Aino Virus

RP Weir

Berrimah Agricultural Research Centre
Berrimah Farm
PO Box 990, Darwin, NT, 0801
richard.weir@nt.gov.au

SUMMARY

Aino virus is an infectious, noncontagious, insect-borne agent that has been implicated in the syndrome of hydranencephaly and arthrogryposis in calves. The virus or specific neutralising antibody can be found in populations of cattle, buffalo, sheep, goats, camels and deer in the western Pacific region from Japan to Australia. Hydranencephaly and arthrogryposis in newborn calves as a result of Aino virus infection cannot be distinguished clinically from that caused by Akabane virus infection. Laboratory confirmation is the only means of differentiating between causative agents. Whereas laboratory-confirmed disease caused by Aino virus in Australia is rare, there are regular and significant outbreaks reported in Japan.

Aino virus infection in adult animals is subclinical. The virus must cross the placenta of a pregnant cow and infect the foetus at a suitable time of gestation. Newborn calves infected with Aino virus during gestation can exhibit a range of skeletal and neurological abnormalities. These abnormalities include: severe hydranencephaly and arthro-gryposis, hydranencephaly or arthrogryposis, unilateral cavitation in the cerebrum, microcephaly and cerebellar hypoplasia. The type of abnormality seen can be related to the time of infection of the foetus; early infection results in hydranencephaly, later infection results in arthrogryposis.

Identification of the agent

Diagnosis of an Aino virus infection is based on virus isolation from an animal or postmortem material or the demonstration of a four-fold rise in specific antibody titre or both. Significantly rising titres may indicate recent infection but are likely to be available only in animals such as sentinels undergoing prospective monitoring. Virus isolation can be attempted on samples of unclotted blood (heparin, EDTA or oxalate) from sentinel animals. In suspect cases of Aino virus infection, virus isolation can be attempted on a variety of postmortem tissues or fluids including brain, spleen, liver, lymph, placenta, cerebrospinal fluid or clotted blood. Isolation of Aino virus from sentinel, clinical or postmortem material should be attempted in embryonated chicken egg, or insect, hamster lung or monkey kidney cell lines. Identification of isolates should be confirmed in a neutralisation test using specific antibody and rise in antibody titre using a known specific viable antigen.

Serological tests

Serological tests for the detection of Aino virus antibody include microneutralisation, plaque reduction neutralisation (PRNT), agarose gel immunodiffusion (AGID), indirect fluorescent antibody (IFA) and enzyme-linked immunosorbent assay (ELISA).

AGID is not a reliable serotype-specific test and will detect antibody to a range of Simbu group viruses that include Akabane, Douglas, Peaton and Tinaroo. The exception being Thimiri virus antibody. The Aino AGID test should be renamed and assumed to be a Simbu virus group AGID test. The most reliable and specific test for the detection of Aino virus antibody is the neutralisation test, either in the microneutralisation or plaque reduction format.

Status of Australia and New Zealand

Serological evidence of infection is demonstrated regularly in northern Australia and in New South Wales (NSW). Clinical disease has been reported from the Atherton Tableland in northern Queensland and the New England region of northern NSW. Infection is restricted to the range of *Culicoides brevitarsis* and *C wadii* assumed to be the major vectors of Aino virus.

There has been no published record of Aino virus infection in New Zealand.

Introduction

Aino virus (originally named Samford virus) was first isolated in Australia from *C brevitarsis* collected at Samford on March 27 1968.[1] Serological evidence and virus isolation indicates that Aino virus is active in northern Australia in most years, with periodic incursions into NSW as far south as the Hunter Valley.[2,3,4,5] No serological evidence of Aino virus has been reported from New Zealand.

The first reports of hydranencephaly (HE) and arthrogryposis (AG) were from NSW and Queensland. Sporadic outbreaks have occurred in the Atherton Tableland[4] and the New England Tableland[5] and numerous isolations of the virus were made from sentinel cattle during the 1980s[3] and 1990s (Melville unpublished data).

