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Recovery of Live Virus after Storage at Ambient Temperature Using ViveST™

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Abstract

Background—A major impediment to performing virological field studies in developing nations is the lack of ultra-low freezers as well as the expense and difficulty of shipping frozen samples. A commercially available product, ViveST™, was developed to preserve nucleic acids at ambient temperature for use in specimen storage and transportation. However, its applications as a viral storage, transport and recovery device have not been evaluated.

Objective—To examine the ability of ViveST to preserve live virus following storage at ambient temperature.

Study Design—A panel of six viruses was stored at ambient temperature (~22°C) in ViveST with fetal bovine serum (FBS), or ViveST with minimal essential media (MEM) and compared with virus stored in universal transport media (M4RT), MEM, and FBS alone. Stored viruses included: human adenovirus (14p), dengue virus 2 (16608), echovirus 3 (Morrissey), human rhinovirus 15 (1734), Cocksackie virus B5 (Faulkner), and herpes simplex virus 1 (HF). After 7 days storage at ambient temperature, virus recovery was measured via titration using viral plaque assays or focus-forming unit assays.

Results—Viral titer studies indicate that ViveST with either FBS or M4RT preserved/recovered 5 different viruses for 1 week at ambient temperature. MEM preserved 4 viruses while FBS and ViveST with MEM preserved 3 viruses each. Statistical analyses indicate that M4RT and ViveST with FBS preserved significantly more virus than the other treatments.

Conclusions—These data suggest that ViveST with either FBS or M4RT may be useful in field specimen collection scenarios where ultra-cold storage is not available.

Keywords

virus; preservation; recovery; storage; transport

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CONFLICT OF INTEREST

Competing interests: None declared.

Ethical approval: Not required.

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BACKGROUND

The most accurate approaches to viral characterization require the collection of viable virus which is amplified in culture. Often, field collection and preservation of circulating virus requires ultra-low freezers or dry ice. Because such cold-chain infrastructure is not available in many developing countries, viral characterization data in these countries can be sparse. Even if virus can be successfully preserved and shipped from the field site to a reference laboratory, there are often long delays in customs where the cold-chain can be lost. These transportation and preservation problems have long been recognized¹⁻³ and the use of alternative storage and transport strategies has been investigated.⁴⁻⁸ Products have been described that preserve viral genomes at ambient temperature for genetic characterization⁹⁻¹¹ however, viable virus was not obtained from these technologies. Studies using cell culture media or viral transport media showed poor recovery of viruses when stored at room temperature.^{4,5}

A commercially available device, ViveST (ViveBio, Norcross, GA) was developed to preserve nucleic acids at ambient temperature for use in specimen storage and transportation as an alternative to frozen plasma or dried blood spots.^{11,12} The ViveST device is composed of a polypropylene (PP), clear tube with a clear high density polyethylene (HDPE) screw cap. The tube contains a proprietary polyolefin fiber based synthetic matrix and a color-indicating DriCap Desiccator. The fibrous matrix is used to retain the sample content for storage, shipping, and eventual reconstitution. The matrix is made from polyolefin fibers that allow it to retain its shape when wet; however, the fibrous nature allows the material to effectively dry at ambient conditions after wetting. During the drying process, water in the sample is evaporated but biological analytes such as proteins and nucleic acids remain in the matrix. The samples can be reconstituted into a liquid state such that the dried analytes are released from the matrix and suspended in water or other elution buffer.

OBJECTIVE

Using *in vitro* viral titer studies, we sought to evaluate the ability of the ViveST device to preserve live virus at ambient temperature in comparison with other viral transport media.

STUDY DESIGN

Viruses

A panel of six viruses was used in this study in order to determine the range of viruses that could be preserved with ViveST. Viruses of various sizes were evaluated and included those with DNA or RNA genomes, with or without envelopes. This panel included Enteric Cytopathic Human Orphan virus 3 (ECHOV), Morrissey; Human rhinovirus 15 (HRV), 1734; Human Cocksackievirus B5 (CBV), Faulkner and Herpes simplex virus 1 (HSV), HF. These viruses are prototype strains of the Center for Disease Control and Prevention (CDC) that were obtained from the American Type Culture Collection (ATCC). Dengue virus (DENV) serotype 2 (16608) was kindly provided by Dr. Barry Alto at the Florida Medical Entomology Laboratory. Human adenovirus type 14p (HAdV), a prototype strain, was kindly provided by Dr. Dean Erdman of the CDC

Cells

Several cell lines were used to culture and titer the viruses used in this study. Cell lines were chosen based on scientific reports suggesting high viral titer production and ability to produce visible viral plaques and included: human lung cells (A549); African green monkey kidney cells, (Vero); Rhesus macaque kidney cells (LLC-MK2); and human cervical cells (HeLa). All cell lines were propagated in Eagle's minimum essential medium (MEM)

(Invitrogen: Calabascas, VA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen: Calabascas, VA), 1% non-essential amino acids (NEAA), 1% glutamine, 1% sodium pyruvate, and 1% penicillin/streptomycin and cultured in a 37°C incubator with 5% CO₂.

