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Seroepizootiological Study on Bovine Leucosis in Korea

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Introduction

Bovine leucosis (bovine lymphosarcoma, BL) is the most common neoplastic disease of the lymphoid tissues in cattle causing considerable economic loss in dairy producing countries.[1,3,13,25] According to clinico-pathological bases, BL is classified into adult form commonly referred to as enzootic bovine leucosis (EBL) and sporadic form including thymic, calf (juvenile) and cutaneous (skin) types.[19,25] The adult form is the most common type of BL with a strong tendency to aggregate in certain geographic areas.[1,3,25] EBL is contagious and induced by bovine leukemia virus (BLV), an exogenous retrovirus.[1,3,17,25] However, the etiological agents of the other three types of the sporadic form are so far unknown.

It is known that EBL is transmitted horizontally to susceptible calves and vertically to progeny.[1,3,18,25] Therefore, it has become a reportable disease in some countries, and attempts are being made to eradicate it by testing and slaughter programs.[1,8,13] Recent epidemiological surveys have shown that BLV infection is widespread in European countries, the United States, Canada, Japan, South America, Israel and most African countries.[1,3,25]

Several virological and serological methods have been designed and used for diagnosis of BLV infection.[3] Recent comparative studies of serological tests revealed that immunodiffusion (ID) test using BLV glycoprotein antigen (gp-ID) was the best test for diagnosis of BLV infection.[11,21,22]

The first survey of BL in dairy herds in Korea was made by Son and Kim[24] in the southern part of the country in 1968. They reported that some clinical cases of BL were occurring in imported dairy herds, and that, of 521 cattle tested hematologically, 15 (2.9%) were positives and 28 (5.4%) were suspects. There have been a few reports[14] on clinical outbreaks of BL, but no epidemiological studies were made until 1980. According to abattoir surveys in 1979, Bak and Lim[2] have reported that 3 (0.7%) out of 452 dairy cows slaughtered in Seoul district revealed typical BL tumor lesions in lymph nodes, heart, liver, etc.

The present paper describes the results of the seroepidemiological surveys on BLV infection in dairy herds throughout the country by using ID test with the glycoprotein antigen of BLV, and etiological studies on the BLV antibody carrying cattle by means of fluorescent antibody test, syncytium assay and electron microscopy.

Materials and Methods

Sera: Sera from 2003 Holstein dairy cattle on 164 farms which are located throughout the country were collected randomly in 1980 and 1981, which represented 1.3% of all dairy cattle in Korea. The number of sera tested by areas are

shown in Tables 1 and 4. Most of the sera were obtained from 17 Provincial Animal Health Centers, collected for detection of BL or brucellosis. The serum samples from 117 breeding bulls from 3 stations in the central region were also obtained through the animal health centers. Reagent serum, negative, weak positive and positive reference sera of bovine origin in Leukassay-B kit (Pitman-Moore, Inc. U.S.A.) were used as the standard control sera for all ID tests. Antisera for bovine syncytial (BS) virus, infectious bovine rhinotracheitis (IBR) virus and parainfluenza-3 (PI-3) virus were kindly supplied by Dr. Onuma at Hokkaido University, Japan.

Immunodiffusion test: Leukassay-B, a glycoprotein antigen of BLV, was used for the detection of BLV infection. The ID test was performed according to the direction of antigen with slight modiffication. In this experiment, ID tests were run with 0.9% solution of Noble's special agar prepared with 0.2% NaOH, 0.9% H_3BO_3 and 7% NaCl. Petri dishes (100mm) were filled with the agar in depth of 2.5mm and cut a central 4.5mm antigen well seperated 3mm from 6 peripheral 4.5 mm serum wells. Wells were filled only once and incubated at room temperature. After 72 hours of incubation, the results were read (Fig. 3).

Immunofluorescent antibody (FA) test: BLV fluorescent antibody for the direct staining method was also supplied by Dr. Onuma. The BLV FA was the fluorescein isothiocyanate conjugated sheep V34 r-globulin, which was prepared from the sheep experimentally infected with BLV[4]. The lymphocytes separated from the peripheral blood of cattle by ficoll-paque densito-gradient centrifugation were cultured in the same manner as previously described[4,6,7] by using Eagle's minimum essential medium (GIBCO) supplemented with 10% fetal calf serum (Difco) and phytohemagglutinin-M (Difco) at a concentration of 0.03 ml/ml for 72 hrs.

