Promotion of Hendra Virus Replication by MicroRNA 146a

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Hendra virus is a highly pathogenic zoonotic paramyxovirus in the genus Henipavirus. Thirty-nine outbreaks of Hendra virus have been reported since its initial identification in Queensland, Australia, resulting in seven human infections and four fatalities. Little is known about cellular host factors impacting Hendra virus replication. In this work, we demonstrate that Hendra virus makes use of a microRNA (miRNA) designated miR-146a, an NF-κB-responsive miRNA upregulated by several innate immune ligands, to favor its replication. miR-146a is elevated in the blood of ferrets and horses infected with Hendra virus and is upregulated by Hendra virus in human cells in vitro. Blocking miR-146a reduces Hendra virus replication in vitro, suggesting a role for this miRNA in Hendra virus replication. In silico analysis of miR-146a targets identified ring finger protein (RNF)11, a member of the A20 ubiquitin editing complex that negatively regulates NF-κB activity, as a novel component of Hendra virus replication. RNA interference-mediated silencing of RNF11 promotes Hendra virus replication in vitro, suggesting that increased NF-κB activity aids Hendra virus replication. Furthermore, overexpression of the IkB superrepressor inhibits Hendra virus replication. These studies are the first to demonstrate a host miRNA response to Hendra virus infection and suggest an important role for host miRNAs in Hendra virus disease.
kidney epithelial Vero cells (ATCC CRL-81) and HEK 293T cells (ATCC CRL-11268) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were incubated at 37°C under a 5% CO2/95% air atmosphere.

**Reagents.** Short interfering RNAs (siRNAs) used in this study were siGENOME siRNA reagents purchased from Dharmacon. Cells were incubated with inhibitors (antagonists) specific to mir-146a (A_A_CCGAAGAAU CAGUUU_C_C-A_C,-Chol [amoNEG]) or a negative-control antagonor (U_A_UUAACCUUACUUGU_Ua_Ua_C,-Chol [amoNEG]) purchased from GeneWorks (Adelaide, Australia); 2 refers to phosphorothioate linkages, A, C, G, and U refer to 2’ O Me A, C, G, and U, respectively. Poly(I-C) was purchased from Invitrogen (San Diego, CA).

**Purification of RNA from Hendra virus animal trials, reverse transcription, and quantitative real-time PCR.** Male ferrets (aged 12 to 18 months) and three adult males were infected with Hendra virus as part of studies described previously (15, 16). Whole-blood samples were collected in EDTA on the indicated days postinfection. RNA was purified using the RNeasy RNA purification kit from Qiagen (Doncaster, Australia). RNA samples (~200 ng) were treated with RNase-free DNase (Promega, Madison, WI) according to the manufacturer’s instructions. For miRNA-specific quantitative real-time PCR (qRT-PCR), sample polyadenylation was performed prior to cDNA synthesis by incubating RNA (~200 ng) with 1 U poly(A) polymerase (Affymetrix, Santa Clara, CA), 0.5 nM rATP (Ambion, Carlsbad, CA) in 1x poly(A) polymerase buffer (In Vitro Technologies) at 37°C for 30 min and then at 95°C for 5 min. cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s guidelines. For miRNA cDNA synthesis, a modified oligo(dT) primer (miR-PTA) was used as described previously (17). qRT-PCR was performed using Sybr green (Applied Biosystems, Foster City, CA) according to the manufacturer’s guidelines. The infectious titer was calculated by the method of Reed and Muench (21).

**Transfections.** HEK cells were seeded in 24-well plates (8 × 104 cells/well) in growth medium. Following day, cells were transfected with siRNAs (40 nM) or plasmid DNA (500 ng/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s guidelines in antibiotic-free medium.

**TCID assessment.** For 50% tissue culture infective dose (TCID50) analysis, 10-fold dilutions of tissue culture supernatants were made in medium and Vero cells added (5 × 105 cells/well) in a 96-well tissue culture. Plates were incubated for 3 days at 37°C and 5% CO2 and scored for cytopathic effect. The infectious titer was calculated by the method of Reed and Muench (21).

