Viral Load Stability of an RNA virus In Stabilized Blood Samples

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Abstract

Background: The stability of viral load in a patient blood sample during storage and shipping is an important pre-analytical variable that affects the accuracy of viral pathogen quantitation. Therefore, there is a need for developing a blood collection tube with stabilizing reagents which can not only inactivate the viral agent but also stabilize the viral nucleic acids for accurate viral load determination in blood samples post blood draw. This study was undertaken to investigate the ability of Cyto-Chex® BCT (BCT) blood collection device to stabilize the nucleic acids of the Mason-Pfizer monkey virus, an RNA virus, in collected blood specimens during storage and shipping for viral load determination at a later time point.

Findings: Blood was drawn from each donor into K3EDTA and BCT tubes, spiked with Mason-Pfizer monkey virus particles and stored at room temperature. Viral RNA was extracted using RNeasy® FFPE Kit. Our results demonstrate that the viral RNA was stable in blood drawn into BCT after storage at 22°C for 7 days. Virus stored in blood drawn into K3EDTA tubes showed a decrease in viral load over time and a statistically significant decrease was observed at day 7. Shipping blood samples which took 3 days did not have adverse effect on viral load measurements for blood samples collected in BCT.

Conclusion: Our results demonstrate that BCT can stabilize viral load in blood during shipping and storage at 22°C for 7 days.

Keywords: Viral load stability; Blood collection tubes; Stability of virus in blood

Introduction

Detection of viral pathogens in peripheral blood samples is important for screening and diagnosing specific viral infections. Viral load testing or viral particle quantitation in blood is used to predict treatment response and monitor antiviral therapy [1]. Such methods typically involve virus specific RNA amplification by reverse transcriptase (RT) PCR to detect and quantify RNA viruses for diagnostic and prognostic purposes [2,3]. The clinical utility of RNA amplification based assays by RT-PCR depends on the specificity, sensitivity, reproducibility, and stability of viral RNA in blood [4]. The stability of viral RNA in blood is dependent on sample storage and shipping conditions and the type of blood collection device [5]. Poor specimen handling, sample processing, storage conditions or use of an incompatible blood collection tube may result in viral RNA degradation which may lead to false-negatives or an underestimation of viral RNA concentration.

Our previous study showed that Cyto-Chex BCT blood collection device, which stabilizes cellular components of the blood [6], can also inactivate human immunodeficiency virus (HIV) and stabilize the viral RNA of HIV in infected patient blood samples [7]. This study was undertaken to investigate if the viral load of another retrovirus, Mason-Pfizer monkey virus (M-PMV), can also be stabilized in Cyto-Chex BCT. M-PMV is the prototype for the D-type beta retroviruses which causes immune deficiencies in Asian macaques.

Materials and Methods

Recruitment of blood donors and blood collection

All human blood donors were recruited from Streck Inc., Omaha, NE. This study was approved by the Institutional Review Board of University of Nebraska Medical Center, Omaha, NE, USA. Informed consent was obtained from all donors prior to blood draw. For each experiment, blood samples were drawn into two different blood collection tubes. Control samples were drawn into K3EDTA tubes (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) and compared to samples drawn into Cyto-Chex BCT (Streck Inc., Omaha, NE). Blood was mixed immediately post draw by inverting 10 times.

Cell culture and preparation of M-PMV spiked blood samples

The CMMT cell line was obtained from American Type Culture Collection (ATCC). CMMT is a cell line derived from a co-cultivated monolayer culture of monkey mammary tumor cells and normal embryo cells [8]. CMMT cells are chronically infected with M-PMV to produce infectious virions that release viral particles into the cell culture medium. CMMT cells were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. As the CMMT cell culture reached 80-90% confluency, cell culture medium was replaced with 10% fetal calf serum. The supernatant was used as the source of M-PMV to spike blood samples. The viral concentration in the supernatant was measured by RT-PCR. Blood collected from each donor was spiked with M-PMV to obtain final virus concentrations of 4.0 × 104, 4.0 × 105 and 4.0 × 106 copies/mL. Blood was mixed immediately after addition of
M-PMV by inverting the tubes 10 times.

