IrIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Use of a DNA probe in the diagnosis of enzootic bovine leukemia

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/124970 since

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

15 October 2024

SCHWEIZ. ARCH. TIERHEILK. 132, 139—145, 1990

Università di Torino – Dipartimento di Patologia Animale[1] e Dipartimento di Oncologia Umana e Scienze Biomediche[2]

USE OF A DNA PROBE IN THE DIAGNOSIS OF ENZOOTIC BOVINE LEUKEMIA

B. PEIRONE[1], B. BIOLATTI[1], G. SAGLIO[2]

SUMMARY

The Authors describe the possibility of adopting a DNA probe in the detection of Bovine Leukemia Virus (BLV), using the molecular hybridization technique. The study was carried out on 18 seropositive Friesian cows taken from different stockfarms during the persistent lymphocytosis phase. The presence of a possible BLV molecular variant was highlighted in one stockfarm.

KEY WORDS: cattle - enzootic leukemia - virus - DNA probe

DIAGNOSE DER ENZOOTISCHEN BOVINEN LEUKOSE MIT HILFE EINER DNS-SONDE

Die Autoren beschreiben die Anwendung einer DNS-Sonde zum Nachweis des bovinen Leukosevirus. Die Proben stammten von 18 seropositiven Holstein-Kühen aus verschiedenen Betrieben. Das Vorkommen einer molekularen Variante des bovinen Leukosevirus konnte in einem Betrieb nachgewiesen werden.

SCHLÜSSELWÖRTER: Rind - enzootische Leukose - Virus - DNS-Sonde

INTRODUCTION

Enzootic bovine leukemia (EBL), which affects the B-cell sub-population of lymphocytes *(Burny* et al., 1986), is the most frequent neoplastic disease in the bovine species.
The viral agent (BLV), which causes the disease, belongs to the Retrovirus family and, up to a few years ago, was classified under the "C" type Oncoviruses subfamily together with other mammalian oncogenic Retroviruses. The functional and structural correlations between BLV and human T-lymphotropic viruses have only recently been highlighted *(Copeland* et al., 1983; *Nagy* et al., 1983; *Rice* et al., 1984; *Burny* et al., 1987; *Ikawa* et al., 1987), suggesting a new BLV classification in the Lentivirus subfamily *(Mayer,* 1986).
BLV's initial phase of infection can give rise to a transitory viraemia that disappears quite rapidly due to a massive antibody response *(Van der Maaten,* 1986); subsequently, the disease may evolve into the so-called "persistent lymphocytosis" phase or into the clinically recognised lymphosarcoma form. In the "persistent lymphocytosis" phase, linked to an expansion of the B-cell population, the BLV provirus integrates polyclonally in the lymphocyte genome *(Kettmann* et al., 1980a; 1980b); while in the tumoural phase, the provirus integration is monoclonal or oligoclonal *(Kettmann* et al., 1980b; *Gregorie* et al., 1984).

The BLV provirus in the tumour appears to be in a state of repression in so far as the presence of viral RNA has never been highlighted *(Gupta* and *Ferrer,* 1982; *Kettmann* et al., 1982).
This could signify that the presence of the virus is needed for the induction of the neoplastic process, but that other mechanisms supporting the neoplastic development intervene later on.
The current diagnostic methods commonly used and officially recognized by the EEC for eradicating the disease is a serological test based on immunodiffusion in agarose gel *(Gauthier* et al., 1982). The test uses purified gp-60 glycoprotein as the antigen for the identification of anti-BLV antibodies. In addition other more refined serological techniques have been tested such as radioimmuno-assay, indirect immunofluorescence and ELISA *(Ferrer* et al., 1976; *Devare* et al., 1976; *Poli* et al., 1981) which have provided excellent precision and reliability.
Neverthless, however sensitive the serological methods based on the identification of anti-BLV antibodies are, they are limited because they cannot identify infected animals which for some reason seroconvert and, above all, they cannot distinguish between antibodies which are the consequence of viral infection and those which have been passively acquired in colostrum *(Ponti* et al., 1985).

