

## ORIGINAL ARTICLE

# The survival of filoviruses in liquids, on solid substrates and in a dynamic aerosol

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**Abstract**

**Aims:** Filoviruses are associated with high morbidity and lethality rates in humans, are capable of human-to-human transmission, via infected material such as blood, and are believed to have low infectious doses for humans. Filoviruses are able to infect via the respiratory route and are lethal at very low doses in experimental animal models, but there is minimal information on how well the filoviruses survive within aerosol particles. There is also little known about how well filoviruses survive in liquids or on solid surfaces which is important in management of patients or samples that have been exposed to filoviruses.

**Methods and Results:** Filoviruses were tested for their ability to survive in different liquids and on different solid substrates at different temperatures. The decay rates of filoviruses in a dynamic aerosol were also determined.

**Conclusions:** Our study has shown that Lake Victoria marburgvirus (MARV) and Zaire ebolavirus (ZEBOV) can survive for long periods in different liquid media and can also be recovered from plastic and glass surfaces at low temperatures for over 3 weeks. The decay rates of ZEBOV and Reston ebolavirus (REBOV) plus MARV within a dynamic aerosol were calculated. ZEBOV and MARV had similar decay rates, whilst REBOV showed significantly better survival within an aerosol.

**Significance and Impact of the Study:** Data on the survival of two ebolaviruses are presented for the first time. Extended data on the survival of MARV are presented. Data from this study extend the knowledge on the survival of filoviruses under different conditions and provide a basis with which to inform risk assessments and manage exposure to filoviruses.

**Introduction**

Viral haemorrhagic fevers can be caused by a range of viral agents. The family *Filoviridae* consists of two genera, *Ebolavirus* and *Marburgvirus*, that contain filoviral species (*Zaire ebolavirus*, *Sudan ebolavirus* and *Bundibugyo ebolavirus* and *Lake Victoria marburgvirus*) that can cause acute and rapidly progressive haemorrhagic fever in humans. Two other *Ebolavirus* species, *Cote d'Ivoire ebolavirus* and *Reston ebolavirus*, cause diseases in primates but are not pathogenic in humans. Zaire ebolavirus (ZEBOV) and Lake Victoria marburgvirus (MARV) have been associated with a number of outbreaks with high fatality rates (up

to 100%) (Feldman *et al.* 1993; Feldman and Klenk 1996). The average lethality for humans is *c.* 76% for ZEBOV and 57% for MARV (figures calculated from Bausch, *et al.* 2008). Both viruses are fast acting, with death often occurring within 7–10-day postinfection, although the incubation period is considered to be 2–21 days (Peters and Khan 1999; Borio *et al.* 2002). To date, the natural reservoir for filoviruses is not known; however reports have shown that species of African fruit bat may be the natural reservoir for the virus as antibodies to ZEBOV and MARV have been found in bat species, and human cases have been linked to bat exposure (Leroy *et al.* 2005; Pourrut *et al.* 2009; Towner *et al.* 2009).

Filoviruses are transmitted through contact with body fluids or tissues of humans, nonhuman primates or infected laboratory animals (Brown 1997; Mwanatambwe *et al.* 2001; Pinzon *et al.* 2004). Historically, nosocomial transmission often occurs through the reuse of incorrectly sterilized needles and syringes, or during nursing of an infected individual through contact with blood, vomit or other infected secretions (Feldman and Klenk 1996). Transmission can also occur during burials and the preparation of bodies for burial (Tukei 1996).

Filoviruses have been reported as being transmitted via the aerosol route, either experimentally or within a biocontainment facility (Jaax *et al.* 1995; Johnson *et al.* 1995; Belanov *et al.* 1996) and are considered to be potential biological warfare or bioterrorism agents (Borio *et al.* 2002; Bray 2003). In addition, there are reports that MARV was previously weaponized (Alibek and Handelman 1999). A recent review of persistence of Category A Select Agents in the environment highlighted how little basic information is known about the filoviruses (Sinclair *et al.* 2008). The stability of MARV within aerosols and dried on various substrates was investigated by workers in the former Soviet Union (FSU) (Belanov *et al.* 1996; Chepurinov *et al.* 1997); however survival characteristics of other filoviruses have not been investigated.

In the United Kingdom, filoviruses are classified as Advisory Committee on Dangerous Pathogens (ACDP) Hazard Group 4 pathogens [equivalent to Biosafety Level 4 (BSL-4)] and are handled under laboratory containment level 4 (CL4) to minimize the risk of infection to laboratory personnel. Filoviruses are also classed as Category A biological agents by the CDC as they have high case fatality rates and are easily disseminated and low infectious doses of filoviruses are sufficient to cause disease in animal models (Borio *et al.* 2002). In the field, the infrastructure to handle the pathogens is not always available, and prevention of spread is reliant on good hygiene and removal of all contaminated and infectious material. It is unknown how long the filoviruses are able to remain viable if waste and contaminated areas are not immediately sterilized. If the filoviruses were aerosolized, either accidentally or as a deliberate release, it is also unknown how long they would survive as aerosols, and therefore how many people might potentially be exposed.

Here, we provide data on the stability and viability of MARV and ZEBOV in both liquid media and on a range of solid substrates at various temperatures, over time. In addition, the stability and decay rate of MARV, ZEBOV and Reston ebolavirus (REBOV) within small-particle aerosols held within a modified version of the Goldberg drum system (Goldberg *et al.* 1958) was investigated.

## Materials and methods

### Virus stocks and containment

ZEBOV strain E718, MARV strain Popp and REBOV were obtained from the Health Protection Agency, Porton Down. ZEBOV strain E718 was a clinical isolate from the 1976 outbreak associated with a case fatality rate of 88% and was one of four isolates (including ZEBOV-Mayinga) taken from the original outbreak. MARV-Popp (Poppinga) was also a clinical isolate from the original outbreak in Germany in 1967. The REBOV stock used in aerosol decay experiments was originally obtained from Fort Detrick (USAMRIID); however the passage history of the strain is unknown and is currently being sequenced.

