

**HENDRA AND NIPAH VIRUS DISEASES**

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**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. In 1994, HeV caused severe respiratory disease and the death of 13 horses and a horse trainer at a stable in Brisbane, Australia. Between September 1998 and April 1999, after spreading unrecognised as a respiratory or encephalitic infection in Malaysian pigs, NiV appeared in the human population there and was the cause of fatal encephalitis. Over one million pigs were culled to stop spread of the disease. HeV has caused the death of two people while it has been reported that there have been 400 cases of NiV in humans, with approximately 200 deaths, in Malaysia, Singapore, Bangladesh and India. Fruit bats (flying foxes) in the genus Pteropus are natural hosts of both viruses.*

*HeV infection of horses is characterised progressively by high fevers, facial swelling, severe respiratory difficulty and, terminally, copious frothy nasal discharge. Some horses display neurological signs. The most common post-mortem observations are dilated pulmonary lymphatics, 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. Laboratory transmission experiments have shown that HeV is not readily transmitted between horses, a finding consistent with the observation that in the original outbreak, the infection did not spread widely to horses in adjacent properties.*

*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. Observations made during the outbreak investigation and during experimental infections confirmed that NiV infection of pigs is characterised by fever with respiratory involvement. In animals showing disease, nervous signs have been frequently reported, but many infections are subclinical. Some infected animals display an unusual barking cough. Abortion is reported in sows. 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. HeV causes pulmonary disease in cats similar to that observed in horses. 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 infection from Pteropid bats. Human-to-human transmission has not been seen with HeV or with NiV in Malaysia and Singapore, but human-to-human transmission is suspected in recent outbreaks of NiV in Bangladesh.*

*HeV and NiV are closely related members of the family Paramyxoviridae. Differences between them and other family members have led to their classification in a new genus, Henipavirus, in the subfamily Paramyxovirinae. HeV and NiV are biosafety level 4 agents and 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 and molecular characterisation. Real-time polymerase chain reaction (PCR) is now available as a diagnostic test.

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 antigens. Submissions for immunohistochemistry should include samples of brain at various levels including meninges, lung, spleen and kidney. 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 of virus should be fresh tissues from the same organs, or urine or throat or nasal swabs.

**Serological tests:** Virus neutralisation tests (VNT) and enzyme-linked immunosorbent assay (ELISA) are available. VNT is currently accepted as the reference procedure. The ability of antisera 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 are no vaccines currently available for either HeV or NiV.

## A. INTRODUCTION

Hendra virus (HeV) and Nipah virus (NiV) occur naturally as viruses of fruit bats commonly known as 'flying foxes', members of the genus *Pteropus*. Antibodies to HeV are found in approximately 50% of the four Australian *Pteropus* species (35). Serological surveys of antibodies to NiV show seroprevalances up to 20% in Malaysian pteropid bats (10, 18). Antibodies to NiV or putative closely related viruses have subsequently been detected in pteropid bats in Bangladesh (15), Cambodia (24, 27), Indonesia (29), Madagascar (19) and Thailand (31). HeV has been isolated from Australian flying foxes (12), and NiV from flying foxes from Malaysia and Cambodia (4, 27). NiV RNA has been detected by polymerase chain reaction (PCR) in pteropid bat urine, saliva and blood in Thailand (30, 31).

HeV disease emerged in Brisbane, Australia, in September 1994 in an outbreak of acute respiratory disease that killed 13 horses and a horse trainer (22). The virus was initially called equine morbillivirus, but subsequent genetic analyses indicated that it did not resemble morbilliviruses closely enough to merit 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 (28). A third horse died in January 1999 with no associated human disease (11). Two further equine cases occurred in 2004, one confirmed and the other unconfirmed, the latter identified by an associated human infection (13). In 2006 Australia reported two further cases in horses, one in Southern Queensland and one in northern New South Wales. All outbreaks since 1995 have involved infection of only one horse at pasture without transmission to in-contact animals.