Currently, with recent biotechnological developments, alternative and highly specific methods can be used based on the detection of the viral genome inside the target cells' DNA, using as a probe the proviral DNA obtained from the molecular cloning of the BLV genome *(Deschamps* et al., 1981; *Sagata* et al., 1983).
The molecular hybridization technique, besides being highly specific, can also provide information on the evolutive phase of the disease and on the molecular characteristics of the viral genome.
We wanted to test the possible applications of this method to a sample population of 18 seropositive cows whose hematology indicated that they were in the so-called persistent lymphocytosis phase.

ANIMALS, MATERIAL AND METHODS

Case histories

For a nine-month period (from October 1987 to June 1988) we monitored three Friesian cattle farms (A, B and C), located in the Borough of Marene (Cuneo, Italy), where cases of enzootic bovine leukemia had been diagnosed using an immunodiffusion in agarose gel serological test. As a negative control, we used three seronegative cows from an uninfected stock-farm (G). Overall, we examined 18 cows, whose data are shown in table 1, and which were periodically subjected to the following examinations.
Blood tests: Every three months a peripheral blood sample was taken from the jugular vein for the hemogram and the differential leukocyte count (table 1). All of the subjects examined showed a constant hematological picture at each sampling, consistent with the so-called persistent lymphocytosis phase.
Serological test: Serological samples were tested every three months for antibodies against the BLV gp-60 glycoprotein external antigen at the Turin Zooprophylactic Institute. The technique used was two dimensional double immunodiffusion in agarose gel (AGID), which uses purified gp-60 antigen and reference positive sera *(Gauthier* et al., 1982). The sera of all the cattle tested were positive on each of the three occasions on which they were sampled (table 1).

Molecular Hybridization

DNA extraction: DNA samples were obtained from peripheral blood leucocytes taken from the jugular vein, through an extraction with chloroform/phenol as previously described *(Saglio* et al., 1988)

Table 1: Hematological and serological parameters

subject No	sex	age	serol.* test	leukocytes** ($x10^3/mm^3$)	lymphocytes** %	lymphocytes** ($x10^3/mm^3$)
			Stock-Farm A			
A– 2	F	7	+	42,8	89	38,0
A– 3	F	7	+	20,8	80	16,0
A– 6	F	4	+	18,8	88	16,5
			Stock-Farm B			
B– 2***	F	7	+	16,3	61	9,9
B– 3	F	8	+	17,0	71	12,0
B–22	F	2	+	15,1	76	11,4
B–23	F	7	+	16,4	73	11,9
			Stock-Farm C			
C– 7	F	6	+	38,1	88	33,5
C–10	F	8	+	16,4	73	11,9
C–19	F	8	+	19,5	82	15,9
C–21	F	11	+	22,6	60	13,5
C–28	F	8	+	18,0	70	12,6
C–29	F	5	+	20,4	72	14,6
C–20	F	4	+	12,7	80	10,6
C–36	F	5	+	16,8	74	12,4
C–40	F	5	+	16,6	84	13,9
C–45***	F	11	+	21,9	77	16,8
C–48	F	7	+	17,2	80	13,7
			Stock-Farm G (uninfected)			
G– 1	F	1	-	10,2	45	4,5
G– 2	F	2	-	9,4	48	4,5
G– 3	F	1	-	11,0	62	6,8

* The serological diagnosis, based on the study of specific antibodies for BLV gp 60 antigen, has been performed using the two dimensional immunodiffusion in agarose gel technique (A. G. I. D.).
** Hematological data – the mean values of the blood samples are shown.
*** The figures relating to these animals are incomplete as the cattle were sold before the experiment was concluded

Digestion of DNA: Each sample of bovine genomic DNA (20 μg) was digested using the following restriction enzymes according to the manufacturers recommendations (Pharmacia): Bam HI (70 International Unit total); Sac I (50 UI); EcoRI (60 UI).

