VECTOR-BORNE AND ZOONOTIC DISEASES
Volume XX, Number XX, 2018

DOI: 10.1089/vbz.2017.2212

ORIGINAL ARTICLE

Application of a Nonpaper Based Matrix to Preserve Chikungunya Virus Infectivity at Ambient Temperature

So Lee Park,[1,2] Yan-Jang S. Huang,[1,2] Stephen Higgs,[1,2] and Dana L. Vanlandingham[1,2]

Abstract

For over 100 years, field studies on arboviruses and the subsequent delivery and administration of live attenuated vaccines have been complicated by the need to maintain a so-called ''cold chain,'' which is the source to destination refrigeration of biological materials. In this study we describe the application of a nonpaper based matrix and demonstrate preservation of chikungunya virus infectivity at ambient temperature for 7 days. The technique was successfully employed using infectious cell culture medium and infected mosquito homogenate samples. This technique provides a simple solution for conducting studies in resource-limited areas, where the maintenance of a cold chain is technically challenging.

Keywords: chikungunya virus, cold chain, sample storage

Introduction

Mosquito-borne viruses cause millions of human infections every year, but despite over 100 years of study, we still do not fully understand natural transmission cycles for many of these viruses. Field studies and surveillance that identify the presence of pathogens before the onset of epidemics are a critical component for an early response, which can limit the spread of emerging and reemerging arboviruses, including, for example, chikungunya virus (CHIKV) which, since 2005, has emerged as a significant threat on a global scale. A major technical challenge in the diagnosis of human infections and detection of viruses in infected arthropods is the preservation of pathogens in the field, where cold chain capacity is limited. Molecular-based techniques that preserve viral nucleic acid and some immunologically-based techniques such as dipstick tests (Hinson et al. 2015, Okabayashi et al. 2015) do not require a cold chain; however, these methods fail to preserve viral infectivity that many regard as the gold standard to prove that an infectious sample is biologically relevant and also to enable virus propagation and identification. Most arboviruses of known human and veterinary public health significance are enveloped RNA viruses. These viruses are sensitive to desiccation and heat and can be quickly inactivated in the absence of a cold chain, thus precluding the isolation of infectious viruses (Johansen et al. 2002, Andrews and Turell 2016). With few antivirals or vaccines available for most mosquito-borne viruses, surveillance of suspected clinical cases and field collected mosquito vectors plays an important role in disease treatment and management. Therefore, the ability to transport samples without the need for a cold chain would reduce costs involved in complex and labor-intensive field studies.

In this study, a simple technique based on a commercially available nonpaper based matrix sample collection and transportation device, ViveST™ (ViveBio; LLC, Alpharetta, GA), was examined for use in the preservation of CHIKV at ambient temperature (Lloyd et al. 2009). The device was originally developed for preservation of the RNA genome of human immunodeficiency virus (HIV) at ambient temperature and provided consistent diagnostic results in combination with Food and Drug Administration-approved HIV diagnostic assays (Lloyd et al. 2009). Infectious CHIKV derived from tissue culture fluid and infected arthropods was successfully recovered after 7 days without any significant decline in viral titer. This method can potentially be used to increase the surveillance and diagnostic capability of CHIKV in resource-limited regions, where a cold chain is unavailable.

Materials and Methods

Cell lines and viruses

Aedes albopictus C6/36 cells and African green monkey kidney Vero76 cells were used for the propagation of virus stocks and the titration of infectious materials, respectively. Both cells were maintained in L-15 media supplemented with

[1]Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas.
[2]Biosecurity Research Institute, Kansas State University, Manhattan, Kansas.

fetal bovine serum (FBS), tryptose phosphate broth, Penicillin, Streptomycin, and L-glutamine as previously described (Huang et al. 2015).

The 2006OPY1 strain of CHIKV used in this study was generated by an additional passage of recombinant virus recovered from baby hamster kidney BHK-21 cells electroporated with infectious RNA in C6/36 cells (Tsetsarkin et al. 2006). Titration of samples was performed with median tissue culture infective dose ($TCID_{50}$) method on Vero76 cells (Higgs et al. 2006).

Infection of mosquitoes

Oral challenge of *Ae. albopictus* was performed to generate samples derived from infected arthropods and to evaluate the feasibility of sample storage at ambient temperature. *Ae. albopictus*, Generation F_2, collected from Trenton, NJ was used for the infection experiment. Seven-to-ten-day-old female mosquitoes were deprived of sugar 24 h before *per os* infection through membrane feeding of viremic blood containing 1:1 volume mixture of defibrinated sheep blood (Colorado Serum Company, Denver, CO) and stocks of CHIKV generated by the procedures described above. Engorged mosquitoes were cold-anesthetized and returned to pint-size cartons. The average titers of blood meals and engorged mosquitoes were 8.52 $\log_{10}TCID_{50}$/mL and 5.07 $\log_{10}TCID_{50}$/mL, respectively. At 7 and 14 days postinfection (dpi), eight orally challenged mosquitoes were collected, stored in 1 mL of L-15 medium supplemented with 20% of FBS, and homogenized at 26 Hz for 4 min before storage at ambient temperature or at −80°C.

