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Establishment of a new bovine leukosis virus producing cell line

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Abstract

Due to the prevalence of different bovine leukosis virus (BLV) species in the cattle population in Europe, problems may arise in the serological diagnosis of BLV infections. In addition, earlier investigations demonstrated that contamination of the BLV antigen-producing cell culture systems by bovine viral diarrhea virus (BVDV) may give rise to misinterpretation of serological test results after BVDV vaccination of cattle. By co-cultivation of peripheral leukocytes of a BLV-infected cow with a permanent sheep kidney cell line, a new BLV-producing cell line named PO714 was established. This line carries a BLV provirus of the Belgian species and has been tested to be free of a variety of possibly contaminating viruses and mycoplasms. Investigations of a panel of well-characterised sera by agar gel immunodiffusion (AGID) and capture ELISA (cELISA) tests using antigen prepared from this new cell line in comparison with antigen of the well-known cell line FLK/BLV yielded comparable results. False positive results caused by BVDV cross-reactions could be eliminated when tests were carried out with antigen derived from the new cell line.

Keywords: BLV species; Cell lines; Antigen production

1. Introduction

Although many attempts have been made either to improve the antigen-producing properties of existing permanent bovine leukosis virus (BLV) expressing cell lines or to generate corresponding new lines (Graves and Ferrer, 1976; Mamoun et al., 1981; Altaner et al., 1985; Altanerova et al., 1989; Wagner et al., 1995), the cell line FLK/BLV still is the one that is used mainly for BLV antigen production for commercial diagnostic test kits. This line was established by co-cultivation of fetal lamb kidney (FLK) cells with lymphocytes of an infected cattle in the USA (van der Maaten and Miller, 1976). Analysis by polymerase chain reaction (PCR), restriction fragment length polymorphism analysis (RFLPA) and sequencing showed a close relation of the FLK/BLV provirus to the Australian and Japanese strains (Fechner et al., 1997; Beier et al., 2001; Licursi et al., 2002). BLV infected cattle in Europe, especially in Germany, however, mostly harbor proviruses closely related to the Belgian provirus line (Rice et al., 1984, 1985; Mamoun et al., 1990; Molteni et al., 1996; Blankenstein et al., 1998). This may cause problems with regard to the sensitivity of serological diagnostic assays.

Furthermore, the cell lines FLK/BLV and fetal lamb spleen FLS/BLV, which are both used for antigen production, are known to be contaminated with bovine viral diarrhea virus (BVDV) (Bolin et al., 1994; Dees et al., 1994; own data, unpublished). This contamination may result in additional diagnostic problems concerning the specificity of the reactions (Roberts et al., 1989). In 1995, an inactivated BVDV vaccine was first applied in bovine leukosis free herds of cattle. Subsequently, an increasing number of positive results

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was observed in routine diagnostic investigations using a distinct ELISA test system for detection of BLV antibodies. Further analysis of these reactions by means of other tests for BLV antibody detection and BLV provirus PCR always yielded negative results. Therefore, it was assumed that cross-reactivity occurred between the BVDV antibodies induced by the vaccine and BVDV in the BLV antigen preparation. In the meantime, this assumption could be confirmed in a vaccination study in BLV-free cattle (Beier and Conraths, 1996). This prompted us to develop a new BLV-producing permanent cell line expressing a European BLV isolate of the Belgian species without contamination by other viruses. This cell line should be useful for antigen production to improve current tests.

2. Materials and methods

2.1. Viruses and cell lines

Peripheral blood mononuclear cells (PBMC) were prepared from EDTA-blood of a naturally infected cow from the Federal Research Centre for Virus Diseases of Animals (FRC, Wusterhausen, Germany) using the Ficoll method according to the manufacturer's recommodation (Ficoll-Paque, Pharmacia). Short time cultures were seeded in RPMI 1640 medium with 10% FCS containing 6–10 $\times$ 10^6 cells/ml.

An established ovine kidney cell line from the Collection of Cell Lines in Veterinary Medicine (CCLV), FRC, Isle of Riems, was used as carrier line for BLV uptake and replication. The PO cell line (PO, CCLV RIE 17) is a fast and stable growing line also suitable for cultivation in roller-bottles.

