Development of anti-leukemia measures in livestock farms of Kalmykia on the basis of complex diagnosis of the disease

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Abstract. The efficiency of diagnostic methods to control cattle leukemia during health measures in the farms of the Republic of Kalmykia was studied and a plan of anti-leukemia measures was developed. As a result, at a negative growth rate (-90.2%) the ill-being index defining the breadth of incidence in the territory of the republic was reduced by 10.2 times over 2003-2018. At the same time, while over 9 years from 2003 to 2008 the average ill-being index was within 0.42, from 2009 to 2018 it decreased from 0.33 to 0.03. The average tension coefficient, which takes into account the rate of acquisition of Bovine Leukemia Virus in the territory of the Republic by the end of the period (2018), remains zero.

1. Introduction
The fight against leukemia of farm animals is key within the national economy and is considered an important component of modern science. The regional features of cattle leukemia are identified in order to study the epizootic process and, in the future, to ensure complete recovery of farms in the territory of Kalmykia from leukemia.

The introduction of the anti-leukemia measures in the context of complex situation prevailing in the region against the background of the long history of this pathology among livestock in public and private animal husbandry sectors requires the use of all available resources, including administrative and veterinary services accompanied by continuous scientific support.

BLV is an exogenous retrovirus, which belongs to Deltaretrovirus genus within Orthoetrovirinae subfamily.

It is structurally and functionally linked to human T-lymphotropic virus 1 and 2 (HTLV-1 and HTLV-2), the main target cells for BLV are B-lymphocytes [1].

Life-time diagnostics is the basis of cattle leukemia epidemic countermeasures. Specificity, sensitivity, ease of technical implementation and low cost determine its efficiency [2].

The immunodiffusion test with glycoprotein antigen in agar gel is quite specific and the reliability of its results is beyond any doubt. However, it takes a lot of time from the moment of the reaction to the accounting of its results – 48 hours. Sometimes, the fall of immunodiffusion reaction is observed
in repeated studies, which is explained by a temporary decrease in antibody titers or a technical error, which is extremely rare. The reliability of immunodiffusion reaction studies increases with repeated or dynamic animal studies, which may well guarantee the efficiency of anti-leukemia measures [3].

Currently, enzyme-linked immunoelectrodiffusion essay (ELISA) deserves special attention as another serological method of leukemia life-time diagnostics. Due to its high level of sensitivity and specificity, it is quite aggressive and is widely used in veterinary practice in many countries of the world in the implementation of cattle leukemia elimination program.

This is a relatively more convenient method characterized not only by specificity, but also by flexibility of operation (the analysis can be carried out manually and automatically). ELISA helps to detect more infected animals earlier since it records lower values of antibody titers [4].

However, when choosing the method of serological diagnostics within the curative measure against cattle leukemia it is necessary to consider epizootic tension on leukemia in this particular farm [5].

The polymerase chain reaction (PCR) provides for the detection of proviral DNA regardless of the age and condition of an animal. Its use allows animals to be divided into healthy and sick in earlier stages, i.e. it is more efficient. An important area of PCR application is the study of imported livestock in quarantine in order to prevent the import of leukemia virus from abroad.

2. Materials and methods

The purpose of the study is to analyze the efficiency of diagnostic methods to control cattle leukemia during curative measure in the farms of the Republic of Kalmykia. The virus-carriage of BLV in cows was determined by immunodiffusion (IDR), ELISA, and PCR reactions. Serological studies were carried out via a set of reagents for IDR in agar gel produced by Kursk Bio-Factory – Biok Company; ELISA – by the test system of NGO Narvak. The reaction was taken into account on enzyme immunoassay AIFR-01 UniPlan followed by coefficient calculation. The dynamics of a titer of specific antibodies to leukemia virus were determined by successive dilutions of blood serum with physiological saline followed by IDR and ELISA

Diatom™ DNA Prep 100 reagent kits (Isogene Lab LLC) were used for DNA isolation. A set of reagents GenPak® DNA PCR test BLV to detect the DNA of a bovine leukemia agent (Isogene Lab LLC) was used for PCR [5].

