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AN INVESTIGATION OF ENZOOTIC BOVINE LEUCOSIS (EBL) INFECTION BY AGAR GEL IMMUNODIFFUSION (AGID), ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) TESTS AND HAEMATOLOGICAL APPLICATIONS ON THE DAIRY COWS IN THE BURDUR REGION

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Haematological tests (alfa nafthyl acetate esterase ANAE activity, May Grünwald Giemsa staining and total leucocyte counts) were applied to 469 dairy cows, where the enzootic form of bovine leucosis was investigated. In the same 469 dairy cows, a search for antibodies directed against bovine leucosis virus (BLV) was carried out using agar gel immunodiffusion (AGID) in blood samples and enzyme linked immunosorbent assay (ELISA) in milk samples.

Among the 469 animals screened, 90 were positive by ELISA and the haematological tests, while 23 were positive by the AGID test. From these results we would advise heavily infected farms to be eliminated. ELISA and haematological methods, in which ANAE activity, May-Grünwald Giemsa staining and total leucocyte counts were used together, were more reliable and sensitive than the AGID test for detecting EBL.

Key words: agar gel immunodiffusion, alfa nafthyl acetate esterase, dairy cows, enzootic bovine leucosis, enzyme linked immunosorbent assay

INTRODUCTION

The BLV is a retrovirus that infects primarily B lymphocytes and causes about 30-70 % of the infected animals to become lymphocytotic (Straub,ss 1987).

The virus is mainly transmitted horizontally by direct exposure to biological fluid contaminated with infected lymphocytes. However, cell-free virus which apparently is shed in blood only during pregnancy, may account for transplacental vertical transmission (Evermann *et al*., 1987).

Bovine leucosis virus causes significant losses to the dairy cattle industry. More and more data are emerging to indicate that chronic BLV infection by itself can lead to reduction in productivity, reproductivity and a shorter life span among high yielding dairy cows (Brenner *et al*., 1989).

The clinical appearance of lymphosarcoma in the herd or in the slaughterhouse constitutes a confirmation of the clinical suspicion. However, in

the past, before the virological aetiology was discovered and of reliable laboratory diagnostic methods developed, histological and haematological keys were the only diagnostic tool for the detection of infected animals (Chiba *et al*., 1995). Nowadays, serological tests developed for the diagnosis of BLV infection include AGID, ELISA, complement fixation, radioimmunoassay and virus neutralization tests (Mammerickx *et al*., 1985).

The aim of the present study was to compare ELISA, AGID and haematological tests in the detection of BLV antibodies in healthy dairy cows.Moreover, this paper reports the first serological and haematological evidence of EBL virus infection in dairy cows in the Burdur region.

MATERIAL AND METHODS

Animals: A total of 469 blood and milk samples were taken from healthy cows of the Holstein breed, between 3 and 10 years old, in the Burdur region. All animals were in late pregnancy (between 7 and 9 months) on 10 different individual private farms.

Blood samples: Peripheral blood was aseptically obtained from the jugular vein with and without anticoagulant (EDTA 0.2 M v/v).

Blood samples for alfa nafthyl acetate esterase activity (ANAE), May Grünwald Giemsa staining and total leucocyte counts were collected into containers with EDTA as anticoagulant.

Milk samples: 0.2 ml rennin and 0.1 ml saturated $CaCl_2$ were added to the milk samples and fat was separated by centrifugation (2000 x g; 10 min.) before the test.

Agar gel immunodiffusion (AGID): The test was carried out using the Bommeli BLV kit (Bern-Switzerland) according to the manufacturer's instructions.

Enzyme linked immunosorbent assay (ELISA): The HerdChek Anti-BLV (milk) (IDEXX, USA) was used to detect antibodies to BLV, according to the manufacturer's recommendations.

