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Strip-dried blood sampling: applicability for bovine leukemia virus detection with ELISA and real-time PCR

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ABSTRACT

We recently proposed a new so-called strip-dried format aimed for convenient use of dried biomaterial in diagnostic purposes. In this work, 334 blood samples obtained in strip-dried form were used for bovine leucosis analysis with ELISA and real-time PCR methods. High percentage of seropositive animals (18.3%) let us estimate both indirect (serological) and direct methods applicability for the analysis of strip-dried blood samples and also to compare them (PCR results concurred with ELISA in 93.4% cases). Parallel analysis of native and corresponding strip-dried samples approved the proposed format as a reliable analytical way of sampling being in 100% concordance with conventional serum/whole blood ELISA and PCR analysis. Even distribution of antibodies against bovine leukemia virus along the membrane carrier was demonstrated by square-to-square analyzing of the sample strip (CV not exceeded 7%). Also, strip-dried blood samples showed enhanced stability at elevated temperatures comparing to liquid serum. The proposed strip-blood format is a promising way of sampling, storage and transportation and can find application in veterinary practice for infectious disease monitoring.

1. Introduction

Enzootic bovine leucosis (EBL) is an infectious persistent retroviral cattle disease caused by bovine leukemia virus (BLV). Cattle may be asymptomatic, and infected animal remain virus carrier for life (Frie and Coussens 2015). Disease is widely spread and eradication measures have been unsuccessful in many countries, for example, 83% of dairy herds contain BLV-infected cattle in the USA (Barlett et al., 2014). Similar situation is observed in Russia, where a third part of dairy cattle in 43 regions is BLV-infected. Ability of BLV to infect humans is a matter of big concern and discussion (Buehring et al., 2015). In most countries government programs against BLV infection are proceeding aimed for rigorous monitoring and eradication of EBL and making restrictions to milk and meat distribution of infected cattle. That leads to great economical loses to livestock industry and raw milk deficit at market. Indirect serological methods such as agar gel immunodiffusion test and enzyme-linked immunosorbent assay (ELISA) are routinely applied for BLV by detecting specific antibodies against virus proteins gp51 or p24 (World Organization of Animal Health (OIE, 2018). To enhance the efficiency of BLV diagnostics a direct method polymerase chain reaction (PCR) is also used (OIE 2018), which is able to detect the proviral DNA 7 days post-infection (Klintevall et al., 1994).

In recent years, Dried Blood Spots (DBS) technology of blood sampling in medical practice is actively growing (Meesters and Hooff 2013), although, in veterinary diagnostics DBS is not widely spread. The technology is based on the application of small amount of whole blood on a porous carrier followed by drying and transportation towards a specialised laboratory where dried samples are analysed. This method shows strong advantages compared to the conventional blood/serum collection and following analysis. DBS requires small blood amount and makes the blood collection minimally invasive. Advantages also include easy and space saving samples storage, transportation to a laboratory often at ambient temperature and significant reduction of costs involved. Application of DBS technology for veterinary screening is really feasible for large countries, such as Russia and the USA, where distances between remote farms and diagnostic laboratories can amount to hundreds of kilometres, but a new affordable and convenient format should be proposed to put it into practice.

Earlier, we proposed a new format of dried blood sampling based on biofluid application onto a narrow strip of fiberglass membrane (Fig. 1) (Samsonova et al., 2016). Such format is considered to be an easy way of sampling, storage and transportation of biofluid material offering some advantages against traditionally used DBS cellulose cards. Previously, principal approaches for applying of strip-dried blood samples

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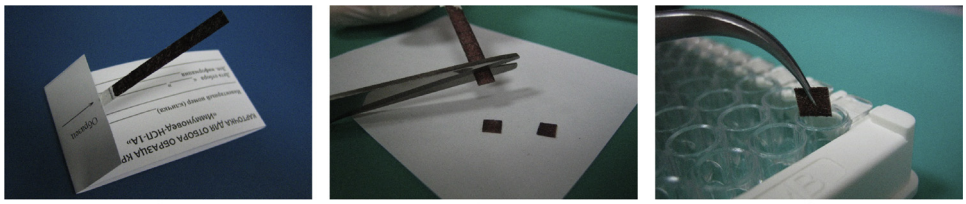

A B C

Fig. 1. General scheme of strip-dried blood sample analysis. (A) – individual strip-sampling card with dried whole blood (up to 10 square pieces can be used in analysis); (B) – cutting a square piece of membrane (0.5 x 0.5 cm) with scissors (equivalent for 15 µl of whole blood); (C) – applying a square piece of strip-dried sample in diagnostic ELISA microplate.

Table 1
Results of parallel investigations of strip-dried and native samples analysed by BLV ELISA and real-time BLV PCR.