The DNA samples were later subjected to electrophoresis in an 0,7% agarose gel in a TBE (Tris Borate EDTA) buffer at 45 volts for 18 hours.

Southern Blotting: Once the electrophoresis had been completed, the DNA samples were transferred onto Hybond extra-C filters (Amersham) according to Southern *(Southern,* 1975) and vacuum dired at 80 °C for 2 hours.

Hybridization was carried out in a 50% Formamide mixture, containing 5x Denhardt's solution, 750 mM of NaCl, 10% of Dextrane Sulphate, 100 μg/ml of a low molecular weight heterologous DNA for 20 hours at 42 °C.

After hybridization, the filters were initially washed in a SSC (Sodium Chloride, Sodium Citrate) 2x, SDS (Sodium Dodecyl Sulphate) 1x saline solution for 10 minutes at room temperature, and then with SSC 0,5x, SDS 0,25x saline solution at 54 °C.

The filters were then dried and exposed for autoradiography to a Kodak X-OMAT film for a period varying from 3 to 5 days at -70 °C.

Molecular probe: As a probe we used a pBR 322 plasmid containing a 9,3 kb Sac I insert, corresponding to the whole BLV provirus genome (kindly supplied by Prof. A. Burny). The pBLV plasmid was amplified and later extracted according to the lysis with alkali method *(Maniatis* et al., 1982).

The probe was then labelled for molecular hybridization, with dCTP ^{32}P according to the Nick-Translation technique at a specific activity of 0,5—1,5 × 10 cpm/μg) and used at a concentration of 2—3 × 10 cpm/ml *(Maniatis* et al., 1982).

RESULTS

Figure 1 shows the restriction map of the probe used, constituted by the BLV proviral genome, which has two Sac I sites near the LTR 5' and 3' regions and three Bam HI sites inside the BLV genome and one EcoRI site close to the 3' extremity. A Bam HI cleavage site is located in the pBR-322 plasmid vector outside the proviral insert.

Figure 2 shows the hybridization picture obtained from the first experiment which was performed at verifying the probe's specificity and at highlighting the bands we should expect in subsequent hybridizations. We diluted 50 pg of

Sac I Sac I
9,3 Kb
EcoRI
5' LTR LTR 3'
2,4 Kb 2 Kb 3,5 Kb 1,4 Kb
Bam HI Bam HI Bam HI

