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SUPPLEMENT ARTICLE

Marburg and Ravn Viruses Fail to Cause Disease in the Domestic Ferret (*Mustela putorius furo*)

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The domestic ferret was recently described as a uniformly lethal model for 3 species of *Ebolavirus*. More importantly, this new model utilizes nonadapted wild-type *Ebolaviruses*. Here, in a proof-of-concept study, we infected ferrets with different variants of the closely related Marburg and Ravn viruses using different doses and routes of exposure. Although ferrets produced a neutralizing humoral response to challenge, we did not observe disease or viremia in any animal. The lack of disease in ferrets underscores the notion that differential mechanisms to immunity among filoviruses exist and may provide a model to better understand how differences contribute to disease.

Keywords. Ebola virus; ferret; filovirus; Marburg virus; Ravn virus.

Marburg virus (MARV) is the etiological agent responsible for several small outbreaks of severe hemorrhagic fever in Germany and Serbia in 1967 in connection with infected nonhuman primates (NHP) originating from Africa [1]. Since then, over a dozen outbreaks have occurred sporadically throughout Central Africa ranging in size from 1 to 252 with case fatality rates (CFR), ranging from approximately 20% to 90% [2]. Most research on MARV has used a small number of isolates including Musoke and Angola and the genetically distinct Ravn virus (RAVV). The Musoke variant was isolated in 1980 from a nonfatal case from an outbreak of only 2 individuals [3]. Ravn virus was first isolated in 1987 from a single case originating in southeastern Kenya, yet it has also been associated with large outbreaks of Marburg hemorrhagic fever (MHF) in circulation with other variants of MARV, thus making CFR calculation impossible [4, 5]. The Angola variant was responsible for the largest documented outbreak (252 cases) with a 90% CFR rate [6]. This epidemiological data coupled with the increased virulence of the Angola variant in primate and rodent models suggest that this isolate may be more virulent than Musoke and RAVV [7, 8].

Currently, there are no regulatory agency approved vaccines or therapeutics for MHF. The recent *Zaire ebolavirus* (EBOV) outbreak in West Africa, where over 11 000 fatalities resulted, underscored the desperate need for approved medical countermeasures for filovirus infections. During the course of this outbreak, a number of novel medical countermeasures were identified; however, screening and validation of these interventions in relevant animal models were limited by the use of host-adapted small animal models or the availability of NHP resources [9].

Historically, NHPs have been considered to be the "gold standard" for modeling filovirus pathogenesis for countermeasure development because they recapitulate many features of human disease including uncontrolled viral replication, unbridled immune response, vascular leak, and coagulopathy without the need for virus adaptation [9]. Many immunocompetent small animal models have been developed for different variants of MARV and RAVV including mice, hamsters, and guinea pigs; however, almost all of these require host adaptation to appropriately serve as models [9]. Some severely immunodeficient mouse models have been described allowing the use of native virus; however, there are considerable limits in the utility of these models in medical countermeasure development where reliance on an intact immune response is essential [9].

The domestic ferret (*Mustela putorius furo*) serves as a lethal animal model for a variety of diseases using viral isolates derived directly from human patients [10]. The ferret has recently been described as a uniformly lethal model for 3 species of *Ebolavirus* known to be pathogenic in humans and NHPs, including the following; EBOV, *Sudan ebolavirus* (SUDV), and *Bundibugyo ebolavirus* (BDBV) [11, 12]. Given the success with *Ebolavirus* infections in ferrets, we conducted a series of experiments to assess the capacity of the ferret to similarly serve as a small animal model for different isolates of MARV and RAVV.

MATERIALS AND METHODS

Animal Challenge, Disease Monitoring, and Biological Sampling

Female ferrets weighing 0.75–1 kg were housed 2–3 per cage per study. Ferrets were anesthetized by intramuscular injection with a ketamine-acepromazine-xylazine cocktail before

