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SUPPLEMENT ARTICLE

Comparison of the Aerosol Stability of 2 Strains of *Zaire ebolavirus* From the 1976 and 2013 Outbreaks

Robert J. Fischer,[1] Trenton Bushmaker,[1] Seth Judson,[2] and Vincent J. Munster[1]

[1]Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Montana; and [2]David Geffen School of Medicine at UCLA

The largest outbreak of Ebola virus disease began in Guéckédou, Guinea, West Africa, in December 2013 and rapidly spread to major population centers in 3 West African countries. Early reports in some scientific and public media speculated that the virus had evolved to more effectively transmit between humans. One route of transmission postulated was aerosol transmission, although there was little epidemiological evidence to support this claim. This study investigates the viability of 2 *Zaire ebolavirus* strains within aerosols at 22°C and 80% relative humidity over time. The results presented here indicate that there is no difference in virus stability between the 2 strains and that viable virus can be recovered from an aerosol 180 minutes after it is generated.

Keywords. Ebola virus; West Africa; aerosol; stability; Goldberg drum.

The largest outbreak of Ebola virus disease to date quietly began in December 2013 with the first cases in Guéckédou, Guinea, West Africa [1]. By August 2014 the disease had become established in large population centers in Guinea, Sierra Leone, and Liberia. The rapid spread of *Zaire ebolavirus* (EBOV) Makona strain over such a wide geographic range led to questions of increased virulence and or human-to-human transmission of the circulating variants of the virus involved in this epidemic. Sequence analysis of early virus isolates collected from patients in Guinea and Sierra Leone was conducted in an attempt to determine whether the Makona strain was changing more rapidly than strains in previous outbreaks. This was critical information because diagnostic assays were dependent on the sequences of the assay reagents matching the sequences of the circulating viruses. These first sequence analysis showed that the West African strains of the virus formed a clade distinct from EBOV sequences collected from previous outbreaks in Central Africa [2]. Further analysis of the new sequences at the nucleotide level revealed a 3% difference among the sequences analyzed, a substitution rate twice that of the rate between outbreaks; in addition, the substitutions were more often nonsynonymous [2, 3]. However, other studies that included more EBOV sequences covering a larger temporal and spatial distribution showed a lower rate of virus evolution, with nucleotide mutations being synonymous or occurring in noncoding regions, similar to what has been observed in previous outbreaks [4, 5].

Correspondence: V. J. Munster, Virus Ecology Section, Laboratory of Virology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 S Fourth S, Hamilton, MT 59840 (munstervj@niaid.nih.gov).

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The pathology of Ebola virus disease induced via inhaled aerosol has been extensively studied in nonhuman primates since 1995. Lethal outcomes using this method have been demonstrated in rhesus and cynomolgus macaques for Ebola virus, Sudan virus, and Marburg virus [6–9]. It is generally accepted that 3 conditions must be met for a natural aerosol transmission to occur: An infected individual must be able to produce aerosols containing viable virus at high enough concentrations to be infectious, the aerosolized virus must remain viable long enough to come into contact with a susceptible individual, and the viable virus must be able to infect the susceptible individual through the point of contact [10, 11].

The current study focused on the second condition—stability of the virus within an aerosol. If a virus can survive for an extended time within an aerosol, there is an increased risk for enhanced transmission between individuals. To test the possibility that the West African EBOV strain is more stable within an aerosol than historic strains, we compared the aerosol stability of EBOV isolated from the Mayinga 1976 outbreak and a strain isolated from a Guinean patient early in the West African outbreak [12, 13].

