METHODS OF LENTIVIRAL INFECTION SURVEILLANCE AND DIAGNOSIS IN SHEEP AND GOATS FARMS: REVIEW

Dan A. Enache[1], Stelian Baraitareanu[1], Marius Dan[2], Maria R. Gurau[1], Doina Danes[1,3]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

[2]Institute for Control of Biological Products and Veterinary Medicines, 39 Dudului Street, District 6, Bucharest, Romania

[3]Section of Veterinary Medicine, Academy of Agricultural and Forestry Sciences, Bucharest, Romania

Abstract

Caprine Arthritis Encephalitis and Maedi-Visna are specific diseases of small ruminants. They are caused by RNA viruses (CAEV and MVV) belonging to the family Retroviridae, gen Lentivirus. These viruses are referred to under the generic name of small ruminant lentiviruses (SRLVs): in this group, five genotypes have been identified (A-E), according to the type of the isolated strains. Meanwhile the viral genome is continuously evolving. The exposure to domesticated ruminants at SRLVs may be very high, if they are subject to high infection pressure as a result of using common areas with small wild ruminants. The choice of the appropriate technique for diagnosis of the SRLVs infections is still a great challenge for many researchers. The aim of the study is to present the most useful methods and the criteria – pros' and cons' – to use, in order to select the appropriate technique for the diagnosis of SRLV infections. In this study, we selected relevant scientific papers, published in the last two decades, and identified the most used methods of diagnostic. Three types of methods were identified and compared: antibody detection (ELISA – enzyme-linked immunosorbent assay – indirect/competitive/home-made, AGID – agar gel immunodiffusion, and WB – westernblotting), identification of specific viral genome by molecular techniques (PCR, RT-PCR and LAMP) and virus isolation by co-cultivation of cell cultures with goat synovial membrane cells/sheep choroid plexus cells. The results were compared in order to decide upon the method of choice for the diagnosis of the lentiviral infection. It can be concluded that all the techniques are useful, but for a result with high confidence, it is important to be decided what will be the targeted purpose (the confirmation of possible outbreaks, the eradication of disease, monitoring of herds/flocks, the criteria to declare/prove a free SRLV herd/flock, or just for the surveillance purpose).

Keywords: *SRLV, infection, diagnosis, methods, viral genome, ELISA, PCR*

1. INTRODUCTION

The Maedi-visna virus (MVV) and Caprine arthritis encephalitis virus (CAEV) belong to *Retroviridae* family, gen *Lentivirus* and referred to both as the group of small ruminant lentiviruses (SRLV). They produce two specific diseases of sheep and goats (maedi-visna and caprine arthritis encephalitis) (Angelopoulou, Brellou, & Vlemmas 2006; Sanjose et al. 2016; Perez et al. 2013; Minguijon et al. 2015; Thomann et al. 2017).

Due to the higher variability of the viral genome, SRLV were classified into five genotypes (A-E) and several subtypes (Shah et al., 2004; Ramirez et al. 2013; Deubelbeiss et al. 2014; L'Homme et al. 2015; Danes et al. 2016; Olech, Valas & Kuzmak 2018; Olech, Murawski & Kuzmak 2019). There is no correlation between geno-groups / subtypes and small ruminant species. Olech et al. 2012 mentioned that subtypes A5, A7 and geno-groups C and D were isolated only in goats, while the A2 subtypes seem to the identified only in sheep. The following subtypes were found in both small ruminant species: A1, A3, A4, A6, B1 and B2. However, a correlation between geno-groups / subtypes and geographic area of spreading seems to be present. The geno-groups A and B are spread in all regions of the world (Ramirez et al. 2013). The C-E geno-groups were isolated in smaller geographic areas. The geno-group C contain isolated strains from Norwegian sheep and goats (Gjerset,

Storset & Rimstad 2006), and the geno-group D was identified in sheep from Spain and Switzerland (Shah et al. 2004). The isolates from the geno-group E (which is different than geno-group A) were identified in the north of Italy and also in Sardinia region (Grego et al. 2007; Reina et al. 2010; Glaria, Reina & Ramirez 2012).

The viral genome is continuously evolving as result of his biological features such as:

- Ability to cross the species barriers and infect domestic and wild population of ruminants (*Capra ibex, Ovis orientalis, Ovis gmelinii, Oreamnos americanus*) (Cruz et al. 2013; Ramirez et al. 2013; Sanjose et al. 2016);
- High mutagenic capacity and genetic diversity: genome is integrated in the host leucocyte DNA and during viral replication, there could be occurred frequently mutations (Cruz et al. 2013; Sanjose et al. 2015).

SRLV are characterized by a very long period of incubation (up to two years), high rate of morbidity, variable mortality, depending on the immune status of animals, and a continuum low rate of production (milk, meat and wool) (Angelopoulou, Brellou, & Vlemmas 2006; Sanjose et al. 2016; Perez et al. 2013; Minguijon et al. 2015; Thomann et al. 2017).

