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Comparison of PCR and ELISA Methods for the Detection of Bovine Leucosis in Dried Blood Spots

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Abstract—PCR and ELISA methods for the detection of bovine leucosis in dried blood spots on porous membranes were compared. Dry samples were analyzed through real-time PCR, using several diagnostic test systems. Nineteen and 20 samples were identified as positive by PCR and ELISA, respectively. Fourteen of these samples were identified as positive by both methods. When using PCR and ELISA, 26 samples were identified as positive for leucosis, which amounted to 47% of the total number of tested samples. The results of the analysis of dried and native samples were in good agreement. The obtained results showed that whole blood sampling in the form of dried spots applied on membrane can be used as a convenient and reliable way to obtain dry samples of biological fluids with the purpose of screening herds for infectious diseases, in particular for bovine leucosis.

Keywords: enzootic bovine leucosis, ELISA, PCR, porous membranes, dried blood spots
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INTRODUCTION

Dried blood spot technology is becoming more widely implemented in human and veterinary medicine [1]. To obtain dried spots, a liquid blood sample collected from a patient is applied dropwise to a special membrane card of a cellulosic material and dried. Even at room temperature, the sample obtained by this procedure is highly stable during storage and transportation. This makes it possible to send dry samples of biological liquids to a diagnostic laboratory by courier or postal service without cold chain requirements. For the analysis, a disc of a certain diameter is punched from the membrane with a special device, after which the dry sample contained on this disc is eluted. The target substances are then determined in the obtained solution using standard analytical methods. We proposed a new sampling format: dried spots on the strips of membrane made of porous fiberglass material [2]. This format was successfully used for the collection, storage, and analysis of dry samples of milk by ELISA for the quantitative determination of progesterone in order to identify nonpregnant cows [3]; it was also successfully used for the detection of proviral DNA of leukemia virus by polymerase chain reaction (PCR) with electrophoretic detection [4]. The present study is devoted to the detection of bovine leukemia virus in dry blood samples; the diagnostics was carried out by real-time PCR and ELISA.

Bovine leucosis is a chronic retroviral infection caused by RNA-containing virus of the Retroviridae family. The source of the pathogen are animals infected with bovine leukemia virus at all stages of the infection process. The virus is transferred via blood, saliva, milk, and other biological fluids containing lymphoid cells. In recent years, enzootic bovine leucosis has become one of the major infectious pathologies in the Russian Federation. Bovine leucosis accounts for more than half of the established cases of infectious diseases. Timely diagnostics and disposal of leucosis-infected animals is essential for preventing the disease spread [5].

The diagnostics of enzootic bovine leucosis is based on serological methods such as agar gel immunodiffusion (AGID) and ELISA. These methods aim at the detection of specific antibodies produced by the organism of an infected animal as an immune response to a foreign protein of the causative virus. In contrast with AGID, which is based on visual detection of precipitating antigen–antibody complexes, ELISA has higher sensitivity and specificity and makes it possible to obtain test results in a few hours. The presence of proviral DNA within the genome of an infected cell is determined by PCR. This method enables the detection of infected animals among sero-

negative animals (i.e., among those animals that have not yet developed an immune response) and animals less than six months of age. Thus, the integrated use of two methods of analysis (PCR and ELISA) provides the most thorough monitoring of herds for the presence of virus infections, such as bovine leucosis, and to take timely sanitary measures [6].

The purpose of this study was to use PCR and ELISA for the bovine leucosis detection in dry blood samples on membrane supports and to compare the results obtained by using different PCR test systems for the detection of proviral DNA of leukemia virus.

EXPERIMENTAL

Collection and Preparation of Dry Blood Samples

The carrier for a dry sample was a 0.5-cm wide marked strip of a fiberglass membrane (a card produced by LLC Immunoved, Moscow, for storage and transportation of biological fluids in the form of dry blood samples). The tests were performed using bovine whole blood samples from cattle farms of the Republic of Tatarstan; the samples were collected during routine monitoring of herds for the presence of animals infected with leucosis. The initial section of a membrane was placed in a liquid blood sample and incubated there for a time required for the liquid to rise and fill the entire strip length. The strip containing the sample was then air-dried for 1.5–2 h at room temperature. The dry samples were stored at 4°C in tightly sealed plastic bags with desiccant.

