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Nipah Virus: A Recently Emergent Deadly Paramyxovirus

K. B. Chua,[1] W. J. Bellini,[2]* P. A. Rota,[2] B. H. Harcourt,[2] A. Tamin,[2] S. K. Lam,[1] T. G. Ksiazek,[2] P. E. Rollin,[2] S. R. Zaki,[2] W.-J. Shieh,[2] C. S. Goldsmith,[2] D. J. Gubler,[3] J. T. Roehrig,[3] B. Eaton,[4] A. R. Gould,[4] J. Olson,[2] H. Field,[5] P. Daniels,[4] A. E. Ling,[6] C. J. Peters,[2] L. J. Anderson,[2] B. W. J. Mahy[2]

A paramyxovirus virus termed Nipah virus has been identified as the etiologic agent of an outbreak of severe encephalitis in people with close contact exposure to pigs in Malaysia and Singapore. The outbreak was first noted in late September 1998 and by mid-June 1999, more than 265 encephalitis cases, including 105 deaths, had been reported in Malaysia, and 11 cases of encephalitis or respiratory illness with one death had been reported in Singapore. Electron microscopic, serologic, and genetic studies indicate that this virus belongs to the family *Paramyxoviridae* and is most closely related to the recently discovered Hendra virus. We suggest that these two viruses are representative of a new genus within the family *Paramyxoviridae*. Like Hendra virus, Nipah virus is unusual among the paramyxoviruses in its ability to infect and cause potentially fatal disease in a number of host species, including humans.

An outbreak of severe febrile encephalitis associated with human deaths was reported in Peninsular Malaysia beginning in late September 1998. The outbreak was associated with respiratory illness in pigs and was initially attributed to Japanese encephalitis (JE) (*1*). JE is a mosquito-borne viral disease that is enzootic in the region, and pigs are among the amplifying vertebrate hosts (*2*). By February 1999, similar diseases in pigs and humans were recognized in other regions in Malaysia, in association with the movement of a large number of pigs from Ipoh southward into the new outbreak areas. In March 1999, a cluster of 11 cases of respiratory and encephalitis illnesses was noted in Singapore in abattoir workers who handled pigs from the outbreak regions in Malaysia. The outbreak in Singapore ended when the importation of pigs from Malaysia was prohibited, and the outbreak in Malaysia ceased when over 1 million pigs were culled from the outbreak area and immediately surrounding areas (*3*). A total of 265 cases of encephalitis, including 105 deaths, were associated with the outbreak in Malaysia.

Because some of the epidemiologic characteristics of the disease in humans were distinct from those of JE [most cases occurred in adult males who worked with pigs, very few case patients were young children, and neither mosquito control nor JE vaccination programs appeared to affect the course of the outbreak (*4*)], investigators in Malaysia expanded attempts to isolate an agent. In early March 1999, Vero cells inoculated with cerebrospinal fluid specimens from three fatal cases of encephalitis developed syncytia.

Electron microscopic (EM) studies of the virus, named Nipah virus (*5*), demonstrated features characteristic of a virus belonging to the family *Paramyxoviridae* (Fig. 1). This family of viruses typically possesses a single-stranded nonsegmented RNA genome of negative polarity that is fully encapsidated by protein. The helical nucleocapsid structure is surrounded by membrane derived from the plasma membrane from which the viruses bud. Virus particles vary in size from 120 to 500 nm. The paramyxovirus envelope contains two transmembrane glycoproteins, a cell receptor binding protein [G (glycoprotein), H (hemagglutinin), or HN (hemagglutinin/neuraminidase)] and a separate fusion (F) protein. Thin-section EM studies of infected cells revealed filamentous nucleocapsids within cytoplasmic inclusions and incorporated into virions budding from the plasma membrane (Fig. 1B). Typical "herringbone" nucleocapsid structures, approximately 1.67 ± 0.07 μm in length (Fig. 1A) and with an average diameter of 21 nm, were observed in infected cells by means of negative stain preparations. Extracellular virus particles were pleomorphic, with an average diameter of 500 nm (Fig. 1C). Surface projections (not shown in the figure) along the virion envelope were only sporadically seen by thin-section EM and measured 10 nm in length.