Transmission of MVV and CAEV could be both horizontally (e.g., respiratory secretions, aerosols, semen, vaginal secretions, faeces) (Minguijon et al. 2015; Highland, 2017) and vertically (e.g. feeding with infected colostrums) (Peterhans et al. 2004; Highland et al. 2017; Minguijon et al. 2015).

The following forms of MV / CAE have been described:

- Respiratory form: progressive losses of weight, dyspnoea, abdominal breathing, opistotonus, expansion of the nostrils and breathing with open mouth (Minguijon et al. 2015; Gomez-Lucia, Barquero & Domenech 2018); the lesions could be lung enlargement, hypertrophic lungs, rounded, rubbery and grayish-yellow lungs, marked lobular pattern (Gomez-Lucia, Barquero & Domenech 2018);
- Mammary form: swelling and pronounced stiffness of the mammary gland, and the reactivity of local lymph nodes; the organoleptic properties of the milk could not be changed; the main recorded lesions are chronic interstitial mastitis, hyperplasia, desquamation of ductal epithelium and sometimes fibrotic mammary structure (Gomez-Lucia, Barquero & Domenech 2018);
- Articular form: multifocal arthritis and progressive lameness; the main lesions are deformed joints, chronically proliferative synovitis and cartilage fibrosis (Minguijon et al. 2015);
- Neurological form: progressive ataxia, hind limb weakness and sometimes paralysis; non-suppurative encephalitis (periventricular or paraventricular) and demyelination of tissues (Minguijon et al. 2015).

The diagnostic of SRLV infection is still a challenge but following the OIE recommendations, it could be done through: (1) antibody detection (ELISA/AGID/WB), (2) virus isolation by co-cultivation of cell cultures with goat synovial membrane cells/sheep choroid plexus cells, and (3) identification of specific viral genome by molecular techniques (PCR/RT-PCR/LAMP/HMA).

In order to control the SRLV spreading between animals/farms, there could be applied a combination of measures such as: the slaughter of the flock and its replacement with non - infected animals / the selective slaughter for only seropositive animals / the replacement of the animals only with subjects belonging to seronegative mothers / the early slaughter of seropositive animals which present clinical signs / the artificial growth of young animals (Reina et al. 2009; Perez et al. 2010). Other measures of SRLV control should be: regular serological tests / forbidding of mother to take any form of contact with kids after parturition / prohibiting of feeding with colostrum / milk (Perez et al. 2013).

In Romania, several studies have been done in small ruminants pathology (Danes et al. 2016; Hotea et al. 2016; Tilibasa & Darabus 2016; Baraitareanu, Ozdemir & Dan 2018; Codreanu & Calin 2018), but the epidemiological data considering the spreading of SRLV infections is still poorly understood

because there were covered only small herds or limited geographical areas (Popescu 2009; Enache et al. 2017, 2018; Mihai et al. 2018).

The aim of the study is to present the most useful methods and the criteria – pros' and cons' – to use, in order to select the appropriate technique for the active surveillance and diagnosis of SRLV infections.

2. MATERIALS AND METHODS

In order to identify and compare the methods of lentiviral infection surveillance and diagnosis in sheep and goats farms, we were selected and analysed 70 bibliographic sources (scientific reports and reviews) which used various serological, virological and molecular biology techniques in detection of MV / CAEV, using different types of samples.

3. RESULTS

In accord with the OIE recommendations, methods of MV / CAEV diagnostic were grouped in three categories: (i) antibody detection, (ii) identification of specific viral genome by molecular techniques and (iii) virus isolation.

i. Antibody Detection

Enzyme-linked immunosorbent assay (ELISA)

ELISA is based on a specific reaction antigen – antibody using specific antibody or antigen. In addition, the complex antigen-antibody also depends on target molecule detection/quantification using an enzyme reaction with its substrate. Two variants of ELISA are usually used in MV / CAEV antibody detection: indirect ELISA (iELISA) and competitive ELISA (cELISA). iELISA involves an antigen-antibody complex but also several elements in plus such as: the addition of unlabeled detecting antibodies, incubation and washing away of excess (unbound) antibodies, the addition of the conjugate, again incubation and washing, the addition of substrate, the stop reaction and the reading in a spectophotometer.

cELISA involves the measurement of either antibody or antigen.

According to the OIE recommendations (OIE 2017), ELISA is proper method for:

- Population freedom of infection;
- Individual animal freedom from infection prior to movement;
- Contribute to eradication policies;
- Prevalence of infection – surveillance.

In the same time, ELISA is not recommended for:

- Confirmation of clinical cases;
- Immune status in individual animals or populations postvaccination.

