Immunodiffusion Studies in Bovine Leukosis

By HIDEO NAKAJIMA, HIROYUKI OIKAWA, SHIGEKI INUMARU
and TAKEO SUGIURA*

The Forth Research Division, National Institute of Animal Health
(Yatabe, Ibaraki, 305 Japan)

Four forms of bovine lymphoid leukosis, that is, the adult, calf, thymic and skin forms have been recognized in the field from epidemiological, clinical and pathological points of view. The latter 3 types are found less frequently and accordingly, called the sporadic form. Their etiological agent still remains unknown. On the other hand, the adult form that occurs in herd aggregations and is called the enzootic form is the most common and frequently seen among cattle in the world. Bovine leukemia virus (BLV) has generally been accepted as the causative agent of the adult type[16,17]. The virus spreads primarily by horizontal transmission[9,22], propagates in the host animal, elicits the formation of antibodies and results in the onset of clinical lymphosarcoma or persistent lymphocytosis in a relatively small percentage of infected animals[7,8].

As cattle once infected with BLV show persistent infection for life and become the source of viral contamination to healthy cattle, specific antibodies in animals have been examined by serological reactions for the diagnosis of BLV infection and for the control of bovine leukosis. It has been demonstrated that most of the animal positive for serological tests retain BLV in their lymphocytes[6,21].

Many serological methods have been developed so far such as agar gel immunodiffusion test, complement fixation test, immunofluorescence, radioimmunoassay, syncytia in-

hibition test as well as enzyme-linked immunosorbent assay[1,10].

Although all of these methods provide means to identify BLV-infected cattle, immunodiffusion has been most widely employed in the world due to its specificity, simplicity and practicality. Antigens for this test are in the market in U.S.A. and West Germany.

The present paper mainly describes the procedures of immunodiffusion test, a method of BLV-antigen preparation and characterization of precipitating antibodies.

Procedures for immunodiffusion test

1) General procedures

Agar gel plates are prepared on microscope slides (26×76 mm) by using 5 ml of 0.8% purified agar (Agar Noble, Difco, U.S.A.) solution dissolved in 0.05 M Tris-HCl buffer (pH 7.2) containing 8.5% NaCl and 0.1% NaN_3. The plate is allowed to harden, the thickness of which is 3 mm.

A seven-well puncher with one central and six peripheral wells in a circle is used for cutting wells in the agar. Wells are 5 mm in diameter and peripheral wells are placed at a distance of 3 mm from the center well. The puncher for this purpose is in the market. Three sets of these combined wells can be prepared on one agar plate (Fig. 1). Disposable immunodiffusion plates are available in the market (Plate 1).

Antigen is filled in the central well and reference positive serum in the left and right peripheral wells as shown in Fig. 1. Test sera are sampled in the other four peripheral

* Present address
  Epizootic Research Station, Equine Research Institute, Japan Racing Association
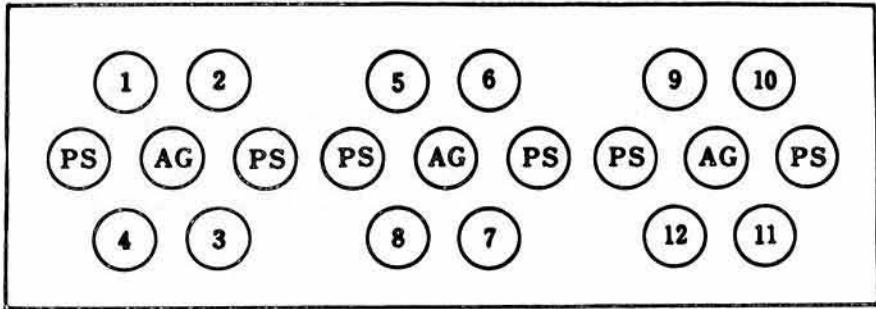 (Shiba, Kokubunji, Shimotsuga, Tochigi, 329–04 Japan)