Solid and liquid survival studies were conducted with the two most pathogenic species (ZEBOV and MARV). REBOV was included in the aerosol survival studies as an apathogenic species for humans but associated with anecdotal evidence of airborne transmission. The stock of Sudan ebolavirus (strain Boniface) held at Dstl Porton Down has been shown to have an altered phenotype (less virulent in immunodeficient mice) and to have undergone substantial mutation, compared to stocks held by the United States (J.E. Farlow and S.M. Ibrahim, personal communication, manuscript in preparation). In addition, there was poor recovery of Sudan ebolavirus upon aerosolization (M.S. Lever, unpublished observation), and therefore Sudan ebolavirus was not used in any studies presented here.

All work was performed within primary containment facilities housed within ACDP CL4 laboratories at Dstl, Porton Down. The majority of the work was performed either within an experimental laboratory consisting of a cabinet line of interconnected microbiological safety cabinets (Lever *et al.* 2008) or interconnected rigid half-suit isolators (for aerosol studies).

### Microtitre TCID<sub>50</sub> end-point dilution assay

African Green monkey kidney cell monolayers (Vero C1008) were prepared in 96-well microtitre plates (Nunc; Thermo Fisher, Basingstoke, UK) at a concentration of  $2 \times 10^4$  cells per well (Vero C1008 cells obtained from ECACC, catalogue number 85020206; Salisbury, UK) and maintained in Dulbecco's modified Eagle's medium, DMEM, supplemented with 10% foetal calf serum (FCS), and 1% penicillin, streptomycin and L-glutamine. Plates were incubated overnight at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere, and 80-µl maintenance media (DMEM plus 2% FCS, 1% HEPES and 1% penicillin, streptomycin and L-glutamine) was added to columns 2–12 of the plate. To column 1 of the plate, 100 µl of virus test

sample (from survival experiments) was added, and 20  $\mu$ l was transferred across the plate (1 : 10 dilution). Control wells (columns 11 and 12) were left in maintenance media, and plates were incubated at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere. At 6-day postinfection, 3  $\mu$ l (final concentration of 1.5%) of neutral red stain (Sigma, Gillingham, UK) was added to each well, and plates were incubated at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere for a further 24 h. On day 7 postinfection, cells were fixed by the addition of 100  $\mu$ l 10% formal saline and left at room temperature for 30 min before removal of the formal saline. Cytopathic effect was then scored for each well. Each column, of eight wells, corresponded to a specific dilution, and the number of positive and negative wells was recorded for each column (dilution). The TCID<sub>50</sub> value was calculated by the method of Reed and Muench (1938). The limit of the assay (under conditions described) was 10 TCID<sub>50</sub> ml<sup>-1</sup>. For solid, liquid and aerosol survival experiments, triplicate samples were taken at each time point to obtain an average count over time.

#### Survival of EBOV and MARV in both guinea pig sera and cell culture media at different temperatures

ZEBOV strain E718 and MARV strain Popp at a starting concentration of  $1 \times 10^6$  TCID<sub>50</sub> were used for liquid survival studies, and each virus was diluted 1 : 10 in either tissue culture media (Dulbecco's modified Eagle's medium with antibiotics, Sigma) or guinea pig sera (Lyophilized guinea pig sera, Sigma). Individual samples were prepared and stored in aliquots at either 4°C or room temperature at a relative humidity of  $55 \pm 5\%$  for a period of 46 days. At day 0, 26 and 46, triplicate aliquots were taken out of storage and titrated to determine TCID<sub>50</sub> ml<sup>-1</sup> values. Samples taken at days 0 and 26 were diluted 1 in 10, in tissue culture media prior to adding to each well in the first column of the microtitre assay plate, and samples taken at day 50 were added (100  $\mu$ l) directly to the first column of each plate to maximize the detection capability of the microtitre assay. Microtitre assays for each of the samples were performed by tenfold dilution across the plate as described previously.

#### Survival of EBOV and MARV on plastic, metal and glass at different temperatures

ZEBOV strain E718 and MARV strain Popp were used for solid substrate survival studies; each virus was diluted at a ratio of 1 : 2 v/v in the appropriate liquid media (guinea pig sera or tissue culture media) prior to being dried onto the substrate. Substrates used were plastic (polyvinyl chloride), metal (316 stainless steel)

and glass (glass coverslips, Fisher, Loughborough, UK). A 20  $\mu$ l suspension of virus was then dropped onto 7 ml-diameter discs of plastic, metal or glass and allowed to air-dry for 30 min. Discs were stored in individual tubes at either +4°C or room temperature at a relative humidity of  $55 \pm 5\%$  for the duration of the study. An initial 14-day survival study was followed by a 50-day study. At predetermined time points, days 2, 7 and 14 for the initial study and days 26 and 50 for the long-term study, triplicate samples were taken out and processed. Processing of samples was carried out in 24-well titration plates (Nunc); individual discs were removed from storage and washed in 1 ml tissue culture media, each disc was washed by aspirating the solution 20 times. The resulting suspension was then added (100  $\mu$ l) to all wells in the first column of corresponding microtitre plate, the sample was then diluted (tenfold) across the microtitre plate as described previously. Time points in all survival studies were dictated by the logistics of working in high containment.

