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Original communication

# Evaluation of the Universal Viral Transport system for long-term storage of virus specimens for microbial forensics

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ABSTRACT

Forensic microbial specimens, including bacteria and viruses, are collected at biocrime and bioterrorism scenes. Although it is preferable that the pathogens in these samples are alive and kept in a steady state, the samples may be stored for prolonged periods before analysis. Therefore, it is important to understand the effects of storage conditions on the pathogens contained within such samples. To evaluate the capacity to preserve viable virus and the viral genome, influenza virus was added to the transport medium of the Universal Viral Transport system and stored for over 3 months at various temperatures, after which virus titrations and quantitative analysis of the influenza hemagglutinin gene were performed. Although viable viruses became undetectable 29 days after the medium was stored at room temperature, viruses in the medium stored at 4 °C were viable even after 99 days. A quantitative PCR analysis indicated that the hemagglutinin gene was maintained for 99 days at both 4 °C and room temperature. Therefore, long-term storage at 4 °C has little effect on viable virus and viral genes, so the Universal Viral Transport system can be useful for microbial forensics. This study provides important information for the handling of forensic virus specimens.



## 1. Introduction

Numerous instances where pathogens have been used as weapons in major conflicts or terrorism have been reported[1] and in the anthrax letter attacks in the United States in 2001, a dried endospore preparation of *Bacillus anthracis* was dispersed.[2–4] Many biocrimes have also been reported, including the transmission of HIV-1 to victims[5,6] and food contamination with *Salmonella* Typhimurium.[7] Forensic microbial specimens that are collected during the investigation of such biocrimes and incidents of bioterrorism include blood, tissue, body fluids, fluid-stained clothes, swabs, water, soil, and aerosols. Although the microbiological analysis of the collected specimens should be performed as soon as practicable after an incident, long-term storage of the specimens before their analysis is often necessary.[8] Even after they are analyzed, it may be necessary to store the residual or unconsumed specimens for a prolonged period in good condition as evidence or for future reanalysis. It is also likely that the frozen storage of specimens immediately after collection will not always be possible. Although it is necessary to understand the effects of the various storage conditions on viruses contained within stored specimens, the optimal long-term storage conditions for viral specimens are not well defined.

The current methods of virological analysis include viral isolation, enzyme immunoassays for antigen and antibody detection, and the direct detection of viruses by immunofluorescence and nucleic acid amplification techniques.[9] Particularly, molecular methods including virus detection by the amplification of specific genes, quantitative monitoring by real-time PCR, and virus identification by sequencing are used widely. The most accurate approaches to viral characterization require the collection and preservation of viable viruses.

Viral transport medium (VTM) generally contains a buffer to control the pH, substances to maintain an appropriate osmotic environment, proteins to stabilize the virus, and antibiotics to prevent the growth of bacterial contaminants. It also allows for the maintenance of a viable, cultivable virus. Although VTM has historically been prepared in-house, many formulations are now commercially available[10] and Universal Viral Transport (Becton, Dickinson and Company, Sparks, MD), packaged with polyester swabs for sample collection, is readily available in Japan. Although

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several studies have investigated the stability of viruses in VTM stored at 4 °C and room temperatures,[11–14] no study has examined the effects of long-term storage for a month or more on virus viability and molecular assays.

In this study, influenza virus was added to the transport medium of the Universal Viral Transport system and stored at various temperatures. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus in the transport medium was measured, and a quantitative analysis of the influenza hemagglutinin (HA) gene was performed with real-time PCR to evaluate the effects of storage on the viral genome. Furthermore, a deposit containing the virus was sampled from the surfaces of inert materials to evaluate the ability to recover the viable virus and the viral genome. This study provides important information on the handling of virus specimens for microbial forensics.

## 2. Materials and methods

### 2.1. Virus and cells

Influenza A virus (H1N1) strain A/PR/8/34 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Madin–Darby canine kidney (MDCK) cells were obtained from the Riken Cell Bank (Riken BioResource Center, Tsukuba, Japan) and maintained at 37 °C under 5% $CO_2$ in Eagle's minimum essential medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 0.1 mM nonessential amino acids (Life Technologies) as instructed.

