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BRIEF REPORT

Persistence in darkness of virulent alphaviruses, Ebola virus, and Lassa virus deposited on solid surfaces

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Abstract Ebola, Lassa, Venezuelan equine encephalitis, and Sindbis viruses were dried onto solid surfaces, incubated for various time periods under controlled conditions of temperature and relative humidity, and quantitatively eluted from surfaces, and viral titers in the recovered samples were determined. The viral inactivation kinetics that were obtained indicated that viral resistance to natural inactivation in the dark follows (in decreasing order of stability) alphavirus > Lassa virus > Ebola virus. The findings reported in this study on the natural decay in the dark should assist in understanding the biophysical properties of enveloped RNA viruses outside the host and in estimating the persistence of viruses in the environment during epidemics or after an accidental or intentional release.

Individual viruses classified within the families *Togaviridae, Arenaviridae,* and *Filoviridae* are associated with high fatality rates [8, 12], making viruses belonging to those families a concern for public health and national security [4, 20]. The survival of viruses outside their vectors or infected host is relevant to human health, since even viruses that are considered to be transmitted mainly by respiratory or fecal-oral routes could remain deposited on fomites before contact or re-aerosolization [3, 15]. Thus, understanding the survival of viruses in the environment is fundamental to risk assessment and to improving current measures to control natural epidemics or to counteract accidental or intentional release of viruses of medical or veterinary interest. Although the inactivation of viruses by ultraviolet radiation in sunlight has been reviewed and modeled [13], the basal decay in the dark of most viruses remains to be characterized. The goal of this study was to determine the inactivation kinetics on solid surfaces in the dark (corresponding to nighttime inactivation) of viruses of particular interest in public health and in biodefense.

The Zaire strain of Ebola virus, isolated from a patient during the 1994–1995 outbreak in the Kikwit District in the Democratic Republic of Congo (former Zaire), was kindly provided by Dr. Peter Jahrling from The United States Army Medical Research Institute for Infectious Diseases (USAMRIID, Fort Detrick, Maryland; currently at the National Institutes of Allergy and Infectious Diseases, Bethesda, Maryland). The virulent strain Trinidad donkey of Venezuelan equine encephalitis (VEE) virus, originally isolated in 1943 from the brain of an infected donkey during a VEE epizootic in Trinidad, was received from Dr. Robert Tesh (University of Texas Medical Branch in Galveston, Texas). The Josiah strain of Lassa virus, isolated in 1976 from the serum of a 40-year-old man in Sierra Leone, Africa, was provided by Dr. Tom Ksiazek (Centers for Disease Control and Prevention [CDC], Atlanta, GA; currently at the University of Texas Medical Branch, Galveston). Sindbis virus strain AR-339 (ATCC# VR-1248), as well as baby hamster kidney cells (BHK-21, ATCC # CCL-10), Vero E6 cells (ATCC # CRL-1586 and ATCC # CCL-81), and Vero-76 cells (ATCC # CRL-1587) were purchased from the American Type Culture Collection (Rockville, MD). Alphaviruses were propagated under biosafety containment level (BSL) 3 on BHK-21 cell monolayers. Ebola and Lassa viruses were grown in VERO