#### Aerosol studies

Aerosol studies were carried out using a modified 40 l rotating Goldberg drum (Goldberg *et al.* 1958). A Collision nebulizer containing 8 ml filovirus (ZEBOV, MARV or REBOV) in DMEM tissue culture media + 2% FCS and without antibiotics was used to generate aerosol particles. *Bacillus atrophaeus* (BA) spores (2 ml) were added to the viral suspension at a concentration of  $1.0 \times 10^9$  ml<sup>-1</sup> to serve as an internal control for physical loss and decay. The particle size of aerosol produced in this manner is c. 1–3  $\mu$ m. The aerosol was conditioned in a modified Henderson apparatus (Druett 1969), and the aerosol stream was maintained at 50–55% relative humidity and  $22 \pm 3^\circ\text{C}$ . Validation experiments were carried out to optimize the fill and mixing times for the 40 l drum system using BA spores. Filoviruses were delivered into the drum for 2 min, prior to a 5-min mixing time, before the start of the experimental run ( $T = 0$ ). Runs were carried out in triplicate for each virus. Samples were taken using mini-impingers (4 l min<sup>-1</sup>) at  $T = 5$ ,  $T = 15$ ,  $T = 30$ ,  $T = 45$ ,  $T = 60$  and  $T = 90$  min. Virus samples were then titrated using a microtitre TCID<sub>50</sub> end-point dilution assay after removing 10% of the sample for dilution and plating for BA counts.

#### *B. atrophaeus* spore quantification

Impinger samples (100  $\mu$ l) were removed prior to addition of antibiotics (penicillin and streptomycin, Sigma) and downstream virological quantification. The impinger sample was serially diluted 1 : 10 in PBS to a dilution of

$10^{-3}$  and plated out on nutrient agar in duplicate and incubated at room temperature for 3 days.

### Decay constant calculation

For the aerosol decay studies, the specific inactivation, or decay, rate ( $k$ ) was calculated using the equation  $Y_t = Y_0 e^{-kt}$  where  $Y_t$  and  $Y_0$  are the virus concentration at time ( $t$ ) and the initial virus concentration ( $Y_0$ ) in the aerosol.

### Graphs and statistical analysis

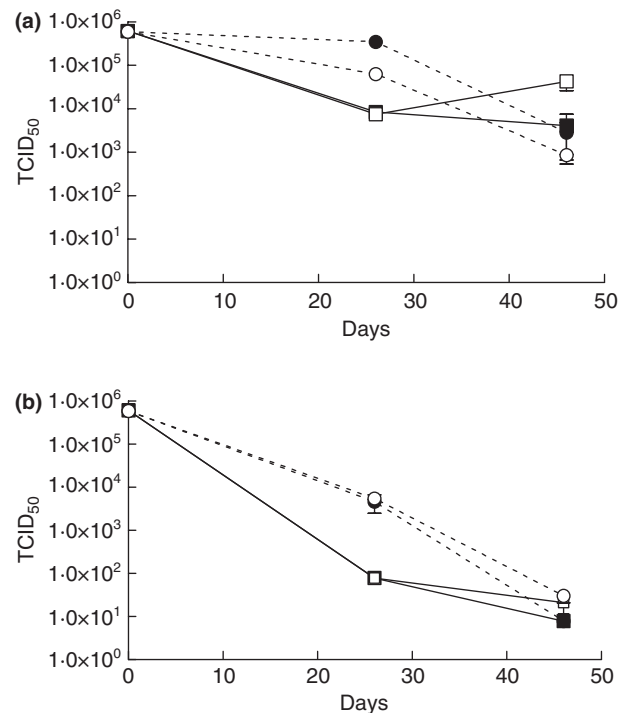
For graphs, mean viral counts from triplicate samples (solid and liquid survival) or triplicate runs in the Goldberg drum were plotted over time. The standard error of the mean is included on the graphs.

Statistical analysis was performed using GRAPHPAD PRISM ver. 4.03 and ver. 5.0 (La Jolla, CA) and Microsoft Excel. For solid and liquid survival studies, one- or two-way ANOVA was carried out with Bonferroni's post-test to compare all data sets against each other to look for statistically significant differences at the 95% confidence level. For aerosol survival curves, nonlinear regression curve fit analysis was performed. Data points were fitted to the decay equation stated earlier. A Runs test was performed on the curves. Using Prism, the  $F$ -test (ANCOVA) was applied to compare the lines and decay rate ( $k$ ) at the 95% confidence level. The null hypothesis was that the lines were the same or had the same decay rate; the alternative hypothesis was that the lines were different or had different decay rates. The  $P$ -value of the ANCOVA/ $F$ -test answers the question: If all the data sets really came from populations with identical slopes, what is the probability that random sampling would result in slopes as disparate as the ones observed in this experiment. For the aerosol decay, Prism analysis could also be used to decide what model best fits a line. In this case, the null hypothesis was for one-phase exponential decay, and the alternative hypothesis was for two-phase exponential decay as the best model for the data points.

## Results

### Survival of EBOV and MARV in liquid media over 46 days

The survival of MARV and ZEBOV in guinea pig sera and tissue culture media was assessed over a 46-day period (Fig. 1). At  $+4^{\circ}\text{C}$  (Fig. 1a), virus viability reduced in titre by a maximum of 3 and 2–3 logs for MARV and ZEBOV, respectively. At room temperature (Fig. 1b), virus viability decreased more rapidly showing a 2- and



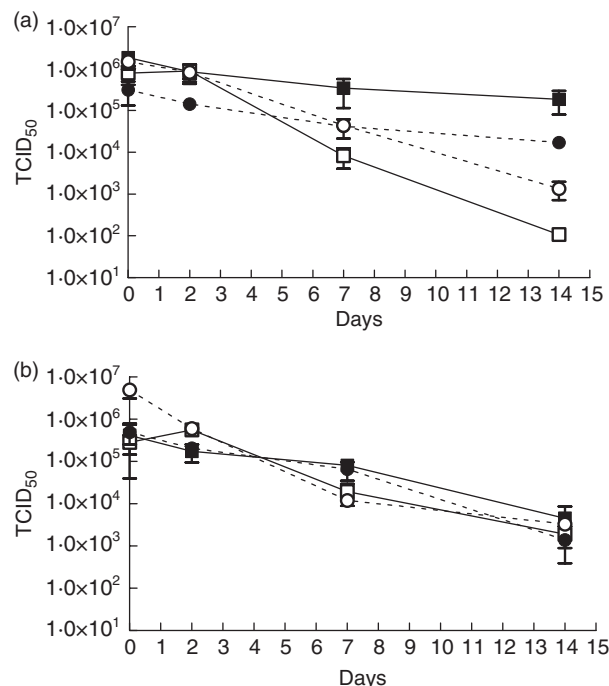
**Figure 1** Survival of Zaire ebolavirus (ZEBOV) and Lake Victoria marburgvirus (MARV) in liquid media at two temperatures. The survival of ZEBOV (—■—) and MARV (---○---) in tissue culture media (closed shapes) and guinea pig sera (open shapes) over 46 days at  $4^{\circ}\text{C}$  (a) and room temperature (b), was assessed by the  $\text{TCID}_{50}$  microtitre plate assay over time. At each time point, triplicate samples were taken; the mean count ( $\text{TCID}_{50}$ ) from triplicate samples, plus the standard error, is shown.

