CHAPTER 3.4.9.

ENZOOTIC BOVINE LEUKOSIS

SUMMARY

Description and importance of the disease: *Enzootic bovine leukosis (EBL) is a disease of cattle caused by the bovine leukaemia virus (BLV), a member of the family* Retroviridae. *Cattle may be infected at any age, including the embryonic stage. Most infections are subclinical, but a proportion of cattle (~30%) over 3 years old develop persistent lymphocytosis, and a smaller proportion develop lymphosarcomas (tumours) in various internal organs. Natural infection has also been recorded in water buffaloes and capybaras. Clinical signs, if present, depend on the organs affected. Cattle with lymphosarcomas may die suddenly, or weeks or months after the onset of clinical signs dependent on the location and number of tumours and the tumour's growth characteristics.*

Identification of the agent: *Virus can be detected in the culture supernatant following* in-vitro *culture of peripheral blood mononuclear cells (PBMC) from infected animals, by BLV antigen detection, by polymerase chain reaction (PCR) or by electron microscopy. Proviral DNA can also be detected in PBMC or tumours of infected animals by PCR.*

Serological tests: *The antibody detection methods widely used are the agar gel immunodiffusion (AGID) assay using serum and the enzyme-linked immunosorbent assay (ELISA) using serum or milk. These tests have formed the basis for successful eradication policies in many countries. Other tests, such as radio-immunoassay, can also be used. A number of AGID and ELISA kits are commercially available.*

Requirements for vaccines: *No vaccine against BLV is available.*

A. INTRODUCTION

There may be several causes of lymposarcomas in cattle, but the only definitely known cause is the retrovirus, bovine leukaemia virus (BLV), which causes enzootic bovine leukosis (EBL). The term sporadic bovine leukosis (SBL) is usually reserved for young animals (calves) as well as cutaneous and thymic types of lymphoma, which is defined by the age of the animal affected and the distribution of the tumours. The cause of SBL is not known. There may also be lymphosarcomatous conditions that do not fall into either the SBL or EBL categories, i.e. adult multicentric lymphoma with sporadic occurrence of unknown aetiology. Only lymphomas caused by BLV infection should be termed leukosis or enzootic bovine leukosis (Gillet *et al.*, 2007).

Although animals can become infected with BLV at any age, tumours (lymphosarcomas) are seen typically in animals over 3 years of age. Infections are usually subclinical; only 30–70% of infected cattle develop persistent lymphocytosis, and 0.1–10% of the infected animals develop tumours. Clinical signs will depend on the site of the tumours and may include digestive disturbances, inappetence, weight loss, weakness or general debility and sometimes neurological manifestations. Superficial lymph nodes may be obviously enlarged and may be palpable under the skin and by rectal examination. At necropsy, lymph nodes and a wide range of tissues are found to be infiltrated by neoplastic cells. Organs most frequently involved are the abomasum, right auricle of the heart, spleen, intestine, liver, kidney, omasum, lung, and uterus. The susceptibility of cattle to persistent lymphocytosis, and perhaps also to tumour development, is genetically determined.

There is growing evidence of the role of the virus in immunological dysfunctions and in increased culling rates. Two large-scale investigations estimated the mean decline in milk production per cow among test-positive BLV herds compared with test-negative herds as very similar at 2.5% and 2.7%, respectively (Emanuelsson *et al.*, 1992; Ott *et al.*, 2003). Such findings have been again confirmed in recent studies (Nekouei *et al.,* 2016; Norby *et al.,* 2016). In addition, a 7% lower conception rate in BLV test-positive cows compared with test-negative cows has

been reported. Increased culling rates and a greater susceptibility to other diseases with infectious aetiology, e.g. mastitis, diarrhoea and pneumonia were also demonstrated among test-positive BLV herds (Emanuelsson *et al.*, 1992). Reduced protective immunity following vaccination in BLV infected cattle has also been reported (Frie *et al.,* 2016; Puentes *et al.,* 2016). Therefore despite no obvious clinical signs during the long subclinical infection period, economic losses caused by persistent BLV infections are relevant.

