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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 K_3EDTA 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 K_3EDTA 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 K_3EDTA 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 cells in culture reached 80-90% confluency, cell culture medium was collected and centrifuged at 300×g for 10 minutes at room temperature to remove cells. 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 4.0×10^4, 4.0×10^5 and 4.0×10^6 copies/mL. Blood was mixed immediately after addition of

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M-PMV by inverting the tubes 10 times.

RNA isolation from M-PMV

M-PMV spiked blood samples were centrifuged at 300×g 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×rpm 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 Proteinase 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 Technologies (Coralville, IA). The sequences for the primers and probe used to quantify M-PMV (accession no. NC-001550) were as follows: forward primer 5′-ACT GTT CCT ACA GTG CCA GCA A -3′; reverse primer 5′-TTG CCA AGT CAG CTC GTT GGT T -3′; probe 6FAM-TAT TGG ACA TGT CAG GGC CCA TTC TGG A-BHQ. RT-PCR was performed on the QX100 Droplet Digital PCR system (Bio-Rad, Hercules, CA) using the One-Step RT-ddPCR™ Kit for probes (Bio-Rad). Briefly, primer and probe final concentrations in the PCR reaction were 900 and 250 nM respectively, in a final volume of 20 µL. RT-PCR reaction mixtures were loaded into the sample wells of an eight-channel droplet generator cartridge. Droplet generation oil (70 µL) was also loaded into the oil wells and droplets were generated using Bio-Rad droplet generator. Droplets were transferred from the droplet wells to a 96-well PCR plate, heat sealed with foil and placed on the C1000 Touch thermal cycler (Bio-Rad). The following RT-PCR conditions were used: reverse transcription reaction was performed at 60°C for 30 min followed by enzyme activation at 95°C for 5 min, then 40 cycles of 30 s denaturation at 94°C and 1 min annealing/extension at 60°C. A final enzyme heat kill step was followed at 98°C for 10 min. After RT-PCR, the PCR plate was loaded on the Bio-Rad droplet reader, which automatically reads the droplets for fluorescence signal. Data analysis was done using Bio-Rad Quanta Soft analysis software.

Assay validation experiments

Verification of the specificity of primers used for the M-PMV digital RT-PCR assay was done using melt curve analysis. Melt curve analysis was carried out using primers described in section 2.7, iQ™ SYBR® Green Supermix and CFX™ Real-Time System (Bio-Rad, Hercules, CA) according to manufacturer instructions. To validate the identity of the RT-PCR product, RT-PCR reaction was carried out on the C1000 Touch™ thermal cycler (Bio-Rad, Hercules, CA) using the Titan One Tube RT-PCR kit (Roche, South San Francisco CA, USA). The PCR product was purified using the GENCLEAN® Turbo Kit (MP 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_3EDTA 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_3EDTA tubes and two 5 mL BCTs. Both tubes were spiked with M-PMV and mixed well by inverting the tube 10 times. One K_3EDTA 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 R^2 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_3EDTA 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 4×10^4, 4×10^5, 4×10^6 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) in K_3EDTA tubes. In Figure 1A the mean viral load for K_3EDTA blood at day 0 was 5.5×10^4 copies/mL plasma and it dropped to 3.78×10^4 copies/mL by day 7 ($P<0.0003$). For the 4×10^5 copies/mL plasma condition, the data showed a mean viral load of 4.4×10^5 copies/mL plasma on day 0 with a 2.1×10^5 copies/mL result on day 7 when stored in K_3EDTA tubes ($P<0.01$) (Figure 1B). For viral load tests using the highest viral titer, the viral load for K_3EDTA

