Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus

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Since it was first described in Australia in 1994, Hendra virus (HeV) has caused two outbreaks of fatal disease in horses and humans, and an isolated fatal horse case. Our preliminary studies revealed a high prevalence of neutralizing antibodies to HeV in bats of the genus Pteropus, but it was unclear whether this was due to infection with HeV or a related virus. We developed the hypothesis that HeV excretion from bats might be related to the birthing process and we targeted the reproductive tract for virus isolation. Three virus isolates were obtained from the uterine fluid and a pool of foetal lung and liver from one grey-headed flying-fox (Pteropus poliocephalus), and from the foetal lung of one black flying-fox (P. alecto). Antigenically, these isolates appeared to be closely related to HeV, returning positive results on immunofluorescent antibody staining and constant-serum varying-virus neutralization tests. Using an HeV-specific oligonucleotide primer pair, genomic sequences of the isolates were amplified. Sequencing of 200 nucleotides in the matrix gene identified that these three isolates were identical to HeV. Isolations were confirmed after RNA extracted from original material was positive for HeV RNA when screened on an HeV Taqman assay. The isolation of HeV from pteropid bats corroborates our earlier serological and epidemiological evidence that they are a natural reservoir host of the virus.

Introduction

A new zoonotic disease affecting horses and humans in Australia was reported by Murray et al. (1995). Two outbreaks of this virus zoonosis occurred within 1 month of each other, at locations 800 km apart (Brisbane, in south east Queensland, and Mackay, in central Queensland) in 1994. A third event involving a single fatal equine case occurred near Cairns in North Queensland in January 1999. To date, two humans and 16 horses have died from this disease (Rogers et al., 1996; Murray et al., 1995; Field et al., 2000). Typically, infected horses had clinical signs of an acute respiratory disease. The first fatal human case also died of an acute respiratory illness (Selvey et al., 1995). The second fatal human case suffered from relapsing encephalitis (O’Sullivan et al., 1997). The causal agent is Hendra virus (HeV), formerly known as equine morbillivirus, a previously undescribed virus belonging to the family Paramyxoviridae (Wang et al., 1998).

Another member of the family Paramyxoviridae emerged in late 1998. Nipah virus was responsible for an outbreak of severe encephalitis in humans in Malaysia and Singapore and is attributed with the deaths of more than 100 people (Lye et al., 1999). Measures to control the concurrent outbreak of respiratory disease in pigs resulted in the culling of over one million pigs (almost half the national pig herd) and had major domestic and international trade repercussions (Aziz et al., 1999). Nipah virus, serological evidence of which was also found in pteropid bats (Field et al., 1999), has been shown to have strong molecular and sequence similarities with HeV, and the two viruses cross-neutralize (Rota et al., 1999). Neither has yet been assigned to a genus; more likely HeV and Nipah virus will form a new genus within the family Paramyxoviridae.

Extensive serological sampling throughout eastern Queensland revealed that of over 5000 sera collected from 46 species, including 34 species of wildlife, antibodies capable of neutralizing HeV have only been detected in pteropid bats (Young et al., 1996). These fruit and nectar feeding bats, commonly known as flying foxes, belong to the Order Chiroptera, Suborder Megachiroptera, Family Pteropidae,
**Methods**

### Samples
Concurrent with serological sampling of pteropid bat populations (Young et al., 1996) was the opportunistic sampling of recently captured sick or injured wild pteropid bats in temporary captivity with wildlife rescue groups. Samples of liver, kidney, lung, spleen and reproductive organs were removed aseptically at necropsy of pteropid bats which had recently died or been euthanized. Additionally, where possible, immediately after euthanasia a blood sample was collected by cardiac puncture for serology.

