

An Intranasal Exposure Model of Lethal Nipah Virus Infection in African Green Monkeys

Joan B. Geisbert,^{1,2} Viktoriya Borisevich,^{1,2} Abhishek N. Prasad,^{1,2} Krystle N. Agans,^{1,2} Stephanie L. Foster,^{1,2} Daniel J. Deer,^{1,2} Robert W. Cross,^{1,2} Chad E. Mire,^{1,2} Thomas W. Geisbert,^{1,2} and Karla A. Fenton^{1,2}

¹Galveston National Laboratory and ²Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston

Due to the difficulty in conducting clinical trials for vaccines and treatments against Nipah virus (NiV), licensure will likely require animal models, most importantly non-human primates (NHPs). The NHP models of infection have primarily relied on intratracheal instillation or small particle aerosolization of NiV. However, neither of these routes adequately models natural mucosal exposure to NiV. To develop a more natural NHP model, we challenged African green monkeys with the Bangladesh strain of NiV by the intranasal route using the laryngeal mask airway (LMA) mucosal atomization device (MAD). LMA MAD exposure resulted in uniformly lethal disease that accurately reflected the human condition.

Keywords. animal model; henipavirus; Nipah virus; pathogenesis; primate.

Over 20 years ago, Nipah virus (NiV) emerged as a previously unknown paramyxovirus, now classified with Hendra virus and Cedar virus within the *Henipavirus* genus. NiV causes febrile encephalitis and severe respiratory illness in humans, with a case fatality rate (CFR) exceeding 75% in some outbreaks [1]. Pteropid fruit bats have been identified as a reservoir for henipaviruses in nature, although other mammalian species can bridge transmission to humans, as was the case with pigs during the first outbreak of NiV in Malaysia [1]. Furthermore, several other domesticated species are susceptible to NiV infection, including dogs, cats, and horses [1]. NiV is classified as a biosafety level (BSL)-4 pathogen because of the high mortality rates associated with infection, lack of approved medical countermeasures, and ease of transmission. NiV is also categorized as a Category C priority pathogen by several US Government agencies because of the concern for deliberate misuse.

Sequencing of isolates from human infections has identified at least 2 strains of NiV responsible for outbreaks in different geographical areas [2]. The Malaysia strain (NiV_M), which has only been responsible for 2 known outbreaks, Malaysia/Singapore in 1998–1999 and the Philippines in 2014, produced CFRs from 40% to 52% [3]. However, the Bangladesh strain (NiV_B) has caused nearly annual outbreaks in Bangladesh and India since 2001, and has had CFRs averaging approximately 75% from 2001 to 2012 [1, 4]. A more recent outbreak in the South Indian state of Kerala resulted in death in 21 of 23 cases

(91%) [5]. In addition, human-to-human transmission of NiV_M seems to have only played a minor role in driving outbreaks; however, contact with infected persons has served as a primary means of transmission with NiV_B [6]. Human cases of NiV_M have primarily presented as neuroinvasive infections, whereas infection with NiV_B has largely resulted in severe respiratory disease [1, 3, 4, 6]; this feature is thought to have facilitated the apparent increased rate of person-to-person transmission observed in NiV_B outbreaks [7]. The quality of supportive care during different NiV outbreaks could also contribute to different CFRs and disease pathogenesis between NiV_M and NiV_B.

Numerous animal models have been developed to study NiV infection [8]. In particular, the African green monkey (AGM) was shown to most accurately recapitulate the neurological and respiratory pathology seen in humans infected with NiV. More importantly, we have developed non-human primate (NHP) models for both NiV_M [7] and NiV_B [9], and we recently showed that NiV_B is more pathogenic in AGMs than NiV_M under identical experimental conditions [9]. We also showed that treatments that protect AGMs against NiV_M were not as effective against NiV_B, demonstrating the importance of medical countermeasures capable of protecting against the more virulent NiV_B [9]. Most animal studies involving NiV have relied on the intratracheal (i.t.) route of virus administration and, more recently, on using small particle aerosols [7, 10, 11]. Instillation by the i.t. route involves injecting small volumes of solutions directly into the trachea of anesthetized animals and results in rapid but localized and uneven distribution of material over a relatively small volume of the lung. It is thus typically not as effective as inhalational techniques in ensuring even pulmonary exposure to a substance. Moreover, volumes administered by the i.t. route must be small to avoid suffocation. Small

Correspondence: T. W. Geisbert, PhD, University of Texas Medical Branch at Galveston, Galveston National Laboratory, 301 University Blvd., Galveston, TX 77550-0610 (twgeisbe@utmb.edu).