Haematology: The proportions of T, B and null cells in dairy cows peripheral blood were determined using ANAE staining, as described by Higgy *et al*. (1977). Differential leucocyte counts were performed as described by Culling *et al*. (1985) on blood smears stained with Giemsa's and May Grünwald's solutions. The total number of leucocytes was determined as described by Konuk (1981).

Statistical analysis: The statistical significance of differences in the total leucocyte, lymphocyte and absolute B and T-lymphocyte counts was assessed with Student's *t* test (Snedecor and Cochran 1967).

RESULTS

Samples of blood serum: 23 (4.9 %) of the 469 blood serum samples collected on farms affected by EBL reacted positively in AGID.

The distribution of seropositive animals according to age was as follows; at 3 years of age 4 (3.23 %), at 5 years of age 12 (6.67 %), at 6 years of age 3 (3.33 %) and at 7 years of age 4 (8.51 %).

Samples of milk serum: 90 (19.2 %) of the 469 milk serum samples collected on farms affected by EBL reacted positively in ELISA.

The distribution of seropositive animals according to age was as follows; at 3 years of age 27 (21.77 %), at 4 years of age 4 (21.05 %), at 5 years of age 29 (16.11 %), at 6 years of age 16 (17.77 %), at 7 years of age 11 (23.40 %) and at 8 years of age 3 (60.0 %).

The result obtained in the AGID test and ELISA are compared in table 1 according to the district of the animals tested.

Table 1. Comparison of AGID and ELISA test result according to the district of the animals tested.

District	Number of samples (blood/milk)	BLV			Negative
		ELISA+ AGID+	ELISA+ AGID-	ELISA- AGID+	
Burdur/ Merkez	220	4 (1.81%)	24 (10.90%)	8 (3.63%)	184 (83.64%)
Burdur/ Yesilova	50	1 (2%)	9 (18%)	1 (2%)	39 (78%)
Burdur/ Tefenni	120	3 (2.5%)	31 (25.83%)	2 (1.66%)	84 (70%)
Burdur/ Aglasun	79	1 (1.26%)	17 (21.51%)	3 (3.79%)	58 (73.41%)
Total	469	9 (8.65%)	81 (77.89%)	14 (13.46%)	365(77.83%)

The comparison of AGID and ELISA for detecting antibodies to BLV showed an overall correlation of 79.74 %.

The distribution of double seropositive animals according to age was as follows; at 3 years of age 2 (1.61 %), at 5 years of age 3 (1.66 %), at 6 years of age 3 (3.33 %) and at 7 years of age 1 (2.12 %).

Haematology: T, B-lymphocytes and null cells, differential leucocyte and total leucocyte counts for ELISA, AGID and double (AGID and ELISA) seropositive and seronegative animals are given in table 3. The haematological parameters are summarized for ELISA seropositive and seronegative cows according to age groups in table 4.

In this study, haematological status (total lymphocyte and total leucocyte) was compared according to the European Economic Community (EEC) key (Levy *et al*., 1977): lymphocytotic, suspect and normal animals in table 2.

Table 2. Comparison of the ELISA seropositive and seronegative animals of peripheral blood lymphocyte and total leucocyte counts using the EEC standard key.

Age	Seropositive/ Seronegative animal	Lymphocytes (%)	Total Leucocytes (mm^3 /blood)	EEC key classification
3	27(+)	57.30±14.2	10826±2036	Leukemic
3	97 (–)	30.95±9.15	5425±1321	Normal
4	4(+)	51.20±17.3	8900±483	Leukemic
4	15 (–)	27.67±7.07	5093±1817	Normal
5	29(+)	54.40±14.8	11255±3566	Leukemic
5	151 (–)	30.54±8.15	5397±1278	Normal
6	16(+)	58.90±13.3	11994±3491	Leukemic
6	74 (–)	31.30±7.86	4804±973	Normal
7	11(+)	58.91±7.66	9381±1797	Leukemic
7	36 (–)	29.14±7.92	4744±1003	Normal
8	3(+)	54.33±4.04	9600±3027	Leukemic
8	2 (–)	39.00±1.41	4850±1344	Normal

Table 3. Statistical analysis of haemoatological parameters for all seropositive and seronegative animals found by ELISA and AGID.

