CHAPTER 3.1.14.

NIPAH AND HENDRA VIRUS DISEASES

SUMMARY

Hendra virus (*HeV*) *and Nipah virus* (*NiV*) *emerged in the last decade of the twentieth century as the causes of outbreaks of respiratory and neurological disease that infected a number of animal species and human. In 1994, HeV caused severe respiratory disease and the death of 13 horses and a horse trainer at a stable in Brisbane, Australia. NiV appeared in the human population between September 1998 and April 1999 in Malaysia as the cause of fatal acute encephalitis, after spreading primarily as a severe respiratory disease of unknown aetiology in the pig population. Over one million pigs were culled to stop spread of the disease. HeV has caused the death of four of seven infected people in Australia while it has been reported that there have been 585 cases of NiV in humans, with approximately 300 deaths, in Malaysia, Singapore, Bangladesh and India. Most recently fatal NiV encephalitis has been reported in the Philippines, where 9 of 17 human cases died. Fruit bats* (*flying foxes*) *in the genus* Pteropus *are natural hosts of both viruses.*

HeV infection of horses is characterised by high fevers, facial swelling, severe respiratory difficulty and, terminally, copious frothy nasal discharge. Ataxia and myoclonus may also be seen. Some horses display neurological signs while others have presented with colic-like signs. The most common post-mortem observations are dilated pulmonary lymphatic vessels, severe pulmonary oedema and congestion. The underlying lesion is generalised degeneration of small blood vessels in a range of organs. Syncytial endothelial cells containing viral antigen are common in capillaries and arterioles. HeV infection of horses is not uniformly fatal and some horses manifesting clinical signs survive infection. HeV does not appear to be highly contagious among horses, and close contact seems to be necessary for it to spread. Infected horses on pastures have rarely transmitted the virus. However, transmission appears to occur more readily in closed environments such as stables.

NiV infection of pigs is highly contagious, but it was not initially identified as a new disease because morbidity and mortality were not marked and clinical signs were not significantly different from other known pig diseases in Malaysia. Based on the observations made during the outbreak and during experimental infections, NiV infection of pigs is characterised by fever with respiratory and often neurological involvement, but many infections are subclinical. Some infected animals display an unusual loud barking cough. Abortion was reported in sows. Sows and boars sometimes died peracutely. Immunohistochemical lesions are found in either or both the respiratory system (*tracheitis and bronchial and interstitial pneumonia*) *and the brain* (*meningitis*) *of infected animals. Syncytial cells containing viral antigen are seen in small blood vessels, lymphatic vessels and the respiratory epithelium.*

Both viruses affect companion animals. Experimentally, HeV causes pulmonary disease in cats similar to that observed in horses while dogs may appear clinically well. Natural infection of dogs with NiV causes a distemper-like syndrome with a high mortality rates; there is serological evidence that some dogs survive infection. Experimentally NiV causes a similar disease to HeV in cats. Syncytial endothelial cells containing viral antigen were demonstrated in both HeV and NiV infections in cats and in NiV infection in dogs.

Infection of humans is from animal contact, usually from an amplifier host rather than directly from the natural, reservoir host: NiV from swine and HeV from horses. However investigations of outbreaks of human NiV in Bangladesh have indicated human infection from Pteropid bats without an intermediary/amplifier host. Human-to-human transmission has not been seen with HeV or with NiV in Malaysia and Singapore, but limited human-to-human transmission is suspected in outbreaks of NiV in Bangladesh.

HeV and NiV are closely related members of the genus Henipavirus*, subfamily* Paramyxovirinae*, family* Paramyxoviridae*. HeV and NiV are dangerous human pathogens such as are designated in laboratory risk management analyses as requiring biosafety level 4* (*BSL4*) *containment. It is important that samples from suspect animals be transported to authorised laboratories only under biologically secure conditions according to international regulations.*

Identification of the agent: *Both HeV and NiV may be propagated in a range of cultured cells. Virus isolation from unfixed field samples should be attempted, but only in situations where operator safety can be assured. Identification procedures following virus isolation include immunostaining of infected cells, neutralisation with specific antisera to the viruses and molecular characterisation. Both reverse-transcription polymerase chain reaction* (*RT-PCR*) *and real-time RT-PCR* (*qRT-PCR*) *are now available as diagnostic tests.*

Viral antigen is present in vascular endothelium, and in the case of NiV in pigs, the respiratory epithelium. A wide range of formalin-fixed tissues can be examined to detect HeV and NiV antigen by immunohistochemistry (*IHC*)*. Submissions for IHC should include samples of lung, spleen, kidney and brain at various levels including meninges. In pregnant animals or in cases of abortion, uterus, placenta and fetal tissues should be included as appropriate. Specimens for virus isolation and molecular detection should be taken from the same fresh tissues of infected organs, and/or urine, throat or nasal swabs.*

Serological tests: *Virus neutralisation tests* (*VNT*) *and enzyme-linked immunosorbent assay* (*ELISA*) *are available. ELISA is currently being used as a screening tool and VNT is currently accepted as the reference procedure and confirmatory test. The ability of antibodies to HeV and NiV to cross-neutralise to a limited degree means that a single VNT using either virus does not provide definitive identification of antibody specificity. Neutralising antibodies to HeV and NiV can be differentiated by the greater capacity to neutralise the homologous compared with the heterologous virus. This may not be a major impediment in outbreak situations where the causative agent is known, but serum samples from suspect cases or from areas of the world other than Australia and Malaysia should be subjected to VNT analyses with both HeV and NiV. The serological relationship between HeV and NiV ensures that ELISAs using HeV or NiV antigen can be used to detect antibodies to both viruses.*

Requirements for vaccines and diagnostic biologicals: *There is a vaccine available for HeV, registered for use in horses in Australia. There is no vaccine currently available for NiV.*

A. INTRODUCTION

Hendra virus (HeV) and Nipah virus (NiV) are classified in the family *Paramyxoviridae* subfamily *Paramyxovirinae*, genus *Henipavirus*. They have morphological and physicochemical properties typical of paramyxoviruses. The viruses are pleomorphic in shape and enveloped, with herringboned nucleocapsids. Virions are 40–600 nm in diameter. Glycoprotein and fusion protein spikes project through a lipid envelope. HeV and NiV have a non-segmented, single-stranded, negative-sense RNA genome (18.2 kb) consisting of six genes which code for six major strucutural proteins, namely: N (nucleocapsid protein), P (phosphoprotein), M (matrix protein), F (fusion protein), G (glycoprotein) and L (large protein).

HeV and NiV occur naturally as viruses of fruit bats commonly known as ‘flying foxes’. These are members of the genus *Pteropus,* family *Pteropodidae*. Antibodies to HeV are found in all four Australian pteropus species with seroprevalence varying over time and location. Serological surveys of antibodies to NiV show seroprevalences up to 75% in Malaysian pteropid bats (Sohayati *et al*., 2011; 2013). Antibodies to NiV or putative closely related viruses have subsequently been detected in pteropid bats over much of their geographical range: in Bangladesh, Cambodia (Olson *et al*., 2002; Reynes *et al*., 2005), Indonesia (Sendow *et al*., 2006), Madagascar (Iehle *et al*., 2007) and Thailand (Wacharapluesadee *et al*., 2005). HeV has been isolated from Australian flying foxes (Halpin *et al*., 2000), and NiV from flying foxes from Malaysia and Cambodia (Chua *et al*., 2002; Reynes *et al*., 2005; Sohayati *et al*., 2011). NiV RNA has been detected by reverse-transcription polymerase chain reaction (RT-PCR) in pteropid bat urine, saliva and blood in Thailand (Wacharapluesadee & Hemachudha, 2007; Wacharapluesadee *et al*., 2005). In Ghana, 39% of *Eidolon helvum*, a non-pteropus fruit bat, had NiV reactive antibodies (Hayman *et al*., 2008). Henipavirus-like sequences were also obtained from *Eidolon helvum* in Ghana (Hayman *et al*., 2008). The detection of antibodies to and sequences of henipaviruses in African bats suggests that the range of potential NiV infections may be wider than previously thought, though no human cases of NiV have been reported from any region other than South-East Asia.