Analysis of representative human and animal specimens from the outbreaks in Malaysia and Singapore provided an etiologic link between the two outbreaks (Table 1). Nipah virus–infected cells reacted strongly with Hendra virus antiserum (*6*) but did not react with antisera against other paramyxoviruses, including measles virus, respiratory syncytial virus, and parainfluenzaviruses 1 and 3, as well as other viruses, including herpes virus, enteroviruses, and JE virus, as indicated by immunofluorescence antibody assays. Cross-neutralization studies (*7*) resulted in an 8- to 16-fold difference in neutralizing antibodies between Nipah and Hendra viruses, indicating that the viruses, though related, were not identical.

Serologic studies in which Hendra virus antigen was used for detection of Nipah virus immunoglobulin M (IgM) and IgG (*8*) suggested that Nipah virus was the principal etiologic agent of the outbreak in Malaysia and Singapore. Virus isolation or serologic testing confirmed Nipah virus infection in all cases from Singapore and in all but one of the initially identified encephalitis cases from Malaysia (Table 1). The most notable illness in pigs implicated in transmitting the virus to humans was respiratory and included a loud and distinctive cough. Although serologic studies found evidence of Nipah virus infec-

[1]Department of Medical Microbiology, University of Malaya Medical Center, 50603 Kuala Lumpur, Malaysia. [2]Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases (NCID), Centers for Disease Control and Prevention (CDC), 1600 Clifton Road, Atlanta, GA 30333, USA. [3]Division of Vector-Borne Diseases, NCID, CDC, Fort Collins, CO 80522, USA. [4]Commonwealth Scientific and Industrial Research Organisation, Australian Animal Health Laboratory, 5 Portarlington Road, East Geelong, Geelong, Victoria 3220, Australia. [5]Department of Primary Industries, Animal Research Institute, 665 Fairfield Road, Yeerongpilly, Queensland 4105, Australia. [6]Department of Pathology, Singapore General Hospital, Outram Road, Singapore 0316.

*To whom correspondence should be addressed.

tion in most of the pigs on farms with infected humans, only a minority of these pigs were noted to be ill and there was only a minimal (~5%) increase in pig deaths. Serologic studies also suggested that Nipah virus infection occurred in dogs, cats, and two polo ponies and in bats in the outbreak area in Malaysia (*9*).

Two key features of this outbreak were the often fatal encephalitis and the nearly universal history of infected humans and other animals, including cats and dogs, having direct contact with pigs. Pathologic studies of infected human and pig tissues provided plausible explanations for these two features of the outbreak. Autopsy studies of humans suggested that the primary pathology was a multiorgan vasculitis associated with infection of endothelial cells. Occasional endothelial cells of affected vessels developed into multinucleated giant cells characteristic of paramyxovirus infections, whereas other endothelial cells were lysed and sloughed into the vessel lumen. Infection was most prominent in the central nervous system, where a diffuse vasculitis was noted in the cerebral cortex and brain stem with extension to parenchymal tissue, where extensive areas of rarefaction (lytic) necrosis were seen. Eosinophilic, mainly intracytoplasmic, viral inclusions with a melted-tallow appearance were seen in the affected neurons and other parenchymal cells. Immunostaining for Nipah viral antigens was initially performed with an anti–Hendra virus hyperimmune serum and later with an anti–Nipah virus hyperimmune serum. Intense immunostaining of endothelial cells and of dead and dying parenchymal cells was seen in the central nervous system (Fig. 2A). Less intense immunostaining was also seen in other tissues, including the lung, heart, spleen, and kidney. These studies demonstrated that Nipah virus infection in humans can cause widespread central nervous system pathology consistent with a severe encephalitis.

