Full Length Research Paper

Selection of ligand peptides with the ability to detect antibodies in enzootic bovine leukosis

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Peptides present in phages were selected using phage display technology and immunoassays to find out the antigenic mimetics of immunodominant epitopes of bovine leukosis virus (BLV). The use of antigenic mimetics may result in the enhancement of the sensitivity and specificity of the serologic diagnosis of enzootic bovine leukosis (EBL), contributing directly to disease control. The selections enabled the choice of clones which can be used as potential antigens in the diagnoses of diseases. The synthetic peptide produced from the selected sequences may be considered as an alternative for antigens in the serologic diagnosis of enzootic bovine leukosis.

Key words: Diagnostic, antigens, enzootic bovine leukosis (EBL), phage display.

INTRODUCTION

The bovine leukosis virus (BLV) is a member of the genus Deltaretrovirus and an etiologic agent of enzootic bovine leukosis (EBL). The EBL shows a worldwide distribution and infects more frequently, the dairy cattle (Modena et al., 1984; Castro et al., 1992; Camargos et al., 2002). The economic damages caused by BLV infection include restrictions on the trading of the animal or its products such as semen and embryos between countries (Burny et al., 1980; Gutiérrez et al., 2009). Other economic damages include bovine deaths due to lymphosarcoma, carcass rejection at the slaughterhouse, and productive and reproductive decrease in infected animals (Camargos, 2005). BLV infection also decreases milk production and causes other losses to farmers (Da et al., 1993).

As there are no protective vaccines against the virus, the control and eradication of EBL are carried out through diagnosis, segregation and slaughtering of the bovine carriers. For this reason, the sensitivity of the diagnostic technique utilized is regarded as a critical factor to prevent the spread of the disease in the flocks (Trono et al., 2001).

Like other retroviruses, the BLV presents envelope glycoprotein gp51 (env) and core viral p24 (gag) as immunodominant proteins *in vivo* (Miller et al., 1981; Portetelle et al., 1989; Callebaut et al., 1993; Gatei et al., 1993; Willems et al., 1995; Doménech et al., 1997). The proteins gp51 and p24 are frequently used in diagnostic tests for the detection of specific BLV virus antibodies (Miller et al., 1981; Kittelberger et al., 1999). The most utilized tests for EBL diagnosis are agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA), but several factors may contribute to the inconsistency of results such that a need arises for the results to be confirmed by other more sensitive and specific techniques (Van Der Maaten, 1974; Portetelle et al., 1989; Reichel et al., 1998; Soutullo et al., 2001; Trono et al., 2001; De Giuseppe et al., 2004; Leroux et al., 2004; Paré and Simard, 2004; Alvarez et al., 2007; Lim et al., 2009). Techniques such as western blot and polymerase chain reaction (PCR) have been

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developed and used as complementary or confirmatory diagnostic tools for EBL and other retroviruses (Payne et al., 1989; Kim and Casey, 1992; Langemeier et al., 1996; Johnson et al., 1998; Reichel et al., 1998; Kittelberger et al., 1999; Llames et al., 2000; Trono et al., 2001; Choi et al., 2002; Alvarez et al., 2007; More et al., 2008).

Even though many assays have been performed to improve antigen production, the traditional cell culture method of production which involves the use of strain FLK/BLV is still the mostly used method in antigen production for commercial diagnostic kits (Van der Maaten et al., 1974; Beier et al., 2004; Lim et al., 2009). The cell strain FLK used in BLV antigen production is also known for being infected with bovine diarrhea virus (Bolin et al., 1994; Dees et al., 1994; Beier et al., 2004; Lim et al., 2009) and this contamination may result in further problems with diagnoses related to the specificity of the reactions (Beier et al., 2004). Other problems common with the antigen productions techniques which utilize cell cultures is that the growth protocols usually includes the addition of bovine fetal serum. This serum contributes to the components that may cause unspecific reactions. Besides these factors, the presence of cell proteins that are not eliminated or that are co-purified during the obtention of BLV proteins may interfere with the viral proteins (Llames et al., 2000), thus, producing mistaken results in EBL diagnosis.

Advances in vaccines and diagnostic tests development require a deeper understanding of pathogen antigenic structures and immunogenic proteins. The synthetic peptides have been utilized for mapping the antigenic sites in several viral systems (Neurath et al., 1990; Callebaut et al., 1991; Ball et al., 1992), presenting a wide potential when utilized as antigens in diagnostic techniques and as efficient vaccine components in the induction of the immunologic memory (Ball et al., 1992; Kabeya et al., 1996; Soutullo et al., 2001, 2005). Peptides selected by a phage display-technology such as antigenic mimetic of natural epitopes may be used to immunize animals. Some of them are capable of inducing new antibodies that present cross-reaction with the natural epitopes which are considered as mimetic immunogenic (Cardoso et al., 2009). In order to avoid the problems presented by use cell culture in the production of antigens from, antigenic mimetics and immunogenic capabilities are utilized as the foundations which help to find epitopes that are used in the development of vaccines and diagnostic techniques for many diseases (Folgori et al., 1994; Pasqualini et al., 1995; Sioud et al., 1996).

In this study, the peptides present in phages were selected from phage display libraries and from immunoassays. These peptides were regarded as antigenic mimetics of the immunogenic epitopes of BLV proteins, having the potential for being used as antigens and in synthetic peptide production for the development of diagnostic techniques for EBL.

METHODOLOGY

Purification of IgG from bovine sera

Bovine sera samples were tested for BLV infection in the Retroviruses Laboratory of Veterinary School, Universidade Federal de Minas Gerais (UFMG), using agar gel immunodiffusion (AGID) test as recommended by Miller and Van der Maaten (1977) with the antigen gp51 produced in FLK cells. In order to obtain total IgG from a positive pool sera for EBL from 20 animals and total IgG from a negative pool sera from 20 different animals, a HP column Hitrap Protein G in ÄKTA system was used following the manufacturer's instructions (GE Healthcare®). The total IgG obtained from 3 ml of each pool were neutralized after the elution of the column with buffer 1 M Tris-HCl of pH 8.0, lyophilized, frozen and diluted in deionized\sterilized water.

The concentrations of purified IgG and gp51 protein were estimated by spectrophotometry at 280 nm and by using the Bradford method (Bradford, 1976). The samples were submitted to electrophoresis in 16% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie-blue R-250. The molecular weight marker Bench Mark Protein Ladder (Invitrogen) was applied to the gel for comparison.

Biological selection (biopanning)

The Ph.D. -12TM library - New England BioLabs® was used for the selection of peptide sequences bound to purified IgG from BLV-positive animal sera. This library contains random and linear peptides which consist of 12 amino acids and presents about 2.7×10^9 possible sequences.

The positive purified IgG were utilized as target for peptide selection. The wells of microtitration plate (MaxiSorpTM - Nunc) were adsorbed using 150 µl of IgG positive for BLV at the concentration of 100 µg/ml in 0.1 M NaHCO$_3$, pH 8.6. Phages at the concentration of 4.0×10^{10} of the original library were diluted in 90 µl of Tris-buffered saline Tween-20 (TBST) solution and added to the wells adsorbed with the targets (total positive IgG). Three selection cycles were performed for the enrichment of the phages containing the linking peptides. From the 2nd cycle, the washing buffer stringency increased from 0.1 to 0.5% with Tween-20 in all the washings. In the 3rd cycle, 15 washings were carried out, and after this step, the phages containing the linking peptides were withdrawn.

The procedures for biopanning, amplification, precipitation and titration of the phages were performed according to the instructions given by the manufacturer (New England BioLabs®).