ViveST inoculation

To test the ability for the ViveST column to preserve virus from laboratory and clinical samples, treatments of virus in ViveST with MEM and virus in ViveST with FBS were performed in effort to mimic field collections of serum, urine, or saliva. There is no accepted method for storage and recovery of live virus at ambient temperature, therefore we used a variety of media used in standard virus culture and transport for control conditions. For positive controls all viruses were stored in aliquot medium MEM, FBS, and M4RTviral transport medium (Remel: Lenexa, KS). We chose M4RT media as we have used it to transport and recover live virus in other experiments M4RT media, like other viral transport media, consist of Hank's balanced salt solution, bovine serum albumin, gelatin, sucrose, glutamic acid, Gentamicin, amphotericin B, cryoprotectants and buffered with HEPES.

Inoculations of the ViveST column were performed according to manufacturer's instructions with the exception of our simulating field conditions by altering product instructions with respect to use of medium (the ViveST device does not require media for use) and drying the specimen overnight in a biosafety cabinet (we did not dry our samples overnight). All matrixes were inoculated with 100 µl virus stock in 400 µl of media, capped, and then stored in the ViveST device at ambient temperature (~22°C) in the dark for 7 days (Figure 1a and 1b). To recover the inoculum from the ViveST column, the column was placed inside a 3 ml syringe and rehydrated with 500 µl of sterile molecular grade water (Figure 1c). The column was allowed to rehydrate for 10 minutes then the inoculum was expressed into a 2.0 ml cryopreservation tube. Recovered samples were stored at -80°C until titration.

Virus titration

All virus titrations were performed using 12-well standard cell culture plates seeded with cells to reach 100% confluent upon infection. Cells were inoculated with 10-fold serial dilutions of the recovered sample and were rocked at 37°C for one hour after which the inoculum was removed and replaced with an overlay of 1 ml of 1% methyl cellulose (Sigma Catalog # M0512) mixed 1:1 with 2x MEM (20% FBS, 2% glutamine, 2% NEAA, 2% penicillin/streptomycin, 2% sodium pyruvate) and was placed in a 37°C incubator with 5% CO₂.

A549 cells were inoculated with HAdV. HeLa cells were inoculated with HRV. LLC-MK2 cells were inoculated with HSV, DENV, and ECHOV. Vero cells were inoculated with CBV. After infection, cells were allowed to incubate for 3 (DENV, CBV, HSV, HRV, and ECHOV), or 7 (HAdV) days. After the appropriate incubation time, the overlay was removed and cells were rinsed with sterile PBS. Cells infected with HSV, HAdV, CBV, ECHOV, or HRV were fixed, permeabilized and stained using a 1:1 acetone/methanol solution containing 1% wt/vol crystal violet. Plates were incubated for 30 minutes at 4°C after which the fixative was decanted. The plates were then rinsed three times in cold tap water and dried overnight at room temperature.

LLCMK-2 cells infected with DENV were fixed and permeabilized using a 1:1 acetone/methanol solution and incubated for 60 minutes at 4°C. Virus foci were detected using a specific mouse monoclonal antibody from hybridoma 2H2 (Millipore catalog #MAB8705), followed by a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Millipore catalog #AP124P), and developed using a 50mg tablet of 3,3'-diaminobenzidine

tetrahydrochloride (Sigma catalog # D5905) dissolved in 20 ml PBS with 8 μ l 30% hydrogen peroxide.

The viral plaque assays and focus-forming unit assays had a limit of detection of as few as 1 plaque forming unit per ml. Results for each virus titration are expressed as the average titer of 3 independent replicates.

