Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication

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Nipah virus (NiV) is a bat-borne zoonotic pathogen that can cause severe respiratory distress and encephalitis upon spillover into humans. NiV is capable of infecting a broad range of hosts including humans, pigs, ferrets, dogs, cats, hamsters, and at least 2 genera of bats. Little is known about the biology of NiV in the bat reservoir. In this study, we evaluate the potential for the Egyptian fruit bat (EFB), *Rousettus aegyptiacus*, to serve as a model organism for studying NiV in bats. Our data suggest that NiV does not efficiently replicate in EFBs in vivo. Furthermore, we show no seroconversion against NiV glycoprotein and a lack of viral replication in primary and immortalized EFB-derived cell lines. Our data show that despite using a conserved target for viral entry, NiV replication is limited in some bat species. We conclude that EFBs are not an appropriate organism to model NiV infection or transmission in bats.

Keywords.   bats; Egyptian fruit bats; experimental infection; Nipah virus.

Nipah virus (NiV) is a zoonotic pathogen that can cause acute respiratory illness and fatal encephalitis upon spillover into human populations. Since its discovery in Malaysia in 1998, NiV has emerged as a persistent public health problem in southeast Asia, warranting inclusion on the World Health Organization Blueprint list of priority diseases for which research toward effective countermeasures is urgently needed [1]. Unlike other paramyxoviruses, NiV has broad host tropism, with multiple taxa supporting viral replication, including ferrets, hamsters, cats, dogs, African Green monkeys, squirrel monkeys, and pigs [2–5]. NiV uses 2 major envelope glycoproteins to enter the host cell: the receptor-binding protein (G) and the fusion protein (F) [6]. After attachment of the NiV G to the host cell receptor, ephrin-B2 or ephrin-B3, NiV F fusogenic activity, is triggered leading to the merger of virion and host cell membranes and subsequent virus infection [7–11]. Ephrins are highly conserved across mammalian taxa given their key role in development of the central nervous system [12], likely contributing to the unusually broad host range of NiV.

Fruit bats in the genus *Pteropus* have been identified as the primary reservoir hosts for NiV [13]. Spillover of NiV from bats to humans is thought to occur through ingestion of food or liquids contaminated with infected bat urine [14]. *Pteropus* spp bats are known to feed from the spigots of date palm sap collection jars, often urinating into and contaminating the collection jars before human consumption [14, 15]. Although *Pteropus*

spp bats are implicated as the primary reservoir for NiV, they comprise only a fraction of the fruit bats that visit to date palm sap collection jars in Bangladesh [15]. Several other fruit bat species overlap with the distribution of *Pteropus* spp bats and visit date palm sap collection jars; if other bats are secondary NiV reservoirs, they may also contribute to NiV spillover.

Less is known about the infection and enzootic transmission dynamics of NiV in reservoir populations or between coroosting bat species. NiV has been isolated from the urine of both wild-caught [16–18] and experimentally challenged [19] *Pteropus* spp bats. NiV RNA has also been detected in an insectivorous bat, *Hipposideros larvatus* [20], and serological evidence of NiV infection has been seen in several other species of frugivorous and insectivorous bats, including *Rousettus leschenaultia* [21, 22].

Despite efforts to understand enzootic transmission dynamics, many questions remain regarding the biology of NiV infection in bats. Studies of wild-caught *Pteropus* spp suggest potential for viral recrudescence [16, 23]; however, the hypothesis that NiV may persist in an individual bat and re-emerge under times of stress has yet to be confirmed experimentally.

Pteropus spp bats are suboptimal model organisms for studying NiV due to size and availability. The Egyptian fruit bat (EFB), *Rousettus aegyptiacus*, belongs to the same taxonomic family as *Pteropus* spp, Pteropodidae, and has been successfully used to model Marburg virus transmission [24, 25] and serological cross-reactivity after filovirus challenge [26]. In contrast to *Pteropus* spp bats, EFB are common in zoological settings because they are small, amenable to handling, and reproduce readily in captivity. The EFB transcriptome is well annotated [27], and there have been recent efforts to analyze the genome

in context of antiviral immunity [28]. In this study, we evaluate EFBs as a model system for NiV infection in bats.