Sequencing

For the sequencing reaction, 500 ng of template DNA (DNA of each phage), 5 pmol of -96 gIII primer (5'-OH CCC TCATAG TTA GCG TAA CG-3' - Biolabs) and Premix (DYEnamic ET Dye Terminator Cycle Kit – Amersham Biosciences) were used. The reaction was carried out in a plate thermocycler (MasterCycler – Eppendorf).
The reading of the sequencing was carried out in a MegaBace 1000 (Amersham Biosciences) automatic sequencer. The DNA sequences obtained from the automatic sequencer were processed by the appropriate software equipment (Sequence Analyzer, Base Caller, Cimarron 3.12, Phred 15).

Translation and analysis of sequences

From the DNA sequences, analysis of deduction *in silico* were performed for the peptide sequences using the software DNA2PRO

(available at https://relic.bio.anl.gov/programs.aspx). The software AAFREQ (https://relic.bio.anl.gov/aafreqs.aspx) was used for calculating the frequency and diversity of amino acids inside the population of selected peptides. The search for similarities between the peptides selected was accomplished using the software Clustal W (version 18.1 available at www.ebi.ac.uk/clustalW/), through multiple alignments (data not shown).

Wild phage

The phage M13KE (New England BioLabs®) was used in the immunoassays as negative control for the reactions. This phage does not present peptides fused to viral capsid. According to the manufacturer's information, this clone may be useful as control or in titration of phage stocks. The procedures of amplification, precipitation and titration of the wild phage were the same as those used for the phages selected from the libraries.

Phage-ELISA (IgG)

High affinity plates (MaxiSorpTM - Nunc) were adsorbed with positive and negative purified IgG. 44 ELISA plate wells were adsorbed with 1 μg of positive IgG diluted in a bicarbonate buffer (0.1 M NaHCO$_3$, pH 8.6) and the other 44 wells of the same plate were adsorbed with 1 μg of negative IgG. In each half of the microplate, four wells were used as controls without IgG adsorption.

The plate was incubated for 18 h at 4°C under agitation, washed twice in 0.05% PBST and blocked with PBS solution with 5% skimmed powdered milk for 1 h at room temperature. After that, the plates were washed three times.

The phage samples were precipitated with polyethylene glycol (PEG)/NaCl and with concentrations estimated by UV absorbance spectrophotometry at the wave lengths of 269 and 320 nm, they were diluted to the concentration of 1x10^{11} phages in the PBS solution with 1% skimmed powdered milk. Each phage sample was added in duplicate to the wells with positive and negative IgG adsorbed and incubated for at least 1 h at 37°C. The wild phage (negative control) was added to the adsorbed and non-adsorbed wells with the IgG according to the layout of plates.

The plates were washed six times with 0.05% phosphate buffered saline with Tween 20 (PBST) and incubated for 1 h at 37°C with anti-M13-peroxidase-conjugated (1:5000, Sigma) in PBS solution with 1% skimmed powdered milk. By the end of the incubation, the plates were washed six times with 0.1% PBST solution and the reaction was initiated by the addition of 0.03% H$_2$O$_2$ and o-phenylendiamin (OPD) 1 mg/mL in 0.1 M citrate-phosphate with pH 5.0. The reaction was interrupted by adding sulphuric acid (2 N) and the optical density (OD) was determined by plate spectrophotometry (Titertek Multiskan Plus, Flow Laboratories, USA) at 490 nm.

The cut-off value was calculated based on the OD obtained for the wild phage (cut-off = wild phage average value + 2X the standard deviation value for wild phage). The ELISA index was calculated by taking the OD average of the duplicates divided by the cut-off value. Values greater than one were considered positive.

In order to verify the difference between the ELISA indexes obtained from the reactivity of positive and negative IgG clones, the paired t-test was performed with the software STATA 10.0.

Dot blot (IgG)

The Dot blot was performed to verify the reactivity of the phages with positive and negative IgG. Nitrocellulose membranes (0.2 μm, Amersham Pharmacia) were sensitized with each phage in the concentration of 10^{11} pfu. The wild phage and 1 μg of gp51 were

also sensitized. After the sensitization, the membranes were blocked with PBS with 5% skimmed powdered milk for 1 h under agitation at room temperature and washed once in 0.05% Tris-buffered saline Tween-20 (TBST) .

Positive and negative IgG were used as primary antibodies in the concentration of 400 μg diluted in block solution and incubated within the membranes for 1 h under agitation at room temperature. After five washings with Tris-buffered saline Tween-20 (TBST) 0.05%, the reaction was initiated by adding 3,3'-diamino-benzidine (DAB) (Sigma Chemical) for visual reading and interrupted with deionized H$_2$O.

Western blot (IgG and sera)

Western blot (WB) was performed to confirm if the peptides fused to protein III of the selected clones were immunoreactive with positive IgG and antibodies from positive sera for BLV, and capable of differentiating the positive and negative samples.

For the WB assays, an electrophoresis in 16% SDS-PAGE was performed with each selected phage (1x10^{12} pfu), wild phage (negative control, 1x10^{12} pfu), and gp51 (10 μg). The samples were electrotransferred for 2 h in 280 mA at 4°C to nitrocellulose membranes (0.2 μm, Amersham Pharmacia) which were blocked by TBS solution with 5% skimmed powdered milk for 1 h under agitation at room temperature. After a wash with 0.05% Tris-buffered saline Tween-20 (TBST) solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween 20), positive and negative IgG (400 μg), and positive and negative sera samples for BLV (1:500) were diluted in blocking solution and incubated in the membranes for 1 h under agitation at room temperature. The membranes were washed three times in 0.05% Tris-buffered saline Tween-20 (TBST) solution. Secondary antibody anti-IgG bovine conjugated to peroxidase (1:5000, Sigma) which was diluted in blocking solution was added to each membrane. They were incubated for 1 h under agitation at room temperature, and afterwards, washed three times in 0.05% Tris-buffered saline Tween-20 (TBST) solution. The reaction was initiated with DAB (Sigma) for visual reading and interrupted with deionized H$_2$O.

ELISA (sera)

Only clones non-reactive with negative IgG and clones reactive with the positive IgG were selected. The immunoreactivity of the phages selected using positive and negative sera samples for BLV infection was tested by ELISA. The bovine sera samples had previously been tested by AGID according to the protocol of Miller and Van der Maaten (1977).

The samples of the selected phages and of the wild phage were diluted to the concentration of 1×10^{11} in 0.1 M NaHCO$_3$ with a pH of 8.6 for the adsorption in the wells of ELISA plates (MaxiSorpTM - Nunc). After adsorption (18 h at 4°C), the plates were washed twice in 0.05% PBST and blocked with PBS with 5% skimmed powdered milk for 1 h. After three washings, 20 positive sera samples and 20 negative sera samples were diluted to 1:100 in PBS solution with 1% skimmed powdered milk and added in duplicates to the wells. The incubation was for 1 h at room temperature. Wells that did not receive sera samples were considered as blank reaction. Six washings were carried out. The secondary antibodies anti-IgG bovine conjugated to peroxidase (1:5000, Sigma) which were diluted in PBS solution with 1% skimmed powdered milk were incubated in the wells for 1 h at room temperature. The plate was washed six times and the reaction was initiated as described for Phage-ELISA with IgG.

