DETECTION OF ANTIBODIES TO BOVINE LEUKEMIA VIRUS IN RANDOMLY SELECTED CATTLE IN OKLAHOMA USING TWO SEROLOGICAL TESTS

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The incidence of antibodies to bovine leukemia virus in sera of cattle from Oklahoma was determined using the agar-gel immunodiffusion test and a rapid syncytia inhibition assay and the sensitivities of the two tests compared. Of the 500 sera samples from randomly selected cattle, 142 (28.4%) were positive by the rapid syncytia inhibition assay and 137 (27%) positive by the agar-gel immunodiffusion test. The incidence of antibodies to bovine leukemia in five herds of cattle (384) with known cases of lymphosarcoma was considerably higher; 165 (43%) were found positive by the rapid syncytia inhibition assay whereas 152 (40%) were found positive by the agar-gel immunodiffusion test. Out of 111 sera samples sent to the Oklahoma Animal Disease Diagnostic Laboratory to be tested for presence of BLV antibodies, 47 (42%) were positive by agar-gel immunodiffusion test and 45 (41%) positive by the rapid syncytia inhibition assay. A good correlation between the results of the two tests for detection of antibodies to bovine leukemia virus was seen; they showed complete agreement of results in 97.8% of the cattle.

INTRODUCTION

Morphological, biophysical and biochemical studies on bovine leukemia virus (BLV) have shown that it is a C-type retrovirus (1, 2). This virus differs from most other C-type viruses, however, in its antigenic properties and its ability to induce cell fusion (syncytia formation) in a number of nontransformed and viral transformed cells (3, 4). BLV is regarded as the etiological agent of the adult or enzootic form of bovine lymphosarcoma but has not been closely associated with other sporadic forms of bovine leukosis such as the calf, thymic, or cutaneous forms (1, 2). Since the transmission of this disease under natural conditions is horizontal (by contact), each infected animal in a herd is a potential focus for spread of the virus which could have potentially serious economic implications (5).

Infection of cattle with BLV results in the production of antibodies to several viral proteins, especially the major envelope glycoprotein (gp60, molecular mass of approximately 51-70×10^3 daltons) and the major core nonglycosylated protein (p24, molecular mass of about 24×10^3 daltons) (1, 2). This immune response has been used as the basis for the development of immunological tests which can be used for the diagnosis of BLV infection in animals. Some of the tests that have been developed are: agar-gel immunodiffusion (AGID) (6, 7); indirect immunofluorescence (IF) (8); complement fixation (CF) (9); enzyme-linked immunosorbent assay (ELISA) (10, 11); and radioimmunoprecipitation (RIA) (12, 13). In most of these tests, either gp60 or p24 is used as the test antigen (13). Recently, a rapid syncytia inhibition assay (RSIA) which is based on the inhibition of early BLV-induced syncytia in cell culture was developed in my laboratory and was found to be very sensitive for the detection of BLV-infected animals (10, 14). The RSIA is similar to the early polykaryocytosis inhibition assay (15) but differs from the virus neutralization assay (16) in that it is based upon the inhibition of early (8-24 hr) syncytia formation rather than late syncytia formation, which requires several days to obtain results. The RIA appears to be the most sensitive assay (13) but requires expensive equipment and elaborate technology and is not practical to use. Since the RSIA and the AGID (using the gp60 as test antigen) are accurate indicators of BLV-infected cattle and are relatively simple to conduct in the laboratory, they were used to detect antibodies to BLV in this study.

The objectives of this study were: (a) to determine the incidence of BLV infection in randomly selected cattle in Okla-

homa as well as in herds with reported cases of lymphosarcoma; and (b) to compare the sensitivity of the RSIA with that of the AGID test.

MATERIALS AND METHODS

Cells, Virus, and Antigens

The F81 feline cell line (17), which contains the murine sarcoma virus genome and was used as indicator for the rapid syncytia inhibition assays, and the fetal lamb kidney (FLK) cells (18) persistently infected with BLV were obtained through the courtesy of Drs. Miller and Van Der Maaten, National Animal Disease Center, Ames, Iowa. Owing to the fact that these cells release relatively large quantities of the major envelope glycoprotein of BLV (gp60) into their culture medium, they were chosen as the source of virus and antigen in the seriological assays employed in this study (19). The FLK and F81 cells were grown, maintained, and harvested as reported previously except that the culture fluids of the FLK cells were collected every 3 days and used immediately (14).