METHODS

Ethics Statement

All work with NiV was conducted in the biosafety level (BSL) 4 facility at the Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, following standard operating procedures as approved by the Institutional Biosafety Committee. EFB were sourced from a US-based zoological facility. All animal experiments were approved by the Rocky Mountain Laboratories Institutional Animal Care and Use Committee (ASP no. 2018-042E) and performed following the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) by certified staff in an AAALAC-approved facility.

Virus Preparation

NiV was obtained through the Special Pathogens Branch of the Centers for Disease Control and Prevention (Atlanta, GA). NiV Bangladesh was isolated from a throat swab from a human patient in 2004 (GenBank accession number AY988601); the virus was propagated on Vero-E6 cells and passaged a total of 2 times. The virus stock was deep sequenced at the Rocky Mountain Laboratories Genomics Core Unit before the start of this study to confirm that no fungal or bacterial contaminants were present.

In Vitro Replication Kinetics on Bat Cell Lines

Primary EFB cell lines were generated as previously described [29], with modifications, from kidney (RAKSM) and lung (RALU) tissue samples obtained from an EFB euthanized under BSL2 conditions. In brief, tissue homogenates were washed in phosphate-buffered saline and resuspended in primary cell culture (D12) media containing Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with non-essential amino acids, 12% fetal bovine serum (FBS), 1 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 μg/mL amphotericin, and 1 mM sodium pyruvate. After 1 passage, amphotericin was excluded from the D12 media. Cells were not maintained after 3 passages.

Immortalized bat cell lines including EFB kidney cells (RoNi), *Hypsignathus monstrosus* kidney cells ([HypNi] provided by Marcel A. Müller at the Berlin Institute of Health, Germany), and EFB fibroblast cells ([RE06] provided by Ingo Jordan at ProBioGen AG, Berlin, Germany) were obtained and grown in D12 media without amphotericin. Vero-E6 cells were used as a positive control. Each cell line was seeded in triplicate in 12-well plates and inoculated with NiV at a multiplicity of infection of 0.1 in DMEM supplemented with 2% FBS, 1 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Supernatants were collected at 0, 24, 48, and 72 hours postinoculation and stored in AVL buffer (QIAGEN) at −80°C until inactivation and RNA extraction. After inactivation

of the virus with AVL and ethanol as described in [30], RNA was extracted performed on the QIAcube (QIAGEN) with the Machery-Nagel Nucleospin Virus Core kit (Machery-Nagel). We then performed quantitative real-time reverse-transcription polymerase chain reaction (qRTPCR) as described in [31] on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with the inclusion of a serially diluted known concentration of NiV on each plate to calculate tissue culture infectious dose ($TCID_{50}$/mL) equivalent for each sample.

Ephrin-B2 and Ephrin-B3 Amino Acid Sequence Alignments

Sequences for the NiV receptors ephrin-B2 and ephrin-B3 that have been experimentally demonstrated to support NiV entry [32] were downloaded from National Center for Biotechnology Information (NCBI) in addition to the EFB ephrin-B2 and ephrin-B3 sequences. The sequences for each ephrin were translated before alignment using the MAFFT v7.388 [33] plugin implemented in Geneious Prime 2019.0.4 (Biomatters Ltd).