Proviral DNA of cattle leukemia were detected on the basis of the molecular genetic laboratory of the Center for Collective Use of Kalmyk State University. In the study of ticks, DNA isolation from tissues was performed using the Ampli Prime DNA Sorb-B kit. To amplify the DNA site of BLV provirus, a PCR test system was used – a set of FRT – 50 F reagents (Central Research Institute of Epidemiology of Rospotrebnadzor). PCR amplification and detection of amplification products was performed using Real-time, Rotor-Gene 6000 (CorbettResearch, Australia). The JOE/Yellow Rotor-Gene 6000 version 1.7 (build 67) software was used to analyze the results of amplification of a specific DNA site of leukemia provirus (Bovine leukosis virus) [6].

3. Results and Discussion

In order to compare the efficiency of two methods of serological diagnostics we conducted parallel IDR and ELISA testing of animals. The results of the studies showed that the enzyme-linked immunoelectrodiffusion essay allows isolating additional up to 10-12% BLV infected animals, which is essential in the eradication of the viral infection.

Table 1 shows indicators of epizootic process of cattle leukemia in the Republic of Kalmykia for the period from 2003 to 2018.

Table 1 shows a significant improvement in the epizootic situation of cattle leukemia. If in 2003-2004 there was 3.72 and 3.33% of prevalence of livestock infection, then by the end of the study (2015-2016) it made 0.40% and 0.17% respectively, which is 9.3 and 19.6 times less the initial stage of the study. In 2018, the prevalence of leukemia infection in the population of cattle of the republic was 0.17%. The prevalence of infection in herds over these years made 3.97% and 5.9%. The
incidence at the beginning of the study (2003) was within 0.82%, while by 2018 it was reduced to 0.001%.

Table 01. Development of epizootic process in 2003-2018

| Years | % coverage in IDR | IDR + | Prevalence | % coverage in hematologic study | Hematologic study + | Incidence rate |
|-------|-------------------|-------|------------|---------------------------------|--------------------|---------------|
| 2003  | 44.32             | 3009  | 3.72       | 46.06                           | 663                | 0.82          |
| 2004  | 47.14             | 2926  | 3.33       | 39.62                           | 352                | 0.40          |
| 2005  | 53.34             | 3149  | 3.96       | 38.85                           | 350                | 0.37          |
| 2006  | 49.14             | 2476  | 2.78       | 40.48                           | 134                | 0.12          |
| 2007  | 47.53             | 2302  | 2.23       | 36.83                           | 253                | 0.17          |
| 2008  | 85.40             | 2227  | 2.39       | 49.25                           | 68                 | 0.04          |
| 2009  | 107.3             | 2910  | 1.73       | 64.50                           | 150                | 0.09          |
| 2010  | 97.01             | 2403  | 0.94       | 143.4                           | 839                | 0.33          |
| 2011  | 81.52             | 2438  | 0.78       | 48.48                           | 322                | 0.10          |
| 2012  | 77.17             | 2833  | 0.75       | 61.77                           | 311                | 0.08          |
| 2013  | 78.12             | 1799  | 0.45       | 74.26                           | 78                 | 0.02          |
| 2014  | 84.78             | 1100  | 0.28       | 85.45                           | 21                 | 0.01          |
| 2015  | 112.3             | 945   | 0.40       | 94.2                            | 38                 | 0.016         |
| 2016  | 101.1             | 650   | 0.17       | 113.8                           | 6                  | 0.001         |
| 2017  | 98.1              | 909   | 0.16       | 92.1                            | 7                  | 0.001         |
| 2018  | 105.6             | 819   | 0.17       | 92.9                            | --                 | --            |
| Average over 16 years | 79.37 | 2056 | 2.83 | 57.44 | 323.2 | 0.30 |

The analysis of serological (IDR) study taking into account the age and sex of cattle showed a particular characteristic. Thus, out of the total number of 151,593 animals studied within IDR, 2,227 heads responded positively, and the specific weight of infected cows made 98.3%, calves and heifers – 1.1%, bulls and bull-calves – 0.4%. At the same time, the specific weight of infected cows and heifers at the age of 2-4 years was 28.1%, and cows of 4-6 years – 70.2%, which is 42.10% more than for heifers and cows.