Tests	T-lymph. (%)	B-lymph. (%)	Null cells (%)	Total lymph. (%)	Monocyte (%)	Neu-trophyle (%)	Eosino-phyle (%)	Basophyle (%)	Total Leucocyte (mm^3/ blood)
Elisa+ N= 90	28.53±12.71	51.82±13.95	19.64±9.69	56.5±13.4	1.70±3.69	33.4±13.0	5.74±5.29	2.70±2.91	10861±2940
Elisa – N=379	49.38±13.38	24.08±11.21	26.72±14.57	30.55±8.31	2.13±3.94	58.6±10.1	5.90±5.55	2.61±3.33	5202±1260
Significant	P=0.0000 **	P=0.0000 **	P=0.0000 **	P=0.0000 **	P=0.33	P=0.0000 **	P=0.81	P=0.80	P=0.0000 **
Agid+ N=23	39.93±16.92	36.91±16.50	23.24±11.91	44.5±16.9	2.00±4.48	47.0±15.5	4.74±4.42	1.70±2.30	8109±3779
Agid – N=446	45.66±15.48	29.01±15.97	25.47±14.15	35.1±13.6	2.05±3.87	54.1±14.5	5.93±5.54	2.67±3.28	6194±2725
Significant	P=0.12	P=0.023 *	P=0.46	P=0.015 *	P=0.96	P=0.043 *	P=0.23	P=0.063	P=0.025 *

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Table 4. Statistical analysis of haemotological parameters for ELISA seropositive and seronegative animals according to age groups.

Age	ELISA (%)	T-lymph. (%)	B-lymph. (%)	Null cells (%)	Total Lymph. (%)	Monocyte (%)	Neutro-phyle (%)	Eosino-phyle (%)	Basophyle (%)	Total Leucocyte (mm^3/ blood)
3 age ELISA+ N=27	21.77	28.93±12.46	51.09±9.70	19.98±7.23	57.3±14.2	1.56±3.25	33.7±13.4	4.89±4.33	2.63±3.21	10826±2036
ELISA– N=97	78.23	50.25±13.40	26.09±11.80	23.66±11.61	30.95±9.15	2.78±4.57	57.4±11.4	5.25±5.08	2.92±4.01	5425±1321
Significant		P=0.0000**	P=0.0000**	P=0.093*	P=0.0000**	P=0.12	P=0.0000**	P=0.72	P=0.70	P=0.0000**
4 age ELISA+ N=4	21.05	43.75±10.34	38.25±19.62	18.00±14.31	51.2±17.3	2.25±3.86	38.7±19.7	5.25±3.59	2.50±1.91	8900±483
ELISA– N=15	78.95	59.13±11.86	15.07±9.27	25.80±13.60	27.67±7.07	4.20±6.17	61.3±10.7	6.60±5.14	1.53±2.26	5093±1817
Significant		P=0.050*	P=0.10	P=0.37	P=0.076 *	P=0.46	P=0.11	P=0.57	P=0.43	P=0.0000**
5 age ELISA+ N=29	16.11	26.19±11.77	54.17±17.04	19.64±11.05	54.4±14.8	1.34±2.21	35.3±16.0	6.10±4.69	3.00±2.96	11255±3566
ELISA– N=151	83.89	48.60±13.81	23.225±10.753	28.32±15.43	30.54±8.15	1.84±3.19	59.38±9.67	5.78±5.50	2.57±3.09	5397±1278
Significant		P=0.0000**	P=0.0000**	P=0.0020**	P=0.0000**	P=0.31	P=0.0000**	P=0.74	P=0.48	P=0.0000**