A crude preparation of the gp60 antigen used in the AGID test was prepared by concentrating it from 3-day-old virus-free culture fluid with ammonium sulfate. Briefly, 1 *l* of culture fluid was clarified by centrifugation ($10^4 \times g$ for 10 min) to remove cells and cellular debris. BLV was then pelletized from the clarified fluid by centrifugation at $10^5 \times g$ for 60 min using a SW 27 swinging bucket rotor in a Beckman ultracentrifuge. The gp60 was precipitated from the resulting supernatant by the addition of 30 g of crystalline ammonium sulfate per 100 m*l* of fluid and the mixture allowed to stand at 4 C for 48 to 72 hr. The precipitate was collected by centrifugation at $10^4 \times g$ for 10 min, resuspended in phosphate buffer saline (PBS) to approximately one-hundredth the original volume, and then dialyzed against PBS for 48 hr with three changes of buffer. This crude gp60 preparation was then diluted as necessary to give a sharp precipitin line in the AGID test using the positive reagent serum obtained in the commercially available AGID test kit obtained from Pitman-Moore, Washington Crossing, N.J.

Sera

Three different groups of sera were tested in this study. With all three sources, the sera came from animals of both sexes and only those samples that were from animals 18 months of age or older were used in order to avoid false positive results due to colostrum in sera from young animals. The first group consists of 500 samples obtained from the United States Department of Agriculture Regional Laboratory for testing brucellosis and were provided through the courtesy of Dr. Paul Woodson. These sera were collected randomly over a period of about four months from cattle of various breeds.

The second group of sera consisting of 384 samples came from five herds of animals with known incidences of lymphosarcoma. These herds consisted of either Holstein, Angus, Santa Gertrudis, or Simmental cattle and the sera samples were supplied through the courtesy of several field veterinarians in Oklahoma. The third group of sera samples were obtained from the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK (supplied by the generosity of Dr. Anthony Castro). This group consisted of 111 samples that had been collected by veterinarians from animals of various bovine herds in the state of Oklahoma and sent to the diagnostic laboratory at Stillwater to be tested for the presence of antibody to BLV.

Rapid Syncytia Inhibition Assay

The RSIA was performed as described previously (14). In brief, cell-free preparations of BLV obtained from the persistently infected FLK cells were mixed with equal volumes of sera (heat inactivated at 56 C for 30 min) that had been diluted 1:5 in Hank's balanced salt solution or minimal essential medium containing 10% fetal calf serum and incubated at 37 C for 30 min followed by incubation at 25 C for 30 min. Samples (0.5 m*l*) of the mixtures were then added to duplicate wells of F81 indicator cells that had been seeded 24 hr previously in multiwell cell culture plates at a concentration of 1×10^5 to 2×10^5 cells per well. After 24 hr of incubation at 37 C, the cells were fixed with methanol and stained with Giemsa stain, and the number of multinucleated cells determined (twenty randomly selected 1-mm^2 fields were counted in duplicate wells and the mean number of syncytia per well calculated). The percent of syncytia inhibition for each serum was determined by dividing

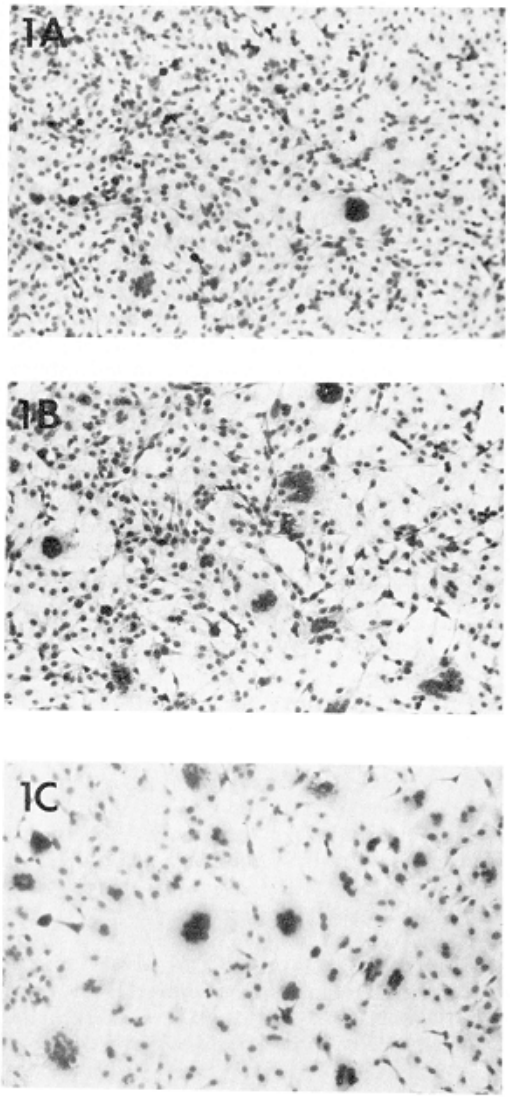

FIGURE 1. Rapid syncytia inhibition assay using F81 cells.
A) F81 cells inoculated with a preparation of BLV that had been incubated with a strongly positive BLV reference serum.
B) F81 cells inoculated with a preparation of BLV that had been incubated with a weakly postitive BLV reference serum.
C) F81 cells inoculated with a preparation of BLV that had been incubated with negative BLV reference serum (See Materials and Methods).

