NIPAH AND HENDRA VIRUS DISEASES

SUMMARY

Hendra virus (HeV) and Nipah virus (NiV) emerged in the 1990s as the causes of outbreaks of zoonotic diseases. The first reported outbreak of HeV occurred in Brisbane, Australia in 1994. In this case HeV caused severe respiratory disease and the death of 13 horses and a horse trainer at a stable. 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 respiratory disease of unknown aetiology in the pig population. HeV has caused the death of four of seven infected people in Australia while it has been reported that there have been more than 600 cases of NiV in humans, with more than 400 deaths, in Bangladesh, India, Malaysia, Philippines and Singapore. Fruit bats (flying-foxes) in the genus Pteropus *are reservoir hosts of both viruses.*

HeV infection of horses does not have a pathognomonic presentation. Horses may present with respiratory, neurological or non-specific clinical signs. Respiratory signs include tachypnoea or frothy nasal discharge. Neurological signs include ataxia, head tilt, circling, seizures, depression or recumbency. Non-specific signs include high fever, tachycardia, inappetence or colic. HeV infection of horses is not uniformly fatal, nor does it appear to be highly contagious among horses. Close contact with infected flying-fox urine, saliva or birth products is necessary for its spill over from flying-foxes to horses. Infected horses on pastures have rarely transmitted the virus to other horses. Transmission appears to occur more readily in closed environments such as stables and veterinary clinics and is associated with contact with bodily fluids from infected horses.

NiV infection of pigs is highly contagious and characterised by fever with respiratory and sometimes neurological involvement, but many infections are subclinical. Some infected pigs display an unusual loud barking cough. Abortion has also been reported.

It is not currently known if the susceptibility of dogs and cats to infection is at a level to have potential for epidemiological significance.

Infection of humans is from animal contact, usually from an amplifier host rather than directly from the reservoir host: NiV from swine and horses and HeV from horses. Investigations of outbreaks of human cases of NiV in Bangladesh have indicated human infection from Pteropid bats directly without an intermediary/amplifier host. Human-to-human transmission has only been seen in outbreaks of NiV in Bangladesh and India.

HeV and NiV are closely related and are the founding members of the genus Henipavirus, *family* Paramyxoviridae. *There are two genotypes of NiV, namely NiV-M (Malaysia) and NiV-B (Bangladesh). Recent genetic characterisation of NiV-B from bat and human cases in Bangladesh 2012–2018 have shown genetic divergence during 1995 forming two sublineages: NiV-B1 and NiV-B2 (Rahman et al., 2021). Two genotypes of HeV have been recognised, namely HeV-g1 and HeV-g2. HeV and NiV are dangerous human pathogens. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level.*

Detection of the agent: *Because of the zoonotic potential and high fatality rates associated with these viruses, diagnostic laboratories may decide, following a comprehensive risk assessment, to use molecular techniques such as real-time reverse transcription polymerase chain reaction (RT-PCR) for agent detection rather than attempting propagation of infectious virus. Both HeV and NiV can be propagated in a wide range of cultured cells. Virus isolation from field samples should be attempted, but only in situations where operator safety can be assured. Identification procedures include RT-PCR, immunostaining of infected cells, and neutralisation with specific viral antisera.*

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).

Serological tests: Following a biological risk assessment, diagnostic laboratories may decide to avoid serological tests that use live virus. Virus neutralisation tests (VNT) and enzyme-linked immunosorbent assays (ELISA) are available. ELISA is currently being used as a screening tool and VNT is 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.

Requirements for vaccines: There is a vaccine available for HeV approved 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 *Orthoparamyxovirinae*, 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 structural 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, also known as flying-foxes. These are members of the genus *Pteropus,* family *Pteropodidae*. Antibodies to HeV have been detected in all four Australian pteropus species with seroprevalence varying over time. To date, all HeV cases in horses have occurred within the geographical range of the *Pteropus* bats, which correlates to the east coast of Australia. Antibodies to NiV or closely related viruses have been detected in pteropid bats over much of their geographical range. HeV-g1 has been isolated from Australian flying-foxes (Halpin *et al.*, 2000), with a novel HeV genotype (HeV-g2) detected in two flying-fox species in Australia (Wang *et al.,* 2021). NiV has been isolated from flying-foxes in Malaysia and Cambodia (Chua *et al.*, 2002; Reynes *et al.*, 2005). In Ghana, a small study showed 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.