Data analysis

Average viral titers were calculated for each virus based upon the standard error for the mean of 3 independent replicates. Mean viral titers from different treatment conditions (e.g. ViveST with FBS compared to M4RT) were compared using a non-paired, homoscedastic t-test. Comparisons with P values less than 0.05 were considered significant. Analyses were performed using SAS version 9.2 (SAS Institute, Inc.: Cary, NC) and Microsoft Excel 2010 (Microsoft: Redmond, WA).

RESULTS

ViveST preserves virus at ambient temperature for 7 days

Viable HAdV, CBV, ECHOV, HSV, and HRV were recovered from the ViveST with FBS treatment after 7 days storage at ambient temperature (Figure 2). Viruses showed an average reduction in infectious units (i.u.) by 2 orders of magnitude when compared to the starting titer of the respective virus (Figure 2). ECHOV exhibited the highest rate of preservation of i.u. when compared with HAdV, HSV, and HRV ($p=0.00006$). While there was a significant loss of i.u. for all viruses ($p = 0.00035$), at least 15,000 i.u./ml were recovered from any given virus which is sufficient for establishing a culture or performing downstream molecular analysis. ViveST with FBS performed equally to ViveST with MEM for preserving HAdV, ($p=0.07$) but showed less preservation with M4RT, FBS, or MEM ($p=0.04$, 0.02 , and 0.01 respectively). ViveST with FBS preserved more HSV and HRV than any other treatment ($p=0.012$ and 0.04 respectively) (Figure 2). ViveST with FBS preserved more CVB than ViveST with MEM and MEM alone ($p=0.006$ and 0.004 respectively) (Figure 2). DENV was not recovered from ViveST with FBS or ViveST with MEM (Figure 2).

Viable ECHOV, CBV, and HAdV were recovered from the ViveST with MEM treatment after 7 days storage at ambient temperature (Figure 2). A reduction in viral titer of 2 orders of magnitude was observed for HSV while a reduction of 3 orders of magnitude was observed for ECHOV and HAdV (Figure 2). This treatment exhibited the lowest recovery rate of all the treatments ($p=0.03427$) and its overall ability to preserve live virus was not statistically significant from MEM alone ($p=0.168$) (Table 1).

M4RT, FBS, and MEM preserve virus at ambient temperature for 7 days

In order to determine if ViveST performed as well or better at ambient temperatures than traditional viral storage media, ViveST was evaluated against standard media used for virus storage in cold chain scenarios (FBS, MEM, and M4RT). Three viruses were recovered from FBS (HAdV, CBV, and DENV), 4 viruses were recovered from MEM (HAdV, CBV, ECHOV, and DENV), and 5 viruses were recovered from M4RT (HAdV, HRV, CBV, ECHOV, and DENV) (Figure 2). M4RT preserved almost twice as many i.u. of HAdV than FBS and triple the i.u. of HAdV in MEM (Figure 2). Though M4RT performed significantly better than FBS or MEM in preserving HSV ($p=0.001$ for both treatments), only an average of 366 i.u. were recovered from a starting population of over 24,000,000 i.u. (0.00001%) (Figure 2). HRV was not recovered from M4RT, FBS, or MEM (Figure 2). However, M4RT preserved more CBV, ECHOV, and DENV than MEM ($p= 0.00000001$, 0.005 , and 0.00004

respectively) (Figure 2). Moreover, M4RT preserved over 5 times as much CBV as FBS ($p=0.001$) and a log more DENV than FBS ($p=0.00004$) (Figure 2).

DISCUSSION

Currently there are no standard methods for the storage and recovery of live virus at ambient temperature and investigations in this subject are sparse. Studies investigating the use of cell culture media and viral transport media for preserving virus at room temperature have documented a low recovery rate for HSV, HAdV, and other viruses tested.^{4,5} Recovery of live HIV from ViveST has been attempted, but was unsuccessful using the standard drying protocol.¹² This may have been a result of the fragile nature of the virus and the fact that it does not survive outside of a host for more than a few hours. The application of ViveST as a viral recovery device should not be based on a single pathogen as viral morphology and physiology are as diverse as the syndromes they cause.