A direct FA test was performed on the cultured cells. The cells were fixed in cold ethanol, stained with V34 conjugate (diluted 1 : 10) at 37°C for 45 mins., and then washed. The cells were examined by a fluorescence microscope, and the percentage of positive cells was determined after counting 100 to 150 cells. The specificity of the BLV FA used in the present experiment has been reported by Driscoll and Olson.[4]

Syncytium assay (SA) : Bovine embryonic splenic cells were used as indicator cells. They were cultured in the same manner as previously described.[9] The second to third passage of the cultured cells were used for SA. One ml of indicator cells(5×10^5 cells/ml) and one ml of lymphocytes (4×10^5 cells/ml) were seeded simultaneously in the test tubes with a cover-slip. After 5 days incubation at 37°C, the cover-slips were fixed in ethanol, stained with hematoxylin-eosin, and observed microscopically for syncytia formation. The appearance of cells with 5 or more nuclei was considered to be positive for syncytium formation. The specificity of the syncytium formation by BLV was tested as prevously described.[6,7,9]

Electron microscopy (EM): EM for detection of BLV in the cultured lymphocytes was carried out as previously described with minor modifications.[16,17] The cell pellets were fixed in 3% glutaraldehyde and postfixed in 1% osmium tetroxide. After embedding and sectioning, the thin sections were stained with 25% uranyl acetate and examined with an electron microscope.

Results

When eight hundreds and fifty two sera collected randomly from 30 dairy herds in 10 districts in the central region were tested, by the ID test 323(37.9%) were positive reactors for BLV antigens, as presented in Table 1. The prevalence of reactors varied from 22.2% to 61.1% according to districts. Sera from Seong-whan, Suweon and Pyeongtaek districts, where clinical cases of bovine lymphosarcoma frequently occur, showed a higher prevalence of reactors (44.4-61.1%), compared with other districts.

Comparison between the results obtained from hematological test by Bendixen Key and ID test are given in Tables 2 and 3. When the results of hematological and ID tests for 400 heads of cattle

were compared by herds (Table 2), 8.6% of the cattle tested hematologically showed lymphocytosis, whereas 45.5% of the cattle tested by ID test were reactive for BLV antigens.

When the relationship between the results of hematological and ID test was analyzed(Table 3), it was found that most (8 out of 9, 88.9%) of the cattle with lymphocytosis were positive for BLV antibodies, but 37.3% (78 out of 209) of the animals normal in the hematological test were also reactive against BLV antigens.

Table 4 presents the results obtained from ID tests for BLV antibodies in dairy cattle from 4 areas-Central, Honam, Youngnam and Youngdong. From the total of 2003 sera tested, 596 sera (29.8%) were positive for BLV antibodies with the rates varying from 23.7% to 41.8%. The prevalence of reactors appeared higher in Gyeonggi and Chungcheong provinces in the Central area, compared with those in the other regions. Distributions of the BLV antibody-positive cattle on a country-wide basis are mapped in Fig. 1. The results of ID tests were analyzed by the age groups and the size of herd. Although a few reactors (11.4%) were found in cattle less than 2 years old, the number of reactors increased gradually with age, showing the highest pesitive rate in the ages between 6 and 8 years (Table 5). The larger size of herd showed a higher prevalence of BLv antibody carriers, with the highest rate in herds of 20 to 50 heads of cattle (Table 6).

One hundred and seventeen breeding bulls from three stations in the central region were tested. As shown in Fig. 2, 4 out of 70 Korean bulls(5.7%) and 14 out of 39 Holstein bulls (35.9%) were positive for BLV antibodies. None of the other 8 breeding bulls, including Hereford, Aberdeen Angus and Charolais, reacted to BLV antigens.