**3’ UTR reporter assay.** 3’ untranslated region (UTR) reporter assays were performed as described previously (22). Briefly, HEK 293T cells were transfected in suspension with 200 ng of 3’ UTR Rm and 15 ng of firefly luciferase control constructs in 6-well tissue culture plates (3 × 106 cells/well). Cells were incubated for 20 h before lysis, and firefly/Renilla assays using the Dual Glo luciferase assay system (Promega) were performed according to the manufacturer’s guidelines.

**Cloning of RNF11.** Human RNF11 was cloned from cDNA of unstimulated HEK cells using the Gateway Recombination Cloning Technology (Invitrogen) according to the manufacturer’s instructions. Primer sequences for wild-type RNF 11 were as follows: forward, ATGGGGAACCGCTACTCTAC; reverse, TCATAGACTTCTCATAGAAG. Cell viability. Cells transfected in a 96-well plate with 40 nM siRNAs were fixed 72 h posttransfection with 4% paraformaldehyde. Cell nuclei were then stained using 4’,6-diamidino-2-phenylindole (DAPI) nuclei stain (Invitrogen), and the number of viable nuclei per treatment group was quantitated using the CellInsight Personal Cell Imager (Thermo Scientific, Waltham, MA).

**Inhibition of NF-κB activity.** The pcMV-MAD-3 expression plasmid, in the absence or presence of the IkBa suppressor (23) (a kind gift of the laboratory of Michael Karin, The University of California at San Di-
Hendra virus induces miR-146a in vivo via RIG-I. To assess the role of miR-146a induction during Hendra virus replication, we next investigated whether miR-146a induction could be replicated in an in vitro model of Hendra viral infection. The human cervix carcinoma cell line, HeLa, supports both Hendra virus replication and RNA interference (RNAi)-mediated gene silencing (C. Stewart and G. Marsh, unpublished observations), providing a model cell to assess miR-146a induction in human cells. A significant increase in miR-146a levels was observed in HeLa cells infected with Hendra virus 3 h postinfection, with a ~60-fold induction observed 8 h postinfection compared to levels in uninfected HeLa cells (Fig. 2A). A relative decrease in miR-146a levels was observed at 24 h.

To investigate other miRNAs regulated by HeV infection in vitro, HeLa cells were infected for 0, 3, 8, and 24 h, at which time small RNAs were purified and levels of 88 miRNAs quantified using a RT2 miFINDER miRNA PCR array system. In addition to miR-146a, we identified 8 miRNAs differentially regulated by HeV in HeLa cells (Table 2).

The induction of miR-146a by VSV requires the cytosolic RNA helicase RIG-I (14). To determine the host receptor for miR-146a during Hendra virus infection, short interfering (si)RNAs were transfected into HeLa cells to reduce expression of RIG-I and the related helicase MDA5. A greater than 85% reduction in expression levels of these genes was observed 48 h posttransfection with siRNAs (Fig. 2B). When cells with reduced expression levels of MDA5 or RIG-I were infected with Hendra virus for 8 h, miR-146a induction was reduced significantly in cells with reduced RIG-I, compared to cells transfected with the siNEG negative-control siRNA (Fig. 2C). miR-146a induction levels were comparable in cells transfected with siNEG and cells with reduced MDA5 levels (Fig. 2C).

The Hendra virus genome has 6 genes expressing 9 proteins, with RNA editing generating multiple proteins (P, V, or W) from the P gene (25). To determine whether miR-146a expression is upregulated by a particular gene or gene product of Hendra virus, individual Hendra virus genes were transfected into HeLa cells, with miR-146a levels measured 24 h posttransfection by qRT-PCR. A significant induction of miR-146a was observed in cells transfected with V (Fig. 2D), while a small miR-146a upregulation was also observed in cells transfected with L. Interestingly, V did not upregulate miR-146a to the same extent as whole Hendra virus (Fig. 2A).