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 was sufficiently different to belong in its own genus. There has been at least one spill-over event each year since 2006, with most events involving only a small number of horses. A retrospective investigation isolated HeV-g2 from a 2015 equine case (Annand *et al.,* 2022). To date seven human cases have resulted in four deaths (case fatality rate 57%). Clinical signs in humans range from an influenza-like infection to severe pneumonia or encephalitis leading to death (Yuen *et al.,* 2021). 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 had 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 in the field, respiratory disease caused by NiV in pigs was often subclinical but highly contagious in these intensively housed animals (Hooper *et al.,* 2001). This led to rapid virus dispersal through the Malaysian pig population with authorities choosing culling as the primary means to control spread (Nor *et al.*, 2000). Over one million pigs were destroyed; approximately 40% of 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). A small number of 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.

Outbreaks of human NiV disease have occurred on an almost annual basis in Bangladesh since 2003, with a few outbreaks in West Bengal, in neighbouring India, and more recently in Kerala, on the western coast of India (Arunkumar *et al*., 2019). Drinking fresh date palm sap contaminated by fruit bat saliva, urine or excreta has been identified as the likely route of transmission from the wildlife reservoir to humans in the Bangladesh outbreaks (Luby *et al.*, 2006). In some outbreaks, there has been human-to-human transmission. As a result of these ongoing outbreaks it is estimated that across Malaysia, Singapore, Bangladesh and India there have now been >600 cases of NiV in humans, with >400 deaths.

In 2014 in the Philippines, an outbreak of human cases of NiV was reported, with 9 deaths from 17 cases (Ching *et al.,* 2015). In this outbreak infected sick horseswere butchered and consumed by the people who later became infected. Subsequent human-to-human transmission was suspected. Cats and dogs were also affected.

Diagnosis of disease caused by henipaviruses is primarily by detection of viral RNA in clinical or post-mortem specimens, and virus isolation or demonstration of viral antigen in tissue samples (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 domestic 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 in 2009. 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). Two henipa-like viruses have been isolated from shrews in the Korea (Rep. of) (Lee *et al.,* 2021).

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	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Virus isolation	–	–	–	+++	–	–
RT-PCR & real-time RT-PCR	+	+	++	+++	+	–
IHC	–	–	–	++	–	–
IFA	–	–	–	++	–	–
Detection of immune response[b]						
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; IHC = Immunohistochemistry;
IFA = Indirect fluorescent antibody; ELISA = enzyme-linked immunosorbent assay; VNT = virus neutralisation test.
[a]A combination of agent identification methods applied on the same clinical specimen is recommended.
[b]Positive ELISA and bead-based assay results should be confirmed by the VNT unless the assay is validated for the purpose.

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. Transport of suspected specimens to laboratories, and 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. Molecular methods for agent detection are preferable as front-line tests as they carry less risk to the laboratory worker than virus isolation. Laboratories that do not have appropriate containment facilities for the handling of samples from suspect cases or for working with infectious virus should avail of the services of an WOAH reference or other specialist laboratory.

Virus propagation in particular must be conducted in high containment facilities under stringent conditions that will prevent accidental infection of personnel in the laboratory. During primary virus isolation from specimens collected 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 level of containment, the class of biosafety cabinet and the appropriate personal protective equipment required. Reverse-transcription polymerase chain reaction (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 1.1.3 *Transport of biological specimens.*

2.   Detection of the agent

2.1. Virus isolation and characterisation

Virus isolation greatly facilitates identification procedures but should only 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 propagation 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.

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.*, 2001). In live animals, swabs (nasal or oro-naso-pharyngeal), EDTA (ethylene diamine tetra-acetic acid) blood 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. In pregnant animals or in cases of abortion, uterus, placenta and fetal tissues should be included as appropriate. 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 CCL181) 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 under appropriate conditions as determined by a thorough biosafety risk assessment. 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* for 3– 5 minutes and 4°C, 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, or paraformaldehyde. The vessel is sealed, and surface sterilised prior to removal to a 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).

2.2.3. Microtitre neutralisation

This procedure is dependent on the availability of anti-serum, specific for HeV and NiV, as well as cell-culture adapted 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. Specimens sampled for PCR should be inactivated as part of the RNA extraction procedure before any further manipulations (PCR or sequencing). Advice on procedures is available from the WOAH Reference Laboratory[1].