The cut-off value was calculated based on the average values of OD from the wild phage with positive and negative sera and the values of all the phages tested with all the negative sera + 2X the
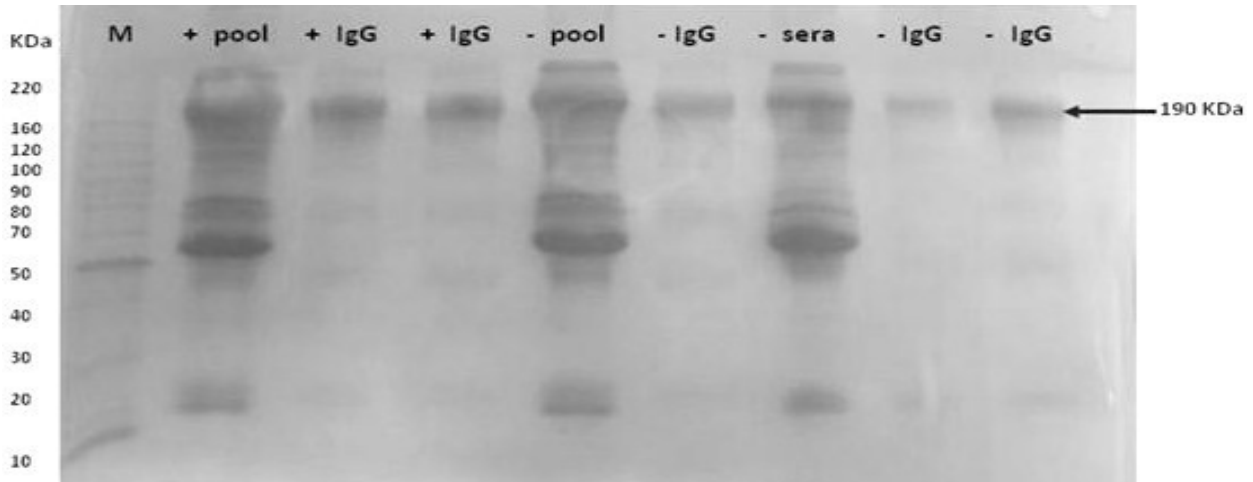
Figure 1. Electrophoresis SDS-PAGE (16%) of the positive and negative samples. M, Molecular weight marker; + pool, positive pool sera for bovine enzootic leukosis (EBL) from 20 animals; - pool, negative pool sera for EBL from 20 animals; + IgG, positive IgG purified from positive pool sera; - IgG, negative IgG purified from negative pool sera; - sera, sera from negative animal for EBL. SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

standard deviation of the same values. From the cut-off, the positive and negative ELISA indexes were calculated thus for each phage sample tested: (i) positive ELISA index for each phage = OD average value obtained with all positive sera/cut-off; (ii) negative ELISA index for each phage = OD average value obtained with all the negative sera/cut-off. The ratio of positive and negative ELISA indexes (EI+:EI-) for each sample was calculated. Phages that presented the highest EI+:EI- were selected.

Dot blot (sera)

Dot blot was performed to verify the immunoreactivity of the phages selected with positive and negative sera for BLV infection. Twenty positive sera samples and 20 negative sera samples were tested at 1:1000 dilution in blocking solution as previously described.

Bioinformatics analysis

The peptides selected by immunoassays were analyzed using bioinformatics tools. The search for the similarities between the sequences was performed via the software Clustal W version 18.1 (www.ebi.ac.uk/clustalW/). The sequences of the selected peptides were analyzed to determine their homology with the sequences of BLV proteins stored in the "GENEBANK" through the software Basic Local Alignment Search Tool (BLAST) (http://www.ncbi. nlm.nih.gov/BLAST/). To find the maximum similarity among all the selected peptides in the immunoassay and the BLV proteins of interest as revealed by Blast, the software MATCH available in the site http://relicbio.anl.gov/match.aspx/ was used.

Synthesis of the peptide containing mimetic epitopes of the BLV

The system of multiple mimetic peptides ("multiple antigens peptides") from the selected sequences was utilized. The synthetic peptide VLB-Ph was planned with repetitions in tandem, presenting the same spacer (GGGS) and had C-terminal amide for

the stabilization of peptide structure. The peptide was synthesized by Genscript (www.genscript.com).

The VLB-Ph presents 60 amino acid residues with molecular weight 6117.1, isoelectric point (IP) 12.4 and a positive charge in pH 7.0 of + 4.4.

The peptide was diluted in sterilized/deionized water and its concentration was determined by spectrophotometry at 280 nm via the method of Bradford (1976). In order to verify the reactivity of the peptide VLB-Ph with the bovine sera samples, ELISA tests were performed.

ELISA VLB-Ph

The 96-wells' high affinity plates (MaxiSorpTM - Nunc) were adsorbed with 1 µg of the diluted peptide in bicarbonate buffer (0.1 M NaHCO$_3$, pH 8.6) for 18 h at 4°C. After the adsorption period, the plates were washed and blocked as previously described.

30 positive sera samples for BLV infection and 30 negative sera samples previously tested by AGID according to the protocol of Miller and Van der Maaten (1977) were diluted 1:100 in PBS solution with 1% skimmed powdered milk and added in duplicates to the wells. After 1 h incubation at room temperature and six washings, secondary antibody anti- IgG bovine conjugated to peroxidase (1:5000, Sigma) which was diluted in PBS solution with 1% skimmed powdered milk was incubated in the wells for 1 hour at room temperature. The plate was washed six times and the reaction was initiated as previously described. The analysis of the results was carried out using the t-test in the software Prisma 5.0 (GraphPad).

RESULTS

Immunoglobulins purification

The IgG purification process from a pool of bovine sera presented efficiency and had an approximate yield of 7 µg/µl. Figure 1 presents the electrophoretical patterns of the purified IgG with respect to the sera pool and
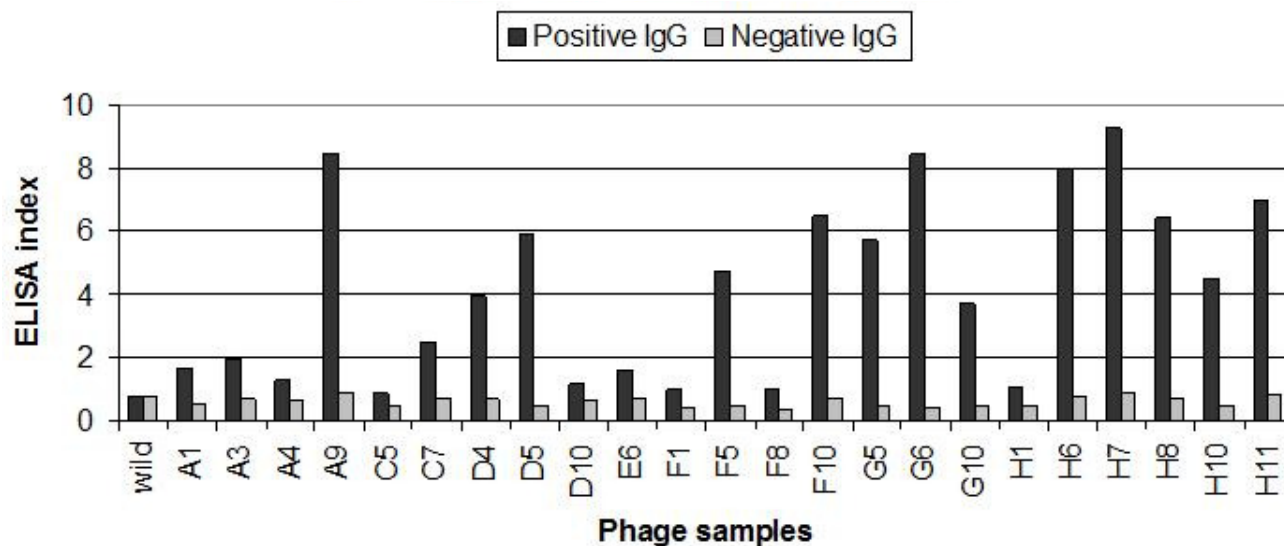
Figure 2. Phage-ELISA: Reactivity index of phages for positive and negative IgG. ELISA, Enzyme-linked immunosorbent assay.

molecular weight marker (M). The presence of a band of about 190 KDa in the sera pool samples as well as on the purified IgG corresponding to the approximated molecular weight of the bovine IgG was verified.

