Global Veterinaria 4 (3): 267-270, 2010
ISSN 1992-6197

Comparative Serological Detection of Enzootic Bovine Leukosis Virus (EBLV) in Cattle Sera

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Abstract: Enzootic bovine leukosis (EBL) is a notifiable disease. Virus screening or monitoring, precautions at borders, control of movement inside the country and stamping out must be carried out as official control measures. The objective of this study was to prepare an enzyme linked immunosorbant assay (ELISA) as a local prepared kits for detection of enzootic bovine leukosis virus (EBLV)-antibodies that is comparable to agar gel immunodifussion (AGID) test, to evaluate the use of this prepared ELISA kits for identification of EBLV-infected herds. Serum samples (n= 1936; from imported cattle under quarantine) and 40 (suffered from enlarged lymph nodes, loss of appetite with severe depletion and drop in milk production) from two dairy farms in Swiss and El-Sharquia governments in Egypt, were collected during the 1st half of 2009. Samples were tested by AGID test and the local prepared ELISA kits. A precipitated line was detected in 10 samples from the dairy farms in AGID test. According to the checkerboard results of ELISA, the best antigen concentration was 4.6 μg/μl and the best serum dilution was 1/160. The prepared ELISA detected 2 samples over than that detected by AGID test. Examination of pooled sera-samples by the prepared ELISA revealed that, this prepared ELISA is more sensitive; it detected 3 sera-samples as one pooled sample. Cohen's kappa test showed good agreement between the measures that the sensitivity value was 90.7%. In conclusion, through the sensitivity between AGID and ELISA tests and for planning purposes, it is preferable to use the ELISA with its high sensitivity. Both tests must be used routinely as they complement each other, especially if sera with low antibody titers are under investigation.

Key words: EBLV · AGID · ELISA

INTRODUCTION

Enzootic bovine leukosis virus (EBLV) (syn. bovine leukemia virus; BLV) is a retrovirus that induces a chronic enzootic bovine leukosis (EBL) in cattle, which develop in three possible pathological forms: asymptomatic course, persistent lymphocytosis (PL) and lymphosarcoma [1]. Most EBLV-infected cattle seldom present with clinical signs of the disease, only about 5% of the infected cows develop a clinical disease. The virus infects T cells, B cells and monocytes, but can be expressed by transcription only in B cells, induces accumulation of B-lymphocytes in the peripheral blood and lymphoid tissues, leading to leukemia/lymphoma [2]. Abnormal accumulation of leukemic B-lymphocytes results from an alteration of different parameters that include cell proliferation and death as well as migration to lymphoid tissues [3, 4]. Allogeneic cells usually become detectable in the blood during the second week after introduction of the virus, rapidly reach a peak number during the third week and decrease soon thereafter [5].

The sanitary and economic impact of EBLV infection is associated with the interference in the international movement of cattle and their germ plasm [6-8]. Producers can incur economic losses through cattle deaths, reduced reproductive efficiency, 3% less milk production (218 kg per cow), increased replacement and veterinary costs and the ineligibility to export live cattle, semen and ova to countries whereas EBLV control efforts are in place [9, 10].

Because no vaccine is available, virus specific antibodies found in serum or milk are a good indicator of exposure and a practical method for disease screening. The agar gel immunodiffusion (AGID) test has been the serological test of choice for routine diagnosis of serum samples [11]. The enzyme linked immunosorbant assay (ELISA) is a highly specific and sensitive assay for the detection of EBLV antibodies in serum and milk samples of cattle [12].

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The objective of this study was to prepare a local ELISA kits for detection of EBLV-antibodies that is comparable to AGID test, to evaluate the use of this prepared ELISA kits for identification of EBLV-infected herds.

MATERIALS AND METHODS

Samples: Serum samples (n= 1936; from imported cattle under quarantine) and 40 (suffered from enlarged lymph nodes, loss of appetite with severe depletion and drop in milk production) from two dairy farms in Swiss and El-Sharquia governments, Egypt, were collected during the 1st half of 2009 and tested.

Agar Gel Immunodiffusion Test: The AGID kits were purchased from Synbiotics Corporation; France and prepared as the manufacturers' leaflet guide. The agar is melted by heating in a water bath and poured into Petri dishes (19 ml per Petri dish of 9 cm Ø). The poured plates were allowed to cool at 4° C for one hour before the holes were cut in the agar. A puncher was used to cuts a hexagonal arrangement of six wells (6 mm Ø) round a central well (4 mm Ø) with 3 mm in between the wells. The reference antigen (32 µl) was placed in the central well and each of the tested sera (73 µl) was placed in one of the outer wells. One control pattern per plate with positive control serum and negative control serum (fetal bovine serum; FBS) in the place of tested sera was done. The plates were incubated at 20° C in a closed humid chamber and examined after 24, 48 and 72 hours.

Quanititation of Antigen Concentration: The AGID antigen concentration was estimated quantitavely using total protein liquicolour reagent, Stanbio laboratory, Boerne, TX USA. The freeze-dried antigen was dissolved in 1.6 ml of supplied diluent buffer. Two ml of total protein reagent and 20 µl of the antigen (1/10 as a primary dilution and then 2 fold serial dilutions) were mixed and incubated at room temperature (RT) for 10 minutes. The optical density (OD) of the mixture was read at 550 nanometer (nm). The antigen concentration (µg/µl) was calculated at the formula: OD of the sample/OD of the standard X concentration of standard (10 gm/dl).

