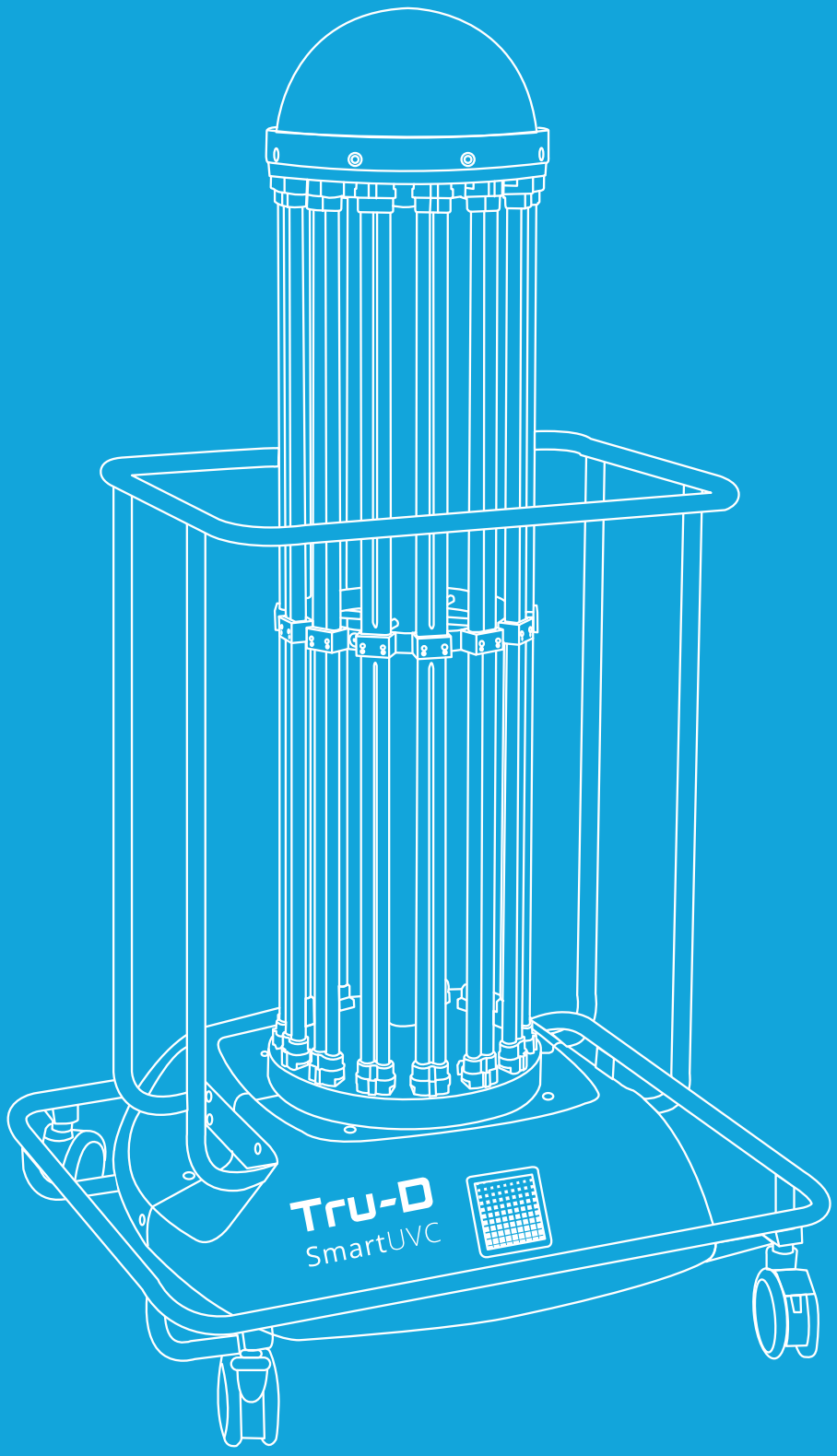

CASE STUDY

SENSITIVITY TO ULTRAVIOLET RADIATION OF LASSA, VACCINIA, AND EBOLA VIRUSES DRIED ON SURFACES

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BRIEF REPORT

Sensitivity to ultraviolet radiation of Lassa, vaccinia, and Ebola viruses dried on surfaces