Biopanning

All the eluates containing amplified and non-amplified phages obtained by biopanning were titrated to estimate the quantity of phages selected in the three cycles using the Ph.D.-12TM library. The titers of entrance of phage in the selection cycles were greater than the output titers which increased gradually from the 1st to the 3rd selection cycles. According to the reading of the sequencing reaction, 44 sequences were obtained from the selection done through Ph.D.-12TM library and were later translated by the software DNA2PRO. All the sequences were different in comparison to each other.

According to the results obtained by the software AAFREQ, the most frequent amino acids were alanine, histidine, proline, threonine and tryptophan, and the search for similarities between the sequences performed using the software Clustal W version 18.1 revealed some common motifs between the selected peptides which include AHKW, AhxW, HKW, HxW, FAPT and WAPT.

The initial selection of the phages by immunoassays using purified IgG

Phage ELISA

The initial selection of the phages by immunoassays was

carried out using ELISA. The aim was to select the phages reactive with positive IgG and those which are non-reactive with negative IgG.

From the 44 phages obtained after sequence analysis, just 23 were reactive with positive IgG and non-reactive to negative IgG. The only phages which were reactive with positive IgG included A1, A3, A4, A9, C5, C7, D4, D5, D10, E6, F1, F5, F8, F10, G5, G6, G10, H1, H6, H7, H8, H10 and H11. The reactivity index of positive IgG varied a lot among the selected phages (Figure 2).

In order to verify the difference between the ELISA indexes obtained, the paired t-test was performed by the software STATA 10.0. The value $p < 0.001$ shows a significant reactivity difference of the phages with positive and negative IgG.

Western blot

The 23 selected phages, wild phage and gp51 protein were electro-transferred to nitrocellulose membranes after SDS-PAGE 16%. No reactivity of the phage samples and gp51 with the negative IgG was observed. In addition, only the phage samples G10 and H1, and wild phage (negative control) did not react with the positive IgG, other phage samples and gp51 reacted with the positive IgG (data not shown).

Dot blot

According to the results of Dot blot (data not shown), the phages A9, C7, G5, D4, F5, H11, D5, H6, D10 and F10 presented the best results in respect of the difference of
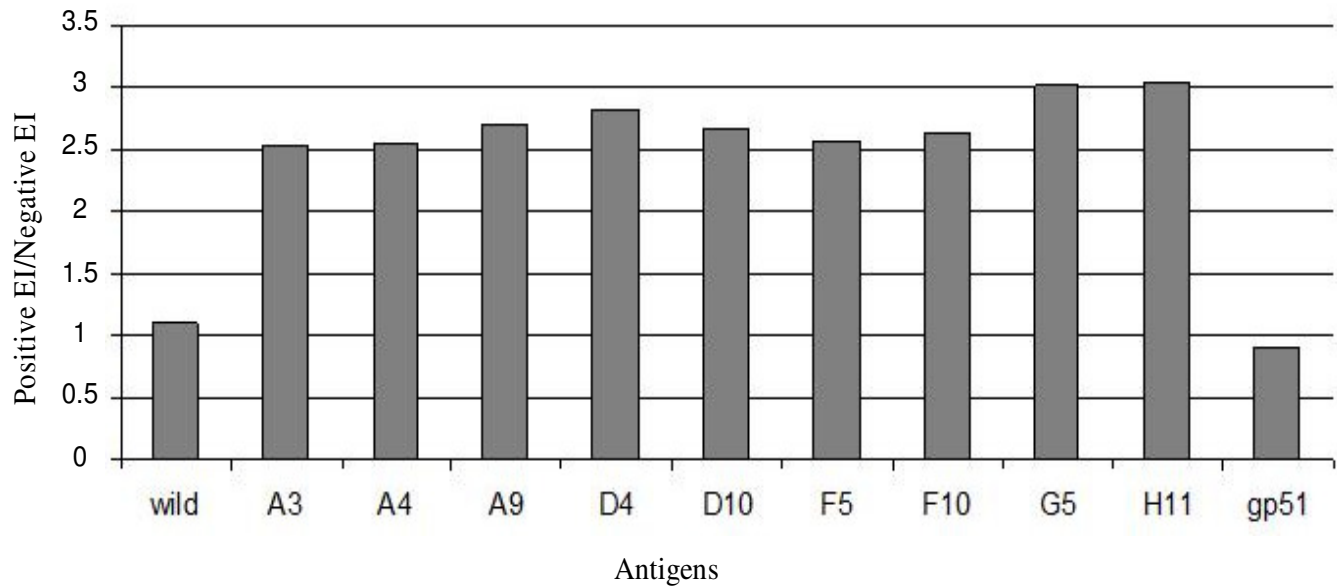
Figure 3. Phages selected by ELISA from the analysis of the reactivity of 20 positive sera and 20 negative sera for EBL. ELISA, Enzyme-linked immunosorbent assay; EBL, enzootic bovine leukosis.

reactivity with the positive and negative IgG. The wild phage did not react in the assay, and the gp51 did not differentiate the positive and negative IgG, but reacted with both.

Reactivity of protein gp51 with purified immunoglobulins and sera samples

The gp51 did not present reactivity in the ELISA experiments performed using positive and negative IgG or the sera samples (Figure 3). Through an electrophoresis SDS-PAGE, it was possible to evaluate the quality of the sample of the protein gp51 utilized in the tests (data not shown). The protein presents mass with an approximate value of 60 KDa (Llames et al., 2000).

The analysis of the reactivity of the phages with sera samples

The 23 phages selected with purified IgG were also evaluated in the immunoassays with positive and negative sera for BLV.

Phage ELISA

The selected phage samples, wild phage and gp51 were adsorbed in the ELISA plate and incubated with sera samples. Positive ELISA indexes (EI +) and negative ELISA indexes (EI -) were calculated and the greater values were used to determined the phage samples

(antigens) with greater ability to differentiate the positive sera and the negative sera. The phages that presented the best results were selected and they include A3, A4, A9, D4, D10, F5, F10, G5 and H11 (Figure 4).

Dot blot

According to the results obtained by Dot blot, the phages that differentiated sharply the positive (coloured dots) and negative sera samples (uncoloured dots) were A3, A4, A9, D4, D10, F5, F10, G5 and H11 (Figure 4), thus, confirming the ELISA results. The wild phage did not react with the sera, and gp51 did not diferentiate the samples (data not shown).