Thus, the age pattern of leukemia is characterized by the fact that cows under 4 years of age belong to increased risk group, and calves under 2 years of age have immunological tolerance under the condition of prenatal or postnatal infection. In case the calves are not infected, they show resistance to infection.

The animals from two years of age and older who respond positively in the diagnostic test system of IDR, ELISA and PCR are subject to hematologic study.

It is worth noting a decline in hematologic studies from 28829 (which made 66.17% of study coverage) in 1988 to 1850 studies (at 22.11% coverage) in 1993 due to increase in serological studies. The hematologic method as a key method mainly remains in the individual sector, where infection exceeded 30%. A sharp decline in hematologic studies to 176 heads per year was noted in 1996. The
coverage of the population by hematologic studies and the incidence rate decreased to 8.2% and 0.1%, respectively. The morbidity growth rate from 1991 to 1996 made 93.0%.

However, since 1997 there has been an increasing tendency of hematologic studies to 799 heads, but at the same time the number of sick animals increased from 45 to 109 heads. By 1999 the hematologic studies increased by 922. In total, 232 sick cows were identified during this year, the incidence made 0.58%.

On average, during the whole period of study, the infection rates and population prevalence made 7.5% and 3.5%. However, taking into account that the prevalence indicators are averaged in the whole republic due to the number of non-infected heads, the values of the percentage of infected animals will be more informative.

The hematologic method of detecting animals with leukemia in the problem of elimination of the disease was not effective for all regions of the republic. After removing sick animals from the herd, the animals with blood changes typical for leukemia appeared again at relatively short intervals.

Besides, cattle leukemia was widespread among livestock of agricultural organizations and farms of the republic. All this gave rise to the search for new ways of health measures in all categories of economic management related to the specifics of beef cattle breeding.

The health measures recommended by us for cattle leukemia in the republic are connected with animal husbandry technology when a calf is with his mother (suckling period) for 7-9 months on a pasture. Such technology implies that the epizootic process of bovine leukemia is naturally supported by the transmission of an agent to sick and infected cows, i.e. it ensues relay transmission of the infectious agent. This means that the agent of this disease is mainly transmitted vertically, which explains the causes of stable ill-being on cattle leukemia in livestock farms of the republic.

It should also be noted that if a breeding farm is ill-behaved regarding this disease, the extent of infection is significantly increased due to the sale of breeding animals.

We carried out PCR diagnostics of blood samples of animals in the farms of Gorodovikovsky district in order to determine the efficiency of diagnostic measures and to improve the epizootic situation of the district.

The blood serum was previously studied within the immunodiffusion reaction followed by a molecular assay (PCR analysis). Table 2 shows the results of the corresponding studies.

| Number of studied animals, n | Farm                          | IDR + | IDR - | PCR + | PCR - |
|-----------------------------|-------------------------------|-------|-------|-------|-------|
| 35                          | Demkin P.V. family farm       | 31    | 4     | 33    | 2     |
| 32                          | Shin-Byadl village (Yuzhny), industrial sector | 26    | 6     | 27    | 5     |
| 21                          | Druzhnoe village, industrial sector | 12    | 9     | 14    | 7     |
| 15                          | BAC KSU Aliev A.M.            | 6     | 9     | 5     | 10    |
| Total                       |                               | 75    | 28    | 79    | 24    |

Table 02. Results of serological and molecular studies

The presence of DNA provirus in 79 animals (PCR+) was isolated by the molecular method of bovine leukemia provirus assay, which made 76.6% of incidence. The PCR diagnosis did not detect DNA provirus in 24 animals, which made 23.3% of the animals.