HAdV is a medium-sized, non-enveloped virus with a nucleocapsid and a double-stranded DNA genome. It is a frequent agent of respiratory infections, gastroenteritis, and conjunctivitis.¹³ Adenoviruses are stable and are commonly resistant to adverse environmental or chemical environments which allow them to survive outside of the body.¹⁴ DENV is a small, enveloped virus with a single-stranded positive sense RNA genome and is the most common human arboviral infection.¹² DENV is an unstable virus and is easily destroyed by chemical and environmental changes.¹⁵ CBV is a small, non-enveloped virus with a single-stranded positive sense RNA genome that causes a range of syndromes from mild gastro-intestinal illness to myocarditis and pericarditis.¹⁶ CBV can remain infectious in spite of a variety of environmental and chemical exposures.¹⁶ Like CBV, HRV is a small, non-enveloped virus with a single-stranded positive sense RNA genome.¹⁴ Rhinoviruses, one of the smallest known viruses, are a frequent cause of rhinorrhea, and can retain infectivity after exposure to organic solvents.^{17,18} ECHOV is a very small, non-enveloped virus. It is a common cause of pediatric respiratory illness and aseptic meningitis.^{19,20} ECHOV is an enterovirus and can survive exposure to most disinfectants, low pH environments, and exposure to high temperatures.¹⁶ HSV is a large, enveloped virus with a double-stranded DNA genome. It is the causative agent of most cold sores.²¹ Like HRV, HSV can remain infectious after being dried on surfaces.²²

Sufficient quantities of 5 of 6 viruses tested were recovered from ViveST with FBS and from M4RT after 7 days storage at ambient temperature. With 2 logs or less in reduction of titer from the original stock virus, ViveST with FBS and M4RT at room temperature may be inexpensive alternatives for shipments of virus on ice packs, dry ice, or in liquid nitrogen. In general, non-enveloped viruses had a higher rate of recovery than enveloped viruses in our study. This may be due to the fact that envelope glycoproteins often mediate fusion with target cells and must remain intact for infection to occur. Alternatively, it may be that the storage process in this experiment may have caused adverse effects to the viral envelope. Enteric pathogens (ECHOV, CBV, HAdV) had the highest rate of recovery for all treatments which may be a function of their inherent environmental stability. The inability to recover HRV from FBS or M4RT was unexpected, and despite repeating the experiment and performing titers in alternate cell lines, viral recovery was not observed. Though it is reported that many rhinoviruses are stable at ambient temperature from hours to days¹⁸, there are many strains of HRV that are not as stable or have not been evaluated for their long-term environmental stability including the strain used in this study.

Overall, virus stored in ViveST with FBS exhibited a higher rate of recovery than virus stored in ViveST with MEM and preserved more i.u. than virus preserved in FBS alone. This could be due to the physical properties of the ViveST matrix in concert with FBS or

one or more of the constituents of FBS. Similar performance of FBS with other additives has been observed to increase the recovery of viable specimens in cryopreservation as the proteins and lipids present in FBS protect the surface of specimens from damage.^{23–25} It is reasonable the data presented here reflects a similar phenomenon.

While too few viruses were evaluated in this work to make conclusions regarding the optimal storage vehicle for a particular class of virus, it does highlight that there are at least 3 alternatives to ultra-low freezers and cold chains for the storage and transport of virus. The data show that ViveST with FBS, FBS, and M4RT can be used to store viruses at ambient temperature. It may therefore be prudent to evaluate the usefulness of M4RT, ViveST, and/or FBS for a specific pathogen before reconciling on using traditional methods for viral storage.

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References

1. Jacobson E, Ginn P, Troutman J, et al. West Nile virus infection in farmed American alligators (*Alligator mississippiensis*) in Florida. *J Wildl Dis.* 2005; 41:96–106.
2. Munster V, Bass C, Lexmond P, et al. Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. *J Clin Microbiol.* 2009;47.
3. Latorre-Margalef N, Gunnarsson G, Munster V, et al. Effects of influenza A virus infection on migrating mallard ducks. *Proc R Soc B.* 2009; 276:7.
4. Jensen C, Johnson F. Comparison of various transport media for the viability maintenance of herpes simplex virus, respiratory syncytial virus, and adenovirus. *Virology.* 1994; 19:137–142.
5. Barnard D, Johnson F, Richards D. Suitability of new chlamydia transport medium for transport of herpes simplex virus. *J Clin Microbiol.* 1985; 24:692–695. [PubMed: 3021811]
6. Runstadler J, Happ G, Selmons R, et al. Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. *Arch Virol.* 2007; 152:9.
7. Forster J, Harkin V, Graham D, McCullough S. The effect of sample type, temperature and RNAlater (TM) on the stability of avian influenza virus RNA. *J Virol Methods.* 2008; 149:5.
8. Evers D, Selmons R, Taubenberger J. Effect of preservative on recoverable RT-PCR amplicon length from influenza A virus in bird feces. *Avian Dis.* 2007; 51:3.
9. Abdelwhab E, Lüscho D, Harder T, Hafez H. The use of FTA filter papers for diagnosis of avian influenza virus. *J Virol Methods.* 2011; 174:3.
10. Gustavsson I, Lindell M, Wilander E, Strand A, Gyllensten U. Use of FTA card for dry collection, transportation and storage of cervical cell specimen to detect high-risk HPV. *J Clin Virol.* 2009; 46:4.
11. Zaroni M, Cortes R, Diaz R, et al. Comparative effectiveness of dried plasma HIV-1 viral load testing in Brazil using ViveST for sample collection. *J Clin Virol.* 2010; 49:3.
12. Lloyd R, Burns D, Huong J, et al. Dried-plasma transport using a novel matrix and collection system for human immunodeficiency virus and Hepatitis C virus virologic testing. *J Clin Microbiol.* 2009; 47:6.
13. Horwitz, M. Adenoviruses. In: Knipe, D.; Howley, P., editors. *Fields Virology*. Vol. 2. New York: Lippincott Williams & Wilkins; 2001. p. 2301–2326.