Jose-Luis Sagripanti · C. David Lytle

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Abstract Germicidal UV (also known as UVC) provides a means to decontaminate infected environments as well as a measure of viral sensitivity to sunlight. The present study determined UVC inactivation slopes (and derived D_{37} values) of viruses dried onto nonporous (glass) surfaces. The data obtained indicate that the UV resistance of Lassa virus is higher than that of Ebola virus. The UV sensitivity of vaccinia virus (a surrogate for variola virus) appeared intermediate between that of the two virulent viruses studied. In addition, the three viruses dried on surfaces showed a relatively small but significant population of virions (from 3 to 10 % of virus in the inoculum) that appeared substantially more protected by their environment from the effect of UV than the majority of virions tested. The findings reported in this study should assist in estimating the threat posed by the persistence of virus in environments contaminated during epidemics or after an accidental or intentional release.

Keywords Ebola · Lassa · Vaccinia · Smallpox · Biodefense · UVC radiation · Environmental inactivation · Microbial fate · Viral persistence

Introduction

Exposure to solar ultraviolet (UV) radiation is a primary means of virus inactivation in the environment, and germicidal (UVC) light is used to inactivate viruses in hospitals and other critical public and military environments [5, 12, 18]. Safety and security constraints have hindered exposing highly virulent viruses to UV and gathering the data needed to assess the risk of environments contaminated with high-consequence (also known as biothreat) viruses [2]. UV sensitivity available for some biothreat viruses has been extrapolated from data obtained with UVC (254 nm) radiation by using a model based on the type, size and strandedness of the nucleic acid genomes of the different virus families [16]. These predictions were based on viruses suspended in liquid solutions, instead of in a dry state. Therefore, there was little or no information to allow accurate modeling, confident extrapolation, and prediction of the UV sensitivity of viruses deposited on contaminated surfaces, conditions more likely to be relevant to public health or biodefense. The goal of this study was to determine the inactivation kinetics produced by exposure to germicidal UV (UVC, 254 nm radiation) of viruses relevant to public health and biodefense that were deposited and dried onto surfaces.

We studied viruses belonging to the families *Arenaviridae, Filoviridae, and Poxviridae* because these are associated with high fatality rates and person-to-person transmission [11, 13]. Ebola virus, a member of the family *Filoviridae*, causes severe hemorrhagic fever in humans and nonhuman primates, with some outbreaks resulting in mortality rates between 80 to 90% [8, 19]. The highly virulent Zaire Ebola virus used in this study was kindly provided by Dr. Peter Jahrling (at the time at 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). Lassa virus is an arenavirus that causes Lassa fever, a widespread disease in West Africa that affects up to 2 million people per year, resulting

J.-L. Sagripanti (✉) · C. D. Lytle
Research and Technology Directorate,
Edgewood Chemical Biological Center,
U.S. Army, Aberdeen Proving Ground, MD, USA
e-mail: joseluis.sagripanti@us.army.mil