RNA isolation from M-PMV

M-PMV spiked blood samples were centrifuged at 300g for 5 minutes at 22°C. The plasma layer was carefully removed, without disturbing the buffy coat, and transferred to a new vial. To isolate M-PMV particles, 200 µL of plasma was treated with 287 µL of 20% sucrose in PBS and centrifuged at 14000×g for 2 hours at 4°C. The supernatant was carefully removed without disturbing the pellet. The pellet containing M-PMV particles was re-suspended with 200 µL of PBS. M-PMV RNA was extracted from blood using the QIAGEN RNeasy® FFPE Kit (Qiagen, Santa Clarita, CA). The manufacturer’s recommended protocol for purification of total RNA from FFPE tissue sections was modified as follows: Steps 1-5, 7 and 10-14 of the protocol were omitted. RNA extraction from M-PMV began at step 6 by treating 50 µL of M-PMV suspended in PBS with 240 µL of buffer PKD. Step 8 was modified by increasing the amount of Protease K from 10 µL to 20 µL. Samples were eluted in 50 µL of sterile nuclease-free water and stored at -80°C until analysis by droplet digital PCR.

Droplet Digital PCR (ddPCR)

The primers and probe were designed using the PrimerQuest online primer design tool and purchased from Integrated DNA Biomedicals, LLC, Solon, OH) and sequence analysis was carried out at the Sequencing Core Facility of the University of Nebraska Medical Center, Omaha NE. The linear dynamic range of the digital RT-PCR assay was determined by constructing a standard curve.

Effect of storage on viral RNA concentration in blood samples

This study was conducted using blood obtained from 6 donors. Blood was drawn from each donor into one 10 mL K EDTA tube and one 5 mL BCT. Both tubes were spiked with M-PMV, mixed well by inverting the tube 10 times and stored at room temperature. Aliquots of blood were taken from both tube types on days 0, 1, 3, and 7. Plasma was separated and virus was isolated by centrifugation as described above. The isolated viral pellet was stored at -80°C until viral RNA was extracted and analyzed by digital PCR.

Effect of shipping on viral RNA concentration in blood samples

For the shipping study, blood was drawn from 6 donors. From each donor, blood was drawn into two 10 mL K EDTA tubes and two 5 mL BCTs. Both tubes were spiked with M-PMV and mixed well by inverting the tube 10 times. One K EDTA tube and one BCT from each donor was shipped round trip via air freight from Lincoln, NE USA to Streck in Omaha, NE USA (elapsed time was 2 days). All blood samples were shipped in an insulated cooler box with a temperature tracking device. On day 3, plasma was separated, virus isolated and viral pellet stored at -80°C until viral RNA extraction and analysis. A control set of unshipped tubes were maintained at room temperature. Similarly, on day 0 and 3, plasma was separated from control samples; virus isolated and viral pellet stored at -80°C until viral RNA extraction. Viral RNA extraction from all samples was carried out with the RNeasy FFPE Kit. Viral RNA concentrations were measured by dd PCR as described above.

Statistical analysis

Statistical analysis was performed using paired, two tailed Student’s t-test. P<0.05 was considered statistically significant.

Results

Effect of storage on viral load

The specificity of the primers used for the M-PMV digital RT-PCR assay was verified using melt curve analysis which resulted in a single melt peak. This indicates that the reaction is specific for one RT-PCR product (M-PMV) and the absence of primer-dimers. Sequence information obtained by sequencing the 125 bp long RT-PCR product matched with the predicted sequence for the RT-PCR product (data not shown). The linear dynamic range of the digital RT-PCR assay was 18 to 120,000 copies/reaction. The efficiency of the assay was 94% and the R² of the standard curve was 0.999.

Figure 1 shows the effect of room temperature sample storage of blood on viral load detection in K EDTA and BCT tubes. This experiment was carried out with three different viral titers. Panel A, B and C illustrates the viral load results for blood spiked with 4×10⁸, 4×10⁹, 4×10⁹ viral copies/mL blood, respectively. Samples maintained in BCT tubes showed a stable viral load for up to 7 days at room temperature for all three viral titers tested (Figure 1A, 1B and 1C). However, there was a statistically significant drop in viral load by day 7 for all viral titers evaluated (Figure 1A, 1B and 1C). For the 4×10⁸ copies/mL plasma, the data showed a mean viral load of 4.4×10⁴ copies/mL plasma on day 0 with 2.1×10⁴ copies/mL result on day 7 when stored in K EDTA tubes (P<0.01) (Figure 1B). For viral load tests using the highest viral titers, the viral load for K EDTA
blood at day 0 was $5.1 \times 10^6$ copies/mL plasma. This dropped to $3.3 \times 10^6$ copies/mL on day 7 ($P < 0.0016$) (Figure 1C).