Aino virus infections can appear concurrently, although rarely, with Akabane virus infections, probably sharing the same vector (*C brevitarsis*) at the same time in the same region. Serological evidence suggests that although the infections can occur concurrently, antibody prevalence rates for Aino and Akabane viruses can vary considerably year to year under the influence of environmental factors.[5] The prime factor would appear to be a prolonged dry period of several years, allowing the development of serologically naive populations. The return of normal weather conditions and the subsequent reintroduction of the vector to its normal range exposes susceptible animals to the virus. Significant HE and AG outbreaks have occurred following prolonged el nino events. [4,5,10]

There has been one laboratory-confirmed instance of concurrent infection of Aino and Akabane viruses in which both viruses were isolated from a single sample of blood from a bull.[6]

There are no reports of a cow giving birth to an affected calf more than once.[6] This suggests that there could be some form of cross-protection between the teratogenic Simbu group viruses eliciting group immunity and preventing further cases of HE and AG or the small number of affected calves makes the possibility of successive infections unlikely.

HE and AG tend not to be seen in the northern regions. Foetal infection is prevented because young animals are exposed annually to the teratogenic Simbu viruses so they are immune when they reach reproductive age.

Aetiology

HE and AG may be the result of an intra-uterine infection with Aino virus, an infectious non-contagious insect-borne virus. This virus causes AG/HE that is indistinguishable from HE and AG caused by Akabane virus. Aino virus is a single stranded, negative sense RNA virus that belongs to the genus *Bunyavirus* of the family Bunyaviridae, order Mononegavirales. The virions are generally pleomorphic or spherical, enveloped, 80 to 120 nm in diameter and show surface projections 5 to 10 nm in height.The virions contain 3 circular nucleocapsids 200 to 300 nm in length. The nucleocapsids contain highly conserved terminal sequences that are specific for the genus.[7]

Clinical disease has been reported from northern Australia and eastern Australia periodically as far south as the New England Tablelands in New South Wales.

Epidemiology

The virus is maintained in the environment by unknown means, but presumably cycling between the insect vector and susceptible hosts. The vector *C brevitarsis* is present in the environment in northern Australia throughout the year. There are insufficient data for Aino virus to determine whether the virus over-winters in adult insects or has an as yet unidentified native animal host. Generally virus isolations occur with the onset of monsoonal rains and the subsequent increase in vector numbers. The first monsoonal activity can begin from November to February. The period during which virus can be isolated from insects is relatively short (3 to 4 weeks) and the viraemia in cattle lasts for less than 1 week.

Clinical signs

Adult animals are not affected by an Aino virus infection. Clinical signs in new-born lambs and calves include: domed skull, sunken eyes, maxillary retraction, skeletal deformation (mild or severe), limbs fixed in flexion or extension, incoordination, lack of sucking reflex or blindness.[8-10] Calves are generally born alive and unassisted, but lack the ability to walk or suck and do not survive for more than a few days without hand feeding.

Pathology

Necropsy can reveal hydranencephalic cavitation of the occipital region of both cerebral hemispheres and a hypoplastic cerebellum.

Histopathological examination can show: mild non-suppurative encephalitis and lack of folial development of the cerebellum, neural lesions of the cerebrum (cysts and malacia, oedema, perivascular cuffing), midbrain (lesions in the pons) or cerebellum (hypoplasia and degeneration, neuronal necrosis and neurophagia). Muscles can be atrophied with some neural degeneration[9].

Diagnostic Tests

Preliminary diagnosis can be made on the basis of gross or histopathological examination but this must be confirmed serologically because Akabane and Aino virus infections have indistinguishable clinical and pathological signs.

Demonstration of specific neutralising antibody or virus isolation from the affected calf before ingestion of colostrum will confirm a diagnosis. Supporting evidence can also be provided by positive maternal serology but is not diagnostic in its own right.

