

Real-time Monitoring of Cardiovascular Function in Rhesus Macaques Infected With *Zaire ebolavirus*

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Nine rhesus macaques were implanted with multisensor telemetry devices and internal jugular vein catheters before being infected with *Zaire ebolavirus*. All animals developed viremia, fever, a hemorrhagic rash, and typical changes of Ebola hemorrhagic fever in clinical laboratory tests. Three macaques unexpectedly survived this usually lethal disease, making it possible to compare physiological parameters in lethally challenged animals and survivors. After the onset of fever, lethal illness was characterized by a decline in mean arterial blood pressure, an increase in pulse and respiratory rate, lactic acidosis, and renal failure. Survivors showed less pronounced change in these parameters. Four macaques were randomized to receive supplemental volumes of intravenous normal saline when they became hypotensive. Although those animals had less severe renal compromise, no apparent survival benefit was observed. This is the first report of continuous physiologic monitoring in filovirus-infected nonhuman primates and the first to attempt cardiovascular support with intravenous fluids.

Ebola and Marburg viruses, members of the family Filoviridae, cause severe hemorrhagic fever in humans, with high case fatality rates. *Zaire ebolavirus* (ZEBOV), the most pathogenic species of Ebola virus, was discovered in 1976, when it caused a large epidemic of hemorrhagic fever (HF) in Zaire (the present Democratic Republic of the Congo [DRC]) [1]. The virus has since caused 9 recognized outbreaks in the DRC and

neighboring countries, with case fatality rates ranging from 47 to 90% [2]. Except for the first outbreak of Marburg HF in 1967, all epidemics of filoviral HF have taken place in areas of central Africa with limited health care capacity. Our knowledge of their pathogenic mechanisms is therefore based largely on studies of infected animals in Biosafety Level-4 (BSL-4) containment laboratories. This research indicates that lethal filoviral HF is associated with unrestricted viral replication in monocytes, macrophages, and dendritic cells, with rapid systemic dissemination, persistently high viremia, and the release of massive quantities of proinflammatory cytokines [3–5].

Of the various animal models that have been developed, filovirus infection of macaques appears to most closely resemble the known features of lethal HF in humans. Rhesus macaques infected with ZEBOV typically become ill on days 4–6 postinfection and die on days 8–10. The rhesus macaque model has been employed to study the pathogenesis of ZEBOV

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HF and to test the efficacy of a number of novel therapeutic agents, including short interfering RNA (siRNA), phosphorodiamidate morpholino oligomers (PMOs), recombinant nematode anticoagulant protein c2 (rNAPc2), and recombinant human activated protein C (rhAPC) [6–10]. However, all studies of hematologic, biochemical, and immunologic changes in infected macaques have been limited to collection of blood samples from sedated animals at 2- or 3-day intervals over the course of illness, and physiological parameters such as the heart rate and blood pressure have not been measured, markedly limiting our understanding of the pathophysiology of filoviral HF.

In this study, we expanded the range of data collection by implanting each of 9 rhesus macaques with an internal radiotelemetry device and introducing a central venous catheter (CVC) into the internal jugular vein, through a tethered jacket that permitted the animal to move freely in its cage. A continuous low-level infusion of normal saline was given to maintain catheter patency. Once the animals had recovered from these procedures, we infected them with ZEBOV and monitored their temperature, respiratory rate, aortic and left ventricular pressures, and electrocardiogram continuously and collected daily blood samples through the CVC without sedation to minimize altering their physiologic parameters. In addition to standard hematology and chemistry analyses, we determined the serum lactate concentration, central venous blood gas, blood urea nitrogen (BUN), and creatinine levels. Four macaques were randomly selected to receive supplemental intravenous fluids once they developed hypotension.

MATERIALS AND METHODS

Laboratory Animals

Nine adult (6 male, 3 female) rhesus macaques (*Macaca mulatta*) weighing between 5.9 and 10.3 kg were acquired from commercial vendors and monitored for health before surgical procedures or being transferred into BSL-4 containment at the US Army Medical Research Institute of Infectious Diseases (USAMRIID). Three experiments were performed, each involving 3 animals; in this report, the animals are identified in the order of the experiments as #1–9.

This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). USAMRIID is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Implantation of Telemetry Devices and Central Venous Catheters

For each round of the study, 3 macaques were surgically implanted with T27-1B multisensor telemetry devices (ITS Inc) and allowed to recover for at least 30 days. They were then implanted with a single-lumen 6.6 French CVC (Bard Access Systems) in

the right internal jugular vein, and normal saline was infused at 3 mL/hour to maintain patency. The animals were placed in tethered jackets (Lomir Biomedical Inc), which allowed them to move freely within the cages without damaging the catheters.

Approximately one week before virus challenge, the macaques were moved into BSL-4 containment. Continuous telemetry monitoring of body temperature, heart rate (HR), left ventricular pressure, aortic pressure, intrapleural pressure, respiratory rate (RR), and the electrocardiogram was begun 5 days before virus infection. The readings were used to calculate the mean arterial pressure (MAP); systolic, diastolic, and end-diastolic blood pressure; and other measures of cardiovascular function. The VR² Data Reporting System (Data Integrated Scientific Systems) was used to verify, reduce, and graph each parameter. Data were tracked for each animal, and baseline levels of MAP and other parameters were determined during the period between virus inoculation and the onset of illness.

Virus Challenge

On day 0, each animal was inoculated intramuscularly with a 1.0 mL suspension of the 1995 Kikwit strain of ZEBOV. Titration of the leftover inoculum showed that each macaque received approximately 50 plaque-forming units (PFUs) of virus.

