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A New Sampling Format for the Diagnostics of Bovine Infectious Diseases in Dried Blood Spots by ELISA

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Abstract—The detection of antibodies to bovine infectious diseases (enzootic bovine leucosis, viral diarrhea, and infectious rhinotracheitis) is performed by ELISA in dry whole blood samples obtained on porous membranes using a new sampling format. Several commercial test systems are used to diagnose the infections. The obtained data are compared to the results of the analysis of liquid samples of blood serum. The results of the analysis of dried and liquid samples are in full concordance. The new sampling format makes it possible to avoid using a cold chain for the transportation of samples to diagnostic laboratories and to simplify the storage of these samples.

Keywords: cattle, enzootic bovine leucosis, viral diarrhea, infectious rhinotracheitis, ELISA, porous membranes, dried blood spots

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INTRODUCTION

Bovine infectious diseases, such as enzootic leucosis (EL), viral diarrhea (VD), and infectious rhinotracheitis (IRT), are common in animal farms and cause serious economic damage [1–3]. These diseases are characterized by a long latent period and many different forms of the disease progression. Under the conditions of the livestock industry, viruses are easily transmitted from one animal to another, leading to rapid spread of infection through herds and a high proportion of infected animals.

A number of diagnostic procedures are used for the early detection of infected animals and the subsequent sanitation of herds. The diagnostics is usually based on using serological methods, such as the agar gel immunodiffusion and ELISA. These methods aim at the detection of specific antibodies produced in animals as an immune response to foreign viral proteins. Methods such as polymerase chain reaction (PCR), designed to directly detect agents of infectious diseases, are also used.

To regularly monitor herds for the presence of infectious diseases and to check the immunity level after vaccination, blood samples are sent to a diagnostic laboratory for analysis. In most cases, it is crucial to freeze the biological material after sampling and then to meet cold chain requirements during its transportation. The problem of storing and delivering biological samples can be solved by using a new sampling technique based on the drying of biological liquids (blood, milk, etc.) applied on a porous membrane support. Such technology (Dried Blood Spot, DBS) for the sampling, storage, and transportation of biological liquids in the form of dried spots (usually blood spots) is relatively common in medicine [4, 5].

When using DBS, a few drops of blood are applied on a special cellulose membrane, air-dried, and sent to a laboratory. The fact that only a few drops of blood are required to obtain a dry sample makes the process less invasive and makes it possible to inflict less injury to an animal during blood collection.

In recent years, the technology based on the preparation of dried samples has become increasingly used in veterinary diagnostics because the preparation of dried samples has a number of advantages over the traditional method of collecting blood and other biological liquids [6, 7]. The advantages of dry spot technology include significant simplification of the sample collection and storage, reduction of transportation costs entailed by the cold chain, and the possibility of preparing samples in the field.

We have previously proposed a new format for the sample preparation on porous membrane supports produced in the form of narrow marked strips. This format was successfully used for the quantitative determination of progesterone by ELISA with the aim of

the early identification of nonpregnant animals [8] and for the determination of proviral DNA of bovine leukemia virus in dried blood samples by PCR [9].

The purpose of the current work is to study the possibility of using the new sampling format for the detection of specific antibodies in dried blood samples by ELISA in the diagnostics of bovine infectious diseases.

EXPERIMENTAL

Sample isolation and preparation. We used blood samples from 62 head of cattle taken in the field conditions at animal farms in the Republic of Tatarstan. Dried samples were prepared by applying whole blood on membrane supports with subsequent drying. The support was a narrow marked strip of 0.5 cm in width fixed on a special card for the storage and transportation of biological liquids as dried spots (OOO Immunoved, Moscow) [10]. To apply the biomaterial, the first part of the membrane was placed into a whole blood sample and incubated to wet the strip completely; the membrane was then removed and air-dried at room temperature for 1.5–2 h. The dried samples were stored at 4°C in tightly sealed plastic bags with a dessicant. To prepare the liquid serum, tubes containing whole blood samples were centrifuged at 3000 rpm for 20 min. The aliquots of the obtained serum were stored at −18°C.