Several ELISA kits have been described for the detection of antibodies anti-MVV and anti-CAEV (de Andres et al. 2005; OIE 2017). Those methods usually use sheep and goats blood sera as biological samples (de Andres et al. 2005), but alternative biological samples (e.g., milk, seminal fluid) can be used in ELISA diagnostic (Plaza et al. 2009; Ramirez et al. 2009; Barquero et al. 2011; Mussi et al. 2015). Also, various types of antigens were used in the SRLVs ELISAs (e.g., whole virus-purified viral proteins, recombinant viral proteins, viral peptides) (Herrmann-Hoesing 2010).

Until present, no single technique or method could be considered as a "gold" one in order to determine the infectious status of animals. Nowadays, there are competitive ELISA methods (cELISA CAEV of VMRD Inc. Pullman, WA) based on monoclonal antibodies, but also there are indirect ELISAs which

are applied more frequently and there are based on polyclonal antibodies (Ramirez et al. 2013). The iELISAs use the whole virus as antigen such as: AG-CHEKIT (CAEV/MVV kit, IDEXX Switzerland AG, Liebefeld, Bern, Switzerland) or recombinant proteins and a peptide (the gag p25 recombinant protein and a TM peptide derived from genotype A) such as: Elitest-MVV: HYPHEN Biomed, Neuville-sur-Oise, France and Pourquier: ELISA Maedi-Visna/CAEV serum verification Institut Pourquier, Montpellier, France (Ramirez et al. 2013). In some cases, specific assays could not cover the whole SRLV antigenic structures and they can fail while trying to detect the genotype E, and subtypes A4 or B2 (Ramirez et al. 2013). Besides the p25 viral protein which is included in most of the serological tests, p14 and p17 proteins can have immunodominant epitopes and they can be used for the differential diagnosis of MVV/CAEV. New genotypes could be studied and identified, other possibilities of diagnosis should be implemented leading to the development of another indirect ELISA tests. A relevant example is the one that use a mixture of gag and env peptides of three different SRLV genotypes (A, B, E) (IN3 diagnostic. Eradikit® SRLV indirect ELISA for Small Ruminant Lentivirus) (Ramirez et al. 2013).

The TM *env* proteins could be useful for serotyping and for the diagnosis of the specific strain due to their variability. The usefulness of synthetic peptide ELISAs is remarkable while trying to detect animals which belong to neurological and arthritic outbreaks (Ramirez et al. 2013).

Several advantages and disadvantages of ELISA using in MVV / CAEV diagnostic have been described (Table 1).

Table 1. Advantages and disadvantages for ELISA kits analyzed in this study

Value	Parameter	Source
Advantages	High sensitivity: allows detection of low antibody titers earlier than AGID.	Torres et al. 2009
	Used in both species (sheep and goats); can test a large number of animals in short time and to detect all viral antigens.	Mussi et al. 2015
Disadvantages	Some kits could be expensive; it is recommended to be used in conjunction with another assay which has a higher specificity (e.g., AGID).	Mussi et al. 2015
	False-negative and false positive results.	Herrmann-Hoesing 2010
	Not detect anytime a seropositive animal due to: the moment of performing the test (the antibodies could be detected after 3 weeks post-infection); the dynamic evolution of antibodies (there is a peak moment and after they can decline causing false-negative results).	de Andres et al. 2005; Mussi et al. 2015

Agarose gel immunodiffusion (AGID)

AGID is based on a serological reaction in which an antigen is precipitated by a specific antibody. The antigen and the antibody can diffuse from wells cut in the agar. A positive reaction means the development of a line of precipitate at the interface.

The specificity of the assay is validated by running of a known antigen in the same time against the same positive antiserum. The control precipitation line is connected with the test line creating a line of identity.

According to the OIE recommendations (OIE 2017), AGID should be used for:

- Individual animal freedom from infection prior to movement;

- Confirmation of clinical cases;
- Prevalence of infection – surveillance.

However, in order to be used for further cases, it should be subjected to several validations.

In the same time, AGID is not so recommended for:

- Population freedom from infection;
- Immune status in individual animals or populations post-vaccination.

There are two major viral antigens for MV and CAE: a surface glycoprotein usually called SU or gp135 and one nucleocapsidal protein called CA or p28. Both proteins are conserved in an antigen containing medium collected from infected cells cultures and concentrated approximately 50 fold by dialysis (OIE 2017). According to the authors of Manual OIE, MVV and CAEV have a nucleotide sequence identity of 73.0-74.4% in the envelope gene and this level of identity could not be enough to produce a sufficient quantity of antibodies for the specific MV and CAE epitopes resulting undetectable precipitation antibody-antigen lines (using MV viral antigen). When it is used a proper antigen, the performance of AGID test is higher. In comparison to ELISA tests, AGID uses the whole virus that has been obtained from culture supernatants. It can detect antibodies to the MVV p24 core antigen and gp135 envelope antigen (de Andres et al. 2005). The precipitation line can be observed due to numerous interactions between antibodies from the serum and epitopes of viral proteins derived from cell culture (Herrmann-Hoesing 2010). This line is available within 24 hours but a concluding result could be seen after 48-72 hours (Abreu et al. 1998; Mussi et al. 2015). The AGID method allows identifying the precipitating antibodies against proteins SU and CA by the naked eye (Minguijon et al. 2015). It was demonstrated that AGID which uses gp135 CAEV – antigen is more sensitive than the one that uses p28 CAEV – antigen (OIE 2017).