The prevalence of BLV antibody-positive cattle was analyzed by the number of herds tested. As the results presented in Table 7, of the 164 dairy

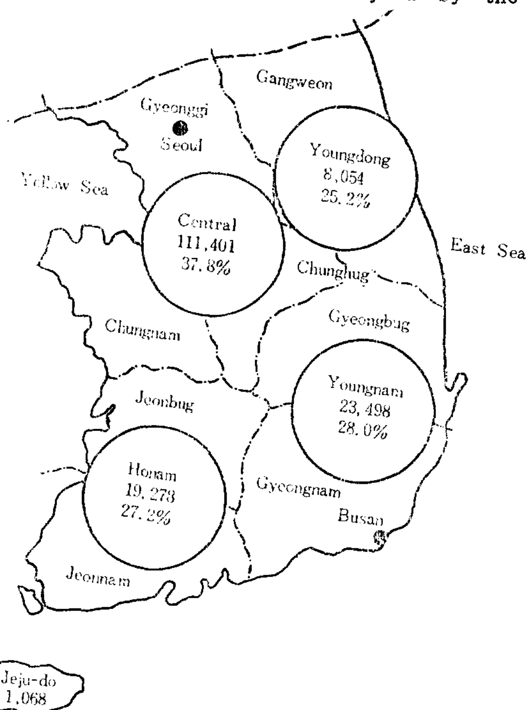

Fig. 1. Prevalence of BLV antibody-positive cattle in various areas in Korea. (Numbers represent total heads of dairy cattle in the areas.)

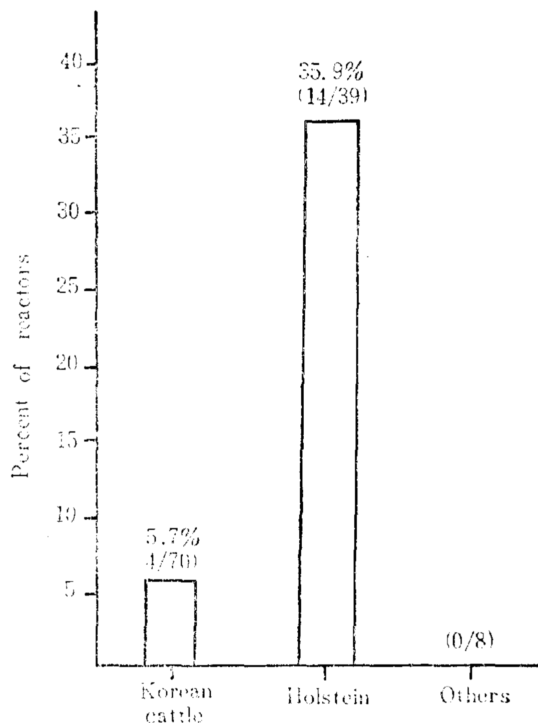

Fig. 2. Prevalence of antibodies to bovine leukemia virus among breeding bulls in three stations.

Korean cattle: 7-10 years old
Holstein :3-8 years old
Others :3-5 years old
(Hereford, AberdeenAngus, Charolais)

Table 1. Results of Immunodiffusion (ID) Test for Antibodies to BLV Antigens in Dairy Cattle from Gyeonggi and Chungcheong Provinces

Districts	No. of herds sampled	Total no. of cattle in herds	No. of sera tested	No. of positive sera	Positive[a] rate (%)
Euijeongbu	4	265	54	19(1)[b]	35.2
Incheon	3	189	68	21(2)	30.9
Anyang	3	352	126	28(3)	22.2
Suweon	2	111	53	26(1)	49.1
Pyeongtaeg	3	187	54	24(1)	44.4
Anseong	2	252	115	47(2)	41.0
Incheon	3	186	65	21(1)	32.3
Seongwhan	4	807	144	88(5)	61.1
Daejeon	3	52	22	8(0)	36.4
Cheongju	3	276	151	41(4)	27.1
Total	30	2,677	852	323(21)	37.9

a: $\frac{\text{No. of positive sera}}{\text{No. of sera tested}} \times 100$

b: The number in parentheses indicates the number of weak positives.

Table 2. Comparison between the Results of Hematological and Serological Tests for Cattle in the Same Herds by Districts

Districts	Positive rate	
	Hematology[a] (%)	ID test[b] (%)
Anseong	10/118(8.5)	47/115 (41.0)
pyeongtaeg	8/84 (9.5)	24/54 (44.4)
Suweon	5/49 (10.2)	26/53 (49.1)
Seongwhan	9/91 (9.9)	68/122 (55.7)
Incheon	0/32 (0)	21/65 (32.3)
Total	32/374 (8.6)	186/409 (45.5)

a: Results of 1st test

b: No. of positive sera/No. of sera tested.