Virus Isolation

Aino virus has been isolated on numerous occasions using a variety of cell culture isolation systems. These systems involve the inoculation of heparinised or EDTA blood or necropsy samples from aborted foetuses directly to cell cultures or embryonated chicken eggs. Mammalian cell cultures were the first to be used to isolate Aino virus. The cell lines used included BHK-21 (ATCC CCL10), HmLu-1[13] and Vero cells (ATCC CCL81). Insect cell cultures (C6/36) (ATCC CRL1660) were introduced as an amplification step in an attempt to make the isolation system more sensitive, followed by subculture to the previously mentioned mammalian cell cultures. A further amplification step was added, which involved the intravenous inoculation of lysed blood into embryonated chicken eggs (ECE), followed by incubation and subculturing to C6/36 and then mammalian cell cultures. The most sensitive isolation system is the ECE isolation system .

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Appendix 1

Isolation of Aino virus

(a) Heparinised whole blood, clotted blood or postmortem samples are collected and should be transported chilled to the laboratory without delay.

(b) Postmortem samples are homogenized in sterile brain heart infusion broth containing penicillin (5 mg/mL), streptomycin (3 mg/mL) and amphotericin B (12 ug/mL) before centrifugation.

(c) Centrifuge at 2000 rpm for 10 min to clarify. Sample is inoculated intravascularly into 3 9–10-day-old embryonated chicken eggs (ECE).

(d) The ECE are incubated at 33°C for 5 days.

(e) The ECE are candled each day. Eggs dying before 24 h are discarded (nonspecific deaths). Eggs dying 48 - 120 h are held at 4°C. At 120 h all remaining eggs are chilled at -20°C for 40 min. Do not freeze.

(f) All embryos are decapitated and harvested (pooling embryos that died at similar times and those that remained alive as separate pools).

(g) Embryos are homogenized in sterile brain heart infusion broth containing antibiotics.

(h) Each sample is inoculated to 2 cell culture tubes containing a confluent monolayer of *Aedes albopictus* cells.

(i) The samples are incubated at room temperature for 7 days.

(j) Each pair of cell culture tubes are sonicated in an ultrasonic cleaning bath for 60 min or scraped from the tubes using a Pasteur pipette before pooling and passage.

(k) Passage each pooled cell culture tube to 2 cell culture tubes containing confluent monolayers of BSR or BHK-21 cells.[11] Other mammalian cell lines (Vero, HmLu-1) appear to be less sensitive than BHK-21 or BSR for the culture of bunyaviruses.

(l) The BHK-21 or BSR cell culture tubes are incubated at 37°C for 7 days. Each tube is inspected for the presence of CPE from day 3 to 7 after tinoculation. If no CPE is detected the tubes are passaged to another lot of BHK-21 or BSR cell cultures.

(m) If no CPE is detected the tubes of the second mammalian cell culture pass the samples are presumed to be negative.

(n) If CPE is detected, 100 µl of the CPE positive supernatant is adsorbed to a 25 cm^2 cell culture flask for 60 min to produce a virus stock that will later be used for virus identification.

(o) Controls should include:
- Cell control (uninoculated monolayer of the same cells).

(p) A valid test requires:
- No CPE in the cell controls
- The presence or absence of CPE in the test monolayers.

NB. as a result of a positive test sample can usually be detected from day 3 post-inoculation of the first mammalian cell culture passage.

OR

The direct inoculation of mammalian or insect cultures can be used if there is difficulty finding a source of ECE.

Essentially virus isolation through the direct inoculation of insect or mammalian cell cultures begins at Step (h) Appendix 1 and follows an identical procedure for the insect cell isolation system. Direct inoculation of mammalian cells substitutes the insect cells for a mammalian cell line[3].

Appendix 2

Identification of Aino virus

All unidentified viral isolates are subjected to either a serogrouping ELISA or a grouping plaque reduction neutralisation test. The test incorporates group monoclonal antibodies or polyclonal antibodies raised to each of the group members.

(a) Serogrouping ELISA [12]

Coat the plate with the unknown antigen (cells from an infected cell culture)

- dilute the pellets (derived from a virus stock flask) 1:100 in Tris buffer (pH 9.0).
- If pellets have been glycerinated remember to dilute 1:50.
- add 50 µl/well for each reference antibody (Bluetongue group, bovine ephemeral fever group, Palyam group, epizootic haemorrhagic disease group and Simbu group) in the panel. Add the appropriate negative control to each plate. A representative positive control panel is required per test.
- incubate at 37^0C, shaking, for 60 min.