Also of interest, the induction of miR-146a by V was significantly not altered by the silencing of RIG-I (Fig. 2E). Collectively, these findings establish that miR-146a is induced as part of the cellular antiviral response to Hendra virus following RIG-I activation.

miR-146a promotes Hendra virus replication. To determine the impact of miR-146a induction on Hendra virus replication, we subsequently tested the effect of blocking miR-146a on Hendra virus replication in HeLa cells. First, it was established that an miR-146a-specific inhibitor, but not a negative-control inhibitor, blocked miR-146a induction in HeLa cells transfected with poly(I-C), a double-stranded RNA mimic that, when introduced into the cell cytoplasm, activates RIG-I (26) (Fig. 3A). In accord with this, the miR-146a-specific inhibitor, but not the negative-
control inhibitor, blocked miR-146a induction caused by Hendra virus infection (Fig. 3B). At multiplicities of infection (MOIs) of 0.01 and 0.1, Hendra virus replication was significantly reduced in cells treated with the miR-146a inhibitor but not in cells treated with the negative-control inhibitor (Fig. 3C). At a higher MOI of Hendra virus (MOI, 1), there was no significant difference in virus titers between control cells and cells with blocked miR-146a. The miR-146a specific inhibitor also significantly reduced cellular virus levels from a 24-h infection with Hendra virus (MOI, 0.1), as measured by qRT-PCR (Fig. 3D). These data suggest that miR-146a induction contributes to Hendra replication.

Modulating miR-146a target gene RNF11 promotes Hendra virus replication in vitro. To characterize the molecular components of miR-146a activity facilitating Hendra virus replication, we next studied whether known targets of miR-146a contributed to Hendra replication. Well-characterized genes targeted by miR-146a for degradation include three members of the type I interferon (IFN) antiviral immune response pathway: IRAK1, IRAK2, and TRAF6 (12, 14). We used short interfering (si)RNAs to examine the effect of specific target gene downregulation on Hendra virus replication. While siRNAs would be expected to downregulate a target gene to a greater extent than an miRNA, this approach has been used previously to study the impact of miRNA target gene downregulation on a biological process (14, 22). RNA interference (RNAi)-based downregulation of IRAK1 and TRAF6 significantly reduced Hendra virus replication, while IRAK1 RNAi did not have any effect (Fig. 4A and B). These results were counterintuitive and suggested that IRAK2 and TRAF6, while being...

TABLE 2 miRNAs differentially regulated by HeV infection in HeLa cells

<table>
<thead>
<tr>
<th>miRNA</th>
<th>3 h (P value)</th>
<th>8 h</th>
<th>24 h</th>
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<tr>
<td>mir-151-5p</td>
<td>3.65 (0.042)</td>
<td>-1.91 (0.34)</td>
<td>-3.74 (0.309)</td>
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<td>mir-128</td>
<td>2.22 (0.048)</td>
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<tr>
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<td>1.43 (0.39)</td>
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<tr>
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<td>-13.4 (0.11)</td>
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<tr>
<td>mir-28-3p</td>
<td>3.05 (0.064)</td>
<td>1.35 (0.53)</td>
<td>-1.47 (0.397)</td>
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<tr>
<td>mir-302c</td>
<td>-1.31 (0.96)</td>
<td>1.71 (0.397)</td>
<td>5.38 (0.023)</td>
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<tr>
<td>mir-150</td>
<td>1.83 (0.73)</td>
<td>3.13 (0.4)</td>
<td>5.83 (0.024)</td>
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<tr>
<td>mir-142-3p</td>
<td>2.29 (0.19)</td>
<td>2.04 (0.379)</td>
<td>9.67 (0.08)</td>
</tr>
</tbody>
</table>

FIG 2 Hendra virus induces miR-146a in vitro via RIG-I (A) miR-146a levels in HeLa cells infected with Hendra virus (MOI, 0.1). *, P < 0.05 compared to time zero samples. (B) MDA5 and RIG-I mRNA levels in HeLa cells measured 48 h posttransfection with siRNAs (40 nM). **, P < 0.01. (C) miR-146a levels in HeLa cells transfected for 48 h with siRNAs (40 nM), followed by 8 h of infection with Hendra virus (MOI, 0.1). **, P < 0.01; N.S., not significant. (D) miR-146a levels in HeLa cells 24 h posttransfection with Hendra virus genes. Mock refers to untreated cells. Lipid refers to cells treated with transfection reagent only. *, P < 0.05 compared to mock samples. (E) miR-146a levels in HeLa cells transfected for 48 h with 40 nM siRNAs, followed by 24 h transfection with V.
repressed following Hendra virus-driven miR-146a induction, significantly contributed to Hendra virus replication.

