AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

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SUB-COMMITTEE ON ANIMAL HEALTH LABORATORY STANDARDS

Caprine Arthritis–Encephalitis

Virology and Serology

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Caprine Arthritis–Encephalitis

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1. History in Australia

O'Sullivan *et al.* (1978) first described a condition similar to viral leukoencephalomyelitis (VLG) (Cork *et al.*, 1974*a*, 1974*b*) in kids in Queensland. Subsequently, some Western Australian goats that were experimentally infected with a cell-free suspension from a goat with chronic interstitial pneumonia and granulomatous encephalitis were shown to develop serum antibodies that reacted with the ovine lentivirus (maedivisna antigen) in agarose gel immunodiffusion tests conducted by the Central Veterinary Institute Diagnostic Laboratory in Holland (Robinson, 1981). The significance of this antibody response was explained by the isolation of a syncytial retrovirus from a sick goat by Coackley *et al.* (1981) and the subsequent demonstration that this virus could induce antibodies reactive with maedivisna virus (Smith *et al.*, 1981). Subsequent isolations of virus were made and characterised by Chung and O'Sullivan (1982) and Ellis *et al.* (1983).

2. Introduction

2.1. Synonyms

(a) VLG
(b) Chronic lymphocytic interstitial progressive pneumonia
(c) Caprine retrovirus infection
(d) Caprine syncytial retrovirus infection (CSR)

2.2. Nature of the Disease

Caprine arthritis–encephalitis (CAE) virus causes chronic progressive diseases in goats characterised by a leucoencephalomyelitis in young kids, and an arthritis–synovitis or bursitis ('big knee'). A chronic progressive interstitial pneumonia and an udder disease ('hard udder') sometimes seen in older animals may also be caused by this virus (Robinson and Ellis, 1986).

Only goats are naturally infected but sheep fed milk from infected does and experimentally inoculated with the virus may become persistently infected and develop antibodies. No clinical symptoms or lesions have been described in Merino or British Breed sheep inoculated with the virus in Australia but in New Zealand, Romney lambs inoculated with the virus developed arthritis–synovitis, as did Suffolk-Hampshire cross lambs in the United States of America.

Infection is naturally transmitted to the newborn kid through the colostrum or milk from an infected dam. *In utero* (transplacental) infection has not been conclusively demonstrated but lateral transmission to animals (of any age) in close contact does occur. This is considered to require intimate contact of mucosal surfaces with milk, vaginal, preputial, anal, nasal, conjunctival or oral discharges from an infected goat.

Goats can develop persistent antibodies from as early as three to five weeks after inoculation with virus by a variety of routes. However, in naturally infected goats seroconversion in some cases was not apparent until five to six months after known exposure. Antibodies are produced to a number of viral proteins but the major detectable antibodies are to a 28 000 dalton (p28) core protein (cross-reactive with maedivisna virus p30) and a viral surface (envelope) glycoprotein of 135 000 dalton molecular weight (gp135). Goats become persistently infected with the virus which can be isolated from peripheral blood leucocytes or explants of organs (particularly synovial membrane, lung or choroid plexus of antibody positive goats) for at least three years and probably for life. Presence of antibody generally indicates the presence of infection although kids up to two to three months of age may have maternally derived antibody to the virus but not become infected from ingested colostrum. As clinical signs develop, or in late pregnancy, the level of antibody especially to p28 may decline or sometimes even become negative in the agarose gel immunodiffusion (AGID) test. The problem of false negative serological results in AGID tests using p28 antigen initially led to the decision in all Australian State veterinary laboratories to routinely test for antibodies to both p28 and gp135 viral antigens in separate AGID tests. Subsequently an enzyme-linked immmunosorbent assay (ELISA) procedure for detection of antibody to CAE virus (Schroeder *et al.*, 1985) was shown to have the improved sensitivity. This procedure was adopted as the preferred test method for diagnostic testing and as the designated test for CAE virus herd accreditation.

With either the ELISA or the dual AGID test procedures it must be stressed that freedom from infection cannot be definitely confirmed without a repeat serological test procedure at an interval of at least six months. Virus isolation or viral genome detection may assist in the determination of an animal's status. The latter procedure is currently only a research tool.