HeV disease emerged in Brisbane, Australia, in September 1994 in an outbreak of acute respiratory disease that killed 13 horses and a horse trainer (Murray *et al*., 1995). The virus was initially called equine morbillivirus, but subsequent genetic analyses indicated that it did not resemble morbilliviruses sufficiently closely for inclusion in that genus. There have been other instances of fatal HeV infection of horses in northern Queensland and further instances of infection of people. Two horses developed an acute disease and died almost 1 month before the Brisbane outbreak, but HeV was determined to be the cause of death only after the horse owner, who probably acquired HeV during necropsy of the horses, died 13 months later with HeV-mediated encephalitis (Rogers *et al*., 1996). Since then there have been more than 40 outbreaks involving more than 75 horses but with only two outbreaks involving more than three horses. The seven human cases have resulted in four deaths (57%). All infected people have had very close contact with infected body fluids from infected horses through performing invasive procedures and/or have not worn fully protective personal equipment.

In Malaysia, retrospective studies of archival histological specimens indicate that NiV has caused low mortality in pigs since 1996, but remained unknown until 1999 when it emerged as the causative agent of an outbreak of encephalitis in humans that had commenced in 1998 (Chua *et al*., 2000; Nor *et al*., 2000). Unlike respiratory disease caused by HeV in horses, which was frequently fatal but characterised by poor transmissibility (Williamson *et al*., 1998), respiratory disease caused by NiV in pigs was often subclinical but highly contagious (Hooper *et al.,* 2001), properties that led to rapid virus dispersal through the Malaysian pig population and forced authorities to choose culling as the primary means to control spread (Nor *et al*., 2000). Over one million pigs were destroyed; 106 of 267 (39%) infected humans, mostly pig farmers in Malaysia and abattoir workers in Singapore who had direct contact with live pigs, died of encephalitis (Chua *et al*., 2000; Paton *et al*., 1999). Cats, dogs and horses were also infected on infected pig farms during that outbreak (Hooper *et al*., 2001; Nor *et al*., 2000) but the infections were not epidemiologically significant.

New outbreaks of human NiV disease have subsequently occurred on an almost annual basis in Bangladesh, with a few outbreaks in West Bengal, in neighbouring India. In outbreaks in 2001 and 2003, a domestic animal source of the human infections was not identified, but pteropid bats, *Pteropus giganteus*, were present and had antibodies capable of neutralising Nipah virus. Clustering of cases and time–sequence studies indicated that there is a possibility of human-to-human transmission at low levels. In another outbreak in 2004 in which 27 of 36 (75%) infected humans died, epidemiological evidence indicated person-to- person transmission and serological studies identified seropositive fruit bats at the location. Drinking fresh date palm sap contaminated by fruit bat saliva, urine or excreta has been identified as likely route of transmission from the wildlife reservoir to humans (Luby *et al*., 2006). As a result of these ongoing outbreaks it is estimated that across Malaysia, Singapore, Bangladesh and India there have now been up more than 585 cases of NiV in humans, with approximately 334 deaths (57%).

In 2014 in the Philippines, an epidemiologically different outbreak of human cases of NiV was reported, with 9 deaths from 17 cases (Ching *et al.,* 2015). In this outbreak infected people were initially exposed to horses having an acute encephalitis that were butchered and consumed by the people who later became infected. Some subsequent person-to-person spread was suspected. Cats and dogs were also affected.

Diagnosis of disease caused by henipaviruses is by virus isolation, detection of viral RNA in clinical or post-mortem specimens or demonstration of viral antigen in tissue samples taken at necropsy (Daniels *et al*., 2001). Detection of specific antibody can also be useful particularly in pigs where NiV infection may go unnoticed. Identification of HeV antibody in horses is less useful because of the high case fatality rate of infection in that species. Human infections of both HeV and NiV have been diagnosed retrospectively by serology. Demonstration of specific antibody to HeV or NiV in either animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of transmission of infection.

The henipavirus genus is expanding, with new viruses recently identified. Cedar virus was isolated from the urine of *Pteropus* bats in Australia, but it remains to be seen if it has the capacity to spill over to other species, and if so, cause disease (Marsh *et al*., 2012). A number of other henipa-like viruses have been detected in wildlife by PCR and sequencing, but have not yet been isolated by traditional virus isolation techniques (Wu *et al*., 2014).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for diagnosis of henipaviruses and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance (no clinical)	Immune status in individual animals or populations post-vaccination
Identification of the agent[1]						
Virus isolation	+	+	–	+++		–
RT-PCR & qRT-PCR	+	+	++	+++	++	–
IHC	–	–	–	++	–	–
IFA	–	–	–	++	–	–
Detection of immune response[2]						
ELISA	+++	+++	+++	+	+++	+++
VNT	+++	+++	+++	+	+++	+++
Bead assays	+++	+++	+++	+	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; qRT-PCR = real-time RT-PCR; IHC = immunohistochemistry; IFA = indirect fluorescent antibody; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation test.

1. Laboratory biosafety

HeV and NiV are dangerous human pathogens with a high case fatality rate and for which there is no human vaccination or effective antiviral treatment (WHO, 2004). All laboratory manipulations with live viral cultures (including serological tests such as virus neutralisation (VN) using live virus) or potentially infected/contaminated material such as tissue and blood samples must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). The safety of the laboratory workers must be assured by the biological risk management strategy adopted. Laboratories may adopt different biological risk management strategies depending on whether they are testing samples by PCR or attempting to propagate the agents.

Virus propagation in particular must be conducted under stringent and appropriate conditions that will prevent accidental infection of personnel in the laboratory. During primary virus isolation from specimens from suspect cases, it must be appreciated and reflected in procedures that if a 'paramyxovirus-like' cytopathic effect (CPE) develops in infected cultures the level of risk has increased. Appropriate biosafety guidelines will emphasise good laboratory practice, the use of class II safety cabinet with appropriate personal protective equipment or a class III cabinet. RT-PCR or immunofluorescent detection of henipavirus antigen in cells fixed with acetone may be used to identify the isolate as a henipavirus. Transfer of cultures to specialist laboratories should follow transportation standards as specified in Chapter 1.13 *Transport of biological specimens.*

1 A combination of agent identification methods applied on the same clinical specimen is recommended.
2 One of the listed serological tests is sufficient.

2. Identification of the agent

2.1. Virus isolation and characterisation

Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by HeV or NiV has not been previously documented. Molecular detection techniques which do not require handling of live virus, can be used to identify the presence of viral genome in samples. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (Daniels *et al*., 2007).

2.1.1. Sampling and submission of specimens

The range of tissues yielding virus in natural and experimental cases has been summarised (Daniels *et al*., 2000). In live animals, swabs (nasal or oro-naso-pharyngeal) and serum should always be submitted. Urine, brain, lung, kidney and spleen are also useful, and can be collected if appropriate biosafety precautions can be taken during sampling. Specimens should be transported at 4°C if they can arrive at the laboratory within 48 hours; if the transport time will be over 48 hours, the samples should be sent frozen on dry ice or nitrogen vapours (≈ –78.5°C). Specimens should not be held at –20°C.

Diagnostic specimens should be submitted to designated laboratories in specially designed containers, in accordance with Chapter 1.1.3.

2.1.2. Isolation in cultured cells

Biosafety considerations are of absolute importance during henipavirus isolation, as noted in Section 1 above.

Virus isolation is aided by the fact that HeV and NiV grow rapidly to high titre in many cultured cells. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. HeV also replicates in suckling mouse brain and in embryonated hens eggs, and laboratories using these isolation systems in the investigation of undiagnosed infections should be aware of this possibility.