Also, for AGID technique several advantages and disadvantages in MVV / CAEV diagnostic have been described (Table 2).

Table 2. Advantages and disadvantages for AGID analyzed in this study

Value	Parameter	Source
Advantages	Sensitivity of AGID is bigger with 35.0% than the sensitivity of radioimmunoprecipitation (using a viral MV antigen) due to multiple antigen-antibody interactions.	OIE 2017
	Availability and acquisition of commercial immunoreagents / Easy ways to implement the method / Easy ways to prepare the staff about it.	Mussi et al. 2015
	Useful to perform screenings / to monitor the results in a control program / to create epidemiological studies / it allows the processing of a large number of samples and also the easy detection of infected animals.	Mussi et al. 2015
Disadvantages	The sensitivity of AGID is dependent on both viral strain and the used antigen.	OIE 2017
	AGID is not proper to be used for alternative biological samples like semen or milk.	Reina et al. 2009
	In goats, false-positive results could occur by passive transfer of antibodies via colostrum. This titre of antibodies is low after the 2-3 months age.	Gouveia 2012
	The use on a large scale for a single type of antigen in order to detect a specific protein causes the loss of its ability to detect all the seropositive animals.	Gouveia 2012

For the adult sheep infected with MVV and the adult goats infected with CAEV, the response of immunoprecipitation antibodies is directed against gp135 antigen. A response anti-p28 is, usually, present on a lower titre than one against gp135. Sometimes, an immune response anti-gp135 is possible to occur in the absence of a response anti-p28 for some goats infected with CAEV. In order to validate the test, there are necessary the reference standard sera producing precipitation line against gp135 and p28 (OIE 2017). The sensibility of AGID depends on the viral strain and the viral antigen. For instance, the number of the infected goats detected with gp135 antigen is higher than that with p28 antigen. The use of both proteins is recommended in order to increase the sensitivity of a test (Mussi et al. 2015). The homologous antigen CAEV provides a bigger sensitivity in comparison with the heterologous one. Both antigens reacted by AGID method and also presented 100% specificity, but sensitivity: 91.0% - CAEV and 56.0% MVV (Peterhans et al. 2004). The homologous antigen is capable to detect a higher number of sera with low titre of antibodies even though there are genetic similarities between both viruses (Cruz et al. 2013).

Western blot (WB)

The principle of the method is based on proteins (serum or plasma antibodies) which are kept on a membrane and subsequently separated by electrophoresis. A complex antigen-antibody could be visualized after adding a conjugate (Mussi et al. 2015). Molecular weights of antibodies can be detected by WB such as: matrix protein, inner capsid protein or transmembrane glycoprotein. A viral capsid protein should be easy determined because of its massive presence in the virus, while gp135 protein is present only in extremely positive sera (Rodrigues et al. 2014; Mussi et al. 2015). Unlike ELISA and AGID, WB has greater sensitivity and specificity. For instance, 4 days post-infection, in experimentally infected goats, there could be identified antibodies to protein p28. Two weeks post-infection, there could be detected antibodies to other viral proteins such as: gp125 and gp90. WB could have other advantages such as: few nonspecific reactions; therefore few false-positive results (Mussi et al. 2015).

Several advantages and few disadvantages of WB using in MVV / CAEV diagnostic have been described (Table 3).

Table 3. Advantages and disadvantages for WB identified in this study

Value	Parameter	Source
Advantages	It can be considered as a gold serological method as long as there are divergent results obtained by ELISA and AGID / It can contribute to determine the sensitivity and the specificity of new tests.	Mussi et al. 2015
	It has greater sensitivity and specificity than AGID and ELISA	Mussi et al. 2015
	It is a solution when there are different results after performing ELISA and AGID. Practically, there will be eliminated the real true seropositive animals from the herds.	Mussi et al. 2015
Disadvantages	Western blot involves long time and laborious steps.	Mussi et al. 2015
	WB is not used in the common control programs of SRLV infections.	Mussi et al. 2015

ii. Identification of specific viral genome by molecular techniques

Molecular techniques used in the diagnosis of SRLVs infections include conventional polymerase chain reaction (PCR), real-time polymerase chain reaction (RT-PCR), the loop-mediated isothermal amplification technique (LAMP) and heteroduplex mobility assay (HMA) which can be useful to genotypic characterization of circulating strains in a flock/herd (Ramirez et al. 2013).

Polymerase chain reaction (PCR) and real-time polymerase chain reaction (RT-PCR)

A polymerase chain reaction (PCR) is based on an ongoing repeated process of a specific DNA target segment using specific primers.