3. Caprine Arthritis–Encephalitis Virus

The virus is an RNA virus, containing a reverse transcriptase, and shares antigenic cross-reactivity with maedivisna virus (Ellis *et al.*, 1983).

4. Serological and Virological Diagnosis of Caprine Arthritis–Encephalitis Virus Infection

4.1. Serological Diagnosis

The preferred serological test for detection of antibody to CAE virus is the ELISA procedure based on that described by Schroeder *et al.* (1985)

in which partially purified CAE virus is used as antigen. This has been shown to have higher relative sensitivity than the AGID test and is recommended for CAE virus testing. However, the ability of ELISA to detect low antibody levels may contribute to loss of sensitivity particularly as the antigen used is only partially purified and may contain various cellular proteins. Non-specific reactions in sheep sera have been attributed to presence of antibody to non-viral proteins in the ELISA antigen by western blotting techniques. Although CAE virus is the only caprine and ovine lentivirus recognised in Australia, both the ELISA and AGID tests based on CAE virus antigens cross-react with other ovine lentiviruses such as maedivisna virus. As some countries still request AGID test for screening export animals for ovine lentivirus infections, the AGID test procedures are included.

4.2. Enzyme-linked Immunosorbent Assay

The ELISA procedure is essentially as described by Schroeder *et al.* (1985).

4.2.1. Preparation of Antigens

The CAE virus is propagated in goat synovial membrane or lamb corneal cell monolayers. When cytopathic effect (CPE) is apparent the medium is changed and thereafter the supernatant fluid is harvested at weekly intervals and stored at -20°C. The harvests are thawed, bulked together and clarified by centrifugation at 8500 *g* for 20 min. Virus is harvested from the clarified supernatant by centrifugation at 8500 *g* for 17 hours at 4°C. The virus pellet is resuspended in Tris–sodium chloride-EDTA (TNE) buffer (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L sodium chloride, 1 mmol/L ethlene diamminetetraacetic acid (EDTA) to a volume of 0.1% of the original fluid. This suspension is clarified at 600 *g* for 5–10 min, the supernatant layered onto a cushion of 25% (w/v) sucrose in TNE-buffer and centrifuged at 42 000 *g* for two hours at 4°C. The virus pellet is resuspended in phosphate buffered saline (PBS) to 0.1% of the original volume. The antigen is stored at -70°C or below.

4.2.2. Test Procedure (see 6.2.)

Optimum concentration of the CAE virus antigen and conjugate should be determined by checkerboard titration using Australian Standard Reference (W and Q) positive sera and the testing laboratories standard negative control serum. Commercial conjugates should be selected to given optimum reactivity with positive and negative reference sera.

The test is performed in 96-well microtitre trays (DYNATECH, IMMULON M129B, LINBRO MICROELISA have all been satisfactory). Optimal dilutions of antigen diluted in carbonate–bicarbonate buffer (pH 9.6, see Bovine Pestivirus Infections 5.9.3.1., this volume) are incubated in the plate (50 µL/well) at room temperature (18–21°C) overnight. Plates are washed three times in wash buffer (distilled water, 0.05% Tween 20).

Sera are diluted 1:10 in high salt diluent [0.5 mol/L sodium chloride, 0.5% Tween 20, 10% foetal calf serum (FCS)] and 50 µL incubated with antigen for two hours at 37°C. Plates are washed six times as above then 50 µL of anti-goat conjugate optimally diluted in high salt diluent is incubated in wells for two hours at 37°C. The plates are washed 12 times then substrate (100 µL/well) is incubated in the wells.

The preferred substrate for the CAE ELISA is 3'-3',5'-5'-Tetramethylbenzidine (TMB) which is a non-carcinogenic compound. ABTS substrate [2,2'-azino-di(3 ethyl-benzthiazoline-6-sulphonic acid)] is also a suitable substrate but the chemical is potentially more dangerous than TMB.

