Title: **Novel Hendra virus variant detected by sentinel surveillance of Australian horses.**

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Article Summary Line: A novel variant of Hendra virus was discovered and isolated from an Australian horse with fatal disease.

Running Title: HeV variant associated with fatal disease in a horse

Abstract: A novel Hendra virus (HeV) variant was identified through multidisciplinary and interagency syndromic surveillance of a horse that suffered acute fatal disease consistent with HeV infection. Novel molecular assays for HeV detection are described in the light of routine testing failure. *In silico* analysis of the variant receptor-binding protein in comparison with prototypic HeV supported that the monoclonal antibody m102.4 used for post-exposure prophylaxis, as well as the equine vaccine, should be effective also against this novel variant. Similarity of this virus (99%) to a partial sequence detected from a South Australian grey-headed flying fox, along with case exposure to this species in Queensland, suggests the variant circulates at least across the range of this flying fox species. Investigation into HeV diversity, comparative kinetics and pathogenicity, reservoir-species associations, viral–host co-evolution and spillover dynamics should be prioritized. Biosecurity practices should be updated to appreciate HeV spillover risk across all regions frequented by flying foxes regardless of species.

Keywords: Hendra Virus; Henipavirus; Henipavirus Infections; Encephalitis Viral; Zoonoses; Severe Acute Respiratory Syndrome; Vasculitis, Central Nervous System, Undiagnosed Diseases; Syndromic Surveillance; Syncytia (entry term for 'Giant Cells'); Sentinel Surveillance; Catastrophic Illness; Critical Illness; Acute Disease; One Health; Communicable Diseases, Emerging; Horse Diseases; Sentinel Species; RNA-Seq; Whole-Genome Sequencing; Reverse Transcriptase Polymerase Chain Reaction; Nested Polymerase Chain Reaction.

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Introduction

Highly pathogenic zoonotic Hendra virus (HeV) and Nipah virus (NiV) are the prototypic members of the genus *Henipavirus*, family *Paramyxoviridae*, with natural reservoirs in pteropodid flying foxes (1). These viruses exhibit wide mammalian host tropism and cause severe acute respiratory and encephalitic disease mediated by endothelial vasculitis in horses, pigs and humans, with high fatality and chronic encephalitis among survivors (2–4). As of March 2021, 63 natural spillovers of HeV have been recognized in Australian horses, resulting in 105 horse deaths, with four of the resultant seven confirmed human cases being fatal (5–7). NiV has caused zoonotic outbreaks in Asia, resulting in more than 700 human deaths, with human case fatality rates as high as 78% (8,9). Henipaviruses are considered to be one of the most significant infectious threats to global health security (1,10).

Vaccination and post-exposure prophylaxis (PEP) strategies have been developed against both HeV and NiV (11) and a subunit vaccine based on the receptor-binding protein (RBP; attachment G glycoprotein) has been licensed and used in horses in Australia since 2012 (12). A human vaccine is under development (13). The monoclonal antibody (mAb) m102.4 has been administrated as emergency PEP in 15 human cases and demonstrated safety and immune efficacy in phase I trials (14). Combinations of cross-reactive humanized antibodies against the fusion (F) protein and the RBP have been described for development as PEP (15–17).

Serological testing has detected anti-HeV antibodies in all four Australian mainland *Pteropus* spp., colloquially known as flying foxes. Reported HeV seroprevalence rates across all these species vary between ~20% and ~65% (18,19), including *P. poliocephalus*, the grey-headed flying fox (GHFF), for which HeV seroprevalence has been reported as ~40% from roosts sampled in South Australia and Victoria (20) and 57% from a roost in south-east Queensland (19). However, HeV transmission from flying foxes to horses is thought

74 to occur primarily via flying fox urine (21) and the spatial distribution of detections of spillover to horses, as
75 well as flying fox testing to detect HeV excretion, suggests that mainly black flying foxes (BFF, *P. alecto*) and
76 spectacled flying foxes (SFF, *P. conspicillatus*) play active roles in disease transmission (21,22).

77 Horses are the only species known to be infected by natural HeV spillover from flying foxes. Humans
78 become infected after close contact with infected horses. Horses are raised and kept for a variety of purposes
79 across Australia, with numbers estimated to exceed 1 million, including several hundred thousand wild horses
80 known as 'brumbies' (23,24). Horses grazing behavior, combined with their large respiratory tidal volume and
81 the large surface area ratio of their highly vascularized upper respiratory epithelium, likely contribute to their
82 vulnerability to respiratory droplet HeV spillover from flying foxes (25,26). Detection of spillover to horses
83 relies on the attending veterinarian recognizing symptoms consistent with disease, appropriate sampling, and
84 laboratory submission for priority state laboratory testing.

85 Passive surveillance via suspect disease testing is affected by strong regional bias for regions where
86 HeV has previously been detected, including coastal north Queensland to northern New South Wales, and
87 where relatively large horse populations overlap with the distribution of BFFs and SFFs (27). Testing for HeV
88 is less commonly performed on horses suffering similar disease further south in Australia due to a perception
89 that the likelihood of disease occurrence is lower in regions without these flying fox species (28).