Detection of Proviral DNA of Bovine Leukemia Virus in Dry Blood Spots by Real-Time PCR

The following kits were used to extract nucleic acids: AmpliPrime RIBO-prep (InterLabService, Moscow), MAGNO-sorb (InterLabService, Moscow), and Universal (Biocom, Moscow). Three equally sized pieces (0.5 × 0.5 cm) were cut, according to the marking, with sterilized scissors from a membrane containing a dry blood sample and placed into a 1.5-mL tube. The scissor blades were re-sterilized before working with each new sample. The tubes were supplied with 300 μL of working buffer and incubated for 20 min with periodical vortexing. After the incubation, the tubes were centrifuged for 5 s at 12000 rpm to remove liquid from the tube walls and lid. The obtained solution was transferred to a clean tube. Further steps for the DNA extraction from the samples were performed according to kit instructions. The analysis of liquid blood was performed in one iteration using a 100-μL aliquot. The extracted DNA was amplified in real time using three test systems: PCR-1 (Leucosis, InterLabService, Moscow), PCR-2 (Biocom, Moscow), and PCR-3 (Federal Center for Toxicological, Radiation, and Biological Safety, Kazan) on a DT-96 (DNA-Technology, Moscow) or a CFX-96 (BIO-RAD, USA) device.

Detection of Antibodies to Bovine Leukemia Virus in Dry Blood Spots by ELISA

A diagnostic BLV Antibody Test Kit (IDEXX, France) was used to perform qualitative ELISA for the detection of antibodies to gp51 protein of bovine leukemia virus. For the analysis, a membrane fragment (0.5 × 0.5 cm) was cut, according to the marking, off a membrane containing a dry blood sample and placed in a well of a 96-well plate. Samples were tested in duplicate. The wells with the samples were supplied with 200 μL of the sample dilution buffer. The samples were shaken for 10 min at 120 rpm. They were then covered with a lid and incubated at 37°C for 1 h. After the incubation, the solution was decanted and the membrane fragments remaining in the wells were removed with forceps. The further steps and the interpretation of the results were performed according to the manufacturer's instructions for the diagnostic kit. Absorbance of liquid samples was measured on an Anthos 2010 spectrophotometer (Biochrom Ltd, UK) at a wavelength of 450 nm. The analysis of liquid samples was performed according to the kit instructions.

RESULTS AND DISCUSSION

For the efficient DNA extraction from blood samples dried on membrane carrier, it was necessary to provide the most complete elution of a sample containing the desired DNA from the membrane strip. Experimentally, it was found that the elution of dry blood should take at least 20 minutes and that the sample should be thoroughly mixed during the elution. Using exogenous control primers, we showed that the solution obtained after eluting the dry sample contained DNA. Table 1 shows test results obtained by analyzing liquid and dry blood samples for the presence of proviral DNA of bovine leukemia virus by real-time PCR with PCR-1 and PCR-3 test systems. DNA was extracted by alcohol precipitation. Both systems produced practically identical results in the analysis of liquid samples. In contrast with the liquid samples, proviral DNA was not identified in two dry samples (no. 8 and no. 22); moreover, sample no. 8 tested negative in both test systems. This is probably due to a small amount of DNA extracted from the dry samples, which, in turn, may be associated with an insufficient amount of dry sample taken for analysis. In the experiment, we used three square pieces of the membrane containing the sample; this is equivalent to half the volume of liquid blood aliquots (100 μL).