Wash

At the end of the coating step, wash the plates three times with PSBT (0.05%) using a wash bottle (discard waste into a dish containing a disinfectant solution) and remove excess liquid. Do not allow plates to dry out.

Add the group-specific antibodies

- dilute the antibody in PBST containing 5% skim milk powder.
- add 50 µL to each well of the appropriate column.
- incubate at 37^0C, shaking, for 60 min.

Wash

At the end of the antibody step, wash the plates three times with PSBT (0.05%) and tap dry. Do not allow plates to dry out.

Conjugate incubation

- dilute the conjugate in PBST with 5% skim milk powder (use protein G-HRP for polyclonal antibodies, and anti-mouse-HRP for mouse immune serum and monoclonal antibodies)
- add 50 µl to each well.
- incubate in the warm room (≈37^0C), shaking, for 30 min.

Substrate preparation

- prepare substrate (Appendix 5). DO NOT ADD H_2O_2**.**

Wash

At the end of the conjugate step, wash the plates three times with PSBT (0.05%) and tap dry. Do not allow plates to dry out.

Substrate incubation

- add H_2O_2 (Appendix 5)
- add 50 µL of substrate (TMB working solution) (Appendix 5) to each well.
- incubate at room temperature for 10 min.

Acid stop

Stop the reaction by the addition of 50 µL of 1M sulphuric acid to each well.

Read the plates

- read the plate on a plate reader at 450 nm.

Nett OD = (virus pellet OD) – (negative cell control OD)

A Nett OD over 0.2 is considered significant.

Cross-reactions do occur between BTV and EHD.

The serogroup with the highest Nett OD is considered the specific serogroup for the virus under investigation.

(b) Serogrouping plaque reduction neutralisation assay

- using 24-well cluster dishes containing 80% confluent BSR, BHK-21 or Vero cells, aseptically discard growth medium.
- Add 250 µL of each pre-titrated polyvalent group antibody (one group antibody per well) across the plate.
- Dilute pre-titrated virus to achieve 100 PFU.
- Add 250 µL of unidentified virus across the plate and mix.
- ALWAYS ADD ANTIBODY BEFORE VIRUS
- Incubate at 37°C for at least 1 h before the addition of the agarose overlay.
- Combine 2x-concentrated medium and 4% agarose (Appendix 3) and add 500 µL to each well of the plate. Mix and allow to cool before returning to the incubator.
- Include antibody, virus and cell controls.

For the test to be valid:

- The cell and antibody controls on the control plate should contain a confluent monolayer of healthy cells.
- Each virus control should have a monolayer displaying CPE.
    - The positive group identification is a well in which 80% or more of plaques have been neutralised when compared with the positive control.

- The test serum controls from the screening test will indicate when a grouping serum is toxic to the cells. If toxicity is encountered, a result cannot usually be reported for that serum.

(c) Serotyping plaque reduction neutralisation assay

- using 24-well cluster dishes containing 80% confluent BSR, BHK-21 or Vero cells, aseptically discard growth medium.
- Add 250 µL of each pre-titrated monovalent antibody (one serotype specific antibody per well) across the plate.

  Testing for five Simbu virus serotypes, use five wells and the sixth and final well can be used as a virus control.
- Dilute pre-titrated virus to achieve 100 $TCID_{50}$.
- Add 250 µL of unidentified virus across the plate and mix.
- ALWAYS ADD ANTIBODY BEFORE VIRUS
- Incubate at 37°C for at least 1 h before the addition of the agarose overlay.
- Combine 2x medium and 4x agarose (Appendix 3) and add 500 µL to each well of the plate. Mix and allow to cool before returning to the incubator.
- Include antibody, virus and cell controls.