90 Over 1000 horses are tested for HeV annually; many of which are suffering disease that is clinically
91 consistent with HeV, such as acute severe respiratory and/or neurological disease often featuring pyrexia and
92 other signs of disease mediated via endothelial vasculitis. However, less than 1% of horses tested annually are
93 found to be positive (29). Studies analyzing Australian flying fox urine have identified multiple novel flying
94 fox-borne rubulaviruses and a novel henipavirus, cedar virus (CedPV), through virus isolation (30,31) and
95 molecular evidence of many more related paramyxoviruses (32). Routine testing for HeV infection is specific
96 for the matrix (M) gene (33). Alternative PCR diagnostic approaches are only routinely applied to HeV-positive
97 samples that undergo further molecular characterization as part of confirmatory testing (34). Importantly, this
98 means that most horse-disease cases found to be negative for HeV are not investigated further. This is consistent

99 with the traditional approach to animal health surveillance globally, which prioritizes an exclusion testing
00 approach for specific and well-characterized disease agents rather than a more open-ended approach pursuing
01 diagnoses in animals with disease.

02 Our 'Horses as Sentinels for New and Emerging Infectious Diseases' research used an innovative
03 multidisciplinary and interagency approach combining clinical–syndromic analysis, and molecular and
04 serological testing, to verify our hypothesis that some severe viral-like diseases in horses considered consistent
05 with HeV, but negative when tested, may result from novel paramyxovirus spill-over from flying foxes,
06 potentially posing similar zoonotic risk. We report the identification of a previously unrecognized variant of
07 HeV (HeV-var) that resulted in severe neurological and respiratory disease in a horse that was clinically
08 indistinguishable from prototypic HeV infection.

09 **Materials and Methods**

10 *Study cohort*

11 We constructed a biobank of diagnostic specimens collected from horses that underwent HeV testing in
12 Queensland between 2015 and 2018, were negative by reverse transcriptase quantitative PCR (RT-qPCR) and
13 for which no causative diagnosis had been determined. Clinical, epidemiological and sample-related data were
14 recorded, and samples archived at –80°C. Information collected on each individual case was drawn from
15 primary laboratory submission documentation and variably included age, sex, breed, geographic location,
16 clinical findings, samples submitted, vaccination status, number at risk, number affected, treatments
17 administered and other notes by the submitting veterinarian such as perceived exposure to flying foxes. We
18 applied a decision algorithm based on analysis of clinical disease and by syndrome and categorized each case
19 by its likelihood of having an infectious viral cause (Table 1). Samples given the highest likelihood of having an
20 infectious cause (priority 1 & 2) were plated for serological screening and high-throughput RNA extraction
21 (EDTA blood, serum, nasal swab, rectal swab) using the MagMAX™ mirVANA kit (ThermoFisher, Australia).
22 Samples from the entire cohort were extracted using the MagMAX™ CORE pathogen kit to obtain total nucleic
23 acid for confirmatory and comparative testing.

24 *Pan-paramyxovirus RT-PCR screening*

25 cDNA was prepared from extracted RNA using Invitrogen SuperScript IV VILO mastermix with
26 ezDNase (ThermoFisher, Australia). A 'nested' pan-paramyxovirus reverse transcriptase-PCR (RT-PCR) assay
27 targeting the L protein gene was adapted from the approach described by Tong et al (35) for use with the
28 Qiagen AllTaq PCR Core kit (QIAGEN, Australia). Positive bands corresponding to the expected size (584 bp)
29 were identified by gel electrophoresis before purification with AMPure XP (Beckman Coulter, Australia). To
30 capture weak hits, pools were also prepared by equal volume mixing of all PCR products across plated rows and
31 purified as before. Next-generation sequencing was performed using an Illumina iSeq with the Nextera XT
32 DNA library preparation kit (Illumina, Australia). For analysis, reads were assembled with MEGAHIT before
33 identification of viral sequence by comparison to NCBI GenBank with BLAST (36).

34 *HeV-var whole-genome sequencing*

35 Samples positive by RT-PCR for the novel HeV-var were subjected to meta-transcriptomic sequencing
36 to determine the complete genome sequence and identify co-infecting agents. RNA was reverse-transcribed
37 with Invitrogen SSIV VILO mastermix (ThermoFisher, Australia) and FastSelect reagent (QIAGEN, Australia).
38 Second-strand synthesis was performed with Sequenase 2.0 before DNA library preparation with Nextera XT
39 and unique dual indexes. Sequencing was performed on an Illumina NextSeq (Illumina, Australia) generating
40 100 M paired reads (75 bp) per library.

41 *Assembly and comparative genomic and phylogenetic analyses*

42 For genome assembly, RNA sequencing reads were trimmed and mapped to a horse reference
43 genome (GenBank GCA_002863925.1) using STAR aligner to remove host sequences. The non-host reads
44 were *de novo* assembled with MEGAHIT (37) and compared with the NCBI GenBank nucleotide and protein
45 databases using blastn and blastx. The putative virus contig was extracted and reads were remapped to this draft
46 genome using bbmap v37.98 (https://sourceforge.net/projects/bbmap) to examine sequence coverage and

47 identify any misaligned reads. The majority consensus sequence was extracted, aligned and annotated with the
48 prototype HeV strain in Geneious Prime v2021.1.1.

49 For classification, we used an alignment of the paramyxovirus polymerase (L) protein sequence
50 according to International Committee on Taxonomy of Viruses (ICTV) guidelines (38). We also prepared an
51 alignment of partial nucleocapsid (N) and phosphoprotein (P) nucleotide sequences with other known HeV
52 strains from the GenBank database. Both phylogenies were prepared using a maximum likelihood approach
53 using MEGA X (https://www.megasoftware.net/) according to the best fit substitution model and 500 bootstrap
54 replicates.