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cell monolayers and analyzed at the Southwest Foundation for Biomedical Research (San Antonio, Texas) under BSL-4 containment. Confluent cell monolayers were inoculated at low multiplicity of infection and incubated at 37 ± 2°C in a 5% CO_2 and 85% relative humidity (RH) atmosphere for at least 1 h to allow virus absorption. Thereafter, the inoculum was removed and cells were rinsed with sterile phosphate-buffered saline (PBS, containing 0.144 g/l KH_2PO_4, 0.795 g/l Na_2HPO_4, NaCl 9.0 g/l, pH 7.4). Fresh minimal essential medium (MEM) was added to the flask, and incubation was continued at 37 ± 2°C in a humidified, 5% CO_2 atmosphere until at least 80% cytopathic effect (CPE) was observed microscopically. The contents of the flask were then transferred to a conical tube, and the cellular debris was removed by centrifugation at 250×*g* for 10 min. The supernatant was isolated and titrated, and aliquots were stored at ≤−70°C. In addition, to increase the titers, the infected cells remaining in the flasks after harvest of Lassa or Ebola viruses were scraped with 2 mL of fresh medium, and the resuspended cells were lysed by three cycles of freeze (in a dry-ice/ethanol bath)-thawing (at 37°C). The resulting lysate was combined with the supernatant from the first flask and centrifuged at 1800×*g* at 10°C to eliminate the cellular debris (in the pellet). The supernatant with viruses was then concentrated 90-fold (determined by titration as described below) using an Amicon Ultra centrifugal filter device (Millipore, Bedford, MA), dispensed into 0.1-mL aliquots and stored at ≤−70°C until use. VEE, sindbis, and Lassa viruses were titrated by serial dilution and infection of Vero cells incubated at 37 ± 2°C in a 5% CO_2 85% RH atmosphere, typically for 24 ± 6 h (VEE virus), 44 ± 6 h (Sindbis virus), or 6 days (Lassa virus). Only plates with plaque numbers below 100 were counted to avoid overlap and underrepresentation of actual titers. The quantity of Ebola virus in concentrated cell supernatants or in experimental samples was estimated by calculating the 50% tissue culture infectious dose ($TCID_{50}$) as described by Reed and Muench [16] after confirming CPE by phase contrast microscopy (typically after 7–10 days of incubation). No wells considered for counting contained visible mold, bacterial contamination or dehydration spots. The preparations of each of the viruses had the following concentrations: Sindbis virus, 1.5×10^7 pfu/mL; VEE virus, 3×10^8 pfu/mL; Ebola virus, 1.2×10^{10} $TCID_{50}$/mL (corresponding to 6.4×10^9 pfu/mL); and Lassa virus, 4.5×10^{10} pfu/mL. Virus suspensions were diluted to comparable concentrations, and any remaining differences were further equalized by calculating the results as percentages relative to the amount of virus in the corresponding inoculum.

We wanted to determine whether the inactivation kinetics were affected by the type of surfaces onto which viruses could be deposited. Three carrier types (5 mm by 5 mm in size) made either of glass, polymeric silicone rubber, or painted aluminum alloy, produced as described previously [19], were selected for study because these materials are representative of smooth, polymeric, or metallic materials often found in urban environments and because of their particular relevance to military settings. Carriers were washed, degreased, rinsed and sterilized before storage in a sterile 50-ml conical tube at room temperature until use. Carriers deposited inside a laminar flow cabinet were contaminated with the different viruses by dispensing an aliquot of virus stock. The total amount of Sindbis, VEE, Ebola, and Lassa viruses deposited on surfaces was 2×10^5 pfu, 3×10^6 pfu, 1.9×10^7 pfu, and 1.4×10^7 pfu, respectively. Samples were allowed to air dry in the dark at ambient laboratory temperatures controlled between 20 and 25°C and 30–40% RH. These parameters were selected to represent conditions frequently found in highly populated temperate zones where the virus could be disseminated during epidemics or intentional releases rather than being typical of the tropical environments where these viruses are naturally prevalent. Immediately after drying (considered time zero), viruses were recovered independently from three carriers from each material type by a quantitative three-step method previously described for bacteria as U.S. ASTM standard E 2414-05 (ASTM 2005) [1, 17, 19]. This method quantitatively recovers microorganisms from contaminated surfaces by progressively increasing the stringency of three elution fractions (steps A, B and C). Viruses that are loosely attached to carriers are released by immersing the carriers (in triplicate in three individual 1.5-ml conical tubes) in 1 ml ice-cold MEM (Step A). Next, the carriers holding viruses bound with higher affinity are transferred to a new 1.5-ml tube containing 1 ml of ice-cold MEM, into which the viruses are released in step B by sonication for 5 min in a low-power water-bath sonicator (rated at 400–500 watts). The carriers are removed with sterile forceps and further transferred into a third tube with 1 ml MEM, where those virions still remaining on the coupons are recovered after a 30-min incubation at 37°C with agitation (at 200 cps) in step C. The viruses recovered in each fraction (A, B and C) were titrated by serial dilution, and the total number of viruses surviving at time zero (immediately after carrier contamination) or after predetermined time periods were calculated by adding the number of virus recovered in fraction A, plus viruses in fraction B, plus viruses in fraction C. The $\log_{10}$ reduction (that is, 90% viral inactivation corresponding to 1 $\log_{10}$ reduction, 99% virus inactivation to 2 $\log_{10}$, etc.) of the total viruses initially on the carriers was calculated by subtracting the total number of recovered viruses from the total number of viruses in the $T = 0$ controls and expressed as a percentage of the number of viruses in the initial inocculum deposited onto the carrier.