	(S/P dried blood sample/ S/P liquid sample) *100 (%); Positive samples	(S/P dried blood sample/ S/P liquid sample)*100 (%); Negative samples	Ct value Whole blood (liquid)	Ct value Whole blood (strip-dried)
Mean	123	117	21.9	22.0
Standard deviation	9	51	5	6
CV, %	7	44	23	27

in ELISA for few bovine infection diseases were developed (Saushkin et al., 2016). In this work the applicability of a strip-dried format for on-site blood sample collection, transportation, storage and screening for BLV-infected cows with ELISA and real-time PCR was investigated. Storage and distribution issues were also addressed.

2. Material and methods

The whole blood samples (n = 334) were taken from tail vein of Holstein Friesian breed cows (Moscow region, Russia) by conventional way using vacuum blood collection tubes. Narrow marked strip (7 x 0.5 cm) of hydrophilic glassfiber material was used as a carrier for whole blood (Fig. 1). Strip-dried blood samples were obtained on-site by immediate immersing the end of membrane carriers into whole blood and desiccating at room temperature for an hour. Strip-dried samples were transported to laboratory 100 km away from farm at ambient temperature.

Strip-dried blood samples and corresponding serum (plasma)/whole blood were analyzed with ELISA and real-time PCR. One or more standard square pieces (0.5 x 0.5 cm) of the membrane with dried sample (equivalent for approximately 15 µl of liquid blood) were cut with scissors and applied for analysis. To perform strip-dried blood samples ELISA a square piece of membrane (0.5 × 0.5 cm) was put into a well of diagnostic plate of BLV diagnostic test kit (IDEXX, France). 200 µl of sample dilution buffer (from the kit) was added into each well. A plate was shaken at 250 rpm for 5 min before the first 1-hour 37^{0}C incubation. Membrane pieces were eliminated while washing a plate after the incubation. Next steps of ELISA protocol were proceeded due to the instruction. For the results interpretation the S/P ratio (%) was calculated as follows:

$$S/P = \left(\frac{ODs - NC}{PC - NC}\right) * 100\%$$

where, ODs – optical density of a sample, NC – optical density of Negative control, PC – optical density of Positive control. Samples with S/P > 60% were considered as positive.

IDEXX ELISA kit is designed to detect anti-BLV antibodies in individual serum and plasma samples or pool of sera (up to ten) from bovines. The kit is standardized to detect the European standard serum for (E5) diluted to 1:100 in negative bovine serum (https://www.idexx.com/en/livestock/livestock-tests/ruminant-tests/idexx-leukosis-serum-screening-ab-test/). For PCR analysis 3 square pieces of the strip were put into 200 µl of washing solution (0.01 M Tris-EDTA buffer) and shaken 3 times during 15 min. Following steps of extraction and purification of DNA were proceeded in common way of phenol-chloroform method both for liquid and dried samples. Amplification was performed with test kit «Leucosis» («InterLabService», Russia). Kit sensitivity was 1×10^3 GE/ml.

3. Results and Discussion

Strip-dried blood applicability for BLV monitoring by ELISA and PCR was investigated by parallel analysis of corresponding liquid samples (serum for ELISA and whole blood for real-time PCR). ELISA revealed 81.7% (273) of both dried and corresponding liquid samples as seronegative and 18.3% (61) as seropositive. No principal difference was observed for the results obtained with dried and native samples in qualitative ELISA according to applied approach. That ratio agrees with BLV prevalence ranges observed in affected dairy herds (Barlett et al., 2014). Nevertheless, some rules should be followed to reach full and repeatable elution of components, such as 5-10 min of dried sample soaking and simultaneous shaking (> 200 rpm) or interval vortexing of the samples. That also contributes to even distribution of biomaterial in solution. Optical density of positive samples was above 1.5, S/P value started from 153%; optical density of negative samples did not exceed 0.5 with S/P values obtained of 38% and less. Variation of mean ratio of S/P (%) values of liquid serum(plasma) vs strip-dried whole blood sample did not exceed 7% for positive samples (Table 1). For negative samples higher level of results variation was due to low value of optical densities observed for these samples. Real-time amplification of DNA material extracted from strip-dried whole blood samples proceeded in common with liquid samples way with the comparable intensity and threshold cycles (Table 1).

Plasma positive samples (n = 8) were sequentially diluted three-fold up to 1:1287 with negative plasma then analysed in liquid and strip-dried from. Limiting dilution of identifying a sample as positive (S/P > 60%) was observed across 1:9-1:81 range. Limiting dilution values were identical for both liquid and dried samples and the results were in full agreement with diluted whole blood strip-dried samples. S/P values for “positive” sample dilution (i.e. giving S/P > 60%) were 95 ± 9%(dried plasma)/100 ± 10%(dried blood) of those for liquid sample. So, limiting dilution (detection threshold) of liquid/strip-dried samples is an agreement with BLV ELISA kit recommendations for analysis of pooled samples (up to 10). For real-time PCR limiting dilutions of positive samples were checked in the range 100-50-15 µl of whole blood and 6-3-1 square pieces of membrane with dried whole blood with Ct values for maximum sample load from 14 to 30 (n = 10). In all cases samples were revealed as positive, however 50 µl/3 square pieces of membrane seems to be an optimal quantity for real time BLV PCR in terms of convenience and optimal sample load.