55 *RT-qPCR assay development*

56 We adapted an existing RT-qPCR assay targeting the HeV M gene (33) to target the novel HeV-var. The
57 duplex assay uses the Applied Biosystems AgPath-ID One-Step RT-PCR kit (ThermoFisher, Australia) and
58 distinguishes prototype and variant HeV strains. Briefly, 4 µL RNA was combined with 10 µL 2× RT-PCR
59 Buffer, 0.8 µL 25× RT-PCR Enzyme Mix, 2 µL nuclease-free water and 3.2 µL primer/probe mix (0.6 µL each
60 primer, 0.3 µL each probe from 10 µM stock; Table 2). The reaction was performed as follows: 10 min at 50°C
61 for cDNA synthesis and 10 min at 95°C for RT inactivation followed by 50 cycles of 95°C for 15s, 60°C for
62 30s using the FAM and HEX channels. As a positive control, gene fragments were synthesized encoding a T7
63 promoter upstream of the partial M gene for both the prototype and variant HeV strains (Supplementary Figure
64 1). RNA transcripts were expressed using the NEB HiScribe T7 High Yield RNA Synthesis kit (New England
65 Biolabs, Australia).

66 *Virus isolation*

67 Positive samples were submitted to the Australian Centre for Disease Preparedness for isolation in Vero
68 cells (ATCC CCL-81) and primary kidney cells derived from *P. alecto* (PaKi; (39) (Supplementary methods).

69 *Serological analysis*

70 Serological analysis was performed using multiplex microsphere immunoassays with a Luminex
71 MAGPIX™ system (ThermoFisher, Australia). Initial screening for IgG antibodies was undertaken using an
72 extensive panel of bacterial (*Leptospira*, *Brucella*) and viral antigens (paramyxovirus, filovirus, coronavirus,
73 flavivirus, alphavirus and hepatitis E virus) coupled to MagPlex beads (Bio-Rad, Australia) for multiplex
74 screening. Briefly, blood or serum, diluted 1:100 was added to the beads, with binding detected following the
75 addition of a combination of biotinylated-Protein-G and -A and streptavidin–R–phycoerythrin. The median
76 fluorescence intensity (MFI) for 100 beads was read. An immunoglobulin (Ig)M assay was also applied in
77 which biotinylated anti-equine IgM antibodies were used instead of the biotinylated Protein A and G.

78 In silico *analysis of the RBP homology and mAb binding*

79 The translated protein sequence of the HeV-var RBP sequence was compared with the X-ray crystal
80 structures of the HeV RBP protein structure bound to mAb m102.4 (40) and to ephrin B2 and B3 using SWISS-
81 MODEL (https://swissmodel.expasy.org/) to assess the ability of m102.4 to neutralize this variant and
82 determine the likelihood of antibodies produced by immunization with the HeV vaccine to be protective against
83 this variant.

84 **Results**

85 *Case report*

86 In September 2015, a 12-year-old Arabian gelding suffered an acute fatal disease that was considered
87 clinically indistinguishable from prototypic HeV infection on the property on which he was bred near Gympie
88 in south-east Queensland. Clinical assessment determined moribund disease featuring depressed (obtunded)
89 demeanor, abnormal gingival mucous membranes (specifically, darkened red–purple with darker periapical line
90 and prolonged capillary refill time), tachycardia (75 beats/min), tachypnoea (60 breaths/min), normal rectal
91 temperature (38.0°C), muscle fasciculations, head pressing and recumbency with rapid deterioration over 24 h
92 resulting in euthanasia. The attending veterinarian, who had previously managed a confirmed case of HeV,
93 recognized the similarity with HeV disease and instigated appropriate biosecurity protocols. Nasal, oral and

94 rectal swab samples were obtained postmortem and combined in 50 mL of sterile saline, and blood was
95 collected in an EDTA tube. The pooled swabs and blood samples were submitted to the Queensland Biosecurity
96 Sciences Laboratory for priority disease investigation HeV testing. Testing for HeV by RT-qPCR targeting the
97 M gene did not detect viral RNA and IgG antibodies against the HeV RBP (G glycoprotein) were not detected
98 by ELISA (41). A flying fox roost near the property fluctuates in size seasonally and annually, hosting BFFs,
99 GHFFs and little red flying foxes (42).

00 *Identification of novel HeV-var*

01 The case received highest categorization by likelihood of infectious cause based on the combination of
02 injected mucous membranes with severe acute respiratory dysfunction and encephalitic signs (Table 1). The
03 polymerase (L protein) gene sequence of a novel paramyxovirus, most closely related to HeV (≈11% nucleotide
04 sequence difference in this region) was detected in both the EDTA blood and pooled swab samples by nested
05 pan-paramyxovirus RT-PCR (35).

06 A near-full length genome of a novel HeV with a mean mapped read depth of 46.9x. (Figure 1A) was
07 obtained by RNA sequencing of EDTA blood RNA. The virus was less abundant in the pooled swab sample,
08 with a mean coverage depth of 0.6X reads spanning 9.9% of the genome (Figure 1B). Importantly, no other
09 viruses were present in either the EDTA blood or pooled swab samples. The only other microbial reads
10 assembled in the blood were from *Staphylococcus aureus* (common microflora) and in the pooled swab were
11 commensal microorganisms including *Aeromonas*, *Veillonella*, *Pseudarthrobacter*, *Streptococcus*,
12 *Acinetobacter* and *Psychrobacter* species.