Clinical Observations and Euthanasia

Following infection, the macaques were examined at least twice daily for their activity level, food intake, evidence of altered mood, development of a rash, conjunctival hemorrhages, and other changes. After the onset of fever ($\geq 38^{\circ}\text{C}$), the animals were observed more frequently, their physiologic parameters were followed more closely, and they were regularly evaluated using a euthanasia scoring system. Animals that reached a total score of 10 for labored breathing, recumbency, and unresponsiveness were humanely euthanized.

Virus Detection

Purification of viral RNA from plasma and the determination of one-step quantitative real-time reverse transcription polymerase chain reaction were performed as previously described [7].

Hematology and Serum Chemistry

Beginning just before virus challenge, and daily thereafter through day 11 postinfection, blood samples were withdrawn through the CVC, which was then flushed with 5 mL of normal saline. Routine hematology and serum chemistry panels were performed using a Coulter AcT 10 (Beckman Coulter), an i-STAT-1 Portable Clinical Analyzer (i-STAT Corporation), and a Piccolo Point of Care Blood Analyzer (Abaxis).

Supplemental Fluid Therapy

In the first round of the study, macaques #1–3 were observed over the course of illness and euthanized when they became moribund; no therapeutic interventions were attempted. In the

second round, animal #6 was randomly chosen to remain untreated, while the others received supplemental normal saline through the CVC. In the third round, animal #7 remained untreated. In both treatment rounds, a macaque was given additional intravenous fluid only after it had become visibly ill and the MAP had fallen by at least 20 mm Hg below the animal's baseline level. Fluid treatment was given by increasing the flow rate of intravenous saline from the maintenance level of 3 mL/hour, so as to deliver an additional 10 or 20 mL/kg over a period of approximately 15 minutes. If no improvement in the MAP was seen above the 20 mm Hg threshold, additional infusions of 10 or 20 mL/kg could be given. Total volumes of supplemental fluid ranged from 568 to 1,121 mL per animal (80–183 mL/kg) over periods ranging from 6.5 to 44 hours. There were significant logistical challenges in coordinating supplemental intravenous fluids in the BSL-4 containment laboratory, leading in some cases to delays in the initiation of supplemental fluids or follow-on boluses.

RESULTS

Disease Course

All 9 macaques developed typical physical and laboratory features of ZEBOV HF, with the onset of lethargy and depression on days 4–6 postinfection and the development of a hemorrhagic petechial rash. The rash appeared 1–2 days earlier in lethally challenged animals. Three macaques survived acute ZEBOV HF; survival did not appear to be related to gender or body weight.

In the first round of the study, macaques #2 and #3 became severely ill and were euthanized on days 9 and 8, respectively, while animal #1 survived the acute illness and was euthanized 5 months later. In the second round, even though macaques #4 and #5 received supplemental fluids, they progressed to severe illness and were euthanized on days 7 and 9. Animal #6, which remained untreated, was euthanized on day 8. In the third round, macaques #8 and #9 received supplemental fluids. Despite receiving fluids, animal #8 became severely ill and was euthanized on day 7, while #9 survived the acute illness and was euthanized 5 months later. Macaque #7, which remained untreated, also recovered from acute illness but subsequently developed a severe eye infection and was euthanized on day 18.

Viremia

All 9 macaques became viremic, but the times of onset and peak plasma levels of viral RNA differed for lethally challenged animals and survivors (Figure 1). Macaques that were euthanized due to severe disease generally showed the earliest appearance and most rapid rise in viral RNA levels, with peak levels exceeding 10^5 relative PFU equivalents/mL. In contrast, 2 of the 3 survivors were the last to show a detectable viremia, and the peak titers of the 3 animals did not exceed 2.5×10^4 relative PFU/mL.

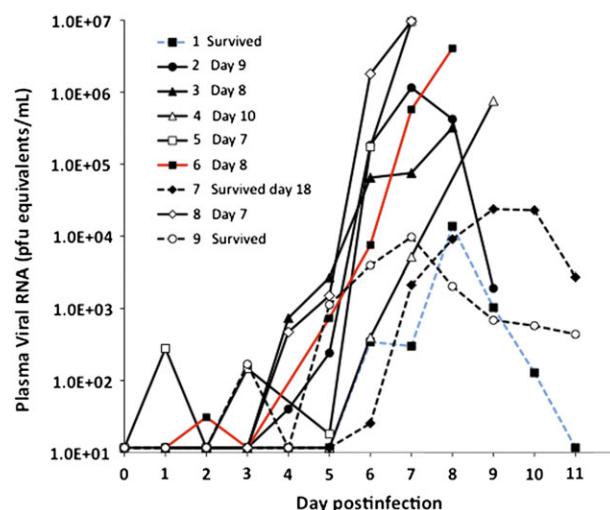


Figure 1. Circulating viral RNA levels, as measured by real-time reverse transcription polymerase chain reaction from citrate plasma. Lethally challenged animals are indicated by solid lines, with the macaque shown in Figure 2 (#6) in red. Macaques that survived infection are shown by broken lines, with the survivor whose telemetry tracings are shown in Figure 3 (animal #1) in blue. Macaques that received supplemental intravenous fluids are indicated by open symbols; the others, by solid symbols.

