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YINGXIA LIU

JIE CAO

JUNJIE ZHANG

KAI HUANG

CHANGMING QI

TÜBİTAK

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Influence of blood storage time on viral RNA extraction for the detection of *bovine viral diarrhea virus* in persistently infected cattle

Yingxia LIU, Jie CAO*, Junjie ZHANG, Kai HUANG, Changming QI
Department of Clinical Veterinary Medicine, College of Veterinary Medicine, China Agricultural University, Beijing 100193, P.R. CHINA

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Abstract: This study aimed to determine the maximum permissible storage times of blood and serum infected with bovine viral diarrhea virus (BVDV). Viral RNA was successfully extracted from blood and serum that were stored at room temperature (RT) for 7 days and was detected by 1-step RT-PCR. The results of this study demonstrate that BVDV-infected blood can be stored at RT for 7 days and that serum can be stored for 10 days without influencing the viral RNA extraction for the detection of BVDV.

Key words: *Bovine viral diarrhea virus*, viral RNA extraction, storage time, blood, serum

Bovine viral diarrhea (BVD), caused by *bovine viral diarrhea virus* (BVDV), has caused significant economic losses in cattle herd management. The diseases and conditions caused by BVDV include fatal mucosal disease, reproductive problems, diarrhea, and the birth of weak, congenitally defective, and persistently infected (PI) calves (1,2). In contrast to the control of acute viral infections, BVDV control focuses on the identification of PI animals, because PI cattle are capable of shedding large quantities of the virus throughout their lives and are considered the primary virus reservoirs (3). With increasing interest in the risk and economic loss from BVDV infection, control and eradication campaigns were authorized in several countries starting in the 1990s (4). Thus, it is important to develop a sensitive method for the detection of PI animals.

BVDV belongs to the genus *Pestivirus* in the family *Flaviviridae*. The viral genome is a single plus-stranded RNA of 12.5 kb in length. The identification of a virus in blood and serum is based on the isolation of viral RNA by reverse transcription-polymerase chain reaction (RT-PCR). Other viral detection methods include antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA) and virus isolation, among which Ag-ELISA and RT-PCR are the most common methods in BVDV eradication programs (2,3,5).

Farms in China are situated in remote rural areas, and it is inconvenient for them to submit samples to diagnostic laboratories in urban centers. For the detection of BVDV, blood and serum samples should be kept cool and transported to the laboratory as quickly as possible. This requirement makes sample submission problematic. Therefore, the effects of storage time on blood and serum samples at room temperature (RT) were studied by viral RNA extraction in combination with RT-PCR for the detection of BVDV.

In the Beijing region of China, 15 PI cattle, detected by ear-notch Ag-ELISA (Herdchek BVDV Antigen/Serum Plus Test Kit, IDEXX, USA), were identified by a BVDV eradication program. Blood samples treated with anticoagulant or left untreated were obtained from the jugular vein. Plasma and buffy coat cells were separated from the anticoagulant blood sample, and the serum was separated from the blood samples without anticoagulant. The BVDV in the plasma, buffy coat cells, and serum was detected by RT-PCR.

First, the serum was separated immediately following the blood collection. After dividing the serum into aliquots of 200 µL/tube, the serum was stored at RT for 6 and 12 h and for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 days. Next, the blood samples with and without anticoagulant were divided into aliquots of 1 mL/tube and were stored at RT for 6, 12, and 24 h and for 2, 3, 4, 5, 6, 7, 8, 9, and 10 days. The plasma and buffy coat cells were separated from the anticoagulant blood, and the serum was separated from the blood without anticoagulant at each time point.

When separating the plasma and buffy coat cells, the tube was centrifuged at 800 × *g* for 5 min. After transferring the plasma to a new tube, the buffy coat layer was transferred to another new tube. The red blood cells in the buffy coat layer were lysed by the addition

* Correspondence: qcm_53@126.com

of 1 mL of erythrocyte-lysing solution, and the solution was centrifuged. The pellet was washed with 1.5 mL of sterile phosphate-buffered saline (PBS) and recentrifuged. The buffy coat cells were suspended in 1 mL of TRIzol and frozen at –80 °C. Samples collected from different time points were stored at –80 °C until the viral RNA was extracted, or they were used to extract viral RNA immediately.

The extraction of the BVDV RNA was carried out directly from the plasma and serum using a commercial kit (TIANamp Virus RNA/DNA Kit, TIANGEN, China) according to the manufacturer's directions. The RNA was extracted from the buffy coat cells using a TRIzol procedure (Invitrogen, USA). The extracted RNA was dissolved in 30 µL of RNase-free H_2O.