13 *Confirmation of HeV infection*

14 A comparison of the primer and probe binding sites used for routine diagnostic assay (33) revealed
15 mismatches in the primers and probe binding sites, explaining the initial failure to detect this variant (Figure 2).
16 An RT-qPCR assay was designed to detect both prototype and variant HeV strains in duplex (Table 2,
17 Supplementary Figures 1, 2), which amplified the viral templates with similar high efficiency (>94%) and

18 sensitivity allowing detection for <100 copies of target RNA (Supplementary Figure 2). The EDTA blood and
19 pooled swabs samples were quantified with this assay and confirmed the RNA sequencing result with higher
20 abundance in the EDTA blood than in the pooled swabs – with a quantification cycle (Cq) of 26.87 from blood
21 versus 30.67 from pooled swabs. Virus isolation was successful from the EDTA blood sample only in Vero
22 cells.

23 Blood was tested for IgM and IgG antibodies against an extensive panel of 33 antigens representative of
24 bacterial and viral zoonoses to which Australian horses might be exposed, including the paramyxoviruses HeV,
25 NiV, CedPV, Mojang, Ghana, Tioman, Menangle, Grove and Yeppoon viruses. No significant reactions were
26 observed to this group in either the IgG or IgM assays, signifying a lack of detectable antibodies consistent with
27 the acute viremic stage of infection.

28 *Genomic analysis of novel HeV-var*

29 A comprehensive phylogenetic comparison of this novel HeV-var was made to other known
30 paramyxoviruses (Figure 3A–C). Comparison of the nucleotide similarity of this novel HeV-var to the HeV
31 prototype strain (GenBank NC_001906) revealed an 83.5% pairwise identity (Figure 3D). Importantly, the
32 phylogenetic analysis of the L protein sequence revealed that branch lengths of the prototype and variant HeV
33 strains back to their common node did not exceed 0.03 (Figure 3A,B) and therefore these should be considered
34 viruses of the same species, consistent with ICTV criteria (38). However, the HeV-var is clearly novel and well
35 outside the known HeV diversity (Figure 3C). Following our finding, comparison with a partial novel HeV
36 sequence derived from a GHFF from South Australia in 2013 (43) revealed 99% similarity between the viruses
37 in the M gene (J Wang, pers. commun.; data not shown), suggesting that this novel HeV-var represents a
38 previous undescribed lineage, with reservoir-host infection at least across the range of this flying fox species.

39 In silico *analysis of the RBP*

40 The pattern of sequence similarity showed greatest variability in the non-coding regions and the mean
41 pairwise identity across the genome was 86.9% for the coding regions (Figure 3D). At the protein level, the

42 variant shared between 82.3% and 95.7% amino acid identity (mean 92.5%) when compared with the HeV
43 prototype (Table 3).

44 Specifically, the HeV RBP showed 92.7% amino acid identity. Modeling of the novel HeV-var based on
45 the comparison of translated protein sequence using the previously published X-ray crystal structure of the
46 prototypic HeV RBP (40) determined that the HeV RBP epitopes for binding of ephrin B2 and B3 receptors
47 (data not shown) as well as for m102.4 should remain functionally unchanged due to consistency between the
48 virus protein sequences at key residues (Figure 4).

49 **Discussion**

50 This research provided an opportunity for diagnostic investigations using an innovative syndromic
51 surveillance and collaborative testing approach beyond what is routinely available. It identified a novel HeV-var
52 that failed detection by routine diagnostic testing. Based on the ICTV criteria (38), the virus identified is
53 considered to be a novel variant rather than a new henipavirus species. This finding highlights the potential for
54 improved novel pathogen detection via a 'One Health' interagency and multidisciplinary approach to syndromic
55 infectious disease surveillance research extending from routine biosecurity operations and prompts recognition
56 of the importance of targeted emerging infectious disease (EID) sentinel surveillance. We have described a new
57 assay suitable for routine human and animal health laboratory diagnosis and surveillance of HeV.

58 Comparison of the translated protein of the RBP of the HeV-var with the prototype HeV *in silico*
59 through computer modeling revealed that the HeV-var shares the same ephrin B2 and B3 receptor binding sites.
60 It is expected that the current PEP using m102.4 will be effective against this virus. Due to the high similarity at
61 the protein level between this variant and the prototypic HeV, it is also expected that the current HeV horse
62 vaccine will elicit protective antibodies and current serological assays would be unable to distinguish between
63 exposure to the variants.

64 The 99% similarity between the HeV-var and a partial sequence generated from a GHFF in Adelaide
65 from 2013 highlights that a greater diversity of HeV variants circulate among flying fox species than has been

66 previously recognized and suggests that this novel variant circulates as a relatively consistent sublineage at least
67 across the range of this flying fox species.

68 These finding prompts urgent reassessment of HeV spillover risk for horses and handlers living in
69 southern NSW, Victoria and South Australia where previously the risk has been considered substantially lower
70 compared with regions within the distribution of BFFs. Updated molecular assays used in screening flying foxes
71 for HeV to reliably detect this HeV-var in both research testing and routine surveillance may afford additional
72 evidence to demonstrate that circulation of this variant of HeV (and possibly others) resolves the previously
73 reported anomaly of high seropositivity despite low HeV detection (18,19).