### Virus isolation
Virus isolations were attempted in RK13 cells. This was found to be the most sensitive cell line to HeV in cell line sensitivity experiments conducted at the Queensland State Health Department (L. Hiley, unpublished results). Samples of liver, kidney, lung, spleen and reproductive organs were ground in a sterile medium consisting of PBS with 160 U/ml benzylpenicillin sodium, 1000 µg/ml streptomycin and 4 µg/ml fungizone. The resulting homogenates were centrifuged at 3000 g for 10 min and the supernatant fluids were retained. Monolayers of RK13 cells grown in 24-well cell culture plates with 10% Eagle’s minimum essential medium with Earle’s salts (10% FCS, 2 µg/ml fungizone and 100 U/ml benzylpenicillin sodium) were inoculated with 0.2 ml of the supernatants of pteropid bat organ homogenates, absorbed for 60 min at 37 °C, washed with sterile medium and then maintained with 10% Eagle’s minimum essential medium with Earle’s salts (2.5% FCS, 2 µg/ml fungizone and 100 U/ml benzylpenicillin sodium). The 24-well plates were incubated at 37 °C with 5% CO₂. Cultures were observed for any signs of cytopathic effect (CPE). If no CPE was detected, inoculated cultures were harvested by a freeze–thaw at intervals of 7 days and passed with attempts terminating after five serial passages. To confirm virus isolation, a range of diagnostic tests were undertaken including the detection of HeV antigen by RT–PCR, immunofluorescent staining of infected cell sheets and a Tagman assay. Direct detection of antigen in tissues by immunostaining was not attempted. Contamination was avoided by observing accepted laboratory protocols and by returning to original tissue for confirmation of the presence of virus antigen.

### RNA extraction
Upon detection of CPE, and removal of some cell culture supernatant for further work (described below), infected cells were lysed by freezing and thawing. RNA was isolated by incubating 0.5 ml of this lysate with extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1% 2-mercaptoethanol, 2 M sodium acetate). Viral RNA was purified by phenol–chloroform extraction and ethanol precipitation, and the RNA was suspended in 18 µl of diethyl pyrocarbonate-treated water (Schorr et al., 1994). For the purpose of the Tagman assay, RNA was also extracted directly from tissue homogenate supernatant, as described above.

### RT–PCR
RT–PCR was performed with the Expand RT pre-amplification system (Boehringer Mannheim) according to the manufacturer’s recommendations. The oligonucleotide primer pair were designed from the sequence published by Murray et al. (1995) and amplified a 200 bp region in the matrix (M) protein (forward primer 5’ GCC TAC AAC GAG AAA TTT GTG 3’; reverse primer 5’ TTC TAG CAT TGT CCT TGG GAT 3’). For RT, 2.5 µl of template RNA was annealed with 10 pmol of reverse-sense primer in a total volume of 5 µl at 65 °C for 10 min. The mixture was then chilled on ice. RT reagents were added to give a total volume of 20.5 µl containing synthesis buffer (250 mM Tris–HCl, 200 mM KCl, 2.5 mM MgCl₂, 2.5% Tween 20, v/v, pH 8.3), 100 mM dithiothreitol, 1 mM (each) deoxycytidine tri- phosphates (dTTP, dCTP, dGTP, dTTP), 20 units of RNase inhibitor and 50 units of Expand reverse transcriptase. This mixture was incubated at 42 °C for 60 min and then chilled on ice. The resulting cDNA was used as a template to amplify a 200 bp region of the M gene of HeV. For PCR, a total volume of 50 µl was prepared, containing synthesis buffer, 2.5 mM MgCl₂, 10 pmol each primer, 2 µl of the reverse-transcribed product and 1.5 units Th DNA polymerase (Biotech International). Each sample was overlaid with mineral oil. Amplification was performed in a DNA thermocycler (DNA Thermal Cycler 480, Perkin Elmer) as follows. Samples were initially heated at 94 °C for 2 min; this was followed by 30 cycles consisting of denaturation (94 °C, 1 min), primer annealing (45 °C, 1 min) and extension (72 °C, 1 min). Samples were finally held at 15 °C. Size fractionation of PCR product was performed by electrophoresis in 1.2% agarose gel prepared in 0.5 × TBE buffer containing 0.5 µg/ml of ethidium bromide at 80 V for 60 min, and was visualized on a UV transilluminator.

### Nucleotide sequencing of the target genomic region
RT–PCR product (40 µl) was separated from the primers and dNTPs by filtration through a QIAquick PCR purification column (Qiagen). All DNA sequencing was performed using the recovered DNA, the oligonucleotide primer pair used in the RT–PCR and dideoxy sequencing chemistry utilizing the ABI PRISM Dye Terminator Sequencing Ready Reaction kit, with AmpliTag DNA polymerase FS according to the manufacturer’s instructions (PE Applied Biosystems). After recovery, sequencing products were resolved on an ABI automated A373 sequencer (PE Applied Biosystems) according to the manufacturer’s instructions.

