animals[5,18,36]. As previously observed, the analysis of a single serum in SU5-ELISA may provide ambiguous results when the specific antibodies bind to SU5 peptides corresponding to different SRLV genotypes with similar affinity. This is the case for a minority of sera containing antibodies binding to epitopes contained in the 5' constant region of the SU5 peptides that is shared between different genotypes (Fig. 4)[18]. Conversely, cross-reactions between different genotypes may occur when key amino acids of the variable region of SU5 are shared between different genotypes[18]. This may well be the case for the virus infecting goat #123. Indeed, the inferred SU5 sequence of this virus showed a Q in position 14 that is shared with the A1 and A4 SU5 sequences and may explain the broad reactivity of this serum (Fig. 4). Similarly, the K to T mutation at position 14 in the virus infecting goat #155 may explain the binding of this serum to the A3 SU5 peptide. Nev-

ertheless, the overall picture obtained while analyzing several sera of the examined flock in parallel is clear and points to SRLV B1 as the infecting subtype (Fig. 3). This was confirmed by virus isolation and sequence analysis. The ambiguous results obtained with these two samples confirm the limitations of the SU5-ELISA when applied to sera showing a broad SU5 reactivity[5].

Viruses were isolated from the PBMC of four goats and the spleen and carpal joints of two additional animals. The ease in isolating viruses from these animals, a hallmark of high viral load, was unusual and contrasted with previous observations pointing to a very low viral load in chronically infected animals[26]. In contrast, the marked cytopathic effects observed in the co-cultures of PBMC and spleen cells with GSM or in GSM explanted from the scarified animals is characteristic of this genotype (Fig. 5) and was described previously[12].

Sequence analysis confirmed the serological results. The gag-pol sequences (Fig. 6), as well as the env sequences (data not shown) obtained from several PCR-generated amplicons, clearly clustered with the prototypic SRLV B1 sequence CAEV-CO. The significant differences (high bootstrap values) observed between sequences obtained from different organs of the same animals is in line with previous reports and suggest that these animals may be infected with multiple viruses, a prerogative for recombination and further increase of the phylogenetic complexity of SRLV circulating in this flock[13,23,24,29]. The elimination of such a flock is certainly warranted as a preventive measure to contain the occurrence and spread of such recombinant forms.

Sequence analysis of the SU4 region of env, which encodes a previously mapped neutralizing epitope of Env, revealed a very high variability in this region, characterized by numerous insertions and deletions[2,32]. Marked differences were detected in sequences obtained from the same animal as well (data not shown). This is most probably the result of a strong selective pressure exerted by neutralizing antibody. This region appears to be an excellent candidate for fingerprinting infecting SRLV, thereby permitting the reconstruction of potential chains of infection.

Conclusions

This unusual case of importation of a large, SRLV-infected goat flock permitted us to test at different levels the efficacy of our response to potential threats to the Swiss CAE eradication program. Except for the pre-import phase, which may be improved, e.g. by encouraging the goat breeders to request a serological testing of the animals to be imported, the other rings of the preventive chain appeared to function as expected. The veterinary in charge immediately recognized arthritis as a potential sign of an SRLV infection, and the coordination between the cantonal and federal authorities with the reference center was smooth and rapid. The different serological, virological and molecular tools at our disposal permitted a precise characterization of the infecting viruses with only a short delay. This notwithstanding, our diagnostic tools need to be further improved to guarantee the detection of a broader spectrum of SRLV genotypes and of single animals showing low viral loads and uncertain serological profiles. In fact, every infected animal escaping diagnostic detection is a potential Trojan horse undermining the SRLV B1 free status of the Swiss goat population.