74 Despite relatively high genetic divergence, the predicted phenotypic similarity of this variant to the
75 prototypic HeV, combined with the observed consistency of equine disease to that seen with prototypic HeV,
76 supports an understanding of equivalent pathogenicity and infection kinetics. Further characterization of HeV
77 strain and variant diversity and any host-species associations should offer a greater understanding of
78 transmission and spillover dynamics, as well as viral–host co-evolution that might have featured co-divergence
79 and/or been subject to founder effects (44). BFFs have rapidly expanded their range southward in response to
80 habitat clearing and other anthropogenic influences (45), increasing their overlap with GHFFs. These changes,
81 along with climatic change and habitat loss, are changing the extent and nature of interspecies interactions.
82 Sampling of multiple species across time and space will be required to understand how the new HeV-var
83 circulates within and among species, and if it interacts with classical HeV within this rapidly changing ecology.
84 Critically, biosecurity practices should be updated to include appreciation of spillover risk in all regions
85 frequented by flying foxes regardless of species.

86 Passive disease surveillance and biosecurity risk management for HeV and EIDs in Australian animals
87 largely relies on recognition and management of suspect disease cases by private veterinarians who play crucial
88 roles that are relevant to animal and human health. In performing these roles, veterinarians have been
89 challenged by tensions between obligations to simultaneously serve the animal patient and animal owner,
90 manage biosecurity risk and meet their *Workplace Health and Safety Act and Biosecurity Act* requirements (46–

91 49). Guidelines to assist in this process have been developed by an Inter-Agency Technical Working Group
92 (50). Prioritization of this case in our research testing pathway was based on the clear description of disease
93 consistent with HeV by the attending veterinarian. This detection highlights the potential for improved EID
94 surveillance via interdisciplinary research targeting novel and divergent priority One Health pathogens
95 extending from government biosecurity significant disease investigations. Systematic interpretation of clinical
96 and field observations guided parallel serology and molecular testing pathways constructed to best suit available
97 sample-types and to target diseases of highest relevance to clinical, species and regional context. The example
98 serves a proof of concept, highlighting need and benefit in further integration of such approaches with routine
99 on-going biosecurity operations.

00 Acknowledging the limitations of this single case and the unavailability of tissue for histopathology and
01 immunohistochemistry, it is nonetheless appropriate that this HeV-var be considered equally pathogenic to the
02 known HeV based on the coherent and consistent available evidence. Specifically: the observed clinical signs of
03 disease and pathology; evidence of viraemia; the phylogenetic analysis indicating that the variant belongs to the
04 HeV species; and the modeling of the interactions of the functional RBP domain to the cell receptors ephrin
05 B2/B3 predicts this is unchanged relative to the prototypic HeV. Moreover, this case fits the case definition for
06 HeV infection in Australia's AUSVETPLAN, which is that an animal tests positive to HeV using one or more
07 of PCR, virus isolation or immunohistochemistry (18).

08 This discovery resulted from our innovative 'Horses as Sentinels for New and Emerging Infectious
09 Diseases' collaborative research approach and serves to demonstrate the value in integrated multidisciplinary
10 and multisector infectious disease research extending beyond routine state biosecurity operations. Updated PCR
11 diagnostics for routine use have been developed and are in use in Australia's animal and human health
12 laboratories.

13 Our findings demonstrate the limitation of exclusion-based testing for emerging zoonoses and a gap in
14 our understanding of how frequently the detection of known zoonoses across a broad range of systems is missed
15 because of the diagnostic tools used.

Further investigations to determine the prevalence of HeV-var circulation among and excretion from all Australian flying fox species should be prioritized. The risk of zoonotic HeV disease in horses and in-contact humans should be interpreted across all regions frequented by flying foxes (regardless of species), particularly in areas previously considered to be low risk for HeV spillover.

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60 **Author Biographies**

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Supplementary Methods

Viral isolation

Positive case samples for the novel HeV were sent to the Australian Centre for Disease Preparedness (ACDP), a World Organisation for Animal Health Reference Laboratory for Hendra and Nipah virus diseases, in line with established national arrangements for confirmatory testing of notifiable disease of animals. Virus isolation was attempted in Vero cells (ATCC CCL-81) and primary kidney cells derived from *P. alecto* (PaKi; (39) on whole blood and pooled nasal, oral and rectal swab samples. Vero cells were grown at 37°C in EMEM (ThermoFisher Scientific) containing 10% fetal calf serum (FCS; ThermoFisher Scientific), supplemented with 1% v/v L-glutamine, 10 mM HEPES, 0.25% v/v penicillin–streptomycin and 0.5% v/v amphotericin B (Sigma-Aldrich). PaKi cells were cultured in DMEM/F-12 media (ThermoFisher Scientific) with 5% FCS and supplemented as above.

For virus isolation, washed monolayers of cells were inoculated with 500 µL of whole blood diluted 1:5 in culture media or 500 µL of pooled swab sample prefiltered (0.45-µm cellulose acetate) to remove bacteria and

24 any residual solid particles. Inoculum was removed after 45 min and cell monolayers were washed with
25 phosphate-buffered saline, then overlaid with culture media containing 1% (v/v) FCS. Flasks were incubated at
26 37°C for 6–7 days and regularly monitored for cytopathic effect by light microscopy. Cells were then frozen,
27 thawed and the cell suspension clarified by centrifugation (1000*g* at 4°C). Supernatant (500 µL) was then
28 passaged onto fresh cell monolayers. A maximum of three passages per sample were performed on each cell
29 line. Final pass samples were tested by RT-qPCR to detect the presence of replicating HeV genome.