Comparative analysis of serological and molecular biological studies shows that the PCR analysis in Demkin P.V. family farm revealed more animals (by 2 heads) with DNA provirus than sero-positive within immunodiffusion reaction. In Shin-Byadl village, Yuzhny industrial sector the molecular
analysis additionally revealed one animal carrier of DNA provirus. In Druzhnoe village industrial sector there were also by 2 carriers of DNA provirus more than among animals detected by carrier antibodies. However, in BAC KSU Aliev A.M. the PCR study revealed one infected animal less than in serological diagnostics.

The analysis of DNA provirus incidence indicates that the percentage of infection based on PCR studies did not correspond to the percentage of IDR-positive animals. In 75 cases, the results of IDR and PCR were the same. At the same time, PCR method revealed 4 cases more carriers of DNA provirus than serological study.

Out of 6 cases of mismatch of serological and molecular methods, one IDR-positive case of PCR was not confirmed by diagnostics, and in 5 cases of PCR-positive case IDR did not reveal antibodies to the virus.

The PCR study revealed 4 more DNA provirus carriers. Therefore, if to separate the calves by PCR method into provirus carriers and healthy ones in the first 2-3 weeks of animal life the farms can be recovered much faster. The molecular method allows carrying out early diagnosis of infection of both adult and young animals and putting them into the category of fattening animals.

Therefore, timely detection of infected animals using molecular genetic diagnostics (PCR) ensures the recovery of animals from this nosology.

The results of phylogenetic analysis on the basis of a pol gene of cattle leukemia pro-virus revealed the identity of isolates 10/5b KALMIKIYA, 10/2D KALMIKIYA with isolate 09/5164 ROSTOV (JQ400141) from Rostov region and isolate 10/8k KALMIKIYA with isolate 10/13 KALUGA (JQ400146) from Kaluga region (Limansky A.P., 2001).

High degree of similarity for the analyzed strains with international isolates on average makes 97-100% depending on the reference sequence. The maximum match was revealed with M16017_USA isolate: 100% similarity is found in case of 10/8k KALMIKIYA and 10/15sh KALMIKIYA strains; for other isolates 10/2d KALMIKIYA, 10/5b KALMIKIYA, 10/2k KALMIKIYA such match makes 98-99%.

In order to detect proviral DNA leukemia of cattle (BLV-provirus), we studied mites as natural reservoirs of infectious agents using PCR with hybridization-fluorescence detection. The study was carried out on Hyalomma scupense mites (collection in January, February) and Hyalomma mardinatum (collection in April, May).

We also conducted real-time PCR studies on cattle leukemia for 187 blood samples of animals, from which mites were removed.

The PCR blood test of cows for the presence of BLV showed that there was a link between the incidence of BLV provirus and the number of cows responding positively to the virus. The highest incidence of BLV provirus in mites is found in Druzhba village of Gorodovikovsky district characterized by the highest infection of cattle with Bovine Leukemia Virus in the republic.

Thus, the studies showed that in the infestation of animals by mites there is a possibility of a transmissive transmission route of Bovine Leukemia Virus. Therefore, we included a set of measures to control animals and premises into the system of anti-leukemia measures.

We have developed a plan of anti-leukemia measures for the next 5 years, thus identifying the following main approaches:

- Serological (IDR) study of cows and heifers 2 times a year – in spring before the mating campaign and in autumn after insemination.
- Serological (IDR) study of bulls and bull-calves 2 times a year – before insemination and after insemination campaign.
- Study of young cattle 1 once a year – in spring before pasture.
- Scientific justification of regulations on PCR use in farms with different epizootic situation for BLV infection.
- Study of genotypic diversity, molecular and genetic structure of cattle leukemia agent in animal population of the Republic of Kalmykia.
- Definition of subspecies of BLV isolates based on phylogenetic analysis.
• Withdrawal of all infected animals from a herd.
• Sale, slaughter, outlet, placement on pastures, and all other movements and regrouping of animals, sale of livestock products only with the permission of veterinary specialists.
• Quarantine of newly arrived animals for 30 days for serological and other studies and treatments.
• Timely monitoring of marking of animals in compliance with all rules of aseptics and antiseptics by veterinary specialists and zookeepers.
• Mandatory animal treatment against mites and blood sucking insects.