Virus can be detected by *in-vitro* cultivation of peripheral blood mononuclear cells (PBMC). The virus is present in PBMC and in tumour cells as provirus integrates into the DNA of infected cells. Virus is also found in the cellular fraction of various body fluids (nasal and bronchial fluids, saliva, milk). Natural transmission depends on the transfer of infected cells, for example during parturition. Artificial transmission occurs, e.g. by blood-contaminated needles, surgical equipment, gloves used for rectal examinations. Lateral transmission in the absence of these contributory factors is usually slow (Monti *et al.*, 2005). In regions where blood-sucking insects occur in large numbers, especially tabanids, these may transmit the virus mechanically. Viral antigens and proviral DNA can be identified in semen, milk and colostrum of infected animals (Dus Santos *et al.*, 2007; Romero *et al.*, 1983). Natural transmission through these secretions, however, has not clearly been demonstrated.

Although several species can be infected by inoculation of the virus, natural infection occurs only in cattle (*Bos taurus* and *Bos indicus*), water buffaloes, and capybaras. Sheep are very susceptible to experimental inoculation and develop tumours more often at a younger age than cattle. A persistent antibody response can also be detected after experimental infection in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelopes, pigs, goats and buffaloes.

BLV was probably present in Europe during the 19th century, from where it spread to the American continent in the first half of the 20th century. It may then have spread back into Europe and been introduced into other countries for the first time by the import of cattle from North America (Johnson & Kaneene, 1992). Despite the global BLV presence, a number of countries, particularly in Western Europe, are recognised as officially free from BLV infection due to continued surveillance programmes.

Several studies have been carried out in an attempt to determine whether BLV causes disease in humans, especially through the consumption of milk from infected cows (Burmeister *et al.*, 2007; Perzova *et al.*, 2000). There was speculation about the involvement in BLV in human breast cancers (Buehring *et al.*, 2015), however such findings were not confirmed by other researchers (Gillet & Willems, 2016; Zhang *et al.,* 2016). Hence with no conclusive evidence of zoonotic transmission, it is now generally thought that BLV is not a hazard to humans.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of enzootic bovine leukosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[a]						
Virus isolation	–	+	–	+	–	–
PCR	+	++	+	++	+	–
Detection of immune response						
AGID	+++	+++	+++	+++	+++	–
ELISA	+++	+++	+++	+++	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ELISA = enzyme-linked immunosorbent assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

BLV is an exogenous retrovirus and belongs to the genus *Deltaretrovirus* within the subfamily *Orthoretrovirinae* and the family *Retroviridae.* It is structurally and functionally related to the primate T-lymphotropic viruses 1, 2 and 3 (STLV-1, -2, -3) and human T-lymphotropic virus[es] 1 and 2 (HTLV-1 and -2). The major target cells of BLV are B lymphocytes (Beyer *et al.*, 2002; Gillet *et al.*, 2007). The virus particle consists of two positive sense single-stranded RNA that encode for the nucleoprotein p12, capsid (core) protein p24, transmembrane glycoprotein gp30, envelope glycoprotein gp51, and several enzymes, including the reverse transcriptase. Proviral DNA, which is generated after reverse transcription of the viral genome, integrates randomly into the DNA of the host cell where it persists without constant production of viral progeny. When infected cells are cultured *in-vitro*, usually by co-cultivation of PBMC with indicator cells, infectious virus is produced, most readily through stimulation with mitogens.

1.1. Virus isolation

PBMC from 1.5 ml of peripheral blood in ethylene diamine tetra-acetic acid (EDTA) are separated on a ficoll/sodium metrizoate density gradient, cultured with 2×10^6 fetal bovine lung (FBL) cells, and subsequently grown for 3–4 days in 40 ml of minimal essential medium (MEM) containing 20% fetal calf serum. Virus causes syncytia to develop in the cell monolayer of the FBL cells. Short-term cultures can be prepared by culturing PBMC in the absence of FBL cells in 24-well plates for 3 days (Miller *et al.,* 1985). The p24 and gp51 antigens can subsequently be detected in the supernatant of the cultures by radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot or agar gel immunodiffusion (AGID), and the presence of the BLV particles or BLV-provirus can be demonstrated by electron microscopy and by PCR, respectively.