Table 4. - continued

Age	ELISA (%)	T-lymph. (%)	B-lymph. (%)	Null cells (%)	Total Lymph. (%)	Monocyte (%)	Neutro-phyle (%)	Eosino-phyle (%)	Basophyle (%)	Total Leucocyte (mm^3/ blood)
6 age										
ELISA+ N=16	17.77	28.751±1.00	54.78±9.14	16.47±9.58	58.9±13.3	1.50±4.98	29.81±9.42	6.31±8.03	3.06±2.86	11994±3491
ELISA– N=74	82.23	50.11±12.30	26.70±11.66	23.86±15.08	31.30±7.86	1.46±3.40	58.61±8.82	5.84±6.42	2.27±2.97	4804±973
Significant		P=0.0000**	P=0.0000**	P=0.045 **	P=0.0000**	P=0.98	P=0.0000**	P=0.83	P=0.33	P=0.0000**
7 age										
ELISA+ N=11	23.40	22.45±9.52	54.95±3.47	22.59±10.89	58.91±7.66	2.73±5.88	29.73±4.29	6.27±5.41	2.36±2.80	9318±1797
ELISA– N=36	76.60	42.60±10.36	22.64±7.70	34.76±13.98	29.14±7.92	1.89±4.06	57.5±10.04	7.67±5.12	3.25±3.47	4744±1003
Significnt		P=0.0000**	P=0.0000**	P=0.010 *	P=0.0000**	P=0.67	P=0.0000**	P=0.46	P=0.40	P=0.0000**
8 age										
ELISA+ N=3	60	48.5±19.0	26.5±23.9	25.00±5.00	54.33±4.04	3.00±2.65	37.00±1.00	5.67±5.51	0.00	9600±3027
ELISA– N=2	40	64.00±2.83	22.00±12.73	14.00±15.6	39.00±1.41	1.00±1.41	55.00±1.41	4.00±2.83	0.00	4850±1344
Significant		P=0.30	P=0.91	P=0.51	P=0.026 *	P=0.39	P=0.041**	P=0.70		P=0.14

*(P<0.05)
**(P<0.001)
N: number of animal

DISCUSSION

The results here constitute a regional survey carried out to detect antibodies to BLV in dairy cows in Burdur-Turkey.

In this study, the prevalence of EBL was found to be 4.9 % (23) with AGID (blood) and 19.2 % (90) with ELISA (milk). Previous seroepidemiological studies using the AGID test and ELISA yielded rather high infection rates, e.g. 63.22-63.47 % (Wawrzkiewicz *et al*., 1988) for the AGID test and 36 % (Prevost *et al*., 1988) for ELISA. These researchers explained that high infection rates were caused by; intensive close physical contact among animals, intensive management practices, importation of dairy cows from the USA, Germany, Austria, Canada and Holland (infection with BLV is widespread in these countries). BLV infection showed high prevalence in herds. Large animal populations and clinical symptoms were investigated before serological tests.

In the present study, the 79.74% correlation between the AGID and ELISA for detecting antibodies to BLV was lower than in other studies (Klintevall *et al*., 1991).

The highest prevalence was detected at 7 years of age (8.51 %) concerning distribution according to age with AGID. The highest number of seropositive animals was found at 5 years of age. The highest prevalence was detected in 7 and 8 year old cows (23.40-60.0 %) concerning distribution according to age with ELISA. The highest number of seropositive animals was found at 3 and 5 years of age. Other studies often included cattle over 5 and 7 years of age (Jacobs *et al*., 1995). The same results were found for two positive (AGID+/ELISA+) tests (at 6 years of age 3.33 %). Thus in parallel to other studies, an increase in infection rate in older animals was observed.

According to the results of this study made in four districts of Burdur, the prevalence varied between 4.00-5.45 % with AGID in blood samples and between 12.70-28.30 % with ELISA in milk samples. For AGID the seroprevalence of BLV infection was found to be less than that of other studies in Turkey (Kandil *et al*., 1989). However, according to ELISA the seroprevalence of BLV infection was found to be the same as in other studies (Iyisan *et al*., 1996). This difference of prevalence can be related to management, sanitary practices, climate, animal breeding, environment, etc.