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all procedures. Before challenge, transponder chips (BioMedic Data Systems) were subcutaneously implanted for identification and temperature monitoring. Subjects were challenged intranasally (IN) or intraperitoneally (IP) with a 1000 plaque-forming units (PFU) dose of MARV variant Angola (n = 5), MARV variant Musoke (n = 2), or RAVV (n = 2), respectively. In a follow-up experiment, a high dose of 100 000 PFU of MARV-Angola was administered IN (n = 2) and IP (n = 2). Passage history of challenge viruses is provided in the Supplementary Methods. For the first experiment involving MARV-Angola IN challenge (1000 PFU), whole blood and ethylenediaminetetraacetic acid plasma samples were collected from the superior vena cava for hematology, serum biochemistry, and viremia determination before virus challenge on day 0 and on postinfection days 4, 6, 8, and at study endpoint on day 21. For all experiments, clinical signs, weights, and transponder-mediated temperatures were recorded daily up to study endpoint of 21 days postinfection. Clinical scores were determined on a scale of 0–12 based on coat appearance, social behavior, and provoked behavior as approved per University of Texas Medical Branch Institutional Animal Care and Use Committee protocol criteria. At study endpoint, gross pathology findings were documented, and portions of select tissues were aseptically removed and frozen at −70°C for virus infectivity assays. Portions of select tissues were also fixed in formalin and processed for histologic and immunohistochemical analyses, as shown in the Supplementary Methods.

Hematology and Serum Biochemistry

Complete blood counts and serum blood chemical analyses were performed on blood and serum specimens obtained from ferrets infected with MARV-Angola (1000 PFU/IN). Analysis details are provided in the Supplementary Methods.

Circulating Infectious Virus and Viral Genome Quantitation

Ribonucleic acid (RNA) was isolated from whole blood utilizing the Viral RNA mini-kit (QIAGEN, Valencia, CA) using 100 µL blood into 600 µL buffer AVL. Primers or probes targeting the NP gene of MARV were used for real-time quantitative polymerase chain reaction, as used previously [13]. Determination of infectious virus in plasma was performed using conventional plaque assays as detailed in the Supplementary Methods.

Humoral Immune Response to Marburg Virus Glycoprotein

Neutralizing antibody titers were determined by performing plaque reduction neutralization titration assays (PRNT). In brief, Vero cells were seeded into 6-well plates to generate a confluent monolayer on the day of infection. Serum dilutions from day 0 before virus challenge and days 4, 6, 8, and 21 postinfection were prepared in Dulbecco's modified Eagle's medium, and 100 µL was incubated with ~100 PFU of MARV-Angola in a total volume of 300 µL. Media was removed from cells, the serum-virus mixture was added, and samples were incubated for 60 minutes at 37°C. The mixture was removed from the cells, and 2 mL 0.9% agarose in Eagle's minimum essential medium with 5% fetal bovine serum was overlayed onto the wells. Cells were observed 7 days postincubation and plaques were counted. The neutralizing antibody titer of a serum sample was considered positive at a dilution showing a ≥50% reduction ($PRNT_{50}$) compared with the virus control without serum.

Statistics Statement

Conducting animal studies in biosafety level 4 severely restricts the number of animal subjects, the volume of biological samples that can be obtained, the ability to repeat assays independently, and thus limits the power of statistical analyses. Consequently, data are presented as the mean calculated from replicate biological samples, not replicate assays, and error bars represent the standard deviation across replicates.

RESULTS

Clinical Scoring Hematology and Serum Biochemistry

Compared with historical controls of EBOV (Kikwit variant, EBOV-Kikwit), SUDV (Gulu variant, SUDV-Gulu), and BDBV, no ferrets in this work succumbed to infection regardless of route, dose, or variant of MARV or RAVV challenge (Figure 1) [11]. No substantial changes from baseline hematology or serum biochemistry were noted at any time after virus challenge (Supplementary Table). No perturbations in clinical score parameters, core temperature, or body weight were noted at any time after any ferret MARV or RAVV challenge (data not shown).

Circulating Virus and Viral Genome Quantitation

No infectious MARV or RAVV was detected on days 4, 6, 8, or 21 postinfection; however, viral genomes were detected on day 6 from 1 animal at 8.35e6 viral genomes/mL blood (data not shown).

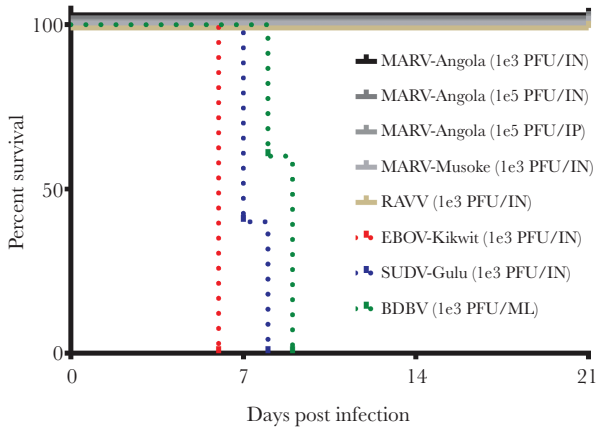

Figure 1. Survival of ferrets infected with filoviruses. *Ebolavirus* data taken from historical controls (N = 5) [11]. Marburg virus (MARV)-Angola 1e3 intranasal (IN) challenge (N = 5). All other *Marburgvirus* or Ravn virus challenges (N = 2).