Table 3. Relationship between the Results of Hematological and Immunodiffusion(ID) Tests

	ID test		Total
	Positive	Negative	
Hematology			
Positive . . .	8	1	9
Negative . . .	78	131	209
Total	86	132	218

Table 4. Prevalence of Bovine Leukosis Virus Antibody-positive Dairy Cattle in Various Regions in Korea

Areas	Provinces	No. of herds tested	No. of sera tested	No. of reactors	%	Mean %
Central	Gyeonggi	31	341	124	36.4	37.8
	Chungcheong	18	122	51	41.8	
Honam	Jeonnam	26	388	114	29.4	27.2
	Jeonbug	21	307	75	24.4	
Youngnam	Gyeongnam	22	346	82	23.7	28.0
	Gyeongbug	30	348	112	32.2	
Youngdong	Gangweon	16	151	38	25.2	25.2
Total		164	2003	596	29.8	

Table 5. Prevalence of BLV Antibody-positive Dairy Cattle* According to Age Groups

Age groups (years)	No. of reactors / No. of tested	%
<2	20/176	11.4
2 - 4	147/594	24.8
4 - 6	94/349	26.9
6 - 8	114/288	39.6
>8	46/133	34.6
Total	421/1540	27.3

* Tested by immunodiffusion method.

Table 6. Prevalence of BLV Antibody-positive Dairy Cattle* in Accordance with the Size of Herd

Herd size (heads)	No. of reactors / No. of tested	%
<20	12/63	19.1
20 - 50	148/476	31.1
50 - 80	67/259	25.9
80 - 110	46/210	21.9
>110	148/532	27.8
Total	421/1540	27.3

* Tested by immunodiffusion method.

Table 7. Distribution of Herds with BLV Antibody-positive Dairy Cattle According to Various Range of Positive Rate by Provinces

Provinces	No. of herds tested	Range of positive rate (%)					
		0	0-<20	20-<40	40-<60	60-<80	>80
Gyeonggi	31	1*	4	9	8	6	3
Chugcheong	18	3	6	3	1	3	2
Jeonnam	26	2	4	7	6	7	0
Jeonbug	21	4	2	7	3	4	1
Gyeongnam	22	2	4	6	7	2	1
Gyeongbug	30	1	8	7	9	3	2
Gangweon	16	4	2	3	4	2	1
Total	164	17	30	42	38	27	10
(%)		(10.4)	(18.3)	(25.6)	(23.2)	(16.5)	(6.1)

* The numberof herds included in the range of positive rate by immunodiffusion test.

Table 8. Detection of Bovine Leucosis Virus Antigens in the Lymphocytes from BLV Antibody-positive Cattle by Immunofluorescent Test, Syncytium Assay and Electron Microscopy

Cattle identity	Breeds[a]	Age	Lymphocyte/mm^3	ID[b] test	Direct FA[c] staning	Syncytium assay	BLV particles by E.M.
BLV-antibody positive							
331	H	4	4,598	++	+(19)	+	None
SC	KN	2	5,026	+	+(8)	NT	NT
SC-A	KN	2	6,283	++	+(12)	NT	NT
285	H	5	3,926	++	+(27)	+	None
312-A	H	6	4,185	+	+(21)	NT	NT
312-B	H	6	3,926	++	+(30)	NT	NT
286	H	5	23,654	++	+(25)	+	Detected
290	H	4	13,246	++	−(0)	−	None
312-C	H	6	3,384	++	+(28)	+	NT
338	H	4	19,426	++	−(0)	+	Detected
297	H	7	3,720	++	−(0)	−	None
BLV-antibody negative							
310	H	5	4,258	−	−(0)	−	None
342	H	3	6,504	−	+(7)	−	None
336	H	4	4,326	−	−(0)	−	None
332	H	5	3,728	−	−(0)	−	NT

a : H=Holstein-Friesian, KN=Korean native.
b : immunodiffusion test; +=weak positive, ++=strong positive,
c : Numbers represent % of cells showing fluorescence. Antisera for bovine leucosis virus, bovine syncytial virus, infectious bovine rhinotracheitis virus and parainfluenza-3 virus were used to prove the specificity of FA staining. NT=not tested.

herds examined, 17 herds (10.4%) have no reactors against BLV antigens, while the most herds(42 herds, 25.6%) were included in the range of 20 to 40% of positive rates, and 10 herds (6.1%) in the range of over 80% of positive rates.