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particle aerosol challenge results in a substantial proportion of the virus being deposited in the lower respiratory tract, where the majority of disease is confined [10]. Neither i.t. nor small particle aerosol exposure accurately mimics human-to-human transmission of NiV, which likely targets the upper respiratory tract. Therefore, direct mucosal exposure to NiV-contaminated droplets or sprays by direct nostril or facial contact can best be modeled by the intranasal (i.n.) route. The LMA mucosal atomization device (MAD) was developed for safe and efficient delivery of test particles and is currently used to administer drugs that are approved by the US Food and Drug Administration for i.n. delivery. LMA MAD delivers atomized particles that range in size from 30 to 100 μm , which is highly consistent with the size of droplets exhaled by humans when coughing [12]. In this study, we describe a lethal i.n. model of NiV_B exposure in AGMs using the LMA MAD.

METHODS

Virus Isolate

The isolate of NiV_B used in the study was 200401066, which was obtained from a fatal human case during the outbreak in Rajbari, Bangladesh in 2004 and passaged on Vero-E6 cells twice [9].

Animal Challenge

Before challenge with NiV_B, all animals were anesthetized by intramuscular injection of ketamine. In the first study, 2 adult AGMs (O9082 and O9070, both male) were exposed i.n. using the LMA MAD (Teleflex) with a target dose of 2000 plaque-forming units (pfu) of NiV_B for O9082 and 20 000 pfu of NiV_B for O9070. The study was repeated using 2 additional adult AGMs (O6720, female and O9267, male); O9267 received a target dose of 2000 pfu of NiV_B and O6720 received a target dose of 20 000 pfu of NiV_B. For both studies, the virus was delivered in a total volume of 0.70 mL (0.35 mL per nostril) 1× Hank's Balanced Salt Solution supplemented with 5% fetal bovine serum. After challenge, animals were monitored for clinical signs of illness, including temperature, respiration quality, and clinical pathology at various times before and after virus challenge.

Ethics Approval and Consent to Participate

The animal studies were performed at the Galveston National Laboratory, University of Texas Medical Branch at Galveston (UTMB) and were approved by the UTMB Institutional Animal Care and Use Committee. This facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Detection of Virus From NiV_B-Infected African Green Monkeys

Ribonucleic acid (RNA) was isolated from blood or tissues and assessed using primers/probe targeting the N gene as described previously [9] and detailed in the [Supplementary Methods](#).

Clinical Pathology and Host Immune Response

Hematology, clinical chemistry, and analysis of circulating levels of cytokines/chemokines are detailed in the [Supplementary Methods](#).

Histopathology and Immunohistochemistry

Necropsy was performed on all subjects. Tissue samples of all major organs were collected for histopathologic and immunohistochemical examination as outlined in the [Supplementary Methods](#).

RESULTS

Mucosal Exposure to NiV_B Via LMA MAD Results in Uniform Lethality in African Green Monkeys

All animals challenged with either low (~2000 pfu) or high (~20 000 pfu) doses of NiV_B using LMA MAD (1) developed illness typical of NiV_B infection induced by higher doses ($\geq 10^5$ pfu) delivered i.t., and (2) succumbed to disease on day 9 (n = 2) or day 10 (n = 2) post infection ([Table 1](#)). Clinical signs appeared during the late or terminal phase of the disease course and included depression, lethargy, anorexia, and progressively severe dyspnea. Increased leukocyte abundance was observed on day of euthanasia in low-dose challenged animals, and granulocytosis was observed in both low-dose challenged animals and 1 high-dose challenged animal, as were increases in hematocrit volume and hemoglobin. Lymphopenia and thrombocytopenia were common to all animals. Changes in serum biochemistry profiles were variable and included hyperglycemia (n = 1), hypoalbuminemia (n = 4), hypoproteinemia (n = 2), and increases in levels of aspartate aminotransferase (n = 3), alanine aminotransferase (n = 1), blood urea nitrogen (n = 2), creatinine (n = 3), and C-reactive protein (n = 4). Plasma levels of proliferative and proinflammatory/anti-inflammatory cytokines and chemokines were measured at several time points throughout the course of disease. Of those profiled, interferon- γ , interleukin (IL)-1RA, IL-6, and IL-10 exhibited the most marked increase in abundance in most animals ([Supplementary Figure 1](#)), whereas other analytes showed no marked changes or too much individual animal variability in response to infection (data not shown).