A 288-bp DNA fragment was amplified using the 324/326 primers previously designed by Vilcek et al. (6). The PCR products obtained using a 1-step RT-PCR kit (Quant One-Step RT-PCR Kit, TIANGEN) were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination.

Virus isolation was attempted using bovine turbinate (BT) cells. Serum samples inactivated at 56 °C for 30 min were inoculated on BT cell monolayers (37 °C, 5% CO_2). The cells were cultivated for 5 to 7 days, followed by a second passage. The replicating virus in the cells was detected by 1-step RT-PCR. To confirm virus isolation, 2 PCR products obtained from the serum and the corresponding BT cultivation were sequenced.

Viral RNA was extracted successfully and amplified by RT-PCR from the serum and plasma using a viral RNA extraction kit and from buffy coat cells using a TRIzol agent. Prior to separation, the serum was demonstrated to be BVDV-positive in all 15 samples at each time point for up to 10 days (including the 10-day point, ≤10 days). BVDV RNA was detected in the plasma and serum separated from the blood up to day 7 (≤7 days), and the buffy coat cells were positive at all of the time points up to day 10 (≤10 days) (Table). Fresh or frozen blood was selected for further experiments, taking into account the storage time and temperature as well as the simple procedure for sample handling. For sample handling, the serum and plasma were used, as they were found to be more convenient than the buffy coat cells.

Two tubes (2/15) of blood began hemolyzing from day 4 and 5 tubes (5/15) developed hemolysis by day 7. Viral RNA was extracted successfully from all 5 plasma samples (5/5) and 5 serum samples (5/5) separated from the hemolyzed blood and was found to be positive by 1-step RT-PCR (Table).

Attempts to isolate live virus from the serum stored at RT and the serum separated from the blood at different time points were successful. The virus retained biological activity in the serum and blood for 7 days. There was no

Table. Results of the BVDV detection of blood samples at different time points by 1-step RT-PCR assays.

Storage time \ Total no. [d]	TP samples [a]				PS samples [b]
	Serum	Plasma	Buffy coat cells	Hemolyzed samples [c]	Serum
	15	15	15	5	15
<7 days	15	15	15	5	15
7 days	15	15	15	5	15
8 days	14	14	15	5	15
9 days	12	11	15	5	15
10 days	10	10	15	5	15
11 days	—	—	15	4	14
12 days	—	—	15	—	12
13 days	—	—	13	—	9
14 days	—	—	8	—	7
15 days	—	—	4	—	6

a Represents the samples separated from blood stored at each time point.
b Represents the samples separated as soon as the blood was collected.
c Represents the samples separated from hemolyzed blood at 7 days.
d Represents the total number of each sample at each time point.

cytopathic effect after 1 serial passage. The sequencing of 2 PCR products prepared from the original serum sample and the corresponding cell suspension yielded identical nucleotide sequences (data not shown).

The results demonstrate that the viral RNA of BVDV can be extracted from infected blood and serum stored for up to 7 days at RT and can be subsequently detected by RT-PCR. Although BVDV is a relatively labile RNA virus, viral genetic material is stable in blood, especially in the serum, outside of the body for 10 days. Blood hemolysis had no effect on the viral RNA extraction. The results of this virus isolation study indicate that the virus can survive in the blood and serum for 7 days.

Stabilization of BVDV in blood and serum is presumably related to the viral envelope. As a lipid membrane, the viral envelope protects the viral genomic material. Some enveloped viruses have been studied with respect to the viral infectivity in the plasma; for example, the infectivity of hepatitis C virus (HCV) was found to be 16 h (7). The survivability of enveloped viruses, such as HIV and HCV, has also been reported by Salvucci (8). Perhaps due to the gradual collapse of the lipid membrane, the viral RNA was stable for 7 or 10 days. Thereafter, the viral RNA could be efficiently extracted and amplified using a RT-PCR method.

A number of diagnostic methods have been described for the use of blood samples; for example, Vilcek et al. (9) studied the immobilization of BVDV-infected blood on filter paper. With both blood storage on filter paper and direct blood and serum storage at RT, the purpose is to increase the usefulness of the sample storage and transport and to make BVDV detection convenient and accurate. However, in an eradication protocol that involves a large number of samples, pooling dozens of samples together is necessary to cut work hours, which makes liquid samples easier to use in these programs. The experiment also revealed that viral RNA is stable in blood and serum for 7 to 10 days, which is sufficient time for inspectors to handle samples.

This study has documented that BVDV-infected blood can be stored at RT for 7 days and that serum can be stored for 10 days without influencing the viral RNA extraction for the detection of BVDV.

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