In the laboratory conducting virus isolation, tissues are handled under sterile conditions, and 10% (w/v) suspensions are generated by grinding the tissues in a closed homogenisation system. All processes should be carried out in a Class III cabinet or a Class II cabinet with appropriate personal protective equipment for the operator. Tubes used should have O-rings, and an external thread. Following clarification of the homogenate by centrifugation in a rotor with safety cap at 300 ***g*** and 4°C, before the supernatant is added to confluent cell monolayers.

A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell.

2.1.3. Methods of identification

i) Immunostaining of fixed cells

The speed with which HeV and NiV replicate and the high levels of viral antigen generated in infected cells make immunofluorescence a useful method to rapidly identify the presence of henipaviruses using either anti-NiV or anti-HeV antiserum. The serological cross reactivity between HeV and NiV means that polyclonal antiserum to either virus or mono-specific antisera to individual proteins of either virus, will fail to differentiate between HeV and NiV.

a) Test procedure

Under appropriate laboratory conditions to manage biological risks, monolayers of Vero or RK-13 cells grown on glass cover-slips or in chamber slides are infected with the isolated virus, and the monolayers are examined for the presence of syncytia after incubation for 24–48 hours at 37°C. It is recommended that a range of virus dilutions (undiluted, 1/10, 1/100) be tested because syncytia are more readily observed after

infection at low multiplicity. Once visible syncytia are detected, infected cells are fixed by immersion completely in a vessel filled with acetone. The vessel is sealed and surface sterilised prior to removal to laboratory environment where the slides on which the virus in now inactivated may be air-dried. Viral antigen is detected using anti-HeV or anti-NiV antiserum and standard immunofluorescent procedures. A characteristic feature of henipavirus-induced syncytia is the presence of large polygonal structures containing viral antigen that fluoresce.

ii) Immuno-electron microscopy

The high titres of HeV and NiV in cells *in vitro* permit their visualisation in the culture medium by negative-contrast electron microscopy without a centrifugal concentration step. Detection of virus–antibody interactions by immunoelectron microscopy provides valuable information on virus structure and antigenic reactivity, even during primary isolation of the virus. Other ultrastructural techniques, such as grid cell culture (Hanna *et al*., 2006), in which cells are grown, infected and visualised on electron microscope grids, and identification of replicating viruses and inclusion bodies in thin sections of fixed, embedded cell cultures and infected tissues complement the diagnostic effort. The details of these techniques and their application to the detection and analysis of HeV and NiV have been described (Hyatt *et al*., 2001).

2.2. Viral identification: differentiation of HeV and NiV

2.2.1. Comparative immunostaining

Further identification of a henipavirus isolate as either HeV or NiV is based on comparative immunostaining as described in this section. It is necessary to compare the isolate with standard cultures of both HeV and NiV, and so all work must be conducted using procedures to manage the biological risks. The control and test viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum. The virus titres are expressed as focus-forming units (FFU)/ml.

2.2.2. Immunofluorescence assay

A virus isolate that reacts with anti-HeV and/or anti-NiV antisera in an immunofluorescence assay is considered to be serologically identical to either HeV or NiV if it displays the same sensitivity to neutralisation by anti-HeV and anti-NiV antisera as do the HeV or NiV positive controls. Anti-HeV antiserum neutralises HeV at an approximately four-fold greater dilution than that which neutralises NiV to the same extent. Conversely, anti-NiV antiserum neutralises NiV approximately four times more efficiently than HeV (Chua *et al*., 2000).

A new version of the differential neutralisation test has been recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres (Bossart *et al*., 2007). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in laboratories without adequate facilities to fully manage the biological risks of culturing HeV and NiV.

2.2.3. Microtitre neutralisation

This procedure is dependent on the availability of anti-serum, specific for HeV and NiV, as well as stock viruses. Stock HeV and NiV and the unidentified henipavirus are diluted and replicates of each virus containing approximately 100 $TCID_{50}$ in 50 µl are added to the test wells of a flat bottom 96-well microtitre plate. The viruses are mixed with an equal volume of either Eagle's minimal essential media (EMEM) or a range of dilutions of anti-HeV or anti-NiV antiserum in EMEM. The mixtures are incubated at 37°C for 45 minutes and approximately 2.4×10^4 cells are added to each well to a final volume of approximately 200 µl. After 3 days at 37°C, the test is read using an inverted microscope and wells are scored for the degree of CPE observed. Those that contain cells only or cells and antiserum should show no CPE. In contrast, wells containing cells and virus should show syncytia and cell destruction. A positive well is one where all or a proportion of cells in the monolayer form large syncytia typical of henipavirus infection.

3. Molecular methods – detection of nucleic acid

The complete genomes of both HeV and NiV have been sequenced, and as more isolates come to hand their sequences have been deposited on Genbank. PCR-based methods are commonly used to detect virus. They have the biosafety advantage of not propagating live infectious virus and they have been validated in a number of laboratories. They are also highly sensitive and specific.

3.1. Real-time reverse-transcription polymerase chain reaction (qRT-PCR)

A particularly sensitive and useful approach to the detection of henipavirus genome in specimens is real-time RT-PCR (see Table 2). Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories (Mungall *et al*., 2006; Wacharapluesadee & Hemachudha, 2007). The HeV M gene (Smith *et al*., 2001) and N gene (Feldman *et al*., 2009) TaqMan assays are the primary tests for HeV disease diagnosis as well as N gene for NiV.

Table 2. Real-time RT PCR (Taqman) assays for the detection of HeV and NiV

Assay	Oligo	Name	Primer sequence (5'–3')	Probe label (5'–3')
HeV_TQM_M	Forward	HeV M 5755F	CTT-CGA-CAA-AGA-CGG-AAC-CAA	
	Reverse	HeV M 5823R	CCA-GCT-CGT-CGG-ACA-AAA-TT	
	Probe	HeV M 5778P	TGG-CAT-CTT-TCA-TGC-TCC-ATC-TCG-G	FAM-TAMRA
HeV_TQM_N	Forward	HeV N119F	GAT-ATI-TTT-GAM-GAG-GCG-GCT-AGT-T	
	Reverse	HeV N260R	CCC-ATC-TCA-GTT-CTG-GGC-TAT-TAG	
	Probe	HeV N198-220P	CTA-CTT-TGA-CTA-CTA-AGA-TAA-GA	FAM-MGBNFQ
NiV_TQM_N	Forward	NiV_N_1198F	TCA-GCA-GGA-AGG-CAA-GAG-AGT-AA	
	Reverse	NiV_N_1297R	CCC-CTT-CAT-CGA-TAT-CTT-GAT-CA	
	Probe	NiV_N_1247comp	CCT-CCA-ATG-AGC-ACA-CCT-CCT-GCA-G	FAM-TAMRA

3.2. Conventional RT-PCR and Sanger sequencing

Two semi-nested conventional PCR assays, targeting the M gene and the P gene, can also be used for the detection of HeV. These two assays are used as supplementary tests to confirm the results from the TaqMan assays when unusual/atypical results arise from the TaqMan assays. They are also used for characterisation of detected HeVs when followed by Sanger (di-deoxy) sequencing using the same primers (see Table 3).