4.2.3. Tetramethylbenzidine Substrate

Dissolve 350 mg of 3'-3',5'-5'-Tetramethyl-benzidine [Catalogue No. T2885 Sigma Chemical Company, PO Box 14508, St Louis, Mo. USA 63178-9916. Tel. (314) 771 5780] in 100 mL of methanol (CH_3OH) by stirring for several hours at room temperature. Store in a dark bottle in a cupboard. In winter keep the solution in a 25°C incubator, as crystals precipitate in the cold. *N.B.* TMB is very light sensitive.

4.2.3.1. Citrate–acetate buffer (0.1 mol/L, pH 6.0)

4.2.3.1.1. Solution 1. Dissolve 8.2 g sodium acetate (CH_3CO_2Na) in 1 L of distilled or deionised water.

4.2.3.1.2. Solution 2. Dissolve 2.1 g of citric acid ($C_6H_8O_7$) in 100 mL of distilled or deionised water.

Add very small quantities of solution 2 to solution 1 adjusting pH to 6.0 (it will require about 10 mL). Aliquot and sterilise by filtering or autoclaving. Store at 4°C.

Immediately prior to use, mix 9.7 mL of citrate–acetate buffer (warmed to room temperature) with 0.3 mL of TMB stock and add 5 µL of 30% hydrogen peroxide (H_2O_2) to activate substrate.

TMB substrate is also available in 1 mg tablet form [3',-3', 5',-5' Tetramethylbenzidin tablets, T3405, Sigma Chemical Company, PO Box 14508, St. Louis, Mo., USA 63178-9916. Tel. (314) 771 5750]. To use, dissolve 1 tablet in 10 mL citrate–phosphate buffer, pH 5.0. Add 2 µL fresh 30% hydrogen peroxide before use.

4.2.3.2. Citrate–phosphate buffer

Citric acid 0.1 mol/L	24 mL
Disodium hydrogen phosphate, Na_2HPO_4, 0.2 mol/L	26 mL
Distilled water to give total of	100 mL

Correct pH to 5.0.

Add 100 µL per well of TMB substrate and incubate for 10 min at room temperature in the dark (in a box or put in a drawer).

Stop the reaction with 100 µL/well of 1 mol/L sulfuric acid (H_2SO_4).
Read at 450 nm on ELISA plate reader.

4.2.4. ABTS® Substrate

Make a stock solution of ABTS [Cat. No. 1112422, Boehringer Mannheim Biochemica, 31 Victoria Avenue, Castle Hill, NSW 2154, Australia. Tel. (02) 899 7999] of 52 mmol/L (28.6 mg/mL) in sterile deionised or distilled water. Aliquot into small volumes and store at 4°C.

4.2.4.1. Substrate buffer — sodium citrate (100 mmol/L)

Prepared by dissolving 21.01 g of citric acid in 900 mL deionised or distilled water and adjusting pH to 4.2 with 2 mol/L sodium hydroxide before making the volume up to 1 L with water. Autoclave at 121°C for 15 min. Store at 4°C.

Working solution of substrate is prepared by adding 1 mL of ABTS stock solution to 50 mL substrate buffer then adding 1 mL of 2.5 mmol/L hydrogen peroxide solution [125 mL of analytical reagent (AR) grade 30% w/v hydrogen peroxide added to 10 mL distilled or deionised water].

Add 100 µL of ABTS substrate per well and incubate for one hour at 22–37°C.

Read at 405 nm on ELISA plate reader.

4.2.5. Controls and Interpretation of Test

The following control sera are included in each ELISA plate.

(a) Negative control serum in duplicate.
(b) Serial dilutions including 1:10, 1:100 and 1:1000 of positive control sera that react with p28 (w) antigen and gp135 (Q) antigen in AGID tests. Either the Australian Reference Positive Sera or appropriate in-house reagents standardised to the reference positives are suitable.

The mean optical density and 99.9% upper confidence limit of the optical density (OD) of a large sample (> 300) of goats from CAE-free flocks should be determined by each laboratory conducting this testing. Pooled serum from these goats should be used as a negative control. For tests where negative and positive controls give results within predetermined limits, reactions of less than the upper 99.9% confidence limit are considered negative. Reactions between this cut-off and the OD of the weakest reaction of the positive control dilutions are considered as suspicious and above this OD is positive.