The results clearly demonstrated that the proportion of T-lymphocytes (ANAE+) was lower in ELISA seropositive animals than in ELISA seronegative cows. B-lymphocytes (ANAE-) were higher in ELISA seropositive animals. T-lymphocyte (ANAE+) percentage was reduced and B-lymphocyte (ANAE-) percentage increased for two positive (AGID+/ELISA+) tests. In parallel to other studies (Paul *et al*., 1979), decrease in T-lymphocyte proportions and an increase in B-lymphocyte proportions was observed. However, the relative numbers of T and B-lymphocytes were not significantly different in AGID seropositive and seronegative animals.

Total lymphocyte and total leucocyte numbers were found to be higher in ELISA, AGID and two test seropositive animals than in ELISA, AGID, and two test seronegative animals. Other researchers (Williams *et al*., 1988) declared similar results.

In this survey the relative numbers of peripheral blood monocytes, eosinophiles and basophiles did not differ in all tests between seropositive and seronegative animals. Only null cells rates were changed a little in ELISA seropositive animals in comparison to seronegative animals. Similar results were obtained by Schalm *et al*., (1975).

B-lymphocytes, total leucocytes and total lymphocyte proportions were found to increase, and T-lymphocytes, null cells and neutrophil proportions to decrease in seropositive cows in all age groups except for 4 and 8 year old cows. Peripheral blood monocyte, eosinophil and basophil proportions were not changed in any age groups.

Total lymphocyte and total leucocyte counts were in accordance with the EEC key according to age groups.

In the present study many animals were seropositive in ELISA (milk) because all animals were in late pregnancy (between 7 and 9 months). Antibodies titers can increase in milk in this period (Perrin *et al*., 1986). Other researchers have also found antibodies increasing in milk in late pregnancy because the colostral period is commencing with changing IgG concentration and ELISA (milk) sensitivity (Gatei *et al*., 1990).

In this survey seropositive herds were kept in overpopulated sheds. We observed mix-common suckling and many treatment applications (contaminated needles, gloves, vaccinations and other operations) on seropositive farms. Since the herds were not grazing on pasture, there was no probability of vectoral transmission (insect or flies).

It was concluded that in the diagnosis of BLV infection ELISA and the haematological methods were more reliable and sensitive than the AGID test.

We consider that control, protection, eradication and detection programmes for EBL, must include large dairy populations in the Burdur region in further studies.

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DIJAGNOSTIKOVANJE ENZOOTSKE LEUKOZE GOVEDA (EBL) AGAR-GEL IMUNODIFUZIJOM, ELISA TESTOM I HEMATOLOŠKIM METODAMA KOD MLEČNIH KRAVA REGIJE BURDUR

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SADRŽAJ

U cilju ispitivanja enzootske forme goveđe leukoze korišćeni su hematološki testovi (aktivnost alfa - naftil acetatesteraze, bojenje razmaza po May-Grunwald Giemzi i ukupan broj leukocita) kod 469 mlečnih krava. Kod krava su utvrđivana direktna antitela na virus goveđe leukoze (BVL) agar-gel imunodifizijom (AGID) u uzorcima krvi, kao i ELISA testom u uzorcima mleka.

Od 469 ispitanih krava, 90 jedinki je bilo pozitivno na ELISA test i hematološke testove, a 23 na AGID test. Na osnovu ovih rezultata preporučeno je da se, u cilju eliminacije izraženih EBL infekcija, koriste ELISA test i hematološke metode. Ako se uporedo sa ELISA testom vrši i određivanje aktivnosti alfa - naftil acetatesteraze, bojenje razmaza po May-Grunwald Giemzi i određivanje ukupnog broja leukocita dobijaju se mnogo pouzdaniji rezultati, nego agar-gel imunodifuzijom.