Pathologic examination of pigs showed extensive involvement of the respiratory tract (Fig. 2B). In the lungs, a giant cell pneumonia with characteristic multinucleated syncytial cells was noted. By immunohistochemistry, Nipah virus antigens were demonstrated in syncytial cells in the lungs, in epithelial cells lining the upper airway passages, and in intraluminal necrotic debris in the upper and lower airways. In a small number of pigs, there was prominent central nervous system pathology with meningeal inflammatory infiltrates, and viral antigen was detected focally in inflammatory cells as well as within the nuclei of rare parenchymal cells. Nipah antigen was also detected in kidneys as focal staining in renal tubular epithelial cells. The presence of extensive infection in the upper and lower airways suggests that the respiratory secretions from infected pigs are likely to be a rich source of infectious virus. Infection in the respiratory tract has proven very efficient for transmission for a number of paramyxoviruses, including measles and respiratory syncytial virus.

Sequence studies of Nipah virus clearly

Table 1. Evidence of Nipah virus infection in tissue and serum specimens tested during the outbreaks in Malaysia and Singapore. Specimens were obtained from fatal (Y) and nonfatal (N) human cases in Malaysia (M) or Singapore (S) or from euthanized (E) pigs (P), a dog, and a cat from Malaysia. Virus was isolated (Isol) on Vero cells from tissue samples described above. IgM or IgG antibody (Ab) was detected in serum samples by enzyme-linked immunoassays, with Hendra virus antigen prepared as described (*8*). For RT-PCR, RNA was extracted from human brain tissue (fatal cases only), human cerebrospinal fluid, porcine lung tissue, canine brain tissue, or feline brain tissue. RT-PCR was performed with primers designed to amplify a region of the N gene of Nipah virus (*12*). In some cases, the nucleotide sequence (Seq) of the PCR products was determined, and the percent identity with the sequence obtained from two cell culture isolates of Nipah virus from M4 and S1 is indicated. Human brain or porcine lung tissue samples were examined by immunohistochemistry (IHC) with the mouse hyperimmune ascitic fluid to Hendra virus; nd, not done.

Source	PCR	Seq	Isol	Ab	IHC	Fatal
M1	+	100	–	+	+	Y
M2	+	100	–	+	nd	Y
M3	+	nd	+	+	+	Y
M4	+	100	+	nd	+	Y
M5, M6	nd	nd	+	nd	nd	N
M7–14	nd	nd	nd	+	nd	N
S1	+	100	+	+	nd	N
S2	+	100	+	+	+	Y
S3–6, 8, 9, 13, 15	–	nd	–	+	nd	N
P1, P5	+	100	+	nd	+	E
P55–56	+	nd	+	nd	–	E
P2	–	nd	–	nd	+	E
P4, P58	+	nd	–	nd	nd	E
Dog	+	100	+	nd	nd	E
Cat	+	100	+	nd	nd	E

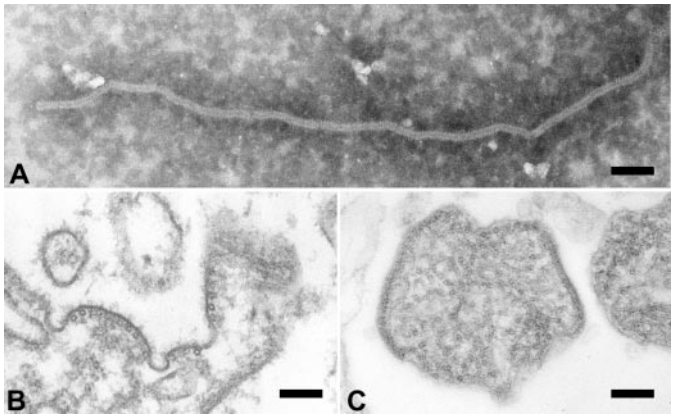

Fig. 1. Ultrastructural characteristics of Nipah virus isolate in cell culture as seen by negative stain (A) and thin-section (B and C) electron microscopy. (**A**) A single nucleocapsid shows the typical herringbone appearance characteristic of the family *Paramyxoviridae*. (**B**) Viral nucleocapsids, as seen in cross and longitudinal sections, aligned along the plasma membrane of Nipah virus–infected Vero E6 cells. (**C**) Extracellular Nipah virus particle showing a curvilinear tangle of nucleocapsids enclosed within the viral envelope. Scale bars, 100 nm.