Effect of shipping on viral RNA concentration in blood samples

To test the impact of sample shipment on viral RNA concentration, blood was drawn from 6 donors into two K3EDTA tubes and two BCTs and spiked with M-PMV ($4 \times 10^5$ copies/mL blood). One K3EDTA and one BCT from each donor were shipped offsite and back to Streck over the course of three days. The unshipped control K3EDTA and BCT tubes from each donor were left at room temperature. Aliquots of control sample blood were removed on day 0 and day 3. Plasma aliquots from shipped samples were removed on day 3. The plasma was separated, virus was isolated and RNA was extracted. Virus maintained in BCT showed no statistically significant change in viral load in initial, not shipped and shipped blood samples ($5.1 \times 10^5$, $4.02 \times 10^5$ and $4.25 \times 10^5$ copies/mL plasma, respectively). Similar results were also obtained for K3EDTA tubes which showed no significant change among initial, not shipped and shipped blood samples ($4.75 \times 10^5$, $4.78 \times 10^5$ and $5.99 \times 10^5$ copies/mL plasma, respectively).

Discussion

Previously, it has been demonstrated that the BCT device is capable of stabilizing white blood cells and immunogenic cell surface markers in HIV-infected patient blood samples at room temperature for 7 days [6]. Our recent study has shown that BCT can also stabilize HIV viral load in HIV-infected patient blood at room temperature for up to 7 days [7]. The present study was undertaken to investigate the efficiency of BCT, in general, to stabilize the viral load of other retroviruses. In this study, we used M-PMV as a model for RNA retroviruses [9, 10]. M-PMV is easy to grow using the CMMT cell line and mature virus is released to cell culture medium, making it easy to concentrate the virus and spike into normal donor blood samples [8].

We used digital PCR technology to quantify M-PMV genomes in blood samples. Digital PCR has several advantages over quantitative real-time PCR. In digital PCR, it is possible to discriminate small differences with great accuracy. For nucleic acid quantification using digital PCR, a standard curve or reference is not required to get absolute copy numbers and the PCR efficiency will not affect the DNA/RNA copy number estimation [11]. We developed a digital PCR based assay.

Figure 1: Effect of storage of blood in Cyto-Chex® BCT and K3EDTA tubes on viral load stability as determined by digital RT-PCR. Blood was drawn from each donor into three BCT and three K3EDTA tubes. Three tubes from each tube type were spiked with three different viral titers. A, K3EDTA and BCT blood were spiked with $4 \times 10^4$ viral copies/mL blood. B, K3EDTA and BCT blood were spiked with $4 \times 10^5$ viral copies/mL blood. C, K3EDTA and BCT blood were spiked with $4 \times 10^6$ viral copies/mL blood. Aliquots of blood were removed at indicated days, the plasma separated and M-PMV viral load or viral concentration was determined using digital RT-PCR as described in the “Materials and Methods” section. Error bars indicate SD. n=6.
capable of amplifying a 125 bp long fragment of M-PMV genome to compare the stability of M-PMV genome in blood drawn into K3EDTA tubes and BCT during sample storage and shipping. Our results show that the M-PMV viral load in K3EDTA blood decreased when samples were stored at 22°C. The decrease was statistically significant at day 7. However, in blood collected into BCT, M-PMV viral load was stable up to 7 days at 22°C. Prior to viral RNA extraction, we separated intact viral particles from plasma by centrifugation and the viral pellet was washed with PBS. This was done to remove all free viral RNA present in plasma released from degraded viral particles. Thus, the viral RNA concentration results in our studies are representative of the intact virus particles in blood sample. Therefore, the stabilized RNA concentration in the blood sample collected in BCT indicates stabilized virus particles. Consequently, our results illustrate that the stabilizing reagent in BCT protects M-PMV and its genome from degradation. Shipping conditions had no significant effect on the quantities of M-PMV RNA in blood drawn either into BCT or K3EDTA. The slight increase in the mean viral RNA concentration seen in the K3EDTA tubes after 3 day shipping is likely due to experimental variations because of the small sample size and the complex viral RNA isolation procedure. A limitation of our study is the use of only one virus. It would be beneficial to study the stability of other clinically important RNA viruses using this stabilizing collection device. Moreover, although PCR-based assays are commonly used to measure viral loads, additional data, e.g. using an Agilent bioanalyzer, could also provide valuable information on viral RNA integrity.

In conclusion, this study provides evidence that BCT device can stabilize the viral nucleic acids of RNA viruses in a blood sample to enable accurate viral load determination even after a prolong sample storage and shipping period.

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References