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 (3, 23). Unlike respiratory disease caused by HeV in horses, which was frequently fatal but characterised by poor transmissibility (33), respiratory disease caused by NiV in pigs was often subclinical but highly contagious, 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 (23). Over one million pigs were destroyed; 106 of 267 infected humans, mostly pig farmers in Malaysia and abattoir workers in Singapore who had direct contact with live pigs, died of encephalitis (3, 26).

New foci of human NiV disease have subsequently been identified in Bangladesh and India. In outbreaks in 2001 and 2003 an animal source of the human infections was not identified (15), 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 human-to-human transmission but at low levels (15). In another outbreak in 2004 in which 27 of 36 infected humans died, epidemiological evidence indicated person-to-person transmission and serological studies identified seropositive fruit bats at the location (1). Drinking fresh date palm sap contaminated by fruit bat saliva, urine or excreta has been identified as one possible route of transmission from the wildlife reservoir to humans (20). As a result of these ongoing outbreaks it is estimated that across Malaysia, Singapore, Bangladesh and India there have now been up to 400 cases of NiV in humans, with approximately 200 deaths.

NiV and HeV are classified taxonomically as paramyxoviruses in the subfamily Paramyxovirinae, and have been grouped in a separate and new genus, the henipaviruses (9).

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 (8). 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.

## **B. DIAGNOSTIC TECHNIQUES**

### **1. Identification of the agent**

#### **a) Virus isolation and characterisation**

HeV and NiV are classified as biosafety level 4 (BSL4) agents, as they are dangerous human pathogens with a high case fatality rate and for which there is no vaccination or effective antiviral treatment. BSL4 is similar to containment group 4 as described in the OIE biosafety guidelines in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities, provide additional information. However, due to the high risk consequences of human infection in the laboratory, BSL4 requirements surpass the OIE containment level 4 requirements. 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. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (7).

##### *i) Sampling and submission of samples*

Diagnostic samples should be submitted to designated laboratories in specially designed containers. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed (17). The requirements are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

The range of tissues yielding virus in natural and experimental cases has been summarised (6). Brain, lung, kidney and spleen should always be submitted. Samples should be transported at 4°C if they can arrive at the laboratory within 48 hours; if shipping time will be over 48 hours, the samples should be sent frozen on dry ice or nitrogen vapours should be used. Samples should not be held at -20°C for long periods.

##### *ii) Isolation in cultured cells*

Virus propagation should be conducted under BSL4 conditions. Strict adherence to this guideline would limit the handling of diagnostic specimens where the presence of HeV or NiV may be suspected but not confirmed to laboratories with BSL4 facilities. Primary virus isolation from suspect samples may of necessity be conducted under BSL3 conditions. However, if this is to be attempted, stringent local guidelines must be developed to ensure operator safety and applied if a 'paramyxovirus-like' cytopathic effect (CPE) develops in infected cultures. Such guidelines will emphasise good laboratory practice, the use of class II safety cabinet with appropriate personal protective equipment or a class III cabinet and may require acetone fixation of infected cells, to destroy infectious virus, followed by immunofluorescent detection of Henipavirus antigen. The culture medium from Henipavirus-positive cells should be transferred to a BSL4 laboratory.

At the recipient laboratory tissues are handled under sterile conditions, and 10% (w/v) suspensions are generated by grinding the tissues in a closed homogenisation system, e.g. stomacher/bag mixer using plastic bag or mixer mills using autoclavable steel balls in closed metal cylinders. All processes should be carried out in a Class III cabinet or a Class II cabinet with appropriate personal protective equipment, with the stomacher operated in the cabinet and the centrifuge pots, with aerosol covers, loaded and unloaded in the cabinet. Following clarification of the homogenate by centrifugation at 300 g, the supernatant is added to cultured cell monolayers. 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 although the former may represent a viable method of primary isolation, there are no data on the relative susceptibility of *in-vivo* systems such as these compared with the more convenient cell culture systems. 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 (16).

iii) *Methods of identification*

o **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. At present the *Henipavirus* genus consists of HeV and NiV and there are no known antigenically related viruses.