1.2. Nucleic acid detection by polymerase chain reaction (PCR)

The use of the polymerase chain reaction (PCR) to detect BLV provirus has been described by various workers (Fechner *et al.*, 1996; Rola-Łuszczak *et al.,* 2013). Primers constructed to match the *gag, pol* and *env* regions of the genome have all been used with variable success. So far, real-time PCR is the most rapid and sensitive method. The methods described are conventional PCRs based on primer sequences from the *env* gene, coding for gp51 and a real-time method based on detection of the pol gene. The technique is restricted to those laboratories that have the facilities for molecular virology, and the usual precautions and control procedures must be in place to ensure validity of the test results (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

PCR is mainly used as an adjunct to serology for confirmatory testing. The detection of BLV infection in individual animals by PCR can be useful in the following circumstances:

i) Young calves with colostral antibodies;

ii) Tumour cases, for differentiation between sporadic and infectious lymphoma;

iii) Tumour tissue from suspected cases collected at slaughterhouses;

iv) New infections, before development of antibodies to BLV;

v) Cases of weak positive or uncertain results in ELISA;

vi) The systematic screening of cattle in progeny-testing stations (before introduction into artificial insemination centres);

vii) Cattle used for production of vaccines, ensuring that they are BLV free.

1.2.1. Sensitivity and reliability of the method

i) *Analytical sensitivity*

Although the PCR assay has a theoretical sensitivity of one target molecule, in practice the analytical sensitivity may be approximately five to ten target molecules of proviral DNA.

ii) *False-positive results*

The high sensitivity of the nested PCR may cause problems of false-positive results due to contamination between samples (Belak & Ballagi-Pordany, 1993). To minimise this, several special procedures are adopted throughout the protocol, such as the use of laminar air-flow hoods, separate rooms for different steps of the procedure, new gloves or the use of special tube openers for each individual assay and negative controls (e.g. water blanks).

iii) *False-negative results*

It should be noted that only a small proportion of the PBMC can be infected, thus limiting the sensitivity of the assay. The presence of inhibitory substances in some samples may cause false-negative results. To detect this, at least one positive control is used on every test run. In addition, assays can use internal controls (mimics) that are added to each sample. The mimic is a modified target molecule that is amplified with the same primers as the real target, but that generates a PCR product with different size, which can be visualised by agarose gel electrophoresis. The mimic is added at a low concentration that favours the amplification of the real target (Ballagi-Pordany & Belak, 1996). However, it is possible for the mimic to compete with the true target. It may therefore be necessary to analyse each sample with or without the mimic.

1.2.2. Sample preparation

PBMC are separated from EDTA blood samples by using a density separation centrifugation method. Alternatively buffy coat may be used, or even whole blood, e.g. where samples have been frozen.

Tumours or other tissues should be homogenised to a 10% suspension.

1.2.3. DNA extraction

Purification of total DNA is a prerequisite for achieving optimal sensitivity. Various purification methods are commercially available and suitable for the assay.

Special precautions should be taken during all steps to minimise the risk of contamination (Belak & Ballagi-Pordany, 1993).

1.2.4. Nested PCR procedure

Several PCR protocols for the detection of BLV provirus sequences have been published; as an example, an assay developed by Fechner *et al.,* 1996 is described in detail. The BLV region used as target is the env gene, encoding for gp51 protein. The sequence used for designing the primers is available from GenBank, accession No. K02120.

1.2.4.1. Method developed by Fechner *et al.* (1996)

i) Primer design and sequences

Oligo	Env-Sequence (5'–3')	Position
BLV-env-1	TCT-GTG-CCA-AGT-CTC-CCA-GAT-A	5032–5053
BLV-env-2	AAC-AAC-AAC-CTC-TGG-GAA-GGG	5629–5608
BLV-env-3	CCC-ACA-AGG-GCG-GCG-CCG-GTT-T	5099–5121
BLV-env-4	GCG-AGG-CCG-GGT-CCA-GAG-CTG-G	5542–5521

The BLV-env-1/BLV-env-2 PCR-product size is 598 bp. The BLV-env-3/BLV-env-4 PCR-product size is 444 bp

ii)   Reaction mixtures

Reaction solutions are mixed (except DNA sample) before adding to the separate reaction tubes. One negative control (double distilled H_2O) per five samples, and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one.