Body Temperature

During the 5 days of baseline telemetry monitoring before virus challenge (not shown), and continuing through the onset of fever, all macaques displayed normal diurnal variation in body temperature, with an increase in the mornings and a fall in the evenings (Figures 2A and 3A). Diurnal variation became less evident or disappeared when the animals developed fever (temperature $\geq 38^\circ\text{C}$) on days 4–6 postinfection. The subsequent course of the fever differed, depending on the disease outcome. As seen in a representative animal (Figure 2A), most lethally challenged macaques remained febrile for 2–3 days and then showed a rapid fall in body temperature, becoming hypothermic at the time of euthanasia. In the 3 surviving animals, by contrast, fever persisted for several days and then gradually resolved (Figure 3A).

Respiratory Rate

Before the onset of fever, all macaques showed diurnal variation in the RR, with an increase during the day and a fall at night (Figures 2A and 3A). In lethally challenged animals (Figure 2A), the RR increased with the onset of fever, and it continued to rise after the body temperature fell below normal, until the animal was euthanized. In survivors, by contrast, the RR rose with the appearance of fever and then declined as body temperature returned to normal during recovery (Figure 3A).

Cardiovascular Function

Before the onset of fever, all animals showed normal diurnal variation in HR, systolic blood pressure (SBP), and diastolic

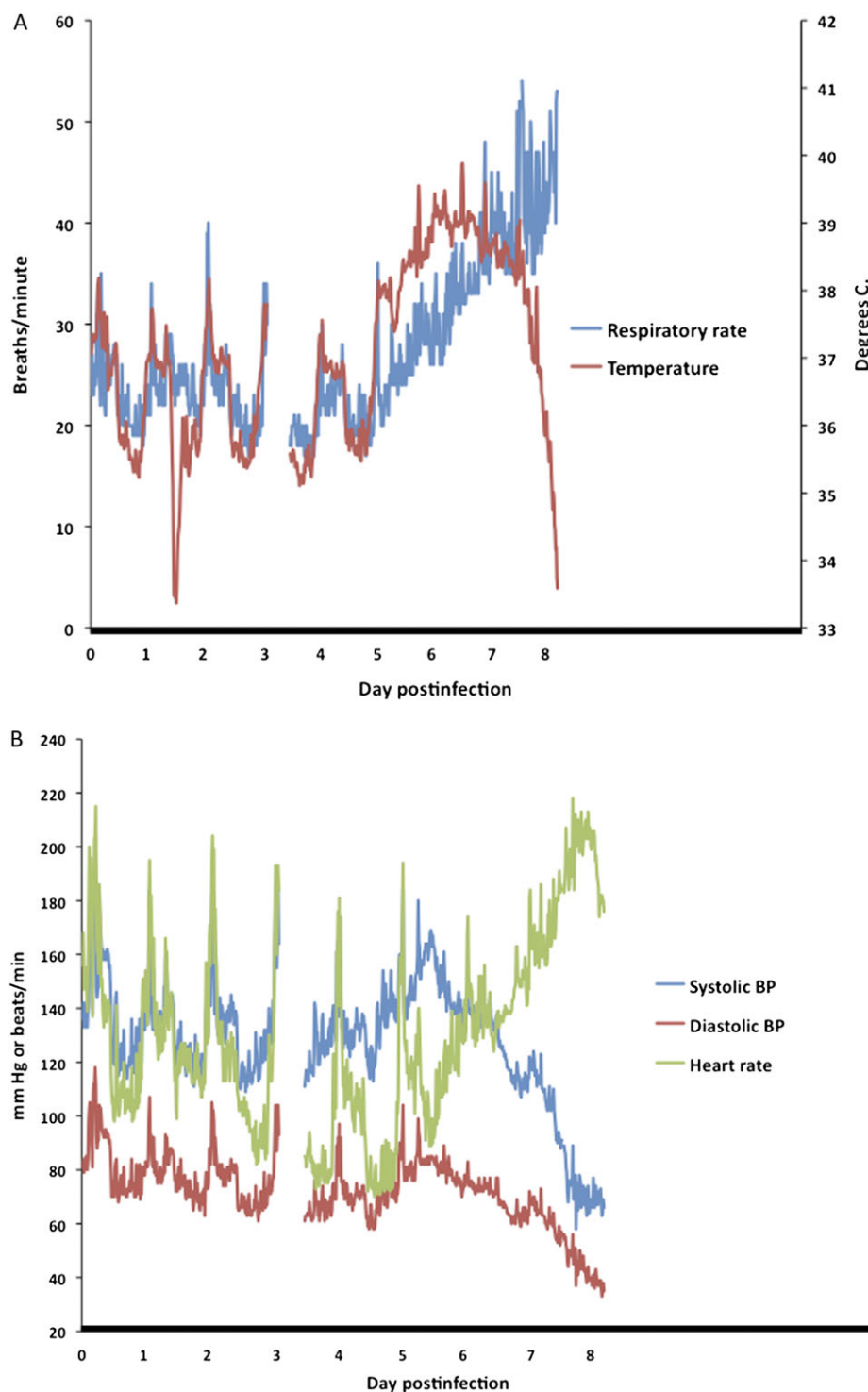


Figure 2. Telemetry data from a fatally infected macaque (#6) shown as 15-minute averages of readings recorded every minute. (A) Body temperature and respiratory rate (RR). With the onset of fever, the RR increases along with body temperature, and it remains elevated as the temperature falls below normal during the last day of life. (B) Cardiovascular function. With the onset of fever, the heart rate (HR) increases, followed by a progressive decline in systolic blood pressure (SBP) and diastolic blood pressure (DBP) that continues until euthanasia.

blood pressure (DBP), with increases during the daytime and declines at night (Figures 2B and 3B). In lethally challenged macaques, the HR began to rise with the onset of fever

and remained elevated until euthanasia, while the SBP, DBP, and MAP began to decline, leading to progressively worsening hypotension during the 24–72 hours before the