Preservation of infectious viruses derived from tissue culture supernatant medium and infected arthropods at ambient temperature

To evaluate the feasibility of using the ViveST device to preserve CHIKV at ambient temperature, two sets of experiments were conducted with infectious viruses derived from tissue culture supernatant medium and infected arthropods.

A frozen stock of CHIKV was thawed and serially diluted with L-15 culture medium supplemented with 10% or 20% FBS, as the previously published study was performed with media supplemented with 20% FBS (Barr et al. 2013). Each column of the ViveST device was inoculated with 500 μL of diluted tissue culture fluid. Three replicates from each dilution were inoculated into individual tubes. The rest of the samples were immediately frozen at −80°C as reference samples to determine the amount of infectious viruses inoculated. The matrix in each column was dried inside a biosafety cabinet, capped, and stored at ambient temperature in the dark for 3 days.

To evaluate the feasibility of preserving isolates of CHIKV derived from infected arthropods, 300 μL of homogenate derived from individual *Ae. albopictus* collected at 7 and 14 dpi was inoculated into each column of the ViveST device, dried inside a biosafety cabinet, capped, and stored at ambient temperature in the dark for 3 or 7 days. The rest of the homogenates were immediately frozen at −80°C to determine the infection status of individual mosquitoes and the quantity of infectious viruses.

Recovery of preserved samples was performed by rehydrating the matrix from each column with 500 μL of molecular grade water. The rehydrated matrix was incubated at ambient temperature for 10 min, followed by sample elution by manually compressing the matrix column. The reconstituted samples were immediately titrated using Vero76 cells to determine the quantity of infectious viruses preserved at ambient temperature.

Statistical analyses

Comparison of titers of infectious viruses derived from tissue culture supernatant medium preserved under two different storage conditions was performed with the Student's *t*-test. Titers of *Ae. albopictus* homogenates stored at −80°C and at ambient temperature for 3 or 7 days were analyzed using one-way analysis of variance. All statistical analyses were performed using SigmaPlot Version 12.0 (Systat Software, Inc., San Jose, CA).

Results

Preservation of infectious viruses generated in vitro

Results from the recovery of infectious viruses suspended in L-15 medium supplemented with 10% or 20% FBS are summarized in Table 1. In the group receiving the highest concentration of infectious viruses, an average titer of 4.7 $\log_{10}TCID_{50}$/mL was recovered when an initial inoculum suspended in L-15 medium supplemented with 10% FBS at an average titer of 7.4 $\log_{10}TCID_{50}$/mL was used. Eluates with average titer of 5.7 $\log_{10}TCID_{50}$/mL were recovered from cultures inoculated using stock virus with known average titers of 7.5 $\log_{10}TCID_{50}$/mL when stored in L-15 medium supplemented with 20% FBS. When an initial input titer of 6.3 $\log_{10}TCID_{50}$/mL suspended with L-15 medium containing 10% FBS was used, the average titer recovered was 3.9 $\log_{10}TCID_{50}$/mL. A 1.6 $\log_{10}TCID_{50}$/mL reduction in titer was observed in the matrix inoculated with stocks having an average titer of 6.4 $\log_{10}TCID_{50}$/mL suspended in L-15 medium with 20% FBS. When the initial input titers of stocks suspended in L-15 medium containing 10% or 20% FBS were 4.8 $\log_{10}TCID_{50}$/mL and 5.7 $\log_{10}TCID_{50}$/mL, respectively, infectious virus with titers of 3.5 $\log_{10}TCID_{50}$/mL and 3.7 $\log_{10}TCID_{50}$/mL was observed after preservation at ambient temperature for 3 days. Although reduction of infectious titers was observed, preservation of infectious viruses at ambient temperature was achieved by inoculating cell culture supernatant medium onto the ViveST device. It is worth emphasizing that while higher titers of infectious viruses were recovered from two groups receiving the inocula suspended in L-15 medium containing 20% FBS, there was no significant difference between virus stocks suspended in L-15 medium supplemented with 20% or 10% FBS.

Recovery of infectious viruses from homogenates of infected mosquitoes preserved at ambient temperature

Based on the above results which demonstrated that the C6/36 cell line is suitable for generating and preserving CHIKV, the next objective was to analyze whether or not homogenates of orally infected *Ae. albopictus* collected at 7 and 14 dpi could be adequately preserved in the ViveST device at ambient temperature for 3 and/or 7 days.