Fig. 1. Arrangement of wells on an agar plate
Antigen (AG) is placed in the central well, reference positive serum (PS) in right and left peripheral wells, and test serum samples in 1 to 12 wells.
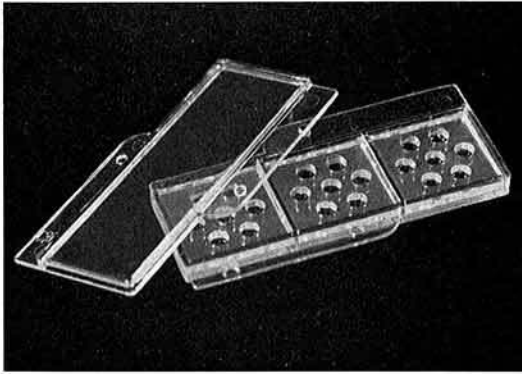

Plate 1. Disposable agar plate for immunodiffusion test in the diagnosis of bovine leukemia virus infection

wells. Accordingly, 12 serum samples can be tested using one agar plate. An amount of 0.05 ml of the material is needed in one well. The plate is incubated at room temperature for at least 24 hr.

In our experiments, three kinds of solution, that is, Tris-HCl buffer, borate buffer and simple salt solution were examined for dissolving the agar. As a result, the clearest precipitation line was obtained when Tris-HCl buffer was used. Salt concentration in agar was also tested. Concentrated salt solution ranging from 7 to 8.5% was better than physical saline for development of precipitation lines. Agarose and agar were compared with each other in the test. It was demonstrated that the agar was rather better than

agarose. The details are fundamentally the same with those reported for the diagnosis of equine infectious anemia[18,19].

Based on these results, immunodiffusion procedures for detecting BLV-precipitating antibodies were established as described above.

2) Reading the reaction

The reaction is recommended to read against a black background. An immunoviewer (Plate 2) is in the market for this purpose.
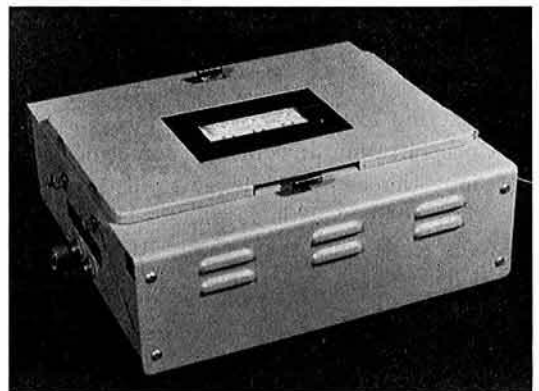

Plate 2. An immunoviewer for reading reactions in immunodiffusion test

A control precipitation line appears between the antigen and the reference positive serum 24 hr after sampling. The control line is the basis for reading the test. If the dis-
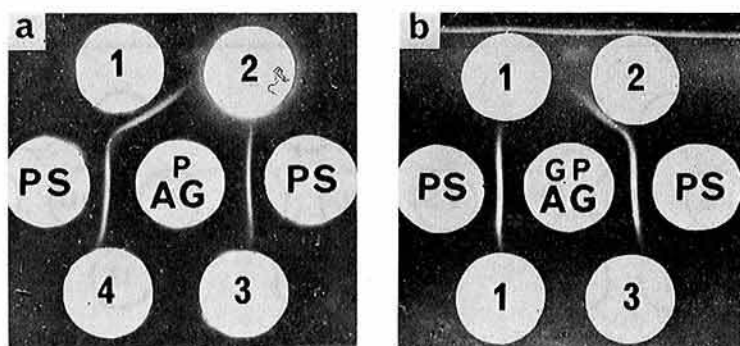
Plate 3. Examples of positive, weak positive and negative
reaction in immunodiffusion test
(a) Protein antigen (P AG) of bovine leukemia
virus is used in the test. Sample 1 is positive,
2 and 4 are negative, and 3 is weak positive.
(b) Glycoprotein antigen (GP AG) is used in
the test. Sample 1 is weak positive, 2 is positive
and 3 is negative.

tinct control line is not formed, the test must be repeated.