Eleven BLV antibody-postive cattle and 4 negative controls were selected by ID tests and tested for BLV antigens or BLV particles by the direct FA test, SA and EM (Table 8). The cultured lymphocytes from 8 cattle (72.7%) out of 11 BLV antibody-positive cattle showed the specific fluorescence for BLV in the cytoplasm and/or cell membrane, with rates of fluorescent cellss varying from 8% to 30%. One of 4 control cattle also revealed the specific BLV fluorescence by the rate of 7% in the lymphocytes. SA was carried out for 7 BLV antibody-positive cattle and 4 controls. The lymphocytes from 5 (71.4%) out of the 7 BLV positive cattle were infected with the syncytium forming BLV (Fig. 4). In electron microscopic examination of the cultured lymphocytes from 6 BLV antibody-positive cattle, cattle Nos. 286 and 338 (33.3%) showed the typical type C virus particles with the size of 90 to 110 nm around microvilli and in intracytoplasmic vacuoles (Fig. 5A, B).

Discussion

Since the recognition of BLV in 1969 in short term lymphocyte culture derived from leukotic cows by Miller *et al*[17] the sensitive and specific virologic and serologic techniques for detection of BLV-infected cattle have been rapidly developed during the 1970s by many researchers.[1,3,4,6,23]

Besides the conventional hematological method, ID test, complement fixation test, SA method, FA test, radio-immunoassay and enzyme-linked Immunosorbent assay (ELISA) have been reported for the diagnosis of EBL. [3,6,7,11] Among various techniques, agar gel ID test, using glycoprotein antigen (gp 51) of BLV was recommended as a Standard method for EBL diagnosis by the commission of European Communities on Bovine Leucosis in 1977, and has been widely used since the test is specific, highly sensitive and extremly simple. [3,13,22,] Recently the ID test has been proved to be equally or more sensitive than the CF test. [11,22,]

In the present experiments, relationship between hematological test and ID test using BLV glycoprotein antigen was compared. It was proved that the serological method seemed to be a more accurate means of dtection of BLV-infected animal, and could efficiently be applied for detection of EBL, replacing the hematological method[13].

The countrywide seroepidemiological survey for EBL was carried out by using the ID test with a BLV glycoprotein antigen, Leukassay-B. The results of antibody survey indicated that BLV is widespread among the dairy herds as well as among the Korean native cattle throughout the country, and EBL may cause a substantial amount of economic loss to dairyi ndustry. In the present survey, the BLV antibody positive rates were ranging from 22.2% to 61.1% by regions, averging 29.8%, and that was found slightly higher than those in Denmark and Japan. The average BLV positive rates were 20.8% in Denmark[8], and 5.8% in dairy cattle and 11.3% in beef cattle in Japan in 1981. In the U.S.A., the prevalence of reactors in dairy cattle varied from 2% to 86% in California,[5,15] and from 23% to 64% in the state of Washington.[2,5] However, the incidence rate of reactors varied according to the geographical conditoin, breeding styles and history of herds. It has been reported in Japan [21,22] that cattle which were grazed together on the same pasture during summer showed higher rates of positive reactors than those from other areas, where the animals were kept individually throughout a year.

In the present studies, the rates also varied depending on regions. the central areas, where frequent occurrence of clinical bovine lyphosarcoma has been reported in the country[2,13,14], showed the highest prevalence of BLV antibody carriers.

The distribution of the reactors by age and herd size was similar with the results of surveys in the U.S.A.[5,12] and Japan.[21,22] The differences could have been caused by the frequency of contact with the infected cattle and the pathogenesis of BLV which depends on the genetic make-up, environmental factors, and immunological surveillance.