The first PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 5 µl (10×) PCR buffer, 20 µl DNA (~1 µg of DNA), 1.25 µl each of the env-specific primers BLV-env-1 and BLV-env-2 (20 pmol/µl), 0.15 dNTP (each 25 mM), 3 µl $MgCl_2$ (25 mM), 0.25 µl Taq polymerase (1.25U), and 19.1 µl of distilled H_2O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

The nested PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 3 µl PCR product of the first PCR, 5 µl (10×) PCR buffer, 1.25 µl each of the env-specific primers BLV-env-3 and BLV-env-4 (20 pmol/µl), 0.15 dNTP (each 25 mM), 0.25 µl Taq polymerase (1.25U), and 36.1 µl of distilled H_2O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

iii)   Laboratory procedure

Mix PCR-reagents for the first or nested PCR and use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Heat the thermoblock to 94°C. Place samples in the thermoblock and start the PCR-programmes accordingly.

iv)   Agarose gel electrophoresis

Load approximately 10 µl of the nested PCR products with 20 µl loading buffer on a 2% agarose gel containing 0.01% ethidiumbromide (or alternative, safer stains for visualising PCR products). Using 0.5 × Tris/borate/EDTA (TBE) buffer, electrophoresis is performed with 90 mA for 2 hours. To control the size of the amplification products, a 100 bp ladder is recommended. Analysis of PCR products is done by UV illumination.

v)   Interpretation of the results

a)   Positive samples should have PCR products of the expected size (444 bp), similar to the positive control.

b)   Negative samples should have no PCR products of the expected size (444 bp).

c)   The assay must be repeated if the positive control remained negative, or if the negative water controls are positive.

vi)   Confirmatory testing

For confirmatory identification, the PCR products can be sequenced, hybridised to a probe, or analysed by restriction fragment length polymorphism (RFLP) analysis (Fechner *et al.*, 1997).

1.2.5. Real-time PCR procedure

Several real-time PCR protocols for the detection of BLV provirus sequences have been published: the Rola-Łuszczak *et al.* (2013) method is described in detail here as an example. The BLV region used as target is the pol gene. The sequence used for designing the primers is available from GenBank, accession No. K02120.

1.2.5.1. Method developed by Rola-Łuszczak *et al.* (2013)

i) Primer design and sequences

Oligo	Pol-Sequence (5'–3')	Position
MRBLVL	CCT-CAA-TTC-CCT-TTA-AAC-TA	2321-2340
MRBLVR	GTA-CCG-GGA-AGA-CTG-GAT-TA	2421-2440
MRBLV probe	6FAM GAA-CGC-CTC-CAG-GCC-CTT-CA BHQ1	2341-2360

PCR-product size: 120 bp

ii) Reaction mixtures

Reaction solutions are mixed (except DNA sample) before adding to the separate reaction tubes. One negative control (double-distilled H_2O) per five samples and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one.

The reaction mixture for each PCR test contains 12.5 µl of 2 × PCR master mix, 0.4 µM of each of the primers and 0.2 µM of the specific BLV probe and 500 ng of extracted genomic DNA, using a final reaction volume of 25 µl. Amplification was performed according to the following conditions: initial incubation and polymerase activation at 95°C for 15 minutes, denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds through 50 cycles.

iii) Laboratory procedure

Mix PCR-reagents and use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Place samples in the thermoblock and run at appropriate parameters.

iv) Interpretation of the results

a) Positive samples are those with Ct less than or equal to 40.95.

b) Negative samples are those without a Ct value or those with a value greater than 40.96.

c) The assay must be repeated if the positive control remained negative, or if the negative water controls are positive. Samples on the borderline of cut-off (i.e. Ct of 40) should be retested and confirmed.

v) Confirmatory testing

For confirmatory identification, the PCR products can be sequenced.

2. Serological tests

Infection with the virus in cattle is lifelong and gives rise to a persistent antibody response. Antibodies can first be detected 3–16 weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear. There is no way of distinguishing passively transferred antibodies from those resulting from active infection. Active infection, however, can be confirmed by the detection of BLV provirus by the PCR. Passive antibody tends to protect calves against infection. During the periparturient period, cows may have serum antibody that is undetectable by AGID because of an antibody shift from the dam's circulation to her colostrum. Therefore, when using the AGID test, a negative test result on serum taken at this time (2–6 weeks pre- and 1–2 weeks post-partum) is not conclusive and the test should be repeated. However, the AGID can be performed at this stage with first-phase colostrum.

The antibodies most readily detected are those directed towards the gp51 and p24 of the virus. Most AGID tests and ELISAs in routine use detect antibodies to the glycoprotein gp51, as these appear earlier. Methods of performing these tests have been published (Dimmock *et al.,* 1987; European Commission, 2009). ELISAs are usually more sensitive than the AGID tests.