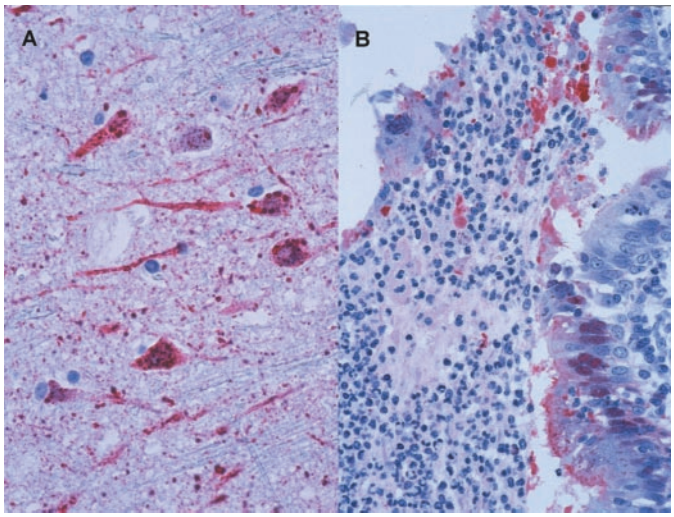

Fig. 2. Detection of Nipah virus antigens in human and porcine tissue samples. (**A**) Immunolocalization of Nipah virus antigens to neurons and neuronal processes of a patient who died with Nipah virus encephalitis. (Immunolocalization was done with an immunoalkaline phosphatase technique using a rabbit antiserum against Hendra virus; tissues were stained with Naphthol/fast red with light hematoxylin counterstain. Original magnification, ×158.) (**B**) Viral antigens as seen in bronchial epithelium of a pig with Nipah virus infection. Note immunostaining in association with necrotic epithelial and inflammatory cells in the bronchial lumen. [Immunolocalization and staining were done as in (A). Original magnification, ×100.]

placed it in the family *Paramyxoviridae*. The RNA was initially amplified by reverse transcription polymerase chain reaction (RT-PCR) assay (*10*) with primers designed to hybridize against regions near the RNA editing site in the phosphoprotein (P) gene (*11*); this region has conserved sequences across many members of the family *Paramyxoviridae* (Fig. 3). The nucleotide sequence obtained from this 141–base pair (bp) PCR product and sequence data from Hendra virus were then used, in a stepwise fashion, to sequence the nucleoprotein (N), P, and matrix (M) genes of Nipah virus. These three genes from Nipah virus share 70 to 78% nucleotide homology with Hendra virus, and this similarity confirms the initial observation that Hendra and Nipah are closely related but distinct viruses (GenBank accession number AF212302).

The N gene region of Nipah virus was identical among several independent cell culture isolates; therefore, a set of unique primers for use in RT-PCR assays was designed from this region. Results of detection assays with this primer set (*12*) in conjunction with nucleotide sequence analysis provided a genetic link between the virus isolates and tissue specimens from infected humans and pigs from the two outbreaks, as well as specimens from cats and dogs from Malaysia (Table 1). All RT-PCR products that were sequenced possessed identical N gene fragment nucleotide sequence, thus linking the genetic makeup of Nipah virus isolates with RT-PCR–amplified DNA from infected tissues.

Within the subfamily *Paramyxovirnae*, the extent of nucleotide homology for N between different viruses in the same genus ranges from 56 to 78%, whereas the extent of nucleotide similarity between viruses from different genera is 39 to 49%. The N genes of Hendra and Nipah viruses have 78% nucleotide homology, but these two viruses have no more than 49% similarity with any other members of the *Paramyxovirinae* (Table 2). Phylogenetic analysis of N gene sequences shows that Hendra and Nipah viruses form a distinct cluster within the subfamily *Paramyxovirinae* and probably represent a new genus in this subfamily (Fig. 4) (*13*). Although the recently described Tupaia paramyxovirus (*14*) was slightly more closely related to Hendra and Nipah viruses than to viruses in the other three genera in *Paramyxovirnae*, phylogenetic analysis suggests that it is distinct from the Hendra virus and Nipah virus cluster. Nipah and Hendra viruses do not fit into existing genera but are most similar to the *Morbillivirus* and *Respirovirus* genera. Both Nipah and Hendra viruses exhibit the ability to infect a broad range of species, a trait that is unusual among the members of the *Paramyxovirinae*.