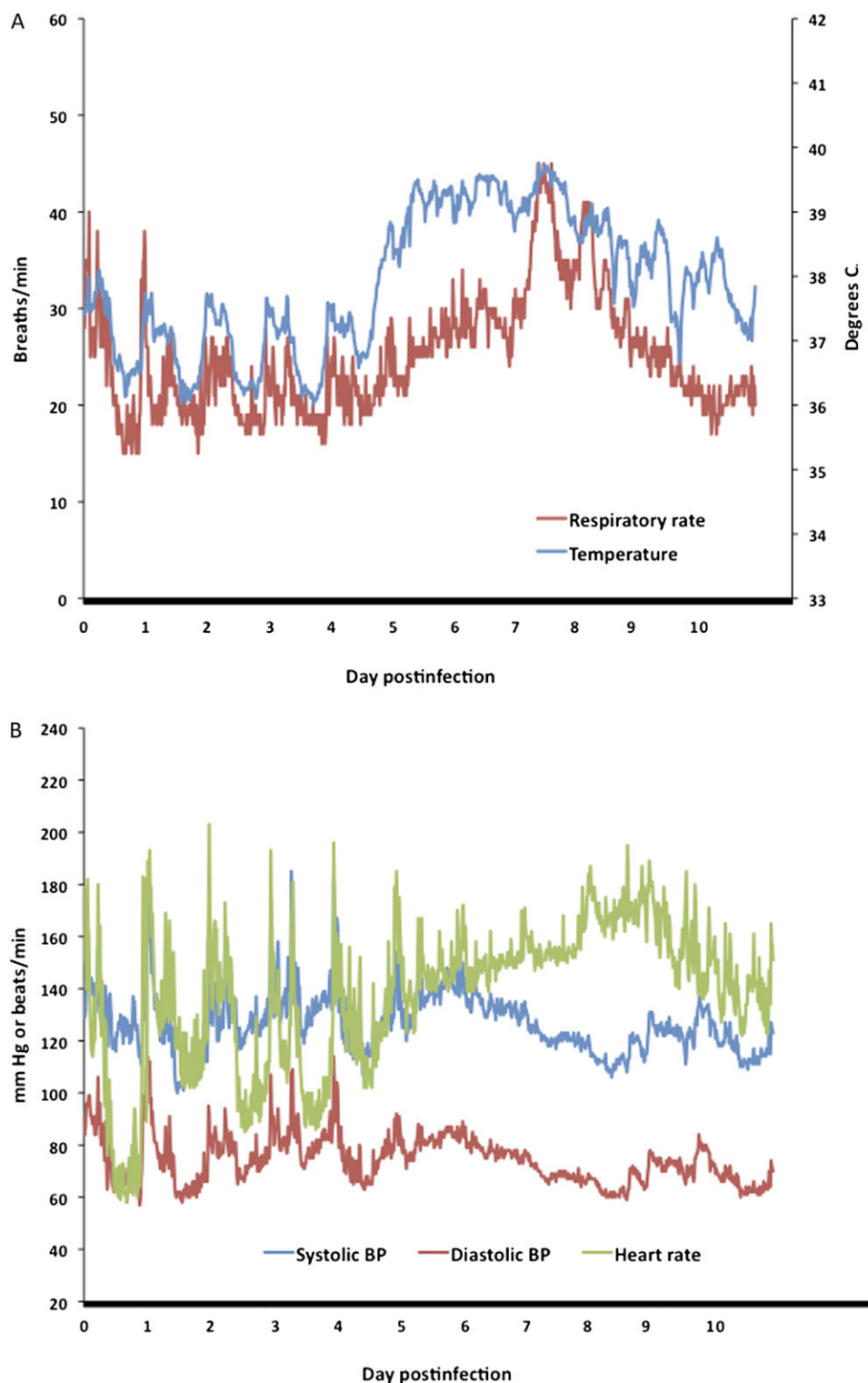


Figure 3. Telemetry data from a macaque that survived infection (#1) shown as 15-minute averages of readings recorded every minute. (A) Body temperature and RR. In contrast to the fatally infected macaque (Figure 2), the temperature and RR rise together and then return to baseline as the illness resolves. (B) Cardiovascular function. Although there is a loss of diurnal variation in HR, SBP, and DBP with the onset of fever, these parameters do not deviate as far from baseline as the nonsurvivor (Figure 2) as illness develops and then resolves.

animal was euthanized (Figure 2B). As hypotension developed, the HR rose in concert, suggesting a picture of compensated shock.

In the 3 macaques that survived infection, the HR also increased after the onset of fever, with a loss of diurnal variation, but with the subsequent return of body temperature to normal it