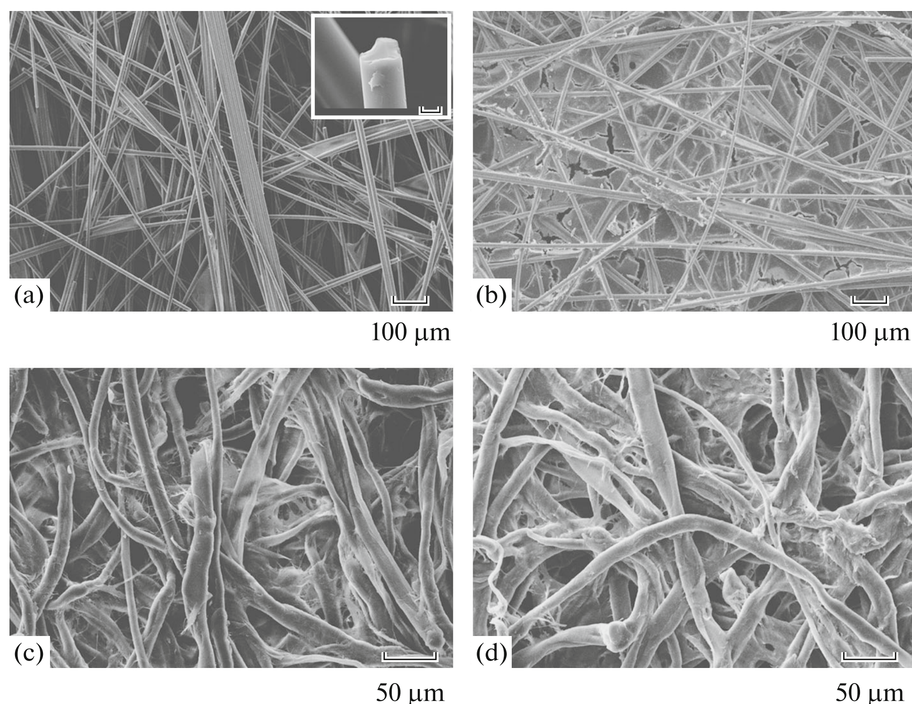
Determination of the antibodies to the agents of bovine infectious diseases in dried blood spots by ELISA. To carry out qualitative enzyme immunoassay aimed at detecting antibodies to the gp51 protein of the bovine enzootic leucosis virus, a BLV Antibody Test Kit (IDEXX, France) was used. From the membrane with a dry blood sample, a 0.5 × 0.5 cm fragment was cut with scissors according to the marking and placed into a well of a 96-well plate. Samples were tested in duplicate. Before cutting off a new sample, the scissors were wiped with a paper towel to remove any remaining particles of the dried blood. The wells containing the samples were supplied with 200 μL sample dilution buffer. Samples were mixed on a shaker at 120 rpm for 10 min, 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. Further actions and interpretation of the results were performed according to the kit instructions. The absorbance of samples was measured on an Anthos 2010 spectrophotometer (Biochrom Ltd, UK) at the wavelength of 450 nm.

To determine the antibodies to the proteins of bovine IRT virus, an IRTSEROTEST diagnostic kit (OOO Vetbiokhim, Moscow) was used. From the membrane with a dry blood sample, a 0.5 × 0.5 cm fragment was cut with scissors according to the marking and placed it into a tube. Before cutting off a new sample, the scissors were wiped with a paper towel to remove any remaining particles of dried blood. The required dilution of the sample was obtained by adding 700 μL dilution buffer to a single marked membrane fragment. Further analysis was performed according to the kit instructions.

To determine the antibodies to the proteins of bovine VD virus, we used a VDKSR-SEROTEST diagnostic kit (OOO Vetbiokhim, Moscow). From the membrane with a dry blood sample, we cut off with scissors two fragments of 0.5 × 0.5 cm in size according to the marking and placed them into a well of a plate. Before cutting off a new sample, the scissors were wiped with a paper towel to remove any remaining particles of dried blood. The samples were mixed in a shaker at 120 rpm for 10 min, covered with a lid, and incubated at room temperature for 2 h. After the incubation, the solution was abruptly decanted and the membrane fragments remaining in the wells were removed with forceps. Further steps of the analysis and the interpretation of the results were performed according to the kit instructions.