Inoculation of EFB and Sample Collection

NiV was diluted in sterile DMEM, and 10^5 $TCID_{50}$/mL was administered to each of 12 adult *R. aegyptiacus* bats via the intraperitoneal route of inoculation in a final volume of 200 μL. Oronasal, urogenital, and rectal swabs were collected daily in addition to swabbing the excreta pan of each cage for the first 14 days followed by twice-weekly sampling through 28 days post inoculation (DPI). Temperature and weight of each bat were taken at the time of sampling. Blood was drawn before inoculation, then at 7 DPI, 14 DPI, and 21 DPI for survivors in addition to terminal blood draws at 3 DPI, 7 DPI, and 28 DPI for serological analyses. Tissue samples were taken at necropsy and either stored at −80°C until RNA extraction or placed in 10% formalin for histopathology and immunohistochemistry analysis. RNA extraction and qRTPCR were conducted as
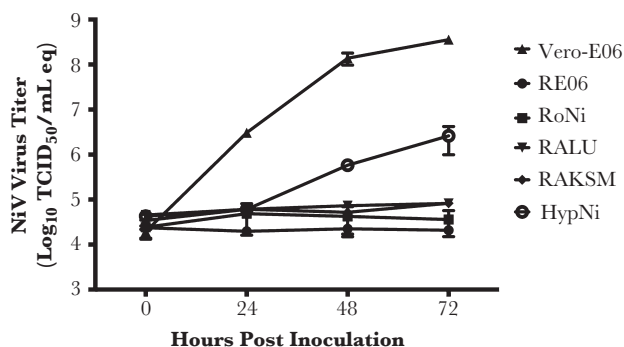

Figure 1. Replication kinetics of Nipah virus (NiV) in Egyptian fruit bat (EFB) primary kidney (RAKSM) and lung (RALU) cell lines, EFB immortalized kidney (RoNi) and fibroblast (RE06) cell lines, and *Hypsignathus monstrosus* bat (HypNi) cell lines relative to African green monkey kidney cell lines (Vero-E6). NiV was added at a multiplicity of infection of 0.1 in triplicate on each cell line and supernatant was collected at 0, 24, 48, and 72 hours post inoculation.

described in [31] and performed on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific).

Serological Analysis

Sera were analyzed for presence of immunoglobulins (Ig) specific to the NiV-G using a Luminex xMAP-based multiplex bead assay adapted from [34]. In brief, blood was collected into serum-separating tubes before centrifugation at 1000 ×*g* for 10 minutes; serum was then collected and frozen at −80°C. Each sample received a dose of 8 MRads irradiation while on dry ice before heat inactivation at 56°C for 30 minutes. Soluble NiV-G (NiV-sG) was produced in a FreeStyle 293-F stable cell-line expression system before purification as described [35] and coupling to Bio-Plex Pro magnetic COOH beads (Bio-Rad). We diluted each serum sample 1:250, and each serum sample was run in duplicate with the Bio-Plex 200 system (Bio-Rad) with purified rabbit IgG against NiV-sG diluted to 1:1000 as the positive control.

RESULTS

In vitro replication kinetics showed no appreciable increase in NiV titer over a 72-hour period on the 4 EFB cell lines tested including the 2 primary EFB cell lines and the 2 immortal EFB cell lines (Figure 1). The hammer-headed (*H. monstrosus*) fruit bat cell line supported moderate NiV replication relative to the Vero-E6 cell line (Figure 1). An alignment of the NiV host receptors ephrin-B2 and ephrin-B3 shows no unique amino acid changes in the critical G protein-binding (G-H) loop between the EFB sequences and the sequences of species that have been confirmed to facilitate NiV entry (Figure 2).