3.1. Real-time reverse-transcription polymerase chain reaction

For the primary detection of henipaviruses, real-time RT-PCR is used (see Table 2). There is a range of test methods and primers published, such as the HeV-g2 M gene assay which is broadly reactive and detects HeV-g1 and HeV-g2 (Wang *et al.,* 2021), whereas the HeV-g1 M gene assay is Hendra-g1 specific (Smith *et al.*, 2001). The HeV P gene assay detects both Hendra and Nipah (Feldman *et al.*, 2009).

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

Assay	Oligo	Name	Primer sequence (5' → 3')	Probe label (5' → 3')
HeV-g1_M gene (Smith *et al.,* 2001)	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-g2 M* gene (Wang *et al.,* 2021)	Forward	HeV-g2-M-F	CTG-ATC-TAC-GTT-ACG-GCA-AAC-CTT	
	Reverse	HeV-g2-M-R	GG-CCC-GCT-TCA-CCA-TCT-CTT-AC	
	Probe	HeV-g2-M-P	CAG-CAT-TGA-ATA-TTG-ACC-CGC-CAG-TCA	FAM-BHQ1

Assay	Oligo	Name	Primer sequence (5' → 3')	Probe label (5' → 3')
HeV-g2 N gene (Wang *et al.*, 2021)	Forward	HeV-g2-N-F	TGC-GAC-AGA-TCC-CAG-TAG-TAT-TAA-AT	
	Reverse	HeV-g2-N-R	GGC-AGC-TTA-TTC-GGC-AAA-AG	
	Probe	HeV-g2-N-P	CTC-TGG-TGA-CGG-AAC-ACA-AAT-GCA-AAT-TTC	FAM-BHQ1
HeV_P** gene (Feldman *et al.*, 2009)	Forward	HeV_P_2698F	ACA-TAC-AAC-TGG-ACC-CAR-TGG-TT	
	Reverse	HeV_P_2794R	CAC-CCT-CTC-TCA-GGG-CTT-GA	
	Probe	HeV_P_2721P	ACA-GAC-GTT-GTA-TAC-CAT-G	FAM-MGBNFQ
HeV_N*** gene (Feldman *et al.*, 2009)	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_L gene**** (Wang unpublished)	Forward	NiV_L_11908F	GGT-ATG-ART-GTT-TTT-TGT-TTT-GGT-TTA-C	
	Reverse	NiV_L_11962R	CGG-CTT-TTG-YGA-ATT-CTT-GA	
	Probe	NiV_L_11937P	ATC-AAA-ACA-GAG-ATG-CGA-GC	FAM-MGBNFQ

*The HeV-g2 M gene assay is broadly reactive and detects HeV-g1 and HeV-g2
**The HeV P gene assay detects both HeV-g1 and HeV-g2 and NiV, but with slightly less sensitivity for HeV-g2.
***The HeV N gene assay detects HeV-g1. There are a few mismatches with
HeV-g2 but this is overcome when viral loads are high.
****The NiV L gene assay detects both strains of NiV, namely NiV-M and NiV-B.

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 real-time assays when unusual/atypical results arise. 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 (Wang *et al.*, 2021)	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	

*The HeV M gene semi-nested conventional PCR detects both HeV-g1 and HeV-g2. The HeV-P gene semi-nested conventional PCR also detects HeV-g1 and HeV-g2, but with much lower sensitivity for HeV-g2.

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

A range of conventional PCRs for NiV have been described, most of which target the N gene. For more details see Wacharapluesadee & Hemachudha (2007). A semi-nested PCR targeting the L gene has been described by Feldman *et al.*, 2009 and is shown below. This assay detects NiV-M and NiV-B and will also detect HeV.

Table 4. Primers used for conventional PCR and sequencing of both strains of NiV (Feldman et al., 2009)

NiV L gene	Primary RT-PCR	Forward	LFWD1	TGA GYA TGT ATA TGA AAG ATA AAG C	364 bp
		Reverse	LREV	TCA TCY TTA ACC ATC CCG TTC TC	
	Semi-nested PCR	Forward	LFWD2	ACC GAR CCA AGA TTG GT	266 bp
		Reverse	LREV	TCA TCY TTA ACC ATC CCG TTC TC	

3.2.2. NiV-L RT-PCR conditions

i)   Primary RT-PCR

    1×   48°C for 30 minutes

    1×   95°C for 15 minutes

    30×   94°C for 30 seconds, 42°C for 30 seconds, 68°C for 1 minute

    1×   68°C for 5 minutes

ii)   Semi-nested PCR

    1×   95°C for 15 minutes

    30×   94°C for 30 seconds, 46°C for 30 seconds, 72°C for 30 seconds

    1×   72°C for 5 minutes

Laboratories wishing to establish molecular detection methods should refer to published protocols or consult the WOAH 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). Antigen is particularly 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 from suspect cases. 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 chromogen. Other methods can be used, with slight variation of the method for different enzymes and chromogens.

