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DURATION OF INFECTIVITY AND RNA OF VENEZUELAN EQUINE ENCEPHALITIS, WEST NILE, AND YELLOW FEVER VIRUSES DRIED ON FILTER PAPER AND MAINTAINED AT ROOM TEMPERATURE

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Abstract. Samples of laboratory propagated Venezuelan equine encephalitis (VEE), West Nile (WN), and yellow fever (YF) viruses were blotted onto filter paper discs, air-dried, and stored at room temperature. At regular intervals over a 90-day period, the dried virus samples were eluted, tested for infectivity by culture and titration in Vero cells, and examined for viral RNA by a reverse transcriptase–polymerase chain reaction. The VEE, WN, and YF viral RNA was detected throughout the 90-day period in all samples examined. Infectious VEE virus could be recovered for up to 40 days; WN and YF viruses were cultured in Vero cells for up to 60 and 90 days, respectively. The results of this study demonstrate that viral nucleic acids and infectious virus can be recovered from arbovirus samples air-dried on filter paper and stored at room temperature for a month or more after collection. This procedure offers a simple and inexpensive method for collecting arbovirus field specimens and transporting them to diagnostic laboratories.

INTRODUCTION

Several recent publications have described techniques for detecting viral nucleic acid sequences in clinical or field samples dried and stored on filter paper. Wacharapluesadee and others[1] reported that rabies virus RNA could be detected by a reverse transcriptase–polymerase chain reaction (RT-PCR) done on canine brain samples blotted on filter paper and stored for up to 222 days at room temperature. Several groups[2,3] have also reported nested PCR techniques for detecting human immunodeficiency virus type 1 (HIV-1) DNA in dried blood spots collected on filter paper in the field. The advantage of collecting such field samples on filter paper is the simplicity, low cost, and ease of transport.

Based on these reports with rabies and HIV-1 viruses, we tested the feasibility of using dried filter paper blots of arboviruses as a diagnostic procedure. The present paper reports preliminary studies comparing the sensitivity of the RT-PCR and isolation in cell culture for detecting Venezuelan equine encephalitis (VEE), West Nile (WN), and yellow fever (YF) virus samples dried on filter paper and maintained at room temperature for up to 90 days.

MATERIALS AND METHODS

Animals. Animals used in this experiment were Institute for Cancer Research (ICR) outbred mice obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice were cared for in accordance with guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC) under an animal-care protocol approved by the University of Texas Medical Branch. All animal work and other procedures with potentially live virus were carried out in biosafety level 3 (BSL-3) facilities.

Preparation of virus suspensions. Three viruses were used in the study: VEE strain TC-83, WN strain Egypt 101, and YF Jimenez strain. Each virus was initially inoculated intracerebrally into a group of 8–10 newborn mice to prepare high-titered virus suspensions. When the mice appeared sick or moribund, they were killed and the brains were removed. Each group of infected mouse brains was triturated in 5.0 mL of phosphate buffered saline (PBS), pH 7.4, containing 25% heat-inactivated (60°C for 20 minutes) fetal bovine serum (FBS), using a sterile Ten Broeck tissue homogenizer (Kontes, Vineland, NJ). After centrifugation at 3,000 × g for 10 minutes to clarify the brain homogenate, a measured 100 mL of the supernatant was added to opposite sides of 5.5-cm filter paper circles (qualitative grade 2; Micro Filtration Systems, Dublin, CA), as shown in Figure 1. The moistened filter paper was allowed to dry overnight at room temperature (22–23°C) and humidity (~70%) within a class II, type A/B3 biologic safety cabinet (Nuaire, Plymouth, MN). The following day, each filter paper circle was cut into two pieces, so that both pieces contained a dried virus spot (Figure 1). Each half circle was inserted with clean forceps into a separate, sterile, 3.9-mL screw-cap glass vial for storage. The vials were held within a BSL-3 laboratory for up to 90 days at the temperature and humidity noted.

Serial sampling for virus detection. The dried virus samples were tested by RT-PCR and by cell culture at the following time intervals: several hours after drying (day 0) and at 2, 4, 7, 14, 21, 28, 40, 60, and 90 days after storage at room temperature and humidity. Two vials with filter paper were sampled at each of these preselected times. One vial was used for the RT-PCR and was transferred to a freezer at −80°C where it was held until the end of the experiment. The sample in the second vial was used for culture. On the preselected day, 1.0 mL of PBS containing 10% FBS was added to the second vial to elute the virus sample from the filter paper. This mixture was held at 5°C for 24 hours to allow the sample to dissolve and diffuse into the PBS solution. After 24 hours, the vial with eluate was transferred to −80°C for storage until testing was done.