EFBs showed no significant change in temperature or weight throughout the study period, although variability was high for both metrics (Figure 3A and B). We did not detect NiV RNA in any of the tissue samples or swab samples tested by qRTPCR, with no samples amplifying within 40 thermal cycles (Table 1, Supplementary Table 1). None of the bats seroconverted against NiV G during the study period, with a cutoff threshold of 3 times the naive mean
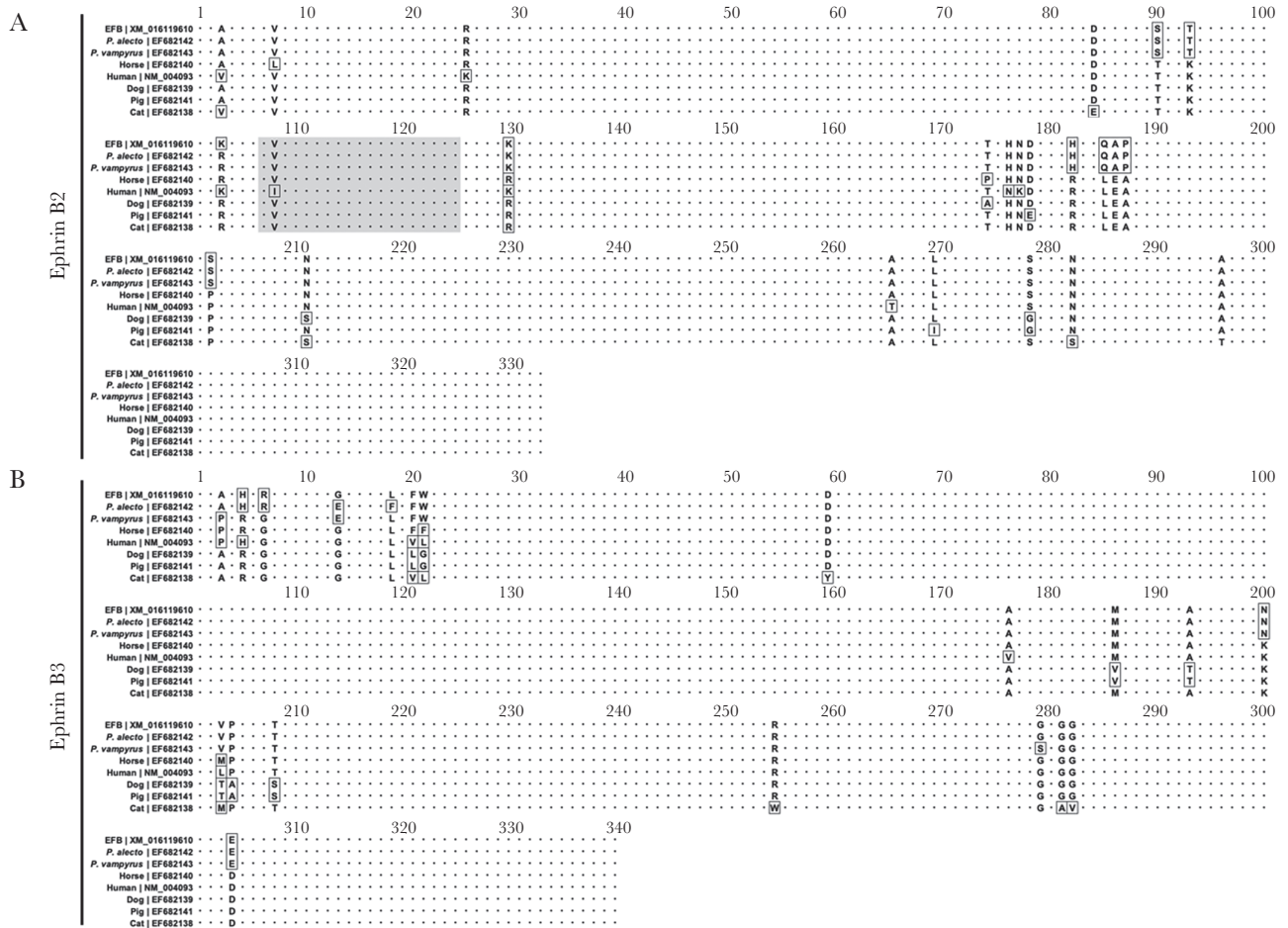