Western blot

All the nine phage samples presented the reactivity of pIII protein (~ 42 KDa) with positive sera (visible coloured bands) and non-reactivity with negative sera (no visible coloured bands) (Figure 5). The wild phage did not present any reaction to the sera, and gp51 did not diferentiate between positive and negative sera (data not shown). According to the results obtained from the immunoassays with purified IgG and with field sera samples, the phages with sequences that presented better antigenic features which differentiated the positive and negative sera were: (i) A3 (ahkwdiplstsg), (ii) A4 (ahkweavqppmt), (iii) A9 (ipastidllppl), (iv) D4 (hkpppqtrlmha), (v) D10 (ghkwspivqpsp), (vi) F5 (lprsaidwlapv), (vii) F10 (wyppmhifapts), (viii) G5
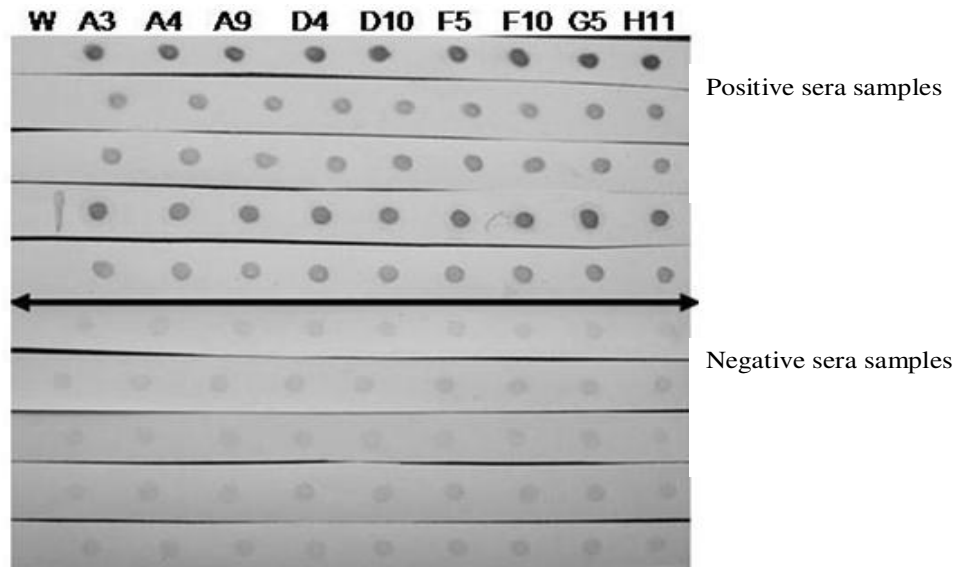
W A3 A4 A9 D4 D10 F5 F10 G5 H11

Positive sera samples

Negative sera samples

Figure 4. Dot blot test of the reactivity of selected phages with the positive and negative sera samples for EBL. The superior part of the black arrow indicates membranes incubated with positive sera samples, while the inferior part of the black arrow indicates membranes incubated with negative sera samples; W, wild phage. EBL, Enzootic bovine leukosis.
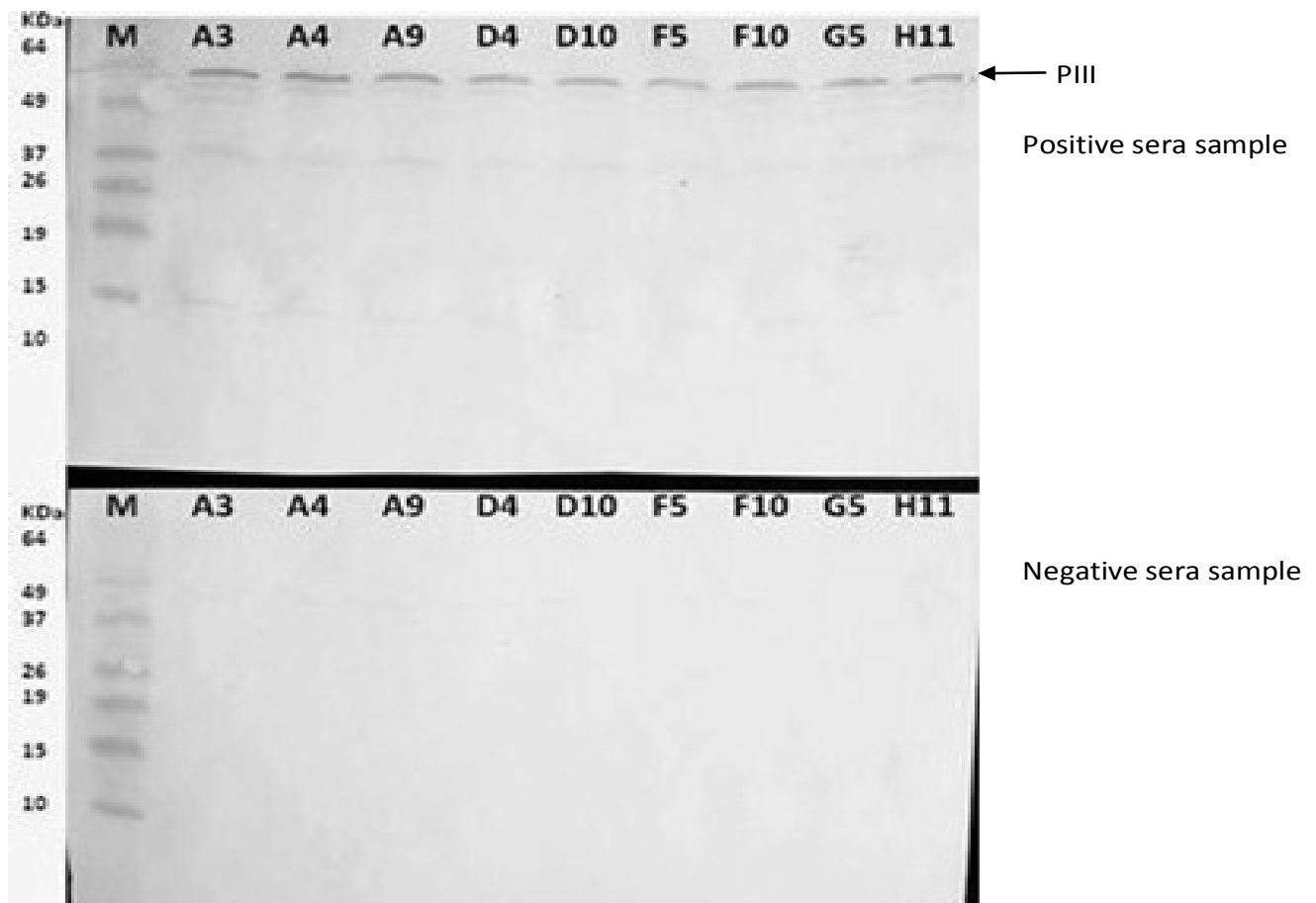
PIII

Positive sera sample

Negative sera sample

Figure 5. Western blot assay for the reactivity test using phages as antigens in positive and negative sera samples for EBL. M, molecular weight marker; superior membrane, positive sera incubation; inferior membrane, negative sera incubation.
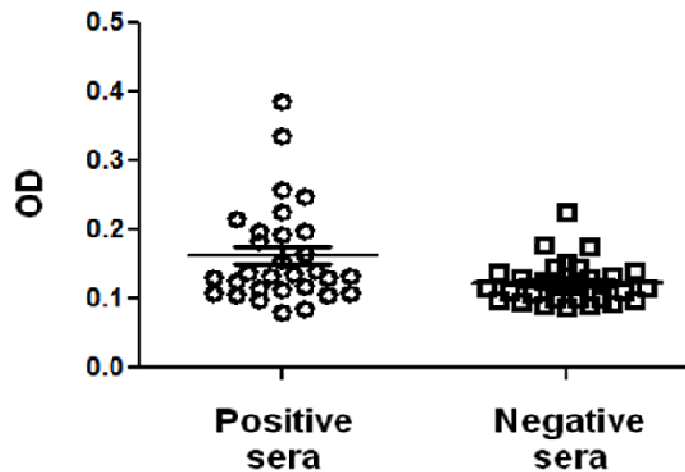
Figure 6. Reactivity analysis of 30 positive sera samples and 30 negative sera samples for BLV infection with the synthetic peptide VLB-Ph in ELISA via the software Prisma 5.0. BLV, Bovine leukosis virus; ELISA, enzyme-linked immunosorbent assay.