Gross pathological examination revealed marked pleural effusion involving fibrinous exudates, necrohemorrhagic interstitial pneumonia involving all lobes of the lungs, lymphadenomegaly, passive congestion of the liver and spleen with occasional splenomegaly (data not shown), and adrenomegaly ([Supplementary Figure 2](#)). In addition, the meninges of all 4 animals were congested, and a single animal exhibited mild multifocal hemorrhage of the urinary bladder (data not shown). All animals displayed histologic lesions consistent with NiV infection ([Supplementary Table 1](#)). Significant histologic lesions included moderate interstitial pneumonia and alveolar hemorrhage, fibrin and edema with endothelial

Table 1. Clinical Description and Outcome of Non-Human Primates Infected With NiV_B^a

Subject No.	Sex	Challenge Dose (Target pfu /Actual pfu)	Clinical Illness	Clinical and Gross Pathology
O9070	M	20 000/22 330	Depression (d10); lethargy (d10); loss of appetite (d4, 7, 9, 10); labored breathing; animal euthanized in AM of d10	Granulocytosis (d10); thrombocytopenia (d6, 10); hypoalbuminemia (d10); AST >3-fold ↑ (d10); BUN >2-fold ↑ (d10); CRE >3-fold ↑ (d10); CRP >5-fold ↑ (d10)
O9082	M	2000/2392	Depression (d10); lethargy (d10); loss of appetite (d2, 9, 10); labored breathing; animal euthanized in AM of d10	Leukocytosis (d10); granulocytosis (d10); thrombocytopenia (d10); lymphopenia (d10); hypoalbuminemia (d10); hypoamylasemia (d10); CRP >4-fold ↑ (d10)
O6720	F	20 000/19 720	Depression (d9); lethargy (d9); loss of appetite (d4–9); labored breathing (d9); animal euthanized in late PM of d9	Thrombocytopenia (d9); lymphopenia (d7, 9); Hct (35% ↑ d9); Hgb (35% ↑ d9); hyperglycemia (d9); hypoalbuminemia (d9); hypoproteinemia (d9); AST >3-fold ↑ (d9); BUN >2-fold ↑ (d9); CRE >3-fold ↑ (d9); CRP >5-fold ↑ (d9)
O9267	M	2000/2190	Depression (d9); lethargy (d9); loss of appetite (d8, 9); mild dyspnea (d8); labored breathing (d9); animal euthanized in AM of d9	Leukocytosis (d9); granulocytosis (d9); thrombocytopenia (d9); lymphopenia (d4, 7, 9); Hgb (41% ↑ d9); hypoalbuminemia (d9); hypoproteinemia (d9); ALT >2-fold ↑ (d9); AST >2-fold ↑ (d9); CRE >3-fold ↑ (d9); CRP >7-fold ↑ (d9)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; CRP, C-reactive protein; d, day; Hct, hematocrit; Hgb, hemoglobin; NiV, Nipah virus; pfu, plaque-forming units.

^aDays after NiV challenge are in parentheses. Lymphopenia and thrombocytopenia are defined by a ≥35% drop in numbers of lymphocytes and platelets, respectively. Leukocytosis and granulocytosis are defined by a 2-fold or greater increase in numbers of white blood cells over base line. Hyperglycemia is defined as a 2-fold or greater increase in levels of glucose. Hypoalbuminemia is defined by a ≥25% decrease in levels of albumin. Hypoproteinemia is defined by a ≥25% decrease in levels of total protein. Hypoamylasemia is defined by a ≥25% decrease in levels of serum amylase.