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. Monoclonal antibodies (MAbs) are currently being generated and tested to fulfil this function both in primary identification of the virus upon isolation and for use in immunohistochemical examination of tissues from suspect cases.

o **Test procedure**

Under BSL4, monolayers of Vero or RK-13 cells grown on glass cover-slips or 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 in a vessel filled completely with acetone. The vessel is sealed and surface sterilised prior to removal to a less secure laboratory environment, for example BSL2, where the slides are 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. These are observed most readily with monospecific and MAbs to the nucleocapsid protein N and phosphoprotein P.

o **Immunoelectron microscopy**

The high titres generated by HeV and NiV in cells *in vitro* permits 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 (13), 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 (16).

b) **Virus neutralisation: differentiation of HeV and NiV**

Neutralisation tests rely on quantification methods and three procedures are available to titre HeV and NiV. In the traditional plaque and microtitre assay procedures, the titre is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells (TCID<sub>50</sub>), respectively. In an alternative procedure, the 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 (5). The virus titre is expressed as focus-forming units (FFU)/ml.

Neutralisation assays using these three methods are described below. 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 HeV or NiV. 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 (3). Virus quantification procedures should be conducted at BSL4. 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 (2). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in countries without BSL4 facilities.

i) *Plaque reduction*

Stock HeV and NiV and the unidentified Henipavirus are diluted in media and replicates of each virus containing approximately 100 PFU in 50–100 µl 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 virus–antiserum mixtures are incubated at 37°C for 45 minutes, adsorbed to monolayers of Vero cells at 37°C for 45 minutes and the number of plaques determined by traditional plaque assay procedures after incubation at 37°C for 3 days.

ii) *Microtitre neutralisation*

Stock HeV and NiV and the unidentified Henipavirus are diluted and replicates of each virus containing approximately 100 TCID<sub>50</sub> 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 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  $2.4 \times 10^4$  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.

iii) *Immune plaque assay*

Vero cells ( $2 \times 10^4$  in 200 µl medium/well) are added to flat-bottom microtitre plates and grown overnight at 37°C. Stock HeV and NiV and the unidentified Henipavirus are diluted and replicates containing about 60 FFU/50 µl are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV and anti-NiV antisera diluted in EMEM. Virus–antiserum mixtures are incubated for 45 minutes at 37°C and adsorbed to Vero cell monolayers for 45 minutes at 37°C. Virus–antiserum mixtures are removed, 200 µl EMEM is added to each well and incubation is continued at 37°C. After 18–24 hours the culture medium is discarded and plates are immersed in cold, absolute acetone for 10 minutes and then placed in plastic bags, which are filled with acetone, heat-sealed and surface sterilised with 4% (v/v) lysol during removal from the BSL4 laboratory. Gluteraldehyde can also be used for sterilisation at concentrations as low as 0.1% for 24 hours. It is recommended that each laboratory determine the concentration of gluteraldehyde required for sterilisation within the time frame required. Acetone-fixed plates are air-dried, the wells are blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 2% skim milk powder, and incubated for 30 minutes at 37°C with antiserum to either HeV or NiV or a monospecific antiserum to a virus protein. Anti-viral antibody binding to syncytia can be detected using alkaline phosphatase-conjugated species-specific antibody and the substrate 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium substrate (NBT/BCIP; Promega, Catalog number S3771). When purple plaques appear against a clear background (10–30 minutes), the substrate is removed and the plates are rinsed with distilled water and air-dried. Plaques are counted using a magnifying glass.

c) **Nucleic acid based recognition methods**

The complete genomes of both HeV and NiV have been sequenced (32), and PCR-based methods have been used to detect virus and are being validated in a number of laboratories.