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 coverslip 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 received from a suspected disease outbreak or from an endemic region should be gamma-irradiated (6 kilograys) or diluted 1/5 in phosphate buffered saline (PBS) containing 0.5% polysorbate 20 and 0.5% octylphenol ethoxylate and heat-inactivated at 56°C for 30 minutes prior to testing. 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 considered when interpreting serological test reactions.

4.1. Virus neutralisation tests

The virus neutralisation test (VNT) 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 (Crameri *et al*., 2002) 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. For laboratories that do not have appropriate biosecure facilities, a neutralisation test using a pseudotype vesicular stomatitis virus expressing green fluorescent protein has been described (Kaku *et al*., 2009).

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 kilograys 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. Now a recombinant expressed soluble form of the Hendra G protein (HeV-sG) (Bossart *et al*., 2005) is the preferred antigen and its use has enabled improvements in Hendra virus immunoassays including the development of a blocking ELISA for multiple species (horses, cats and dogs) (Di Rubbo *et al.,* 2019). An IgM antibody capture (MAC) ELISA for the detection of IgM HeV antibodies for use in horses has been recently described (McNabb *et al*., 2021). The HeV IgM MAC ELISA is intended to supplement other molecular and serology test results, with selective use, and is the only serology test which can provide an indication of recent infection.

In the national swine surveillance programme in Malaysia in 1999 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. For NiV, an ELISA using a recombinant nucleocapsid antigen has also been described (Yu *et al*., 2006), which is configured to detect either IgG or IgM.

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.

4.2.1. Hendra sG I-ELISA method for use with horse serum – test procedure

A recombinant expressed soluble form of the Hendra G protein (Bossart *et al.,* 2005) has been used in the assay of choice. The Hendra soluble G indirect ELISA eliminated almost all false-positive results from the previously used HeV I-ELISA, which used a crude SDS (sodium dodecyl sulphate) viral preparation, with marginally decreased relative sensitivity (Colling *et al.,* 2018).

Assay robustness was evaluated in inter-laboratory and proficiency testing panels (Colling *et al.,* 2018). This assay is considered to be fit for purpose for serosurveillance and international movement of horses when virus neutralisation is used for follow-up testing of positive or inconclusive serum samples.

i)   I-ELISA method

    a)   Coat a 96 well microtitre flat bottom hard ELISA plate with HeV sG recombinant protein diluted 1/3000 in PBS A coating buffer (50 μl/well equivalent to a coating concentration of 0.23 μg per ml) for 1 hour at 37°C on a plate shaker

    b)   Proceed to step c or seal plate with tape and store at 4°C (no longer than overnight).

    c)   Block plate by adding 50 μl/well of blocking buffer (5% skimmed milk powder [SMP]in PBS A) (no wash at this step).

    d)   Incubate for a further 30 minutes to 1 hour at 37°C on a plate shaker.

    e)   Wash plate 4× with PBS containing 0.05% polysorbate 20 (PBST) using plate washer.

    f)   Dilute test sera and controls 1/100 in ELISA diluent (1% SMP in PBST) and add 50 μl/well. Note: dilute samples 1/20 if they have been octylphenol ethoxylate/polysorbate 20 treated.

    g)   Incubate for 1 hour at 37°C on a plate shaker, cover with plate sealer.

    h)   Wash plate 4× with PBST using plate washer.

    i)   Add anti-equine-horseradish peroxidase conjugate 1/5000 in ELISA diluent (1% SMP in PBST).

    j)   Incubate for 30 minutes at 37°C on a plate shaker, cover with plate sealer.

    k)   Wash plate 4× with PBST using plate washer

    l)   Add 50 μl/well TMB (tetramethylbenzidine) chromogen/substrate and incubate at room temperature for 7–10 minutes.

    m)   Stop reaction with 50 μl/well 1 M H_2SO_4.

    n)   Read optical density at 450 nm on plate reader.