It was shown to be free of 11 bovine, 2 ovine and 7 porcine viruses[1] by immunofluorescence tests (IFT). All viruses but BVDV were investigated by direct IFT using commercially available FITC labelled antibodies (Gamakon, Bioveta, Nitra), or in-house preparations (Entero-, Rota- and Coronavirus, BRSV, BSV, BLV). The test for BVDV was carried out indirectly using the pan-pesti specific monoclonal antibody WB103/105 (c.c.pro GmbH, Neustadt/Germany), 1:500 diluted, and anti-mouse IgG (Sigma/Germany), 1:200 diluted. For BVDV, BDV, BLV and BRSV, the results were confirmed by PCR assays. BVDV PCR assay was done as described (Vilcek, 2001). No virus particles were detectable by electron microscopy (EM). Tests for mycoplasm using the culture method, DNA staining and PCR were repeatedly negative.

FLK/BLV cells (FLK/BLV, CCLV, RIE 44-1) served as reference line for BLV antigen production. This line is contaminated with BVDV (data not shown).

[1] Bovine viruses: BHV1; BHV4; rotavirus, type A; coronavirus; BRSV; BFV; BVDV; PI3; BLV; reovirus, type 1; adenovirus; porcine viruses: CSFV; TGEV; adenovirus; rotavirus, type A; HE virus; parvovirus; enteroviruses; sheep viruses: MVV; BDV.

2.2. Co-cultivation and replication of PO714 cells

Short time PBMC cultures and PO cells were co-cultivated at a ratio of about 1:0.3 using Eagle's MEM in Earle's BSS containing 1% non-essential amino acids, 120 mg/l Na-pyruvate, 10% FCS, pH 7.2–7.4. When the monolayer was confluent, we used a split ratio of 1:3 for the first three subcultures. Subsequently, cells were subcultivated regularly twice a week at a split ratio of 1:6 using the same culture medium. The cells could easily be detached by an EDTA/trypsin solution.

The monolayer was stable for at least 10 days, if the medium was changed every other day after confluence. After cryopreservation of 2–4 $\times$ 10^6 cells/ml culture medium containing 7.5% DMSO and 10% FCS, the thawed cells show a viability of more than 90%. The new cell line was named PO714 (PO714, CCLV, RIE 714). Tests for contamination by foreign viruses and mycoplasms were negative. Thereafter, several experiments were carried out to show whether multiple virus harvests from one cell culture were possible without antigen loss. Cultures of different passage ranges were included. Cell culture supernatant was harvested on day 3, 6, 9 and 12 after subcultivation, and antigen titres were determined as described.

2.3. Antigen preparation

In initial experiments, BLV was pelleted from the cell culture supernatant by ultracentrifugation (rotor TFT45.94 (Kontron), 17,000 rpm/min, 3 h, 10 °C) avoiding any previous freeze-thawing step. The pellet was resuspended in 1/200 of the starting volume (V_0) in TEN-Zwittergent buffer (0.01 M Tris–buffer, 0.15 M NaCl, 0.025 M Na–EDTA, 0.056 M Zwittergent 3.08 (Calbiochem), pH 7.2). The remaining antigen in the supernatant was concentrated by ultrafiltration (regenerated cellulose membrane YM10, Millipore Corp., USA) to 1/100 V_0. For further investigations, antigen was prepared by ultrafiltration of the native cell culture supernatant after removal of cell debris by low speed centrifugation.

2.4. Antigen yield of PO714 over 10 passages detected by cELISA

Tissue culture flasks were seeded with 1 $\times$ 10^5 cells/ml in Leibowitz (L15)/Dulbecco's modified Eagle's medium (DMEM) [Seromed], corresponding to 5 $\times$ 10^5 cells per 25 cm^2 flask, and were examined for BLV proteins for 3 days over 10 passages. Samples of cell culture supernatant (0.5 ml) were collected daily, centrifuged at 1500 rpm for 10 min and supernatants were stored at −20 °C. The presence of BLVp24 was determined as an indicator for viral gene expression using a capture enzyme immunoassay as described by Platzer et al. (1990) with some modifications. The amount of antigen (BLV gp51 and p24) in cell culture supernatants was determined using a standard BLV preparation. This was adjusted to quantified BLV preparations kindly provided by C.

Platzer. A duplicate set of serial dilutions of this reference was included in each test run and a standard curve of relative antigen concentration versus optical density was developed. The limit detection was about 5 ng/ml.