RESULTS AND DISCUSSION

To collect, store, transport, and analyze the dry blood samples, a new sampling format was used. As a support for the biological material, a narrow marked strip of membrane made of porous hydrophilic fiberglass material was used. In comparison with the traditionally used cellulose support, a fiberglass membrane has a number of mechanical (higher strength and deformation resistance) and structural (solid fiber structure) advantages. The electron micrographs obtained on a Tescan Vega 3SB scanning electron microscope (Jeol Ltd., Japan) for two membrane supports clearly demonstrate the structural differences between the cellulose and fiberglass membranes (see figure). Electronic photos were obtained for free membranes and for the membranes with a dried blood sample (parts b and c in the figure). In contrast to hollow cellulose fibers (parts c and d in the figure), the solid structure of the fiberglass strands makes it impossible for a biological liquid to penetrate the fibers (parts a and b in the figure) and also increases the efficiency of elution of the analyzed components from the membrane. Moreover, the structure of the fiberglass membranes makes it possible to eliminate the chromatographic effect of the distribution of components during the biological liquid absorption and to overcome the difficulties in the quantitative analysis of whole blood that are associated with the hematocrit effect and are typical of cellulose membranes [5]. The volume percent of cellular elements in blood (hematocrit) can vary greatly depending on the age, physical condition, and life cycle of an animal, resulting in errors in the measurements performed using cellulose membranes. Uniform distribution of a biological liquid in a fiberglass membrane makes it possible to avoid inaccuracies during the analysis of complex biological



Scanning electron microscopy images of membranes: a—fiberglass membrane without a sample; b—fiberglass membrane with a dried blood sample; c—cellulose membrane without a sample; and d—cellulose membrane with a dried blood sample.

liquids such as blood and milk independent of the formed elements of blood.

Adaptation of commercial diagnostic kits for the detection of antibodies in dry samples of biological liquids depends on the final dilution of the studied sample desired in the given diagnostic system and on the type of the biological liquid applied on the membrane. In this study, we used samples of bovine dried whole blood adsorbed on a fiberglass membrane with a capacity of about 15 μL liquid for each marked square region (0.5 × 0.5 cm). Given that the average value for bovine hematocrit is 30–50%, we can assume that each membrane fragment contains 7–9 μL of dried plasma. To prepare the dilution (100 times) desired in the analysis of dried blood samples using the IRT-SEROTEST diagnostic kit (OOO Vetbiokhim, Moscow), we added 700 μL dilution buffer to one marked fragment. The analysis of liquid blood serum samples using a BLV Antibody Test Kit (IDEXX, France) required 20-fold dilution of the sample. This was achieved by adding 160 μL dilution buffer to one square fragment containing 15 μL dried blood, which is less than the liquid volume required for the analysis (a well must contain liquid with a total volume of 200 μL). To avoid further dilution of the samples, a membrane fragment was placed directly into a plate well and then supplied with 200 μL dilution buffer. This approach resulted in dilution that was close to the design value and there were no additional steps to complicate the procedure. For a correct interpretation of the results, we varied the volume of supplied positive and negative controls from 5 to 15 μL. Varying the negative control had almost no effect on the interpretation of the results. Varying the positive control volume from 7 to 10 μL had a proportional effect on the measured absorbance in the control well. The most accurate interpretation of the results of the dried blood sample analysis (in comparison with that for the liquid samples of serum) was achieved using a 7-μL aliquot of the positive control.

The table presents the final results of the testing of dry samples for the presence of antibodies to the antigens of viral infections. The ELISA results obtained using dry blood samples on membrane supports completely agreed with the results obtained from testing of liquid samples of serum. It turned out that 22 of 62 (35%) samples were ELISA-positive for leucosis virus proteins. Such a high degree of herd infection clearly requires the isolation of infected animals. It is prohibited to sell the milk of cows suffering from leucosis; for livestock farms, this leads to serious economic losses. It is known that enzootic bovine leucosis is incurable and the infected animals are slaughtered to prevent the spread of infection. Planned vaccination is performed to control bovine VD and IRT. To test the efficiency of vaccination, the immunity level is assessed by measuring the level of post-vaccination antibody titer. Analysis of test samples isolated from vaccinated animals for the presence of antibodies to IRT virus proteins showed that 98% of animals have a high level of IRT

Results of the analysis of dried blood samples for infectious bovine diseases performed by ELISA