4-log reduction in virus titre, respectively, within the first 26 days. After 46 days, titres of virus were only just detectable above the detection limit of the microtitre assay in this study ( $10 \text{ TCID}_{50} \text{ ml}^{-1}$ ). There was no significant difference ( $P > 0.05$ , two-way ANOVA) in survival of virus in either guinea pig sera or tissue culture media, however there was a significant difference between survival during storage at  $+4^{\circ}\text{C}$  compared with storage at room temperature ( $P < 0.001$ , two-way ANOVA).

### Survival of EBOV and MARV dried onto solid substrates over 14 days

An initial recovery experiment showed that no virus could be recovered from any substrate stored at room temperature (results not shown). All results reported are for  $+4^{\circ}\text{C}$ . Neither MARV nor ZEBOV could be recovered from metal substrate at any time.

The survival of MARV and ZEBOV on plastic stored at  $+4^{\circ}\text{C}$  showed a decrease in viral titre over time, with a of 3- or 4-log reductions in titres over 14 days for MARV



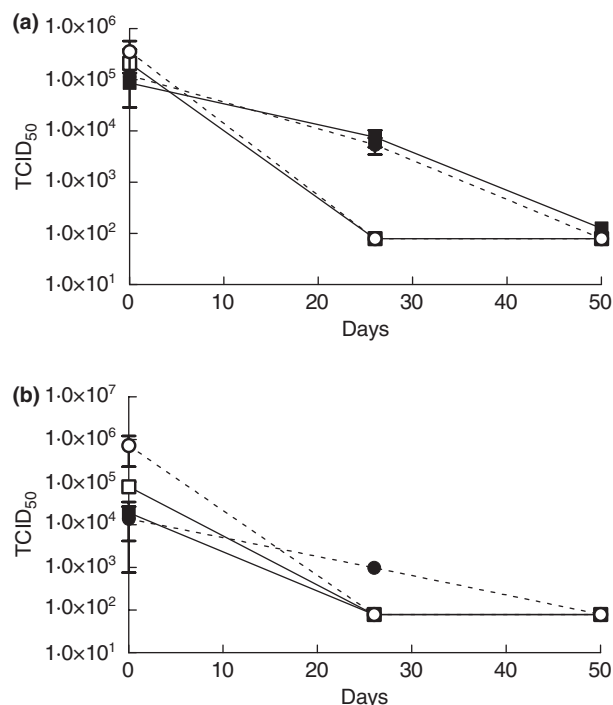
**Figure 2** Survival of Zaire ebolavirus (ZEBOV) and Lake Victoria marburgvirus (MARV) dried onto different solid substrates over a 14-day period. The survival of ZEBOV (—■) and MARV (---○) on different substrates when dried in tissue culture media (closed shapes) or guinea pig sera (open shapes) over 14 days was assessed by the TCID<sub>50</sub> microtitre plate assay. Samples were recovered, in triplicate, from the surfaces at various time points. (a) Survival on glass at 4°C, (b) Survival on plastic at 4°C. Each point shows the mean count (TCID<sub>50</sub>) from triplicate samples, plus the standard error.

and ZEBOV, respectively (Fig. 2b). When dried in tissue culture media onto glass and stored at +4°C, ZEBOV decreased in titre by less than a log, MARV by a log (Fig. 2a). Virus dried onto glass in guinea pig sera decreased by 3 logs (MARV) or 4 logs (ZEBOV) (Fig. 2a).

There was no significant difference in the initial recovery of either virus suspended in either sera or tissue culture media dried onto glass or plastic ( $P > 0.05$ , two-way ANOVA). Further statistical analysis showed that there was no significant difference in the decay rates of the two viruses across the range of substrates and liquid storage media ( $P > 0.05$ , using ANCOVA analysis) over 14 days.

#### Survival of EBOV and MARV dried on to solid substrates over 50 days

The survival of MARV and ZEBOV on plastic and glass substrates was assessed when stored at +4°C, over 50 days (Fig. 3). No significant differences in the initial recovery of either virus across the two substrates and liquid media



