Isolation and Full-Genome Characterization of Nipah Viruses from Bats, Bangladesh

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Despite molecular and serologic evidence of Nipah virus in bats from various locations, attempts to isolate live virus have been largely unsuccessful. We report isolation and full-genome characterization of 10 Nipah virus isolates from Pteropus medius bats sampled in Bangladesh during 2013 and 2014.

Nipah virus (NiV) is an emerging zoonotic virus carried by bats. It is considered a global health priority by the World Health Organization and has pandemic potential because of its zoonotic nature, human-to-human transmissibility, wide geographic distribution of bat reservoir species, high case-fatality rate in humans, and lack of available vaccine or therapeutic agents (1). Although NiV or NiV-related infections have been demonstrated by serologic surveillance or PCR detection in several bat species across extensive areas, attempts to isolate live NiV have been unsuccessful; there have been only 3 successful reports: Pteropus hypomelanus bats (2) and P. vampyrus bats (3) in Malaysia and P. lylei bats in Cambodia (4).

Bangladesh has reported seasonal outbreaks of infectious NiV almost annually since 2001, and India has reported 2 outbreaks in neighboring West Bengal, the last in 2007 (5,6). In May 2018, India reported an outbreak in Kerala State, which is >1,800 km southwest of West Bengal. NiV genomic data from the region have come primarily from human cases (7,8,12). Spillover might not be limited to humans in Bangladesh; nonneutralizing antibodies against NiV in cattle, goats, and pigs (13) underscore the urgency of characterizing diversity of henipaviruses in P. medius bats and other possible animal reservoirs in the region.

We report isolation of NiVs from P. medius bats in Bangladesh. We performed full-genome characterization of these viruses by using enrichment-based next-generation sequencing (NGS).

The Study
During January 2011–April 2014, we collected 2,749 bat samples from various ongoing projects in the region. We collected samples nondestructively from individual bats as described (7) and collected environmental urine samples from underneath roosts by using polyethylene sheets. In early 2013, an outbreak of infection with NiV occurred in 13 districts of Bangladesh (Gaibandha, Jhinaida, Kuri- gram, Kushtia, Magura, Manikganj, Myzensingh, Naog- aon, Natore, Nilphamari, Pabna, Rajbari, and Rajshahi). In April 2013, we collected only urine samples during an outbreak investigation in Raipur, Manikganj. We also tested 944 underroost urine samples, 829 throat swab specimens, and 976 urine samples from 2 confirmed bat species (P. medius and Rousettus leschenaultia) for NiV. Samples were collected with permission from the Government of Bangladesh Forestry Office and under Institutional Animal Care and Use Committees (Tufts University no. G2011-12 and University of California at Davis no. 19300).

We also collected bat samples from 5 sites in Rairpur that showed NiV spillover and 1 district (Sylhet) that had no reported cases (Figure 1). Samples were tested at the CSIRO Australian Animal Health Laboratory under Biosafety Level 4 containment. Individual samples were pooled into groups of 4 for initial extraction and PCR analysis. Of 688 pooled samples tested, 20 pools were positive by a nucleoprotein gene–specific 1-step reverse transcription

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quantitative PCR. The 80 individual samples from positive pools were thawed for virus isolation and RNA reextracted for PCR. Of 80 samples (urine and throat swab specimens) tested by nucleoprotein gene–gene specific 1-step reverse transcription quantitative PCR, only 20 urine samples were positive. We used these 20 urine samples for virus isolation.

We performed virus isolation under BioSafety Level 4 containment. For virus isolation from PCR-positive samples, we prepared Vero cells in 96-well plates in Eagle minimum essential medium (EMEM) containing 10% fetal bovine serum and 1× antibiotic–antimycotic mixture (GIBCO, http://www.biosciences.ie/gibco). We added 50 µL of each sample to 2 wells, incubated each sample for 90 min, removed the inoculum, and added 200 µL EMEM to each well. Plates were incubated at 37°C, checked at 7 days postinfection for a cytopathic effect (CPE), and frozen at –80°C.

A total of 11 wells showed CPE, and putative virus culture from these wells was passaged a second time by inoculation of a 24-well plate containing 80% confluent Vero cells in EMEM with 80 µL of culture supernatant from each positive well. Plates were incubated at 37°C for 90 min; inoculum was removed and 1 mL EMEM added. Plates were then incubated at 37°C for 5 days. All 11 samples remained CPE positive after this second passage.

For a third passage, we harvested culture supernatant and added 300 µL to a 25-cm flask containing 80% confluent Vero cells. Flasks were incubated for 90 min at 37°C before inoculum was removed and 5 mL EMEM added. We incubated the flasks at 37°C and harvested...
virus supernatants at 2–3 days postinfection. Supernatants were clarified by centrifugation at 10,000 × g for 5 min and frozen at –80°C.

To confirm the identity of isolated viruses, we submitted each sample to electron microscopy and PCR. All isolates had morphology consistent with NiV and were positive by NiV-specific PCR. Virus stocks were amplified in Vero cells, and supernatants were harvested and clarified by centrifugation at 10,000 × g for 10 min, followed by pelleting virus from supernatant by centrifugation at 200,000 × g for 30 min. Virus pellets were resuspended in 200 μL of Magmax Buffer (Applied Biosystems, https://www.thermofisher.com/us/en/home/brands/applied-biosystems.html) for RNA extraction according to the manufacturer’s protocol.