Humoral Immune Response to Marburg Virus Glycoprotein

We performed a $PRNT_{50}$ assay to assess whether any neutralizing antibody response was present in MARV-challenged ferrets. It is interesting to note that a significant amount of pre-existing neutralization capacity was present in lower dilutions (≥1:40) up to day 6 postinfection. Neutralization capacity of serum increased successively from day 8 (1:80) and study endpoint (day 21, 1:320) and thus verifying successful challenge by initiation of a MARV-specific humoral immune response (Figure 2).

Pathology

No significant lesions were observed on gross examination of tissues of any MARV-Angola challenged ferret at necropsy. In addition, no significant lesions or immunolabeling was noted for hematoxylin and eosin- and immunohistochemistry-stained liver and spleen tissue sections for MARV-Angola challenged ferrets. Conversely, historical control tissues of EBOV-infected ferrets were characterized by lymphohistiocytic and neutrophilic necrotizing hepatitis and necrotizing splenitis. Immunolabeling of EBOV antigen was noted in hepatic sinusoidal mononuclear cells, hepatocytes, and mononuclear cells within the red and white pulp of the spleen (Figure 2).

DISCUSSION

Ferrets have largely been used to study respiratory infections such as influenza, respiratory syncytial virus, and henipaviruses; in all of these viruses, the mucosal routes of challenge