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Diagnostic response to a cross-border challenge for the Swiss caprine arthritis encephalitis virus eradication program

E. De Martin et al.

Réponse diagnostique à un défi transfrontalier pour le programme suisse d'éradication du virus de l'arthrite encéphalite-encéphalite caprine

Le commerce d'animaux entre pays où le statut infectieux des lentivirus des petits ruminants est différent constitue un danger potentiel pour la réintroduction de génotypes éradiqués. Ce fut le cas en 2017 avec l'importation d'un grand troupeau de chèvres séropositives en Suisse. Le traitement de cette affaire nous a permis de tester les mesures préventives mises en place. La coordination entre le vétérinaire local et les autorités vétérinaires cantonales et fédérales a été efficace et a impliqué rapidement le centre de référence national dans les enquêtes. Ce cas a constitué un défi pour le centre de référence et a permis d'examiner de près les tests de diagnostic appliqués. Les tests ELISA et Western blot ont fourni des résultats cohérents et ont mis en évidence un taux d'infection anormalement élevé dans le troupeau. Cela a été confirmé par l'isolement de plusieurs virus provenant d'organes et de cellules différents, démontrant que la rate est particulièrement bien adaptée à l'isolement des lentivirus des petits ruminants. Le SU5-ELISA, conçu pour prédire le sous-type du virus infectant, désignait correctement un sous-type B1 en tant qu'agent infectieux. Nous avons confirmé qu'avec ce test, il était nécessaire d'analyser un nombre représentatif d'échantillons d'un troupeau et pas seulement des sérums individuels pour obtenir des résultats fiables. Cette analyse nous a permis d'identifier des résidus d'acides aminés particuliers dans les peptides SU5 qui pourraient jouer un rôle crucial dans la détermination de la spécificité de sous-type de la liaison à l'anticorps. Différentes régions gag-pol et env ont été amplifiées par PCR en utilisant des amorces conçues à cet effet. L'analyse phylogénétique a révélé une hétérogénéité étonnamment élevée des séquences, indiquant de multiples infections chez les animaux isolés et dans l'ensemble du troupeau.

En conclusion, cette affaire a montré que la défense du statut négatif CAEV de la population de chèvres suisses vis-à-vis du virus virulent, sous-type B1 des lentivirus des petits ruminants, nécessite, entre autres mesures, un système de diagnostic capable d'effectuer une analyse approfondie des échantillons collectés.

Mots clés: lentivirus des petits ruminants, SRLV, CAEV, diagnostic, analyse phylogénétique, épizootie.

Risposta diagnostica transfrontaliera per il programma svizzero di eradicazione dell'artrite encefalite virale caprina

Il commercio di animali tra paesi con diversi stati di indennità da lentivirus dei piccoli ruminanti (SRLV) è un potenziale pericolo per la reintroduzione di genotipi eradicati. Questo è avvenuto nel 2017, in seguito all'importazione di un gregge di capre sieropositive in Svizzera. La gestione di questo caso ha permesso di testare le misure preventive in vigore. Il coordinamento tra il veterinario locale e le autorità veterinarie cantonali e federali ha funzionato in modo efficiente, con un rapido coinvolgimento del centro di referenza nazionale. Questo caso ha rappresentato una sfida per il centro di referenza e ha permesso di verificare i test diagnostici applicati. Il test ELISA e il Western Blot hanno fornito risultati coerenti e hanno evidenziato un tasso di infezione insolitamente elevato nel gregge. Ciò è stato confermato dall'isolamento di numerosi virus da cellule e diversi organi, tra i quali la milza si è dimostrata particolarmente adatta all'isolamento di SRLV. Il test ELISA-SU5, volto a identificare il sottotipo del virus infettante, ha correttamente identificato come agente eziologico un sottotipo B1. Abbiamo inoltre constatato che, per ottenere risultati affidabili con questo test, è necessario analizzare un numero rappresentativo di campioni, e non singoli animali. Questa analisi ci ha permesso di identificare particolari residui di amminoacidi nei peptidi SU5, determinanti la specificità di reazione tra i vari sottotipi e i relativi anticorpi. Diverse regioni Gag, Pol e Env sono state amplificate mediante PCR utilizzando primers appositamente disegnati. L'analisi filogenetica ha rivelato un'elevata eterogeneità di sequenze, indice di infezioni multiple sia a livello di gregge che di singoli animali. In conclusione, questo caso ha dimostrato che la difesa dello stato CAEV-negativo della popolazione caprina svizzera richiede, per quanto concerne il sottotipo B1 (un prototipo virulento di SRLV), tra le altre misure, una struttura diagnostica in grado di effettuare un'analisi approfondita dei campioni raccolti.

Parole chiavi: lentivirus nei piccoli ruminanti, SRLV, CAEV, diagnostica, analisi filogenetiche, focolaio.

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