Weak positive and negative WOAH reference sera for use in ELISA are available in freeze-dried, irradiated form from the WOAH Reference Laboratory in Germany[1]. The calibration of these sera is based on the accredited WOAH reference serum, named E05, which has been validated against the former reference serum E4 by different AGID and ELISAs.

2.1. Enzyme-linked immunosorbent assay

Either an indirect or blocking ELISA may be used. Assays based on both of these are available commercially; different kits may be required for serum or milk samples. Some ELISAs are sufficiently sensitive to be used with pooled samples. ELISAs are carried out in solid-phase microplates. BLV antigen is used to coat the plates either directly or by the use of a capture polyclonal or monoclonal antibody (MAb). The antigen is prepared from the cell culture supernatant of persistently BLV-infected cell lines. Fetal lamb kidney (FLK) cells are most commonly used for commercial tests (Van der Maaten & Miller, 1976). Since 2004, a new BLV-producing cell line, PO714, which is free from other viral infections and contains a provirus of the Belgian subgroup, has been made available (Beier *et al.*, 2004). The antigen is used at a predetermined dilution (e.g. 1/10) in phosphate buffered saline. In kit form, the plates are sometimes purchased precoated. Some preservatives may be added to milk samples to prevent souring. Preserved samples will not usually deteriorate significantly if stored for up to 6 weeks at 4°C.

2.1.1. Blocking enzyme-linked immunosorbent assay – serum ELISA

The following method is suitable for antibody detection in single or pooled serum samples.

2.1.1.1. Test procedure

i) Coating the plate

All wells are coated with BLV antibody, prediluted in coating buffer (100 µl/well), the plate is sealed and incubated for 18 hours at 4°C. A wash cycle (standard wash) is performed, which is three washes filling wells to the top, with a 3-minute soak in between each wash, and then the plate is blotted. BLV antigen is added, prediluted in wash buffer (100 µl/well), the plate is sealed and incubated for 2 hours at 37°C. A standard wash cycle is performed.

ii) Preparation and addition of samples and controls

The positive and negative control sera are prediluted (1/2) in wash buffer and the solution is added to four wells per control (100 µl/well). For testing pooled samples, 80 sera may be bulked then diluted (1/2) using wash buffer and the solution is added to two wells (100 µl/well) per sample. Single samples should be diluted 1/100 using wash buffer and the solution added to two wells (100 µl/well) per sample. After plating out the samples, the plate is sealed and incubated for 18 hours at 4°C. A brief wash is performed by filling the wells and immediately emptying them.

iii) Preparation and addition of conjugates and substrate

Prediluted biotinylated antibody is added (100 µl/well) to all wells – predilute using wash buffer + 10% fetal calf serum – the plate is sealed and incubated on a rocking table for 1 hour at 37°C. A standard wash is performed as described earlier. The peroxidase-conjugated avidin is prediluted in wash buffer and the solution is added to all wells (100 µl/well). The plate is sealed and incubated on a rocking table for 30 minutes at 37°C. A standard wash is performed. 100 µl orthophenylamine diamine substrate is added to all wells, the plate is covered and left in the dark for 9 minutes. The reaction is stopped with 100 µl of 0.5 M sulphuric acid per well.

2.1.1.2. Reading and interpretation of results

The plate reader is blanked on air and the absorbance is read at 490 nm. For dual wave-length readers a reference filter between 620 nm and 650 nm is used. Results are read within 60 minutes after the addition of stop solution.

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1   https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

The absorbance of the negative control should be about 1.1 ± 0.4; if the absorbance is below 0.7, the colour development time in step iii above (preparation and addition of conjugates and substrate) should be increased. Conversely, the time should be shortened if the absorbance is above 1.5. The absorbance of the positive control should be less than the absorbance of the negative control × 0.25.

A sample is positive when the absorbance of each of the two test wells is identical with or less than the mean absorbance of the four negative wells × 0.5.

A sample is negative when the absorbance of each of the two test wells is identical with or higher than the mean absorbance of the four negative control wells × 0.65.

For samples giving values between the absorbance of the negative control × 0.5 and × 0.65, it is recommended to retest the animal, using a sample taken 1 month later.

