

# Menangle Virus Infections

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## Part 1. Diagnostic Overview

### Summary

Menangle virus was first recognised as a new pathogen in 1997. An outbreak of severe reproductive failure and congenital defects was observed in a large piggery in New South Wales. The disease syndrome was characterised by reduced conception rates, reduced litter size, and the delivery at term of a large number of mummified and stillborn foetuses including some with severe skeletal and craniofacial defects. There was no evidence of disease in pigs of any age after birth. This new virus was found to be zoonotic, causing a severe febrile illness in two farm workers. Investigations of possible hosts revealed that the natural reservoirs of this virus are several species of fruit bats (flying foxes). Serological surveys confirmed that the virus was confined to the main pig breeding complex and two remote contract growing farms, also in NSW. Epidemiological studies conducted on the main farm elucidated probable methods of spread and mechanisms for the persistence of the virus in a large population. Control measures were subsequently implemented and the virus was eradicated from the pig population.

This virus is not known to exist any more as an infection of terrestrial animals in any country. The diagnostic methods that have been developed have been used to exclude this virus during the investigation of disease outbreaks and for health certification for regulatory purposes. Testing is also requested to exclude Menangle virus as a cause of illness in people, especially those with contact with flying foxes. The virus neutralisation test is the preferred method to detect antibodies to this virus and virus isolation in cell cultures has been used to isolate the virus in situations where serological tests are not useful or possible.

### 1. Aetiology

Menangle virus (MenV) is a newly recognised virus belonging to the genus *Rubulavirus* in the family *Paramyxoviridae*<sup>1,2</sup>. Virions are pleomorphic with both spherical and elongated forms that range in size between 150-350 nm. There is a single layer of surface spikes approximately 17 nm in length. Ruptured particles reveal long herringbone-shaped nucleocapsids, which have a diameter of approximately 19 nm in diameter<sup>3</sup>. Another closely related rubulavirus, Tioman virus, has more recently been identified in flying fox populations in Malaysia<sup>4</sup> but it has not been associated with any disease.

### 2. Clinical Signs

In the pig population in which there was infection with MenV, disease was observed only where there were breeding females. The reproductive disease was characterised by reduced conception rates, reduced litter size, the delivery of a large number of mummified and

stillborn foetuses including some with severe skeletal and craniofacial defects<sup>5</sup>. Congenital defects included arthrogryposis, hydranencephaly, hypoplasia of the spinal cord and lungs and degeneration of the spinal cord, cerebellum and brain stem<sup>6</sup>. There was no evidence of disease in pigs of any age after birth. MenV is zoonotic, causing a severe febrile illness in farm workers following close contact with infected pigs<sup>7</sup>. Affected people also experienced headaches and significant weight loss.

### 3. Epidemiology

This virus does not appear to be as highly contagious as some porcine pathogens as it took some time to spread through the affected pig population, even though all pigs were presumed to be naive. The prevalence of high antibody titres to MenV in finishing age and adult pigs exceeded 95%. Once the virus had spread through the breeding population, signs of reproductive disease and foetal infection were no longer apparent but the virus remained endemic in the population due to a continuous cycle of infection in young growing age pigs as they lost protection afforded by maternal antibodies<sup>8</sup>. It has not been possible to establish evidence for persistent infections. Although the route of infection is not known, it is believed that the virus may be spread by the oral-faecal route. Transmission of the virus in an endemically infected population was interrupted by the implementation of control measures that were directed to breaking the cycle of infection in young pigs<sup>8</sup>. These measures were disinfection of the environment and a brief period of depopulation of an individual unit followed by restocking with pregnant sows that were expected to be immune. The only young animals to be raised in this environment were the progeny of these sows. These piglets were then in turn protected by maternal antibodies for a period of at least 6 weeks. Collectively these measures were believed to provide a period of several months in which the virus would need to survive in the environment before susceptible piglets would become available for infection and continuation of the endemic cycle<sup>8</sup>. These measures were successful and the virus was eventually eradicated from the population without complete depopulation of the entire piggery at one time. The manner in which the virus was spread from flying foxes to pigs remains unknown.