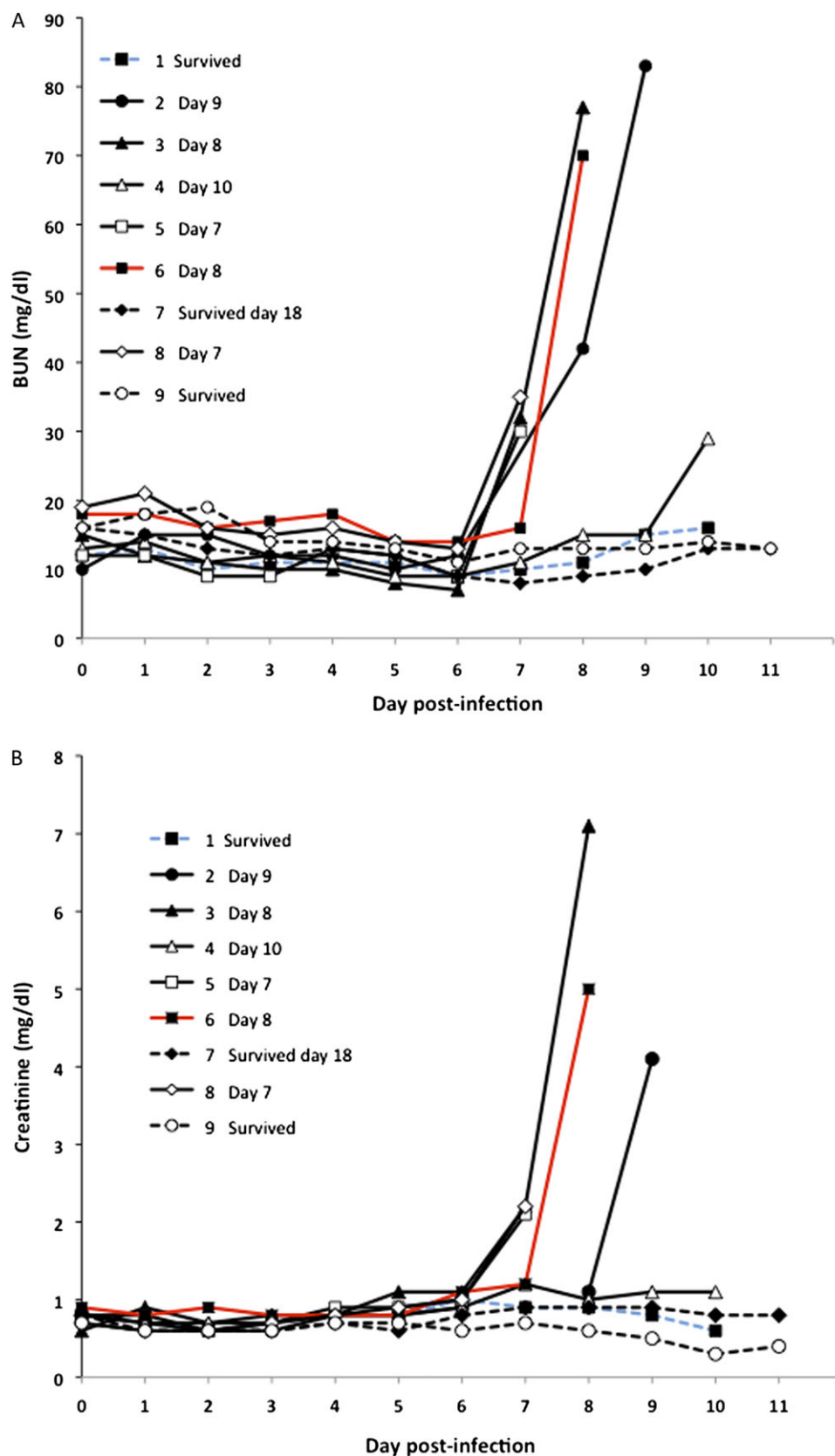


Figure 4. Indices of renal function. Fatally infected, surviving, fluid-treated, and untreated macaques are indicated as described in the legend of Figure 1. (A) Blood urea nitrogen. (B) Creatinine. Evidence of terminal renal failure is most prominent in 3 of the 6 fatally infected animals that did not receive supplemental intravenous fluids.