2.5. Identification of the BLV provirus isolate

2.5.1. By PCR assay

Genomic DNA was extracted from the cell line PO714 using a commercially available extraction kit (Macherey-Nagel, Düren/Germany).

The DNA was subjected to PCR, followed by a nested PCR (N-PCR) that amplifies the *env* gene between nucleotides (nt) 5099 and 5521 (Fechner, 1995; Beier et al., 1998).

Primers were designed based on published sequence data (Sagata et al., 1985). Forward primers were:

$^{env}5032$5′-TCTGTGCCAAGTCTCCCAGATA-3′
$^{env}5099$5′-CCAACAAGGGCGGCGCCGGTTT-3′

Reverse primers were:

$^{env}5521$5′-GCGAGGCCGGGTCCAGAGCTGG-3′
$^{env}5608$5′-AACAACAACCTCTGGGAAGGGT-3′

Primers $^{env}5099$ and $^{env}5521$ were identical with those previously used for BLV provirus detection by PCR (Naif et al., 1990, 1992; Brandon et al., 1991). All primers were custom synthesised (MWG Biotech, Ebersberg/Germany). The PCR protocol has been described previously (Fechner et al., 1996). The primer pair $^{env}5032$/$^{env}5608$ was used for the first round of amplification resulting in a 598 bp fragment. For the N-PCR, 3 μl of the product was transferred into a new tube containing fresh PCR mix and primer pair $^{env}5099$/$^{env}5521$. The N-PCR product consists of 444 bp. PCR assays were carried out using a DNA Thermal Cycler 480 (Perkin–Elmer Cetus Inc., Weiterstadt/Germany). In order to visualise PCR products, 15 μl of the total assay volume was run on a 1.5% agarose gel followed by ethidium bromide staining.

2.5.2. By RFLPA

Direct digestion of 10 μl *env* nested PCR product (444 bp) with the restriction endonucleases BclI, PvuII and BamH1 (Boehringer, Mannheim/Germany) was done at 37 °C for 2 h to verify the specificity of the amplicons and to determine the BLV subgroup (Fechner et al., 1997).

2.5.3. By DNA sequencing

PCR products were separated from primers and nucleotides using QIAquick columns (Qiagen, Hilden/Germany). Sequences of the purified amplicons were determined using the fluorescent dye deoxy-terminator cycle sequencing kit (Perkin–Elmer Cetus Inc., Weiterstadt/Germany) and analysed by an ABI Prism 377 DNA sequencer (Applied Biosystems, Weiterstadt/Germany) as previously described (Marquardt and Haas, 1998). The primers were the same as used for nested PCR. The resulting sequences were aligned to published sequences from different BLV provirus species over 374 bp.

In addition, BLV provirus sequences of cattle from Germany were included in the phylogenetic analysis. The phylogenetic tree was established using the software package DNASIS for Windows 1.1 (Hitachi, Japan).

2.6. Detection of BLV antigens by serological methods

2.6.1. Test sera

Standard and reference sera from the National Reference Laboratory for Bovine Leukosis, located at the Federal Research Centre for Virus Diseases of Animals (FRC), and from an agar gel immunodiffusion (AGID) test kit producer were used for comparative examination of the different serological test systems. In addition, sera from naturally infected cattle kept at the Federal Research Centre and field sera with contradictory results in serological detection and PCR as well as in several serological tests were investigated.

Blood samples for serological tests were obtained together with the blood used for DNA preparation.

2.6.2. Agar gel immunodiffusion test

Antigen preparations were characterised by AGID as described (Wittmann, 1993). Sera from two cattle, which had been investigated clinically, virologically and serologically over several years, served as internal reference for detection of BLVp24 and gp51, respectively. Serial lg2-dilutions of the antigens were prepared with Tris–NaCl buffer pH 7.2 and were tested against the two reference sera in parallel with an internal standard antigen, using 50 μl of each. AGID plates were read after 24 and 48 h (p24 test) and 48 and 72 h (gp51 test). All tests were at least done in duplicate. The titre of the last antigen dilution causing a visible reaction was used as measure for the relative p24 or gp51 content in the preparation. Standard and field sera were investigated in parallel with a commercially available test kit (Riemser Rinderleukose Test Kit, RTAM/Germany) in order to compare sensitivity and specificity of the antigens from the cell lines PO714 and FLK/BLV.