The course of the disease is such that the amount of infected lymphoid cells in the blood of an infected animal is individual for each animal and strongly depends on the stage of the disease. For example, the virus may infect only the internal organs and tissues and the amount of peripheral blood cells containing proviral DNA may then be insufficient to be amplified in the aliquot used for the isolation of genetic material. To increase the sensitivity of the method, more mem-

Table 1. Results of the analysis of dry and liquid samples of bovine whole blood by PCR (alcohol precipitation) and ELISA

Test system	PCR-1		PCR-3		ELISA
Sample	dry	liquid	dry	liquid	dry
1	neg	neg	neg	neg	neg
2	neg	neg	neg	neg	neg
3	neg	neg	neg	neg	neg
4	neg	neg	neg	neg	neg
5	neg	neg	neg	neg	neg
6	neg	neg	neg	neg	neg
7	neg	neg	neg	neg	neg
8	neg	pos	pos	pos	pos
9	neg	neg	neg	neg	neg
10	neg	neg	neg	neg	pos
11	neg	neg	neg	neg	pos
12	neg	neg	neg	neg	pos
13	neg	neg	neg	neg	pos
14	pos	pos	pos	pos	pos
15	neg	neg	neg	neg	pos
16	neg	neg	neg	neg	pos
17	neg	neg	neg	neg	pos
18	neg	neg	neg	neg	pos
19	neg	neg	neg	neg	pos
20	neg	neg	neg	neg	pos
21	pos	pos	pos	pos	pos
22	pos	pos	neg	pos	pos
23	pos	pos	pos	pos	pos
24	pos	pos	pos	pos	pos
25	neg	neg	neg	neg	neg
26	–	–	pos	pos	pos
27	–	–	neg	neg	neg
28	–	–	pos	pos	pos
29	–	–	pos	pos	pos
30	–	–	pos	pos	neg
31	–	–	neg	neg	neg
32	–	–	neg	neg	neg
33	–	–	neg	neg	neg
34	–	–	neg	neg	neg
35	–	–	pos	pos	neg

Notation: pos are positive tests, neg are negative tests, and dash (–) means that no tests were performed.

brane fragments should be used for the elution of a dry sample and the analysis should be performed in several replicates. Comparison of the PCR results with the results obtained by the serological study (ELISA) showed that two PCR-positive samples turned out seronegative, whereas four seropositive samples were PCR-negative. In the first case, the result is due to the direct detection of an adverse factor (proviral DNA) by PCR as opposed to the indirect serological test, which cannot detect antibodies in animals at the early stages of infection. In the second case, the discrepancy between the results may be associated with a lack of PCR sensitivity in the detection of proviral DNA resulting from its low content in the blood at certain stages of the disease. The discrepancy may also emerge from an error in the ELISA test, which could provide false positive results. False positive results in ELISA may be caused by elevated levels of antibodies in adult animals after vaccination or calving or in calves (less than six months of age) born from infected cows and having a so-called colostral immunity. These samples should be retested by ELISA and PCR a few months

Table 2. Results of the analysis of dry and liquid samples of bovine whole blood by PCR performed using two DNA extraction methods and by ELISA

Sample number	Test system					
	PCR-1* (liquid)	PCR-1* (dry)	PCR-2* (liquid)	PCR-2* (dry)	PCR-2** (dry)	ELISA (dry)
1	pos	pos	pos	pos	pos	pos
2	pos	neg	pos	neg	pos	pos
3	neg	neg	neg	neg	pos	neg
4	neg	neg	neg	neg	neg	neg
5	neg	neg	neg	neg	neg	neg
6	neg	neg	pos	neg	neg	pos
7	neg	neg	neg	neg	neg	neg
8	neg	neg	neg	neg	pos	neg
9	neg	neg	neg	neg	pos	neg
10	neg	neg	neg	neg	neg	neg
11	neg	neg	neg	neg	neg	pos
12	neg	neg	neg	neg	neg	neg
13	neg	neg	neg	neg	neg	pos
14	neg	neg	neg	neg	neg	neg
15	neg	neg	neg	neg	neg	neg
16	neg	neg	neg	neg	pos	neg
17	neg	neg	neg	neg	neg	neg
18	pos	pos	pos	pos	pos	pos
19	neg	neg	neg	neg	neg	neg
20	pos	neg	neg	neg	pos	pos

Notation: pos are positive tests and neg are negative tests.

* Sorbent-based extraction and ** alcohol precipitation.

later and the suspect animals should be isolated from the herd.