530 **Table 1**. Infectious disease prioritization categories (with examples) used in this study to identify Hendra-negative equine disease cases with
531 highest likelihood of similar undiagnosed viral cause from larger cohort for further investigation

Infectious disease priority	**Description**	**Example**
Category 1 Highest infectious disease suspect	Case features 'pyrexia' or 'abnormal mucous membranes AND one or more other clinical signs related to infectious disease OR the presence of either change AND 'epidemiological observation indicative of infectious cause' based on temporal and/or spatial relationship to similar disease cases	Pyrexia with tachycardia and acute onset respiratory consolidation and/or secretions. Pyrexia and neurological symptoms. Pyrexia and 'injected/congested' mucous membranes. 'Congested/injected mucous membranes' with acute severe respiratory dysfunction. Clustering of similar cases on same or neighboring properties
Category 2 High infectious disease suspect	Pyrexia OR other clinical signs associated with infectious disease of interest	Acute onset abnormal respiratory secretions. Fever of unknown origin. Colic with the presence of neurological symptoms
Category 3 Moderate infectious disease suspect	Clinical signs may be associated equally with infectious and non-infectious causes	Colic with the presence of dehydration and mucous membrane changes. Ataxia with the absence of pyrexia or known trauma
Category 4 Low infectious disease suspect	Non-infectious etiologies more common or most likely on differential diagnosis list, but infectious cause still possible	Ataxia following known traumatic event. Traumatic wounds following unusual behavioral event. Acute lethargy following chronic non-infectious disease condition
Category 5 No infectious disease suspect	No clinical signs of illness or no infectious cause considered likely	Traumatic wounds in the absence of underlying disease. Screening in unvaccinated horses to manage biosecurity risk prior to invasive procedures addressing non-infectious disease such as is a common requirement for dentistry or admission to equine hospitals in Australia
Category 6 Confirmed infectious disease	Other infectious disease confirmed via diagnostic testing	A case submitted for HeV testing, found negative and then testing positive for alternative known infectious disease such as ABLV, WNV, EHV or RRV*

532 *ABLV, Australian bat lyssavirus; EHV, Equine herpes virus; HeV, Hendra virus; RRV, Ross River Virus; WNV, West Nile virus

533 **Table 2**. Oligonucleotides used for new HeV duplex RT-qPCR targeting matrix gene

Virus	Name	Sequence (5'-3')[1]	Reference
Prototype	Mr_fwd_1	CTTCGACAAAGACGGAACCAA	34
	Mr_rev_1	CCAGCTCGTCGGACAAAATT	
	Mr_prb_1	FAM-TGGCATCTT-ZEN-TCATGCTCCATCTCGG-IABk	
Variant	Mv_fwd_1	TCTCGACAAGGACGGAGCTAA	This study
	Mv_rev_1	CCGGCTCGTCGAACAAAATT	
	Mv_prb_1	HEX-TGGCATCCT-ZEN-TCATGCTTCACCTTGG-IABk	

534 [1]FAM and HEX 5′ reporter dyes were combined with ZEN Internal Quencher and the 3′
535 quencher Iowa Black, and supplied by Integrated DNA Technologies, Singapore.

536 **Table 3**. Novel HeV variant protein length and similarity to prototype strain (NCBI GenBank
537 accession NC_001906)

Protein	Length (AA)	Similarity (%)
Nucleoprotein	532	96.6
Phosphoprotein	707	82.3
Matrix	352	95.7
Fusion	546	95.4
Glycoprotein	603	92.5
Large	2,244	95.7