Figure 2. Amino acid sequence alignments for NiV host cell receptors ephrin-B2 and ephrin-B3 from the EFB and sequences of ephrins from species in which NiV entry has been confirmed. A dot indicates that the amino acid sequence is conserved among all aligned sequences. Species and accession numbers are listed to the left of each (*A*) amino acid alignment for ephrin-B2 with the GH loop highlighted in gray and (*B*) amino acid alignment for ephrin-B3.
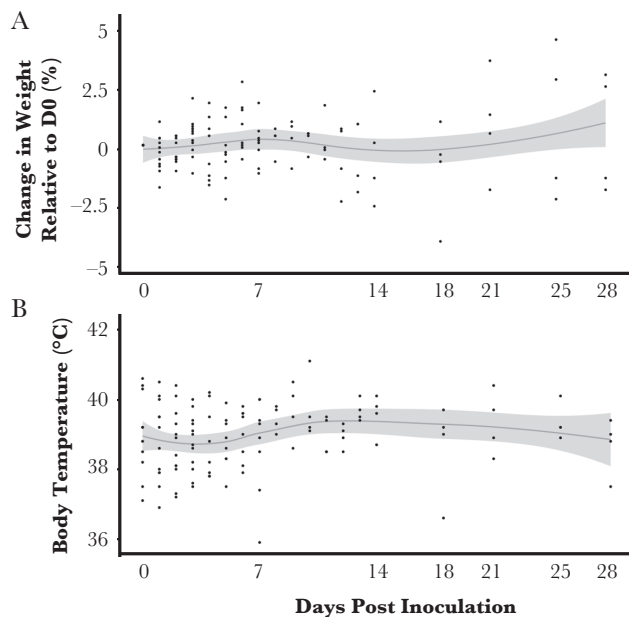
Figure 3. Changes in bat weight and body temperature after inoculation with NiV. (*A*) Bat weights plotted relative to the naive weight of each individual and (*B*) body temperature plotted through 28 days post inoculation with NiV.

Table 1. Tissues Samples Tested for Presence of NiV RNA by qRTPCR

Sample	D3	D7	D28
Lung	>40 (4)	>40 (4)	>40 (4)
Kidney	>40 (4)	>40 (4)	>40 (4)
Bladder	>40 (4)	>40 (4)	>40 (4)
Brain (frontal)	>40 (4)	>40 (4)	>40 (4)
Brain (cerebellum)	>40 (4)	>40 (4)	>40 (4)
Brainstem	>40 (4)	>40 (4)	>40 (4)
Nasal turbinates	>40 (4)	>40 (4)	>40 (4)
Neutralizing antibody titer	0 (4)	0 (4)	0 (4)

Cq values listed, followed by number of individual bats sampled (in parentheses)
Abbreviations: Cq, quantitation cycle; D, day; NiV, Nipah virus; qRTPCR, quantitative real-time reverse-transcription polymerase chain reaction.

fluorescence intensity; however, 1 bat showed a slight increase in mean fluorescence intensity relative to the naive bat serum at 21 and 28 DPI (Figure 4). We confirmed that the inoculum contained 10^5 TCID$_{50}$/mL through back-titrations of the diluted viral stock (data not shown). None of the sectioned tissue samples showed pathology associated with NiV infection, and no NiV antigen was detected via immunohistochemistry staining (Figure 5).

DISCUSSION

Our data show a lack of productive NiV replication in EFBs. Viral RNA was not detected in swab or tissue samples at all time points, suggesting that the bats did not shed virus, nor
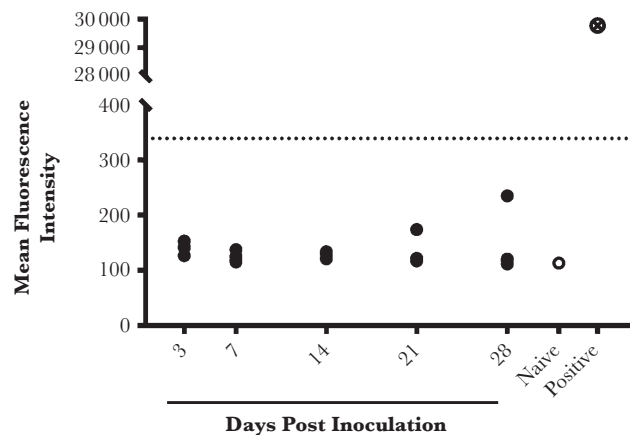