Agarose overlay can be stained with 1% neutral red enabling easier visualisation of the plaques

For the test to be valid:

- The cell and antibody controls on the control plate should contain a confluent monolayer of healthy cells.
- Each virus control should have a monolayer displaying CPE.
- The positive serotype identification is a well in which 80% or more of plaques have been neutralised when compared with the positive control.

A method replacing agarose with carboxymethyl cellulose can be derived from the Bluetongue diagnostic technique.

Appendix 3

Agarose gel overlay

To prepare 200 mL of a 1% gel overlay:

(a) Make a 2x concentration of culture medium. Dispense 100 mL to a sterile bottle and place at 37°C.

(b) Prepare a 4% agarose (Seaplaque agarose) solution by adding 4 g Seaplaque agarose to 100 mL distilled water and microwave until dissolved and sterilised. Place dissolved agar in a 56°C water bath and allow to stand for 30 min.

(c) Combine the culture medium and agarose and dispense an equal volume to each well of the test.

Appendix 4

Virus neutralisation test (VNT)

This test is used only for testing serum that has been screened positive for the presence of Aino antibody.

The virus neutralisation test (VNT) for Aino virus is carried out using aseptic technique in flat bottomed 96 well microtitre plates that support the growth of cell cultures. Two types of tests are performed: a screening test done in triplicate at a serum dilution of 1:4, and a titration of positive sera (determined from the screening test) from a dilution of 1:4 and done in quadruplicate.

Materials

Serum for Test:

The serum is heated at 56°C for 30 min before testing.

Screening test – 50 µL of each serum is aseptically dispensed into a microtitre well using a suitable pipettor. The serum is diluted 1:4 in the well by adding 150µl of appropriate diluent. Then 50µl of each diluted serum is pipetted into the next three wells, allowing the first well to be a serum control with three test wells. Diluent is added to the serum control wells and virus is added to the test wells.
The screening test serum control also acts as the serum control for the titration.

To titrate a serum - dilutions of each serum are made in quadruplicate from 1:4 to 1:128, using columns 1-6 or 7-12 with columns 1 or 7 being used for a serum control. Serum dilution is as described above. Diluent is added to the next 5 wells and serial two-fold dilutions are made across the plate using a multichannel pipette. 50 µL is discarded from the last dilution well (6 or 12). All wells should now contain 50 µL of diluted serum; up to 4 sera can be tested per plate.

Cells: Vero, BSR or BHK-21 cell lines.

Media/Diluent:

Vero - Medium 199 containing 10% inactivated foetal bovine serum and antibiotics as required.
BSR - BME containing 5% inactivated foetal bovine serum and antibiotics as required.
BHK-21 - BME containing 5% inactivated foetal bovine serum and antibiotics as required.

Virus: The virus to be used is Aino virus diluted to contain 100$TCID_{50}$ in 50µL at 5 days after inoculation. The virus is diluted in 10 fold dilutions from 10° to 10^{-5}

Positive control serum: Positive field sera.
Negative control sera: Negative field sera.

Procedure

(a) Heat test sera at 56°C for 30 min.

(b) Using flat-bottomed, 96 well, sterile, cell culture plates, dispense 50µL of diluent to each well of the plate, except those of column 1 and column 7.

(c) Dispense 100 µL of 1:4 diluted test serum in column 1 and column 7 as necessary. Run each sera in quadruplicate (Rows A, B, C, D etc.)

(d) Serially dilute test serum across the plate from column 1 to column 6 (dilutions 1:4 – 1:128) using a multichannel pipette, thoroughly mixing each dilution and discarding 50 µL from column 6 when the dilution series is complete.

ALTERNATIVELY IF SERA ARE TITRATED DIRECTLY RATHER THAN UNDERGOING AN INITIAL SCREENING TEST

Add 150 µL of media to Row 1 of the labelled tissue culture plates.

Add 50 µL of media to Rows 3 - 8 of the tissue culture plates.

In duplicate add 50 µL of respective test serum to Row 1 (→ 1/4 serum dilution) of the tissue culture plates.

Mix well, then transfer 50 µL diluted serum from Row 1 to Row 2 (1/4 serum dilution) and Row 3.