Virus assay in cell culture. The viability of each VEE and WN virus sample was initially tested by inoculation of 200 μL of the eluate into a 12.5-cm^2 flask of Vero cells. If the culture was positive (viral cytopathic effect and confirmation by immunofluorescence), the titer of the virus suspension was determined by plaque assay in 24-well microplate cultures of Vero cells, as previously described.[4] Serial 10-fold dilutions of each virus eluate were prepared from undiluted to 10^{-9} in PBS containing 10% FBS. Four microplate culture wells were inoculated (100 μL/well) with each dilution. Plaques were

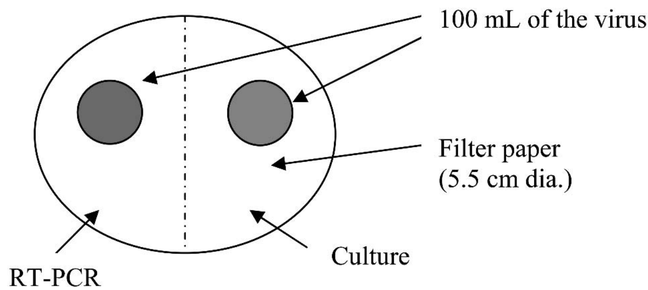

FIGURE 1. Illustration of the filter paper disc used for drying virus suspensions. After storage, the filter paper was cut in half (along the hatched line); half was used for the reverse transcriptaase–polymerase chain reaction (RT-PCR) and the other half was used for cell culture. dia. = diameter.

read 3–4 days after inoculation and the infectivity (viability) of the viruses was expressed as the number of plaque-forming units (PFU) per milliliter of eluate. Samples of the original (wet-frozen) brain homogenates were also titrated to determine the amount of virus in the original filter paper inocula.

Infectivity of the YF virus eluates was initially tested in 12.5-cm^2 cultures of the C6/36 clone of *Aedes albopictus* cells. If the initial culture was positive (determined by immunofluorescence), the virus eluate was titrated in microplate cultures of C6/36 cells, as previously described.[5] Serial 10-fold dilutions of the YF virus suspensions were made as above; virus endpoints were determined by indirect fluorescent antibody test and the detection of YF virus antigen in the mosquito cells.[5] Titers of viable YF virus were calculated as the 50% tissue culture infectious dose ($TCID_{50}$) units per milliliter of eluate.

Recovery of viral RNA for the RT-PCR. Prior to RNA extraction, the frozen filter paper samples with dried virus in the first set of vials were eluted overnight at 5°C in 1.0 mL of PBS. The following day, 100 μL of each eluate were added to sterile vials containing 900 μL of TRIzol® LS reagent (Life Technologies, Carlsbad, CA). After vigorous mixing and incubation for 5 minutes at room temperature, 200 μL of chloroform was added. The solution was mixed and incubated for another 10 minutes at room temperature, and then centrifuged at 10,000 × *g* for 15 minutes. The colorless aqueous phase was transferred to a fresh tube, mixed well with 500 μL of isopropanol, and held at –30°C for 20 minutes. The mixture was then centrifuged at 12,000 × *g* for 10 minutes. After discarding the supernatant, 1.4 mL of 75% ethanol was added, the solution was mixed thoroughly and then centrifuged at 7,500 × *g* for 10 minutes. The pellet was air-dried for 15 minutes at 37°C, 10 μL of RNase-free water was then added, and the RNA was dissolved by pipetting.

Reverse transcriptase–polymerase chain reaction. The primer sequences used for the three viruses are listed in Table 1. For each reaction, 5 μL of RNA were added to a 0.5-mL Eppendorf (Hamburg, Germany) tube, mixed with 2 μL of the primer mixture (1 μL of forward primer and 1 μL of reverse primer), denatured at 94°C for 3 minutes, and annealed at 42°C for 3 minutes. For cDNA synthesis, the following was added: 4 μL of 5× RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 μL of 10 mM dNTP, 0.5 mL of reverse transcriptase (SuperScript II™ RNase H^-Reverse Transcriptase; Invitrogen, Carlsbad, CA), 1 μL of RNase inhibitor, and 6.5 μL of water. This mixture was incubated at 42°C for 1 hour, and at 94°C for 3 minutes, to inactivate the reverse transcriptase. The PCR was carried out in a 0.5-mL Eppendorf tube containing 31 μL of distilled water, 5 μL of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1.0% Triton X-100), 2 μL of 10 mM dNTP, 3 μL of 24 mM $MgCl_2$, 2 μL each of the forward and reverse primers (10 pmole/μl), 0.3 μL of *Taq* DNA polymerase (5 units/μL) (Promega, Madison, WI), and 5 μL of the cDNA sample. The PCR temperature cycles included denaturation at 94°C for 3 minutes, and 35 PCR cycles. For YF virus, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; for WN virus, 94°C for 30 sec, 38°C for 30 sec, and 72°C for 30 sec; for VEE virus, 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min, the last cycle followed by an extension at 72°C for 5 min. Reverse transcription and PCRs were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, Roche Molecular Systems, Alameda, CA).