the number of syncytia seen in virus-test serum inoculated cells by the number of syncytia seen in virus-negative serum inoculated controls. The negative serum used as negative control was a pool of six negative BLV reference sera. Each plate consisted of 9 test sera, one positive and one negative (pool) reference serum, and two wells containing only minimal essential medium plus 10% fetal calf serum for a cell control. A serum causing greater than 50% inhibition was considered strongly positive for antibodies to BLV (Figure 1A); 25 to 50% weakly positive (Figure 1B); and less than 25% inhibition, negative (Figure 1C).

Agar-Gel Immunodiffusion Test

The AGID test for antibodies to the BLV glycoprotein (gp60) was performed and interpreted as reported by Miller and Van Der Maaten (7) using the commercially available AGID test kit (Pitman-Moore) except that our preparation of gp60 was used as the test antigen. In each immunodiffusion test plate which contains 6 peripheral wells and one central well, the BLV antigen was placed in the center well, the positive reagent control serum was placed in alternate peripheral wells, and test sera placed in the remaining 3 peripheral wells (Figure 1). Sera were not diluted when tested in the AGID test. The plates were read and interpreted after incubation at 25 C for 48 to 72 hr. A strong positive serum formed a precipitation line similar to the one from the positive reagent control test serum but a weak positive serum did not always form a distinct precipitation line. If a serum caused an inward deflection of precipitation lines produced by the positive reagent control serum in adjacent

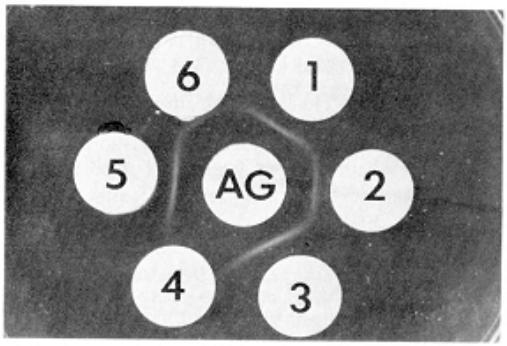

FIGURE 2. Agar-gel immunodiffusion of bovine sera using BLV glycoprotein as test antigen (center well). Peripheral wells contain strongly positive BLV serum (well 2), weakly positive BLV serum (well 6), reagent test sera (wells 1, 3 and 5) and a negative serum (well 4).

wells, it was considered weakly positive. Interpretation of the results of weak positive sera was often difficult and in many cases, the sera in question were tested several times in order to allow for adequate interpretation.

TABLE 1. *Presence of BLV antibodies in sera of randomly selected cattle obtained from the brucellosis testing laboratory.*

Experiment	Number tested	Serological evaluation[a] AGID	RSIA
1	200	57 (29)[b]	60 (30)[b]
2	120	29 (24)	32 (27)
3	100	26 (26)	27 (27)
4	80	23 (29)	23 (29)
Total	500	135 (27)	142 (28.4)

[a]The agar-gel immunodiffusion (AGID) test and rapid syncytia inhibition assay (RSIA) conducted as described in Materials and Methods.
[b]Number (per cent) positive.

RESULTS

The presence of antibodies to BLV in sera of randomly selected cattle obtained from the brucellosis testing laboratory was examined using the AGID test and RSIA. Out of 500 sera samples, 135 (27%) were found positive by the AGID test and 142 (28.4%) by the RISA (Table 1). There was a 98.6% agreement of results between these two serological tests for this population of animals. Of the 384 cattle obtained from herds located in various regions of Oklahoma which were known to have reported cases of lymphosarcoma, 152 (40%) were positive by the AGID test and 165 (43%) by the RSIA (Table 2). There was 96.7% agreement of results obtained by the two serological tests for these sera samples. In the last group of samples tested, those obtained from the diagnostic laboratory, 47 out of 111 (42%) were positive by the AGID test whereas 45 out of 111 (41%) were positive by the RSIA (Table 3). Again, there was good agreement (98.2%)

TABLE 2. *Presence of BLV antibodies in sera of cattle from herds with incidence of lymphosarcoma.*

Herd	Number tested	Breed	Serological evaluation[a] AGID	RSIA
A	80	Holstein	29 (36)[b]	30 (37)[b]
B	107	Santa Gertrudis	44 (41)	46 (43)
C	48	Angus	28 (58)	32 (67)
D	84	Simmental (Beef)	26 (31)	29 (35)
E	64	Holstein	25 (38)	28 (43)
Total	384		152 (40)	165 (43)

[a]The agar-gel immunodiffusion (AGID) test and rapid syncytia inhibition assay (RSIA) conducted as described in Materials and Methods.
[b]Number (per cent) positive.