538

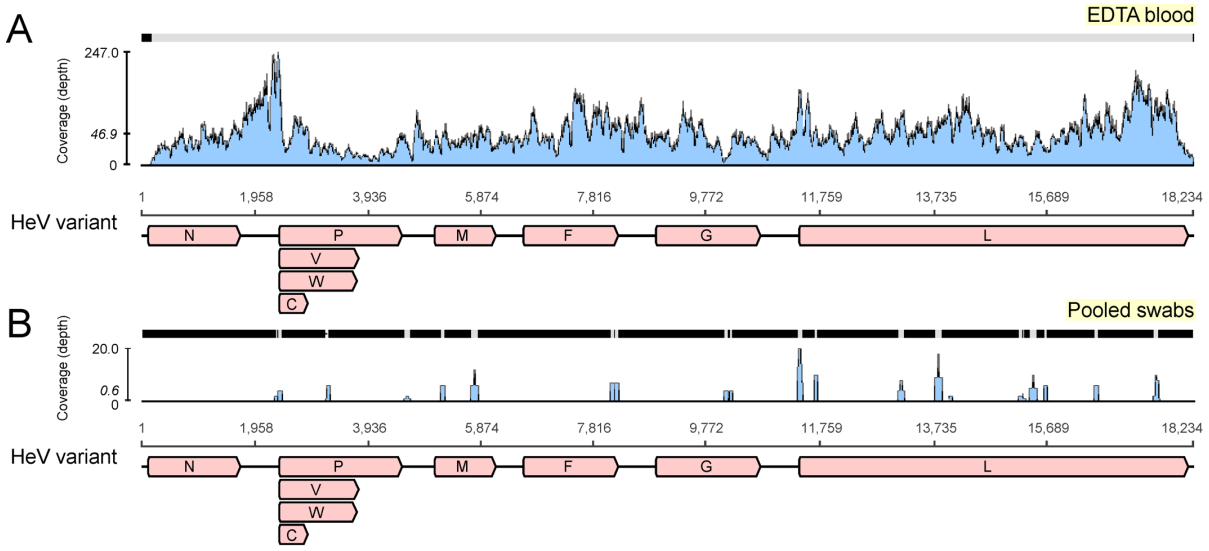

Figure 1. Sequence coverage of novel Hendra virus (HeV) variant. The RNA sequencing reads were mapped to the novel HeV variant genome to examine coverage across the genome and depth for the EDTA blood and pooled swab samples in panels A and B, respectively. The x-axis shows the genome position with genes annotated and the y-axis shows the sequence read coverage (depth). The italicized coverage value indicates the mean coverage depth for that sample.

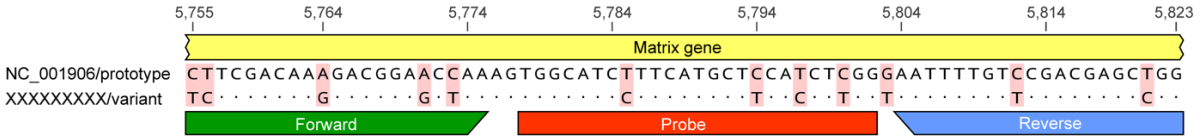

Figure 2. Genomic variation in the Hendra virus (HeV) matrix gene assay primer/probe binding sites. The genomic region targeted by the commonly used HeV matrix gene RT-qPCR assay (Smith et al *J Virol Methods* 2001;98:33–40) was aligned and compared for the prototype and variant HeV strains. The genomic positions relative to the prototype strain (NCBI GenBank accession NC_001906) are shown at the top. Primers (forward and reverse) and probe binding sites are indicated by the colored bars. Mismatches between the sequenced have been highlighted with red shading.