Homogenates of orally challenged mosquitoes, incubated for either 7 or 14 dpi and then stored at −80°C, were titrated

Table 1. Recovery of Chikungunya Virus Serially Diluted with L-15 Medium Containing 10% Fetal Bovine Serum or 20% Fetal Bovine Serum. Serially Diluted Chikungunya Virus Stocks with L-15 Medium Supplemented with 10% or 20% Fetal Bovine Serum Were Preserved at −80°C or in the ViveST Device for 3 Days

	Dilution 1		*Dilution 2*		*Dilution 3*	
Concentration of FBS in L-15 media	10%	20%	10%	20%	10%	20%
Average titer of infectious viruses inoculated	7.4 ± 0.5	7.5 ± 0.0	6.3 ± 0.3	6.4 ± 0.5	4.8 ± 0.2	5.7 ± 0.2
Average titer of infectious viruses recovered	4.7 ± 0.4	5.7 ± 0.2	3.9 ± 0.6	4.8 ± 0.2	3.5 ± 0.0	3.7 ± 0.2
Loss of average infectious titer	2.7 ± 0.6	1.9 ± 0.2	2.5 ± 0.5	1.6 ± 0.8	1.3 ± 0.2	2.0 ± 0.4

Titers of infectious viruses recovered after storage were determined by titration by $TCID_{50}$ method. All titers are shown in $\log_{10}TCID_{50}$/mL.
FBS, fetal bovine serum.

revealing that 87.5% (7/8) of these mosquitoes were positive at 7 and 14 dpi. Additional samples of these homogenates were preserved in the ViveST device and incubated at ambient temperature for 3 or 7 days. Infection rates remained at 87.5%. Thus, as summarized in Table 2, the infection status of individual mosquitoes determined by titration of homogenates remained unaltered following storage at ambient temperature for 3 or 7 days.

In addition to the consistency in the infection rates derived from the titration of three different storage conditions, there was no demonstrable difference in the titers of homogenates of infected mosquitoes stored under those three different conditions. The average titer of homogenized mosquitoes collected at 7 dpi and stored at −80°C was 5.3 ± 0.6 $\log_{10}TCID_{50}$/mL, which is comparable to the infectious titers of samples stored at ambient temperature for 3 (4.7 ± 0.5 $\log_{10}TCID_{50}$/mL, $p = 0.26$) or 7 (5.0 ± 0.6 $\log_{10}TCID_{50}$/mL, $p = 0.75$) days. Although minor reductions in infectious titers were observed, similar concentrations of infectious viruses were obtained after homogenates of mosquitoes collected at 14 dpi were stored at ambient temperature for 3 (4.0 ± 0.7 $\log_{10}TCID_{50}$/mL) or 7 (4.5 ± 0.6 $\log_{10}TCID_{50}$/mL) days compared to the average titer of samples stored at −80°C (4.8 ± 0.7 $\log_{10}TCID_{50}$/mL) ($p = 0.06$).

Discussion

Our results demonstrated the feasibility of using a non-paper based matrix to preserve infectious viruses of CHIKV derived from tissue culture supernatant medium and infected arthropods. No significant reductions in infectious titers of mosquitoes were observed, and titers of ViveST samples kept at ambient temperature were comparable to those stored at −80°C. In addition to the preservation of CHIKV, the method also preserves a broad range of human viral pathogens, including HIV, hepatitis C virus, enteric cytopathic human orphan virus 3, human rhinovirus, human coxsackievirus B5, and herpes simplex virus, as demonstrated by previously published studies (Lloyd et al. 2009, Barr et al. 2013). Our and others' findings demonstrate the feasibility of the incorporation of such a technique into the existing surveillance and diagnostic methods when cold chain or cryopreservation capability is not available.

Although our report is not the first study demonstrating that the solid matrix can be used to preserve infectious virions of alphaviruses, the technique developed in this study provides a significant advancement of currently available methods. For example, the previously developed techniques based on cellulose materials such as filter paper have a significant technical constraint. To preserve infectious viruses of alphaviruses, large volumes of samples must be used as inoculum yet only a small volume can be eluted, limiting the possibility of further application in preserving samples with limited volumes in the field (Guzman et al. 2005). While small-volume inocula have also been evaluated for the preservation of CHIKV, the filter paper technique has only been demonstrated to successfully preserve purified viral RNA extract (Matheus et al. 2015).