Fig. 1: Restriction MAP of bovine leukemia virus (BLV) genome

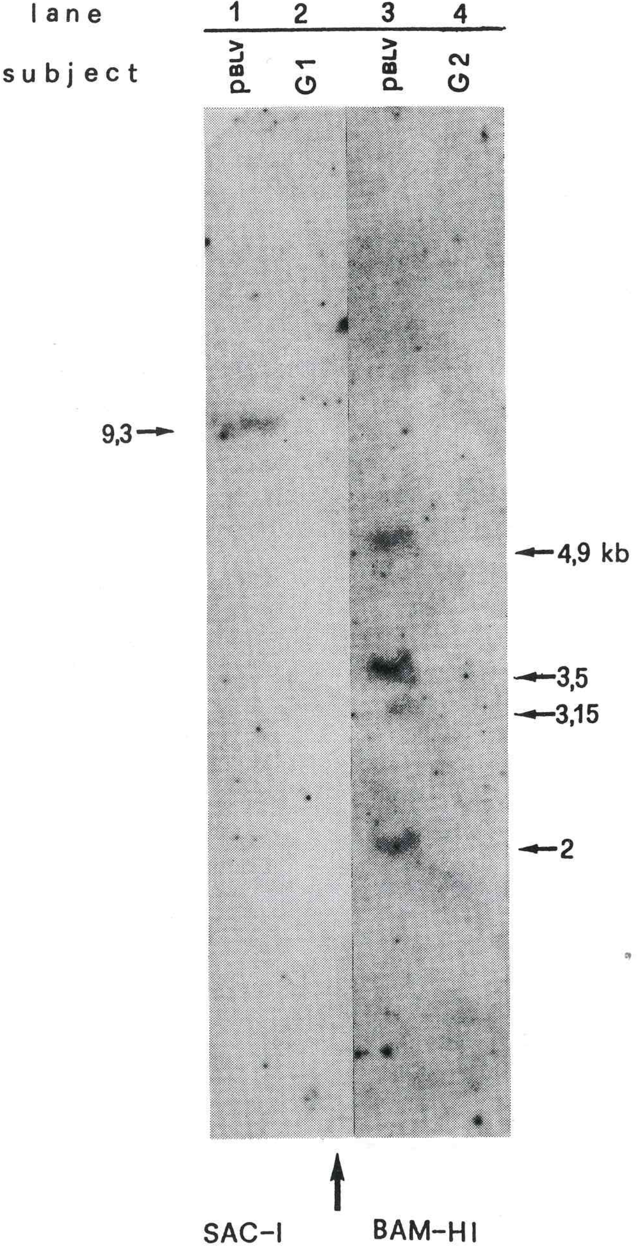

Fig. 2: Hybridization patterns of pBLV ^{32}P restriction fragments with mixtures of 50 pg of pBLV-cDNA and 20 μg of c-DNA from seronegative cattles "G-1" and "G-2" (lanes 1 and 3); and with 20 μg of c-DNA from seronegative cattles "G-1" and "G-2" (lanes 2 and 4). DNAs were exhaustively digested by SAC I (lane 1 and 2) and by Bam HI (lane 3 and 4), submitted to electrophoresis in a 0,7% agarose gel and analysed by hybridization. The molecular weight marker on the side of the gel represents the positions of fragment of known length derived by digesting lambda phage DNA simultaneously with EcoRI and Hind III.

p-BLV plasmid in 20 μg of DNA extracted from the leucocytes of the seronegative cow "G-1" from the uninfected stock-farm (G) and we subjected the sample, digested with Sac I and Bam HI enzymes, to hybridization with the probe radiolabelled with dCTP ^{32}P. Only the 9,3 kb band corresponding to the "Sac I-Sac I" proviral insert was observed in lane 1, while in lane 3 we detected the presence of four bands of 4,9 Kb, 3,5 Kb, 3,1 Kb and 2 Kb in size respectively.
The second and fourth bands (3,5 Kb and 2 Kb) corresponded to two central fragments of the proviral genome, while the first and third bands (4,9 Kb and 3,15 Kb) corresponded to two lateral viral genome fragments (2,4 Kb and 1,4 Kb) respectively summed to the two regions originating inside the pBR 322 vector as result of its sole Bam HI site (see BLV restriction map in fig. 1). In lanes 2 and 4, where only DNA from a seronegative control sample was digested, no hybridization bands were seen.
Then the samples from the three stock-farms (A, B and C) were analyzed.