TABLE 3. *Presence of BLV antibodies in sera of cattle obtained from the Oklahoma Animal Disease Diagnostic Laboratory.*

Number tested	Breed	Serological evaluation[a] AGID	RSIA
111	Mixture	47 (42)[b]	45 (41)[b]

[a]The agar-gel immunodiffusion (AGID) test and rapid syncytia inhibition assay (RSIA) conducted as described in Materials and Methods.
[b]Number (per cent) positive.

between the two serological tests. As one would expect, the sera samples sent to the diagnostic laboratory to be tested for antibodies to BLV (suspected infection) and the sera from herds with reported cases of lymphosarcoma had greater numbers of positive reactions than those sera obtained from randomly selected cattle.

The relative sensitivities of the AGID test and the RSIA for detecting BLV antibodies were compared by using the results obtained from all of 995 sera samples tested. There was total agreement of results in 97.8% of the sera samples (332 positive and 641 negative). There was disagreement in the results from only 22 sera. Most of these 22 samples that showed disagreement in their results were weakly positive by one of the two serological tests. The greater number of positive sera were detected by the RSIA (352) as compared to the AGID test (334). In only two cases were the AGID results positive and the RSIA negative. These two sera were from the 111 samples obtained from the diagnostic laboratory. The RSIA appears to be more sensitive than the AGID test for detecting BLV antibodies in sera.

DISCUSSION

The findings of the present study on 500 random cattle in Oklahoma establish the frequency of BLV infection in the domestic cattle population to be about 28%. In a study by Devare and Stevenson (12) using the RIA and examining approximately 6000 random cattle from various geographic regions of the United States, the frequency of positive animals ranged from 2 to 41%. They reported that 29% of the cattle in Oklahoma to be positive reactors. Their data correlates very closely to what was found in this study; 27% positive by AGID test and 28.4% positive by RSIA.

In the study of herds where cases of lymphosarcoma had been clinically diagnosed, the frequency of infection with BLV appeared to be about 43%. Because of the small number of herds sampled, one can not predict from this study whether beef cattle have a higher incidence of infection than dairy cattle. In a very recent study by McGuire, et al. (20), a higher percentage of the positive sera samples screened at the Oklahoma Diagnostic Laboratory were from beef rather than dairy cattle. These authors indicated that this probably reflects a greater number of submissions of beef breeds to the diagnostic laboratory because of their greater numbers within the state of Oklahoma (20). Further studies are needed on various cattle populations in Oklahoma to determine the incidence rate in dairy cattle versus beef cattle.

It is also interesting to note that even though slaughterhouse reports and clinical studies show a low incidence of bovine lymphosarcoma in the United States, about 0.02% in dairy cattle and somewhat lower in beef cattle, a considerably higher percentage of the cattle population are infected with BLV (21). The fact that most cattle with BLV infection remain clinically healthy and only rarely develop lymphosarcoma can account for this discrepancy. Animals that are sero-positive for BLV antibodies have probably been exposed to BLV and are potential carriers of the virus but will not necessarily develop the lymphosarcoma (21). Development of the malignancy probably depends upon factors other than the presence of BLV only. Since lymphosarcoma can occur in herd and geographic aggregations, the genetic makeup of the animal probably plays some role (21).

In a recent study by McGuire, et al. (20) using the AGID test, 130 (41%) out of 317 sera that were field submissions to the Oklahoma diagnostic laboratory were found to be positive for BLV antibody. Some of the 111 sera samples obtained from the diagnostic laboratory and used in this study were among the same 317 samples tested by these investigators. It is interesting to note that out of the 111 sera tested in this study, 42% were found positive by the AGID test and 130 (41%) out of 317 were found positive in McGuire's study. A good correlation between their results and the results obtained in this study can be seen.

Discrepancies between the AGID and RSIA existed with 22 sera. There were 20 sera found positive by RSIA but negative by AGID test. The most logical explanation for these discrepancies is that the RSIA is a more sensitive assay and can detect lower levels of BLV antibody than the AGID test. However, a second possibility is that syncytia formation was inhibited by some of these 20 sera owing to the presence of

antibodies to other antigens other than gp60 of BLV. It has been reported that antiserum of p24 can cause syncytia inhibition (3, 15). Still another possibility for this discrepancy is that an inhibitory substance other than an immunoglobulin was present in the sera that inhibited the early syncytia formation.

There were only two sera that were found weakly positive by the AGID test and negative by the RSIA. The AGID test and RSIA were repeated several times using these two sera and similar results were obtained each time. The discrepancies with these two sera are difficult to explain.

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