MATERIALS AND METHODS

Viruses and Cells

Ebola virus H.sapiens-tc/COD/1976/Mayinga and Ebola virus H.sapiens-tc/GIN/2014/Makona-C07 were used; they are described elsewhere [14]. Stock cultures were diluted in Dulbecco's minimum essential medium (DMEM) supplemented with 2% fetal bovine serum (FBS), and 2 mmol/L L-glutamine. All work using the viruses was conducted in a class 2 type IIA biosafety cabinet within a biosafety level 4 (BSL-4) laboratory. Ebola viruses were titrated by end-point titration in quadruplicate in Vero E6 cells cultured in DMEM supplemented with 2% fetal calf serum, 1 mmol/L L-glutamine, 50 U/mL penicillin and

50 μg/mL streptomycin. Cells were inoculated with 10-fold serial dilutions of virus and scored for cytopathic effect 14 days later. The median tissue culture infective dose ($TCID_{50}$) was calculated by the method of Spearman-Karber [15].

Aerosol Generation and Sample Collection

The aerosols were generated by passing air at a flow rate of 7.5 L/min through a 3-jet Collison nebulizer (BGI), as described elsewhere [16]. The aerosol thus generated was mixed with humidified air flowing at 3 L/min before introduction into the Goldberg drum (Biaero) to achieve an initial relative humidity of 70%–80% [17]. The initial conditions inside the drum were a temperature of 22°C–23°C and a relative humidity of 70%–80%. The drum was rotating at 3 rpm during the entire experiment, including the coating and clearing process. The samples were collected via passing air at 6 L/min for 30 seconds from the Goldberg drum through an all-glass air sampling impinger (Ace Glass) containing 10 mL of DMEM supplemented with 10% FBS and 40 μL of SE-15 antifoam (Sigma); humidified make-up air was introduced simultaneously into the drum at 6 L/min. Both generator and sampler were calibrated using a frictionless bubble meter (Gilibrator; Sensidyne) to ensure that the desired flow rates and pressure were achieved. The airflow and humidity were controlled and airflow, humidity and temperatures monitored using the AeroPlus aerosol management platform (AeroMP; Biaera Technologies).

Particle Sizing

The particle size range of the generated aerosol was measured using a Model 3321 aerodynamic particle sizer spectrometer (TSI). The drum was loaded as described above. In the coating process, aerosolized DMEM with 10% FBS was introduced into the drum for 5 minutes, and then humidified air was passed through the drum for 5 minutes to clear it, at a flow rate of 10.5 L/min. After coating, the drum was loaded with aerosolized virus-free medium for 10 minutes, the inlet and exit valves were closed, and the aerosol was allowed to mix for 5 minutes. At the end of the 5 minutes (T_0) the valves were opened, and a sample was analyzed. At T_0 and at 30 and 60 minutes, a 40-second (3.3-L) sample was analyzed at a flow rate of 5 L/min.

Virus Stability

The Goldberg drum was coated, cleared, and loaded as described above, except that the Collison nebulizer was loaded with DMEM/FBS containing 5×10^4 $TCID_{50}$/mL virus. Samples were collected from the drum at T_0 and at 30, 60, 120, and 180 minutes by flowing 6 L/min of humidified air through the drum for 30 seconds. The virus was captured in 10 mL of DMEM/FBS with anti-foam SE-15 in the all-glass air sampling impinger. Samples were collected for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and virus titration.

qRT-PCR Analysis

Viral RNA was extracted from 140 μL of each of the collected samples using a Corbett robotics extraction robot (Qiagen) with Macherey-Nagel reagents, according to the manufacturer's instructions after the samples had been inactivated with AVL buffer (Qiagen) and absolute ethanol [18]. One-step qRT-PCR targeting the Ebola virus L gene was performed on a Qiagen Rotor-Gene Q PCR cycler using QuantiFast reagents (Qiagen), according to the manufacturer's instructions [19]. The steps for qRT-PCR were as follows: reverse-transcription at 63°C for 3 minutes and initial PCR activation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 40 seconds.

The RNA genome copy number was calculated using in vitro transcribed copies of a section of the L gene. To obtain the genome copy number a sample was analyzed using a NanoDrop spectrophotometer (Thermo Scientific). The genome copy number was calculated for the specific transcript using Beer's law and the molar absorptivity calculated for the transcript.