2.1.1.3. Sensitivity of the enzyme-linked immunosorbent assay

The sensitivity of pooled milk ELISAs can be evaluated using the WOAH weak positive and reference sera. Assays should give a positive result on WOAH reference sera E05 diluted in negative milk 250 times more than the number of individual milks in the pool (EU Directive 88/406). For example, for pools of 60 milks, E05 should be diluted 1/250 × 60 = 1/15,000. For individual milk samples the positive WOAH reference sera E05 diluted 1/250 in negative milk must be positive.

Where pooled serum samples are tested, the WOAH reference serum E05 must test positive at a dilution 10 times higher than the number of individual animals in the pool. For example, for a pool of 50 individual samples, the WOAH reference serum diluted 1/500 in negative serum should give a positive result. In assays where serum samples are tested individually, WOAH reference serum E05 diluted 1/10 must be positive.

For some ELISA kits, a positive result is not recommended as the sole determinant of individual animal disease status; verification by a secondary method is recommended.

2.1.2. Indirect enzyme-linked immunosorbent assay – Milk ELISA

The following method is suitable for antibody detection in pooled milk samples.

2.1.2.1. Controls

Strong positive, weak positive, negative milk and diluent controls should be included in each assay. A strong positive control should be prepared by diluting the WOAH reference serum E05 1/25 in negative milk. A weak positive control should be prepared by diluting, in negative milk, the WOAH reference serum E05 25 times the number of individual milk samples in the pool under test. The milk used for diluting the WOAH reference serum controls should be unpasteurised, cream free and preserved.

2.1.2.2. Example test procedure

i)   Milk samples must be stored, undisturbed in a refrigerator until a definite cream layer has formed (24–48 hours), or alternatively, centrifuged at 2000 rpm for 10 minutes, the cream layer should be removed prior to testing.

ii) A BLV antigen and a control negative antigen are precoated in alternate columns in the plate. 100 µl of test sample is added to 100 µl wash buffer in the plate to make a 1/2 dilution, adding to two control antigen wells and two BLV antigen wells.

iii) The plate is sealed and mixed on a shaker.

iv) The plate is incubated between 14 and 18 hours at 2–8°C.

v)   300 µl per well of wash diluent is added and discarded, and then 200 µl per well wash diluent is added, shaken for 10 seconds and discarded. Finally, 300 µl of wash diluent is added and soaked for 3 minutes and discarded.

vi)   200 µl per well of anti-bovine IgG-horseradish peroxidase affinity-purified conjugate diluted in wash diluent is added and the plate is incubated for 90 minutes at room temperature.

vii) The plate is washed by adding 300 µl of wash diluent per well; this is then discarded and a further 300 µl of wash diluent is added. This is left to soak for 3 minutes and discarded. Steps vi and vii are repeated.

viii) 200 µl of ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) substrate (prewarmed to 25°C) is added and the plate is incubated for 20 minutes at room temperature in the dark. The reaction may be stopped by adding 50 µl of stopping solution.

2.1.2.3. Reading and interpreting the results

The plate reader is blanked on air and the absorbance is read at 405 nm. All microplate wells must be read within 2 hours of addition of stopper. The absorbance readings of the wells containing negative antigen are subtracted from the readings of wells containing the positive antigen. The two net absorbance values for each test sample should be averaged. The same applies for the replicate weak positive controls. Replicates should be within 0.1 absorbance units of each other.

For the test to be considered valid, the averaged net absorbance of the weak positive (WP) controls should be 0.2–0.6 absorbance units. The net absorbance of the strong positive control should be >1.0 absorbance units. The net absorbance of the negative and diluent controls should be less than the lower limit of the inconclusive range.

Assuming that the above criteria are met:

i)   Test samples are positive if their net absorbance value is greater than or equal to that of the WP control.

ii)   Test samples are inconclusive if their net absorbance value is 75% or less of the net absorbance value of the WP control.

i.e. if the WP control net absorbance = 0.40

then the lower limit of the inconclusive range = 0.40 × 0.750 = 0.30

the inconclusive range in this example would be 0.30–0.39

and samples of ≥0.40 are considered positive.

iii) Test samples are negative if their net absorbance value is less than the lower limit of the 'inconclusive' range (<0.30 in the example).

2.2. Agar gel immunodiffusion

The AGID test is a specific, but not very sensitive, test for detecting antibody in serum samples from individual animals. It is, however, unsuitable for milk samples (except first colostrums) because of lack of specificity and sensitivity. The AGID is simple and easy to perform and has proven to be highly useful and efficient as a basis for eradication schemes. Reference sera are included with commercial AGID test kits.