### Immunofluorescent antibody (IFA) staining
When CPE was detected, cell culture supernatant was used to inoculate 16-well glass chamber slides (Nunc) containing RK13 cells (2 x 10⁶ cells per well). The chamber slides were incubated at 37 °C in a humid atmosphere containing 5% CO₂. When CPE was detected in these wells, supernatant was aspirated and the cell sheet was fixed in cold 90% acetone for 15 min, and then stained by an indirect IFA technique. Antiserum to HeV collected from clinical horse and human cases was used as the primary antibody. A fluorescein isothiocyanate-labelled protein G conjugate (Zymed) was used to stain the preparations. Uninfected cells, overlaid with horse serum positive for HeV antibodies and stained with the conjugate as described above, were used as indicators of specificity. Immunofluorescence was examined with a fluorescence microscope (Olympus).

### Constant-serum varying-virus neutralization test
Supernatant collected from CPE-producing wells was diluted from 10⁻¹ to 10⁻⁶ in PBS. Horse serum positive for HeV antibodies was held constant at 1/40. Horse serum negative for HeV antibodies, also diluted at 1/40, was
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Fig. 1. Positive immunofluorescent staining of RK13 cells infected with HeV isolated from the uterine fluid of Bat 1.

used as a negative control. Serum and supernatant were incubated for 1 h at 37 °C and subsequently inoculated onto RK13 cell monolayers in a 24-well plate (100 µl per well). Wells were monitored for signs of CPE.

**Taqman assay.** This two-step assay was developed at Queensland State Health Laboratories (I. Serafin, K. Halpin & G. Smith, unpublished data). The first step involved conventional cDNA production from RNA (extraction method described previously) using random primers. The HeV Taqman primers and probes were designed using the program Primer Express (PE Applied Biosystems) and reviewed based on the design guidelines. The 69 bp target region was based on the sequence of the HeV M gene. The PCR involved 50 cycles of denaturation at 95 °C for 15 s followed by a combined annealing/extension step for 1 min at 60 °C (PE Applied Biosystems).

**Serology.** Blood, collected by cardiac puncture immediately after euthanasia, was allowed to clot at room temperature and centrifuged at 3000 r.p.m. for 10 min. Serum was removed from this preparation and stored at −20 °C until used. Serum was initially screened by ELISA. All incubations took place at 37 °C. The 96-well microtitre plates (Nunc) were coated with inactivated HeV-infected cell extract treated with 10% SDS at a 1/400 dilution with PBS (50 µl per well) by incubation at 37 °C for 1 h. Following a brief rinse (3 × 200 µl) with PBS containing 0.1% Tween 20 (PBST), the plates were blocked for 1 h by the addition of 200 µl per well of 1% skim milk powder (SMP) in PBS. Plates were washed again, as described above. Pteropid bat serum (50 µl), diluted 1/100 with PBST containing 1% SMP, was added and incubated for 30 min, followed by another wash. The conjugate (ProteinG–HRP, ICN Biomedicals) was diluted at 1/10000 and incubated for 30 min. After a final washing of the plates, 50 µl of TMB peroxidase substrate (K-blue, Neogen Corporation) was added to each well and the plates were incubated for 10 min. At this point the reaction was stopped by the addition of 50 µl of 1 M sulfuric acid and the absorbance was read at 450 nm. Positive and negative control horse sera were included on each plate.

Additionally, aliquots of serum were sent to the Australian Animal Health Laboratories, Geelong, Victoria, Australia where serum neutralization tests using HeV were carried out in a biosafety level 4 laboratory.

**Electron microscopy.** Preparations of supernatant collected from CPE-producing wells were mounted on grids previously rendered hydrophilic by glow-discharge and were negatively stained in 2% phosphotungstic acid and viewed in a Philips CM 10 microscope operated at 80 kV, using a magnification of × 25000 plus the × 10 binocular.
Results

Between July and December 1996, 465 pteropid bats were sampled, providing 652 tissues for virus isolation. Most of these animals came from south-east Queensland. Many pteropid bats were taken into captivity as a result of physical injury, commonly electrocution on power transmission cables, or entanglement in barbed-wire fences. No lesions, other than those from trauma, were detected at necropsy of the pteropid bats.