The test serum sample is judged to be positive when a precipitation line formed between the antigen and the test sample continuously joins the control line. The sample is regarded as weak positive case when the control line bends slightly toward the inside of the test serum well but it does not continue on to form a complete line. This sample may contain antibody at a low level. When the control line reaches straightly the test serum well or bends toward the outside of the well, this is judged to be negative. Examples of the reaction are presented in Plate 3.

A precipitation line formed, sometimes, does not join the control line smoothly, or cross the control line. This is due to the reaction between antigen other than BLV and its antibody. These reactions are regarded as nonspecific ones.

3) Preparation of reference positive serum

As described later in detail, two antigens, BLV-structural glycoprotein (gp) and inner protein (p), have been commonly used for immunodiffusion reaction. Accordingly, reference positive serum to each antigen is needed. Serum containing antibody only to gp antigen can be easily obtained from cattle

infected with BLV and used as reference positive serum. There is, however, not such serum material as contains antibody only to p antigen. Therefore, the serum with antibodies both to gp and p antigens has to be used as positive serum to p antigen in the reaction.

Optimal precipitating antibody titer as reference positive serum in immunodiffusion was determined by box titration between serially diluted antigen and antiserum (Table 1). As a result, reaction between antigen and antibody both of which contain approximately 8 units seemed to be the best.

Characteristics of immunodiffusion antigen

1) Antigenic substance

Two kinds of antigenic substance are widely known for determining antibodies against BLV in immunodiffusion test. One is glycoprotein (gp) antigen with a molecular weight of 51,000 daltons, derived from the envelope of BLV. The other is major internal protein (p) of BLV with a molecular weight of 24,000 daltons[3,5,7,20]. The immunodiffusion antigen available in the market is dual antigen containing both gp and p antigens. As a consequence, a second precipitation line appears

Table 1. Determination of optimal concentration of gp antigen and its precipitating antibody by box titration

		Antibody dilution								
		1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512
Antigen dilution	1 : 1	╫	╪	+	+	+	+	−	−	−
	1 : 2	╫	╫	╪	+	+	+	−	−	−
	1 : 4	╫	╫	╪	+	+	+	+	−	−
	1 : 8	╫	╫	╫	╪	+	+	+	+	−
	1 : 16	╫	╫	╫	╪	+	+	+	+	−
	1 : 32	╫	╫	╫	╫	╪	+	+	+	−
	1 : 64	╫	╫	╫	╫	╫	╪	−	−	−
	1 : 128	−	−	−	−	−	−	−	−	−

╫ : The strongest reaction in immunodiffusion test
╪ : Strong reaction
+ : Positive reaction
− : Negative reaction

between the antigen and test serum when it contains antibodies to both antigens, and it is often confused whether the line is specific or not. In addition, precipitation lines seemed to be clearer and easy to read when purified antigen were employed in the test. It is, therefore, recommended to use separated and purified antigen for diagnostic means.

2) Preparation of antigens

By the reason described above, each antigen is separated and partially purified by concanavalin A affinity chromatography in our laboratory using the culture fluid of Bat_2Cl_1 cells which were established by Professor J. F. Ferrer and have been persistently infected with BLV[11]. Spherical viral particles are observed in and around the infected culture cells by electron microscope. The process of budding is also found (Plate 4). These cells have been confirmed to produce a large amount of BLV antigens in our laboratory.

The infected culture fluid was first ultracentrifuged at 100,000 × g for 60 minutes. Then, gp antigen which is in soluble state in the supernatant was adsorbed to concanavalin A Sepharose 4B (Con A Sepharose, Pharmacia Fine Chemicals, Sweden). The antigen was eluted with 0.1 M α-methyl-D-mannoside. This fraction was transparent, mainly contained gp antigen and no p antigen
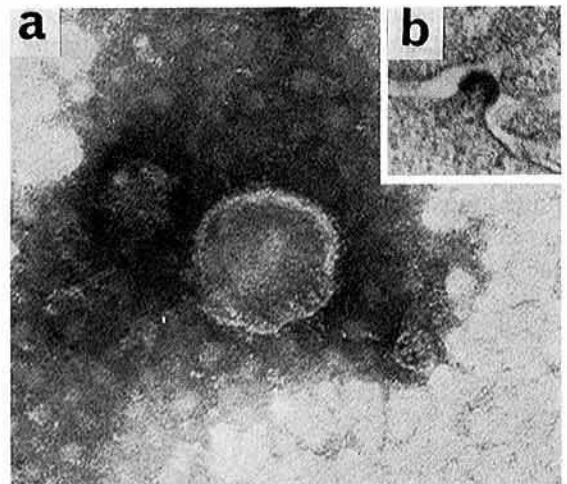