RESULTS

Table 2 summarizes our results and shows the duration of viral RNA, as determined by RT-PCR, and of infectious virus, as determined by culture and titration in Vero cells.

Venezuelan equine encephalitis virus. The initial titer of the virus inoculum was $10^{9.8}$ PFU/100 μL. After drying (day 0), the titer decreased to $10^{8.0}$ PFU. During the next seven days at room temperature, the virus titers decreased 100-fold. By day 28, the titer of infectious VEE virus on the filter paper had decreased to $10^{3.0}$ PFU. On day 40, infectious virus could still be detected in culture, but no plaques were observed upon titration of the eluate. This suggests that only a small amount of viable virus remained, since virus assay in cell culture is generally slightly more sensitive than the plaque assay.

The RT-PCR for VEE virus RNA remained positive for the full 90-day period (Figure 2). In summary, infectious VEE virus could be detected for up to 40 days after storage dried on filter paper at room temperature, but the RT-PCR

TABLE 1
Primers used for RT-PCR amplification of viral RNA*

Virus	Primer sequences	Sense	PCR products (bp)
YF	5′-CATGGTCGATTCATGGGAAAG-3′	Forward	162
	5′-CGCACAGCTTGTCTTGTCTC-3′	Reverse	
WN	5′-GACATCTGGTGTGGCAGCCTG-3′	Forward	170
	5′-CAGTACTGTGTCCTCAACC-3′	Reverse	
VEE	5′-AAGAGAGACGTGAAAGTGACTCCAGGAAC-3′	Forward	428
	5′-GAACATTCCAGATTTCATCATGGCTCCGAA-3′	Reverse	

* RT-PCR = reverse transcriptase–polymerase chain reaction; bp = basepairs; YF = yellow fever; WN = West Nile; VEE = Venezuelan equine encephalitis.

TABLE 2

Duration of RNA and infectivity of Venezuelan equine encephalitis (VEE), West Nile, and yellow fever viruses air-dried on filter paper and stored at room temperature, as determined by RT-PCR and cell culture, respectively*

Storage time (days)	VEE virus		West Nile virus		Yellow fever virus	
	RT-PCR	Culture (titer)	RT-PCR	Culture (titer)	RT-PCR	Culture (titer)
Original inoculum	+	P (9.8)	+	P (9.0)	+	P (8.3)
0	+	P (8.0)	+	P (6.5)	+	P (8.7)
2	+	P (7.2)	+	P (6.0)	+	P (8.3)
4	+	P (6.3)	+	P (6.3)	+	P (8.0)
7	+	P (6.0)	+	P (5.8)	+	P (7.5)
14	+	P (4.8)	+	P (5.0)	+	P (6.5)
21	+	P (3.8)	+	P (5.3)	+	P (6.0)
28	+	P (3.0)	+	P (3.7)	+	P (5.5)
40	+	P (< 1.7)	+	P (1.8)	+	P (5.0)
60	+	N	+	P (< 1.7)	+	P (< 1.7)
90	+	N	+	N	+	P (< 1.7)

* RT-PCR = reverse transcriptase–polymerase chain reaction; + = positive RT-PCR; P = positive; N = negative. Titer = $\log_{10}$ plaque-forming units or 50% tissue culture infectious dose of virus/mL of filter paper eluate, or per 100 mL of the original virus inoculum.

was still positive after 90 days of storage at the same conditions.

West Nile virus. Results with WN virus were similar. Based on titration of the original WN virus stock, the virus inoculum was estimated to contain $10^{9.0}$ PFU. Immediately after drying (day 0), the titer of infectious virus decreased to $10^{6.5}$ PFU. Thereafter, the titer of the dried virus showed little change for the next 21 days. Viable WN virus was still detected in culture after storage for 60 days at room temperature, but no plaques were observed at that time, suggesting that very small amounts of infectious virus remained.

The RT-PCR was still positive after 90 days of storage at room temperature.