2.6.3. Indirect and capture ELISA

ELISA tests were carried out either by using the CHEKIT-Leucotest kit (Bommeli AG, Bern, Switzerland) according to the manufacturer's recommendations, or by a capture ELISA (cELISA). The cELISA was done using two monoclonal antibodies (mAb), kindly provided by Dr. C. Platzer (Faculty of Medicine, University of Jena, Germany). MAb gp51/22 was directed against epitope B of the BLV gp51, and mAb p24/07 against p24 antigen. Capture ELISA was performed as described previously (Platzer et al., 1990). Cell culture supernatant of FLK/BLV or PO714, used as antigen, was diluted in phosphate buffer with 0.5% Tween 20 and 10% horse sera.

The cut off for sera is twice the OD of the negative control. Sera with an OD between 1.5 times and twice of OD of neg-

ative control are counted as suspicious. The capture ELISA was also used for quantitation of BLV antigen in cell culture supernatant as described above.

2.6.4. Western blot analysis

Cells were lysed with two volumes RIPA buffer (1% Triton X-100, 1% sodium desoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 1 mM PMSF, 0.1% gelatine) by three cycles of freezing and thawing. Twenty micrograms whole protein from each sample was separated on a SDS–15% polyacrylamide gel. The FLK/BLV control lysate was adjusted with standard antigen preparation and contained about 0.5 ng gp51 and 0.5 ng p24 in 1 μg whole protein. Western blots were developed with mAb gp51/22 and p24/07 (Platzer et al., 1990) and goat anti-mouse alkaline phosphatase. For detection of bound antibodies, NBT/BCIP color substrate (PROMEGA) was used.

3. Results

During the first co-cultivation, PO and PBM cells were observed without any mutual interference. However, already after two subcultures, lymphocytes, macrophages and dendritic cells could no longer be seen microscopically. The newly established cell line PO714 started to show small syncytia typical for BLV replication in the 13th passage. In the 14th passage, cell culture supernatant was investigated for BLV by cELISA. The amount of gp51 and p24 was about 20–30% compared to FLK/BLV. Higher passages (20th–40th passage) showed a higher BLV antigen expression, which was nearly comparable with that of FLK/BLV under the same conditions. As shown in Table 1, the p24 content in the supernatant was relatively stable for 3 days after passaging for at least 10 subcultivation steps. In addition, Western blot analysis of cell lysates from FLK/BLV and PO714 showed that both cell lines express similar amounts of gp51, p24 and different precursor proteins.

Table 1
Dynamic of excretion of BLV p24 protein [ng/ml] by PO714 during 10 subcultures within a period of 3 days each

BLV p24 excretion (ng/ml)			
Number of passage	24 h	48 h	72 h
28	47 ± 1.9	130 ± 12.3	134 ± 30.7
30	35 ± 1.8	107 ± 4.9	141 ± 4.8
31	40 ± 2.4	106 ± 5.0	143 ± 0.3
32	40 ± 1.3	96 ± 9.6	107 ± 8.8
34	25 ± 3.9	93 ± 0.9	168 ± 19.3
36	50 ± 1.0	n.d.	150 ± 4.2
37	34 ± 1.2	n.d.	161 ± 3.8
38	17 ± 3.6	117 ± 5.6	152 ± 2.7

n.d.: not determined. The cells were seeded in duplicate at each passage (28–38) in tissue culture flasks (25 cm^2) with 1 × 10^5 cells/ml. Samples of cell culture supernatant were collected daily between days 1 and 3 after passage. The amount of p24 was titrated by cELISA. The results of p24 expression represent the average of two assays.

Cells of the 20th subculture were shown to express BLV by IFT and EM. Particles with typical retrovirus morphology were detectable in ultrathin sections. Cells and cell culture supernatant of the 63rd and 65th subculture revealed positive PCR results (earlier passages were not tested by PCR assay). The RFLPA products from *env* amplicons showed a high homology with the Belgian subtype (Fig. 1). Fur-