### 4. Occurrence and Distribution

MenV is only known to have caused one disease outbreak<sup>3</sup>, which was near Sydney, NSW, in 1997. However, in addition to the main breeding farm, there was also evidence of active virus transmission on two contract growing farms that received weaned pigs from the breeding farm. The growing farms were located several hundred kilometres from the main farm. A small number of finishing age pigs and gilts that had been supplied by the breeding farm to other small farms nearby were also shown to be seropositive but there was no evidence of spread of the virus because all non-introduced pigs remained seronegative. There was no other evidence of MenV infection found in Australia during subsequent serological surveys of pigs in NSW and other States, except for two positive sera that had been collected at different times from a small farm in northern coastal NSW. This farm had experienced a reproductive problem and was near a flying fox colony. If MenV had been on this farm, it is quite possible that the infection had become self-limiting in a small population.

### 5. Infections of Other Species

There has been no evidence of infection of terrestrial species other than pigs and humans. Samples of serum and tissues from rats, mice, cats, sheep, cattle, birds and a dog on the

affected farm gave negative results. The reservoir of the virus appears to be several species of flying fox<sup>3</sup>.

## 6. Pathology

Many foetuses infected with MenV are mummified at birth and specific lesions cannot be found. However, there were also many stillborn and aborted foetuses, which showed a range of gross pathological changes including arthrogryposis, craniofacial and spinal deformities, pulmonary hypoplasia and degeneration of the brain and spinal cord<sup>6</sup>. Lesions in the central nervous system (CNS) included marked reduction in size of the cerebral hemispheres, cerebellum, brain stem and spinal cord as well as hydranencephaly. The most frequent change was reduced size or absence of the cerebellum. Histologically, changes were most frequently observed in the CNS where there was extensive degeneration, necrosis, infiltration of macrophages and gliosis<sup>6</sup>. However, in many cases, there was insufficient remnant CNS for a meaningful examination to be conducted. Perivascular cuffs of lymphocytes and macrophages were also evident in the brain and spinal cord. A multifocal, nonsuppurative myocarditis was evident in some piglets. Intranuclear and intracytoplasmic eosinophilic inclusion bodies were observed in neurones and other cells in the brain and spinal cord. Intranuclear inclusions tended to be single, small and centrally placed. Intracytoplasmic inclusions tended to be large and refractile, occupying most of the cytoplasm, and were often crescent shaped, enveloping the nucleus. Some piglets had mild nonsuppurative leptomeningitis. Typical pleiomorphic paramyxovirus particles with herringbone-shaped nucleocapsids were observed in these inclusions.

## 7. Diagnostic Tests and Specimens

The diagnostic methods that are available have been used to exclude this virus during the investigation of disease outbreaks and for health certification for regulatory purposes. Testing is also requested intermittently to exclude MenV as a cause of illness in people, especially those with contact with flying foxes.

The virus neutralisation (VN) test is the preferred method to detect antibodies to this virus, and virus isolation in cell cultures has been used to isolate the virus in situations where serological tests are not useful or possible.

### 7.1 Disease Diagnosis

As MenV is believed to be no longer present in the Australian pig population, the most rapid method of excluding this virus as a cause of reproductive loss or congenital defects in pigs is to test a collection of serum samples from sows that have reduced reproductive performance or have produced affected piglets. As the prevalence of antibody in an infected population is relatively high, if 10-15 samples are collected, negative results in serological tests should be significant for exclusion purposes.

When piglets are to be examined, samples should be collected from freshly aborted or stillborn foetuses, usually for virus isolation in cell culture and histopathology. Investigations<sup>6</sup> have shown that MenV is most likely to be isolated from stillborn foetuses with gross and/or histological evidence of degeneration of the brain. The virus has been consistently isolated from brain, lung and myocardium. The detection of antibody in fluids from a stillborn or aborted foetus that has been infected in the second half of pregnancy is also of significance.

Testing of humans to exclude MenV is most readily achieved by comparative serological tests on acute and convalescent sera. As long as the convalescent serum is collected at least 4 weeks after the onset of illness, it is probable that a negative result in a serological test will be significant.