in 5,000–10,000 fatalities annually [17]. The virulent Josiah strain of Lassa virus, originally isolated from a human patient in Sierra Leone [4], was provided by Dr. Tom Ksiazek (at the time at the Centers for Disease Control and Prevention [CDC], Atlanta, Ga, currently at the University of Texas Medical Branch, Galveston, TX). The etiological agent of smallpox, variola virus, causes 20-30% mortality [10] and persists in an infectious state for many days in dried crusts from skin lesions as well as in fluid from vesicles [6]. Like variola (smallpox) virus, vaccinia virus is also a poxvirus and has been used to vaccinate against smallpox [9]. It is often used as a less virulent experimental surrogate for variola virus, due to limited availability of the latter. The WR strain of vaccinia virus (a strain adapted to tissue culture by NIH scientists) was obtained from the collection at the Southwest Foundation for Biomedical Research (SFBR, San Antonio, TX). Viruses were propagated on Vero cell monolayers (purchased from the American Type Culture Collection Rockville, MD; catalogue no. CCL-81). Experiments with Ebola virus and Lassa virus were carried out under biosafety-level-4 containment at the SFBR. Confluent cell monolayers were inoculated at low multiplicity of infection (MOI ranging between 0.001 for Lassa virus and 0.01 for Ebola virus) and incubated at 37 ± 2°C in a 5% CO_2 and 85% relative humidity (RH) atmosphere for at least one hour to allow virus absorption. The inoculum was then removed, and cells were rinsed with saline solution. Fresh medium was added to the flask, and the cells were incubated at 37 ± 2°C in a humidified, 5% CO_2 atmosphere until at least 80% cytopathic effect (CPE) was observed microscopically. The liquid contents of the flask were then transferred to a conical tube, and the cellular debris was removed by centrifugation at 250 x g for 10 minutes. To increase the titers, the infected cells remaining in the flasks after harvest of Lassa or Ebola virus were scraped with 2 mL of fresh DMEM-10. Following lysis of the cells by three rounds of freeze-thaw cycling in a dry-ice/ethanol bath and a 37 °C water bath, the resulting lysate was combined with the supernatant from the first flask, transferred to sterile centrifuge tubes, and centrifuged at 1800 x g at 10°C to pellet the cellular debris. This supernatant with viruses was then concentrated 90-fold using Amicon Ultra centrifugal filters (Millipore, Bedford, MA), dispensed into 0.1-mL aliquots and stored at ≤ –70 °C until used as working stock for the survival studies. Before being employed in the survival experiments described below, the stock preparations of each of the viruses were titrated by serial dilution and subsequent infection of cell monolayers with the following concentrations of viruses: vaccinia virus, 1 x 10^9 pfu/mL; Lassa virus, 4.5 x 10^{10} pfu/mL; Ebola virus, 1.2 x 10^{10} $TCID_{50}$/mL (corresponding to 6.4 x 10^9 pfu/mL; see below).

An aliquot (3 µl) of stock virus was deposited onto each of 24 sterile glass cover slips (5 mm x 5 mm, prepared as described previously [22]), allowing the sample to air dry for approximately 5 minutes at 25 °C and 30% relative humidity. Samples were considered dry when liquid was no longer observed on the slide. Sets of three cover slips were randomized and placed into the middle of seven inverted UV-transparent 50-mm Petri dishes (Lumox petriPERM, Greiner Bio-One, Inc., Longwood, FL, product no. 96077303) that allowed UV transmission through the gas-permeable bottom. Because of the biohazard risk associated with these viruses, the virus samples were contained inside closed and sealed UV-transparent Petri dishes while they were being kept in the dark until UV exposure and during irradiation. Radiation exposure was performed by placing a covered Petri dish containing three virus samples on a platform below the UV bulb and removing the cover for selected times up to 30 seconds. The source of UV radiation was a 15-watt G15T8 low-pressure mercury vapor (germicidal) lamp that emitted over 90% of its radiation at 254 nm (UVC), as confirmed with a RPS900 spectroradiometer. A UVX-25 digital radiometer was calibrated with an International Light Technologies ILT400 radiometer for the 254 nm wavelength. The exposures were performed with the samples 12 inches directly below the germicidal lamp, where the UV flux was approximately 4 W/m^2. The UV opaque cover was replaced after the UV exposure was completed. The Petri dishes were used upside down (because the dish bottoms are UV transparent, while the tops are UV opaque) allowing 88% (ratio of the UVC flux measured with the petri dish bottom directly on top of the radiometer sensor to the flux with nothing between the UV lamp and the sensor) of the 254 nm UV radiation emitted by the lamps to be transmitted through the UV-transparent dishes to the virus samples. The Petri dishes with the irradiated samples were kept in the dark until all exposures were performed. Radiation flux measurements were made before each exposure at the exact location where the samples were irradiated. The UV flux varied by 1.5% or less over the area occupied by the Petri dish, less so for the irradiated samples located near the center of the Petri dishes. After all UV exposures were completed, the cover slips containing the dried, UV-irradiated virus samples were individually placed in sterile microcentrifuge tubes containing 200 µl of phosphate-buffered saline. Cover slips were soaked for 5 minutes and then agitated by moderate vortexing for 10 seconds. The amount of Lassa or vaccinia virus eluted and recovered from each carrier after various irradiation doses was determined by serial dilution and subsequent infection of Vero cells in 25-mm well plates. Wells with uninfected control cells formed confluent monolayers, while plaques appeared in wells containing infected monolayers.