Table 3. *Primers used for conventional PCR and sequencing of HeV*

Target	Assay	Type	Name	Primer Sequence (5'-3')	PCR product
HeV M gene	Primary PCR	Forward	HeV M 5481F	GCC-CGC-TTC-ATC-ATC-TCT-T	300 bp
		Reverse	HeV M 5781R1	CCA-CTT-TGG-TTC-CGT-CTT-TG	
	Semi-nested PCR	Forward	HeV M 5481F	GCC-CGC-TTC-ATC-ATC-TCT-T	211 bp
		Reverse	HeV M 5691R2	TGG-CAT-CTT-TCA-TGC-TCC-ATC-TCG-G	
HeV P gene	Primary PCR	Forward	HeV P 4464F1	CAG-GAG-GTG-GCC-AAT-ACA-GT	335 bp
		Reverse	HeV P 4798R	GAC-TTG-GCA-CAA-CCC-AGA-TT	
	Semi-nested PCR	Forward	HeV P 4594F2	TCA-ACC-ATT-CAT-AAA-CCG-TCA-G	205 bp
		Reverse	HeV P 4798R	GAC-TTG-GCA-CAA-CCC-AGA-TT	

3.2.1. HeV RT-PCR conditions

i) Primary RT-PCR

1× 48°C for 30 minutes, 94°C for 2 minutes

40× 95°C for 30 seconds, 53°C for 30 seconds, 68°C for 45 seconds

1× 68°C for 7 minutes

ii) Semi-nested PCR

1× 95°C for 5 minutes

30× 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds

1× 72°C for 7 minutes

Table 4. *Primers used for conventional PCR and sequencing of NiV*

NiV M gene	Primary RT-PCR	Forward	NiV M 5659F	TGG-AAT-CTA-CAT-GAT-TCC-ACG-AAC-CAT-G	279 bp
		Reverse	NiV M 5942R1	TAA-TGT-GGA-GAC-TTA-GTC-CGC-CTA-TG	
	Nested PCR	Forward	NiV M 5659F	TGG-AAT-CTA-CAT-GAT-TCC-ACG-AAC-CAT-G	250bp
		Reverse	NiV M 5909R2	GTG-AAA-ACT-GCA-ATT-TCA-TCC-TAT-CAA-TC	

3.2.2. NiV RT-PCR conditions

i) Primary RT-PCR

1× 48°C for 30 minutes

1× 94°C for 2 minutes

40× 94°C for 30 seconds, 52°C for 30 seconds, 68°C for 45 seconds

1× 68°C for 7 minutes

ii) Nested PCR

1× 95°C for 5 minutes

30× 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 45 seconds

1× 72°C for 7 minutes

A range of conventional PCRs for NiV have been described, most of which target the N gene. For more details see the following publication – Wacharapluesadee & Hemachudha (2007). A hemi-nested PCR targeting the L gene has been described by Feldman *et al*., 2009.

Laboratories wishing to establish molecular detection methods should refer to published protocols or consult the OIE Reference Laboratory.

3.3. Henipavirus antigen detection in fixed tissue – immunohistochemistry

Immunohistochemistry is a powerful tool that allows the visualisation of viral antigen within cell and tissue structures. Nucleoprotein viral antigen in usually located within particulate structures of variable size and form within the cytoplasm. Because of the morphological aspect to the interpretation, colour signal can be effectively evaluated for its specificity. The test is done on formalin-fixed tissues, allowing the procedure to be done safely under non-microbiologically-contained conditions.

Henipavirus antigen replicates in a range of cell types, including endothelium, vascular smooth muscle, lung parenchyma, kidney glomeruli, neuron cell bodies, lymphoid tissues and connective tissues (Hooper *et al.,* 2001; Marsh *et al*., 2011; Middleton *et al*., 2002; Mungall *et al*., 2006). Antigen is particular dense in syncytia and in macrophages within lesions. Therefore, suitable tissues for diagnosis of henipavirus infection include lung, brain, lymph nodes, spleen and kidney. In the absence of these tissues, it is worthwhile examining any tissue type, as antigen can be found in occasional blood vessels throughout the vascular bed. Unless full protective clothing can be worn and suitable disinfection protocols be

implemented, it is safer to remove only small pieces of tissue through 'keyhole' sampling. Lung tissue and sub-mandibular lymph nodes are good tissues to remove in this manner.

Rabbit polyclonal antisera raised against recombinant henipavirus nucleoprotein are highly reliable for use as primary antibodies for diagnostic immunohistochemistry. Detection of phosphoprotein antigens is also suitable for diagnostic purposes, although phosphoprotein tends to be less expressed than nucleoprotein. There are various secondary detection systems on the market that can be used. The following is an example of an immunohistochemical procedure using an immunoperoxidase system and AEC chromagen. Other methods can be used, with slight variation of the method for different enzymes and chromagens.

3.3.1. Test procedure

i) Process the fixed tissues according to routine histological procedures into paraffin wax blocks and cut sections onto glass slides. Cut positive control sections and negative controls, if appropriate.

ii) Dewax the slides by immersion in three consecutive xylene baths for 3 minutes each. Hydrate sections through two changes of 98–100% ethanol, one change of 70% ethanol and running tap water to remove residual alcohol.

iii) Antigen retrieval can be done through heating in a citrate buffer (pH 9) for 20 minutes at 97°C, or by proteinase K digestion for 5 minutes.

iv) At this point and between each successive step till after step vii, wash the slides in TRIS buffer (pH 7.6) multiple times.

v) Block endogenous compound at this stage. This will depend on the detection system used, for example, if an immunoperoxidase system is used then endogenous peroxidase needs to be blocked with 3% aqueous H_2O_2 for 10 minutes.

vi) Add the primary antibody at a pre-characterised dilution for 45 minutes.

vii) Add the secondary antibody conjugate. Many different systems are available: the simplest and most robust consist of a single step. Consult the manufacturer's product guidelines for the correct use.

viii) Add the chromagen (for example, 3-amino-9-ethylcarbazole (AEC), or 3,3' diamino-benzidine (DAB) for 10 minutes. Refer to the product guidelines for the correct use.

ix) Wash in distilled water to stop colour development.

x) Counterstain in haematoxylin for 30 seconds to 3 minutes (depending on type).

xi) Rinse in tap water. Add Scott's solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), for 1 minute and wash well in running tap water.

xii) Mount with a cover-slip using aqueous mounting medium.

xiii) Viral antigen can be visualised by the brown/ red stain, the colour depending on the chromagen used.

All the above test methods should be considered as a guide only; each test parameter will need to be optimised for each testing laboratory, as they will vary according to specific laboratory conditions.

4. Serological tests

In laboratories doing serological testing, particularly in outbreak situations, several strategies have been adopted to reduce the risk of exposure of laboratory personnel to HeV and NiV. Sera may be gamma-irradiated (6 kiloGreys) or diluted 1/5 in phosphate buffered saline (PBS) containing 0.5% Tween 20 and 0.5% Triton-X100 and heat-inactivated at 56°C for 30 minutes. The process used will be based on a risk assessment. Specimens for surveillance testing and testing for animal movement certification may be considered a lesser biohazard than those for disease investigation during an outbreak. In some circumstances heat inactivation may be adopted as a sufficient precaution. However there is value in having a standardised approach for all samples in managing a test, rather than be maintaining multiple test methods.

In Australia, the introduction of equine vaccination against Hendra virus has affected the possible range of purposes of testing of assays that detect antibody to the G protein. The test may be used to detect immune responses to vaccination, and detection of antibodies no longer necessarily indicates prior infection in situations where vaccine may have been used. The possibility of vaccination must be taken into account when interpreting serological test reactions.

4.1. Virus neutralisation tests

The virus neutralisation test (VNT) (Kaku *et al*., 2009; Tamin *et al*., 2009) is accepted as the reference standard. The most commonly used is the microtitre assay which must be performed under appropriate conditions of biological risk management. Test sera are incubated with either HeV or NiV in the wells of 96-well microtitre plates prior to the addition of Vero cells. Sera are screened starting at a 1/2 dilution although this may lead to problems with serum-induced cytotoxicity. Where sample quality is poor or sample volumes are small, as may be the case with flying fox or microbat sera, an initial dilution of 1/5 may be appropriate. Cultures are read on day 3, and those sera that completely block development of CPE are designated as antibody positive. If cytotoxicity is a problem an immune plaque assay approach would have merit because the virus/serum mixtures are removed from the Vero cell monolayers after the adsorption period, thereby limiting their toxic effect. VNT results are considered positive if virus neutralisation is observed at any of the dilutions used in the test. If neutralising antibodies are present for both HeV and NiV, the higher titre >four-fold is considered the positive and if titres differed by <four-fold the serum is considered positive for an unspecified henipavirus.