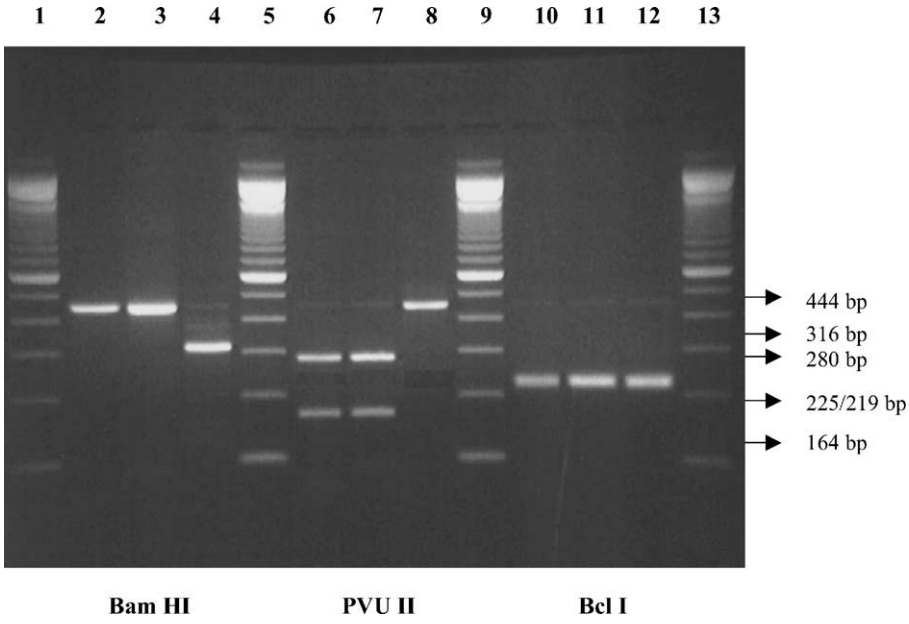

Fig. 1. RFLPA products of partial BLV provirus *env* gene (444 bp) after nested PCR. Lane 1, 5, 9, 13: 100 bp DNA Ladder (Gibco BRL); lane 2, 3, 4: BamHI digestion; lane 6, 7, 8: PVU II digestion; lane 10, 11, 12: Bcl I digestion (all Encymes: Boehringer Mannheim, Germany). In each case, lane 2, 6, 10: reference sample (Belgian clone); lane 3, 7, 11: PO714 (Belgian clone); lane 4, 8, 12: FLK/BLV (Australian clone).

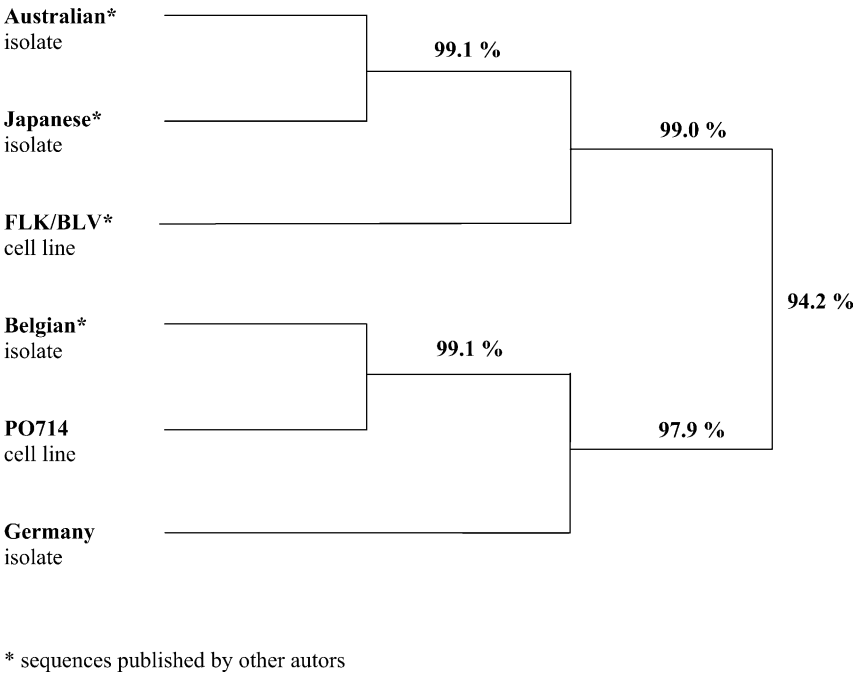

Fig. 2. Phylogenetic tree based on *env* fragment (374 bp) sequence analysis. PO714 derived BLV (accession number AY062265) was compared to sequences from BLV of different geographical origin (Japanese: Sagata et al., 1985; Australian: Coulston et al., 1990; Belgian: Rice et al., 1984; FLK/BLV and a cow from Germany of Belgian provirus type: Fechner et al., 1997).

thermore, the sequence of the *env* PCR product (GenBank accession number: AY062265) exhibited a 99.1% homology with the Belgian subtype over the analysed 400 bp (Fig. 2).