Plate 4. Electron micrographs of bovine leukemia virus
(a) A purified virus particle with diameter of approximately 100 nm (× 150,000). An outer envelope is clearly shown.
(b) A virus particle in the process of budding from the plasma membrane (× 46,000).

was demonstrated by immunodiffusion. Finally, an optimal antigenic concentration was determined by titration between serially diluted antigen and antiserum (Table 1). Antigen with 8 units was shown to react with antibodies ranging from high and low levels of titer. This has been stocked at −80°C as

the standard antigen. Antigen for practical use is prepared by the same procedures. Antigenic titer in the eluate from Con A Sepharose is quantitatively adjusted to that of the standard antigen by single radial immunodiffusion[14].

On the other hand, p antigen was prepared from the precipitates. The pellet was suspended in phosphate buffered saline containing 0.1% Triton X-100 so that the virus particles became solubilized. Then, concanavalin A affinity chromatography was carried out. The adsorbed fraction was combined with that from the supernatant. Non-adsorbed fraction was collected and centrifuged. The clarified supernatant contained p antigen and no demonstrable gp antigen. After determining an optimal concentration as stated in gp antigen, the fraction was used as p antigen in immunodiffusion. These procedures are briefly summarized in Fig. 2.

3) Stability of antigens

Stability of immunodiffusion antigen partially purified by concanavalin A affinity chromatography was examined against physical and chemical treatments. Generally, gp antigen is unstable and sensitive to various treatments. At temperatures below 4°C, gp antigen was stable but not at temperatures over 37°C. The antigen was almost inactivated at 56°C for 2 min. It was stable at pH 6 to 7 but was unstable when incubated at the other pH ranges. The antigen was rather sensitive to 0.1% Triton X-100 treatment. The antigenic activity reduced by half when gp antigen was treated with ether once. However, it did not decrease any more against further treatment. Freezing and thawing, and lyophilization did not give any influence on activity of gp antigen.

On the contrary p antigen was more stable and resistant to any treatment described above even to incubation at 56°C for 30 min.

Characterization of precipitating antibody

1) Appearance and development of antibodies

Precipitating antibodies have been detected in almost all cattle infected with BLV[10,21]. Their appearance and developing pattern to gp and p antigens are obviously different from each other.

Antibody to gp antigen appears 3 weeks in some cattle, 5 to 6 weeks in most cases and 3 months at the latest after inoculated with BLV. The antibody generally persists for life with only a few exceptions.

On the contrary, it usually takes more time before appearance of precipitating antibody to p antigen. The antibody is not detected at all in some cases, and it becomes sometimes undetectable even in positive cases. Accordingly, serum samples obtained from infected cattle have been demonstrated to be positive both to gp and p antigens in 60% of the cattle but positive only to gp antigen in the other 40% (Table 2). There has not been such serum material that shows positive only to p antigen and not to gp antigen so far examined.

From the result described above, gp antigen is more sensitive for detecting antibody and seems better for field survey of BLV