Sequence analyses of the P and M genes are consistent with that of the N gene in suggesting that Nipah and Hendra viruses are closely related members of a new genus in the *Paramyxovirinae.* The sequence of the P gene of Nipah virus shares 70% nucleotide homology with the P gene of Hendra virus (*15*). The arrangement of coding regions within the P gene of Nipah virus is similar to that of Hendra virus, as well as to those of the morbilliviruses and the respiroviruses (*11*). The P gene of Nipah virus is predicted to encode a C protein from an overlapping reading frame and a V protein from an edited mRNA (Fig. 3). In addition, the sequence of the RNA editing site of Nipah virus is identical to that of the morbilliviruses and Hendra virus. However, an open reading frame (ORF) predicted to encode a short basic protein in the P gene of Hendra virus (*15*) is not present in Nipah virus. Studies of the M gene provide

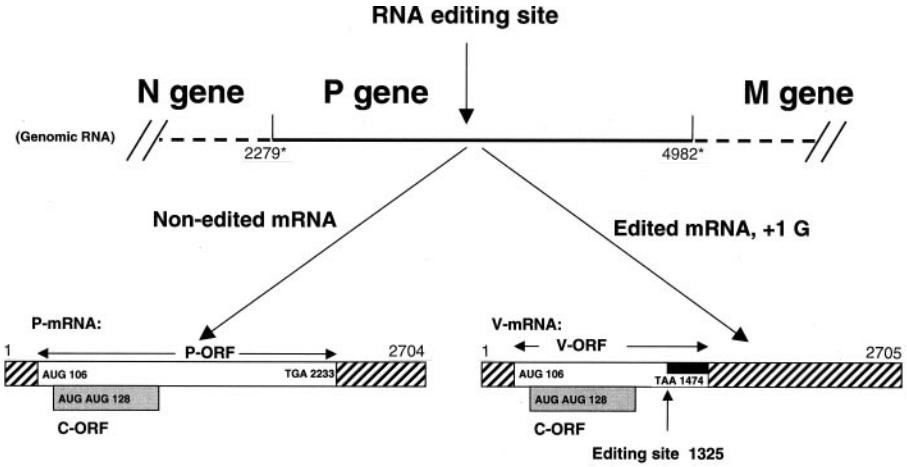

Fig. 3. The organization of the P gene of Nipah virus is similar to those of Hendra virus, the morbilliviruses, and the respiroviruses. The figure is a schematic representation of the coding strategy found in the P gene of Nipah virus. The predicted P mRNA is 2704 nucleotides in length (nucleotides with asterisks indicate the location of the P gene sequence within GenBank accession number AF212302). The P protein is encoded by a faithful transcript of the viral genomic RNA from an ORF beginning at nucleotide 106 of the mRNA. The mRNA coding for the V protein is generated by a process known as pseudotemplated addition of G nucleotides or RNA editing. The RNA editing sites (vertical arrows) in the P genes of the paramyxoviruses, including Hendra and Nipah viruses, are highly conserved. In Nipah virus, the addition of a G nucleotide at the RNA editing site (nucleotide 1325) allows access to a different reading frame (–1 relative to P). Therefore, the V protein contains the amino-terminal domain of the P protein joined to the cysteine-rich domain that is unique to the V protein (black box). The C protein (gray box) is expressed from an ORF that begins at nucleotide 128 (or 131) and overlaps P in the +1 frame. Hatched boxes indicate nontranslated regions of the mRNA.