The only serological test that has been frequently used for testing for MenV antibodies is the VN test. It has the advantage of being suitable to test serum samples from any species. However, it does suffer from a requirement to use cell cultures and hence high quality, sterile samples.

## **7.2 Health Certification**

The purpose of health certification testing is to demonstrate that pigs for export or those that are semen donors have not been exposed to MenV. As pigs infected with MenV seroconvert quickly and antibody titres persist for many years, freedom can be most readily demonstrated by negative results in serological tests.

## **7.3 Specimen Storage**

Blood and serum samples should be chilled during transport to the laboratory and can be stored for about four weeks at 4°C without significant decline in antibody titre. However, as the serum has to be tested by the VN test, it is important that the serum is aseptically decanted from the blood clot as soon as separation of serum and red cells has taken place. Freezing at -20°C or lower is preferred for longer storage but repeated freeze-thaw cycles should be avoided.

When virus isolation is to be conducted, tissues, if not examined within a few days of receipt, should be stored frozen at -70°C or lower.

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### *Part 2: Diagnostic Test Methods*

**NOTE: Although laboratory acquired infections have not occurred with Menangle virus, it is known to be a zoonotic agent after contact with infected pigs under field conditions. Therefore, extreme care should be exercised at all times when undertaking necropsies, handling specimens, preparing homogenates, during the inoculation and all subsequent handling of potentially infected cell cultures and during the conduct of VN tests. Work should be conducted in a Class 2 biosafety cabinet at all times. Suitable personal protective equipment (especially gloves) should be used, strict aseptic techniques should be followed, and procedures that may generate aerosols should be minimised.**

#### **1. Detection of Menangle Virus**

Specimens are only likely to be examined for evidence of MenV infection during the investigation of specific disease incidents when MenV cannot be excluded by serological tests on samples collected from sows that have produced abnormal piglets.

##### **1.1 Selection of Specimens**

During the investigation of the initial MenV outbreak, virus isolation procedures were initially unsuccessful, but with the benefit of hindsight, it is probable that tissues of piglets that are born in the early stages of an outbreak are unlikely to contain infectious virus. Rather, they are more likely to be seropositive and serum and body fluids should be tested for antibodies. However, virus isolation is more likely to be successful from freshly aborted or stillborn foetuses with gross and/or histological evidence of degeneration of the brain<sup>5</sup>. These piglets have been infected earlier in gestation (at perhaps less than 60 days of development) when an immune response was less developed. In such animals, virus has been consistently isolated from brain, lung and myocardium.

##### **1.2 Selection of Cell Cultures**

MenV has been shown to replicate in cell cultures from a wide range of mammalian species. While hamster kidney (BHK<sub>21</sub>) cells were used for the initial isolation of MenV, it is likely that many of the other continuous cell lines that are held in diagnostic laboratories are also suitable. These cell lines include hamster lung (HmLu-1), monkey kidney (Vero, CV-1), porcine kidney (PK15), canine kidney (MDCK) and humans (HeLa, Hep-2).

##### **1.3. Virus Isolation Methods**

As this virus is infectious for humans, ensure that all work is conducted in a Class 2 biosafety cabinet. Suitable personal protective equipment (especially gloves) should be used, strict aseptic techniques should be followed, and procedures that may generate aerosols should be minimised.

Once amplified in cell culture MenV produces very distinctive cytopathological changes, allowing easy detection of virus replication. The isolation of MenV in cell culture can be attempted in two stages:

- (a) inoculation and passage of the specimen in susceptible cell cultures; and
- (b) identification of cytopathogenic agents.

##### **1.3.1 Reagents for virus isolation**

***Antibiotics – cell culture grade:***

Penicillin  
Streptomycin  
Amphotericin B

### ***Phosphate buffered gelatin saline (PBGS)***

Phenol red	0.4 mg
Gelatin	5.0 g
PBS (pH 7.4)	1 L

Dissolve gelatin in PBS at 56°C, add phenol red, dispense in 5 mL volumes and autoclave at 121°C for 20 min.