Serially dilute each serum from Row 3 (1/8 serum dilution) to Row 8 (1/256 serum dilution) using a 50 µl single or multichannel pipettor. Discard 50 µL from Row 8.

Row 1 is a serum control (1/4 serum dilution) and tests for cytotoxicity of the test sera.

(e) Preparation of a Control Plate:

(i) a known positive serum is titrated;

(ii) a known negative serum is tested (at least in quadruplicate at a 1/4 dilution, but this may be titrated. Alternatively, a known negative serum can be included on one of the standard test plates and treated in the same manner as a 'test' serum);

(iii) a cell control (containing only culture medium to a volume to replace all diluent, test serum and virus) (i.e. 100 µL per well) to which 100 µL of cells are added;

(iv) a 'back' titration of the stock virus dilution used in the test (see h.).

(f) Dilute virus using the appropriate diluent to give $100TCID_{50}/50\mu l$.

(g) Add 50 µL of diluted virus ($100TCID_{50}/50$ µL at day 5 after inoculation) to each well excluding the cell control, serum control and back titration wells.

(h) Prepare a back titration by making 6 ten-fold dilutions of the test virus ($100TCID_{50}/50$ µL). The dilutions are made using the test diluent. Each dilution is inoculated in quadruplicate across the plate. A virus titration of $2\log_{10} \pm 0.5\log_{10}$ is an acceptable result.

(i) All test and control plates are then incubated at 37°C in 5% CO_2 for 1 h.

(j) Add 100 µL of cells to all wells at a seeding rate of $2x10^5$ cells per ml ($2x10^4$cells/well) using a multistepper pipette.

(k) Incubate at 37°C in 5% CO_2 for up to 10 days.

For the test to be valid:

- The cell controls on the control plate should contain a confluent monolayer of healthy cells.

- The virus titre employed in the test should not vary by more than $\pm 0.5\log_{10}$ (ie. 1.5 - 2.5 $\log_{10}/50$ µL). If the virus titre is low, negative results may be accepted, but positives should be retested. When the virus titre is high, positive results will give a guide to titre, but all negatives should be retested.

- The positive serum control should not vary more than one dilution from its known titre in any one test.

- The test serum controls from the screening test will indicate when a serum is toxic to the cells. If toxicity is encountered, a result cannot usually be reported for that serum.

- A titre of 1:4 or greater is considered to be positive.

General

- The VNT can be difficult to standardise because of fluctuations of the titre of the virus. Titres may vary depending on the growth rate, passage level of the Vero cells or strain of Aino virus being used. If a consistently low titre is occurring the Vero cells can be replaced with BSR cells. The BSR cell line consistently yields a higher titre than Vero cells.
- Each batch of foetal bovine serum should be tested for specific neutralising antibodies to he target virus. FCS found to contain antibody should not be used for that test or rejected outright in preference to a more compatible batch of FCS.
- It is preferable to have several technicians able to do Aino VNTs.
- If the media becomes too alkaline (pink/purple), Aino virus replication is affected. This should not be a problem if plates are incubated in a well-maintained and calibrated humidified CO_2 incubator.

Appendix 5

Reagents

PBST 20x concentrate

Ingredients	Amt / 2000 mL
Na_2PO_4 anhydrous	42.8 g
NaH_2PO_4. $2H_2O$	15.6 g
NaCl	340 g
Tween 20	20 mL
Distilled Water	QS to 2000 mL

Dissolve all ingredients in glass distilled water to a final volume of 2000 mL. Adjust pH 7.4.
For use, dilute concentrate to 1x with distilled water

TMB substrate

Ingredients	Amt / 10 ml
TMB	0.101 g
DMSO	10 ml

Dissolve TMB powder in DMSO. Dispense 1ml volumes into 5 ml vials wrapped in alfoil and store at 4°C.

TMB Substrate: Working

Ingredients	4 plates	7 plates
Distilled water	18 mL	27 mL
Citric acid	2 mL	3 mL

TMB	200 µL	300 µL
H_2O_2 25 µL of 30% H_2O_2 to 225 µL Distilled water	25 µL	37.5 µL