Calculating survival as a percentage allowed comparison of virus survival using virus stocks of different titers.

The average inactivation of the three-step method used to recover viruses at time zero from all inoculated carriers resulted in a relatively low inactivation (0.172 ± 0.089 $\log_{10}$ reduction [average $\pm$ standard deviation, $n = 12$] for viruses on the three carriers). No significant differences in recovered titers were observed by varying the virus drying time (between 10 and 60 min) on any of the three different material surfaces (glass, polymeric rubber or painted metal) and thus, 60 min was selected for drying all viruses onto surfaces during all of the subsequent experiments. The amount of alphavirus recovered from each carrier after surviving in the dark for various lengths of time after inoculation, was determined by plaque assay, with the results displayed in Fig. 1. No significant difference was observed in the survival kinetics of either VEE or Sindbis virus that remained up to 7 days on the three different material surfaces.

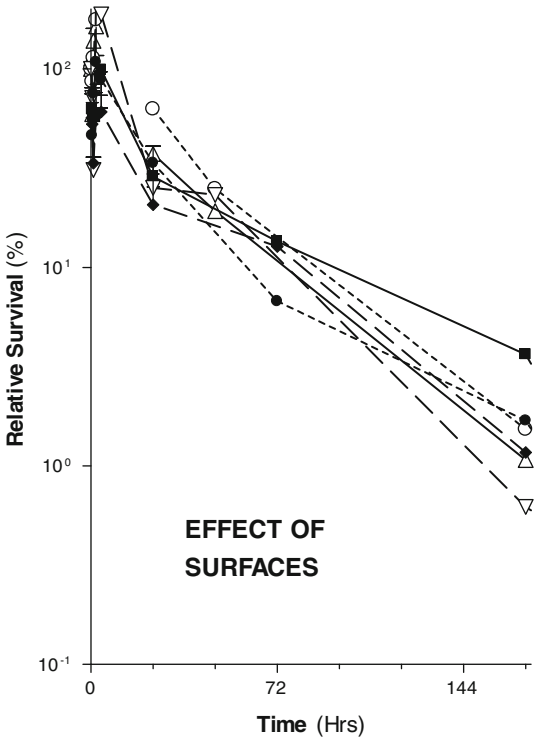

Fig. 1 Lack of surface effect on virus survival. VEE virus (*dark symbols*) or Sindbis virus (*empty symbols*) were dried for 1 h onto either glass (*squares* and *upward-pointing triangles*), rubber (*diamonds* and *downward-pointing triangles*) or metal (*circles*). The total amounts of VEE and Sindbis virus deposited on surfaces were 3×10^6 pfu and 2×10^5 pfu, respectively. After incubating in the dark at 20–25°C and 30–40% RH for the times indicated on the *x*-axis, the surviving virus was quantitatively recovered from the carriers, and the relative survival (ratio of the titer of samples recovered from glass carriers at the time indicated on the *x*-axis to the titer recovered at time zero $\times$ 100) was plotted on the *y*-axis. Each data point represents the average obtained in three independent experiments, in which average values were determined in triplicate. The slopes of the linear regression of virus inactivation ($\log_{10}$) as a function of incubation time were fit to the individual data points by the method of least squares