A particularly sensitive and useful approach to the detection of henipavirus genome in specimens is real-time PCR. This method has the biosafety advantage of not propagating live infectious virus. Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories (21, 30). The virus-specific reagents used in one such assay (21) based on Taqman chemistry are as follows:

<b>For Hendra virus detection:</b>		
Primer # 1	(HENDRA-N1433F)	5'-ATC-TCA-GAT-CCA-GAT-TAG-CTG-CAA-3'
Primer # 2	(HENDRA-N1572R)	5'-ATC-ATT-TTG-GGC-AGG-TTT-GG-3'
TaqMan Probe	(HENDRA-N1510T-FAM)	5'-6FAM-AAC-CGC-CCT-CAG-GCA-GAC-TCA-GGA-TAMRA-3'
<b>For Nipah virus detection:</b>		
Primer # 1	(Nipah-N1198F)	5'-TCA-GCA-GGA-AGG-CAA-GAG-AGT-AA-3'
Primer # 2	(Nipah-N1297R)	5'-CCCCTTCATCGATATCTTGATCA-3'

TaqMan probe	(Nipah-1247comp-FAM):	5'-6FAM-CCT-CCA-ATG-AGC-ACA-CCT-CCT-GCA-G-TAMRA-3'
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Laboratories wishing to establish molecular detection methods should refer to published protocols or consult the OIE Reference Laboratory.

#### d) **Henipavirus antigen detection in fixed tissue - immunohistochemistry**

Immunohistochemistry is also a useful test in HeV and NiV detection. Performed on formalin-fixed tissues or formalin-fixed cells, it is safe and has allowed retrospective investigations on archival material. As virus replication and the primary pathology occur in the vascular endothelium (14), there is a wide range of tissues in which HeV and NiV antigen can be detected (8). It is thought that HeV antigens may be cleared from lung tissue early in the course of infection and so the sample submitted should include a range of tissues, not just lung. HeV antigen has been detected in the kidney of a horse 21 days post-infection (33) and so this organ should always be submitted. Ideally a submission for immunohistochemistry would include samples of the brain at various levels, lung, mediastinal lymph nodes, spleen and kidney. In pregnant animals the uterus, placenta and fetal tissues should be included.

A range of antisera to HeV and NiV may be used in immunohistochemical investigations of HeV- and NiV-infected tissues, but rabbit antisera to plaque-purified HeV and NiV have been found to be particularly useful. Some MAbs are also available. The Nipah Virus Pathology Working Group has described a detection system (34). A biotin–streptavidin peroxidase-linked detection system has also been used successfully (14). The following detection system is an anti-rabbit/anti-mouse dextran polymer-linked reagent conjugated with alkaline phosphatase.