Infected culture fluid
Ultracentrifugation
Sup.   Ppt.
Con A affinity   Suspend in PBS
chromatography   containing 0.1%
                             Triton-X
Collect eluate with
α-methyl-D-mannoside   Con A affinity
                             chromatography
Concentrate
Centrifugation   Collect non-
                             adsorbed fraction
Sup.
                             Centrifugation
Adjust antigenic
titer   Sup.
Glycoprotein (GP)   Adjust
antigen   antigenic titer
                             Inner protein (P)
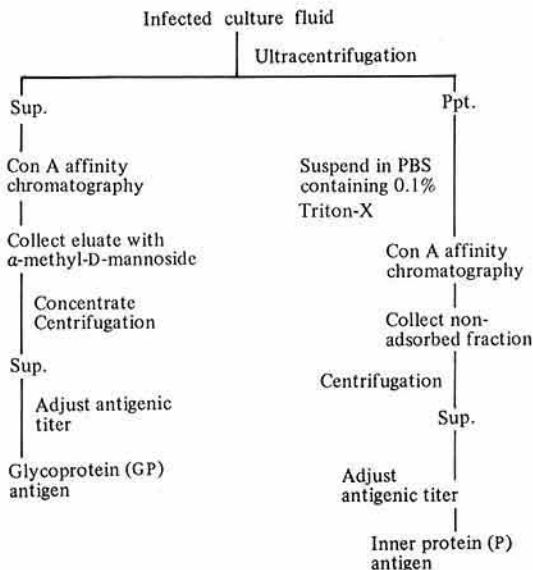   antigen

Fig. 2.   Preparation procedures of immunodiffusion antigens of bovine leukemia virus

Table 2. Prevalence of antibody to bovine leukemia virus in cattle from clinically normal herds, from contact herds and with lymphosarcoma

Cattle	No. tested	Antibody positive %	
		to gp antigen (%)	to p antigen
Normal	1,391	51 (3.7)	34 (2.4)
Lymphosarcoma*	24	22 (91.7)	22 (91.7)
Contact	145	28 (19.3)	25 (17.2)

* Pathologically classified as adult form of bovine leukosis

infection among cattle.

2) Prevalence of antibodies

A national survey of BLV infection among cattle in Japan has been performed by determining the prevalence of antibody to BLV-gp antigen starting from 1980. It was 3.7% in approximately 10,000 dairy cattle and 7.4% in approximately 3,500 beef cattle, respectively, by the result obtained until now. These values are quite different from those of U.S.A. which were between 10 to 30% in dairy cattle and 2.6% in beef cattle[4,12].

There were great differences in the antibody prevalence among districts of Japan. It was the greatest in the cattle of Tohoku district, showing 12.3% in dairy cattle and 22.5% in beef cattle. On the contrary, there was no seropositive case among serum samples from 4 prefectures, namely, Yamanashi, Shiga, Kochi and Okinawa.

Relationship between the prevalence of BLV antibody and the environment has been studied. The prevalence has been closely related to age, calving frequency, herd size, location, mountain pasturing and the previous occurrence of bovine leukemia in the herd.

Prevalence of BLV antibody was also tested in cattle from contact herds and with lymphosarcoma which was pathologically diagnosed as the adult type of leukosis, and compared with that in clinically normal dairy cattle of the central district of Japan (Table 2).

The prevalence of antibodies to gp and p antigens was 3.7% and 2.4%, respectively, in clinically normal cattle. These values are

almost the same with those obtained by a national survey as described above. On the other hand, 22 of 24 cases with lymphosarcoma (91.7%) were positive to both antigens. By the result, involvement of BLV in adult type of bovine leukosis was positively demonstrated. Out of 145 serum samples from contact herds where the adult type of bovine leukosis had occurred before, 28 samples (19.3%) were positive to gp antigen and 25 samples (17.2%) to p antigen, respectively. The prevalence of precipitating antibody was obviously higher than that of clinically normal herds, and the fact suggests horizontal transmission of BLV due to contact with clinically infected cattle with adult form of bovine leukosis.

3) Antibody titer

For antibody titration serum samples were serially diluted twofold with saline and reacted in agar plates. Antibody titer was expressed as reciprocal of the highest dilution showing complete precipitation line. The mean value of antibody titers to gp and p antigens in serum samples from clinically normal herds was 1:4.4 and 1:0.6, and maximum titer to gp and p antigens was 1:16 and 1:4, respectively. Seventeen samples (33.3%) were positive only to gp antigen and negative to p antigen (Table 2).