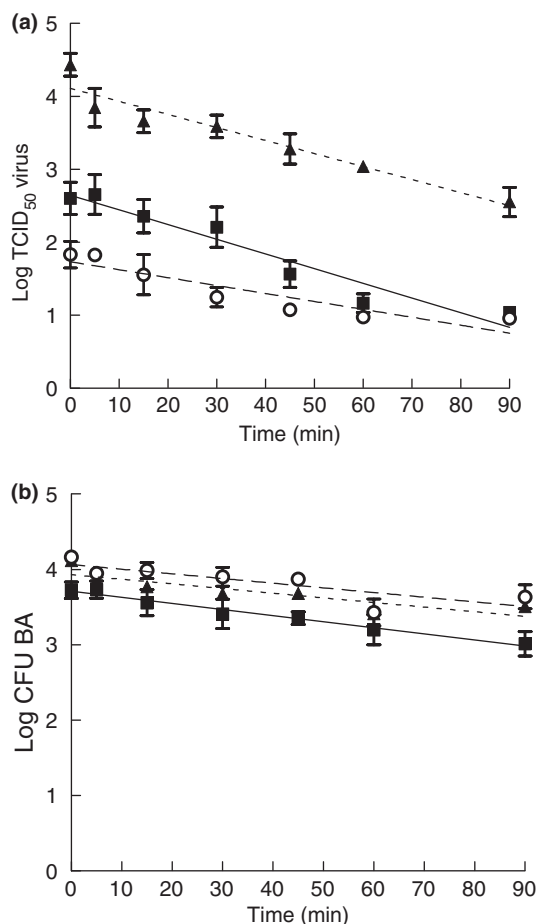
**Figure 3** Survival of Zaire ebolavirus (ZEBOV) and Lake Victoria marburgvirus (MARV) dried onto different solid substrates over a 50-day period. The survival of ZEBOV (—■) and MARV (---○) on different substrates when dried in tissue culture media (closed shapes) or guinea pig sera (open shapes) over 50 days was assessed by the TCID<sub>50</sub> microtitre plate assay. Samples were recovered from the surfaces, in triplicate, at various time points over a longer time period. (a) Survival on glass at 4°C, (b) Survival on plastic at 4°C. Each point shows the mean count (TCID<sub>50</sub> ml<sup>-1</sup>) from triplicate samples, plus the standard error.

were detected ( $P > 0.05$ , two-way ANOVA). At day 26, there were only three samples from which virus could be recovered; ZEBOV in tissue culture media on glass and MARV in tissue culture media on both glass and plastic. At day 50, the only sample from which virus could be recovered was that of ZEBOV from tissue culture media dried onto glass.

#### Inactivation rates of MARV, ZEBOV and REBOV within an aerosol

The stability of MARV, ZEBOV and REBOV was assessed over 90 min using a 40-l Goldberg drum. For each virus, three replicates were carried out for each time point using BA as an internal control. The average decay rate of BA spores during the filovirus runs was 1.42% min<sup>-1</sup>. This value represents the physical decay rate in the system used. Statistical analysis performed on the BA data showed no significant difference in decay rates ( $P > 0.05$ , ANCOVA) between runs for different viruses (Fig. 4b),

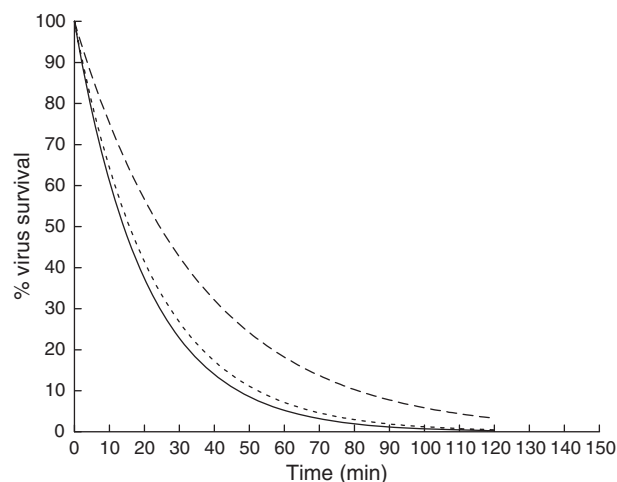




**Figure 4** Aerosol decay of filoviruses and *Bacillus atropheus* (BA) spores in the Goldberg drum. (a) Lake Victoria marburgvirus (MARV) (—■—), Zaire ebolavirus (ZEBOV) (---▲---) and Reston ebolavirus (REBOV) (---○---) were held in a 40-l Goldberg drum as a dynamic aerosol over a period of 90 min at 50–55% relative humidity and  $22 \pm 3^\circ\text{C}$ . At various time points, samples were taken and the viral titre was determined by TCID<sub>50</sub> assay. Each virus was run in the drum on three occasions, and nonlinear regression was performed to obtain the decay curve fitted to the equation of one-phase exponential decay. (b) BA spores were aerosolized in the drum in parallel with the filoviruses to control for internal loss and physical decay. The decay curves for BA run in triplicate with MARV (—■—), ZEBOV (---▲---) and REBOV (---○---) are shown. Each point shows the mean count (TCID<sub>50</sub> ml<sup>-1</sup> or CFU ml<sup>-1</sup>) from triplicate runs, plus the standard error.

ensuring that any difference in decay rates of the filoviruses can be attributed to differences between the aerosol characteristics of the different virus species. All three filoviruses under investigation could be detected after 90 min in a dynamic aerosol (Fig. 4a). ZEBOV and MARV had similar decay rates and REBOV appeared to decay at a slower rate (Fig. 4a).

Data from replicate runs of each virus were fitted to the equation for one-phase exponential decay, and the total decay rates for MARV, ZEBOV and REBOV were



**Figure 5** Theoretical survival of filoviruses in an aerosol over time. Based on the decay rates of the three filovirus obtained from the Goldberg Drum analysis, the theoretical survival curves of Lake Victoria marburgvirus (—), Zaire ebolavirus (---) and Reston ebolavirus (---) were plotted as a percentage of initial virus titre over time. The starting amount for each virus was 100%, and the amount of virus remaining, as a percentage, over time is shown.

calculated as  $4.81$ ,  $4.29$  and  $2.72\% \text{ min}^{-1}$ , respectively (composite of biological and physical decay). Taking into account the physical decay rates observed for BA, this suggests the biological decay rate of the filoviruses is  $3.04\% \text{ min}^{-1}$  for MARV,  $3.06\% \text{ min}^{-1}$  for ZEBOV and  $1.55\% \text{ min}^{-1}$  for REBOV. Statistical analysis showed no significant difference between the decay rates of ZEBOV and MARV ( $P > 0.05$ , ANCOVA); however there was a significant difference between the decay rates of ZEBOV and MARV compared to REBOV ( $P < 0.01$ , ANCOVA). GRAPHPAD PRISM analysis also showed that one-phase exponential decay was the more appropriate model to fit the data for all three viruses, compared to two-phase exponential decay ( $P = 0.3451$  to  $0.5832$  for one-phase decay).