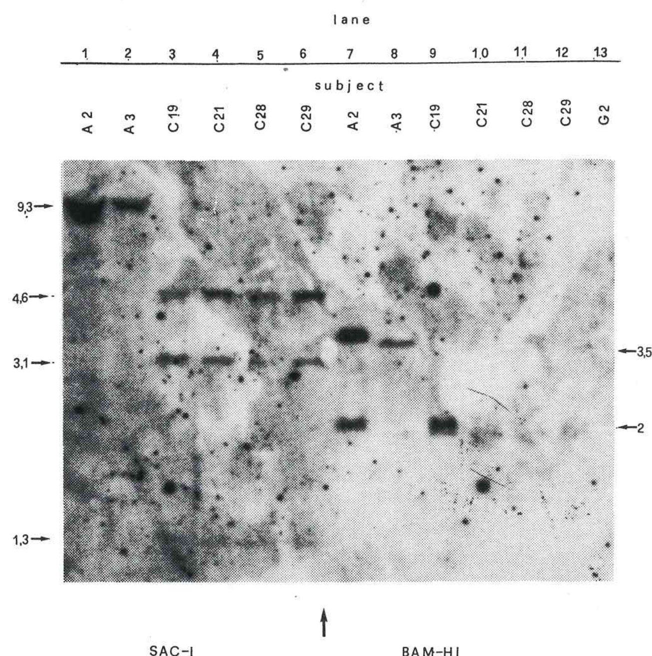

Fig. 3: Hybridization patterns of pBLV ^{32}P restriction fragment from circulating leucocyte (stock-farm A, C) of BLV infected animals. DNAs (20 μg each) of A-2, A-3, C-19, C-21, C-28, C-29 (animals in persistent lymphocytosis), and G-2 (normal control) were exhaustively digested by SAC-I (lane 1 to 6) and by Bam HI (lane 7 to 13), submitted to electrophoresis in 0,7% agarose gel and analysed by hybridization. The letter and number (A-2) on the top of each lane refers to the rank list on table 1.

The hybridization pictures for stock-farm A and B corresponded perfectly to those expected, while all the subjects from the third stock-farm (C) gave a different hybridization picture (Fig. 3; table 2). The sample from stock-farm "A" digested with Sac I enzyme (lanes 1—2) shows a single hybridization band of 9,3 Kb corresponding to the whole viral genome.

Table 2: Differences in cleavage sites highlighted within the two BLV proviral genome

		Sac-I		Bam-HI	
stock-farms	subjects number	cleavage sites No	Kb bands	cleavage sites No	Kb bands
A-B	7	2	9,3	3	3,5 2,0
C	11	4	4,6 3,1 1,3	2	2,0

Digesting the same samples with Bam-HI enzyme (lanes 7—8) there were two fairly pronounced hybridization bands of 3,5 Kb and 2 Kb, corresponding to the two central portions, Bam HI proviral genome fragments, while the two lateral provirus 5' and 3' regions were not in evidence (see BLV restriction map shown in Fig. 1). This is explained by considering that the BLV integration in cattle in the persistent lymphocytosis phase is polyclonal and thus entails a notable difference in the insertion site of the virus from cell to cell and consequently a variable distribution of the Bam HI sites in the different DNA genomic fragments in the provirus extremity.
The hybridization results of samples from stock-farm "B" were identical to those obtained from cattle from stock farm "A".
However the samples from stock-farm "C" (Fig. 3), digested with Sac-I enzyme, (lanes 3—6), gave three hybridization bands of 4,6 kb, 3,1 kb and 1,3 kb respectively, in place of the expected 9,3 Kb band. The same samples, digested with Bam-HI enzyme (lanes 9—12), had a sole hybridization band of 2 Kb, the same as the expected one, but they did not display the second 3,5 Kb band.

DISCUSSION

The hybridization fragments obtained, related to stock-farms "A" and "B", corresponded perfectly to those expect-