4.2. Enzyme-linked immunosorbent assay

Henipavirus antigens derived from tissue culture for use in the enzyme-linked immunosorbent assay (ELISA) are irradiated with 6 kiloGreys prior to use, a treatment that has negligible effect on antigen titre. In the indirect ELISA developed in response to the initial outbreak at Hendra in 1994, antigen was derived from HeV-infected cells subjected to several cycles of freezing and thawing and treatment with 0.1% (w/v) sodium dodecyl sulphate. More recently, the use of a recombinant expressed soluble form of the Hendra G protein (Bossart *et al*., 2005) has been applied for improvements in Hendra immunoassays (McNabb *et al*., 2014).

In the national swine surveillance programme in Malaysia in 1999 (Daniels *et al*., 2000) an indirect ELISA format was used in which antigen was derived by non-ionic detergent treatment of NiV-infected cells. Subsequently, to control for high levels of nonspecific binding activity in some porcine antisera, a modified ELISA was developed based on the relative reactivity of sera with NiV antigen and a control antigen derived from uninfected Vero cells. At the Centers for Disease Control (CDC), Atlanta, USA, the approach has been to not only have an indirect ELISA for detection of IgG but also to use a capture ELISA for detection of IgM. For NiV, an ELISA using a recombinant nucleocapsid antigen has also been described (Yu *et al*., 2006), which is also configured to detect either IgG or IgM.

The specificity of the indirect NiV ELISA (98.4%) (Ong *et al*., 2000) means that in surveillance programmes the test will yield false positives. This may not be a significant problem in the face of a NiV outbreak where a high proportion of pigs are infected and the purpose of the surveillance is to detect infected farms. However, this level of test specificity creates a problem in non-outbreak specimens or if the number of samples to be tested is limited. If a positive ELISA result was indicative of a bona fide infection, failure to respond may lead to virus spread and human fatalities. In contrast, initiating control measures in response to a false positive ELISA result would be wasteful of resources (Daniels *et al.,* 2001). The current approach is to test all ELISA reactive sera by VNT, with sera reacting in the VNT considered to be positive. Confirmatory VNT should be done under conditions where the risks of working with live virus are adequately managed and this may entail sending the samples to an internationally recognised laboratory with established procedures for such work.

The following procedure for the NiV ELISA has been developed at Australian Animal Health Laboratory (AAHL) for porcine sera and standardised after collaborative studies in the Veterinary Research Institute, Ipoh, Malaysia.

4.2.1. Test procedure

Detailed methodology for production and/or supply of irradiated NiV and uninfected Vero cell antigens are available from the Australian Animal Health Laboratory.

i) Preparation of test sera

   a) Preparation of blood samples prior to centrifugation should be done in a biological class II safety cabinet with appropriate personal protective equipment or a class III cabinet.

   b) Dilute test serum 1/5 in PBS containing 0.5% (v/v) Triton X-100 and 0.5% (v/v) Tween 20 in the wells of a 96-well microtitre plate. Seal the microtitre plate. Laboratory personnel should wear gowns and gloves and spray both their hands and the sealed microtitre plate with suitable disinfectant (e.g. 1% Virkon) before removing the microtitre plate from the biosafety cabinet to heat at 56°C for 30 minutes.

c) Mix 22.5 µl heat-inactivated serum with an equal volume of uninfected Vero cell antigen diluted 1/100 in PBS. Mix thoroughly and incubate at 18–22°C for 30 minutes.

d) Add 405 µl blocking solution (PBS containing 5% chicken serum and 5% skim milk powder) to give a final serum dilution of 1/100 and incubate at 18–22°C for 30 minutes. Aliquots of 100 µl are added to two wells containing NiV antigen and two wells containing uninfected Vero cell control antigen as described in step vi.

ii) ELISA procedure

a) Dilute Vero cell control and NiV antigens in PBS to ensure that control and virus antigen wells are coated in parallel and at a similar concentration of protein. Antigen is usually diluted 1/1000 to 1/4000, but a specific dilution factor must be determined for each batch of antigen. Add 50 µl virus and cell control antigen to the wells of a 96-well microtitre plate as follows: virus antigen in columns 1, 3, 5, 7, 9 and 11 and cell control antigen in columns 2, 4, 6, 8 10 and 12. Incubate at 37°C for 1 hour with shaking. Plates can be also incubated at 4°C overnight.

b) Wash ELISA plates three times with PBS containing 0.05% Tween 20 (PBST) (250 µl/well) and block with PBS containing 5% chicken serum and 5% skim milk powder (100 µl/well) for 30 minutes at 37°C on a shaker.

c) Wash plates three times with PBST and add 100 µl of inactivated, absorbed sera from step iii to each well as indicated in the format below. Add 100 µl PBS containing 5% chicken serum and 5% skim milk powder to conjugate and substrate control wells. Incubate the plates without shaking for 1 hour at 37°C and wash three times with PBST.

d) Dilute protein A/G-horseradish peroxidase conjugate in PBST containing 1% (w/v) skim milk powder. The dilution factor is approximately 1/50,000. Mix well and add 100 µl protein A-conjugate to all wells except the substrate control wells. Add 100 µl PBST containing 1% skim milk powder to the substrate control wells. Incubate the plates for 1 hour at 37°C without shaking and wash four times with PBST.

e) Prepare the substrate (3,3',5,5'-tetramethylbenzidine; TMB) by dissolving one tablet (1 mg) in 10 ml of 0.05 M phosphate citrate buffer, pH 5.0, and add 2 µl of fresh 30% (v/v) H_2O_2. Add 100 µl of the TMB substrate to each well. Incubate for 10 minutes at 18–22°C and stop the test by adding 100 µl 1 M sulphuric acid to each well.

f) Read plates after blanking on a substrate control well. The optical density (OD) at 450 nm on NiV antigen and control Vero cell antigen are used to calculate an OD ratio for each serum (OD on NiV antigen/OD on Vero control antigen).

iii) Interpretation of results

Samples with NiV antigen OD value less than 0.20 are negative. Samples with NiV antigen OD value greater than 0.2 are assessed by OD ratio (antigen/control) value accordingly as:

a) an OD ratio >2.0 are considered positive

b) an OD ratio between 2.0 and 2.2 should be considered doubtful

Doubtful and positive sera should be tested by VNT.

4.3. Bead-based assays

Bead-based assays can be used. The validated methods below are examples of such assays[3].

Two multiplexed bead-based serological assays have been developed using magnetic bead-based technology (Luminex) and incorporate identification of antibodies to both HeV or NiV in a single test (Bossart *et al*., 2007; McNabb *et al*., 2014). Both assays measure antibodies to recombinant expressed soluble glycoprotein (sG) of HeV and NiV. One assay measures antibodies that bind directly to sG (binding assay) and the other assay measures the ability of antibodies to block the henipavirus receptor EphrinB2 binding to sG (blocking assay). The recombinant HeV or NiV sG proteins are first coupled to individually identifiable magnetic beads. The coupled beads are then mixed with test sera. For the binding assay, bound sera are then detected using a biotinylated protein A/G secondary conjugate and Streptavidin-phycoerythrin (S-PE). For the blocking assay, sera must compete with biotinylated ephrinb2 for binding to the sG and S-PE is again used to quantify the reaction. The beads are then interrogated by lasers in a Luminex machine and the results recorded as the median fluorescent intensity (MFI) of

3 Commercial reagents are specified to assist laboratories wishing to establish this technique. It does not imply any endorsement of particular products by OIE, and validated alternatives may be used.

100 beads. The assays use completely recombinant reagents and can be performed with relative biosafety, whereas the traditional ELISA requires more stringent attention to biological risk management, particularly for the production of the antigen. Similar to the approach taken with ELISA, any suspect positive sera are then tested by VNT.

4.3.1. Bead-coupling procedure

i) Bead activation

a) Bring the bead activation buffer (0.1 M NaH_2PO_4, pH6.2) to room temperature prior to use.