Figure 4. Mean fluorescence intensity values for individual bat serum samples screened with Nipah virus glycoprotein in a Bio-Plex assay (Bio-Rad). Dashed line shows ×3 naive value cutoff. Values for the naive bat serum sample and positive control serum sample are highlighted.

was viral replication detected in any of the tested tissue types (Table 1). Viral replication and shedding are qualities associated with natural hosts [36, 37]. Back-titrations of the viral inoculum confirmed that the EFBs received 10^5 TCID$_{50}$ of NiV, which is higher than the 5×10^4 TCID$_{50}$ NiV challenge that resulted in productive viral replication in guinea pigs and *Pteropus* bats [19]. The lack of detectable virus in any EFB tissue samples 3 days post inoculation suggests that the virus was rapidly cleared; Halpin et al [13] report that henipavirus inoculum is cleared within 48 hours. The bats did not seroconvert against NiV G in the given timeframe of 28 days, although 1 individual had a slight increase in mean fluorescence intensity at 21 and 28 DPI, which may have increased above our cutoff if given more time (Figure 4). Previous studies have demonstrated that EFB cells are permissive to Ebola virus, but EFB challenged here did not shed virus or support productive replication [38, 39] despite compatibility between the Ebola virus glycoprotein and the host receptor, NPC1 [40]. These data suggest that productive viral replication is blocked by a mechanism other than compatibility with the host receptor. Likewise, van Doremalen et al [41] reported a lack of efficient viral replication in EFBs challenged with bat severe acute respiratory syndrome-like coronavirus WIV1 despite in vitro receptor compatibility. Given the lack of unique variation in either EFB ephrin-B2 or ephrin-B3 relative to compatible host ephrin sequences (Figure 2), it is likely that NiV virus replication is not inhibited by poor binding between NiV G and EFB ephrin-B2 or ephrin-B3, nor by the subsequent F-mediated activation and membrane fusion process. Indeed, NiV F and G can mediate productive cell-cell fusion in a variety of different mammalian species, including several that are negative or refractory to productive infection, such as rabbit and mouse [32, 42].
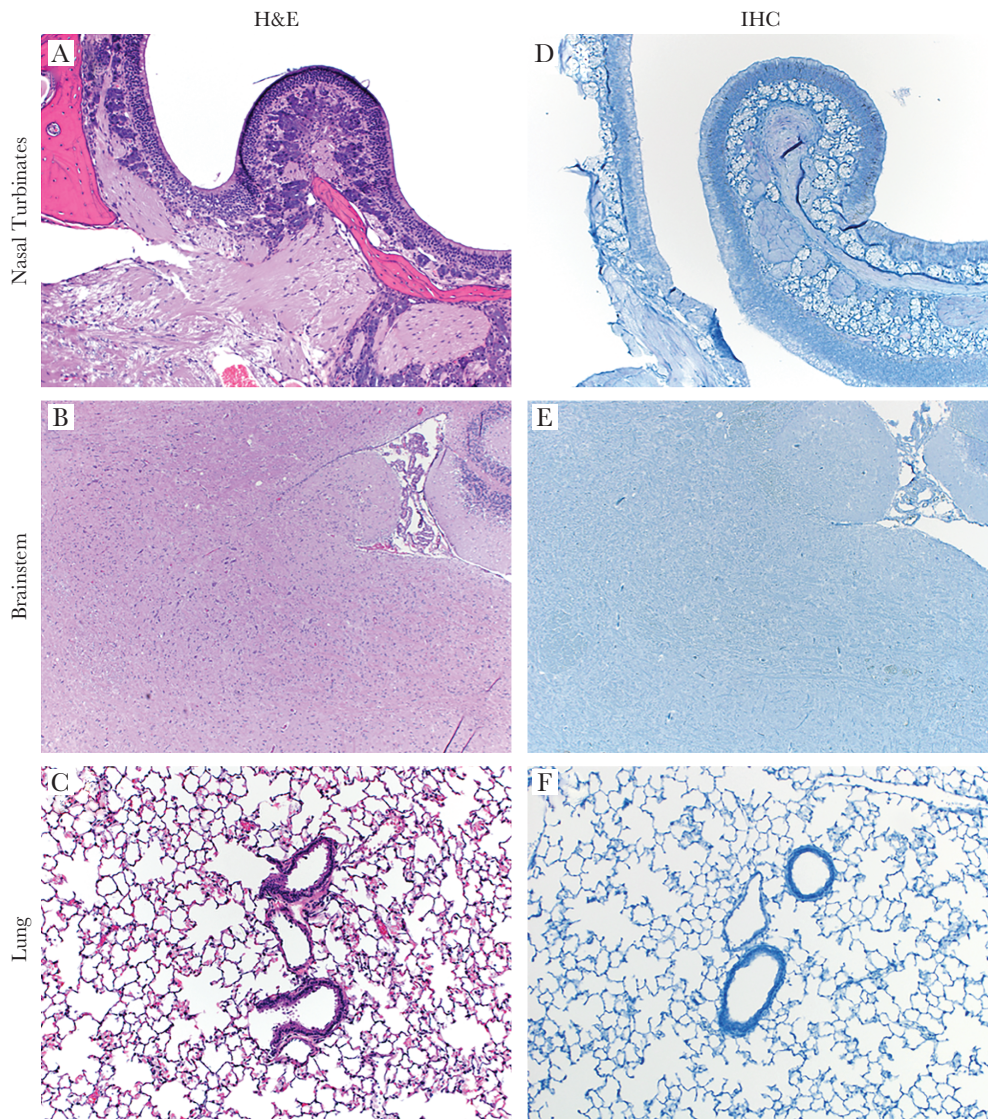
H&E   IHC