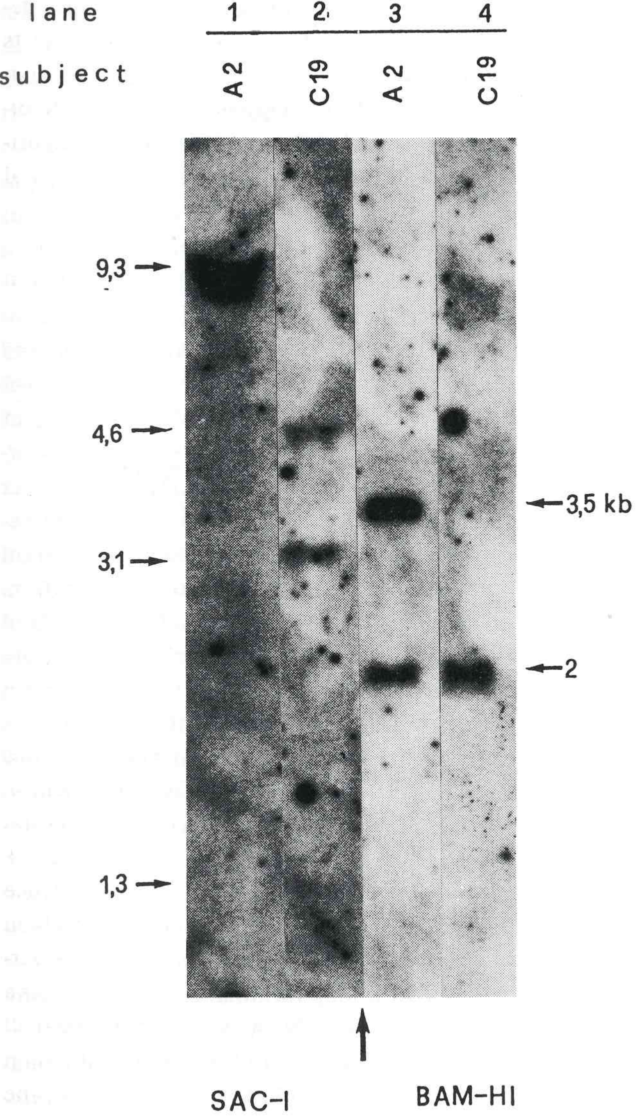

Fig. 4: Comparative molecular hybridization patterns between a cow (A-2) coming from stock-farm A and a cow (C-19) coming from stock-farm C.
DNAs (20 μg each) of A-2 and C-19 ere exaustively digested by Sac I (lane 1 and 2) and by Bam HI (lane 3 and 4), submitted to electrophoresis in a 0,7 agarose gel and analysed by hybridization.

ed and the results are the same as those previously obtained by *Gaudi* et al. (1988), and testify to the probe's reliability and the technique therefore proposed (see fig. 2; 3 and table 2).
With respect to the hybridization pictures related to stock-farm "C" samples (fig. 3; table 2), we have tried to interpret them assuming the presence of at least three polymorphisms located inside the BLV provirus genome at stock-farm "C", which would create new cleavage sites for the enzymes used (Sac I and Bam HI).
We can presume that this hypotetical BLV molecular viariant presents, inside its genome, two new cleavage sites for the Sac I enzyme which would justify the three 4,6 Kb, 3,1 Kb, 1,3 Kb bands; while, as regards the hybridization pictures obtained with the detection of the Bam HI enzyme, we can explain the detection of the sole 2 Kb central fragment by hypothesizing the loss of the third cleavage site related to the afore-mentioned enzyme (fig. 4; table 2).
Our hypothesis is supported by the detection of the same molecular hybridization picture in all the subjects examined from the same stock-farm (C) and our hypothetical BLV variant presents substantial structural modifications compared to the standard BLV genome, when it is considered that we obtained substantially different hybridization bands to those expected with both the enzymes used.
A study of literature (*Kettmann* et al., 1981; 1982; *Ikawa* et al., 1987), showed that a limited number of BLV provirus molecular variants have been described, above all for the Xba I enzyme, but the molecular pattern related to Bam HI and Sac I enzymes were not comparable to those we obtained.
Preliminary data from our epidemiological study suggests that this BLV molecular variant could have a significant incidence in Piedmont (Italy). We are investigating whether the carriers of this viral variant can present hematological, serological and, above all, clinical modifications of the progress of the disease in its terminal stage.
All of the seropositive subjects examined displayed obvious, discrete hybridization bands (table 2, 3), while in control subjects, marked by the letter "G", no hybridization fragments have ever been observed.

Table 3: Comparative diagnosis between hematology, serology and molecular hybridization in BLV infected subjects

Stock-farms	subjects number	blood test	serological test	molecular hybridization
A	3	1p	+	+
B	7	1p	+	+
C	11	1p	+	+

The diagnostic possibilities of the probe need to be examined in a wider range of cases and, in particular, it should be tested in those "false negative" and "false positive" subjects where serological diagnosis is not able to offer a valid discrimination.
The molecular investigation provides information of great interest on the viral genome's molecular structure and on possible modifications that could arise internally.
Lastly, this technique, highlighting the integration modalities of the provirus inside the lymphocytes' DNA, is able to discriminate the precocious phase of the disease, represented by persistent lymphocytosis, from the tumoural or tardive phase.