##### o **Test procedure**

- i) Dewax slides containing formalin-fixed, paraffin-embedded test material and positive and negative control tissue sections by immersion three times in xylene for 1 minute. Hydrate sections through two changes of 98–100% ethanol, one change of 70% ethanol and running tap water to remove residual alcohol.
- ii) Rinse slides in distilled water, immerse in 0.01 M CaCl<sub>2</sub> (adjusted to pH 7.8 with 0.1 M sodium hydroxide) containing 0.1% (w/v) trypsin (Difco Trypsin 250) for 20 minutes at 37°C and wash in distilled water.
- iii) Lay slides flat in a humid chamber and rinse with PBS for 5 minutes. Add 200 µl 3% aqueous H<sub>2</sub>O<sub>2</sub> to each slide for 20 minutes at room temperature to block endogenous peroxidase. Rinse slides in PBS for 5 minutes.
- iv) Add 200 µl of an appropriate dilution of rabbit anti-Nipah or anti-Hendra antibody in PBS containing 0.1% (w/v) skim milk powder to test tissue slides and positive and negative control slides. To a duplicate set of test and positive and negative control slides add rabbit antibody to an unrelated pathogen. Cover the slides and incubate at 37°C for 1 hour.
- v) Rinse slides in PBS for 5 minutes and apply 2–3 drops of Envision™ solution (anti-rabbit Ig conjugated to peroxidase-labelled dextran polymer [DAKO Corporation, 6392 Via Real, Carpinteria, CA 03013]). Incubate at 37°C for 20 minutes.
- vi) Prepare the substrate by dissolving 2 mg 3-amino-9-ethylcarbazole (AEC) in 200 µl dimethyl formamide (Merck) and add to 10 ml 0.02 M acetate buffer, pH 5.0. Add 5 µl H<sub>2</sub>O<sub>2</sub> (30% w/v) and mix. Check the positive control slide for sufficient staining, usually 2–5 minutes, and stop the reaction by rinsing in distilled water. The substrate solution should be made fresh prior to use.
- vii) Counterstain the slides in haematoxylin for 1–3 minutes, rinse in tap water, add Scott's solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), and wash well in running tap water. Rinse the slides in distilled water and mount with a cover-slip using aqueous mounting medium.
- viii) Read the slides for cytoplasmic deposition of the chromogen indicating the presence of viral antigen. Brown/red granular staining will be seen in the cytoplasm of positive cells. The cell nuclei are blue and this facilitates identification of tissue morphology and assists in the location of viral antigen within the tissue.

## 2. 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 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 biosafety risk than those for disease investigation. 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.

### a) Virus neutralisation tests

Henipaviruses can be quantified by plaque, microtitre or immune plaque assays and these assays can be modified to detect anti-virus antibody (see above). The virus neutralisation test (VNT) is accepted as the reference standard. In the most commonly used microtitre assay, which is performed under BSL4 conditions, sera are incubated with virus 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 used. Cultures are read at 3 days, and those sera that completely block development of CPE are designated as positive. If cytotoxicity is a problem the immune plaque assay described above has merit because the virus/serum mixtures are removed from the Vero cell monolayers after the adsorption period, thereby limiting their CPE.

### b) 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 (P. Selleck, unpublished data). In the national swine surveillance programme in Malaysia in 1999 (6) a similar 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 (36), which is also configured to detect either IgG or IgM.

The specificity of the indirect NiV ELISA (98.4%) (25) 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 the absence of an outbreak 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 (8). 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 BSL4 conditions and this may entail sending the samples to an internationally recognised laboratory.

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.

#### o Test procedure

##### *Preparation of NiV antigens*

- i) Grow Vero cells until confluent in roller bottles in EMEM containing 10% (v/v) fetal calf serum (FCS). To infect with virus, pour off all but 5 ml of the medium from each roller bottle and, in a BSL4 laboratory, add low passage, plaque-purified NiV to a multiplicity of infection of 0.1 TCID<sub>50</sub>/cell.
- ii) Rotate roller bottles for 30 minutes at 33°C to adsorb virus, add 60 ml EMEM containing 10% FCS to each bottle and roll for a further 48 hours at 33°C. The multiplicity of infection, incubation time and temperature are chosen so that although the majority of cells become infected and are incorporated into syncytia within 48 hours, few cells detach into the culture medium. The culture medium of cells infected under these conditions is an excellent source of virus for further purification.

- iii) Wash monolayers of virus-infected cells once with cold 0.01 M PBS and, using a large scraper, scrape cells from each roller bottle into 5–10 ml ice-cold PBS.
- iv) Pool scraped cells into 50 ml tubes kept in ice and pellet the cells at 300 **g** for 5 minutes at 4°C. Pour off PBS and resuspend cells in ice cold TNM (10 mM Tris, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, pH 7.2), approximately 0.5 ml TNM per roller bottle.
- v) Add NP40 (non-ionic detergent, Nonidet P40) to 1% (by addition of 1/10 volume of 10% [v/v] NP-40 in water) and lyse cells using 5–10 strokes of a Dounce homogeniser. This also releases from the cytoskeleton viral antigen that would otherwise be removed by centrifugation (step vi).
- vi) Pellet the nuclei at 600 **g** for 10 minutes at 4°C. The nuclei will not lyse under these conditions and should form a tight white pellet.
- vii) Gently remove the supernatant cytoplasmic extract into a clean tube and add ethylene diamine tetra-acetic acid to 1.5 mM. Make up to 10 ml with TNE, aliquot in small amounts, freeze at –80°C and gamma-irradiate with 6 kiloGreys. Store aliquots at –80°C.