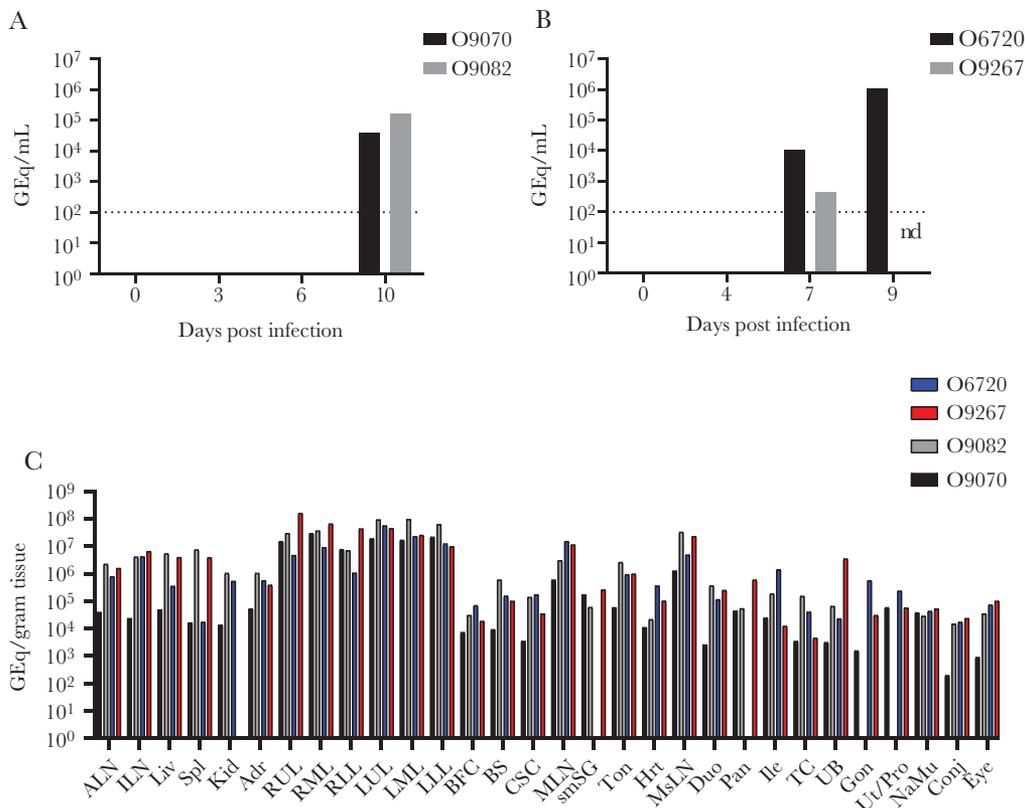


Figure 1. RT-qPCR detection of NiV vRNA from whole blood and selected tissues from NiV_B-infected African green monkeys (AGMs). RT-qPCR targeting NiV vRNA was performed on RNA extracted from whole blood at various time points through the course of infection (A, B) or tissues harvested at necropsy (C). Dashed line in (A) and (B) indicates limit of detection for the assay; blood samples prior to 6 days post infection were below this limit. A value is not reported for the 9-day post-infection sample from subject O9267 due to poor quality of the RNA preparation and is denoted as “nd” for “not determined.” Abbreviations for tissues in (C): ALN: axillary lymph node, ILN: inguinal lymph node, Liv: liver, Spl: spleen, Kid: kidney, Adr: adrenal gland, RUL: right upper lung, RML: right middle lung, RLL: right lower lung, LUL: left upper lung, LML: left middle lung, LLL: left lower lung, BFC: brain frontal cortex, BS: brain stem, CSC: cervical spinal cord, MLN: mandibular lymph node, smSG: submandibular salivary gland, Ton: tonsil, Hrt: heart, MsLN: mesenteric lymph node, Duo: duodenum, Pan: pancreas, Ile: ileum, TC: transverse colon, UB: urinary bladder, Gon: gonad, Ut/Pro: uterus/prostate, NaMu: nasal mucosa, Conj: conjunctiva.

syncytial cell formation, moderate lymphoid necrosis with syncytial cell formation of the splenic white pulp, and diffuse gliosis of the brain (Supplementary Figure 3A, C, and E). Strong immunoreactivity for NiV N antigen was present within the pulmonary endothelium (including syncytial cells), alveolar septae and scattered alveolar macrophages, the splenic endothelium, syncytial cells, and scattered mononuclear cells in the red and white pulp and in scattered small caliber vessels within the meninges and parenchyma of the brain (Supplementary Figure 3B, D, and F).