Sample number	EL	BD	IRT	Antibody titer to IRT virus	Sample number	EL	BD	IRT	Antibody titer to IRT virus
1	neg	neg	pos	>1/3200	32	neg	neg	pos	>1/3200
2	neg	pos	amb	1/100	33	neg	pos	pos	>1/3200
3	neg	pos	pos	>1/3200	34	neg	pos	pos	>1/3200
4	neg	pos	pos	>1/3200	35	neg	pos	pos	>1/3200
5	neg	pos	pos	>1/3200	36	pos	pos	pos	>1/3200
6	neg	pos	pos	>1/3200	37	pos	pos	pos	>1/3200
7	neg	pos	pos	>1/3200	38	neg	pos	pos	>1/3200
8	pos	pos	pos	>1/3200	39	neg	neg	pos	>1/3200
9	neg	pos	pos	>1/3200	40	pos	neg	pos	>1/3200
10	pos	pos	pos	>1/3200	41	neg	pos	pos	>1/3200
11	neg	pos	pos	>1/3200	42	neg	pos	pos	>1/3200
12	neg	pos	pos	>1/3200	43	pos	pos	pos	>1/3200
13	neg	pos	pos	>1/3200	44	neg	pos	pos	>1/3200
14	pos	pos	pos	>1/3200	45	neg	pos	pos	>1/3200
15	neg	pos	pos	>1/3200	46	pos	pos	pos	>1/3200
16	neg	pos	pos	>1/3200	47	neg	pos	pos	>1/3200
17	neg	pos	pos	>1/3200	48	neg	pos	pos	>1/3200
18	pos	pos	pos	>1/3200	49	pos	pos	pos	>1/3200
19	pos	pos	pos	>1/3200	50	neg	pos	pos	>1/3200
20	pos	pos	pos	>1/3200	51	pos	neg	pos	>1/3200
21	pos	pos	pos	>1/3200	52	pos	pos	pos	>1/3200
22	pos	neg	pos	>1/3200	53	neg	pos	pos	1/1600
23	pos	pos	pos	>1/3200	54	neg	pos	pos	>1/3200
24	pos	pos	pos	>1/3200	55	neg	pos	pos	>1/3200
25	neg	pos	pos	>1/3200	56	pos	pos	pos	>1/3200
26	pos	pos	pos	>1/3200	57	neg	pos	pos	>1/3200
27	neg	pos	pos	>1/3200	58	neg	pos	pos	>1/3200
28	pos	pos	pos	>1/3200	59	neg	pos	pos	>1/3200
29	pos	pos	pos	>1/3200	60	neg	pos	pos	>1/3200
30	neg	pos	pos	>1/3200	61	neg	pos	pos	1/800
31	neg	pos	pos	>1/3200	62	neg	pos	pos	>1/3200

immunity. For bovine VD, the percentage of positive samples was 90%. The obtained data indicate that vaccination against these infections was successful but seronegative animals should be revaccinated and retested.

The use of dried samples of bovine blood resulted in a high analytical reliability of the obtained data of qualitative and semi quantitative ELISA. In the analysis of dried samples, the antibody titers (limiting dilution of a sample at which the presence of antibodies can be detected) to the IRT virus were in the same range as in the analysis of liquid samples. A discrepancy between the values of the antibody in the case of serial twofold dilutions is acceptable at the boundaries of the twofold dilution range. The desorption of analytes from the membrane into the buffer is rather efficient during the preliminary dilution both in a tube and in plate wells. For the analysis, liquid blood samples should be precentrifuged to obtain serum or plasma. When using dried blood samples in ELISA, the need for the preliminary isolation of the liquid blood fraction is eliminated. To obtain a sample in which the analyzed components (in this case, antibodies) are uniformly distributed within its entire volume, it is sufficient to place

the membrane fragment containing the sample into the elution buffer and mix them in a shaker.

CONCLUSIONS

The applied sampling and analysis format for the samples dried on fiberglass membrane supports is suitable for the preparation, storage, and transportation of biomaterial for further analysis by ELISA with the aim of diagnosing such infectious diseases as bovine IRT, VD, and EL.

The new approach has several advantages over the traditional collection of liquid blood: whole bovine blood can be applied directly to a membrane support and used for analysis without an additional step for the separation of serum and the transportation of dried samples requires no special temperature conditions. In comparison with the existing cellulose analogues, fiberglass membranes have better structural and physicochemical properties because of the solid structure of their fibers and high capillary absorption, capacity, and deformation resistance. The use of fiberglass supports in veterinary medicine for the collection of biomaterial will provide for more efficient monitoring of herds for the presence of infectious diseases and make it possible to carry out a complex of measures for their sanitation in a timely manner.

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