### **1.3.2 Preparation of Specimens**

**Tissues** - Fresh brain, lung and myocardium are the preferred tissues. Prepare a 10% (w/v) homogenate of the tissue in cell culture growth medium (no serum) or PBGS containing 1000 units/mL of penicillin and streptomycin (50,000 units/mL), and 4 ug/mL of amphotericin B. Centrifuge at approximately 4°C at 800 g for 10 min, discard the cell pellet and centrifuge the supernatant again. The supernatant is then ready to be cultured.

### **1.3.3 Inoculation of Cultures**

Generally, specimens are inoculated onto slightly subconfluent monolayers of the cell type chosen (usually BHK<sub>21</sub>). These cells should be grown in sealed plastic or other unbreakable tubes rather than microplates to ensure biosecurity and to facilitate medium changing after inoculation with potentially cytotoxic specimens. All cultures should be incubated for 5 to 7 days after inoculation, and then passaged 4 more times (a total of 5 passages in cell culture) in the absence of cytopathological changes.

#### ***1.3.3.1 Inoculation of Tube Cultures***

- (a) Inoculate 0.2 mL of sample (clarified homogenate of tissue) to at least duplicate tubes. Tube cultures should be slightly subconfluent, with tissue culture medium freshly changed to maintenance medium without serum before inoculation.
- (b) After rolling the tubes for two hours, the medium is again changed by decanting the tissue culture fluid into separate containers for each specimen to prevent cross-contamination of tubes. If specimens are toxic to the monolayer, cell damage can be reduced by adsorbing the specimen for one hour. Add new maintenance medium containing serum and antibiotics, and incubate tubes, preferably rolling, at 37°C.
- (c) At seven days after inoculation, sub-culture into new tubes by scraping cells from the tube and passaging the cell/culture supernatant suspension.
- (d) Passage up to 4 more times at weekly intervals before considering the cultures are negative.

#### ***1.3.3.2 Examination of cultures for cytopathological changes***

Cell cultures should be examined regularly to ensure that there is no microbial contamination or toxicity from the inoculum and to check for evidence of cytopathological changes. Replication of MenV may initially be apparent as cell rounding, lysis and detachment. As infection progresses, vacuolation of cells, multinucleated giant cells and the formation of large syncytia will be seen. The syncytial cells may involve a significant portion of the monolayer before it is completely destroyed and cells dislodge from the surface of the culture vessel.

## **1.4 Virus Identification**

Initially, the presence of a paramyxovirus may be suspected by the extensive vacuolation and the formation of multinucleated giant cells and syncytia. Electron microscopic examination of culture supernatants is the most rapid method of provisional confirmation of the presence of a paramyxovirus. There are spherical to pleomorphic virus particles 150 nm to over 300 nm long, containing herringbone-shaped nucleocapsids with a diameter of  $19 \pm 4$  nm and a pitch of  $5.8 \pm 0.4$  nm, surrounded by an envelope with a single fringe of surface projections  $17 \pm 4$  nm long. Unlike many paramyxoviruses, MenV is non-haemadsorbing and non-haemagglutinating using erythrocytes from several species, including humans, guinea pigs and chickens. Definitive confirmation of the identity of a possible isolate can be obtained by neutralisation of the virus in a neutralisation test with reference polyclonal antiserum or by immunoperoxidase staining of infected monolayers with a reference antiserum.

## 2. Serology

The only serological test that has been routinely used for testing for antibodies to MenV is the virus neutralisation test. Although the test depends on the availability of cell cultures and the use of live virus, it does allow serum from any species to be tested without modification of the test.

As this virus is infectious for humans, ensure that all work is conducted in a Class 2 biosafety cabinet. Suitable personal protective equipment (especially gloves) should be used, strict aseptic techniques should be followed, and procedures that may generate aerosols should be minimised.