2.2.1. Agar gel

A 0.8–1.2% solution of agar or agarose is prepared in 0.2 M Tris buffer, pH 7.2, with 8.5% NaCl. One method of preparing the agar is to dissolve 24.23 g of Tris methylamine in 1 litre of distilled water and adjust to pH 7.2 with 2.5 M HCl. Sodium chloride (85 g) is dissolved in 250 ml Tris/HCl and made up to 1 litre. Agarose (8 g) is added and the mixture is heated in a pressure cooker or autoclave at 4.55 kg/sq. cm for 10 minutes. The mixture is dispensed in 15 ml aliquots, which can be stored at 4°C for up to approximately 6 weeks.

2.2.2. Antigen

The antigen must contain specific glycoprotein gp51 of BLV. Antigen is prepared in a suitable cell culture system, such as permanently infected FLK cell monolayers. The cells used to produce the BLV antigen should be free from noncytopathic bovine viral diarrhoea virus and of bovine retroviruses, bovine immunodeficiency-like virus (lentivirus), and bovine syncytial virus (spumavirus). After 3–4 days' culture at 37°C, the growth medium is replaced with maintenance medium. The cells are harvested after 7 days using standard trypsin/versene solution. The cell suspension is centrifuged at 500 g for 10 minutes. Cells are resuspended in growth medium; 30% of the cells are returned to the culture vessel and the remainder is discarded. All culture supernatants are collected. The supernatants are concentrated 50–100-fold by available methods. This can be done by concentration in Visking tubing immersed in polyethylene glycol, or by precipitation with ammonium sulphate followed by ultrafiltration, or by precipitation in polyethylene glycol followed by desalting and size separation on a polyacrylamide bead column. The antigen contains gp51 predominantly, but may also contain p24.

The antigen may be standardised for glycoprotein gp51 by titration against the WOAH reference serum E05 as follows: a twofold dilution of the antigen preparation is made. The highest dilution that, when tested against undiluted WOAH reference serum E05, gives a precipitation line equidistant between the antigen and the serum will contain one unit. Two units of antigen are used in the test.

2.2.3. Known positive control serum

The positive control serum comes from a naturally or experimentally infected animal (cattle or sheep). The precipitation line formed should be a sharp distinct line midway between the antigen and the control serum wells. A dilution of the control positive serum that gives a weak positive result should be included in the test as an indicator of the test's sensitivity.

2.2.4. Known negative control serum

Serum from uninfected animals (cattle, sheep) is used.

2.2.5. Test sera

Sera from any species of animal are suitable.

2.2.6. Test procedure

i) The agar is melted by heating in a water bath and poured into Petri dishes (15 ml per Petri dish of diameter 8.5 cm). The poured plates are allowed to cool at 4°C for about 1 hour before holes are cut in the agar. A punch is used that cuts a hexagonal arrangement of six wells round a central well. Various dimensions of wells can be used; one satisfactory pattern has been produced using wells of 6.5 mm in diameter with 3 mm between wells. For best results, agar plates are used the same day that they are poured and cut.

ii) Antigen is placed in the central wells of the hexagonally arranged patterns. Test sera are placed alternately with positive control serum in the outer wells. There should be one control pattern per plate with positive control serum, weak positive control serum and negative control serum in the place of test sera.

iii) The test plates are kept at room temperature (20–27°C) in a closed humid chamber, and read at 24, 48 and 72 hours.

iv) *Interpretation of the results:* A test serum is positive if it forms a specific precipitation line with the antigen and forms a line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and if it does not bend the line of the control serum. Nonspecific lines may occur; these do not merge with or deflect the lines formed by the positive control. A test serum is a weak positive if it bends the line of the control serum towards the antigen well without forming a visible precipitation line with the antigen; the reaction is inconclusive if it cannot be read either as negative or positive. A test is invalid if the controls do not give the expected results. Sera giving inconclusive or weak positive results can be concentrated and retested.

C. REQUIREMENTS FOR VACCINES

Despite advances in research on experimental vaccines, there is as yet no commercially available vaccine for the control of EBL.

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NB: There are WOAH Reference Laboratories for enzootic bovine leukosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratory for any further information on
diagnostic tests and reagents for enzootic bovine leukosis

NB: First adopted in 1991. Most recent updates adopted in 2018.