From the total decay rates, the theoretical decay (as a percentage of original titre) over time was calculated (Fig. 5). The time taken for 50% of the initial virus concentration to decay (i.e. the half-life of the virus) is 14, 15 and 24 min for MARV, ZEBOV and REBOV, respectively. The time for 99% of the initial virus concentration to decay was estimated as 93 min for MARV, 104 min for ZEBOV and 162 min for REBOV.

## Discussion

This study has demonstrated that filoviruses are able to survive and remain infectious for cell culture, for extended periods when suspended within liquid media and dried onto surfaces. In addition, decay rates of a

range of filoviruses, within small-particle aerosols, have been calculated, and these rates suggest that filoviruses are able to survive and remain infectious for cell culture for at least 90 min.

Recovery of virus from liquid media (tissue culture media and sera) was significantly higher in samples stored at +4°C compared to room temperature. MARV and ZEBOV, dried onto solid substrates, were recovered in high titres from both plastic and glass surfaces. It has also been shown that low titres of virus could be recovered from samples suspended in tissue culture media and dried onto both plastic and glass until day 26, but only virus dried onto glass substrate was recovered by day 50, when stored at +4°C. The only significant differences that could be detected across the range of conditions were attributable to the suspending liquid dried onto glass, where virus in tissue culture media could be recovered for significantly longer than virus dried in guinea pig sera.

Data from researchers of the FSU indicated that MARV in human blood was able to survive on steel, glass and cotton wool for at least 6 days (Belanov *et al.* 1996). Results from our study extend these findings to include survival data for EBOV as well as MARV and also include data for the survival of filoviruses on plastic and glass substrates over a longer period of time.

There was no survival of MARV on metal surfaces in our study, contrary to the results recorded by scientists of the FSU (Belanov *et al.* 1996). The two studies were performed at different relative humidities, and this may have influenced the survival rates. In addition no details of the steel used in the study conducted in the FSU were provided, and therefore no details of any possible virucidal components within the metal were available. The lack of recovery of virus from metal substrates may be attributed to several factors; previous work (Sommer *et al.* 1999; Pawar *et al.* 2005) has shown the binding of micro-organisms to metal surfaces to be poor because of the high surface energy, high electronegative and hydrophilic properties of metal. Other research has also shown that positively charged metallic ions such as copper and silver have a high bactericidal activity (Friedman and Dugan 1968; Bitton and Freihofer 1977; Slawson *et al.* 1990) and hence by extrapolation may also have a high virucidal activity. Short survival times for several haemorrhagic fever viruses on metal surfaces have been observed; on aluminium discs, the time taken for the initial virus titre to decay by 90% for hantavirus 76–118, Sicilian virus Sabin and Crimean–Congo haemorrhagic fever virus was between 1·08 and 1·45 h (Sinclair *et al.* 2008).

Viruses display a range of survival times within the environment. Variola virus, the causative agent of smallpox, for example, can remain infectious in dust and on tissue for up to 1 year, whereas influenza virus has a low

level of survival and only retains its infectivity on surfaces for a matter of days (Harper 1961). Data generated in this study suggested that both EBOV and MARV could be recovered from contaminated substrates for at least 50 days. No comparable data have been published by other groups; however in a recent study undertaken to assess the risk of transmission of Sudan ebolavirus from bodily fluids and fomites, virus was detected by PCR in a number of samples. No data were recorded however, as to the length of time virus was able to survive in these samples (Bausch *et al.* 2007).

Data on the survival of filoviruses on substrates and in liquid media presented in this study should be set within the context of the infectivity of the viruses. It has been reported that the infectious dose of filoviruses for mice and nonhuman primates is low (e.g. 400 PFU, Johnson *et al.* 1995; 1 PFU, Bray *et al.* 1999). Studies with the wild-type ZEBOV and MARV strains used in these survival studies in a susceptible mouse model demonstrated 100% mortality with <10 TCID<sub>50</sub> of either virus by the aerosol or intraperitoneal challenge route (M.S. Lever, personal communication). These low infectious dose reports suggest that if the initial viral titre is high, infectious quantities of viable virus could be recovered from samples stored at +4°C for periods of up to 46 days in liquid media, and from samples dried onto glass at both 26 and 50 days. This demonstrates the need for good control measures when handling and disposing of clinical samples that may be contaminated with filoviruses.

Data produced in this study have shown that MARV, ZEBOV and REBOV have total decay rates in small particle aerosols of 4·81, 4·29 and 2·72% min<sup>-1</sup>, respectively. There was a significant difference between the decay rates of ZEBOV and MARV when compared to REBOV ( $P < 0·01$ , ANCOVA), but no significant difference between the decay rates of ZEBOV and MARV ( $P > 0·05$ , ANCOVA). To our knowledge, this is the first time the decay rates of the genera *Ebolavirus* have been reported.

Many factors affect the inactivation rate of viruses in aerosols, such as relative humidity, the suspending fluid from which the virus is sprayed and the system used to measure aerosol decay. It is challenging to therefore compare directly the inactivation rates determined in this study with published data generated under different conditions for other viruses. However, in this study the average decay rate of the bacterial tracer (used to distinguish between physical and viability losses within the Goldberg Drum) was 1·42% min<sup>-1</sup>, which was comparable to the 1·5% reported elsewhere (Ehrlich and Miller 1971; Larson *et al.* 1980). Inactivation rates for filoviruses within aerosols determined in this study were much lower than values for MARV in aerosols reported by the FSU scientists (Belanov *et al.* 1996), but comparable with values

reported for other lipid-containing viruses, known to be transmissible via the airborne route. Such viruses include, Venezuelan equine encephalomyelitis virus (VEEV) ( $1.3\text{--}2.99\%$   $\text{min}^{-1}$ ), influenza A virus ( $1.9\%$   $\text{min}^{-1}$ ) and vaccinia virus ( $0.34\%$   $\text{min}^{-1}$ ) (Harper 1961). Other quoted decay rates recorded for viruses at a similar relative humidity (50%) and temperature ( $20\text{--}25^{\circ}\text{C}$ ) as was used in this study include Japanese encephalitis virus ( $3.3\%$   $\text{min}^{-1}$ ) (Larson *et al.* 1980) and yellow fever virus ( $7.04\%$   $\text{min}^{-1}$ ) (Mayhew and Hahon 1970).