### 2.1. Virus Neutralisation Test

- (a) Inactivate sera to be tested by heating at  $56^{\circ}\text{C}$  for 30 minutes. Dilute sera in 96-well tissue culture microplates. Sera are tested in duplicate starting at a 1:4 dilution. Allow 2 sets of wells for the neutralisation test and a third set of wells as a 'serum control' to monitor for toxicity of the test samples. Add 75  $\mu\text{L}$  of diluent (complete growth medium) to the first row of wells and 50  $\mu\text{L}$  to all other wells. Make doubling dilutions, using a multichannel pipette, by adding 25  $\mu\text{L}$  of test serum to the first row, discarding tips and transferring 50  $\mu\text{L}$  to the next row to finally give 50  $\mu\text{L}$  of diluted serum per well. Discard 50  $\mu\text{L}$  from the last row of wells. Include as a positive control a serum with a known neutralising titre.
- (b) Add the virus (50  $\mu\text{L}/\text{well}$ ), diluted in tissue culture medium to contain 100  $\text{TCID}_{50}/50$   $\mu\text{L}$ , to all wells containing serum other than the 'serum control' wells. The final dilution will vary depending on batch of virus stock and cell type used. The titre of the stock virus should be measured in the same cell type used for the VN test.
- (c) Incubate at  $37^{\circ}\text{C}$  for one hour.
- (d) In each test, titrate the virus used to check the amount of challenge virus used per well. Leave four to eight wells uninfected as a cell culture control. A known positive serum should also be included in each test for standardisation purposes.
- (e) Add cells in growth medium (100  $\mu\text{L}/\text{well}$ ) at the appropriate seeding rate to achieve confluence in about 24 hours.
- (f) Incubate plates at  $37^{\circ}\text{C}$  in 5% carbon dioxide, 85% relative humidity, for five days.
- (g) Examine the cells in the plates by light microscopy. Firstly, check the control cell wells to confirm that there is a uniform monolayer and no abnormalities, then check the virus titration to see that the virus titre is acceptable. Any well in which cytopathological changes are detected, whatever the extent, should be scored as positive, indicating no specific antibody. Read endpoints as the highest dilution at which there are no cytopathological changes in at least 50% of the wells.

#### 2.1.1. Acceptance Criteria

- (a) The control virus titration should be correct to within 0.5 log<sub>10</sub> (preferably within 0.3 log<sub>10</sub>) of the preferred challenge dose (2.0 log<sub>10</sub>) for the results to be accepted.
- (b) If the titration exceeds 10<sup>2.5</sup>, positive results can be accepted but the actual titres may be higher than currently observed.
- (c) If the titre is too low (<10<sup>1.5</sup>), only negative results should be accepted and tests of all other sera repeated.
- (d) The positive reference serum should not vary by more than four-fold.

### 2.1.2. Interpretation

In pigs and humans, titres of 16 are considered to be inconclusive and titres >16 are positive. Peak titres may reach as high as 4096 and sometimes 8192. In affected humans, titres have remained at levels ranging from 1024 to 4096 for more than 12 months. In other species, such as flying foxes, titres of 8 are considered to be inconclusive and 16 or higher to be positive.

### 2.1.3 Notes

- (a) Use a cell line that supports growth of MenV to high titre, such as BHK<sub>21</sub> or Vero cells.
- (b) Stock virus should be prepared and stored in small aliquots at approximately -70°C, then diluted before use.

## 4. References

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4. Chua KB, Wang LF, Lam SK et al. *Tioman virus*, a novel paramyxovirus isolated from fruit bats in Malaysia. *Virology* 2001;283: 215-229
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8. Kirkland PD, Love RJ, Philbey AW et al. Epidemiology and control of Menangle virus in pigs. *Aust Vet J* 2001;79:199-206.

### **Part 3: Reagents and Test Kits for Menangle Virus Diagnosis in Australia and New Zealand**

The list of suppliers provided below may not be exhaustive but includes all materials that have been evaluated and found to be suitable for use under Australian or New Zealand conditions. These reagents have been approved only for use in Australia or New Zealand for the purposes described below. Other suppliers who have materials that may be used for the purposes described below are welcome to submit reagents and kits for evaluation by contacting the Executive Officer, SCAHLS <[www.scahls.org.au](http://www.scahls.org.au)>.

#### **3. Reference Sera and Viruses**

The following reagents are available from the Elizabeth Macarthur Agricultural Institute:

##### **3.1. Virus neutralisation tests**

Reference viruses and control sera (positive and negative) are available.

#### **4. Test Kits**

No diagnostic test kits or other reagents are available from commercial suppliers.