REFERENCES

Burny A., Bruck C., Cleuter Y., Couez D., Deschamps J., Ghysdael J., Gregorie D., Kettmann R., Mammerickx M., Marbaix G., Portelle D., Willems L. (1987): Bovine leukemia virus, a distinguished member of the human T lymphotropic viruses family, pp 202—216, in Retroviruses and Human Pathology. Gallo R., Stehelin D., Varnier O. edit., Humana Press, Clifton, New Jersey. — *Burny A., Bruck C., Clueter Y., Couez D., Gregorie D., Kettmann R., Mammerickx M., Marbaix D., Portelle D., Willems L.* (1986): Bovine leukemia virus as an inducer of bovine leukemia. pp 108—119, in Saltzman L. A., Animal model of Retrovirus infection and their relationship to AIDS. Academic Press. — *Copeland T. D., Oroszlan S., Kalyanaraman V. S., Sarnfadharan M. G., Gallo R. C.* (1983): Complete aminoacid sequence of human T-cell leukemia virus structural protein p 15. F. E. B. S. Lett. *162,* 390—395. — *Deschamps J., Kettmann R., Burny A* (1981): Experiments with cloned complete tumor-derived bovine leukemia virus information prove that the virus is totally exogenous to its target animal species. J. Virol. *40,* 605—609. — *Devare S. G., Stephenson, J. R., Sarma P. S., Aaronson S. A., Chandler S.* (1976): Bovine lymphosarcoma: development of a radioimmunologic technique for detection of the etiologic agent. Science *194,* 1428—1430. — *Ferrer J. F., Baliga V., Diglio C., Graves D.* et al. (1976): Recent studies for the characterization of bovine leukemia virus (BLV): development of new methods for the diagnosis of BLV infection. Vet. Microb. *1,* 159—184. — *Gaudi C., Agresti A., Marozzi A., Ponti W., Bonizzi L., Poli G., Ginelli E.* (1988): Rilevamento del virus della leucosi bovina (BLV) mediante ibridazione molecolare. La Clinica Vet. *110,* 207—213. — *Gauthier T., Guillemain B., Astier T., Mamoun R.* (1982): Sensitivity of an immunodiffusion test for the detection and quantitation of bovine leukemia virus induced antibodies. IV International Symposium on bovine leukosis, Bologna 5—7 novembre 1980, Ed O. C. Straub, Published C. E. C. Luxembourg. — *Gregorie D., Couez D., Deschamps J., Heuertz S., Hors-Cayala M. C., Szpirer J.* (1984): Different bovine leukemia virus induced tumors harbor the provirus in different chromosomes. J. Virol. *50,* 275—279. — *Gupta P., Ferrer J. F.* (1982): Expression of bovine leukemia virus genome is blocked by a non-immunoglobulin protein in plasma from infected cattle. Science *215,* 405—407. — *Ikawa Y., Sagata N., Yasunaga T.* (1987): Close structural similarities between the LTRs of bovine leukemia virus and Human T-cell leukemia viruses. pp 333—342, in Retroviruses and Human Pathology, Gallo R., Stehelin D., Varnier O. edit. Humana Press, Clifton, New Jersey 1987. — *Kettmann R., Marbaix G., Cleuter Y., Portelle D., Mammerickx M., Burny A.* (1980a): Genomic integration of bovine leukemia provirus and lack of viral RNA expression in the target cells of cattle with different responses to BLV infection. Leukemia Res. *4,* 509—519. — *Kettmann R., Ceuter Y., Mammerickx M., Meunier Rotival M., Bernardi G., Burny A., Chantrenne H.* (1980b): Genomic integration of bovine leukemia provirus: Comparison of persistent lymphocytosis with lymph node tumor form of enzootic bovine leukosis. P. N. A. S. *77,* 2577—2581. — *Kettmann R., Couez D., Burny A.* (1981): Restriction endonuclease mapping of linear unintegrated proviral DNA of Bovine Leukemia Virus. J. Virol. *38,* 27—33. — *Kettmann R., Deschamps J., Cleuter Y., Couez D., Burny A., Marbaix G.* (1982): Leukemiogenesis by bovine leukemia virus: Proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3' proximate cellular sequences. P. N. A. S. *79,* 2465—2469. — *Mayer B.* (1986): The causal agent of AIDS and comparative retroviruses of animals. Pro Veterinario *1,* 2. — *Maniatis T., Fritsch E. F., Sambrook J.* (1982): Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring N. Y. — *Nagy K., Claphman P., Cheinsong-Popov R., Weiss R.* (1983): Human T-cell leukemia virus type I: Induction of syncytia and inhibition by patient's sera. Int. J. Cancer *32,* 321—328. — *Poli G., Balsari A., Toniolo A., Ponti W., Vacirca G.* (1981): Microplate enzyme-linked immunosorbent assay for bovine leukemia virus antibodies. J. Clin. Microbiol. *13,* 46—48. — *Ponti W., Lopalco L., Poli G.* (1985): Leucosi bovina enzootica: il vecchio e il nuovo. Clin. Vet. *108,* 357—369. — *Rice N. R., Stephens R. M., Couez D., Deschamps J., Kettmann R., Burny A., Gilden R. W.* (1984): The nucleotide sequences of the env and post env region of bovine leukemia virus. Virology *138,* 82—93. — *Saglio G., Camaschella C., Serra A., Guerrasio A., Giai M., Peirone B., Ga-*