RESULTS

Particle Sizing

Aerosols have been described as particles suspended in air that are <20 μm in diameter. They can be further divided into small droplets (5–20 μm in diameter) and droplet nuclei (<5 μm) [20]. At 0 minutes the composition of the aerosol was 4% small droplets and 96% droplet- nuclei. This ratio shifted to 99% droplet nuclei and 1% small droplets at 1 hour. The total concentration of particles dropped from 7169/cm^3 to 6456/cm^3 over the same time period [21, 22].

Virus Stability

The aerosols were generated from approximately 2 mL of virus at concentrations of 5.6×10^4 and 1.3×10^4 $TCID_{50}$/mL for EBOV Mayinga 1976 and EBOV Makona 2014, respectively; 105 L of humidified aerosol was generated and passed through the drum to create an aerosolized environment inside the drum, with virus concentrations of 8.1×10 and 1.17×10 $TCID_{50}$/L for EBOV Mayinga 1976 and EBOV Makona 2014, respectively. EBOV RNA was used as an internal control for physical loss of aerosol owing to sampling and or settling of the aerosol over the course of the experiment. qRT-PCR was used to monitor the genome copy number of the aerosolized virus suspension as a proxy for the amount of aerosol still in suspension (Figure 1*A*). Virus titration of the sample collected in the all-glass impinger was carried out in 96-well microtiter plates, and the data were used to back calculate the $TCID_{50}$ per liter in the drum aerosol (Figure 1*B*). All viability measurements were then normalized to the viral genome copies that remained in suspension (Figure 1*C*).

A nonlinear regression model was used to analyze the data for each virus and the slopes were compared. There was no significant difference between the slopes of the $TCID_{50}$ per liter of