Lack of a consensus on the best way to express viral inactivation kinetics on environmental samples resulted previously in data being presented as distinct but related parameters (see the footnotes of Table 1). The average virus inactivation rates (IR) as $\log_{10}$ virus inactivated per day ($\log_{10}$/day), were calculated from the slope of the line that best fit (by the method of least squares) the linear curve obtained by plotting ($\log_{10}$) inactivation versus incubation time as described previously [9]. The IR values for VEE on glass, rubber, and metal carriers were 0.24, 0.28, and 0.29 $\log_{10}$/day, respectively. IR values for Sindbis virus were 0.28, 0.30, and 0.25 $\log_{10}$/day on glass, rubber and metal, respectively. The T_1 values (time needed to decrease the viral load by 90%) obtained for both alphaviruses on the three surfaces (79 h $< T_1 <$ 98 h) were within the range of T_1 values previously reported for a herpesvirus deposited on rubber (42 h) or on steel (108 h) [15]. The similar recovery of alphaviruses from three materials agrees with similar findings reported previously for *Bacillus* spores [19] and apparently disagree with a reported difference on the survival of influenza virus on copper or stainless steel [14]. This apparent disagreement can be explained by the absence in the relatively less reactive glass, rubber or painted aluminum surfaces studied here of the damaging free radicals generated by copper [reviewed in 18]. Although it cannot be ruled out that future examination of additional materials and viruses could indeed identify some surface effects, care should be taken not to assume that any apparent differences in virus decay actually result from surface effects when the variation could be related to differential retention rather

Table 1 Inactivation kinetics of dry viruses in the dark

Family	Virus[a]	IR[b] (r^2)	T_1^c (T_4)	D_{37}^d
Togaviridae	VEE	**0.24** (0.9386)	98.3 h (11.4 days)	**42.7** h
	Sindbis	**0.28** (0.9350)	84.8 h (14.6 days)	**36.9** h
Arenaviridae	Lassa	**0.41** (0.8291)	58.2 h (9.7 days)	**25.3** h
Filoviridae	Ebola	**0.68** (0.9068)	35.3 h (5.9 days)	**15.4** h

[a] Viruses were deposited and dried over glass carriers (5 $\times$ 5 mm in size) and incubated at 20–25°C and 30–40% RH inside covered dishes that prevented exposure to light

[b] The inactivation rate (IR) was calculated as the slope obtained by plotting log virus inactivation versus time and expressed as $\log_{10}$ inactivation per day. The fitting of the data to a straight inactivation line is reflected by the correlation coefficient r^2, in parentheses

[c] T_1 corresponds to the time required to decrease the viral load by 90% (1 $\log_{10}$ inactivation), and T_4 is the time required to decrease the viral load by 4 $\log_{10}$

[d] D_{37}, corresponding to the time (in hours) necessary to reduce the amount of virus to 37% of the burden at the beginning of the experiment, was calculated by dividing T_1 by the natural logarithm base 2.3 [5, 13]

than inactivation of the viruses on different surface materials.

VEE and Sindbis viruses decayed nearly twofold faster on all three of the surfaces tested ($0.24\ \log_{10}$/day $<$ IR $<$ $0.30\ \log_{10}$/day) than these viruses had been reported to decay (inactivation rate $= 0.12\ \log_{10}$/day) in liquid suspensions [9]. The inactivation rate of aerosolized VEE virus at room temperature was previously reported to be approximately 2 $\log_{10}$/day, 5 $\log_{10}$/day, or 15 $\log_{10}$/day (as can be calculated by plotting the data reported in Table 1 of [11], from the background decay without SO_2 or solar radiation in the figures of [2], or from Figs. 1 and 2 in [6]).