Although we were able to successfully apply this technique to preserve infectivity of the alphavirus, CHIKV, we were unable to preserve the 17D vaccine strains of the flavivirus, yellow fever, using this technique. This may, in part, reflect

Table 2. Recovery of Infectious Viruses Derived from Infected *Ae. albopictus* After Storage of Homogenates at Room Temperature for 3 or 7 Days

		Mosquito									
Time of collection	*Storage conditions*	*#1*	*#2*	*#3*	*#4*	*#5*	*#6*	*#7*	*#8*	*Average titer*	*Infection rates*
7 dpi	−80°C	4.5	6.0	5.0	5.0	5.0	6.0	5.5	ud	5.3 ± 0.6	87.5% (7/8)
	AT for 3 days	4.0	5.0	4.5	4.5	4.5	5.3	5.5	ud	4.8 ± 0.5	87.5% (7/8)
	AT for 7 days	6.0	5.0	5.0	5.5	5.0	4.5	4.0	ud	5.0 ± 0.6	87.5% (7/8)
14 dpi	−80°C	5.5	5.0	3.5	4.0	5.0	4.0	5.0	ud	4.8 ± 0.7	87.5% (7/8)
	AT for 3 days	5.0	5.0	4.5	4.0	4.5	3.5	5.0	ud	4.5 ± 0.6	87.5% (7/8)
	AT for 7 days	4.3	4.0	4.0	4.5	4.0	2.5	4.5	ud	4.0 ± 0.7	87.5% (7/8)

Homogenates of orally challenged *Ae. albopictus* were stored at −80°C or ambient temperature for 3 or 7 days. Titers of infectious viruses recovered are shown in $\log_{10}TCID_{50}$/mL.
AT, ambient temperature; ud, samples without detectable level of infectious viruses; dpi, days postinfection.

differences in the stability of alphaviruses and flaviviruses (Fang et al. 2009, Barr et al. 2013, Huang et al. 2015). The next logical step of evaluating the feasibility of this method for field application in alphavirus diagnosis will be to determine if other alphaviruses, with well-defined thermostability characteristics, are preserved using this technique (Park et al. 2016).

Acknowledgment

The authors thank Ms. Susan M. Hettenbach for technical assistance.

Author Disclosure Statement

S.H. is the editor-in-chief of Vector-borne and Zoonotic Diseases. All other authors declare no conflicts of interest.

References

Andrews ES, Turell MJ. Effect of holding conditions on the detection of chikungunya and venezuelan equine encephalitis viruses in mosquito pools. J Am Mosq Control Assoc 2016; 32:51–54.

Barr KL, Messenger AM, Anderson BD, Friary JA, et al. Recovery of live virus after storage at ambient temperature using ViveST. J Clin Virol 2013; 56:57–61.

Fang Y, Brault AC, Reisen WK. Comparative thermostability of West Nile, St. Louis encephalitis, and western equine encephalomyelitis viruses during heat inactivation for serologic diagnostics. Am J Trop Med Hyg 2009; 80:862–863.

Guzman H, Ding X, Xiao SY, Tesh RB. Duration of infectivity and RNA of venezuelan equine encephalitis, West Nile, and yellow fever viruses dried on filter paper and maintained at room temperature. Am J Trop Med Hyg 2005; 72:474–477.

Higgs S, Vanlandingham DL, Klingler KA, McElroy KL, et al. Growth characteristics of ChimeriVax-Den vaccine viruses in Aedes aegypti and Aedes albopictus from Thailand. Am J Trop Med Hyg 2006; 75:986–993.

Hinson JM, Dave S, McMenamy SS, Dave K, et al. Immunochromatographic wicking assay for the rapid detection of chikungunya viral antigens in mosquitoes (Diptera: Culicidae). J Med Entomol 2015; 52:699–704.

Huang YJ, Hsu WW, Higgs S, Vanlandingham DL. Temperature tolerance and inactivation of chikungunya virus. Vector Borne Zoonotic Dis 2015; 15:674–677.

Johansen CA, Hall RA, van den Hurk AF, Ritchie SA, et al. Detection and stability of Japanese encephalitis virus RNA and virus viability in dead infected mosquitoes under different storage conditions. Am J Trop Med Hyg 2002; 67:656–661.

Lloyd RM, Jr., Burns DA, Huong JT, Mathis RL, 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; 47:1491–1496.

Matheus S, Huc P, Labeau B, Bremand L, et al. The use of serum spotted onto filter paper for diagnosing and monitoring Chikungunya virus infection. J Clin Virol 2015; 71:89–92.

Okabayashi T, Sasaki T, Masrinoul P, Chantawat N, et al. Detection of chikungunya virus antigen by a novel rapid immunochromatographic test. J Clin Microbiol 2015; 53: 382–388.

Park SL, Huang YJ, Hsu WW, Hettenbach SM, et al. Virus-specific thermostability and heat inactivation profiles of alphaviruses. J Virol Methods 2016; 234:152–155.

Tsetsarkin K, Higgs S, McGee CE, De Lamballerie X, et al. Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies. Vector Borne Zoonotic Dis 2006; 6:325–337.

Address correspondence to:
Dana L. Vanlandingham
Biosecurity Research Institute
Kansas State University
1041 Pat Roberts Hall
1900 Denison Avenue
Manhattan, KS 66506

E-mail: dlvanlan@vet.k-state.edu