NOTE: Be careful to protect the beads from light as they photobleach (cover tubes with foil where possible).

b) Select the MagPlex carboxylated beads (Luminex corp., supplied as 1.25×10^7 beads/ml) for the protein coupling reaction (usually HeV: Bead#29 & NiV: Bead#30). Vortex the beads for 30 seconds at medium speed, then sonicate the beads by bath sonication for ~30–60 seconds. It is important that the beads are completely resuspended as single monodisperse particles.

c) Transfer 300 µl of MagPlex carboxylated beads #28 & #30 (3.75×10^6 beads) into 2 ml sarstedt tubes. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

d) Wash beads by adding 300µl of PBS-T to the tubes and vortexing. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet. Repeat.

e) Add 600 µl of bead activation buffer to the tubes and vortex. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet. Repeat.

f) Add 240 µl of bead activation buffer to the tubes, cover with foil and shake for 3 minutes.

g) Prepare EDC (Pierce) and S-NHS (Pierce) in bead activation buffer immediately prior to use to a concentration of 50 mg/ml (20 µl buffer/mg powder). Add 30 µl of the freshly made 50 mg/ml EDC into the tubes, closely followed by 30 µl of the freshly made 50 mg/ml S-NHS into the tubes. NOTE: Discard unused portion and make fresh each time.

h) Cover the tubes with aluminum foil and shake the beads at room temperature for 20 minutes.

i) While beads are incubating, prepare sG proteins. Use 90 µg each of HeV sG & NiV sG and use PBS (do not use PBS-T, as it blocks carboxy groups) to bring proteins up to a final volume of 300 µl.

j) After incubation, the beads are now activated and ready for coupling. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

ii) Protein coupling

a) Wash beads by adding 300 µl of PBS to the tubes and vortexing (do not use PBS-T as it blocks carboxy groups). Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

b) Add all of the 300 µl of prepared protein, above, to the activated beads.

c) Cover the tubes with aluminium foil and shake the beads moderately at room temperature for 2 hours.

d) The protein is now coupled to the beads. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the

magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

e) Wash the beads twice with 300 µl of PBS-T as described above. Place the tubes into a magnetic separator and allow separation to occur for 30–60 seconds. With the tubes still positioned in the magnetic separator, remove the supernatant with a pipette; take care not to disturb the bead pellet.

f) Resuspend the coupled beads in 1.8 ml bead storage buffer (10 ml PBS, 1% BSA, 0.05% sodium azide and 1 protease inhibitor tablet (Roche) and store at 4°C.

NOTES: Check reactivity of sG with panel of henipavirus sera before use. Use 1 µl of coupled beads per well for henipavirus binding and blocking serological assays (this procedure couples enough beads to test around 1800 sera). Coupled beads are able to be stored at 4°C for at least 1 year and maintain reactivity.

4.3.2. Henipavirus luminex binding assay procedure

i) Test procedure

a) Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds at maximum speed, then sonicate the beads by bath sonication for ~30–60 seconds.

b) Dilute beads in blocker (2% skim milk in PBS-T) at an appropriate concentration for the number of sera to be tested (1 µl of each bead set/well).

c) Add 100 µl of diluted beads to appropriate wells of a 96-well NUNC TC flat bottom plate.

d) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

e) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

f) Wash twice with PBST or alternatively, use automated magnetic plate washer.

g) Add 100 µl of control and test sera diluted 1/100 in PBS-T to the wells (bat sera dilute 1/50).

NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

h) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

i) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

j) Wash twice with PBST or alternatively, use automated magnetic plate washer.

k) Dilute biotinylated protein A (Pierce) 1/500 (2 ug/ml) and biotinylated protein G (Pierce) 1/250 (2 µg/ml) in the same tube in PBS-T and add 100 µl to the wells.

l) Cover plate in foil and shake at RT for 30min on a plate shaker.

m) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

n) Wash twice with PBST or alternatively, use automated magnetic plate washer.

o) Add 100 µl of Streptavidin R-PE (QIAGEN) diluted 1/1000 (1 ug/ml) in PBS-T to the wells.

p) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

q) Read plate using an appropriate Luminex machine and software.

ii) Interpretation of results

The results can be interpreted from the raw MFI values or can be transformed into a percentage relative to the MFI for the positive control (%P) using the following formula:

(MFI test serum/MFI positive control) × 100

A sample giving an MFI >1000 or %P >5 should be first retested in the Binding assay and Blocking assay. If the sample is still positive it should be tested further by VNT for confirmation.

4.3.3. Henipavirus luminex blocking assay procedure

i) Test procedure

a) Select previously coupled HeV and NiV sG beads. Vortex the beads for 30 seconds at max speed, then sonicate the beads by bath sonication for ~30–60 seconds.

b) Dilute beads in blocker (2% skim milk in PBS-T) at an appropriate concentration for the number of sera to be tested (1 µl of each bead set/well).

c) Add 100 µl of diluted beads to appropriate wells of a 96 well NUNC TC flat-bottom plate.

d) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

e) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

f) Wash twice with PBST. Or, alternatively, use automated magnetic plate washer.

g) Add 100 µl of control and test sera diluted 1/50 in PBS-T to the wells (bat sera dilute 1/25).

NOTE: All sera should be heat-inactivated for 35 minutes at 56°C before testing.

h) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

i) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

j) Wash twice with PBST or alternatively, use automated magnetic plate washer.

k) Dilute biotinylated ephrinB2 (RnD Systems) 1/1000 (50 ng/ml) in PBS-T and add 100 µl to the wells.

l) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

m) Place plate on magnetic holder and allow separation to occur for 30–60 seconds. With the plate still in the magnetic holder, flick contents into the sink and gently blot on paper towel, remove plate from magnetic holder.

n) Wash twice with PBST or alternatively, use automated magnetic plate washer.

o) Add 100 µl of streptavidin R-PE (QIAGEN) diluted 1/1000 (1 ug/ml) in PBS-T to the wells.

p) Cover plate in foil and shake at RT for 30 minutes on a plate shaker.

q) Read plate using an appropriate Luminex machine and software.

ii) Interpretation of results

For the blocking assay, the raw MFI readings are converted into percentage inhibition (%I) using the following formula: (1 - [MFI test serum/MFI NSC]) × 100

A sample giving a %I >15 should be first retested in the binding assay and blocking assay. If the sample is still positive it should be tested further by VNT for confirmation.

4.4. DIVA

Now that a vaccine exists for HeV for use in horses, the ability to differentiate vaccinated horses from unvaccinated naturally infected horses may seem desirable. Traditionally DIVAs have been applied on

the premise that vaccinated animals will only have antibodies to the viral protein(s) used in the vaccine (in the case of HeV, that is the G protein) whereas naturally infected animals will have antibodies to all viral proteins, both structural and non-structural. However, caution must be exerted when interpreting such serological results. Experimentally it has been shown in ferrets that not all individuals mount an immune response to all of the viral proteins of HeV that are detectable using conventional assay systems (Middleton D., unpublished data). In particular, serological profiles of unvaccinated infected animals may be indistinguishable from those that are vaccinated.

C. REQUIREMENTS FOR VACCINES

Veterinary vaccines for henipaviruses

1. Background

The original outbreak of NiV in Malaysia and Singapore was linked to transmission of the virus from pigs to humans, and all of the human infections with Hendra virus in Australia have been linked to contact with sick horses. Development of veterinary vaccines against henipaviruses is important both to protect susceptible domestic animal species (i.e. porcine, equine, feline, and canine) and to reduce transmission from domestic animals to humans. This was the rationale for development of the vaccine for HeV which is currently available for use in horses in Australia.

2. Soluble G henipavirus vaccine

Studies using NiV in cats (Mungall *et al*., 2006;) and monkeys (Bossart *et al*., 2012) and HeV in ferrets (Pallister *et al*., 2011) provided strong evidence that a HeV soluble G (HeVsG) glycoprotein subunit–based vaccine could prevent not only disease but often infection in animals exposed to otherwise lethal doses of NiV or HeV. The horse vaccine has been formulated using a proprietary adjuvant (Zoetis). The henipavirus surface-expressed G glycoprotein has the critical role of initiating infection by binding to receptors on host cells, and antibodies directed against this protein can neutralize virus. The vaccine was released under a Minor Use Permit for use in Australia in November 2012, and is only available for administration by accredited veterinarians. For primary immunisation two doses of vaccine should be administered 3 weeks apart in horses four months of age or above. For continued effect, a booster dose every 6 months is currently recommended by the manufacturer.