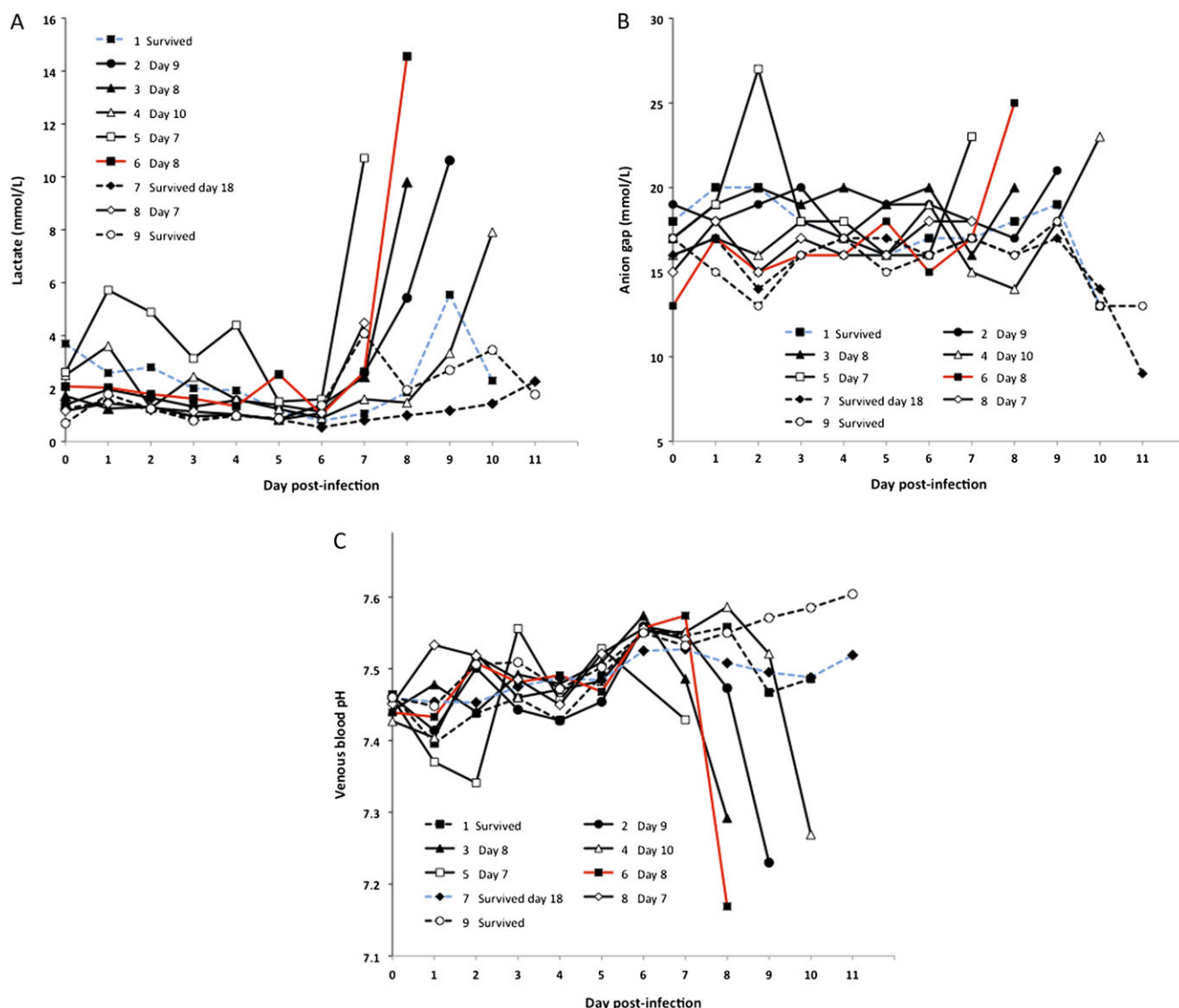


Figure 5. Indices of acid–base balance. Fatally infected, surviving, fluid-treated, and untreated macaques are indicated as described in the legend of Figure 1. (A) Serum lactate concentration. (B) Anion gap, calculated as $([Na^+] + [K^+] - [Cl^-] - [HCO_3^-])$. (C) Central venous blood pH. Lactic acidosis, with an increase in the anion gap and a drop in central venous pH, is seen in both fluid-treated and untreated animals.

began to decline, and HR returned to baseline by day 11 (Figure 3). Two to 5 days after the onset of fever, the SBP, DBP, and MAP declined to subnormal levels, matched by a concomitant rise in heart rate, but they then stabilized and gradually returned to normal.

Renal Function

Most lethally challenged macaques showed a sharp terminal rise in serum BUN and creatinine, as previously observed in ZEBOV-infected laboratory animals [6, 11–13]. In contrast, the survivors maintained a BUN below 20 mg/dL and a creatinine level below 1.0 mg/dL (Figure 4A and Figure 4B). On the day of euthanasia, the lethally challenged animals that had not received supplemental intravenous fluids had average BUN levels more than twice as high (77 versus 31 mg/dL) and average creatinine

levels 3 times as high (5.4 versus 1.8 mg/dL) as those that had received supplemental fluids, suggesting that fluid treatment helped to maintain renal perfusion.

Lactate, Anion Gap, and Venous Blood pH

Beginning on day 7, lethally challenged animals showed a rapid increase in serum lactate, reaching levels above 8 mmol/L on the day they were euthanized (Figure 5A). In contrast, only one surviving animal developed a lactate level greater than 4 mmol/L. The anion gap, calculated as $([Na^+] + [K^+] - [Cl^-] - [HCO_3^-])$, increased as lactic acidosis developed (Figure 5B). The central venous pH began to rise in all animals at the onset of fever, but in lethally challenged macaques it then fell below normal as serum lactate rose (Figure 5C).

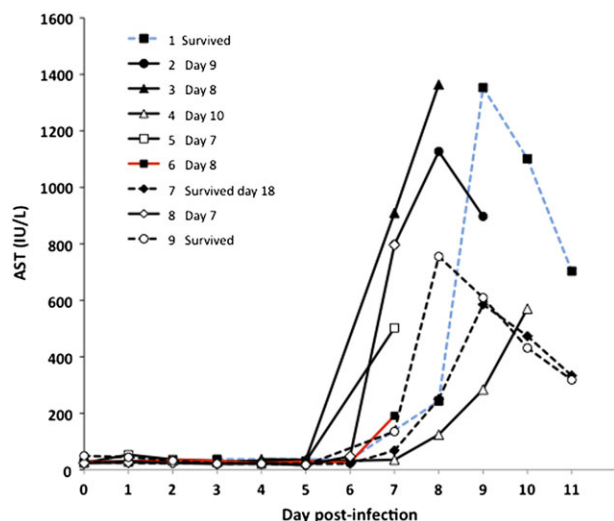


Figure 6. Serum aspartate aminotransferase (AST), an index of hepatocellular and other organ injury. Fatally infected, surviving, fluid-treated, and untreated macaques are indicated as described in the legend of Figure 1.

In contrast to tests of renal function, there was no clear difference in serum lactate levels between lethally challenged animals that did or did not receive supplemental fluids. All 3 survivors showed an abrupt decrease in the anion gap on days 10 and 11, corresponding with a rise in the serum bicarbonate and venous pH. In 2 of the 3 animals, this corresponded with a decline in serum lactate.

Liver Function Tests

Changes in the serum concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) resembled those previously observed in ZEBOV-infected laboratory animals [6, 11–13]. The difference in AST levels between lethally challenged animals and survivors was less clear-cut than for parameters such as BUN or lactate (Figure 6), possibly because AST is not specific for a single organ but is found in numerous tissues. In all but one animal, the peak AST level was higher than the peak ALT level (not shown). On the day of the highest AST level, all lethally challenged macaques showed an AST/ALT ratio >2.0 , but the ratio was <2.0 in 2/3 survivors.