The time taken for initial viral titres to decrease by 90 or 99% has been reviewed for an arenavirus and an alphavirus (Lassa and VEEV), and two flaviviruses (Japanese encephalitis virus and Saint Louis encephalitis virus). The times varied greatly and were dependant on the humidity and temperature, from 0.7 of an hour to over 2000 h (Sinclair *et al.* 2008) with corresponding inactivation coefficients of between 0.02 and 1.41. Inactivation coefficients from this study for the filoviruses of 0.027 to 0.049 fall within the range observed for other haemorrhagic fever viruses; however it would be expected that decay constants and survival times would vary dependent on humidity and temperature, as has been observed for other enveloped viruses.

Data generated by FSU scientists found that the decay rate of MARV (strain Popp) was  $11.5\%$   $\text{min}^{-1}$  (Belanov *et al.* 1996), which was much higher than values suggested by this study. Data from the two studies, however, cannot be compared directly because of differences in the equipment and conditions used. The work carried out by the FSU researchers used human saliva as a suspension fluid, and no mixing of the aerosol after generation was undertaken. Such factors may in part have contributed to the increased rate of decay. In addition, differences in enumeration methods (guinea pig lethal doses, compared to tissue culture infectious doses) make direct comparisons difficult.

The infectious dose of filoviruses, via the aerosol route, in nonhuman primates (reviewed in Leffel and Reed 2004) and in susceptible mice (M.S. Lever, personal communication), is very low. Such data, obtained from experimental animal models, combined with the aerosol decay rates determined in this study, would suggest that filovirus, at infectious levels, may remain a potential aerosol threat for at least one and a half hours. Epidemiological evidence, however, would suggest that during outbreaks, filoviruses are rarely transmitted by the airborne route. The lower decay rate observed for REBOV in our study may support observations made during the original outbreak in captive primates and subsequent studies where airborne transmission may be involved in the spread of filoviruses (Jaax *et al.* 1995; Johnson *et al.* 1995 and Jahrling *et al.* 1996). The ZEBOV and MARV used in the

studies are from human clinical isolates, whereas REBOV is nonpathogenic in humans. This might suggest that the factors that cause a decrease in virulence in humans also contribute to an increase in aerostability. In the future, sequence analysis, protein structural information and characterization of protein expression from the filoviruses after aerosolization may yield further insight into the survival characteristics of the viruses.

This study has shown that human pathogenic filoviruses may survive in an aerosol in the dark to detectable levels for at least 1.5 h. If filoviruses were deliberately (Borio *et al.* 2002; Leffel and Reed 2004), or accidentally aerosolized during normal laboratory or clinical practices (Dimmick *et al.* 1973; Bennett and Parks 2006), they may pose a significant threat to humans, as they are able to remain infectious over a significant period of time. The results presented in this study are able to provide basic survival data on which hazard management, risk assessments, decontamination and control measures can be implemented to help prevent infection and transmission of disease.

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## References

- Alibek, K. and Handelman, S. (1999) *Biohazard*. New York, NY, USA: Random House.
- Bausch, D.G., Towner, J.S., Dowell, S.F., Kaducu, F., Lukwiya, M., Sanchez, A., Nichol, S.T., Ksiazek, T.G. *et al.* (2007) Assessment of the risk of Ebola virus transmission from bodily fluids and fomites. *J Infect Dis* **15**(196 Suppl 2), S142–S147.
- Bausch, D.G., Sprecher, A.G., Jeffs, B. and Boumandouki, P. (2008) Treatment of Marburg and Ebola hemorrhagic fevers: a strategy for testing new drugs and vaccines under outbreak conditions. *Antiviral Res* **78**, 150–161.
- Belanov, E.F., Muntianov, V.P., Kriuk, V.D., Sokolov, A.V., Bormotov, N.I., P'iankov, O.V. and Sergeev, A.N. (1996) Survival of Marburg virus infectivity on contaminated surfaces and in aerosols. *Vopr Virusol* **41**, 32–34.
- Bennett, A. and Parks, S. (2006) Microbial aerosol generation during laboratory accidents and subsequent risk assessment. *J Appl Microbiol* **100**, 658–663.
- Bitton, G. and Freihofer, V. (1977) Influence of extracellular polysaccharides on the toxicity of copper and cadmium towards *Klebsiella aerogens*. *Microb Ecol* **4**, 119–125.
- Borio, L., Inglesby, T., Peters, C.J., Schmaljohn, A.L., Hughes, J.M., Jahrling, P.B., Ksiazek, T., Johnson, K.M. *et al.*