sparini P., Ceppellini R. (1988): Distribution of haplotypes of the human Ha-ras proto-oncogene in breast cancer patient and in normal population. Breast Cancer Res. Treat. *11,* 147–153. – *Sagata N., Ogawa Y., Kawamura J., Onuma M., Izaua H., Ikawa Y.* (1983): Molecular cloning of bovine leukemia virus: DNA integrated into the bovine tumor cell genome. Gene *26,* 1–10. – *Southern E. M.* (1975): Detection of specific DNA sequences among DNA fragments separated by electrophoresis. J. Mol. Biol. *98,* 503–517. – *Van der Maaten M.* (1986): Pathogenesis of Bovine Retrovirus infection. pp 213–222, in Saltzman L. A.: Animal models of Retrovirus infection and their relationship to AIDS. Ac. Press, 1986.

Impiego di una sonda di DNA provirale per la diagnosi di leucosi bovina enzootica

Gli Autori descrivono la possibilità di impiego di una sonda di DNA provirale per rilevare la presenza del virus della Leucosi Bovina (BLV) mediante tecnica di ibridazione molecolare. La ricerca è stata condotta su 18 vacche di razza Frisona, provenienti da diversi allevamenti, sierologicamente positive ed in fase di linfocitosi persistente. Viene descritta la presenza di una variante molecolare del virus evidenziata in un allevamento.

Le diagnostic de la leucose bovine à l'aide d'une sonde de DNA

Les Auteurs décrivent la possibilité d'emploi d'une sonde de DNA proviral pour déceler le virus de la leucose bovine (BLV) en employant l'hybridation moléculaire.
L'étude a été conduite sur 18 vaches de race frisonne, qui provenaient de différents élévages et qui résultaient séropositives et en phase de lymphocytose persistante. On décrit la présence d'une variante moléculaire du virus, qui a été évidenciée dans un des dits élévages.

ACKNOWLEDGMENTS

We gratefully acknowledge Prof. A. Burny and Dr. L. Willems, Dep. de Biologie Moléculaire, Université Libre de Bruxelles, who kindly provided the pBLV plasmid.

Address: B. Peirone
Università di Torino
Dipartimento di Patologia Animale
Via Nizza 52
I-10126 Torino

Manuskripteingang: 18. April 1989