Only wells with plaque numbers below 100 were counted to avoid plaque overlap, which would result in underrepresentation of actual titers. No wells considered for counting contained visible mold, bacterial contamination or dehydration spots. The quantity of Ebola virus in samples eluted from exposed glass cover slips was estimated by calculating the 50% tissue culture infectious dose ($TCID_{50}$) in 96-well tissue culture plate seeded with Vero cells, as described by Reed and Muench [21].

The virus samples were irradiated with UVC between 0 and 30 seconds, with the longest time that the viruses might be on the cover slips in the experiments being less than one hour after drying. The inactivation of unirradiated virus on cover slips kept in the dark for 1 hour after drying ranged from less than 5% for vaccinia virus to an estimated inactivation of 1.2% of the initial inoculum, and similar results were obtained for Lassa and Ebola viruses (data not shown). Thus, the recovery after drying and eluting the viruses from glass slides was higher than 95 %. Survival curves were constructed by plotting the percentage surviving (ratio of the titer of an irradiated sample to the titer of the unirradiated control x 100) versus UV fluence. The slopes of the resulting survival curves obtained from the linear portions of the graphs were used to calculate D_{37} values. The D_{37} value equals the reciprocal of the slope on the semi-logarithmic graph and corresponds to the UV fluence that produced, on average, one lethal hit to the virus, resulting in a survival of 37%. D_{37} can be calculated by dividing the fluence that results in a 1-Log_{10} reduction in virus load (as obtained from the linear portion of the graphs) by 2.3 (the natural logarithmic base). A lower value for the D_{37} indicates a higher sensitivity to inactivation by UV radiation.

The inactivation by UVC of vaccinia virus was measured in four independent experiments, and the data obtained are shown in Figure 1A. The initial slope of the biphasic survival curve indicated a D_{37} of 6.0 J/m^2 for vaccinia virus dried onto surfaces. The final slope had a D_{37} of 25 J/m^2 (Table 1). Extrapolation of the final slope to zero fluence occurred at 4.2% surviving virus, indicating that 4-5% of the virus particles contributed that segment of the survival curve. The sensitivity of 95-96 % of the virions of vaccinia virus on glass surfaces (6.0 J/m^2 from the initial slope) was similar to that reported in suspension after infecting mouse L cells (D_{37} of 6.2 J/m^2 in ref. 3) or CV-1 cells (D_{37} of 7.7 J/m^2 in [1]), a line of African green monkey kidney cells similar to the Vero cells used in these experiments.

The biphasic survival curves obtained here in each experiment were unexpected because previously published curves describing the effect of 254-nm UV radiation on vaccinia virus in liquid suspensions were linear [3, 14, 15]. However, our results showing a biphasic decay after UVC irradiation of vaccinia virus dried onto surfaces correlates with the environmental behavior of variola major (smallpox) virus, whose infectivity in dried scabs or in fluid from vesicles also decreased in a biphasic fashion – first fairly rapidly and then remaining detectable at low titers for many months [6].

The effect of UVC on virulent Lassa virus was studied, and the resulting survival curve is presented in Figure 1B, where the initial slope corresponded to a D_{37} of 16 J/m^2 (Table 1). A final slope corresponding to a D_{37} of 88 J/m^2 could be estimated for inactivation of Lassa virus. Extrapolation of the final slope to zero fluence occurred between 9 and 10% surviving virus, indicating that 9-10% of the virus particles dried onto surfaces contributed to the final segment of the survival curve. Thus, the data presented indicate that most (90%) of the dried Lassa virus had a UVC sensitivity (D_{37} = 16 J/m^2) remarkably close to the value (13 J/m^2) previously predicted by virus inactivation modeling [16].

The UVC survival data obtained for Ebola virus were also biphasic (Figure 1C), similar to those obtained for vaccinia virus. The first data point that was obtained for Ebola virus survival corresponded to a nearly 2-Log_{10} inactivation, making the determination of the initial slope less precise than for the other viruses in this study. The D_{37} of the initial slope, however, was no greater than 4 J/m^2. This D_{37} value for dried Ebola virus was lower than previously predicted by modeling (7.4 J/m^2), indicating that Ebola virus may be more sensitive to UVC radiation than estimated based on the UV-sensitivities in liquid medium of viruses whose genome consists of a single-stranded RNA molecule [16]. The higher sensitivity to UVC observed here for Ebola virus compared to Lassa virus (Table 1) correlates with the relative sensitivities to gamma irradiation reported previously for both viruses [7]. The final slope indicated a D_{37} of 17 J/m^2, and extrapolation to zero fluence indicated that about 3 to 4 % of the Ebola virus population contributed to the resistant portion of the survival curve (Table 1).