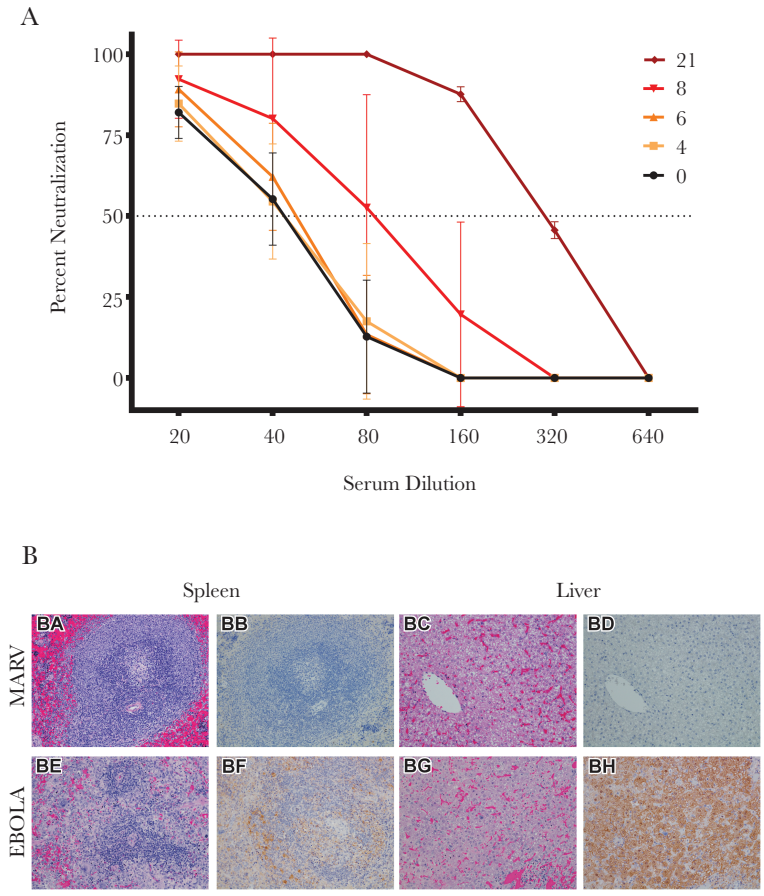

Figure 2. (A) Reciprocal Marburg virus (MARV)-Angola serum neutralizing antibody titers: neutralizing antibody titers that reduce viral plaque formation are shown for serum taken from MARV-Angola-infected ferrets (1000 plaque-forming units/intranasally (IN), N = 5) on select days. Days 0, 4, and 6 demonstrate an approximate plaque reduction neutralization titration ($PRNT_{50}$) at 1:40 dilution. Beginning day 8 and subsequently to study endpoint on day 21, progressively higher neutralizing titers are observed. Lines with symbols represent days postinfection. Dashed line represents $PRNT_{50}$ threshold. Error bars represent the standard deviation from the mean. (B) Histology and immunohistochemistry: histopathology of filovirus-infected ferrets. (BA–BH) Histopathology of spleen and liver of MARV-Angola-infected ferrets: hematoxylin and eosin (H&E) (BA and BC), corresponding anti-*Marburgvirus* immunohistochemistry (IHC) (brown) (BB and BD). Marburg virus-Angola-infected ferret spleen (BA) and liver (BC) H&E had no significant lesions, corresponding spleen (BB) and liver (BD) with anti-*Marburgvirus* IHC had no significant immunolabeling. Histopathology of spleen and liver of Ebola virus (EBOV)-infected ferrets: H&E (BE and BG) and corresponding anti-*Ebolavirus* IHC (brown) (BF and BH). The EBOV-infected ferrets had splenitis with lymphoid depletion, tingible body macrophages, and apoptotic cellular debris within the white pulp (BE) diffuse cytoplasmic anti-*Ebolavirus* immunolabeling of mononuclear cells in red and white pulp (BF). The EBOV-infected ferrets had lymphohistocytic and neutrophilic necrotizing hepatitis (BG) with corresponding diffuse cytoplasmic anti-*Ebolavirus* immunolabeling of mononuclear cells and sheets of hepatocytes (BH). All H&E and IHC images are ×20.

have been the most pertinent means of infection in the context of human infection [10]. In recent studies, ferrets have been shown to be particularly sensitive to *Ebolavirus* infection to the extent that even infection with BDBV (25%–51% CFR in humans and 67%–75% in NHP) is uniformly lethal [8, 14]. Although rodent models for MARV and RAVV have been described, these models require serial passage to achieve uniform lethality. Thus, this work attempted to build on the success of previous groups with establishing an *Ebolavirus* ferret model. Despite several attempts, we present the surprising finding that regardless of dose, route, or variant, MARV and RAVV do not cause observable disease in ferrets.

Mucosal infection has been suggested as the most likely means of filovirus transmission in humans and has also been demonstrated as an effective method of artificial infection in animal models [11, 15]. Previous successes with IN infection of ferrets with *Ebolaviruses* suggested that this might be a viable route to start with; thus, we first carried out a pilot study with a dose previously shown to be effective with ferrets and 3 species of *Ebolavirus* [11]. After failing to produce disease by this route, we attempted to validate this finding using another commonly used variant of MARV (Musoke) and RAVV using the same route and dose. After this second failure, we attempted a high-dose exposure via IN instillation as well as IP injection because the latter route has been shown to be important for virulence in mouse and guinea pig models of filovirus infection [9]. Despite these multiple attempts, none of these approaches produced discernable disease in ferrets, and no evidence of residual virus or significant pathology remained in tested tissues known to be targets of filovirus infection.

It is uncertain why the ferret is resistant to MARV and RAVV infection yet particularly sensitive to infection by *Ebolaviruses*. It is tempting to speculate that contributions from the many secreted or shed forms of the *Ebolavirus* glycoprotein are key to this difference: Marburg virus and RAVV infections are not known to produce similar forms of their respective glycoprotein [2]. There are also several mechanistic differences in cellular subversion of the innate immune system by *Ebolaviruses* versus MARV and RAVV, which may also contribute to resistance. The availability of reverse genetics systems for these viruses may prove useful for isolating the key viral contributions to differences in susceptibility, and the availability of a draft ferret genome may assist investigators in understanding how the host differentially responds to each filovirus genus.

CONCLUSIONS

Taken together, the ferret model not only offers a new and sensitive model for understanding *Ebolavirus* pathogenesis, but it also opens the possibility to further understand how differences between *Ebolaviruses* and *Marburgviruses* contribute to virulence in different species. To this end, despite the uniform lethality observed after infection with EBOVs, these results suggest that the ferret may not be a reliable model for generalized assessment of filovirus pathogenesis as it relates to the human condition.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Disclaimer. The opinions, interpretations, conclusions, and recommendations contained herein are those of the authors and are not necessarily endorsed by UTMB.

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Potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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