It has been known that EBL is transmitted horizontally to susceptible new born calves, and the disease could also spread vertically from dam to offspring through the placenta and the spermatozoa[1,3,10,18,20]. Therefore, it may be predicted that the high incidence of BLV carriers in the breeding bulls may cause the propagation of the virus among the cattle population.

For detection of BLV in the peripheral lymphocytes, EM as a direct method, and FA techniques, SA and ELISA techniqques as indirect methods, were applied[3,4,9,17]. In the present study, FA test, SA and EM techniques were used to verify the etiological aspects of the BLV antibody carrying cattle in Korea. It was evident that serologically positive animals for BLV were closely associated with the presence of the BLV in the lymphocytes. Therefore, it may be speculated that a considerable proportion of the cattle in korea are affected with the virus and act as carriers of BLV, and also playing an inportant role in the spread of the virus.

Under the prevailing circumstances in this country, the authors recommend four strategies for the control and prevention of FBL; a) isolation of BLV carrier from the uninfected herds, b) isolation of negative calves from the BLV carriers, c) restriction on the import of BLV infected animals, d) establishment of culling programs in accordance with the results of serological tests.

Summary

Since bovine lymphosarcoma causes considerable economic loss to the dairy industry, seroepidemiological survey on bovine leucosis virus (BLV) was carried out for the dairy herds throughout the country to observe the epidemiological situation of the disease by using immunodiffusion test. Attempts were simultaneously made to detect bovine leucosis virus in the lymphocytes from BLV antibody-positive cattle by means of fluorescent antibody techniques, syncytium assay and electron microscopy.

In immunodiffusion test for BLV antibody in 2003 heads of dairy cattle selected randomly from 164 herds, the prevalence of positive reactors by regions were 37.8% in Central, 27.2% in Honam (Southwest), 28.0% in Youngnam (Southeast) and 25.2% in Youngdong (East coast)and averaging 29.7%. By provinces, Chungcheong appeared the highest prevalence of BLV antibody carriers (41.8 %), while Jeonbug revealed the lowest incidence rate (24.4%).

When the results of serological studies were analyzed by age groups and the sizes of herds, the number of reactors increased gradually with the advance in the age of cattle and the herd size. The highest rate of BLV carriers was found in the ages between 6 and 8 years, and in the size of herds with 20 to 50 heads.

One hundred and seventeen breeding bulls from the central regions were tested for BLV antibody. Four out of 70 bulls (5.7%) of Korean cattle and 14 out of 39 bulls (35.9%) of Holstein were reactive for BLV antigens. Of 164 dairy herds examined, 17 herds (10.4%) have no BLV antibody-positive cattle, while 42 herds (25.6%) were included in the range of 20 to 40% of the positive rate and 10 herds (6.1%) in the range of over 80% of the rate.

When the lymphocytes from the BLV antibody carrying cattle were cultured in the presence of phytohemagglutinin and stained with FITC-conjugated sheep anti-BLV serum, 8 out of 11 cases (72.7%) of BLV positive cattle revealed specific fluorescence for BLV in the lymphocytes. In syncytium assay of the peripheral lymphocytes of the cattle, 5 out of 7 (71.4%) lymphocytes from BLV antibody carriers induced syncytia in the indicators of bovine embryonic splenic cells. The cultured lymphocytes were examined with an electron microscope to detect the BLV particles. Two out of 6 specimens (33.3%) from the reactors showed the typical type C virus with the size of 90 to 110 nm around microvilli and in intracytoplasmic vacuoles.

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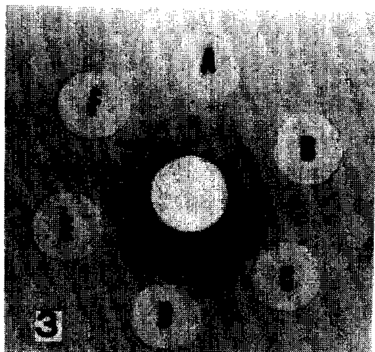
Legends for Figures

Fig. 3. Agar gel immunodiffusion test for BLV antibody. Center well: BLV glycoprotein antigen; Peripheral wells: F,C -positive control serum, A,B,D,E- test sera. B and D show positive reaction.