Determination of NiV_B Viral Load and Tissue Tropism

Circulating virus in blood plasma was not detectable by plaque assay titration until days 6–10 post infection in all animals and remained undetectable by plaque assay in a single animal immediately preceding euthanasia (data not shown). Likewise, NiV genomic RNA (gRNA) was not detected in whole blood by RT-quantitative PCR (qPCR) until after day 6 post infection (Figure 1A and B). NiV is largely understood to infect the endothelium; thus it was unsurprising that NiV gRNA was detectable in all tissues sampled for most animals by RT-qPCR, with the highest viral burden found in highly vascularized tissues such as the lungs and lymph nodes (Figure 1C).

DISCUSSION

The AGM has proven to be a valuable model of human NiV infection and has shown utility for elucidating the mechanisms of NiV pathogenesis and assessing the efficacy of experimental vaccines and treatments. Most work to date has used i.t. instillation for delivery of high doses ($\geq 10^5$ pfu) of NiV to AGMs, whereas a few studies have combined i.t. instillation with direct i.n. delivery of small volumes of culture fluid containing NiV. In addition to physiological concerns regarding the i.t. route as previously mentioned, those performing the i.t. technique must be proficient at intubating the animals and ensuring proper placement of the tube into the trachea and not the esophagus. The i.t. technique also presents special challenges in working in BSL-4 containment, particularly with NHPs, where additional time and skill are required in placing tubes into the trachea of anesthetized animals with less mobility than in a non-BSL-4 environment. Although i.t. instillation of NiV has been routinely and safely done in BSL-4 facilities, easier and more rapid techniques are advantageous. In this study, we have refined the AGM model of NiV_B infection using the LAM MAD technology for i.n. delivery of NiV_B, which is a rapid and easy method for mucosal exposure of NHPs to infectious agents. More importantly, we achieved uniform lethality and a primarily respiratory clinical presentation consistent with i.t. delivery of NiV_B to AGMs and consistent with natural NiV_B infection of humans. In addition, lower doses of NiV_B achieved similar results when compared with much higher doses of NiV_B used in i.t.-based models.

CONCLUSIONS

The progression of disease observed in the LAM MAD AGM model of NiV_B infection was very rapid from the onset of clinical illness until death, which was previously reported by us and others for AGM models of NiV_B using much higher doses of virus [9, 13, 14]. This is consistent with human NiV infection, in which disease progression is rapid, with an average time from onset of symptoms to death of 5 days and as little as 1 day [15]. The earliest indication of illness in the LAM MAD AGM NiV_B model was loss of appetite, whereas most changes in any other parameter, such as circulating virus, clinical pathology, or levels of circulating cytokines or chemokines generally were not detected or substantially elevated until the terminal stage of disease. Dysregulation of the proinflammatory response as indicated by increased or decreased levels of circulating cytokines and/or chemokines has not been assessed in human cases of NiV infection. However, another study looking at NiV_M infection of NHPs also reported similar findings including late-stage changes in levels of some of the same cytokines/chemokines, as we have noted in the current study [10]. Future studies need to address whether the disease course in AGMs can be prolonged by further reducing the dose of NiV_B used, as well as identifying other clinical parameters or biomarkers that would indicate infection closer to the time of actual exposure.

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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Author contributions. J. B. G. and T. W. G. conceived and designed the study. J. B. G., D. J. D., R. W. C., C. E. M., and T. W. G. performed the Nipah challenge experiments. K. N. A. performed the clinical pathology assays. J. B. G. and V. B. performed the Nipah virus infectivity assays. K. N. A. performed the polymerase chain reaction and multiplex assays. J. B. G., V. B., A. N. P., K. N. A., D. J. D., S. L. F., R. W. C., C. E. M., T. W. G., and K. A. F. analyzed the data. K. A. F. performed gross pathologic, histologic, and immunohistochemical analysis of the data. J. B. G., A. N. P., T. W. G., and K. A. F. wrote the paper. All authors had access to all of the data and approved the final version of the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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