According to the OIE recommendations (OIE 2017), PCR is suitable for:

- Contribute to eradication policies;
- Confirmation of clinical cases;
- Prevalence of infection – surveillance.

However, there should be done more validations in order to be performed on a large scale.

In the same time, PCR is not recommended for:

- Population freedom from infection;
- Individual animal freedom from infection prior to movement;
- Immune status in individual animals or populations postvaccination.

All the small ruminant lentiviruses present LTR genes, *gag* gene, *pol* gene and *env* gene. Every gene is responsible of coding different viral elements as follows: gag – nucleocapsid, capsid and matrix; pol – reverstranscriptase, protease and integrase; env – transmembranare and surface glicoproteins (Minguijon et al. 2015).

The target sequences for the design of PCR primers are spread in the SRLV genome including regions as: LTR, *gag*, *pol* and *env* having different values of sensitivity and specificity (Ramirez et al. 2013).

The first conventional protocols PCR were created in the early 1990s. PCR can detect the viral nucleic acids in the infected cell as provirus (DNA) or in exudates which can contain free viral elements (RNA). In order to increase the sensitivity of the method, there are more rounds of amplification such as: nested-PCR or semi-nested PCR. The efficiency of PCR depends on the specificity of the primers, the choice of a proper viral region for amplification and the sensitivity. A low level of viral quantity in the infected animals, a low level of permissive cells and the high genetic heterogeneity could influence the PCR diagnosis of SRLVs infection (Ramirez et al. 2013).

The studies based on the diagnosis of *gag*-PCR technique, present the fact that this method can detect the infection in 88.3% of seropositive goats and sheep previously detected by AGID and 11% of seronegative animals. Comparative studies on the sensitivity of PCR showed that LTR-PCR is lower sensitive than ELISA and AGID. However, PCR presented a specificity of 100% like AGID, while ELISA was less specific (59.0%). The specificity of LTR-PCR remains 100% in different tissues, but the values of sensitivity can be different according to the type of tissue: peripheral blood leukocytes, milk cells or other tissues (Ramirez et al. 2013).

The development of the adapted techniques for specific regions can help to resolve the high rate of mutation in the lentiviral genome. Sequencing SRLV strains is a mandatory step for this area of interest. All the PCR protocols can guide to obtain proper results in a short time (de Andres et al. 2005).

The methods of nucleic acids identification can be used for the detection, quantification and identification DNA provirus MV and CAE using PCR reaction followed by hybridization *in situ*, cloning and/or sequencing the obtained positive result. Moreover, molecular techniques such as PCR, cloning, or sequencing can provide useful information for SRLV strains from a specific region /

country. This aspect can influence what serological technique and what antigen MV or CAEV can be used. Phylogenetic analyses of proviral DNA (MVV and CAEV) suggested that in some areas, the cross barrier species should be happen (OIE 2017).

Advantages and disadvantages of PCR in MVV / CAEV diagnostic have been described (Table 4).

Table 4. Advantages and disadvantages for PCR and RT-PCR identified in this study

Value	Parameter	Source
Advantages	RT-PCR is useful to determine and to quantify the viral nucleic acids in different cells and tissues, although its use is not so common for the routine diagnosis.	Ramirez et al. 2013
	RT-PCR presents a low risk of cross-contamination and has a higher sensitivity using SYBR Green I or TaqMan methods.	Ramirez et al. 2013
	RT-PCR has a higher specificity due to the use of oligonucleotide probes.	Ramirez et al. 2013
	PCR techniques could identify infected animals before the occurrence of the seroconversion / PCR methods can be used in order to verify even seronegative animals / In the programmes of small ruminants farming, it could be useful to check biological samples as blood or semen from seronegative males previously tested by ELISA.	de Andres et al. 2005
	RT-PCR methods are used to determine the infectious status of a herd or to quantify de provirus MVV and CAEV level for an animal.	OIE 2017
	The positive PCR reactions are observed in many seronegative animals because of the delayed seroconversion. Therefore, the serological methods could be less sensitive than PCR.	Ramirez et al. 2013
Disadvantages	RT-PCR can be used for a limited number of samples and its use on a large scale can present obstacles because of the variation in the binding site of primer.	Ramirez et al. 2013
	PCR techniques tend to be lower sensitive than serological ones.	de Andres et al. 2005
	In some studies, there were used peripheral mononuclear blood cells as target cells for PCR. Some PCR molecular reactions can fail because only $1/10^6$ of leukocytes can contain the virus and, practically, the viral load is under the detection limit.	de Andres et al. 2005

Loop-mediated isothermal amplification technique (LAMP)

LAMP was performed by Notomi et al. 2000. This technique uses 4-6 primers which amplify 6-8 regions of the target gene. The amplification and the detection of the target gene could be done in one single step by subjecting the mix (sample, primers, buffer solutions and DNA polymerase) at a constant temperature between 60º-65ºC. This number of primers work simultaneously and the DNA can be amplified 10^9-10^{10} in 15-60 minutes with high efficiency and specificity. It is an alternative method which not requires too much time and the devices should be easy purchased (Balbin et al. 2014). LAMP was also developed for the detection of CAEV targeting p25 protein (Huang et al. 2012).