We used a virus enrichment strategy (14) to obtain the full-genome sequence for NiV isolates. Total RNA was used to prepare NGS libraries (New England Biolabs, https://www.neb.com) according to the manufacturer’s instructions. DNA libraries were subjected to a liquid-based...
Table. Characteristics of 11 bat samples tested for Nipah virus, Bangladesh*

| Sample no. | Sample ID   | Date sampled | Location         | Sample type | Tagman PCR C<sub>t</sub> | NGS       | Isolate designation                  |
|------------|-------------|--------------|------------------|-------------|------------------------|----------|--------------------------------------|
| 1          | PGB-1401B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.9                   | Yes      | NiV/BD/BA/2013/Raipur1401            |
| 2          | PGB-1402B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 30.1                   | Yes      | NiV/BD/BA/2013/Raipur1402            |
| 3          | PGB-1403B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.0                   | Yes      | NiV/BD/BA/2013/Raipur1403            |
| 4          | PGB-1404B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.3                   | Yes      | NiV/BD/BA/2013/Raipur1404            |
| 5          | PGB-1405B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.0                   | Yes      | NiV/BD/BA/2013/Raipur1405            |
| 6          | PGB-1406B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.9                   | Yes      | NiV/BD/BA/2013/Raipur1406            |
| 7          | PGB-1408B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.3                   | Yes      | NiV/BD/BA/2013/Raipur1408            |
| 8          | PGB-1409B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.7                   | Yes      | NiV/BD/BA/2013/Raipur1409            |
| 9          | PGB-1410B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 31.0                   | Yes      | NiV/BD/BA/2013/Raipur1410            |
| 10         | PGB-1411B   | 2011 Apr 13  | Raipur, Manikganj | ORU         | 30.8                   | No       | NiV/BD/BA/2013/Raipur1411            |
| 11         | PGB-191     | 2006 Jan 13  | Sylhet           | RU          | 39.0                   | Yes      | NiV/BD/BA/2013/Sylhet191             |

*C<sub>t</sub>: cycle threshold; ID: identification; NGS, next-generation sequencing; NiV, Nipah virus; ORU, outbreak roost urine; RU, roost urine.

**Table**

| Sample no. | Date sampled | Location | Sample type | Taqman PCR C<sub>t</sub> | NGS       | Isolate designation |
|------------|--------------|----------|-------------|------------------------|----------|----------------------|
| 1          | 2011 Apr 13  | Raipur   | ORU         | 31.9                   | Yes      | NiV/BD/BA/2013/Raipur1401 |
| 2          | 2011 Apr 13  | Raipur   | ORU         | 30.1                   | Yes      | NiV/BD/BA/2013/Raipur1402 |
| 3          | 2011 Apr 13  | Raipur   | ORU         | 31.0                   | Yes      | NiV/BD/BA/2013/Raipur1403 |
| 4          | 2011 Apr 13  | Raipur   | ORU         | 31.3                   | Yes      | NiV/BD/BA/2013/Raipur1404 |
| 5          | 2011 Apr 13  | Raipur   | ORU         | 31.0                   | Yes      | NiV/BD/BA/2013/Raipur1405 |
| 6          | 2011 Apr 13  | Raipur   | ORU         | 31.9                   | Yes      | NiV/BD/BA/2013/Raipur1406 |
| 7          | 2011 Apr 13  | Raipur   | ORU         | 31.3                   | Yes      | NiV/BD/BA/2013/Raipur1408 |
| 8          | 2011 Apr 13  | Raipur   | ORU         | 31.7                   | Yes      | NiV/BD/BA/2013/Raipur1409 |
| 9          | 2011 Apr 13  | Raipur   | ORU         | 31.0                   | Yes      | NiV/BD/BA/2013/Raipur1410 |
| 10         | 2011 Apr 13  | Raipur   | ORU         | 30.8                   | No       | NiV/BD/BA/2013/Raipur1411 |
| 11         | 2006 Jan 13  | Sylhet   | RU          | 39.0                   | Yes      | NiV/BD/BA/2013/Sylhet191 |

**Conclusions**

We report isolation of NiV from *P. medius* bats, a natural virus reservoir in Bangladesh. With an improved enrichment-based NGS strategy, we generated complete genome sequences for 10 bat NiV isolates with higher efficiency than for traditional PCR-based sequencing methods. NiV has been difficult to isolate from bats and, similar to results of previous studies of Hendra virus (10), we observed that successful virus isolation does not correlate with cycle thresholds. The complete sequence identity match among isolates obtained during the outbreak investigation in Raipur suggests that multiple strains were not co-circulating in the bat population at the time, supporting results of a previous study in Faridpur (10). None of the bat NiV isolate sequences were identical with any previously detected human NiV isolate sequences, suggesting that NiV spillover into humans is a rare event. However, the genetic diversity of bat NiV isolates needs to be fully identified.

**Acknowledgments**

We thank Ina Smith and Vicky Boyd for providing assistance and support during this study, Sandy Crameri for performing the electron microscopy, and Jordan Menscher for his help collecting bat roost urine samples. We also thank the Forest Department, Government of Bangladesh, for their support and for providing permission and the appropriate permits to conduct the study.

This study was supported by the National Institutes of Health Fogarty International Center (R01TW005869: P.D., J.H.E., A.I., S.P.L., and E.S.G.), the US Agency for International Development, PREDICT Project (J.H.E., A.I., and P.D.), and a National Science Foundation Research Coordination Network Award (EcoHealthNet DEB-0955897). L.-F. W. was supported by the National Research Foundation (NRF2012NRF-CRP001-056), the Ministry of Health (CDPHRG/0006/2014) and the Ministry of Defence (DIRP2015-9016102060) in Singapore. D.E.A. and I.H.M. were supported by New Investigators Grants from the National Medical Research Council of Singapore (NMRC/BNIG/2030/2015 and NMRC/BNIG/2005/2013).

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