3. Experimental vaccines

Current experimental vaccines for protection from NiV infection have focused on the use of NiV glycoprotein (G) and/or fusionprotein (F) as immunogens in various platforms, including DNA vaccines, subunit vaccines, non-replicating vectors, as well as replicating vectors. Efficacy of most candidates required a prime/boost(s) approach, which would not favour their use in an emergency situation for rapid dissemination during an outbreak. A live-attenuated vaccine vector based on recombinant vesicular stomatitis viruses (rVSV) expressing NiV glycoproteins (G or F) or nucleoprotein (N) has been evaluated. Vaccination of Syrian hamsters with a single dose of the rVSV vaccine vectors resulted in strong humoral immune responses with neutralizing activities found only in those animals vaccinated with rVSV expressing NiV G or F proteins suggesting that these may be prime candidates for emergency vaccines to be utilised in NiV outbreak management. A similar construct consisting of a replication-defective vesicular stomatitis virus (VSV)-based vaccine vectors expressing either the NiV fusion (F) or attachment (G) glycoproteins protected hamsters from over 1000 times LD_{50} NiV challenge when vaccinated with a single dose.

REFERENCES

BOSSART K.N., CRAMERI G., DIMITROV A.S., MUNGALL B.A., FENG Y.R., PATCH J.R., CHOUDHARY A., WANG L.F., EATON B.T. & BRODER C.C. (2005). Receptor binding, fusion inhibition, and induction of cross-reactive neutralizing antibodies by a soluble G glycoprotein of Hendra virus. *J. Virol*., **79**, 6690–6702.

BOSSART K.N., MCEACHERN J.A., HICKEY A.C., CHOUDHRY V., DIMITROV D.S., EATON B.T. & WANG L.F. (2007). Neutralization assays for differential henipavirus serology using Bio-Plex protein array systems. *J. Virol. Methods*, **142**, 29–40.

BOSSART K.N., ROCKX B., FELDMANN F., BRINING D., SCOTT D., LACASSE R., GEISBERT J.B., FENG Y.R., CHAN Y.P., HICKEY A.C., BRODER C.C., FELDMANN H. & GEISBERT T.W. (2012). A Hendra virus G glycoprotein subunit vaccine protects African green monkeys from Nipah virus challenge. *Sci. Transl. Med*., **4** (146).

CHING P.K.G., DE LOS REYES V.C., SUCALDITO M.N., TAYAG E., COLUMNA-VINGNO A.B., MALBAS F.F., BOLO G.C., SEJVAR J.J., EAGLES D., PLAYFORD G., DUEGER E., KAKU Y., MORIKAWA S., KURODA M., MARSH G.A., MCCULLOUGH S. & AFOXWELL R. (2015). Outbreak of henipavirus infection, Philippines, 2014. *Emerg. Infect. Dis*., **21**, 328–331.

CHUA K.B., BELLINI W.J., ROTA P.A., HARCOURT B.H., TAMIN A., LAM S.K., KSIAZEK T.G., ROLLIN P.E., ZAKI S.R., SHIEH W.J., GOLDSMITH C.S., GUBLER D.J., ROEHRIG J.T., EATON B., GOULD A.R., OLSON J., FIELD H., DANIELS P., LING A.E., PETERS C.J., ANDERSON L.J. & MAHY B.W.J. (2000). Nipah virus: A recently emergent deadly paramyxovirus. *Science,* **288**, 1432–1435.

CHUA K.B., KOH C.L., HOOI P.S., KONG, F.W., KHONG J.H., CHUA B.H., CHAN Y.P, LIM M.E. & LAM S.K. (2002). Isolation of Nipah virus from Malaysian Island flying foxes. *Microbes and Infection*, **4**, 145–151.

DANIELS P., AZIZ J., KSIAZEK T.G., ONG B.L., BUNNING M.J.B., FIELD H., OLSON J., HOFFMAN D., BOLOU J. & OZAWA Y. (2000). Nipah virus – developing a regional approach. *In:* Comprehensive Reports on Technical Items presented to the International Committee or Regional Commissions. OIE, Paris, France, 2207–2217.

DANIELS P., HALPIN K., HYATT A. & MIDDLETON D. (2007). Infection and disease in reservoir and spillover hosts: determinants of pathogen emergence. *Curr. Top. Immunol. Microbiol.*, **315**, 113–131.

DANIELS P., KSIAZEK T. & EATON B.T. (2001). Laboratory diagnosis of Nipah and Hendra virus infections. *Microbes Infect.,* **3**, 289–295.

FELDMAN K.S., FOORD A., HEINE H.G., SMITH I.L., BOYD V., MARSH G.A., WOOD J.L.N., CUNNINGHAM A.A. & WANG L.F. (2009). Design and evaluation of consensus PCR assays for henipaviruses. *J. Virol. Methods*, **161**, 52–57.

HALPIN K., YOUNG P.L., FIELD H.E. & MACKENZIE J.S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *J. Gen. Virol.,* **81**, 1927–1932.

HANNA, J.N., MCBRIDE W.J., BROOKES D.L., SHIELD J., TAYLOR C.T., SMITH I.L., CRAIG S.B. & SMITH G.A. (2006). Hendra virus infection in a veterinarian. *MJA*, **185**, 562–564.

HAYMAN D.T., SUU-IRE R., BREED A.C., MCEACHERN J.A., WANG L., WOOD J.L. & CUNNINGHAM A.A. (2008). Evidence of henipavirus infection in West African fruit bats. *PLoS ONE*, **3**, e2739.

HOOPER P., ZAKI S., DANIELS P. & MIDDLETON D. (2001). Comparative pathology of the diseases caused hy Hendra and Nipah viruses. *Microbes Infect.,* **3**, 315–322.

HYATT A.D., ZAKI S.R., GOLDSMITH C.S., WISE T.G. & HENGSTBERGER S.G. (2001). Ultrastructure of Hendra virus and Nipah virus within cultured cells and host animals. *Microbes Infect. ,* **3**, 297–306.

IEHLE C., RAZAFITRIMO G., RAZAINIRINA J., ANDRIAHOLINIRINA N., GOODMAN S.M., FAURE C., GEORGES-CORBOT M-C., ROUSSET D. & REYNES J-M. (2007). Henipavirus and Tioman virus antibodies in pteropid bats, Madagascar. *Emerg. Infect Dis.,* **13**, 159–161.

KAKU Y., NOGUCHI A., MARSH G.A., MCEACHERN J.A., OKUTANI A., HOTTA K., BAZARTSEREN B., FUKUSHI S., BRODER C.C., YAMADA A., INOUE S., WANG L.F. (2009). A neutralization test for specific detection of Nipah virus antibodies using pseudotyped vesicular stomatitis virus expressing green fluorescent protein. *J. Virol. Methods*, **160**, 7–13.

LUBY S.P., RAHMAN M., HOSSAIN M.J., BLUM L.S., HUSAIN M.M., GURLEY E., KHAN R., AHMED B.N., RAHMAN S., NAHAR N., KENAH E., COMER J.A. & KSIAZEK T.G. (2006). Foodborne transmission of Nipah virus, Bangladesh. *Emerg. Infect. Dis.,* **12**, 1888–1894.

MARSH G.A., HAINING J., HANCOCK T.J., ROBINSON R., FOORD A.J., BARR J.A., RIDDELL S., HEINE H.G., WHITE J.R., CRAMERI G., FIELD H.E., WANG L.F. & MIDDLETON D. (2011). Experimental infection of horses with Hendra virus/Australia/horse/2008/Redlands. *Emerg. Infect. Dis*., **7**, 2232–2238.