Fig. 4. The syncytium formation containing about 6 to 9 nuclei (arrows) in the co-cultures of bovine embryonic splenic cells and peripheral lymphocytes from the BLV antibody positive cattle, H&E × 100.

Fig. 5. Electron micrograph of the cultured lymphocytes from BLV antibody positive cattle. C-type virus particles (arrows) with the size of 90 to 110 nm appear around microvilli (A) × 25,000, and in intracytoplasmic vacuoles (B) × 15,000.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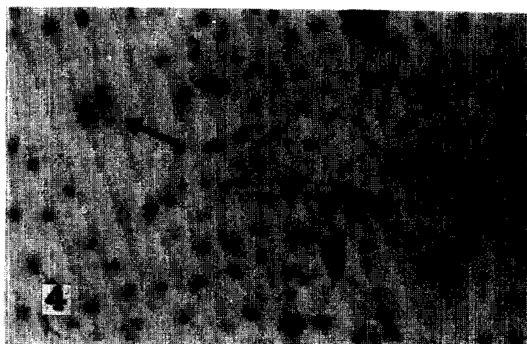
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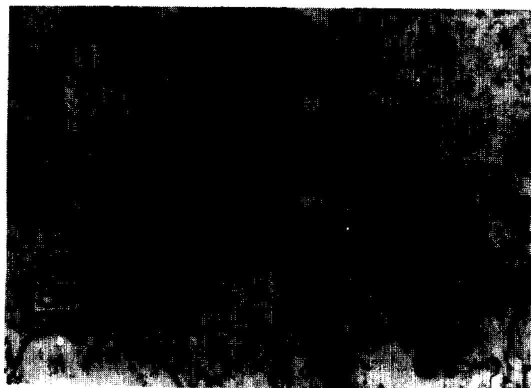

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韓國에서의 소白血病의 血淸疫學的 硏究

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抄 錄

소白血病바이러스 封入體蛋白을 抗原으로 이용한 免疫擴散法으로 전국에 산재해 있는 164개 牧場에서 사육중인 2003頭의 乳牛에 대한 소白血病바이러스抗體 조사결과, 陽性率은 忠淸道가 41.8%로 가장 높았고, 全北은 24.4%로 가장 낮았다. 地域別 陽性率은 中部地域이 37.8%, 湖南地域이 27.2%, 嶺南地域이 28.0%, 그리고 嶺東地域이 25.2%였다. 전국의 평균 陽性率은 29.8%였다.

血淸學的 檢査結果를 分析하였던 바 陽性率은 소의 年齡이 높을수록, 飼育規模가 클수록 높은 경향이 있었고, 소의 年齡群이 6내지 8세에서, 飼育規模가 20내지 50頭의 牛群에서 陽性率이 가장 높았다. 中部地域에 사육중인 117頭의 種牡牛에 대해 조사한 결과 韓牛에서는 5.7%(4/70), 홀스타인 種牡牛에서는 35.9% (14/39)의 陽性率을 나타냈다. 牛群別로는 血淸檢査를 한 164개 牛群중에 陽性牛가 전혀 없는 牛群이 17個群(10.4%), 20～40%의 陽性牛가 있는 牛群이 42個(25.6%)였고, 80%이상의 陽性牛가 있는 牛群은 10個群(6.1%)이었다.

소白血病바이러스抗體 陽性牛에서 分離한 淋巴球를 phytohemagglutinin을 첨가한 培地에 短期培養한 후 BLV 螢光抗體를 이용하여 淋巴球내의 BLV抗原 證明을 시도한 바, 陽性牛 11頭중 8頭(72.7%)에서 特異한 BLV抗原이 糾明되었다. BLV 抗體陽性牛 7頭와 陰性牛 4頭에서 분리한 淋巴球의 牛胎兒脾臟細胞에 대한 syncytium 形成能을 시험한 바, 陽性牛 7頭 중 5頭(71.4%)의 淋巴球가 syncytium을 形成하였다. 培養된 淋巴球를 電子顯微鏡으로 검사한 결과 6頭의 陽性牛 중 2頭에서 90～110nm. 크기의 典型的인 C型 소白血病바이러스가 證明되었다.