All tests for foreign viruses and mycoplasma were negative.

An important result of the investigations regarding antigen production was the fact that from one culture, up to four virus harvests were possible without loss of antigen content. In addition, the amount of p24 antigen clearly increased within that time. The content of gp51 antigen did not increase as strongly as that of p24 but showed the same tendency. Furthermore, it is possible to use a wide range of passages of cell line PO714 for BLV antigen production: there were no significant differences between the antigen yields of the 23rd and the 69th subculture (Table 2). This is valid for p24 as well as for gp51. Comparable results were obtained using maintenance medium with 10 and 5% FCS, respectively. The cell line is stable and produces BLV, so far up to the 100th subculture.

AGID antigen prepared from cell line PO714 was compared to antigen from a commercial AGID test kit (Table 2). The precipitation lines of the PO714 antigens were confluent with those of the control antigen showing homology between both preparations. The lines were long, clear and distinct and, thus, offer the same quality as the commercial antigen. On an average, the final titres were slightly lower, which, however, had no influence on the test results. Twenty-seven standard

Table 2
Analysis of BLV antigen prepared from cell line PO714 after the 23rd and 69th subculture by means of AGID using gp51- and p24-specific reference sera

Antigen prepared from/reciprocal dilution	p24		gp51		
	23rd subculture	69th subculture	23rd subculture	69th subculture	Commercial antigen[a]
Undiluted	++	++	++	++	++
2	++	++	++	++	++
4	+	++	++	+	++
8	(+)	++	+	(+)	+
16	(+)	+	(+)	−	(+)
32	−	(+)	−	−	−

Antigens presented in the table were prepared after the second (23rd subculture) and fourth (69th subculture) harvest of cell culture supernatant, respectively. Serial lg2-dilutions of the antigens were prepared with Tris–NaCl buffer (pH 7.2), and the intensity of the precipitation bands in the agar gel was evaluated using a classification from ++ (strong) to (+) (weak).

[a] Does not contain p24.

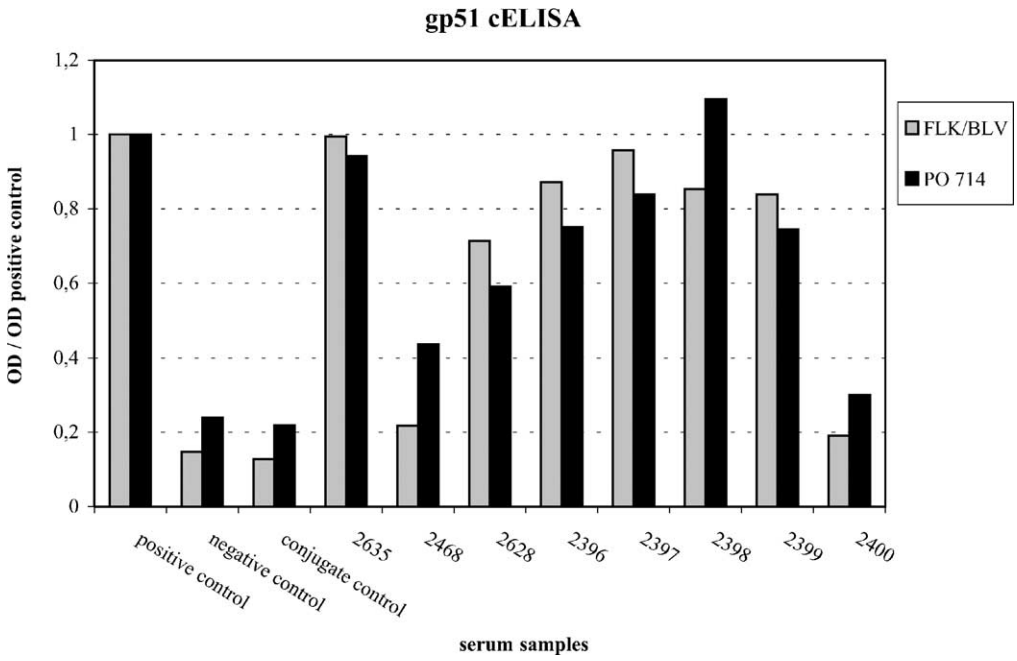

Fig. 3. Comparative investigations of sera from cattle infected with BLV of different provirus subtypes with antigen from both cell lines using the gp51 cELISA.