(nmlqiappfpas) and (ix) H11 (tvtfaptydsss).

Bioinformatics analysis

The similarity between the sequences were analyzed by multiple alignments with the software Clustal W version 18.1 which identified the common motifs AHKW, HKW or HKx, FAPT and PPM. The selected peptide sequences were also analyzed for homology with BLV proteins sequences deposited in the "GENEBANK" through the software BLAST. According to these results, the sequences of the peptides show similarity with the BLV sequences G4 (ABE28439.1), tax (ACR15161.1), gp51 (CAR64532.1), p34 (AAC82590.1), gp30 (AAP32011.1), gp60 (NP777385.2), p24 (AAA42784.1), protease (AAB50411.1), integrase (ACR15158.1), reverse transcriptase (ACR15158.1), rex (ABE28498.1), env (ACR15160.1) and pol (AAA42785.1). The software MATCH was used to calculate the maximum point of similarity between some of the selected peptides and the BLV proteins of interest from Blast search. The peptides from phages D10, G5 and A9 showed maximum similarities with p24, the main BLV capsid protein encoded by the gene gag, while the peptide from phage D4 showed maximum similarity with glycoprotein gp51 of the viral envelope encoded by gene env.

Synthesis of the peptide containing epitopes mimetics of the bovine leukosis virus (BLV)

According to the results obtained from the immunoassays and bioinformatics analysis, the peptide sequences

displayed by phages D4 and G5 were selected for the development of a synthetic peptide.

The synthetic peptide was designed with two repetitions of each sequence intercalating between the linker -GGGS- and C-terminal amidation for the stabilization of the peptide structure. The peptide called VLB-Ph was produced, presenting the sequences hkpppqtrlmha and nmlqiappfpas which correspond to the sequences of phages D4 and G5, respectively in the following manner:

VLB-Ph
HKPPPQTRLMHAGGGS**NMLQIAPPFPAS**GGGS**HKPP PQTRLMHA**GGGS**NMLQIAPPFPAS**- NH_3

The reactivity of the synthetic peptide VLB-Ph in ELISA with sera samples

The statistics which invovled the use of the software Prisma 5.0 for t-test with the values of the ODs indicated a difference among the results for positive and negative sera samples with the peptide VLB-Ph in ELISA. The p value was 0.0114, showing a significant difference between the results (Figure 6).

DISCUSSION

Phage display techniques which were utilized in the discovery of epitopes are important for the development of diagnostic platforms and vaccines (Smith and Petrenko, 1997; Ziegler et al., 1998; Casey et al., 2009). This methodology enables the identification of new antigens with no previous information on the properties of

the antibodies, avoiding the preparation and characterization of the individual monoclonal antibodies (Parmley and Smith, 1988, 1989).

In this study, selected peptides were regarded as epitopes mimicking the proteins of bovine leukosis virus (BLV). The peptides were obtained by immunologic selection from library Ph.D.-12TM presented in phages against purified immunoglobulins of a pool of sera from animals naturally infected by BLV. The isolation of peptides that present similarities with the BLV proteins was carried out through repeated selection cycles of the peptides by immunologic affinity to the biologic target (positive IgG). So, the selection cycles and amplification tend to generate specific peptides recognized by the antibodies showing higher titers, producing specific subpopulations of clones with affinity for IgG (Parmley and Smith, 1988, 1989). After biologic selection (biopanning), the phages clones were characterized and validated through the usage of specific criteria. Phage titration in the beginning and at the end of the selection cycles were carried out to obtain the enrichment of the eluates during the selective process. Presumably, the phages with higher affinity to the positive IgG remained bound and phages with low affinity were removed in the washing processes. The output titers gradually increased from the 1st to the 3rd round, indicating the enrichment of positive clones and that the selections were directed towards the target (Ziegler et al., 1998; Kirsch et al., 2008; Leong et al., 2008).

Based on the results of the selection done through the library Ph.D.-12TM, 44 different sequences were obtained with high frequencies of the amino acids alanine, histidine, proline and tryptophan, and common motifs between the selected peptides AHKW, AhxW, HKW, HxW, FAPT and WAPT, suggesting that these are the main participants in the interactions with target for biological selection. The initial selection of the phages through immunoassays was performed with the aim of identifying phages that react only with positive IgG and not with negative IgG. From the 44 peptide sequences obtained, only 23 were selected based on the results of ELISA, Dot blot and Western blot with purified IgG. Based on the large ratios of positive and negative indexes (EI+:EI-) in both ELISA and Dot blot serum assays, nine phages were selected because of their potential to differentiate between positive and negative sera samples. The results of the assays using the wild phage (negative control) and the results obtained from the Western blot proved that only peptides fused to protein III were specifically recognized by purified IgG and antibodies from sera samples.

Based on the similarity analysis, these peptide sequences were found to be similar to protein p24 and glycoproteins gp51 and gp30 which show immunedominant epitopes as well as important proteins such as Tax, Rex and G4 which are related to the regulation of viral expression and tumor growth during the development of the infection (Burny et al., 1980; Miller et al., 1981; Walker et al., 1987; Willems et al., 1993, 1995). The peptides from phages D10, G5 and A9 presented great similarities with the protein p24 which is considered as the main protein of the BLV capsid (Miller et al., 1981), while the peptide from phage D4 presented a great similarity with the glycoprotein gp51 of the viral envelope. These two proteins are considered the most immunegenic during BLV infection since many researchers have shown that the infected bovines developed a primary response to specific antibodies directed to the envelope glycoprotein gp51 and the core viral p24 protein (Deshayes et al., 1980; Portetelle et al., 1980; Walker et al., 1987). Using bioinformatics analysis, common motifs AHKW, HKW or HKx, FAPT and PPM were found in the nine selected peptides, indicating the importance of certain amino acids in distinghuishing between peptides and IgG (Cortese et al., 1995) as they potentially mimic the BLV protein epitopes.

The sequences of the nine peptides selected showed similarities with various BLV proteins and played important regulatory, structural and immunodominant roles during the infection. Also, based on the results of the study, the phage display technique may be considered as a major tool to obtain a database of epitopes selected by immunologic interaction with antibodies that recognize the BLV proteins, but this technique needs to be verfied by different techniques.

The immunoreactivity of the antigenic protein gp51 which is produced in FLK cells was evaluated in the assays with purified IgG and with sera samples. In the ELISA tests, the protein gp51 did not show reactivity with the positive or negative IgG, but presented an almost insignificant reactivity with the sera samples. Due to this, it was not possible to use gp51 as a control or a competitor in phage ELISA assays. In the Western blot and Dot blot, the gp51 showed reactivity, but it did not differentiate positive and negative samples. The lack of reactivity of gp51 may be related to the absence of standardization of this kind of antigenic material to the techniques utilized since this antigen is prepared for AGID. Considering the low differentiation ability of gp51 in the immunoassays, these mimetic peptides are potentially much better antigens for EBL diagnostics.

With the immunoassay results and similarities found between the peptide sequences obtained from the selection with the library Ph.D.-12TM and the BLV immunodominant proteins, one can consider the selected nine peptides as mimetics of immunodominant epitopes of the virus. The mimetic antigens may work as specific probes for antibodies in the diagnoses of diseases in the same way that viral proteins are used in tests for illnesses caused by retroviruses. Moreover, they enable the emphasis on a specific recognition, excluding signals not related to the disease diagnosis and avoiding cross-reactions (Smith and Petrenko, 1997).