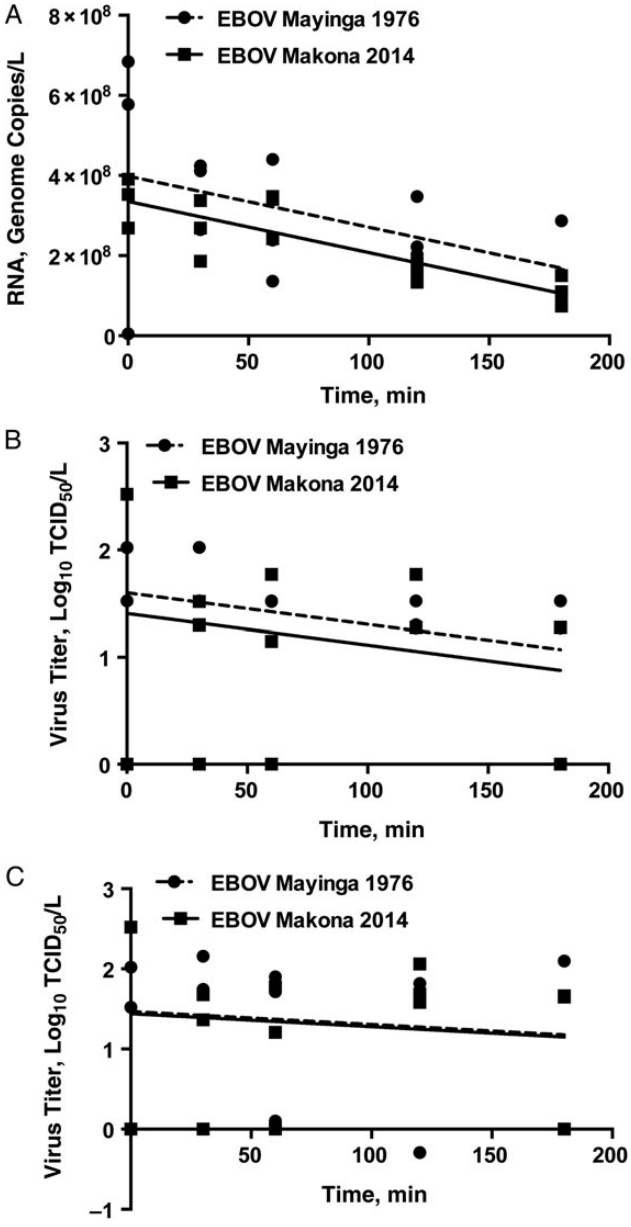

Figure 1. Nonlinear regression model calculated for 3 separate experiments using GraphPad Prism version 6.05 software for each of the following conditions: *Zaire ebolavirus* (EBOV) RNA level over time (*A*), calculated virus titer of viable EBOV (*B*), and the virus titer normalized to RNA level, used to reflect the loss of aerosol due to sample withdrawal and settling (*C*). The limit of detection in this assay is 1.28 $\log_{10}$ median tissue culture infective dose ($TCID_{50}$)/L. Slopes were compared for each graph using the GraphPad analysis of covariance function. In each case, there is no significant difference between slopes.

aerosolized virus, regardless of whether the data were normalized to the RNA copy number data; therefore, in both cases the nonlinear regression model was fitted to the slope derived from all of the data. The slope of the nonlinear regression of the nonnormalized data is −0.0029, and the slope of the nonlinear regression for the normalized model is −0.0016. Using the single-slope regression model generated for the data normalized to genome copy number, the time required for a reduction of 1 log $TCID_{50}$ was calculated to be 619 minutes, or 0.43 days. This value is similar to the decay rate reported elsewhere for virus dried on stainless steel or plastic at 27°C (0.45 days for both substances) [23]. Viable virus was recovered from the aerosol after 180 minutes, at an average concentration of just over 1×10^1.

DISCUSSION

In this study we compared the viability of 2 temporally separated strains of EBOV to determine whether the virus had become more stable within an aerosol, which could potentially increase the ability for human-to-human transmission. The viruses EBOV Mayinga 1976 and EBOV Makona 2014 were aerosolized and kept in suspension in a Goldberg rotating drum. Samples were collected over the course of 3 hours, and the $TCID_{50}$ per liter was calculated and plotted to compare the slopes of the viability over time. There was no statistically significant difference in viability between the 2 strains over 3 hours. This held true regardless of whether or not the viability was normalized to the number of RNA copies in the aerosol sample.

In a previous study of the stability of aerosolized filoviruses using a Goldberg drum, viable EBOV E718 was recovered after 90 minutes, which was the length of the experiment. Direct comparison with that study is problematic because, although a $TCID_{50}$ was reported, there was no reference to units of volume. The volume of aerosol sampled and that of the medium in the mini-impinger were also not stated, making it impossible to calculate the concentration of virus in the aerosol inside the drum [21].

The rate at which the virus lost viability in an aerosol remained the same between the 2 viruses, indicating that the scope of the West African outbreak was not due to an increased stability of aerosolized virus. This is in agreement with epidemiological findings that showed no evidence for increased transmission via the aerosol route.

One limitation of this our is the low starting titer. Although the starting titer in the aerosol generated for this study may be more biologically relevant, for the purposes of this study a higher starting titer would allow a more accurate assessment of the loss of virus viability over time. A second limitation is that within the drum the aerosol is confined to the volume of the drum. That is necessary for the parameters of this study, but under real-world conditions the virus would be expected to dissipate, thus lowering its concentration over time. In addition, the aerosols were generated using virus in cell culture medium and not a body fluid, as they would be in a natural setting.

The results of our study suggest that the ability of EBOV Makona 2014 to survive within an aerosol is not greater than that of EBOV Mayinga 1976, a genetically distinct and temporally separated virus strain. They also show that EBOV can survive for 3 hours as an aerosol at 22°C and 80% relative humidity. From these data, we calculated a decay rate of 1 Log_{10} $TCID_{50}$/619 min

reduction in virus; this number is in line with the stability of EBOV dried on surfaces at 27°C.