and reference sera were investigated in a first AGID test. They included 11 highly positive, 8 weakly positive and 7 negative sera and the EU standard serum "E4" (1:10). All highly and weakly positive sera were detected in this test, and no false positive results occurred. In the following, 79 sera from field and laboratory animals infected with BLV of different provirus subtypes were tested in parallel with a commercially available test kit and PO714 antigen. The sera had been characterised using up to five commercial ELISA systems and full blood was tested by *env* nested PCR. Out of these sera, 20 were positive (9 strongly and 11 weakly positive sera), 57 were negative and 2 were doubtful. Both AGID test systems revealed the same results.

Furthermore, these serum samples were investigated in gp51- and p24-cELISA tests using cell culture supernatant of FLK/BLV as well as of PO714. Fig. 3 shows representative results of the gp51-cELISA. The sera reacted in the same manner when testing by the p24-assay. Thus, both cell lines are suitable antigen sources for cELISA tests. Most of the sera reacted similarly with both antigens. In a few cases, differences occurred in the strength of the reactions: some sera reacted better with FLK/BLV antigen, but others showed a stronger reaction with PO714 antigen. This should not be important for clearly negative or strongly positive sera, but in particular cases (see sample 2468 in Fig. 3 and Table 3), it may result in differences in the positive/negative-grading of the sample. This means that weakly positive sera might be detected by only one of the antigens and, thus, might be missed if tested using only one system. Different reactivities were also seen between FLK/BLV and PO714 antigen in p24 cELISA (data not shown).

Table 3

Serumbank number	Commercial ELISA	*env* PCR	BLV subgroup corresponding to the RFLP result
2635	Positive	Positive	Belgian
2468	Negative	Positive	Belgian
2628	Positive	Positive	Belgian
2396	Positive	Positive	Australian
2397	Positive	Positive	Belgian
2398	Positive	Positive	Belgian
2399	Positive	Positive	Australian
2400	Negative	Positive	Australian

Comparative investigations of sera from cattle infected with BLV of different provirus subtypes with antigen from both cell lines using the gp51 cELISA.

4. Discussion

Efforts to establish new BLV-producing cell lines free of extraneous agents and attempts to isolate additional BLV strains in cell culture continue. The new permanent cell line PO714 productively infected with BLV was created by cocultivation of PO cells with a short time culture of leukocytes from a BLV-infected cow. PO714 was of special interest because it is free of other contaminating infectious agents and because it carries a BLV provirus which is different from that of FLK/BLV with an *env* region closely related to the Belgian species. This is the dominant provirus subtype among cattle in Germany (Fechner, 1995). Further attempts to isolate BLV from cattle with indifferent serological but positive PCR results and from tumour cells of an intensely tumourous

cow killed in a moribund state were not successful. The lymphocytes as well as the tumour cells were contaminated with BVDV and/or bovine foamy virus (BFV) which in each case caused a massive infection of the carrier cells.

The yield of gp51 antigen produced by cell line PO714 as analysed by AGID test was slightly lower than that of the commercially available antigen. This fact had no consequences for the sensitivity of the test. Sensitivity and specificity of PO714-antigens prepared and used for AGID are comparable to those of commercial test kits making the cell line PO714 attractive for production. The amount of p24-antigen appears to be stable and comparable to that produced by the most commonly used FLK/BLV. This is an important parameter for the production of ELISA antigen. Cell culture supernatant of both cell lines was proven to be useful as antigen in cELISA tests for serological investigations. Most of the sera showed the same reaction with both antigens. Only a few sera reacted differently in the two tests demonstrating that there are differences in antigenicity. Thus, antigen from both cell culture systems should be used to reliably demonstrate the absence of BLV during the last stage of eradication programs and to confirm the BLV-free status of a herd.

To improve viral yield, further investigations need to be performed to optimise culture conditions as well as subcloning in order to enhance the antigen titres. On the other hand, PO714 cells represent a good production cell system because they are easy to handle and do not require special media conditions. They grow fast and stable also in roller-bottles, and several harvests of supernatants are possible from one cell culture.

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