So, obtained 100% agreement between dried and liquid samples analysis in both methods makes strip-dried format a reliable alternative

Table 2
Mean, standard deviation and coefficient of variation (CV, %) of optical density for sequential square pieces (from the 1st to the 8th) of strip-dried blood samples in qualitative ELISA.

	Sample 1	Sample 2	Sample 3	Sample 4
Mean (n = 8)	1,720	1,620	1,559	1,560
Standard deviation	0,077	0,098	0,109	0,107
CV, %	4,5%	6,0%	7,0%	6,8%

for conventional blood analysis for infectious disease monitoring. Concordance between the results of ELISA and PCR in this work was 93.4% (79.6% both negative and 13.8% both positive). Real-time PCR detected proviral DNA in 75.4% of seropositive samples. This percentage is close to results obtained for herds with high BLV prevalence (Nagy et al., 2003). Seven PCR positive samples were revealed among seronegative samples. Results of comparative serological/PCR studies for the BLV detection revealed that more positive animals can be detected by PCR than by ELISA (Eaves et al., 1994, Fechner et al., 1996, Martin et al., 2001, Khudhair et al., 2016, Lee et al., 2016). It is suggested that the results can be attributed to late seroconversion after BLV infection or biased sampling in field conditions (Khudhair et al., 2016, Lee et al., 2016). It was reported earlier that naturally infected animals can exhibit periodically or permanently low titres of BLV antibodies, for instance in the pre- and postpartum period (World Organization of Animal Health (OIE, 2018). Moreover, some BLV provirus carrying animals (being PCR positive) can show no anti-BLV antibodies titres detectable for months and years (Eaves et al., 1994, Fechner et al., 1996). On the other hand, the proviral DNA wasn't recovered in 15 seropositive samples. Such result can depend on special aspects of disease progress when small amount of infected mononuclear cells circulates in peripheral blood along with insufficient PCR sensitivity. In case of BLV monitoring, PCR is considered as an additional method for BLV diagnostic of seronegative cattle, especially calves, which can be BLV-infected, but with no observable immune response to the virus (Martin et al., 2001).

Several strips of seropositive samples were square-to-square analyzed with ELISA to study distribution of antibodies against BLV along the strip. Coefficient of variation did not exceed 7% when analyzing 8 sequential square pieces of the fiberglass membrane strip with dried blood sample (Table 2). Similar experiment with a strip made of cellulose membrane, utilized for traditional DBS cards, showed significant decreasing of analytical signal detected from the 1 st to the 8th square piece (data not shown). As chromatographic effect was observed, cellulose material cannot be applied for strip-dried sampling. The thermostability of 9 seropositive strip-dried blood samples was also investigated (Ct values 15-26). Storage at 37°C for a week (the conditions are considered to be equivalent for a year storage at 4°C) led only to 5 ± 2% reducing of colorimetric signal in ELISA for BLV specific antibodies comparing to 4°C storage of respective samples. At the same time for corresponding liquid serum 17 ± 5% signal reducing was obtained. Storage of strip-dried blood at 60°C for 24 hours resulted just in 9 ± 4% reduction while liquid serum denatured in 5 hours. For PCR, no marked difference between 4°C and 37°C storage of dried samples was observed. Elevating the storage temperature to 60°C led to 10-15% increased value of threshold cycles in real-time PCR detection, while all dried samples were still defined as positive. The results demonstrated that analyzed components of strip-dried blood such as specific antibodies or BLV proviral DNA are much more stable that in liquid form, so the samples can be stored at ambient temperature for a long period. That makes strip-dried samples easy for storage and transportation eliminating costs for cold chain maintenance.

4. Conclusion

The strip-dried format of blood collection offers some advantages against traditionally used cellulose cards where only drop application is available. The format provides easy sample collection by its application onto the end of the rigid strip with ability of direct blood saturation from puncture. Application of membrane strips to animal's ear, jugular vein, or coccygeal vein allows to obtain dried blood samples without additional tubes or micropipettes reducing costs of sampling. Small blood volume sampled with membrane strip (100-150 µl) also minimizes animal injuring and the risk of infection distribution. Hydrophilic glassfiber material of membrane used contributes to equal distribution of biofluid along the strip with capillary force preventing haematocrit effect which is inherent for cellulose material. In veterinary practice qualitative analysis is primary used for infection disease monitoring, but the use of strip-dried format can promise good results also in quantitative analysis (Samsonova et al., 2016, 2017). BLV and other infectious diseases monitoring can benefit from strip-dried sampling due to low price of sampling cards and ease collection in field conditions. One strip (up to 10 square membrane pieces) can be used both in ELISA and PCR due standard instructions with slight modification excluding the stage of serum preparation. The OIE reference serum E05 was not evaluated through strip-dried form, so, presently whole blood strip-dried samples can be recommended just for screening surveys. The effectivity of BLV and other infectious diseases monitoring in remote areas can benefit from strip-dried sampling due simplified delivery to laboratory (space saving, no cold chain), low price of sampling cards and ease collection in field conditions excluding the stage of serum preparation.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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