The results of the immunoassays and bioinformatics

were utilized as criteria for selecting peptide sequences that constitute the synthetic peptide VLB-Ph, and ELISA tests performed with the VLB-Ph showed significant differences between positive and negative sera samples, although a great number of the samples need to be evaluated before the standardization of the use of this peptide in serologic tests.

This study demonstrated that mimetic antigen selection of the BLV proteins from the phage display libraries may represent an important tool to define epitopes that are recognized by antibodies during viral infections, which avoids the expensive and complex preparation and characterization of monoclonal antibodies. The use of mimetic peptides which are obtained from the phage display libraries selection as antigens in diagnostics has been suggested as an alternative for the use of gross viral antigens. Using such peptides may eliminate the high ratio of unsuitable epitopes present in the preparations of gross antigens, producing a more efficient and specific diagnosis (Casey et al., 2009). The use of mimetic peptides may also work as a positive control precisely defined and suitable for diagnostic tests, and as an alternative whenever the original antigen is hard to obtain, unstable, or in the case of a virus with erratic distribution (Ziegler et al., 1998).

The mimetic peptides selected in this work can be potentially utilized as antigens in EBL diagnosis, development of vaccines, and as a basis for the production of synthetic peptides. According to the results obtained, the synthetic peptide VLB-Ph has the potential for use as antigen in ELISA for the diagnosis of EBL. This study provided alternatives for the development of new methodologies for EBL diagnosis via the production of new antigens that may be used in routine and large scale procedures, contributing directly to the control and prevention of the disease studied.

REFERENCES

Alvarez I, Gutierrez G, Vissani A, Rodriguez S, Barrandeguy M, Trono K (2007). Standardization and validation of an agar gel immunodiffusion test for the diagnosis of equine infectious anemia using a recombinant p26 antigen. Vet. Microbiol. 121: 344-351.
Ball JM, Rushlow KE, Issel CJ, Montelaro RC (1992). Detailed Mapping of the Surface Unit Glycoprotein of Equine Infectious Anemia Virus by Using Synthetic Peptide Strategies. J. Virol. 66(2): 732-742.
Beier D, Riebe R, Blankenstein P, Starick E, Bondzio A, Marquardt O (2004). Establishment of a new bovine leukosis virus producing cell line. J. Virol. Methods, 121(20): 239-246.
Bolin SR, Ridpath JF, Black J, Macy M, Roblin R (1994). Survey of cell lines in the American Type Culture Collection for Bovine Viral Diarrhea Virus. J. Virol. Methods, 48(2): 211-221.
Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(7): 248-254.
Burny A, Bruck C, Chantrenne H, Cleuter Y, Dekegel D, Ghysdael J, Kettmann R, Leclerq M, Leunen J, Mammerickx M, Portetelle D (1980). Bovine leukemia virus: mol. boil. epidemiol. In: Klein, G. (Ed.), Viral Oncol. New York, Raven Press. pp. 231-289.
Callebaut I, Burny A, Krchnák V, Gras-Masse H, Wathelet B, Portetelle D (1991). Use of Synthetic Peptides to Map Sequential Epitopes Recognized by Monoclonal Antibodies on the Bovine Leukemia Virus

External Glycoprotein. Virol. 185(1): 48-55.
Callebaut I, Vonèche V, Mager A, Fumière O, Krchnak V, Merza M, Zavada J, Mammerickx M, Burny A, Portetelle D (1993). Mapping of B-neutralizing and T-helper cell epitopes on the bovine leukemia virus external glycoprotein gp51. J. Virol. 67(9): 5321-5327.
Camargos MF, Melo CB, Leite RC (2002). Frequência de soropositividade para a Leucose Enzoótica Bovina em rebanhos de Minas Gerais. Cienc.Vet. Trop. 5(1): 20-26.
Camargos MF (2005). Vírus da Leucemia Bovina: Epidemiologia Molecular e Diagnóstico. 95f. Tese (Doutorado em Ciência Animal) - Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte.
Cardoso R, Homsi-Brandeburgo MI, Rodrigues VM, Santos WB, Souza GRL, Prudencio CR, Siquieroli ACS, Goulart LR (2009). Peptide mimicking antigenic and immunogenic epitope of neuwiedase from Bothrops neuwiedi snake venom. Toxicon, 53(2): 254-261.
Casey JL, Coley AM, Parisi K, Foley M (2009). Peptide mimics selected from immune sera using phage display technology can replace native antigens in the diagnosis of Epstein-Barr virus infection. Protein Eng. Des. Sel. 22(2): 85-91.
Castro RS, Leite RC, Abreu JJ (1992). Prevalence of antibodies to selected viruses in bovine embryo donors and recipients from Brazil, and its implications in international embryo trade. Trop. Anim. Health Prod. 24(3): 173-176.
Choi KY, Liu RB, Buehring GC (2002). Relative sensitivity and specificity of agar gel immunodiffusion, enzyme immunosorbent assay, and immunoblotting for detection of anti-bovine leukemia virus antibodies in cattle. J. Virol. Methods, 104(1): 33-39.
Cortese R, Monaci P, Nicosia A, Luzzago A, Felici F, Galfré G, Pessi A, Tramontano A, Sollazzo M (1995). Identification of biologically active peptides using random libraries displayed on phage. Curr. Opin. Biotechnol. 6(1): 73-80.
Da Y, Shanks RD, Stewart JA, Lewin HA (1993). Milk and fat yields decline in bovine leukemia virus-infected Holstein cattle with persistent lymphocytosis. Proc. Natl. Acad. Sci. USA. 90(14): 6538-6541.
Dees C, Godfrey VL, Foster JS, Schultz RD, Travis CC (1994). Stabilization of the p53 gene product in two bovine leukemia virus infected cell lines. Cancer Lett. 86(1): 33-40.
De Giuseppe A, Feliziani F, Rutili D, De Mia GM. (2004). Expression of the bovine leukemia virus envelope glycoprotein (gp51) by recombinant baculovirus and its use in an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. 11(1): 147-151.
Deshayes L, Levy D, Parodi AL, Levy JP (1980). Spontaneous immune response of bovine leukemia virus infected cattle against five different viral proteins. Int. J. Cancer, 25(4): 503-508.
Doménech A, Llames L, Goyache J, Suárez G, Gómez-Lucía E (1997). Macrophages infected with bovine leukaemia virus (VLB) induce humoral response in rabbits. Vet. Immunol. Immunopathol. 58(3): 309-320.
Folgori A, Tafi R, Meola A, Felici F, Galfre G, Cortese R, Monaci P, Nicosia A (1994). A general strategy to identify mimotopes of pathological antigens using only random peptide libraries and human sera. EMBO J. 13(9): 2236-2243.
Gatei MH, Good MF, Daniel RC, Lavin MF (1993). T-cell responses to highly conserved CD4 and CD8 epitopes on the outer membrane protein of bovine leukemia virus: Relevance to vaccine development. J. Virol. 67(4): 1796-1802.
Gutiérrez G, Alvarez I, Fondevila N, Politzki R, Lomónaco M, Rodríguez S, Dus Santos MJ, Trono K (2009). Detection of bovine leukemia virus specific antibodies using recombinant p24-ELISA. Vet. Microbiol. 137(3): 224-234.
Johnson M, Rommel F, Moné J (1998). Development of a syncytia inhibition assay for the detection of antibodies to bovine leukemia virus in naturally infected cattle; comparison with Western blot and agar gel immunodiffusion. J. Virol. Methods 70(2): 177-182.
Kabeya H, Ohashi K, Ohishi K, Sugimoto C, Amanuma H, Onuma M (1996). An effective peptide vaccine to eliminate bovine leukaemia virus (VLB) infected cells in carrier sheep. Vaccine, 14(12): 1118-1122.
Kim CH, Casey JW (1992). Genomic variation and segregation of equine infectious anemia virus during acute infection. J. Virol. 66(6):

3879-3882.