Data transformation: Mean OD values are calculated for the test and control results. The mean OD for the negative control serum, OD C(–) is subtracted from all mean OD values. These are converted to a signal-to-positive ratio (S/P) of the median low positive serum (C+).

$$S/P = (OD_{TEST} - OD_{NHS})/(average\ OD_{L\_POS} - OD_{NHS})$$

ii)   Acceptance criteria

The test is valid if all of the following criteria are met

OD C(+) – OD C(–)	0.30 to 1.0
OD C(–)	< 0.25
S/P C(++)	1.4 to 2.4

iii)   Interpretation of results

Samples with S/P less than 0.25 are negative.

Samples with S/P between 0.25 and 0.4 are considered inconclusive and a VNT is required to clarify status. Samples with S/P between > 0.4 are considered positive and a VNT is required to confirm this result.

One limitation of this assay is that it does not distinguish between antibodies due to natural infection and those due to vaccination (due to soluble G being present in the current Hendra virus vaccine). Any positive result must be interpreted in the context of the horse's vaccination history. Also, this assay can only be used with horse serum due to the use of an anti-equine horseradish peroxidase conjugate in the ELISA.

4.2.2. Hendra B-ELISA method

A blocking Hendra virus ELISA has been validated for use for equine, feline and canine sera (Di Rubbo *et al.,* 2019).

i) B-ELISA method

    a) Pre-treatment of sera with octylphenol ethoxylate/polysorbate 20 (1/5 dilution in PBS containing 0.5% [v/v] octylphenol ethoxylate and 0.5% [v/v] polysorbate 20).

    b) Coat a 96 well microtitre flat bottom hard ELISA plates with 50 µl/well of Hendra virus soluble G antigen in PBS, equivalent to 4.4 ng. Incubate at 37°C for 1 hour on a plate shaker.

    c) Block plates by adding 50 µl of ELISA diluent (1 × blocking buffer) directly to the wells without washing the plate. Place the plate on the plate shaker for 30 minutes at 37°C.

    d) Wash the plates 3 × with PBST.

    e) Dilute serum (30 µl of sera + 120 µl of blocking buffer) and add 50 µl per well. If using octylphenol ethoxylate/polysorbate 20 treated samples add 50 µl directly to wells. Place on plate shaker for 1 hour at 37°C.

    f) Add monoclonal antibody (MAb) 1.2 diluted in blocking buffer 50 µl per well. Do not add MAb to the blank wells. Place on plate shaker for 1 hour at 37°C.

    g) Wash the plates 3 × with PBST.

    h) Add diluted conjugate (Jackson anti-mouse horseradish peroxidase) in blocking buffer 50 µl per well.

    i) Wash the plates 3 × with PBST.

    j) Add 50 µl of TMB chromogen/substrate. Colour development occurs in 7–10 minutes.

    k) Add 50µl of stop solution (1 M sulphuric acid) to all wells.

    l) Read using a plate reader at 450 nm within 5 minutes of stopping.

Results are expressed as percentage inhibition (PI) relative to the average OD of the negative control sera:

$$PI = \{100 \times (1 - [\text{Test OD}_{AVG} / \text{NEG CONTROL OD}_{AVG}])\}$$

ii) Interpretation of results

If MAb1.2 PI is less than 33% the test result is negative. If the PI is greater than or equal to 33% the test result is positive. Positive results should be confirmed by the VNT. Negative results are reported without the need for further testing.

This Hendra B-ELISA also does not distinguish between antibodies due to natural infection and those due to vaccination (due to soluble G being present in the current Hendra virus vaccine used in horses). Any positive result must be interpreted in the context of the animal's vaccination history.

The following procedure for the NiV ELISA has been developed at Australian Centre for Disease Preparedness (ACDP) for porcine sera and standardised after collaborative studies in the Veterinary Research Institute, Ipoh, Malaysia. Other ELISA protocols for henipavirus diagnostics in pigs have been published.

4.2.3. Nipah indirect ELISA (NiV I-ELISA) for use with pig serum

Detailed methodology for production and/or supply of irradiated NiV and uninfected Vero cell antigens are available from the WOAH Reference 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) octylphenol ethoxylate and 0.5% (v/v) polysorbate 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 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% SMP) 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 ii) *ELISA method* below.

ii) ELISA method

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% polysorbate 20 (PBST) (250 µl/well) and block with PBS containing 5% chicken serum and 5% SMP (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 i) *Preparation of test sera* above to each well. Add 100 µl PBS containing 5% chicken serum and 5% SMP 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% SMP 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 chromogen/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 inconclusive

Inconclusive and positive sera should be tested by VNT to either confirm or clarify a result.