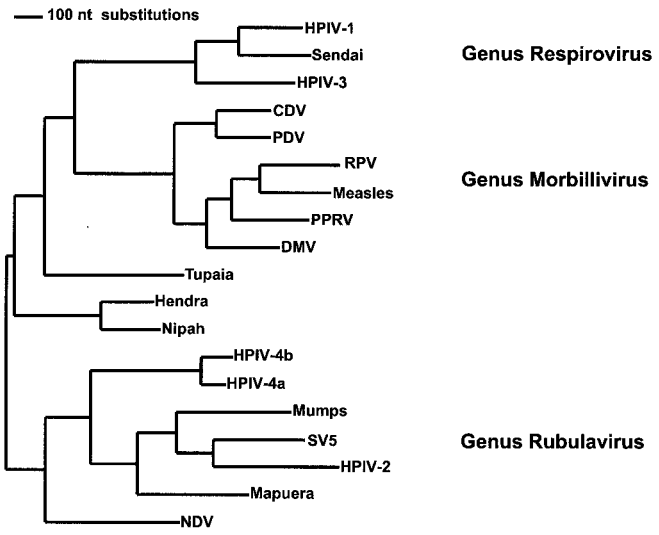

Fig. 4. Phylogenetic analysis of the N ORF from members of the subfamily *Paramyxovirinae*. A scale representing the number of nucleotide changes is shown at top left. Accession numbers used are as follows: canine distemper virus (CDV), AF014953; dolphin morbillivirus (DMV), X75961; Hendra virus, AF017149; human parainfluenza virus 1 (HPIV-1), D01070; human parainfluenza virus 2 (HPIV-2), M55320; human parainfluenza virus 3 (HPIV-3), D10025; human parainfluenza virus 4a (HPIV-4a), M32982; human parainfluenza virus 4b (HPIV-4b), M32983; Mapuera virus, X85128; mumps virus, D86172; measles virus, K01711; Newcastle disease virus, AF064091; peste-des-petits-ruminants virus, (PPRV), X74443; phocid distemper virus, (PDV), X75717; rinderpest virus (RPV), X68311; Sendai virus, X00087; simian virus 5 (SV5), M81442; and Tupaia virus, AF079780.

Table 2. Relatedness of Nipah and Hendra viruses to other members of the family P*aramyxoviridae*, subfamily *Paramyxovirinae*. Table lists percentage of nucleotide homology within the N genes. Bold numbers represent the extent of homology between viruses within a genus: respiroviruses (HPIV1, human parainfluenza virus 1; HPIV3, human parainfluenza virus 3; Sendai virus), rubulaviruses (mumps virus; SV5, simian virus 5; Mapuera virus), morbilliviruses (CDV, canine distemper virus; PDV, phocine distemper virus; MV, measles virus), and Tupaia paramyxovirus.

	HPIV1	Sendai	HPIV3	Mumps	SV5	Mapuera	CDV	PDV	MV	Nipah	Hendra	Tupaia
HPIV1	**100**											
Sendai	**72**	**100**										
HPIV3	**63**	**61**	**100**									
Mumps	40	40	42	**100**								
SV5	40	40	39	**59**	**100**							
Mapuera	40	39	40	**58**	**56**	**100**						
CDV	42	42	40	40	40	42	**100**					
PDV	43	40	43	40	40	41	**77**	**100**				
MV	42	44	42	42	41	40	**64**	**62**	**100**			
Nipah	40	40	43	42	42	43	46	44	44	**100**		
Hendra	43	40	43	41	42	43	46	46	45	**78**	**100**	
Tupaia	41	40	42	41	43	42	47	47	47	48	49	**100**

some data on variation in Hendra virus sequences over time, which highlights the differences between Nipah virus and Hendra virus sequences. The nucleotide sequence of the original Hendra virus isolate (1994) and Hendra virus from an infected horse in Cairns, Australia (1999), differ by only 0.38% (5 out of 1301 bp). In contrast, the Hendra and Nipah virus sequences differed by 31% (408 out of 1301 bp) over the same region of the M gene (*16*).

In addition, the genes coding for fusion (F) and attachment (G) envelope glycoproteins of Nipah virus share 74 and 70% nucleotide homology, respectively, with the F and G genes of Hendra virus. Phylogenetic analyses of these F and G sequences confirm the observations made for the N, P, and M genes and show that Nipah and Hendra viruses represent a unique group within the *Paramyxovirinae* (*17*).