Nasal Turbinates — Brainstem — Lung (row labels)

Figure 5. Histopathology and immunochemistry on bat tissue samples after challenge with NiV. (*A–C*) Hematoxylin and eosin (*H&E*) showing no pathological changes and (*D–F*) showing no immunoreactivity with NiV antibody. Nasal turbinates and lung shown at ×100 magnification and brainstem shown at ×40 magnification.

Because we did not detect viral replication or shedding, we conclude that EFBs are not a suitable system for modeling NiV transmission dynamics in bats. However, follow-up studies to determine the mechanism of inhibition of viral replication in EFBs would be valuable in elucidating the evolution of NiV in its natural reservoirs. Few controlled studies have been conducted using bats as a model organism, and, as such, there are few reagents commercially available for studying the immunobiology of bats in response to viral infection. Further research is urgently needed to expand upon the current capacity to conduct research in bats, particularly when considering that 5 of 9 viruses associated with the World Health Organization's Blueprint list of priority diseases [1] likely originated as bat-borne zoonoses.

CONCLUSIONS

Recent studies have applied machine learning algorithms to prioritize surveillance for high-impact pathogens such as NiV and Ebola virus using data on ecological traits, life history, demographic traits, and species distributions [43, 44]. Although it is important to consider broader ecological characteristics in determining potential for a host species to contribute to virus spillover and maintenance, understanding the limitations to reservoir potential on a mechanistic level would further improve predictive modeling work for high-impact pathogens like NiV. Testing viral entry and replication through in vitro assays are important first steps in determining host potential, but should be followed by in vivo experiments when possible.

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