Yellow fever virus. Under the conditions of this experiment, YF virus was the most stable. The amount of YF virus initially added to the filter paper was estimated to be $10^{8.3}$ $TCID_{50}$. Drying had little effect on the virus titer (viability), and after 40 days at room temperature, a significant amount of infectious virus ($10^{5.0}$) was still present. Infectious virus was recovered in culture after 90 days, although the titer of the filter paper eluate was < $10^{1.7}$ $TCID_{50}$/mL, the limit of our cell culture assay.

The RT-PCR with YF was also still positive after 90 days at room temperature (Figure 3).

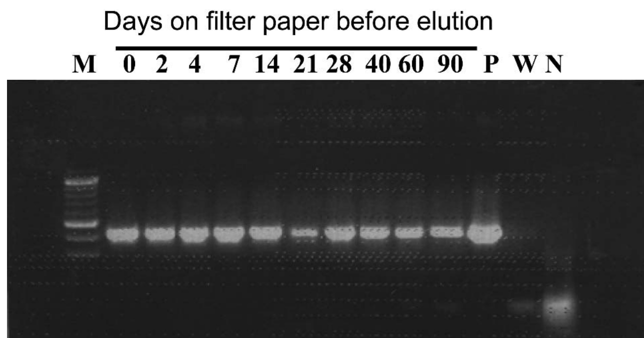

FIGURE 2. Reverse transcriptase–polymerase chain reaction results on Venezuelan equine encephalitis (VEE) virus RNA extracted from the filter paper eluates. Lane M = molecular mass marker (100-basepair ladder); lane P = positive control (VEE virus RNA); lane W = negative control (West Nile virus RNA); lane N = negative control.

DISCUSSION

The results of this study were surprising, especially the prolonged survival of infectious virus at room temperature. Previous studies[6–9] on the thermal inactivation of alphaviruses (VEE and Semliki Forest) and flaviviruses (dengue types 1-4 and WN) indicate that these RNA viruses rapidly lose their infectivity when held in liquid media at temperatures in the range of 22–37°C. For example, a recent study[8] on the survival of dengue virus serotypes 1-4 reported a rapid loss of infectivity when the viruses were held in cell-free culture medium with 10% FBS at 37°C. The calculated half-life times for the four dengue virus serotypes varied from 2.5 to 7.5 hours under these conditions. The addition of protein (FBS or bovine albumin) to the solution prolonged virus survival slightly. In the aforementioned dengue study, infectious virus could not be detected after 48 hours at 37°C, although the RT-PCR was still positive on the non-infectious virus suspensions.[8] These results concur with our own and suggest that viral RNA remains intact for some time after infectivity is lost.

Lyophilization (freeze-drying) is a common method to preserve viruses and other biologic products for long periods of time. Removal of water to form a solid generally improves storage stability.[10] Some of the alphaviruses and flaviviruses in our arbovirus reference collection have been stored lyophilized for more than 65 years at −20°C, and many are still viable. These samples were prepared on a free-drying apparatus under vacuum, and the glass ampules containing the dried virus were filled with nitrogen gas prior to sealing. However, freeze-drying is not a practical procedure for the preservation of clinical or diagnostic samples collected in the field.

The survival of VEE, WN and YF viruses, air-dried on filter paper and held at room temperature for more than one month indicates that this technique might be a feasible method to collect and preserve clinical samples (i.e., blood or impression smears of tissues) obtained during epidemiologic field investigations in remote areas. It might also be a simple and cheap method of transporting viruses. Upon receipt of the dried samples, they could be stored at −80°C for later study or processed immediately. Of the two techniques tested,

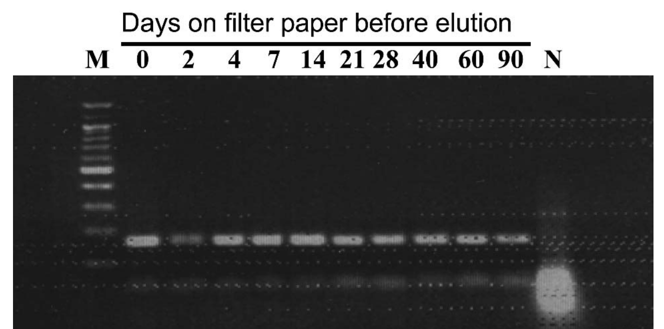

FIGURE 3. Reverse transcriptase–polymerase chain reaction results on yellow fever virus RNA extracted from the filter paper eluates. Lane M = molecular mass marker (100-basepair ladder); lane N = negative control.

the RT-PCR proved to be more sensitive and gave a positive result longer.

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