The emergence of Hendra virus in two outbreaks of severe respiratory disease in thoroughbred horses in Brisbane and MacKay, Queensland, Australia, in 1994 provided the first glimpse of a zoonotic paramyxovirus with an expanded host range that includes humans. Three human cases (two were fatal) resulted from exposure to infected horses. Under experimental conditions, Hendra virus was shown to infect cats, guinea pigs, horses, and fruit bats. In fact, Hendra virus has been isolated from uterine fluids of a female grey-headed fruit bat, suggesting a reservoir for the virus in *Pteropus sp.* (*18*). Like Hendra virus, Nipah virus exhibited an extended host range, with natural infections in Malaysia that included swine, humans, cats, and dogs, as well as serological studies that imply that infection also occurred in horses and bats. The finding of neutralizing antibodies to Nipah virus in two species of fruit bats (*Pteropus vampyrus* and *P. hypomelanus*) and the data implicating fruit bats as a reservoir for Hendra virus led us to suspect a similar reservoir for Nipah virus. To date, however, neither virus isolation nor RT-PCR analysis of over 300 bats representative of many Malaysian bat species has proven successful; thus, the reservoir of Nipah virus and the source of the virus that initiated this outbreak remain open questions.

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7. In a biosafety level 4 (BSL-4) laboratory, stocks of plaque-purified Hendra and Nipah viruses were diluted to produce approximately 50 plaque-forming units per well in 96-well plates containing Vero cell monolayers. Replicates (n = 8) of diluted Hendra and Nipah viruses were then mixed with an equal volume of doubling dilutions (in phosphate-buffered saline) of either homologous or heterologous antisera generated by experimental infection of a horse and a cat with Hendra and Nipah viruses, respectively. Control wells contained virus alone. After incubation at 37°C for 30 min, virus-antiserum mixtures were transferred to Vero cell monolayers and incubated for 30 min at 37°C before removal of the inoculum and addition of Eagle's minimal essential medium containing 10% fetal calf serum. Twenty-four hours later, infected cell monolayers were fixed with methanol and removed from the BSL-4 laboratory, and virus antigen in syncytia were detected by using a monospecific rabbit antiserum made to a portion of the Hendra virus P protein (B. Eaton, unpublished data). The primary antibody was detected with alkaline phosphatase–linked anti-rabbit IgG (Promega, Madison, WI), as previously described [L. F. Wang *et al.*, *J. Immunol. Methods* **1678**, 1 (1995)].
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10. RNA was extracted from infected cells or tissues by means of the guanidinium acid-phenol method [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. RT-PCR was performed with primers specific for the P gene of *Paramyxoviridae* (5′-CATTAAAAAGGGCACAGACGC-3′ and 5′-TGGACATTCTCCGCAGT-3′), using reaction conditions that have been described previously [J. S. Rota *et al.*, *Virus Res.* **31**, 317 (1994)]. PCR primers for the initial amplification of a portion of the N gene were 5′-ACTATCAGATTCGGTCTTGAA-3′ and 5′-GATATCTAGATCACACGTCTGCTCTAAC-3′, and primers for the M gene were 5′-ACTATCACAAAGATCAATGATTC-3′ and 5′-CACCATTGATCTTAATGTGGAG-3′. PCR products were purified by means of the Promega PCR Clean-Up kit (Promega), and cycle sequencing was done with one of the amplification primers in the ABI (Big Dye) Cycle Kit. Sequencing reaction products were analyzed with an ABI 373 DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequence of Hendra virus and of the initial PCR products from the Nipah virus N, P, and M genes were used to design a series of PCR primers that were used to amplify the complete N, P, and M genes. Sequences of the N, P, and M genes from Nipah virus were deposited in GenBank (accession number AF212302).
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12. Primers used for the Nipah virus N-specific PCR assay were 5′-CTGCTGCAGTTCAGGAAACATCAG-3′ and 5′-ACCGGATGTGCTCACAGAACTG-3′. These primers amplify a PCR product of 228 bp. Reactions were performed using the EZ rTth RNA PCR kit (Perkin-Elmer–Applied Biosystems, Norwalk, CT). Reactions were analyzed by electrophoresis through 1.5% agarose gels that were stained with ethidium bromide.
13. Phylogenetic analysis using PAUP 4.02 was performed on N ORF sequences taken from members of the subfamily *Paramyxovirinae*. The tree shown in Fig. 4 was based on maximum parsimony; however, analysis of the same data using maximum likelihood gave a tree with nearly identical topologies (*9*).
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