4.3. Bead-based assays

An advantage of using bead-based assays is that serum can be tested for both Hendra and Nipah virus antibodies in the same well using one experiment instead of multiple tests (ELISAs and VNTs) as validated by McNabb *et al.* (2014). The validated methods below are examples of such assays.

Two multiplexed bead-based serological assays have been developed using magnetic bead-based technology and incorporate identification of antibodies to both HeV or NiV in a single test (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 bead-based multiplexed immunoassay system and the results recorded as the median fluorescent intensity (MFI) of 100 beads. 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 magnetic carboxylated beads supplied as 1.25×10^7 beads/ml for the protein coupling reaction for the relevant virus (HeV or NiV). 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 magnetic carboxylated beads (3.75×10^6 beads) into 2 ml screw capped microtubes. 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 300 µl of PBST 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 crosslinkers (1-ethyl-3-[3-dimethylamino-propyl] carbodiimide [EDC] = a carboxy- and amine-reactive zero-length crosslinker and S-NHS = sulfo-N-hydroxysulfosuccinimide) 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 aluminium 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 PBST, 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 PBST 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 PBST 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% bovine serum albumen [BSA], 0.05% sodium azide and 1 protease inhibitor tablet and store at 4°C.

NOTES: Check reactivity of sG with 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 binding assay procedure

i) Test method

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 PBST) 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 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 PBST 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 1/500 (2 ug/ml) and biotinylated protein G 1/250 (2 μg/ml) in the same tube in PBST 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 diluted 1/1000 (1 ug/ml) in PBST 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 bead-based multiplexed immunoassay system and software.

ii) Interpretation of results

The results can be interpreted from the raw mean fluorescence intensity (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 >1500 or %P >5 should be first retested in the binding assay. If the sample is still positive, it should be tested further by VNT for confirmation.

4.3.3. Henipavirus blocking assay procedure

i) Test method

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 PBST) 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 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 PBST 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 1/1000 (50 ng/ml) in PBST 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 diluted 1/1000 (1 ug/ml) in PBST 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 bead-based multiplexed immunoassay system and software.

ii) Interpretation of results

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

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

4.4. DIVA

A HeV DIVA assay is used at the Australian Centre for Disease Preparedness to help distinguish between horses that are infected with Hendra virus and horses that have been vaccinated with the Hendra soluble G vaccine. It is currently undergoing validation (please contact the WOAH Reference Laboratory for details).

C. REQUIREMENTS FOR VACCINES

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. There is no vaccine approved for the prevention of HeV in humans.

2. Soluble G henipavirus vaccine

A preliminary study using HeV in ferrets (Pallister *et al.*, 2011) provided strong evidence that a HeV soluble G (HeVsG) glycoprotein subunit–based vaccine could prevent disease in animals exposed to an otherwise lethal dose of HeV. 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 neutralise virus. A Hendra virus horse vaccine has been formulated using HeVsG and a proprietary adjuvant by the vaccine manufacturer. The vaccine, released in Australia in November 2012, is only available for administration by registered veterinarians. For primary immunisation two doses of vaccine should be administered 3–6 weeks apart in horses four months of age or above, followed by a third vaccine 6 months after the second dose. For continued effect, a booster dose every 12 months is recommended by the manufacturer.

3. Experimental vaccines

Recently WHO included Nipah virus as one of 11 prioritised pathogens most likely to cause severe outbreaks in the near future. Alongside this has been the development of an R&D Blueprint for Action to Prevent Epidemics, which establishes a platform for R&D preparedness that is intended to accelerate research and product development in advance of and during epidemics (WHO, 2019). At least 13 Nipah virus vaccine candidates have been confirmed to be under development in preclinical stages (Gouglas *et al.,* 2019). Candidates have focused on the use of NiV glycoprotein (G) and/or fusion protein (F) as immunogens in various platforms, including DNA vaccines, subunit vaccines, non-replicating vectors, as well as replicating vectors. A vaccine based on NiV-B G protein in a replication-

deficient simian adenovirus vector in Syrian hamsters (ChAdOx1 NiVB) has shown promising results (Van Doremalen *et al.,* 2019). A prime-only as well as a prime-boost regime protected Syrian hamsters against challenge with a lethal dose of NiV-B and NiV-M.

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NB: There is a WOAH Reference Laboratory for Hendra and Nipah virus diseases
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH 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 2022.