*Preparation of control, uninfected Vero cell antigen*

- viii) Grow Vero cells in roller bottles in EMEM containing 10% FCS. When confluent, wash monolayers once with cold PBS and scrape the cells from each roller bottle into 5–10 ml ice-cold PBS. Proceed as described for virus-infected cells in steps iv–vii above.

*Preparation of test sera*

- ix) In a biological class II safety cabinet with appropriate personal protective equipment or a class III cabinet, 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 1% Virkon before removing the microtitre plate from the biosafety cabinet to heat at 56°C for 30 minutes.
- x) 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.
- xi) 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 xiv.

*ELISA procedure*

- xii) Dilute Vero cell control and NiV antigens in PBS to ensure that control and virus antigen wells are coated with 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 Nunc Maxisorp 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 (Fig. 1). Incubate at 37°C for 1 hour with shaking. Plates can be also incubated at 4°C overnight.
- xiii) Wash ELISA plates three times with PBS containing 0.5% 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.
- xiv) Wash plates three times with PBST and add 100 µl of inactivated, absorbed sera from step xi 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.
- xv) Dilute protein A/G-horseradish peroxidase conjugate (Protein-A/G-Conjugate Supplied by Pierce, through Progen Biosciences Product No. 32490) 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.
- xvi) Prepare the substrate (3,3',5,5'-tetramethylbenzidine; TMB; Sigma, catalogue number T 3405) 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<sub>2</sub>O<sub>2</sub>. 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.

- xvii) 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).

*Interpretation of results*

- xviii) An OD ratio >2.0 with an OD on NiV antigen >0.20 is considered positive.  
 xix) An OD ratio >2.0 with an OD on NiV antigen <0.20 is considered negative.  
 xx) Sera displaying an OD ratio between 2.0 and 2.2 should be considered doubtful.  
 xxi) Doubtful and positive sera should be tested by VNT.

**Fig. 1.** ELISA plate format and result sheet.

	Ni	Ni	U	U	Ni	Ni	U	U	Ni	Ni	U	U
	1	2	3	4	5	6	7	8	9	10	11	12
A	Test serum #1	Test serum #1	Test serum #1	Test serum #1								
B	Test serum #2	Test serum #2	Test serum #2	Test serum #2								
C	Test serum #3	Test serum #3	Test serum #3	Test serum #3								
D	Test serum #4	Test serum #4	Test serum #4	Test serum #4								
E	Test serum #5	Test serum #5	Test serum #5	Test serum #5					H+	H+	H+	H+
F	Test serum #6	Test serum #6	Test serum #6	Test serum #6					L+	L+	L+	L+
G	Test serum #7	Test serum #7	Test serum #7	Test serum #7					N-	N-	N-	N-
H	Test serum #8	Test serum #8	Test serum #8	Test serum #8					CC	TMB	CC	TMB

Nunc Maxisorp 96 well plate; Ni: Nipah virus infected cell antigen; U: uninfected Vero antigen (control antigen);  
 H+: High positive control sera e.g. LAF pig 6 sera; N: Negative control sera e.g. negative pig sera;  
 L+: Low Positive control sera e.g. LAF Pig 6 sera 1:800

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines currently available for either HeV or NiV.

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**NB:** There is an OIE Reference Laboratory for Hendra and Nipah virus diseases (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).