NF-κB participates in the induction of the interferon responses and affects cell survival. We therefore measured the effect of RNAi-mediated knockdown of IRAK1, IRAK2, and TRAF6 on IFN-β production and cell survival. RNAi-mediated silencing of these genes did not alter basal levels of IFN-β mRNA expression at the time of Hendra virus infection (Fig. 4C). To demonstrate that HeLa cells were capable of upregulating IFN-β mRNA in response to appropriate stimuli, cells were also transfected with poly(I·C) for 8 h, resulting in significant IFN-β upregulation. We detected no significant change in the viability of cells transfected with these siRNAs, compared to those in the negative control (Fig. 4D). As a positive control in this experiment, cells were also transfected with a siRNA targeting polo-like kinase 1 (PLK), a gene with an established role in regulation of apoptosis (27). RNAi-mediated silencing of PLK1 caused a significant reduction in HeLa cell viability. Levels of IRAK2 and TRAF6 mRNA were not significantly altered in HeLa cells by Hendra virus infection (Fig. 4E).

IRAK2 and TRAF6 are members of the NF-κB transduction pathway. The effect of downregulating these genes on Hendra virus replication (Fig. 4B) led us to speculate that NF-κB activation following Hendra virus infection could in fact be beneficial to the virus. As such, we analyzed predicted targets of miR-146a previously involved in the repression of NF-κB signaling, under the rationale that miR-146a induction would repress them and thereby potentiate NF-κB signaling. Ring finger protein (RNF)11, which is part of the A20 ubiquitin editing complex (28), was predicted to be targeted by miR-146a in horse, human, and mouse (Fig. 5A).

To determine whether miR-146a inhibits translation of RNF11, the 3′ UTR of RNF11 (22), in addition to the 3′ UTR regions of IRAK2 (a validated miR-146a target gene) and F-box and leucine-rich repeat protein 11 (Fbxl11, not an miR-146a target gene), was cloned downstream of the Renilla luciferase reporter gene. Cells expressing 3′ UTR reporter constructs were transfected with a synthetic miR-146a mimic, and reporter expression was measured 24 h later by luminescence. miR-146a caused a significant decrease in RNF11 expression (Fig. 5B), comparable to miR-146a-mediated downregulation of IRAK2, the positive control in this experiment. In contrast, miR-146a did not influence expression levels of Fbxl11. We next tested the effect of miR-146a on RNF11 mRNA levels in HeLa cells. HeLa cells were either infected with Hendra virus or transfected with synthetic miR-146a for 24 h to induce miR-146a, and RNF11 mRNA levels were measured by qRT-PCR. Both Hendra virus infection and miR-146a transfection resulted in small decreases in RNF11 mRNA levels (Fig. 5C and D, respectively) that were not statistically significant, suggesting that the effect of miR-146a on RNF11 may be exerted at the posttranscriptional level.

We then tested whether modulation of RNF11 impacted Hendra virus replication. First, it was demonstrated that siRNA-mediated silencing of RNF11 reduced RNF11 mRNA levels by more than 80% in HeLa cells (Fig. 5E). Second, the impact of reducing RNF11 levels on Hendra virus replication was tested. Compared to cells transfected with a negative-control siRNA, Hendra virus replication was significantly elevated in cells with reduced RNF11 (Fig. 5F). We also measured the impact of overexpressing RNF11 on Hendra virus replication. Cells overexpressing RNF11 showed a significant reduction in Hendra virus titers compared to control cells (Fig. 5G). Collectively, these results suggest that RNF11 is a
restriction factor of Hendra virus replication and that Hendra virus makes use of miR-146a induction to decrease RNF11 levels and favor its replication.