LAMP is based on the auto-cycling strand displacement DNA synthesis which is performed by the DNA polymerase. LAMP rapidly amplifies DNA with high sensitivity and efficiency in isothermal

conditions. It uses *Bst* DNA polymerase with strand displacement activity, two outer primers (F3 and B3) and two inner primers (FIP and BIP) which recognize six specific regions from the target sequence. Moreover, other two additional primers (LoopF and LoopB) are used in the reaction to accelerate the amplification and to spread the specificity and efficiency (Huang et al. 2012).

LAMP reaction can be done by the incubation of the reagents, specific primers and sample in one single tube. The simple equipment: the heat block and a water bath can be used for this molecular technique in order to detect SRLVs. The property of the test is the possibility to analyse the results by naked eye and this method can be performed outside the laboratory (Balbin et al. 2014).

Advantages of LAMP using in MVV / CAEV diagnostic are described in Table 5.

Table 5. Characteristics for LAMP identified in this study

Value	Parameter	Source
Advantages	LAMP is easy to be done due to the lower costs than PCR.	Huang et al. 2012
	The amplification reaction can be done in a heat block and, practically, a thermo-cycler becomes unnecessary anymore.	Huang et al. 2012
	The identification of positive amplified samples by LAMP can be performed visually based on the turbidity (correlated with DNA level) caused by the magnesium pyrophosphat, a secondary product of the amplification reaction / In the presence of calcein (fluorescent intercalating dye) magnesium pyrophosphate combines magnesium ion and it is generated fluorescence or a colour reaction (from orange to green) visible by naked eye.	Huang et al. 2012; Babin et al. 2014
	The sensitivity and specificity of the method could be due to the presence of primers which recognize specific regions of the sequence / The activity of *Bst* polymerase, activated to a high temperature, consolidates the high specificity of LAMP / The rapidity of the assay is due to the 4-6 primers which work simultaneously to amplify the specific sequence in isothermal conditions.	Balbin et al. 2014
	The primers recognize the sequences and can cause multiple stem-loop DNA structures with some inverted repeats of the target region. These are multiple lines in the agarose gel obtained by electrophoresis.	Balbin et al. 2014

Heteroduplex mobility assay (HMA)

HMA is an efficient solution for genotypic characterization of circulating strains from a herd/flock (Ramirez et al. 2013). The first attempts to perform this method were noted by Germain, Croise & Valas (2008). It could discriminate between among SRLV groups and it was described for V1-V2 region of the *env* gene. Subsequently, HMA was developed for gag region of SRLV genome. Germain, Croise & Valas (2008) mentioned that HMA analysis of SRLV field isolated from mixed flocks were confirmed by genetic and phylogenetic analyses.

iii. Virus Isolation

SRLVs can be propagated *in vitro* from peripheral blood leukocytes and from the target organs by cell cultures obtained from the host's. The most common cells are the fibroblasts derived from the synovial goat membrane and from sheep choroid plexus. Some cellular lines allow the SRLV infection and they provide technical advantages facilitating virus culture (Matsuura et al. 2011). The infection has cytopathic effect and different degrees of cellular lyses. The growth of SRLVs must be confirmed by other methods as: electron microscopy or the amplification of viral DNA (Minguijon et al. 2015).

After the isolation of the virus in tissue cultures, it is important to be made a procedure for manipulation of the virus in the laboratory in a safety manner (Thormar 2013).

The discovery of the fact that Visna virus could be transmitted to sheep by the inoculation of brain filtrates allows the idea that the infectious agent was a filterable virus. The next step was the isolation of the virus in the tissue culture. The fact that subependymal inflammation could be a visible lesion in lateral ventricles, led to the idea that there could be used cultures of healthy subependymal tissue in chicken plasma clots. The fibroblastic cells were obtained from an explant and there were obtained monolayers in the tube tests. The sheep brain extracts with Visna were inoculated in tissue cultures, and after 1-3 weeks the cytopathic effects were visible in cell monolayers, characterised by multinucleated cell syncytia. The passages of tissue culture fluids caused cytopathic changes in several days, leading to the replication of virus in cultures. In other experiments, the extracts of the sheep choroid plexus from lateral ventricles grew in the tissue culture rather than sub-ependymal tissue. The direct explantation of sheep choroid plexus from sheep with Visna was proved to be a more sensitive method than the inoculation of brain extracts for the virus isolation. In order to answer the question if the isolated virus in the tissue culture was the agent of form Visna, the filtrated fluids from the tissue culture were inoculated intracerebrally to young sheep. There were identified as the cause of characteristic symptoms of this form disease. The early pleocytosis was observed together with typical brain lesions belonging to Visna form disease (Thormar 2013).