The main component of health measures is the production of offspring free of cattle leukemia virus by using the reproductive potential of intact, uninfected animals. The only solution to the problem can be early diagnosis of leukemia in young cattle thus allowing timely rejection and eliminating the cost of growing an infected low-productive calf, or at least isolating it from the common herd to avoid the infection of healthy animals.

4. Conclusion
Thus, the proposed methodology allowed, first of all, improving all tribal farms of the republic. Currently, the ill-being status of breeding farms is confirmed by annual single studies (Table 3).

| Table 03. Results of effective measures to combat cattle leukemia |
|----------------------|----------------|----------------|----------------|
| Years | Ill-being index | Focality ratio | Tension coefficient | For slaughter |
|------|----------------|----------------|----------------|--------------|
| 2003 | 0.54 | 3.97 | 0.121 | 3009 |
| 2004 | 0.44 | 2.55 | 0.034 | 2926 |
| 2005 | 0.36 | 2.63 | 0.033 | 3149 |
| 2006 | 0.37 | 1.04 | 0.003 | 2476 |
| 2007 | 0.33 | 2.16 | 0.006 | 2302 |
| 2008 | 0.41 | 0.53 | 0.000 | 2227 |
| 2009 | 0.33 | 1.28 | 0.002 | 2910 |
| 2010 | 0.17 | 11.4 | 0.035 | 2403 |
| 2011 | 0.20 | 4.74 | 0.004 | 2438 |
| 2012 | 0.14 | 9.15 | 0.006 | 2833 |
| 2013 | 0.09 | 4.33 | 0.000 | 1799 |
| 2014 | 0.04 | 1.31 | 0.000 | 1100 |
| 2015 | 0.03 | 1.28 | -- | 945 |
| 2016 | 0.02 | 1.19 | -- | 650 |
| 2017 | 0.03 | 1.27 | -- | 909 |
| 2018 | 0.03 | 1.25 | -- | 819 |
| Average | 0.26 | 3.34 | 0.04 | 2193 |
| Growth rate | -90.2 | 18.1 | -99.85 | -51.9 |

The ill-being index, which determines the breadth of infection in the territory of the republic, at a negative growth rate (-90.2%) from 2003 to 2018 was reduced 18 times. At the same time, while over 6 years from 2003 to 2008 the average ill-being index was within 0.41, from 2009 to 2018 this indicator decreased from 0.33 to 0.03.

The average tension factor, taking into account the level of BLV contamination in the territory of the republic, at the beginning of the study was within 0.121 (2003) and by the end of the period (2018) it equaled zero. At the same time, if from 2005 to 2009 there was a decrease of epizootic process tension index from 0.033 to 0.002, respectively, in 2010 there was again an increase of the same values to the level of 0.035 with subsequent reduction of the tension index to zero. This
situation is explained by the increase in the coverage of the population with diagnostic study in the republic by 2010 and the increase in anti-leukemia measures by the end of the study period.

There is also a decrease in the indicators of focality coefficient from 3.97 in 2003 to 1.25 in 2018, while the growth rate of indicators has decreased, but still remains positive (18.1). However, similar to the tension coefficient in 2010, there is also an increase of focality coefficient to 11.4 followed by a decrease in these values, which is also associated with an increase in diagnostic studies, detection and elimination of infected and sick animals. The use of PCR in a complex of anti-leukemia measures ensures earlier and complete detection of infected animals and reduces the time of herd recovery.

The study resulted in a steady decrease in the indicators of herd ill-being at a negative growth rate of the tension coefficient (-99.8). In 2013 and 2014 the tension coefficient equaled zero, which confirms the ill-being for leukemia infection in the farms of the republic.

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