These results suggested that modulation of NF-$\kappa$B activity may impact Hendra virus replication. We tested the impact of blocking NF-$\kappa$B activity on Hendra virus replication in two ways. First, HeLa cells were transfected with the IkB superrepressor gene, which sequesters NF-$\kappa$B in an inactive conformation (23). Upon infection with Hendra virus, virus titers were significantly reduced in HeLa cells expressing the IkB superrepressor compared to control cells (Fig. 6A). We next measured Hendra virus replication in cells treated with two chemical compound inhibitors of NF-$\kappa$B activity, Bay11-7082 (29) and MG-132, which also inhibits the 26S proteosome (30). Hendra virus replication was significantly reduced by MG-132 and reduced (not significantly) by Bay11-7082 (Fig. 6B). At these concentrations, Bay11-7082 and MG-132 were not adversely impacting HeLa cell viability (Fig. 6C), suggesting that inhibition of Hendra virus replication by MG-132 was not due to toxicity. Both chemical compounds (Fig. 6D) and the IkB superrepressor (Fig. 6E) significantly inhibited NF-$\kappa$B activity in HeLa cells. Furthermore, increasing levels of miR-146a in HeLa cells, by intracellular transfection of an miR-146a mimic, significantly increased NF-$\kappa$B activity in HeLa cells, compared to that in cells transfected with a negative-control miRNA mimic (Fig. 6F). Collectively these results suggest that Hendra virus replication is favored by increased NF-$\kappa$B activity.

**DISCUSSION**

We have shown that miR-146a promotes replication of Hendra virus. While miR-146a has previously been shown to promote replication of two other viruses—VSV (14) and Epstein-Barr virus (13)—the mechanisms of proviral miR-146a activities differ.
the previous two studies, miR-146a downregulated IRAK1, IRAK2, and TRAF6; this, in turn, impacted type I IFN production and virus replication. However, human cells infected with Hendra virus do not upregulate expression of the type I IFN cytokines (31). Antagonism of IFN signaling by paramyxoviruses has been demonstrated in several studies. The Nipah virus phosphoprotein \((P)\) binds and inhibits STAT1 (32), which would be expected to block interferon-stimulated gene (ISG) production following IFN-dependent activation of the IFN-\(\gamma\) receptor. Furthermore, nuclear translocation of the Nipah W protein inhibits phosphorylation and activation of IFN regulatory factor 3 (IRF-3) (33). Hendra virus and Nipah virus inhibit these pathways in infected cells (31). The apparent lack of a type I IFN response to Hendra virus would suggest that proviral properties of miR-146a observed in the present study are not related to regulation of the type I IFN response.

Rather, we are proposing a unique context whereby miR-146a acts in a proinflammatory manner to aid Hendra virus replication. In a prototypic antiviral immune response, RIG-I activation prompts activation and nuclear translocation of the three transcription factors required for type I IFN production—NF-\(\kappa\)B, IRF3/7, and AP-1 (reviewed in reference 34). The transient production of type I IFN is regulated in part by the deubiquitinating complex A20, induced by NF-\(\kappa\)B and type I IFN, which feeds back to inhibit the interaction of RIG-I with the mitochondrial adaptor protein MAVS (34), leading to the termination of NF-\(\kappa\)B and IRF3/7 activation.

We hypothesize that Hendra virus modifies the type I IFN pathway to aid its replication cycle. First, as mentioned above, Hendra virus blocks type I IFN production. Consistent with these published reports, HeLa cells infected with Hendra virus did not upregulate IFN-\(\beta\) at 3 h or 8 h postinfection (data not shown).
However, despite an absence of type I IFN production during Hendra virus infection, part of the type I IFN signaling pathway remains active, as illustrated by the RIG-I-dependent induction of the NF-κB-responsive miR-146a.

miR-146a inhibits translation of RNF11, a member of the A20 complex that is required for suppression of NF-κB activity (35). We therefore hypothesize that miR-146a aids Hendra virus replication by impacting the timing and transient nature of NF-κB regulation by the A20 complex. Our results are therefore suggestive of proinflammatory properties for miR-146a to accompany previously described anti-inflammatory properties (11, 12, 36).