Kirsch MI, Hülseweh B, Nacke C, Rülker T, Schirrmann T, Marschall HJ, Hust M, Dübel S (2008). Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). BMC Biotechnol. 8(66): 1-15.

Kittelberger R, Reichel MP, Meynell RM, Tham K, Molloy JB (1999). Detection of antibodies against the core protein p24 of the bovine leukaemia virus in cattle for confirmatory serological testing. J. Virol. Methods, 77(1): 109-114.

Langemeier JL, Cook SJ, Cook RF, Rushlow KE, Montelaro RC, Issel CJ (1996). Detection of Equine Infectious Anemia Viral RNA in Plasma Samples from Recently Infected and Long-Term Inapparent Carrier Animals by PCR. J. Clin. Microbiol. 34(6): 1481-1487.

Leong YH, Devi S, Lin TK (2008). Mimotopes of heat shock proteins of Salmonella enterica serovar Typhi identified from phage-displayed peptide library. J. Infect. Dev. Ctries, 2(4): 313-323.

Leroux C, Cadoré J, Montelaro RC (2004). Equine Infectious Anemia Virus (VAIE): what has HIV's country cousin got to tell us? Vet. Res. 35(4): 1-19.

Lim SI, Jeong W, Tark DS, Yang DK, Kweon CH (2009). Agar gel immunodiffusion analysis using baculovirus-expressed recombinant VLB envelope glycoprotein (gp51/gp30T-). J. Vet. Sci. 10(4): 331-336.

Llames L, Gómez-Lucia E, Doménech A, Suárez G, Goyache J (2000).. et al. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot of nonspecific and specific viral proteins frequently detected in different antigen preparations of bovine leukemia virus. J. Vet. Diagn. Invest. 12(4): 337-344.

Miller JM, Van Der Maaten MJ (1977). Use of glycoprotein antigen in the immunodiffusion test for bovine leukemia virus antibodies. Eur. J. Cancer, 13(12): 1369-1375.

Miller JM, Schmerr MJF, Van Der Maaten MJ (1981). Comparison of four serologic tests for the detection of antibodies to Bovine Leukemia Virus. Am. J. Vet. Res. 42(1): 5-8.

Modena CM, Gouveia AMG, Azevedo NA (1984). Leucose enzoótica bovina: I-Prevalência em rebanhos de alta linhagem no Estado de Minas Gerais. Arq. Bras. Med. Vet. Zoot. 36(1): 39-45.

More SJ, Aznar I, Bailey DC, Larkin JF, Leadon DP, Lenihan P, Flaherty B, Fogarty U, Brangan P (2008). An outbreak of equine infectious anaemia in Ireland during 2006: Investigation methodology, initial source of infection, diagnosis and clinical presentation, modes of transmission and spread in the Meath cluster. Equine Vet. J. 40(7): 706-708.

Neurath AR, Strick N, Lee ESY (1990). B cell epitope mapping of human immunodeficiency virus envelope glycoproteins with long (19- to 36-residue) synthetic peptides. J. Gen. Virol. 71(1): 85-95.

Paré J, Simard C (2004). Comparison of commercial enzyme-linked immunosorbent assays and agar gel immunodiffusion tests for the serodiagnosis of equine infectious anemia. Can. J. Vet. Res. 68(4): 254-258.

Parmley SF, Smith GP (1988). Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene. 73(20): 305-318.

Parmley SF, Smith GP (1989). Filamentous fusion phage cloning vectors for the study of epitopes and design of vaccines. Adv. Exp. Med. Biol. 251(1): 215-218.

Pasqualini R, Koivunen E, Ruoslahti E (1995). A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. J. Cell Biol. 130(5): 1189-1196.

Payne SL, Rushlow KE, Dhurva BD, Issel CJ, Montelaro RC (1989). Localization of conserved and variable antigenic domains of equine infectious anemia virus envelope glycoproteins using recombinant env-encoded protein fragments produced in *Escherichia coli*. Virology, 172(1): 609-615.

Portetelle D, Bruck C, Mammerickx M, Burny A (1980). In animals infected by bovine leukemia virus (VLB) antibodies to envelope glycoprotein gp51 are directed against the carbohydrate moiety. Virology, 105(1): 223-233.

Portetelle D, Dandoy C, Burny A, Zavada J, Siakkou H, Gras-Masse H, Drobecq H, Tartar A (1989). Synthetic peptides approach to identification of epitopes on bovine leukemia virus envelope glycoprotein gp51. Virology, 169(1): 34-41.

Reichel MP, Tham KM, Barnes S, Kittelberger R (1998). Evaluation of alternative methods for the detection of bovine leukaemia virus in cattle. N. Z. Vet. J. 46(4): 140-146.

Sioud M, Forre O, Dybwad A (1996). Selection of ligands for polyclonal antibodies from random peptide libraries: potential identification of (auto) antigens that may trigger B and T cell responses in autoimmune diseases. Clin. Immunol. Immunopathol. 79(2): 105-114.

Smith GP, Petrenko VA (1997). Phage Display. Chem. Rev. 97(2): 391-410.

Soutullo A, Verwimp V, Riveros M, Pauli R, Tonarelli G (2001). Desing and validation of an ELISA for equine infectious anemia (EIA) diagnosis using synthetic peptides. Vet. Microbiol. 79(2): 111-121.

Soutullo A, García MI, Bailat A, Racca A, Tonarelli G, Borel IM (2005). Antibodies and PBMC from EIAV infected carrier horses recognize gp45 and p26 synthetic peptides. Vet. Immunol. Immunopathol. 108(3): 335-343.

Trono KG, Perez-Filgueira DM, Duffy S, Borca MV, Carrillo C (2001). Seroprevalence of bovine leukemia virus in dairy cattle in Argentina: comparison of sensitivity and specificity of different detection methods. Vet. Microbiol. 83(3): 235-248.

Van der Maaten MJ, Miller JM, Boothe AD (1974). Replicating C-type virus particles in monolayer cell cultures of tissues from cattle with lymphosarcoma. J. Natl. Cancer Inst. 52(2): 491-497.

Walker PJ, Molloy JB, Rodwell BJ (1987). A protein immunoblot test for the detection of bovine leukemia virus p24 antibody in cattle and experimentally infected sheep. J. Virol. Method, 15(3): 201-211.

Willems L, Kettmann R, Dequiedt F, Portetelle D, Voneche V, Cornil I, Kerkhofs P, Burny A, Mammerickx M (1993). In vivo infection of sheep by bovine leukemia virus mutants. J. Virol. 67(7): 4078-4085.

Willems L, Gatot JS, Mammerickx M, Portetelle D, Burny A, Kerkhofs P, Kettmann R (1995). The YXXL signaling motifs of the bovine leukemia virus transmembrane protein are required for in vivo infection and maintenance of high viral loads. J. Virol. 69(7): 4137-4141.

Ziegler A, Mayo MA, Torrance L (1998). Synthetic antigen from a peptide library can be an effective positive control in immunoassays for the detection and identification of two Geminiviruses. Virology, 88(12): 1302-1305.