Notes

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References

1. Baize S, Pannetier D, Oestereich L, et al. Emergence of Zaire Ebola virus disease in Guinea. N Engl J Med **2014**; 371:1418–25.
2. Gire SK, Goba A, Andersen KG, et al. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. Science **2014**; 345:1369–72.
3. Basler CF. Portrait of a killer: genome of the 2014 EBOV outbreak strain. Cell Host & Microbe **2014**; 16:419–21.
4. Hoenen T, Groseth A, Rosenke K, et al. Nanopore sequencing as a rapidly deployable Ebola outbreak tool. Emerg Infect Dis J **2016**; 22:331.
5. Carroll MW, Matthews DA, Hiscox JA, et al. Temporal and spatial analysis of the 2014-2015 Ebola virus outbreak in West Africa. Nature **2015**; 524:97–101.
6. Johnson E, Jaax N, White J, Jahrling P. Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. Intern J Exp Pathol **1995**; 76:227–36.
7. Twenhafel NA, Mattix ME, Johnson JC, et al. Pathology of experimental aerosol *Zaire ebolavirus* infection in rhesus macaques. Vet Pathol Online **2013**; 50:514–29.
8. Zumbrun EE, Bloomfield HA, Dye JM, et al. A characterization of aerosolized Sudan virus infection in African green monkeys, cynomolgus macaques, and rhesus macaques. Viruses **2012**; 4:2115–36.
9. Alves DA, Glynn AR, Steele KE, et al. Aerosol exposure to the Angola strain of Marburg virus causes lethal viral hemorrhagic fever in cynomolgus macaques. Vet Pathol Online **2010**; 47:831–51.
10. Tang JW. Investigating the airborne transmission pathway—different approaches with the same objectives. Indoor Air **2015**; 25:119–24.
11. Jones RM, Brosseau LM. Aerosol transmission of infectious disease. J Occup Environ Med **2015**; 57:501–8.
12. Johnson KM, Lange JV, Webb PA, Murphy FA. Isolation and partial characterisation of a new virus causing acute haemorrhagic fever in Zaire. Lancet **1977**; 309:569–71.
13. Hoenen T, Groseth A, Feldmann F, et al. Complete genome sequences of three Ebola virus isolates from the 2014 outbreak in West Africa. Genome Announc **2014**; 2:e01331–14.
14. Marzi A, Feldmann F, Hanley PW, Scott DP, Günther S, Feldmann H. Delayed disease progression in cynomolgus macaques infected with Ebola virus Makona strain. Emerg Infect Dis J **2015**; 21:1777.
15. Hierholzer J, Killington R. Virus isolation and quantitation. In: Mahy, Brian WJ, Kangro HO, eds. Virological Methods Mannual. 1st ed. San Diego: Academic Press, **1996**:25–46.
16. de Wit E, Prescott J, Baseler L, et al. The Middle East respiratory syndrome coronavirus (MERS-CoV) does not replicate in Syrian hamsters. PLoS One **2013**; 8:e69127.
17. Goldberg LJ, Watkins HMS, Boerke EE, Chatigny MA. The use of a rotating drum for the study of aerosols over extended periods of time. Am J Epidemiol **1958**; 68:85–93.
18. Fischer RJ, Judson SD, Miazgowicz K, Bushmaker T, Munster VJ. Ebola virus persistence in semen ex vivo. Emerg Infect Dis J **2016**; 22:289.
19. Qiu X, Wong G, Audet J, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. Nature **2014**; 514:47–53.
20. Judson S, Prescott J, Munster V. Understanding Ebola virus transmission. Viruses **2015**; 7:511.
21. Piercy TJ, Smither SJ, Steward JA, Eastaugh L, Lever MS. The survival of filoviruses in liquids, on solid substrates and in a dynamic aerosol. J Appl Microbiol **2010**; 109:1531–9.
22. Srikanth P, Sudharsanam S, Steinberg R. Bio-aerosols in indoor environment: composition, health effects and analysis. Indian J Med Microbiol **2008**; 26:302–12.
23. Fischer RJ, Judson SD, Miazgowicz K, Bushmaker T, Prescott JB, Munster VJ. Ebola virus stability on surfaces and in fluids in simulated outbreak environments. Emerg Infect Dis J **2015**; 21:1243.