### 2.2. Virus preparation and titration

Viral stocks were obtained by inoculating MDCK cells with the influenza A virus. Confluent cells were infected with a dilution of the virus and maintained at 35 °C in ATCC-recommended viral growth medium: Eagle's minimum essential medium supplemented with 0.125% BSA fraction V (Life Technologies), 10 mM HEPES (Life Technologies), and 1 μg/mL TPCK-treated trypsin (USB Corporation, Cleveland, OH, USA). Four days after virus inoculation, the cytopathic effect (CPE) was observed, and the culture fluid was harvested and centrifuged briefly to pellet the cellular debris. Aliquots of the supernatants were stored as stock at −80 °C until use. To determine the $TCID_{50}$ values, approximately $2 \times 10^4$ cells were plated in each well of a 96-well microtiter plate. After 24 h, the cells were infected with serial dilutions of the viral sample and maintained at 35 °C for 6–7 days in the viral growth medium. After the CPE was observed, the $TCID_{50}$ value was calculated with the Behrens–Kärber method.[15]

### 2.3. Storage conditions

The Universal Viral Transport Standard Kit (Becton, Dickinson and Company) was used to store the virus. The accompanying polyester swab was inoculated with 100 μL of stock solution containing $10^{6.4}$ $TCID_{50}$ of the virus and placed in 3 mL of the transport medium in the capture-cap test tube (final concentration: $10^{5.9}$ $TCID_{50}$/mL). The capture-cap test tube is designed to secure the shaft of the swab to the cap. The test tubes containing the virus were stored at room temperature (~23 °C) or 4 °C for 0, 1, 3, 5, 7, 14, 29, 64, or 99 days (about 3 months), or at −20 °C, −30 °C, or −80 °C for 0, 28, 90, or 182 days (about 6 months). At each time point, the two test tubes per temperature were used for independent experiments. A part of the transport medium was collected after vortexing for 15 s (after thawing if frozen). As a negative control, viral growth medium was added to the transport medium instead of the virus.

### 2.4. RNA extraction and cDNA synthesis

Viral genomic RNA was extracted from the transport medium stored at 4 °C and at room temperature using the High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol, as previously reported.[16] 200 μL of the transport medium was mixed with 400 μL of binding buffer supplemented with poly(A) carrier RNA. The RNA was eluted in 50 μL of elution buffer. As an extraction negative control, the transport medium containing the viral growth medium was used. cDNA was synthesized from 2 μL of the eluate using a random hexamer mix as the reverse transcription primer and the PrimeScript® RT Reagent Kit (Takara Bio Inc., Otsu, Japan), according to the manufacturer's protocol.

### 2.5. Quantitative real-time PCR

The cDNA fragments were used as the templates to amplify the influenza virus HA gene using SYBR® *Premix Ex Taq*™ (Takara Bio Inc.) and the LightCycler™ 1.1 system (Roche Diagnostics GmbH) with quantitative real-time PCR. HA gene fragments of 87 base pairs (bp) and 1205 bp were amplified using the HA primer set[17] and the F6-R1193 primer set,[18] respectively. In each glass capillary tube, 2 μL of 10-fold diluted cDNA was added to 23 μL of PCR mixture containing 12.5 μL of SYBR® *Premix Ex Taq*™ (2 × concentration; containing Takara Ex Taq® HS, dNTP mixture, $Mg^{2+}$, and SYBR® Green I), 1 μL of primer mixture (5 μM each primer), and 9.5 μL of $H_2O$ (PCR grade). The 87-bp fragment was amplified according to the standard shuttle PCR protocol of SYBR® *Premix Ex Taq*™. The following PCR conditions were used to amplify the 1205-bp fragment: an initial denaturation step of 30 s at 95 °C, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s. The amplification reactions and data acquisition were performed with the LightCycler™ system. The cDNA derived from the 0 day sample without storage were diluted from 5 times to 640 times. The serial dilutions of the cDNA were included as standards for each PCR run, and HA gene levels relative to the 0 day sample were estimated using a standard curve. To avoid cross-contamination, the pre-PCR sample processing was performed in a different room. EASY Dilution (Takara Bio Inc.) was used to dilute the cDNA.

### 2.6. Sampling from inert material surfaces

Stainless-steel (mirror-like and hairline finish) and polypropylene plates (each 25 $cm^2$) were used as the inert materials. Stock solution (100 μL) containing $10^{6.4}$ $TCID_{50}$ of virus, 25 spots of 4 μL each, was attached to the material surfaces in Petri dishes in a class II biological safety cabinet (n = 2 each). One hour after deposition, the wet spots on the materials were wiped up using the polyester swabs supplied with the kit. Seventeen hours after deposition, the dried spots on the materials were sampled using polyester swabs wetted with 100 μL of Hank's solution (Sigma–Aldrich). The swabs were placed in 3 mL of transport medium in the capture-cap test tubes. Using a part of the transport medium, the $TCID_{50}$ values and the HA gene levels relative to a non-deposition sample were determined as described above. In this case, the 1205-bp fragment of the HA gene was amplified. For the non-deposition sample, the polyester swab was inoculated with 100 μL of the viral solution and placed in the transport medium. After the viral solution was set in the tube for 17 h, another swab was inoculated and placed similarly. As a negative control, the viral growth medium was used instead of the viral solution.