On the other hand, the mean value of the titer in cattle with lymphosarcoma was 1:44 to gp antigen and 1:22 to p antigen, respectively. Maximum titer was 1:128 to both gp and p antigens. The result indicates that antibody titer especially against p antigen

was strikingly higher in the material from the cattle with lymphosarcoma compared with subclinically infected cases. Similar results were reported in elsewhere[13].

Based on these data, 95% confidential ellipse and 95% tolerance ellipse on antibody titers of each group were calculated (Fig. 3). Significant difference was shown between two groups with 5% confidence limits.
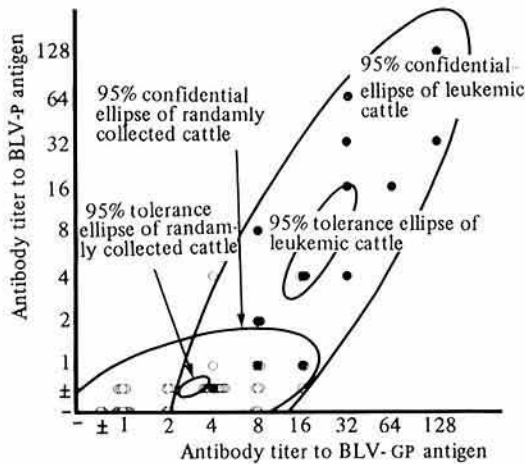

Fig. 3.   Relationship of antibody titers to gp and p antigens in cattle with lymphosarcoma (●) and those subclinically infected with bovine leukemia virus (BLV) (○)

4)   Physicochemical properties

Precipitating antibodies to BLV antigens present in cattle suffering from various stages of leukosis have been identified as immunoglobulins of IgG_1 subclass and IgA class[15]. Each of these precipitating immunoglobulins exhibited several electrical charges as well as molecular sizes. Both antibodies to gp and p antigens were fractionated in 19S and 7S fractions by gel filtration using Sephacryl S-200. According to our experiments, however, both antibodies were eluted in only 7S fraction by Sephadex G-200 filtration (Fig. 4). This disagreement is probably due to the difference of stages of the disease. Precipitating antibodies in subclinically infected cases were also found in 7S fraction.
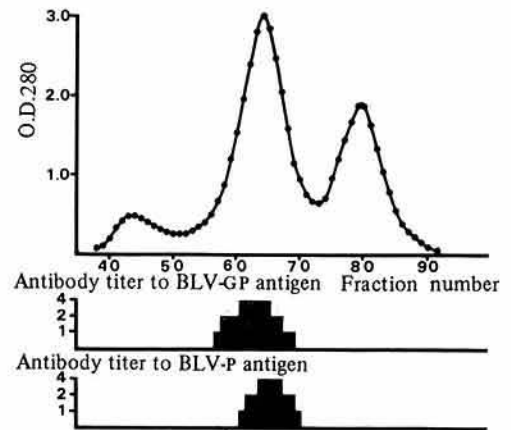

Fig. 4.   Elution profile of precipitating antibodies to gp and p antigens in the serum from a cow with lymphosarcoma by Sephadex G-200 filtration

5)   Maternal antibodies

Only 18% of calves born from infected cows are observed to be antibody positive right after the birth and proved to be infected with BLV by prenatal transmission[23]. A majority of the calves which are not infected and antibody negative, however, becomes positive soon after the birth by absorbing colostrum. Antibodies remain positive for variable periods ranging from 2 to 7 months (mean 3.7 months) before becoming negative. This indicates residual maternal antibodies acquired through colostrum[2]. Thus, immunodiffusion test is not useful in the diagnosis of BLV infection in calves under 8 months of age.

Acknowledgements

sion of their institute for taking electron micrographs of bovine leukemia virus.

References

1) Burny, A. et al.: Bovine leukemia virus involvement in enzootic bovine leukosis. In: Klein, G. & Weinhouse, S. eds., Advances in Cancer Research, Vol. 28, 251–311, Academic Press, New York (1978).

2) Chander, S. et al.: BLV-antibodies in serial sampling over five years in a bovine leukosis herd. *Ann. Rech. Vet.*, 9, 797–802 (1978).