The demyelination of the white matter of the central nervous system was observed in the Visna form disease. Moreover, there were concerns about the implication of this disease in any human pathology. The most interested in this hypothesis were the persons who were working in the laboratory and with high titres. There were tested primary tissue cultures from human choroid plexus and also cultures from other species as: small ruminants, swine, cats or dogs. The cytopathic effect was observed on small ruminants and bovine cultures, but not in the human cultures (Thormar 2013).

4. DISCUSSION

In order to choose the most useful methods for diagnosis of lentiviral infections, it is important to draw specific directions. First of all, the confirmation of MV and CAE could be certified following the OIE recommendations (ELISA, AGID and PCR methods). Secondly, there must be implemented specific methods and procedures in the confirmed outbreaks. Practically, the eradication of MV and CAE could contain steps which allow the elimination of all the seropositive animals from herds/flocks; methods of monitoring in order to eliminate the certain positive animals; and criteria to declare free SRLV areas/herds/flocks. Last but not least, it should be taken into consideration the surveillance of free SRLV animals knowing to easy ways of contamination and the high level of mutation rate in the viral genome.

The confirmation of MVV and CAEV could contain some steps:

a) The isolation from an alive animal co-cultivating sheep choroid plexus cells or goat synovial membrane cells;

b) The isolation from animal tissues (pulmonary tissues, synovial membranes or mammary tissues) (OIE 2017).

The specificity is an important aspect in any procedure of diagnosis, the more as so the first step is the confirmation of virus presence in biological samples. Once the virus has been isolated, the nucleic acid recognition methods should be taken into consideration (PCR/RT-PCR). Although, the specificity of molecular methods is better known compared to the serological ones. Even so, the most used methods are AGID and ELISA, the letter ones have many properties in SRLV control or eradication (OIE 2017).

An eradication program could be a certain measure in order to eliminate any source of contamination in a specific area. The first objectives of controlling the viral spread could be: the determination of

prevalence by specialized studies or other information sources; the reducing seroprevalence no matter the level and the consolidation of a negative serological status (Peterhans et al. 2004).

The control of SRLV infections is correlated with the knowledge about the epidemiology of these diseases. The control programs started in some countries and in some of them, these diseases were eradicated by early slaughters and replacement of animals by: other animals from free SRLV areas, by seronegative ones or by gradual slaughter of the infected animals as in Finland or in the Netherlands (Reina et al. 2009; Perez et al. 2010).

The eradication of diseases should begin with the elimination of all the seropositive animals from any herd/flock. Due to the fact that ELISA method allows the use of many types of biological samples (serum/milk/semen) could be possible. Barquero et al. 2011 observed that 90.0% agreement was obtained in their results using serum and milk. However, ELISA could have disadvantages because of its lower specificity. Therefore, c-ELISA or AGID method could be used in the detriment of i-ELISA (Hermann-Hoesing 2010; Mussi et al. 2015).

Olech, Murawski & Kuzmak 2019 extended the knowledge regarding to the genetic variability of viral genome. Their study combined serological investigations (indirect ELISA assay) with molecular investigations (PCR and sequencing). In Poland, there are 3 new subtypes which could be identified in small ruminant populations (A16, A17 and A18). The latter one was tentatively designated for the first time (Olech, Valas & Kuzmak 2018; Olech, Murawski & Kuzmak 2019). Such studies should help authorities to start local eradication programs and to improve knowledge regarding to SRLVs.

On a different note, there are a lot of other important serological investigations which could have different properties:

a) The identification of seropositive subjects in order to eliminate them from herds/flocks;

b) The creation of a start point for researchers in order to isolate the viruses and to characterize them;

c) Contribute to declare some countries/regions as SRLV free areas.

Such studies were performed by different types of ELISA/AGID:

- Savic et al. 2012 (21.0% – 1500 sheep by i-ELISA => Serbia);
- Hamza & Ozkan 2017 (65.0% – 100 sheep by i-ELISA => Iraq);
- Giardinis et al. 2015 (43.86% – 57 sheep by i-ELISA => Greece);
- Popescu 2009 (33.1% – 2922 sheep by i-ELISA => Romania, Covasna county);
- Enache et al. 2017 (43.905% – 1145 sheep by i-ELISA => Romania);
- Enache et al. 2018 (66.67% – 98 sheep by i-ELISA => Romania);
- Albayrak et al. 2012 (23.5% – 583 sheep by i-ELISA => Turkey);
- Ali WH et al. 2016 (39.23% – 368 sheep and goats by c-ELISA => Sudan);
- Heinrichs et al. 2017 (3.1% – 725 sheep by c-ELISA => Canada);
- Jesse et al. 2018 (8.8% – 91 goats by c-ELISA => Malaesia);
- Michiels et al. 2018 (9.0% – 555 sheep / 6% – 401 goats by i-ELISA => Belgium);
- Muz et al. 2013 (51.04% – 911 sheep by i-ELISA => Turkey);
- Norouzi et al. 2015 (34.5% – 220 sheep by i-ELISA => Iran);
- Oem et al. 2012 (2.73% – 658 black goats by i-ELISA => South Korea);
- Reina et al. 2010 (positive results belonging to genotype E in >50.0% from herds/flocks of the study – 504 goats and sheep by home-made ELISA - Italy);
- Sanchez et al. 2016 (undetermined – 57 sheep: 25 adult females and 32 foetuses by c-ELISA/i-ELISA/home-made ELISA => Mexico);