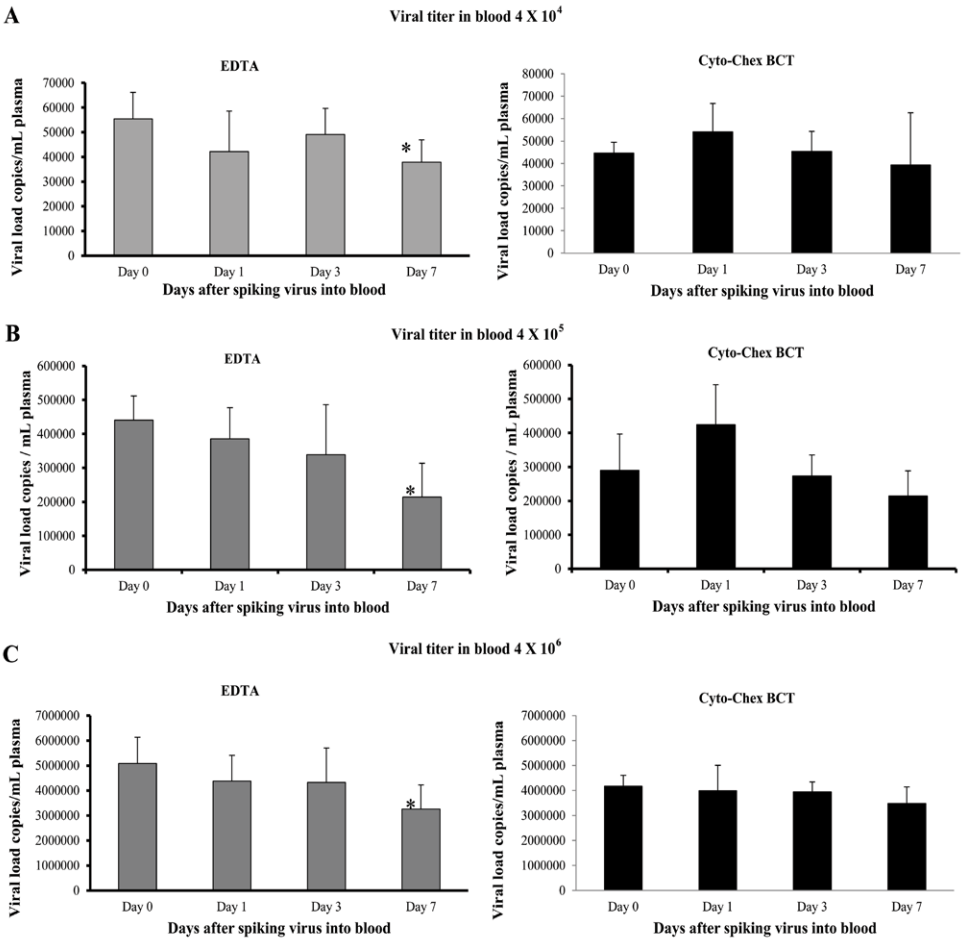

Figure 1: Effect of storage of blood in Cyto-Chex® BCT and K_3EDTA tubes on viral load stability as determined by digital RT-PCR. Blood was drawn from each donor into three BCT and three K3EDTA tubes. Three tubes from each tube type were spiked with three different viral titers. **A.** K_3EDTA and BCT blood were spiked with 4×10^4 viral copies/mL blood. **B.** K_3EDTA and BCT blood were spiked with 4×10^5 viral copies/mL blood. **C.** K_3EDTA and BCT blood were spiked with 4×10^6 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.

blood at day 0 was 5.1×10^6 copies/mL plasma. This dropped to 3.3×10^6 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 K_3EDTA tubes and two BCTs and spiked with M-PMV (4×10^5 copies/mL blood). One K_3EDTA and one BCT from each donor were shipped offsite and back to Streck over the course of three days. The unshipped control K_3EDTA 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 (5.1×10^5, 4.02×10^5 and 4.25×10^5 copies/mL plasma, respectively). Similar results were also obtained for K_3EDTA tubes which showed no significant change among initial, not shipped and shipped blood samples (4.75×10^5, 4.78×10^5 and 5.99×10^5 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

capable of amplifying a 125 bp long fragment of M-PMV genome to compare the stability of M-PMV genome in blood drawn into K_3EDTA tubes and BCT during sample storage and shipping. Our results show that the M-PMV viral load in K_3EDTA 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 K_3EDTA. The slight increase in the mean viral RNA concentration seen in the K_3EDTA 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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