The most likely explanation for the biphasic nature of the survival curves found in the experiments reported here should relate to the environmental conditions surrounding the dried virus particles, i.e., the presence of significant amounts of dried protein from serum and cellular debris from the growth medium. In addition to the intrinsic sensitivities determined by the initial inactivation slopes, all three dried viruses studied on surfaces showed a relatively small but significant population of virions dried in growth medium (from 3 to 10 % of virus in the inoculum) to be more protected from UV radiation than the majority of virions tested. Comparison between the D_{37} derived from the final resistant slope and the D_{37} from the initial slope indicated that the protected virus population had between

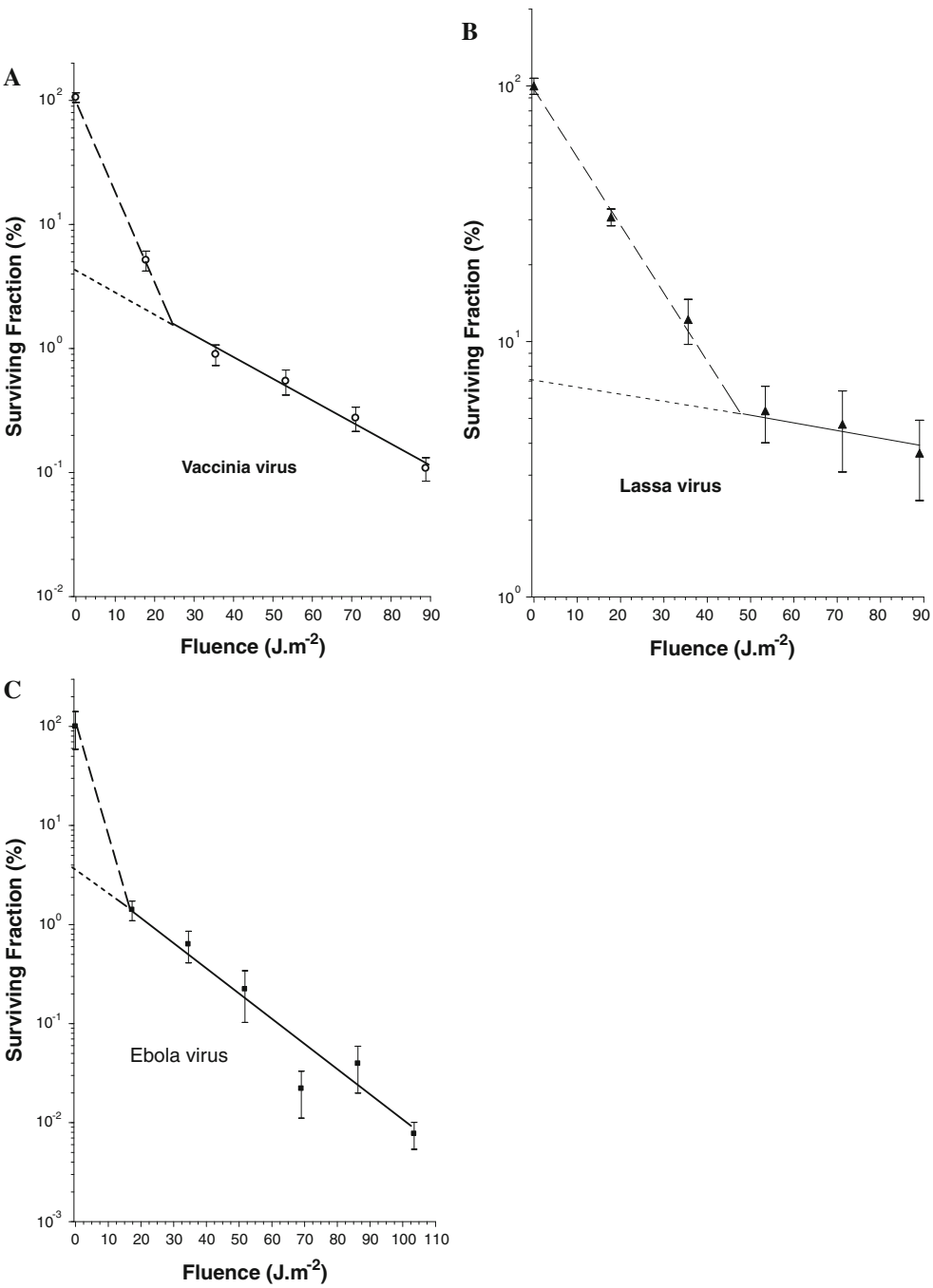

Fig. 1 Survival of viruses dried onto glass carriers following exposure to 254-nm radiation for various periods of time, providing the respective fluences indicated on the x-axis. The curve in panel A corresponds to the inactivation of vaccinia virus (3 X 10^6 pfu/carrier), with the averages of six to twelve data points determined by plaque formation represented by circles and the standard error of the mean (SEM) shown as brackets above and below the mean. The average survival of virulent Lassa virus (1.3 x 10^8 pfu/carrier) in panel B is represented by triangles, with SEM corresponding to six data points each The survival of virulent Ebola virus (1.9 x 10^7 pfu/carrier) is represented by squares, corresponding to the average of six data points determined by TCID50. Trace and solid lines represent the initial and final slopes of the curve, respectively. The protected virus population responsible for the final inactivation slope was obtained by extrapolation (dotted line) at fluence = 0

four- and sixfold lower UVC sensitivity than the general virus population. Thus, drying the virus in growth medium containing 10% fetal calf serum and some cellular debris resulted in a protection rate of several-fold, but only for only 3-10 % of the total virus particles irradiated. Virions that were less shielded (lying on or near the top of the protein layer, etc.) would contribute to the initial portion of the inactivation curve, while virus particles shielded from the UV radiation by other virions, proteins, and additional components of the medium, would correspond to the resistant (final) portion of the survival curve. Although the initial (intrinsic) sensitivity of unshielded viruses in various environments should remain relatively constant, the resistance of the viral population corresponding to the final portion of the curve could vary with the amount of chemical or cellular components able to shield virus. This could be particularly important on the survival of a fraction of the virus in blood dried on surfaces, where the effect of UV radiation remains to be fully characterized.