Enzyme Linked Immunosorbant Assay: As the standard protocol that described briefly by Crowther [13], the ELISA technique was carried out on individual as well as pooled sera-samples to evaluate the sensitivity of this prepared kits in the diagnosis. According to the checkerboard results, the best antigen concentration was 4.6 µg/µl and the best serum dilution was 1/160. Briefly, the ELISA plates were coated with the antigen at the recommended concentration (4.6 µg/µl) in 100 µl coating buffer (1.59 g Na_2CO_3, 2.94 g $NaHCO_3$ pH 9.6 in 1 liter deionized water) and stored at 4° C overnight. In the next day and after removal the coating buffer, wells were blocked with 200 µl/well blocking buffer (2% bovine serum albumin; BSA, 0.05% tween 20 in phosphate buffered saline; PBS pH 7.2) and incubated at 37° C for 2 hours. For 3 times, the wells were washed thoroughly with the washing buffer (0.05% tween 20 in PBS pH 7.2). In the diluting buffer (0.5% BSA, 0.05% tween 20 in PBS pH 7.2), the tested serum was diluted at the recommended dilution (1/160) and added as duplicated as 100 µl/well and incubated at 37° C for one hour before washing as previously. After that, 100 µl/well of the diluted horseradish peroxidase conjugated anti-bovine IgG ($1:10^4$) in PBS pH 7.2 (Bethyl laboratories, INC, Germany) was added and incubated at 37° C for one hour. Plates were washed as previously and 100 µl/well of the substrate (0.4 mg O-phenylenediamine-OPD and one drop of 30% H_2O_2 per ml of 0.01 M citrate buffer pH 5) were added. After the color development, the reaction was stopped by 50 µl/well of 0.2 N sulfuric acid and the optical density (OD) at 492 nm wavelength was read. Positive and negative controls were included in each plate as duplicated.

Statistical Analysis: Concordance between the 2 tests was calculated as described by Cohen [14].

RESULTS

Agar Gel Immunodifusion Test: A precipitated line was developed in 10 samples from the dairy farms.

ELISA Results

According to the checkerboard results, the best antigen concentration was 4.6 µg/µl and the best serum dilution was 1/160. The prepared ELISA was more sensitive that detected 2 samples over than the AGID test. In the pooled sera-samples, this prepared ELISA detected 3 sera-samples as one pooled sample.

Statistical Analysis: Cohen's kappa test showed good agreement between the measures that the sensitivity value was 90.7%.

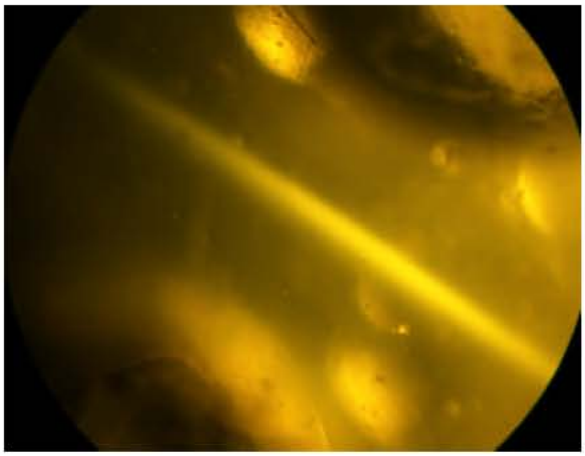

Fig. 1: AGID test for detection of antibodies against EBLV in serum samples using gp51 antigen. A precipitated line was developed between antigen and positive serum sample

Table 1: Quanititation the antigen concentration of 1/10 as a primary dilution and then 2 fold serial dilutions.

Antigen dilution	1/10 (a primary dilution)	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
OD	0.036	0.014	0.014	0.012	0.011	0.010	0.008	0.006
Conc. µg/µl	13.9	5.4	5.4	4.6	4.2	3.8	3.0	2.3

DISCUSSION

Herd testing for EBLV infection can be very helpful for three reasons: 1) to determine which animals are infected, 2) to monitor overall progress toward control or eradication and 3) to determine if and where horizontal transmission is occurring within a herd. By frequent testing (every 6 months), one can determine if any new infections are still occurring and if so, whereas these infections are most likely to develop as animals progress from calf hood to yearling heifers to milk cows. When the weak links in the program are identified through testing, additional management changes can be implemented and subgroups can be tested to determine if these changes are effective [15].

Many virological tests were proposed. The sensitivity of all the diagnostic methods is sufficient to do an early detection of EBLV infection on an individual base. Advantages of the highly sensitive methods like ELISA appear when the samples to be tested have naturally very low antibody titers (individual milk, bulk milk, pooled sera) [16].

The methods most widely used are AGID on serum and the ELISA on serum or milk. These tests have formed the basis for successful eradication policies in many countries [11]. The antibodies most readily detected are those directed towards the glycoprotein (gp51) and the core protein (p24) of the virus. Most AGID and ELISA tests detect antibodies to the gp51, as these appear earlier [17].

The AGID test is a highly specific, but not very sensitive, simple and easy to perform and has proven to be highly useful and an efficient technique as a basis for detection schemes. In the present study, a precipitated line was developed in 10 sera-samples from the dairy farms (Fig.1).

The ELISA is a highly specific, sensitive, reliable, practical and economic assay for identification of EBLV-infected herds [12]. In this study, the stock antigen of AGID was diluted 80 times, used for coating the ELISA plates and subsequently increases the numbers of tested samples. Also, the tested serum was diluted to 1/160 of the original, so the ELISA can detect the lower titer of antibodies as compared to the AGID test that use the serum sample as it is without dilution. The prepared ELISA was more sensitive that detected 2 sera-samples over than the AGID test. In the pooled sera-samples, this prepared ELISA detected 3 sera-samples as one pooled sample.

In conclusion, through the sensitivity between AGID and ELISA tests and for planning purposes, it is preferable to use the ELISA with its high sensitivity. Both tests must be used routinely as they complement each other, especially if sera with low antibody titers are under investigation.

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