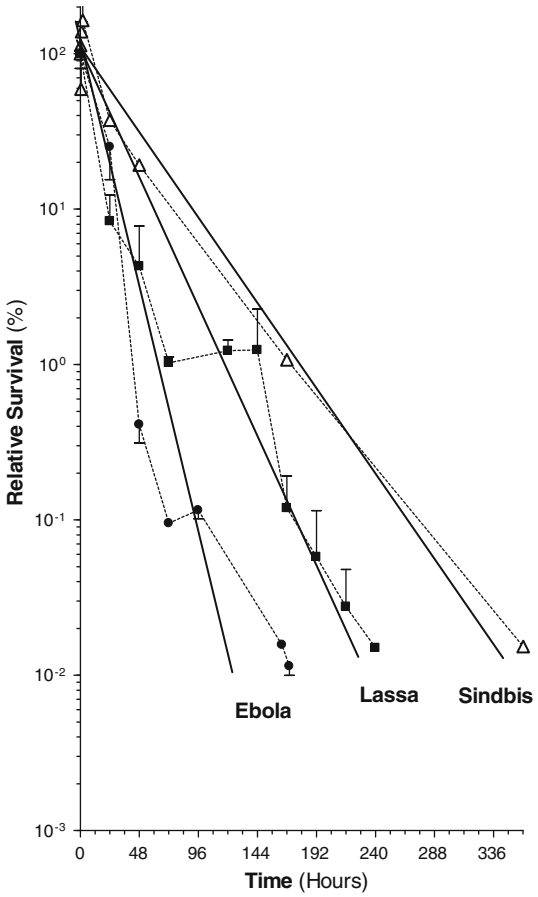

Fig. 2 Inactivation kinetics of viruses in the dark. The total amount of Ebola and Lassa viruses deposited on surfaces was 1.9×10^7 pfu and 1.4×10^7 pfu, respectively, and the amount of Sindbis virus was similar to that used in the experiments shown in Fig. 1. After drying, the viruses were incubated in the dark. At the times indicated on the *x*-axis, the viruses were recovered from the carriers, and the virus concentration was determined for at least three replicate carriers in two or more independent experiments. Survival curves were constructed by plotting relative survival versus time as in Fig. 1. Average values obtained for Lassa virus are represented by *black squares*, Ebola virus by *black circles*, and Sindbis virus by *empty diamonds*. The data points represent mean values, with the standard error indicated by the *bar* when extending beyond the data symbols. The inactivation curves ($\log_{10}$) were fitted as a function of incubation time as in Fig. 1

Thus, the inactivation rate at room temperature for VEE on surfaces ($0.24\ \log_{10}$/day $<$ IR $<$ $0.30\ \log_{10}$/day) is between 8- and 60-fold slower than previously reported for VEE in aerosols and between 2.0- and 2.5-fold faster than VEE in liquid suspensions.

Similar data obtained for both alphaviruses studied here indicate that Sindbis virus could be employed as an adequate experimental simulant or surrogate for VEE during studies on environmental persistence, as proposed previously [7].

Although an effect of the different carrier materials tested here on other viruses cannot be ruled out, the lack of a detectable difference on the survival of alphaviruses justified that experimentation with Lassa and Ebola viruses be pursued only on the glass carriers. Glass carriers contaminated with either Ebola virus or Lassa virus were kept (at 20–25°C and 30–40% RH) inside dishes covered with aluminum foil to prevent illumination until testing. At each experimental time point, three carriers were transferred using sterile forceps from the covered dishes into a sterile (1.5 ml) microcentrifuge tube. The relative amount of virus eluted from the carriers is presented in Fig. 2. The ($\log_{10}$) virus survival as a function of time fit a linear regression (as indicated by the correlation coefficients in Table 1), indicating that the samples in our study represented a homogeneous population of viruses (without sensitive or more resistant subpopulations, which would have resulted in graphical tails or shoulders [5]).

The decay rate obtained for Ebola virus (5.9 days for T_4) appeared similar to the previously reported inactivation rate of other highly virulent RNA viruses such as Hantaan and Crimean-Congo viruses (bunyaviruses), for which the time to achieve a 4-$\log_{10}$ inactivation in liquid suspension can be estimated as approximately 6.2 days (calculated from the previously reported data shown in Fig. 1 of [10]). The same bunyaviruses deposited on aluminum disks were not detectable when tested after 24 h, but reported clumping of viruses before titration and relatively low recoveries (reported to be between 6 and 14% of the input concentration [10]) may prevent further comparison with the decay of Ebola virus observed in our study.

The kinetic parameters presented in Table 1 suggest that viral resistance to environmental inactivation follows (in decreasing order of stability) alphavirus $>$ Lassa virus $>$ Ebola virus. The inactivation kinetics parameters obtained in this work indicate that relatively little overnight inactivation of alphaviruses, arenaviruses, or filoviruses should be expected when deposited on glass. These results support the notion that viruses are hardier and survive, at least in a non-porous material like the glass studied here, for longer periods than often expected. The relatively long time required to inactivate in the dark even relatively small amounts of the viruses analyzed in this study highlights the

challenge to remediate hospitals, schools, and any other civilian or military infrastructure that could be contaminated with viral agents. However, the same results also demonstrate that viruses do not persist indefinitely in the environment, even in total darkness. These results should contribute to understanding the biophysical properties of enveloped RNA viruses outside the host. It is hoped that the findings reported in this study on natural decay in the dark will assist in estimating the threat posed by virus persistence during epidemics or after an accidental or intentional release.

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