14. Shenk, T. Adenoviridae: the viruses and their replication. In: Knipe, D.; Howley, P., editors. *Fields Virology*. Vol. 2. New York: Lippincott Williams & Wilkins; 2001. p. 2265-2300.
15. Lindenbach, B.; Rice, C. Flaviviridae: The viruses and their replication. In: Knipe, D.; Howley, P., editors. *Fields Virology*. Vol. 1. New York: Lippincott Williams & Wilkins; 2001. p. 991-1042.
16. Pallansch, M.; Roos, R. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knipe, D.; Howley, P., editors. *Fields Virology*. Vol. 1. New York: Lippincott Williams & Wilkins; 2001. p. 723-776.
17. Racaniello, V. Picornoviridae: The viruses and their replication. In: Knipe, D.; Howley, P., editors. *Fields Virology*. Vol. 1. New York: Lippincott Williams & Wilkins; 2001. p. 685-722.
18. Couch, R. Rhinoviruses. In: Knipe, D.; Howley, P., editors. *Fields Virology*. Vol. 1. New York: Lippincott Williams & Wilkins; 2001. p. 799-840.
19. Viral meningitis associated with increase in echovirus type 13. Communicable Disease Surveillance Center; 2000.
20. Birenbaum E, Handsch R, Kuint J, et al. Echovirus type 22 outbreaks associated with gastrointestinal disease in a neonatal intensive care unit. *Am J Perinatol*. 1997; 14:469–473. [PubMed: 9376008]
21. Roizman, B.; Knipe, D. Herpes simplex viruses and their replication. In: Knipe, D.; Howley, P., editors. *Fields Virology*. Vol. 2. New York: Lippincott Williams & Wilkins; 2001. p. 2399-2460.
22. Turner R, Shehab Z, Osborne K, Hendley J. Shedding and Survival of Herpes Simplex Virus from 'Fever Blisters'. *Pediatrics*. 1982; 70:547. [PubMed: 6289234]
23. Beesley S, Costanzo J, Lee R. Cryopreservation of spermatozoa from freeze-tolerant and intolerant anurans. *Cryobiology*. 1998; 37:155–162. [PubMed: 9769166]
24. Harrison R, Dott H, Foster G. Effect of ionic strength, serum albumin and other macromolecules on the maintenance of mobility and the surface of mammalian spermatozoa in a simple medium. *J Reprod Fertil*. 1978; 52:65–73. [PubMed: 621699]
25. Watson P. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*. 1995; 7:871–891. [PubMed: 8711221]

Figure 1. The use of ViveST to preserve virus at ambient temperature
The process of A. inoculation, B. storage, and C. recovery of virus from the ViveST device.

Figure 2. Preservation of 6 viruses for 1 week at ambient temperature in different storage conditions

Average virus recovery of 3 independent replicates ($\pm$ SEM) of initial titer recovered after seven days at ambient temperature. Treatments include viruses stored in the ViveST device, FBS, M4RT, or MEM.

Table 1

Two-sample t-test comparing the recovery rates of the viral preservation treatments: p-value grid. Treatments were analyzed comparing differences in total infectious units recovered for all viruses

	UTM	MEM	ViveST + MEM	ViveST + FBS
FBS	0.1483	0.177163	0.032155	0.400772
UTM		0.0202	0.015042	0.314365
MEM			0.168487	0.037834
ViveST + MEM				0.007297