- (2002) Working Group on Civilian Biodefense. Hemorrhagic fever viruses as biological weapons: medical and public health management. *JAMA* **287**, 2391–2405.
- Bray, M. (2003) Defense against filoviruses used as biological weapons. *Antiviral Res* **57**, 53–60.
- Bray, M., Davis, K., Geisbert, T., Schmaljohn, C. and Huggins, J. (1999) A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. *J Infect Dis* **179**(Suppl 1), S248–S258.
- Brown, D.W. (1997) Threat to humans from virus infections of non-human primates. *Rev Med Virol* **7**, 239–246.
- Chepurinov, A.A., P'iankov, O.V., Chepurnova, T.S., Makhova, N.V., Bakulina, L.F. and Tiunnikov, G.I. (1997) Methods for controlling colonization of air and laboratory surfaces by pathogens of certain especially dangerous viral infections. *Vopr Virusol* **42**, 189–191.
- Dimmick, R.L., Vogl, W.F. and Chatigny, M.A. (1973) Potential for accidental microbial aerosol transmission in the biological laboratory. In *Biohazards in Biological Research* ed. Hellman, A., Oxmand, M.N. and Pollack, R., pp. 246–266. New York: Cold Spring Harbor Laboratory.
- Druett, H.A. (1969) A mobile form of the Henderson apparatus. *J Hyg (Lond)* **67**, 437–448.
- Ehrlich, R. and Miller, S. (1971) Effect of Relative Humidity and Temperature on Airborne Venezuelan Equine Encephalitis Virus. *Appl Microbiol* **22**, 194–199.
- Feldman, H. and Klenk, H.D. (1996) Marburg and Ebola viruses. *Adv Virus Res* **47**, 1–52.
- Feldman, H., Klenk, H.D. and Sanchez, A. (1993) Molecular biology and evolution of filoviruses. *Arch Virol Suppl* **7**, 81–100.
- Friedman, B.A. and Dugan, P.R. (1968) Concentration and accumulation of metallic ions by the bacterium *Zoogloea*. *Dev Ind Microbiol* **9**, 381–387.
- Goldberg, L.J., Watkins, H.M., Boerke, E.E. and Chatigny, M.A. (1958) The use of a rotating drum for the study of aerosols over extended periods of time. *Am J Hyg* **68**, 85–93.
- Harper, G.J. (1961) Airborne micro-organisms: survival tests with four viruses. *J Hyg (Camb)* **59**, 489–493.
- Jaax, N., Jahrling, P., Geisbert, T., Geisbert, J., Steele, K., McKee, K., Nagley, D., Johnson, E. *et al.* (1995) Transmission of Ebola virus Zaire strain to uninfected control monkeys in a biocontainment laboratory. *Lancet* **346**, 1669–1671.
- Jahrling, P.B., Geisbert, T.W., Jaax, N.K., Hanes, M.A., Ksiazek, T.G. and Peters, C.J. (1996) Experimental infection of cynomolgus macaques with Ebola-Reston filoviruses from the 1989–1990 U.S. epizootic. *Arch Virol Suppl* **11**, 115–134.
- Johnson, E., Jaax, N., White, J. and Jahrling, P. (1995) Lethal experimental infections of rhesus monkeys by aerosolised Ebola virus. *Int J Exp Pathol* **76**, 227–236.
- Larson, E.W., Dominik, J.W. and Slone, T.W. (1980) Aerosol Stability and Respiratory Infectivity of Japanese B Encephalitis Virus. *Infect Immun* **30**, 397–401.
- Leffel, E.K. and Reed, D.S. (2004) Marburg and Ebola viruses as aerosol threats. *Biosecur Bioterror* **2**, 186–191.
- Leroy, E.M., Kumulungui, B., Pourrut, X., Rouquet, P., Hassanin, A., Yaba, P., Délicat, A., Paweska, J.T. *et al.* (2005) Fruit Bats as reservoirs of Ebola virus. *Nature* **438**, 575–576.
- Lever, M.S., Howells, J.L., Bennett, A.M., Parks, S. and Broster, M.G. (2008) The microbiological validation of a new containment level 4 cabinet line. *J Appl Biosafety* **13**, 98–105.
- Mayhew, C.J. and Hahon, N. (1970) Assessment of Aerosol Mixtures of Different Viruses. *Appl Microbiol* **20**, 313–316.
- Mwanatambwe, M., Yamada, N., Arai, S., Shimizu-Suganuma, M., Shichinohe, K. and Asano, G. (2001) Ebola hemorrhagic fevers (EHF): mechanism of transmission and pathogenicity. *J Nippon Med Sch* **68**, 370–375.
- Pawar, D.M., Rossman, M.L. and Chen, J. (2005) Role of curli-fimbriae in mediating the cells of enterohaemorrhagic *Escherichia coli* to attach to abiotic surfaces. *J Appl Microbiol* **99**, 418–425.
- Peters, C.J. and Khan, A.S. (1999) Filovirus diseases. *Curr Top Microbiol Immunol* **235**, 85–95.
- Pinzon, J.E., Wilson, J.M., Tucker, C.J., Arthur, R., Jahrling, P.B. and Formenty, P. (2004) Trigger events: enviroclimatic coupling of Ebola hemorrhagic fever outbreaks. *Am J Trop Med Hyg* **71**, 664–674.
- Pourrut, X., Souris, M., Towner, J.S., Rollin, P.E., Nichol, S.T., Gonzalez, J.P. and Leroy, E. (2009) Large serological survey showing cocirculation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus*. *BMC Infect Dis* **28**, 159.
- Reed, I. and Muench, H. (1938) A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493–497.
- Sinclair, R., Boone, S.A., Greenberg, D., Keim, P. and Gerba, C.P. (2008) Persistence of category A select agents in the environment. *Appl Environ Microbiol* **74**, 555–563.
- Slawson, R.M., Lee, H. and Trevors, J.T. (1990) Bacterial interactions with silver. *Biol Met* **3**, 151–154.
- Sommer, P., Martin-Rouas, C. and Mettler, E. (1999) Influence of the adherent population level on biofilm population, structure and resistance to chlorination. *Food Microbiol* **16**, 503–515.
- Towner, J.S., Amman, B.R., Sealy, T.K., Carroll, S.A., Comer, J.A., Kemp, A., Swanepoel, R., Paddock, C.D. *et al.* (2009) Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog* **5**, e1000536.
- Tukei, P.M. (1996) Threat of Marburg and Ebola viral haemorrhagic fevers in Africa. *East Afr Med J* **73**, 27–31.