Table 2 shows the comparison of the results obtained for liquid and dry samples using PCR-1 and PCR-2 systems to amplify DNA obtained by two extraction methods. When using sorbent-based extraction, the target DNA was not found in two dry samples (no. 2 and no. 20); however, these dry samples, similar to the respective liquid samples, were identified as positive when using alcohol precipitation. The extraction of genetic material from dry samples by sorption on magnetic particles turned out less efficient than DNA extraction by alcohol precipitation. Four additional positive samples were identified in the second case. Moreover, the DNA extraction steps of the method based on alcohol precipitation require significantly less time than those of the sorbent-based extraction, which comprises many sorbent washing steps. Each washing of the sorbent can lead to a loss of the DNA adsorbed on this sorbent affecting the final results. According to Table 2, the results of amplification of the extracted DNA in some cases discord for different PCR test systems, which is probably due to the dissimilar set of primers used in these systems. The selection of primers in the test system design is based on the nucleotide sequence in a certain region of proviral DNA of different viral genotypes. The selection can be targeted on conserved or variable regions. There are eight different genotypes of bovine leukemia virus and these genotypes have pronounced geographic distribution [7]. However, several virus genotypes, introduced together with infected animals from other countries and regions, can be present even within one farmstead. This makes it harder to develop a universal set of primers and leads to false negative results in the PCR analysis, thereby reducing its efficiency.

In this study, we tested 55 bovine blood samples. We identified 19 positive samples (35%) and 37 negative samples (65%) by PCR performed with different test-systems. Using ELISA assays, antibodies to bovine leukemia virus were found in 20 samples (36%), whereas 35 samples (64%) were identified as seronegative. Both methods produced similar number of positive tests; however, the test results discord for 12 samples (22%) (Table 3). When accounting for the results obtained by both methods, 26 out of 55 samples (47%) are identified as positive for bovine leucosis indicating a high level of animal infection in the herd.

Therefore, samples should be tested by both methods for a more thorough identification of infected animals.

Table 3. Concordance of the results obtained by PCR and ELISA in the analysis of 55 bovine blood samples

Result	Number of samples	%	Concordance and discordance of the results	
PCR (pos) ELISA (neg)	14	25	Concordance of the results obtained by PCR and ELISA	78%
PCR (pos) ELISA (neg)	29	53		
PCR (pos) ELISA (neg)	6	11	Discordance of the results obtained by PCR and ELISA	22%
PCR (pos) ELISA (neg)	6	11		

This approach will increase the herd sanitation effectiveness and, by enabling timely isolation of infected animals, will reduce the likelihood of the infection transmission to healthy animals. The comparison of the results obtained by PCR and ELISA once again confirms that it is impossible to unambiguously identify infected animals based solely on the results of indirect serological studies because of the nature of the disease. However, serological methods such as AGID and ELISA remain the cheapest and the most convenient for routine monitoring of a large number of cattle.

The use of dried spot technology for the sampling and subsequent analysis of biomaterial has several advantages over the traditional sampling of liquids. One of these advantages is that blood can be sampled directly on the support, thereby eliminating the step of isolating the serum. As is well known, only specific biological fluids, such as serum of blood plasma, are used in ELISA assays. Whole blood is not used in ELISA assays because of the difficulties in storing and collecting aliquots; these difficulties result from an uneven distribution of analytes in the aliquot volume and a large volume of cellular material that complicates the handling of a sample. By collecting animal whole blood directly on the membrane support, it becomes possible to evenly distribute analytes contained in the blood along the strip of membrane material and, after washing the dry sample from the membrane, to obtain a homogeneous solution suitable for ELISA. Moreover, dry blood samples are characterized by a high storage stability.

CONCLUSIONS

We showed that dry blood samples can be used to monitor herds for the presence of bovine leukemia virus by PCR and ELISA. When analyzing dry blood samples by real-time PCR to identify proviral DNA, a DNA extraction method should be carefully considered and a test system that best detects infected animals should be used. The combined use of PCR and ELISA makes it possible to carry out diagnostic measures for sanitizing herds more effectively. The use of dry samples of bovine whole blood on membrane carrier as test samples simplifies the procedure for storing and shipping the samples to a laboratory and makes the veterinary diagnostics based on promising research methods more accessible.

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