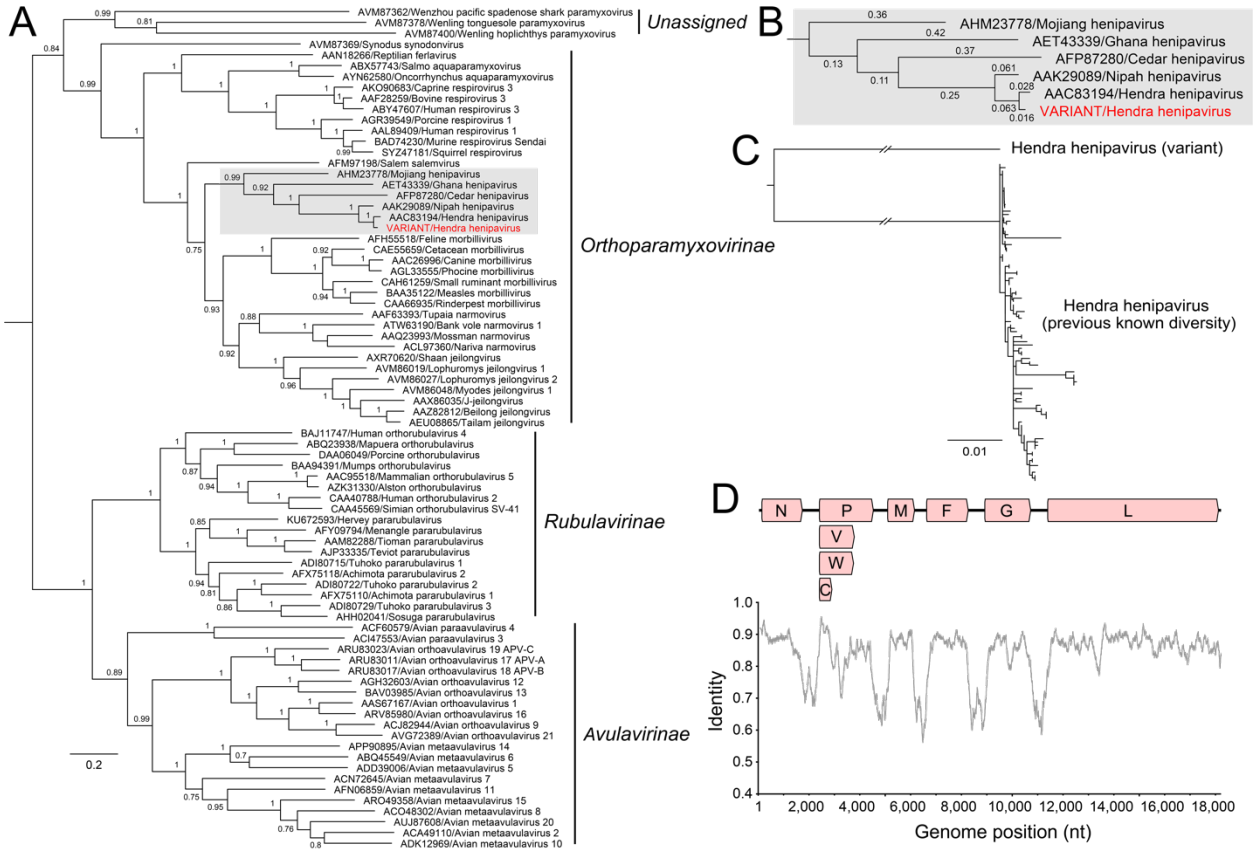

Figure 3. Phylogenomics of novel Hendra virus (HeV) variant. Panel A shows a maximum-likelihood phylogeny of paramyxoviruses using complete L protein sequences with henipaviruses highlighted in grey. The novel HeV (colored red) groups with the prototypic HeV. Bootstrap support values as proportions of 500 replicates are shown at nodes with values less than 0.7 hidden. Panel B shows the branch lengths for the henipavirus clade, showing that the branch leading back to the common ancestor of all known HeVs and this novel virus does not exceed 0.03; thus they are considered variants of the same species. For the phylogenies, the scale bars indicate the number of substitutions per site. Panel C shows a maximum-likelihood phylogeny of partial N and P where deep branch lengths have been collapsed for visualization only to demonstrate that the variant is well outside the known diversity of HeV. Panel D shows nucleotide genomic similarity of the variant compared with the prototypic HeV strain.

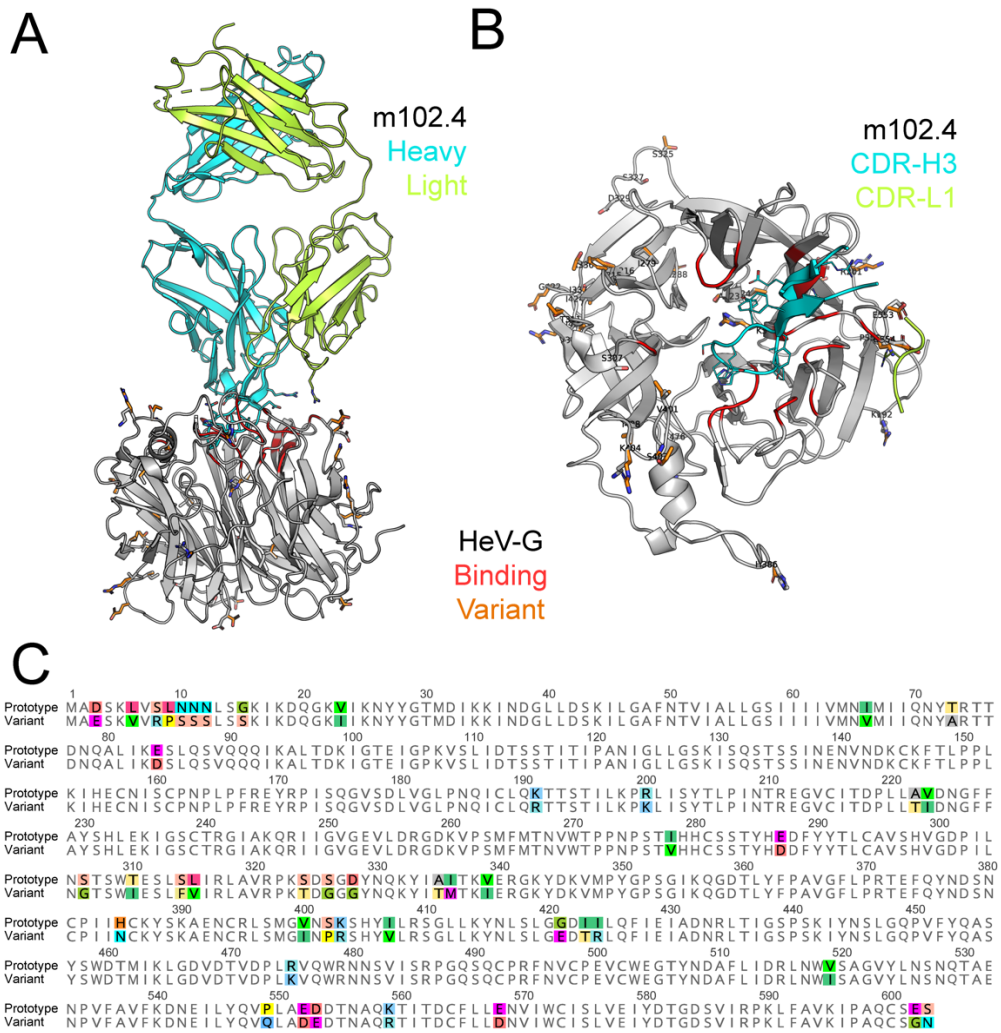

Figure 4. *In silico* modeling of Hendra virus (HeV) variant G protein (receptor-binding protein) with humanized monoclonal antibody. The translated protein sequence encoded by the HeV variant G gene was modeled using the known protein structure of the prototype virus bound to the humanized monoclonal antibody m102.4. Panel A shows the side view of the interactions between the HeV G protein and m102.4, highlighting key binding residues (red) and the variant positions (orange) relative to the m102.4 heavy (cyan) and light (green) chains. Panel B shows a zoomed top view at the HeV G and m102.4 binding interface, highlighting specific interactions between the complementarity-determining regions. These data show that variable positions do not occur at critical epitopes at the HeV G and m102.4 binding interface. Panel C shows an alignment of the prototypic and variant HeV strain G proteins with variable positions highlighted in color.