Blood Cell Counts

All 9 animals showed a decline in hemoglobin and hematocrit over the course of illness (not shown). Changes in leukocyte and platelet counts resembled those previously reported for ZEBOV infection of laboratory animals [6, 11–13]. There was an early leukocytosis, peaking at 12 000–25 000 cells/mL on days 4–7; values over 15 000 cells/mL were seen only in lethally challenged animals (Figure 7A). Both nonsurvivors and survivors showed an increased percentage of neutrophils and a decline in the percentage of lymphocytes over the course of illness (Figure 7B).

The platelet count fell significantly in all animals, beginning on days 2–4 (Figure 7C). When collection of blood samples ceased on day 11, all 3 survivors were still thrombocytopenic.

Effects of Supplemental Fluid Administration

As noted, provision of supplemental fluids to 4 randomly chosen macaques did not result in an appreciable change in survival or time to euthanasia. Three treated animals were euthanized on days 7, 7, and 10, and one survived, while 3 untreated animals were euthanized on days 8, 8, and 9, and 2 survived. Although investigators sometimes observed transient improvements in MAP or HR during supplemental fluid administration, no sustained effects were observed. The provision of supplemental fluid did not result in appreciably increased respiratory distress or respiratory rates, except in one survivor (#9) that received the most fluids (1121 mL; 183 mL/kg over 40 hours) and somewhat earlier in the disease course. Fluid therapy did appear to benefit renal function, as the BUN and creatinine of the 3 untreated, lethally challenged macaques ranged from 70 to 83 and 4.1 to 7.1 mg/dL, respectively, on the day of euthanasia, compared with 29–35 and 1.1–2.2 mg/dL for the 3 treated, lethally challenged animals (Figure 4A and 4B). Although the number of animals is too small for meaningful statistical analysis, the trend toward preservation of renal function suggests that these parameters should be examined in a larger study.

DISCUSSION

This is the first published report to describe the use of telemetry to monitor physiological parameters in filovirus-infected non-human primates, much as severely ill patients are monitored in an intensive care unit. It is also one of the first studies to obtain blood samples from infected macaques without subjecting them to repeated sedation. By tracking body temperature, RR, HR, SBP, and DBP over the entire course of illness, together with daily viral titers and clinical laboratory values, we obtained the most detailed picture to date of the clinical course of ZEBOV HF in nonhuman primates.

Ideally, we would attempt to validate this animal model by comparing our data with the features of illness in patients with filoviral HF, but because so few data from human cases have been published, only a limited comparison can be made [2]. Both macaques and humans show the abrupt onset of fever, development of a rash, leukocytosis with lymphocytopenia, thrombocytopenia, increase in the serum AST level, development of renal failure, and a terminal decline in blood pressure. We find it especially interesting that tachypnea (presumably corresponding with metabolic acidosis), which occurred in all febrile macaques and progressively increased in lethally challenged animals, is also a feature of terminal ZEBOV HF in humans [14, 15], because it means that the RR may provide a useful index of disease severity in patients for whom