MARSH G.A., DE JONG C., BARR J.A., TACHEDJIAN M., SMITH C., MIDDLETON D., YU M., TODD S., FOORD A.J., HARING V., PAYNE J., ROBINSON R., BROZ I., CRAMERI G., FIELD H.E. & WANG LF. (2012). Cedar virus: a novel Henipavirus isolated from Australian bats. *PLoS Pathog*, **8**, e1002836.

MCNABB L., BARR J., CRAMERI G., JUZVA S., RIDDELL S., COLLING A., BOYD V., BRODER C., WANG L.F. & LUNT R. (2014). Henipavirus microsphere immuno-assays for detection of antibodies against Hendra virus. *J. Virol. Methods*, **200**, 22–28.

MIDDLETON D.J., WESTBURY H.A., MORRISSY C.J., VAN DER HEIDE B.M., RUSSELL G.M., BRAUN M.A. & HYATT A.D. (2002). Experimental Nipah virus infection in pigs and cats. *J. Comp. Pathol*., **126**, 124–136.

MUNGALL B.A., MIDDLETON D., CRAMERI G., BINGHAM J., HALPIN K., RUSSELL G., GREEN D., MCEACHERN J., PRITCHARD L.I., EATON B.T., WANG L.F., BOSSART K.N. & BRODER C.C. (2006). Feline model of acute nipah virus infection and protection with a soluble glycoprotein-based subunit vaccine.*J. Virol*., **80**, 12293–12302.

MURRAY K., SELLECK P., HOOPER P., HYATT A., GOULD A., GLEESON L., WESTBURY H., HILEY L., SELVEY L. & RODWELL B. (1995). A morbillivirus that caused fatal disease in horses and humans. *Science,* **268**, 94–97.

NOR M.N.M., GAN C.H. & ONG B.L. (2000). Nipah virus infection of pigs in peninsular Malaysia. *Rev. sci. tech. Off. int. Epiz.,* **19**, 160–165.

OLSON J.G., RUPPRECHT C., ROLLIN P.E., UNG S.A., NIEZGODA M., CLEMINS T., WALSTON J. & KSIAZEK T.G. (2002). Antibodies to Nipah-like virus in bats (Pteropus lylei), Cambodia. *Emerg. Infect. Dis.,* **8**, 987–988.

ONG B.L., DANIELS P.W., BUNNING M., AZIZ J., WHITE J., MUNIANDY M., OLSON J., CHANG K.W., MORRISSY C., LIM Y.S., KSIAZEK T. & NORDIN M.N. (2000). A surveillance program for the detection of pig herds exposed to Nipah virus infections in peninsular Malaysia. *In:* Proceedings of the 9th International Symposium on Veterinary Epidemiology and Economics (ISVEE). Abstract ID 63. Colorado, USA.

PALLISTER J., MIDDLETON D., WANG L.F., KLEIN R., HAINING J., ROBINSON R., YAMADA M., WHITE J., PAYNE J., FENG Y.R., CHAN Y.P. & BRODER C.C. (2011). A recombinant Hendra virus G glycoprotein-based subunit vaccine protects ferrets from lethal Hendra virus challenge. *Vaccine*, **29**, 5623–56230.

PATON N.I., LEO Y.S., ZAKI S.R., AUCHUS A.P., LEE K.E., LING A.E., CHEW S.K., ANG B., ROLLIN P.E., UMAPATHI T., SNG I., LEE C.C., LIM E. & KSIAZEK T.G. (1999). Outbreak of Nipah-virus infection among abattoir workers in Singapore. *Lancet,* **354**, 1253–1256.

REYNES J., COUNOR D., ONG S., FAURE C., SENG V., MOLIA S., WALSTON J., GEORGES-COURBOT M.C., DEUBEL V. & SARTHOU J. (2005). Nipah virus in Lyle's flying foxes, Cambodia. *Emerg. Infect. Dis.,* **11**, 1042–1047.

ROGERS R.J., DOUGLAS I.C., BALDOCK F.C., GLANVILLE R.J., SEPPANEN K.T., GLEESON L.J., SELLECK P.N. & DUNN K.J. (1996). Investigation of a second focus of equine morbillivirus infection in coastal Queensland. *Aust. Vet. J.,* **74**, 243–244.

SENDOW I., FIELD H.E., CURRAN J., DARMINTO, MORRISSY C.J., MEEHAN G., BUICK T. & DANIELS P.W. (2006). Henipavirus in *Pteropus vampyrus* bats, Indonesia. *Emerg. Infect. Dis*., **12**, 711–712.

SMITH I.L., HALPIN K., WARRILOW D. & SMITH G.A. (2001). Development of a fluorogenic RT-PCR assay (TaqMan) for the detection of Hendra virus. *J. Virol. Methods*, **98**, 33-40.

SOHAYATI A.R., HASSAN L., EPSTEIN J.H., ZAINI C.M., AZIZI M.Y., SHARIFAH S.H., FIELD H.E., TOM H., JUSTIN W., NAIM S.N., ARSHAD S.S., AZIZ JAMALUDDIN A., DASZAK P. & HENIPAVIRUS ECOLOGY RESEARCH GROUP (2013). Risk factors for Nipah virus infections among pteropid bats, Peninsular Malaysia. *Emerg. Infect. Dis.*, **1**, 51–60.

SOHAYATI A.R., HASSAN L., SHARIFAH S.H., LAZARUS K., ZAINI C.M., EPSTEIN J.H., SHAMSYUL NAIM N., FIELD H.E., ARSHAD S.S., ABDUL AZIZ J., DASZAK P. & HENIPAVIRUS ECOLOGY RESEARCH GROUP (2011). Evidence of Nipah virus recrudescence and serological pattern of captive *Pteropus vampyrus*. *Epidemiol. Infect*, **10**, 1570–1579.

TAMIN A., HARCOURT B.H., LO M.K., ROTH J.A., WOLF M.C., LEE B., WEINGARTL H., AUDONNET J.C., BELLINI W.J. & ROTA P.A. (2009). Development of a neutralization assay for Nipah virus using pseudotype particles. *J. Virol. Methods*, **160**, 1–6.

WACHARAPLUESADEE S. & HEMACHUDHA T. (2007). Duplex nested RT-PCR for detection of Nipah virus RNA from urine specimens of bats. *J. Virol. Methods*, **141**, 97–101.

WACHARAPLUESADEE S., LUMLERTDACHA B., BOONGIRD K., WANGHONGSA S., CHANHOME S., ROLLIN P., STOCKTON P., RUPPRECHT C.E., KSIAZEK T.G. & HEMACHUDHA T. (2005). Bat Nipah Virus, Thailand, *Emerg. Infect. Dis*., **11**, 1949–1951.

WILLIAMSON M.M., HOOPER P.T., SELLECK P.W., GLEESON L.J., DANIELS P.W., WESTBURY H.A. & MURRAY P.K. (1998). Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. *Aust. Vet. J.,* **76**, 813–818.

WORLD HEALTH ORGANIZATION (WHO) (2004). Laboratory Biosafety Manual, Third Edition. WHO, Geneva, Switzerland. http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

WU Z., YANG L., YANG F., REN X., JIANG J., DONG J., SUN L., ZHU Y., ZHOU H. & JIN Q. (2014). Novel Henipa-like virus, Mojiang Paramyxovirus, in rats, China, 2012. *Emerg. Infect. Dis.,* **20**, 1064–1066.

YU F., KHAIRULLAH N.S., INOUE S., BALASUBRAMANIAM V., BERENDAM S.J., TEH L.K., IBRAHIM N.S., ABDUL RAHMAN S., HASSAN S.S., HASEBE F., SINNIAH M. & MORITA K. (2006). Serodiagnosis using recombinant Nipah virus nucleocapsid protein expressed in *Escherichia coli*. *J. Clin. Microbiol.,* **44**, 3134–3138.

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NB: There is an OIE Reference Laboratory for Hendra and Nipah virus diseases
(please consult the OIE Web site:
https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Hendra and Nipah virus diseases

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2015.