How NF-κB activity positively regulates Hendra virus replication will be the focus of future work. Increased NF-κB activity has been observed for other paramyxovirus infections. Sendai virus induces activity of the IκB kinase (IKK) complex that phosphorylates and activates the latent NF-κB/IκB complex (37), while respiratory syncytial virus infection results in persistent NF-κB activation and downstream interleukin-8 production in lung epithelial cells (38). NF-κB activity is associated with both negative and positive regulation of apoptosis in virally infected cells. NF-κB activity induced by hepatitis C virus, encephalomyocarditis virus, and Epstein-Barr virus blocks apoptosis (39–41), while conversely, apoptosis of dengue virus-infected cells is promoted by NF-κB activity (42). NF-κB activity can also have a more direct impact on virus replication, as observed for HIV-1, which relies on NF-κB activity for viral gene expression during monocyte differentiation due to the presence of κB binding sites in the HIV promoter (43).

Whether miR-146a-mediated regulation of NF-κB activity impacts the replication of viruses other than Hendra virus is also of interest. A genome-wide microRNA agonist–antagonist screen demonstrates that miR-146a promotes the replication of murine gamma herpesvirus 68 in mouse fibroblast cells (44). Further-
more, our own preliminary results show that herpes simplex virus-1 (HSV-1) replication is positively regulated by miR-146a in HeLa cells (data not shown). As HSV-1 gene expression is regulated by NF-κB (45), the data from our study and others are suggestive of a role for miR-146a in the replication of viruses associated with NF-κB activity.

The kinetics of miR-146a induction during Hendra virus infection are intriguing compared to other viral stimuli. In mouse peritoneal macrophages infected with VSV, miR-146a levels are most elevated 36 h postinfection and not to the extent observed for Hendra virus. The rapid induction of miR-146a by Hendra virus in HeLa cells resembles more the rapid induction observed upon LPS stimulation of THP-1 macrophages (12). This difference between Hendra virus and VSV may be explained by the high pathogenicity of Hendra virus or the high MOI used in this study. Another interesting result is the relative decrease in miR-146a levels observed after maximum induction by Hendra virus, observed here in vitro and in vivo. Given the known stability of miRNAs (46), including in serum (47), this result is intriguing and would suggest that some other mechanism, such as degradation or secretion, may occur after miR-146a production. The cellular export of miRNAs via exosomes (48) may explain the presence of miR-146a in the blood of infected horses and ferrets.

miRNA expression profiles are emerging as tools to assist clinical diagnoses, including those of viral infections (49, 50). For diagnostic decision support, miRNAs are attractive for several reasons: they can be detected from easily collected bodily fluids such as blood and urine, are stable once secreted into bodily fluids, and can be detected using common laboratory techniques such as PCR (51). Horses infected with Hendra virus do not display signs of illness (for example, elevated heart rate, temperature) or detectable Hendra virus genome in most biological samples until the onset of florid disease, which can be up to 16 days postexposure (15). However, during the incubation period, horses replicate virus in the upper respiratory tract as early as day 2 postexposure (15). At this time these horses represent a severe risk to humans, with one reported human case most likely having received exposure during this period (52). A finding of major significance from the present study is that levels of miR-146a increased during early stages of virus exposure in horses and ferrets, suggesting that measuring miRNA levels could assist the early detection of Hendra virus infection in humans and in horses. However, as it is well established that miR-146a induction is not unique to Hendra virus (12–14), a genome-wide analysis of miRNAs responsive to Hendra virus in vivo would be required to assess miRNAs as tools to aid the definitive diagnosis of Hendra virus disease.

This study exemplifies how rational analysis of miRNA targets presents novel avenues in the characterization of cellular factors aiding in viral replication. It also presents novel opportunities for treatment and diagnosis of particular virus diseases. Targeting of RNF11 could present novel therapeutic opportunities against Hendra virus infections, while miRNA profiling may aid in the confirmation of Hendra virus disease.

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