The initial UVC inactivation slopes determined for the viruses dried onto surfaces obtained in this study (Table 1) were similar to the values obtained previously using

Table 1 Sensitivity of biothreat viruses to UVC (254 nm) radiation

Virus	Predicted[1] or reported[2] Liquid suspension (D_{37}, J/m^2)	Measured Dried on surfaces		%[5]
		Initial (J/m^2)[3]	Final (J/m^2)[4]	
Vaccinia virus (VACV)	7.7[2]	**6.0**	25	4-5
Lassa virus (LASV)	13[1]	**16**	88	9-10
Ebola virus (EBOV)	7.4[1]	**<4.0**	17	3-4

1. Predicted previously by inactivation modeling [16]

2. D_{37} determined in CV-1 African green monkey kidney cells [1]

3. Values expressed with two significant figures as obtained from the initial slope between 0 and 20 J/m^2

4. Obtained from the final, linear slope at higher exposure levels

5. Percent of UV-protected irradiated virus particles, obtained by extrapolating the resistant component of the survival curve back to 0 fluence

vaccinia virus or predicted by modeling of arenaviruses and filoviruses in liquid suspension ref. 16, thus suggesting that the sensitivity to UV of viruses in liquids and on surfaces is comparable. These results indicate that predictions of the UV sensitivity of untested viruses (as estimated in [16]) can be relied upon for risk assessment of viruses in liquid suspensions or for the bulk of viruses dried on surfaces. However, a correcting safety factor of four to six should be applied to estimate the UVC fluence needed to inactivate the relatively small but apparently highly protected population of viruses identified for samples dried onto surfaces. Although the protected virus population corresponds to a relatively small fraction (10% or less) of the total viral population, this protected fraction of virus dried on fomites and environmental surfaces could be high in absolute numbers, posing a serious risk to human health within hospital infectious units or in the environment contaminated after a natural or intentional viral release. The results of this study should assist in predicting the length of time viruses remain a viable threat after natural broadcast from infected patients during epidemics or after accidental or intentional release into the environment.

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