

ISOLATION OF HENDRA VIRUS FROM PTEROPID BATS: A NATURAL RESERVOIR OF HENDRA VIRUS

Samples of liver, kidney, lung, spleen and reproductive organs were removed aseptically at necropsy of pteropid bats which had recently died or been euthanized. Blood samples were collected by cardiac puncture.

Sample

Virus Isolation

- 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 passaged with attempts terminating after five serial passages.

- Upon detection of CPE, infected cells were lysed by freezing and thawing. RNA was isolated by incubating 0.3 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.

RNA Extraction

- RT-PCR was performed with the Expand RT preamplification system according to the manufacturer's recommendations.
- 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) deoxynucleoside triphosphates (dATP, 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 Tth 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.



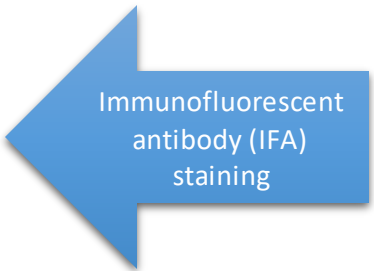
RT - PCR



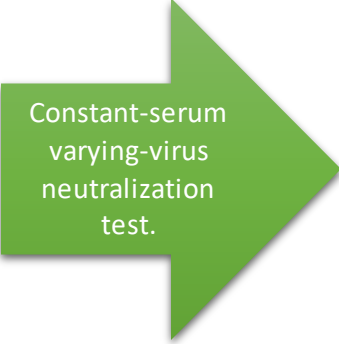
Nucleotide sequencing of the target genomic region

- 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.
- After recovery, sequencing products were resolved on an ABI automated A373 sequencer (PE Applied Biosystems) according to the manufacturer's instructions.

- When CPE was detected, cell culture supernatant was used to inoculate 16-well glass chamber slides (Nunc) containing RK13 cells (2×10⁴ 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.
- 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.




Immunofluorescent antibody (IFA) staining



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 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 μ l per well). Wells were monitored for signs of CPE.

- The first step involved conventional cDNA production from RNA (extraction method described previously) using random primers.
- 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



Taqman assay



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 μ l per well) by incubation at 37 °C for 1 h.
- Following a brief rinse (3 \times 200 μ l) with PBS containing 0.1% Tween 20 (PBST), the plates were blocked for 1 h by the addition of 200 μ l per well of 1% skim milk powder (SMP) in PBS.
- Plates were washed again.
- Pteropid bat serum (50 μ l), 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 μ l 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 μ l of 1 M sulfuric acid and the absorbance was read at 450 nm.
- Positive and negative control horse sera were included on each plate.

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 60 kV, using a magnification of $\times 25000$ plus the $\times 10$ binocular.



Electron microscopy