3) Devare, S. C., & Stephenson, J. R.: Biochemical and immunological characterization of the major envelope glycoprotein of bovine leukemia virus. *J. Virol.*, 23, 443–447 (1977).

4) Evermann, J. F. et al.: Prevalence of bovine leukemia virus antibody in seven herds of Holstein-Friesian cattle. *J. Amer. Vet. Med. Assoc.*, 177, 549–550 (1980).

5) Ferrer, J. F. et al.: Recent studies on the characterization of bovine leukemia virus (BLV): development of new methods for the diagnosis of BLV infection. *Vet. Microbiol.*, 1, 159–184 (1976).

6) Ferrer, J. F. & Diglio, C. A.: Development of an in vitro infectivity assay for the C-type bovine leukemia virus. *Cancer Res.*, 36, 1068–1073 (1976).

7) Ferrer, J. F. et al.: Diagnosis of bovine leukemia virus infection: evaluation of serologic and hematologic tests by a direct infectivity detection assay. *Amer. J. Vet. Res.*, 38, 1977–1981 (1977).

8) Ferrer, J. F. et al.: Relationship between lymphosarcoma and persistent lymphocytosis in cattle.: a review. *J. Amer. Vet. Med. Assoc.*, 175, 705–708 (1979).

9) Ferrer, J. F.: Bovine leukosis: Natural transmission and principles of control. *J. Amer. Vet. Med. Assoc.*, 175, 1281–1286 (1979).

10) Ferrer, J. F.: Bovine lymphosarcoma. In: Brandly, C. A. & Cornelius, C. E. eds., Advances in Veterinary Science and Comparative Medicine. Vol. 24, 1–68, Academic Press, New York (1980).

11) Graves, D. C. & Ferrer, J. F.: In vitro transmission and propagation of the bovine leukemia virus in monolayer cell culture. *Cancer Res.*, 36, 4152–4159 (1976).

12) House, C. et al.: Antibodies to the glycoprotein antigen of bovine leukemia virus in the cattle population of five states. *Cornell Vet.*, 67, 510–522 (1977).

13) Kono, Y. et al.: Antibody titers in cattle clinically and subclinically infected with bovine leukemia virus. *Vet. Microbiol.* 6, 167–170 (1981).

14) Mancini, G. et al.: Immunological quantitation of antigens by single radial immunodiffusion. *Immunochem.*, 2, 235–254 (1965).

15) Matthaeus, W.: Heterogeneous properties of BLV-precipitating immunoglobulins in bovine leukemic sera. *Ann. Rech. Vet.*, 9, 635–641 (1978).

16) Miller, J. M. et al.: Virus particles in phytohemagglutinin-stimulated lymphocyte culture with reference to bovine lymphosarcoma. *J. Nat. Cancer Inst.*, 43, 1297–1305 (1969).

17) Miller, J. M. & Olson, C.: Precipitating antibody to an internal antigen of the C-type virus associated with bovine lymphosarcoma. *J. Nat. Cancer Inst.*, 49, 1459–1461 (1972).

18) Nakajima, H. & Ushimi, C.: Immunodiffusion studies of purified equine infections anemia virus. *Infect. Immun.*, 3, 373–377 (1971).

19) Nakajima, H.: Diagnosis of equine infections anemia by immunodiffusion test. *JARQ*, 8, 47–53 (1974).

20) Onuma, M. et. al.: An ether-sensitive antigen associated with bovine leukemia virus infection. *J. Nat. Cancer Inst.*, 55, 1155–1158 (1975).

21) Onuma, M. et al.: Detection of bovine leukemia virus by syncytium assay. *Cand. J. Comp. Med.*, 44, 289–293 (1980).

22) Piper, C. E. et al.: Seroepidemiological evidence of the horizontal transmission of the bovine C-type virus. *Cancer Res.*, 35, 2714–2716 (1975).

23) Piper C. E. et al.: Postnatal and prenatal transmission of the bovine leukemia virus under natural conditions. *J. Nat. Cancer Inst.*, 62, 165–168 (1979).