- Stonos et al. 2013 (80.4% dairy farms/17.0% meet farms – 607 goats by i-ELISA => Canada);
- Tabet, Hosri & Abi- Rizk 2015 (13.13% – individual/51.67% - farm level – 952 goats by i-ELISA => Liban);
- Tolari et al. 2013 (36.4% sheep/15.9% goats – 434 sheep and goats by home-made ELISA => Iordania);
- Villagra – Blanco et al. 2015 (1.95% – 359 sheep by i-ELISA => Costa Rica);
- Yang et al. 2017 (61.7% – individual/98.5% farm level – 3437 goats by i-ELISA => Taiwan);
- Mihai et al. 2018 (31.86% – 295 French Alpine goats by AGID => Romania).

Nationwide images belonging to a region/country could be obtained after performing regular serological studies.

The animals from a herd / flock should be regularly tested because of the fluctuations levels of antibodies. They could appear because of: (1) the intermittent responses in serological investigations (Mussi et al. 2015); (2) the sensitivity of the serological test/method (de Andres et al. 2005); (3) the genetic factors; (4) the differences of the humoral response in sheep and goats (Rachid et al. 2013); (5) the SRLV genotype which could be determinant or not for viral replication (Mussi et al. 2015).

The costs of AGID method are higher and nowadays, it is used only to confirm / to invalidate the preliminary result, even if the specificity of AGID is higher than any type of ELISA (de Andres et al. 2005; Hermann-Hoesing 2010; Mussi et al. 2015; Panneum & Rukkwamsuk 2017).

It is not enough to identify seropositive animals. A proper result should be obtained performing serological and molecular procedures. Even if there could be identified preliminary seronegative animals in a herd/flock, it is recommended to be tested also by PCR/RT-PCR because of many reasons (seroconversion, too diluted serum samples, fluctuate levels of antibodies, a low level of sensibility and specificity of serological techniques) (Hermann-Hoesing, 2010; Mussi et al. 2015). For instance, Panneum and Rukkwamsuk, 2017 tested 29 dairy goats by different type of methods and they obtained: 69.6% sensitivity, 100% specificity for PCR and 95.7% sensitivity, 83.3% specificity for ELISA. Studies based on *gag*-PCR technique present the fact that the infection could be detected in 88.3% of goats and sheep which are previously seropositive by AGID and in 11.0% of seronegative ones. There were performed comparative studies regarding to the PCR sensitivity and the serological tests (ELISA and AGID) were more sensitive than LTR-PCR. PCR has 100% specificity (as AGID), while ELISA is lower specific (59.0%). LTR-PCR specificity remains 100% in different type of tissues, but the values of sensitivity could be changed according to the type of the tissue (83.5% - peripheral blood leukocytes; 66.7% - milk cells; 88.0% - other tissues). Therefore, a molecular method could be more specific than a serological one, but lower sensitive (Ramirez et al. 2013). Even so, the sensitivity of PCR tests could be improved by performing a nested-PCR reactions, and the specificity of nested-PCR could be validated by hybridization and sequencing (OIE 2017).

The control and elimination of SRLV strains could offer the possibility to obtain the free SRLV – status in every herd/flock. It is not enough to arrive at this level, but it is essential to remain at the same level. Therefore, regular serological tests should be performed and preventive measures should be taken in any exploitation. For instance, an individual program should have the following points:

- MV and CAE should not be seen as individual diseases;
- Seropositive animals and seronegative animals should not be kept in the same area. It is recommendable to be performed regular serological tests and according to the results, they have to be separated;
- The animal transport must be controlled;
- Every test has to be performed by official laboratories (Peterhans et al. 2004).

5. CONCLUSIONS

The diagnosis of SRLV infection still remains a challenge for most of the researchers. On the one hand, it should be seen as the most important step for eradication of the disease, but on the other hand, it should be upgraded permanently because of the viral genome mutations. The serological methods are useful in order to apply the measures of a control program. Moreover, according to their sensitivity and specificity, they should be supplemented by molecular biology techniques to get a certain results. Regular tests performed on the small ruminant herds/flocks could prevent financial losses of farmers and they could create an overview of the herd's heath status.

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