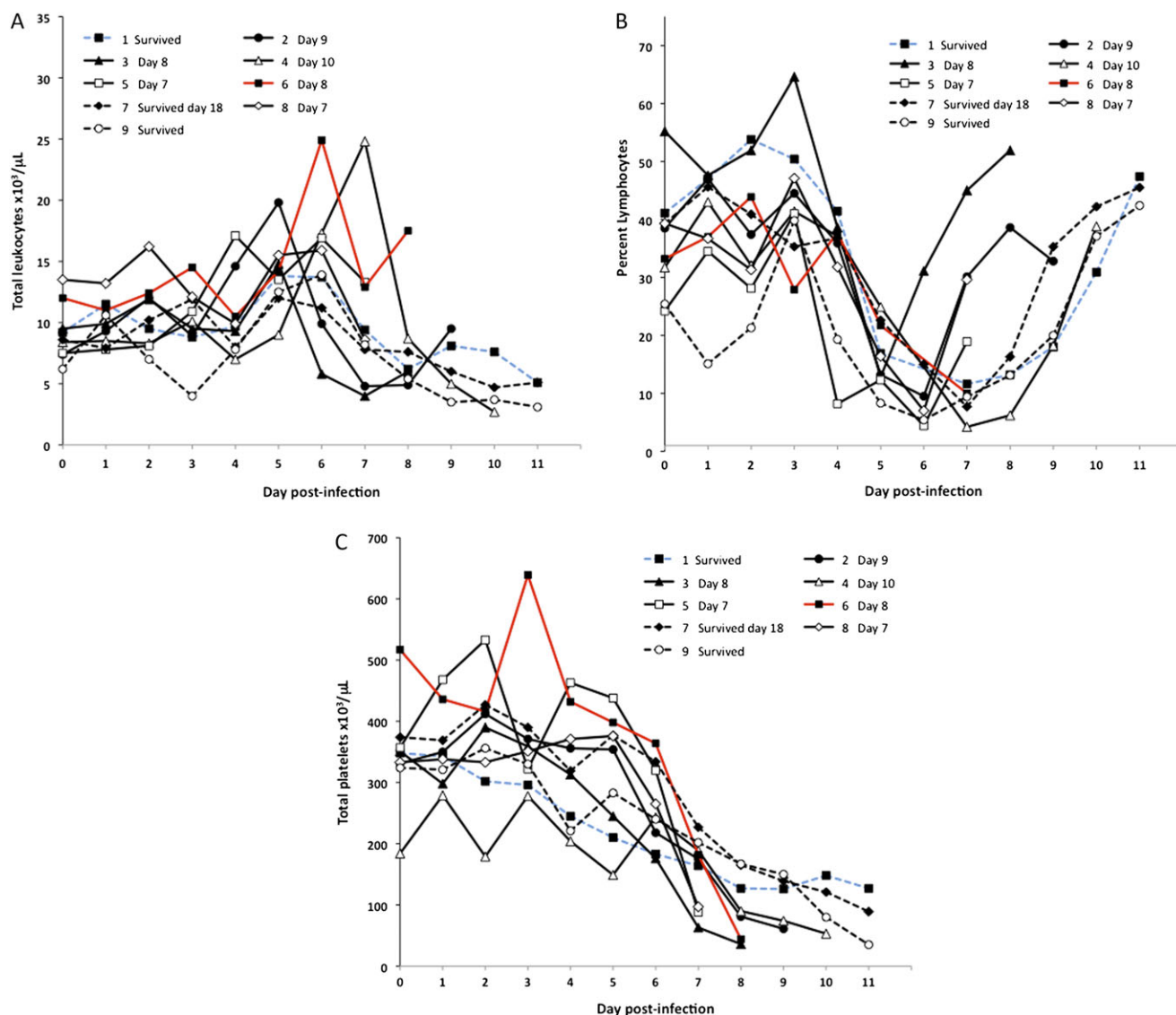


Figure 7. Changes in blood cell counts. Fatally infected, surviving, fluid-treated, and untreated macaques are indicated as described in the legend of Figure 1. (A) Total leukocyte count. (B) Percentage of lymphocytes in the total leukocyte count. (C) Platelet count.

more advanced monitoring is not possible. The HR, SBP, and DBP are also critically important indexes for clinical management, but we cannot comment on similarities between macaques and humans, because so few data from patients have been published.

An unanticipated aspect of our study was the survival by 3 animals of ZEBOV HF, which until now has been considered a uniformly lethal disease in nonhuman primates. In the above-mentioned studies in which rhesus macaques were used to assess novel forms of postexposure prophylaxis, more than 25 animals served as concurrent or historical controls for ZEBOV infection, and all of them died, with a mean time to death of about 8 days [7–10]. In 2 of those studies, both experimental and control animals received a constant low-level infusion of isotonic saline through a CVC, so we cannot attribute the survival of our

3 animals to catheter maintenance fluids [8, 9]. Instead, the major difference between our study and previous experiments appears to be the lower challenge dose of virus, as other investigators have inoculated macaques with 1000 PFU of ZEBOV, while we gave approximately 50 PFU. Although a challenge dose as low as 6 PFU is lethal for cynomolgus macaques [16], it is possible that rhesus macaques, which show a later onset of illness and longer time to death after ZEBOV challenge [17, 18], are somewhat more resistant to the virus. The earlier studies of rNAPc2 and rhAPC treatment in rhesus macaques also identified “responders” and “nonresponders” to therapy [7, 8], suggesting that these outbred animals differ in their response to infection.

The clearest evidence of susceptibility or resistance to ZEBOV infection is the extent of systemic viral replication, as reflected

in the level of viremia. In our study, the lowest peak viral titer of a lethally challenged animal exceeded the highest peak titer of a survivor by more than 10-fold, and mean peak values for nonsurvivors and survivors (2.4×10^6 and 1.6×10^4 relative PFU equivalents/mL, respectively) differed more than 100-fold. These findings resemble those in the previously cited studies of postexposure prophylaxis, in which peak serum titers of infectious virus were significantly lower in treated animals that survived infection [7, 8]. Similarly, reports from African outbreaks have shown that the persistence of high viral titers in blood samples is predictive of a fatal outcome [19, 20]. If low-dose ZEBOV challenge regularly results in a range of outcomes in rhesus macaques, it may prove to be a useful model for studying human variation in resistance to filoviral HF. In the only study of this question to date, the survival or death of patients with *Sudan ebolavirus* HF was linked to 2 human leukocyte antigen-B loci [21].

The progressive decline in blood pressure that began on days 5–6 in lethally challenged macaques is presumably the result of multiple physiologic changes, similar to septic shock in humans, in which organ hypoperfusion is manifested by the development of lactic acidosis and renal failure. Although the logistics of coordinating therapy in a BSL-4 laboratory limited optimal timing of intravenous fluids, the persistent hypotension we observed despite supplemental fluids is consistent with septic shock [22]. We would therefore not expect that supplemental intravenous fluids alone would be sufficient to salvage the majority of severe cases of filoviral HF, although the improved renal parameters in the treated animals indicates that further study of intravenous fluids, perhaps earlier in illness before hypotension, is warranted. In addition, any successful treatment regimen in macaques will probably resemble the currently recommended approach for septic shock: early, multifaceted, goal-directed therapy, in which vasopressors such as norepinephrine are given to maintain blood pressure and adequate tissue perfusion, while intravenous fluids are given to replenish and maintain intravascular volume [23–25]. Thanks to recent advances in experimental therapy, treatment of ZEBOV HF in macaques might also include a medication that directly targets the infectious agent, such as siRNA or PMOs, and an anticoagulant or immunomodulator, such as rNAPc2 or rhAPC, all of which have been beneficial when begun in the early post-exposure period [7–10]. The availability of these therapeutic agents, and our demonstration that physiological parameters can be monitored in real time in infected animals, means that opportunities to model the treatment of ZEBOV HF are now within reach.

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