Isolation of HeV

Through the RK13 cell culture system, three virus isolates were obtained. The first two isolates were from a female grey-headed flying-fox (Bat 1), which was euthanized due to extensive injuries. She had recently aborted twin foetuses after becoming entangled on a wire fence, in Brisbane. CPE was noted in the RK13 cells approximately 60 h after inoculation with uterine fluid collected from this animal. Reisolation of this first isolate was attempted and CPE was noted in the RK13 cells on third passage. The second isolate was from a pooled sample of liver and lung collected from the aborted foetuses of Bat 1. This material underwent three serial passages before CPE was noted in the RK13 cells. The third isolate was from the lung of a Pteropus alecto foetus, collected from a female (Bat 2) which was euthanized following the diagnosis of severe spinal injuries. Bat 2 was also found in Brisbane. This material also underwent three serial passages before CPE was noted in the RK13 cells. All other tissues collected from these bats were negative for virus isolation.

Amplification of genomic region and sequencing

The three isolates all produced a PCR product of the expected size (200 bp), and when this region was sequenced, it was found that the sequences were identical to that of HeV.

IFA staining

Infected cells showed very intense fluorescence throughout the cell sheet (Fig. 1). Fig. 1 also illustrates the typical CPE observed. The CPE was characterized by extensive fusion and formation of syncytia, features which are commonly observed with other paramyxoviruses. The IFA specificity was confirmed by the absence of fluorescent staining in uninfected cells.

Constant-serum varying-virus neutralization test

No cytopathic changes were observed at any dilution where horse serum positive for HeV antibodies was incubated with the virus isolates. In the control, where horse serum negative for HeV antibodies was incubated with the virus isolates, CPE was observed up to the $10^{-4}$ dilution.

Taqman assay

These isolates were positive when tested with the Taqman assay. RNA extracted from the uterine fluid of Bat 1, and RNA extracted from the tissue homogenates of both foetuses (foetal lung and liver pool from Bat 1, and foetal lung from Bat 2) as well as supernatant from different passages all gave positive results, confirming the original isolation.

Serology

Bat 1 tested positive by HeV ELISA and by the HeV serum neutralization test (titre 1:40). Serum collected from pooled organs of the foetuses of Bat 1 was also ELISA-positive, but toxic on serum neutralization test. Blood was not collected from Bat 2, or from the foetus of Bat 2.

Electron microscopy

Preparations of supernatant collected from CPE-producing wells, examined by negative contrast electron microscopy, revealed virus particles with typical paramyxovirus morphology. Fig. 2 shows an example of the nucleocapsid filaments which were observed in the infected supernatant. The ‘herring-bone’ morphology of the nucleocapsid filaments produced by
the close association of the N protein with the genomic RNA, which is characteristic of the Paramyxoviridae family, is clearly visible. Surface projections were also visible on the virions.

Discussion

Following the first reported outbreak of HeV infection of horses (Murray et al., 1995), the Queensland Department of Primary Industries (DPI) Animal and Plant Health Service undertook a search for the natural reservoir host of the new virus. A wide range of domesticated and native animals were surveyed for serological evidence of infection, but none was found. Subsequent to the second reported outbreak (Rogers et al., 1996), a multidisciplinary group including representatives from the Queensland DPI, the University of Queensland, and the Australian Quarantine Inspection Service reviewed the available laboratory and epidemiological data. As a result we postulated that pteropid bats were a plausible natural host as they best fulfilled the proposed characteristics of the most likely reservoir host of HeV (Young et al., 1996).

As previously described, our targeted serological surveillance of pteropid bats revealed antibodies capable of neutralizing HeV in all four mainland pteropid species (Young et al., 1996). However, the possibility existed that these animals were infected with a related paramyxovirus and it was therefore important to isolate virus from pteropid bats and compare it to HeV.

In targeting tissues for virus isolation, we developed the hypothesis that virus excretion might be associated with the birthing process. In forming this hypothesis, we firstly observed that a serological study of people with close and prolonged contact with pteropid bats had revealed no evidence of infection (Selvey et al., 1996). We interpreted these findings as possibly indicating that the route of excretion of the virus was novel. Secondly, the outbreaks of HeV infection in horses occurred during the birthing season of three of the four outbreaks.

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