It is recommended that goats giving suspicious reactions are retested in two months by which time infected goats will give clear cut positive results.

The Australian Standard Reference Positive sera for this test can be obtained from Australian National Quality Assurance Programme, VIAS Attwood, 475-485 Mickleham Road, Attwood, Vic. 3049, Australia.

4.3. Agarose Gel Immunodiffusion Test

Serum should be tested in individual gel diffusion tests using both the viral p28 antigen (W-AGID) and the viral gp135 antigen (Q-AGID). To be classified as negative a serum must not react in either test.

4.3.1. Preparation of Antigen

4.3.1.1. W–AGID

Two methods currently in use in Australia are:

(a) Weekly or fortnightly harvest of supernatant from persistently CAE virus-infected goat synovial membrane (GSM) cell or lamb corneal (LC) cell cultures. The medium is concentrated 15–20 times by dialysis then mixed with an excess of diethyl ether ($C_2H_5OC_2H_5$) for 5–10 min. Ether is removed after centrifugation by suction and evaporation to leave the W–AGID test antigen.
(b) Supernatants from persistently CAE virus-infected LC or GSM cell cultures harvested at 21–28 day intervals filtered through AMICON spiral wound or hollow fibre cartridge of 100 000 m.w. cut-off. The filtrate is collected and then filtered and concentrated through a 10 000 m.w. cut-off cartridge. The retentate concentrated about 15–20 times is used as antigen without diethyl ether treatment.

4.3.1.2. Q–AGID

Two methods currently used in Australia are:

(a) CAEV-infected GSM or LC cell cultures harvested 21–28 days after infection by freezing and thawing are clarified by centrifugation and concentrated 45–100 times by dialysis. The antigen is used without ether treatment.
(b) Supernatants from persistently infected GSM or LC cell cultures are harvested 21–28 days post infection and filtered and concentrated through AMICON spiral-wound or hollow-fibre cartridges with 100 000 m.w. cut-off. The retentate (concentrated about 50 times) is ultracentrifuged (120 000 *g* for 90 min) to remove whole virus and the supernatant containing partially purified gp135 viral antigen is used in the Q–AGID test.

4.3.2. Reference Positive Antisera

Both antigen and reference sera for W–AGID and Q–AGID tests are available from the Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle, NSW 2570, Australia.

4.3.3. Test Procedure

The test procedure is carried out by standard gel diffusion test procedures using a seven-well pattern cut in Petri dishes containing 1% agarose medium. Antigen is placed in the central well

and reference positive serum is placed in each alternate peripheral well. Undiluted test sera are added to the remaining wells. Precipitin reactions are recorded as negative, weak positive or positive (1+, 2+, 3+, 4+) depending on the intensity and position of the line of identity compared to the reference positive antiserum reaction when the test is read at 48 hours.

4.4. Virus Isolation

4.4.1. Isolation from a Live Animal that is Serologically Positive or Exhibiting Clinical Signs

Virus can be isolated from peripheral blood leucocytes. About 50 mL blood is collected in 7 mL sodium citrate solution (Ellis, 1990) and the leucocyte fraction (buffy coat) separated by centrifugation at 2000 rpm/30 min. The leucocytes are harvested in a small volume (3 mL) and 15 mL distilled water added to lyse the erythrocytes. After gentle agitation for about 45 s, 15 mL of 0.3 mol/L sodium chloride solution is added to restore isotonicity. The leucocytes are separated by centrifugation at 2000 rpm/10 min, resuspended in 10 mL of growth medium with 10% FCS and counted. About 3 x 10^7 leucocytes are added to a 25 cm^2 bottle of goat synovial membrane (GSM) cells (see 6.1.) prepared from a healthy serologically negative goat by explant culture. Syncytia become apparent from 10 days onwards. If no syncytia are observed after 30 days incubation, one blind passage is carried out before discarding as negative.

4.4.3. Isolation from a Recently Dead Animal

Virus can be isolated in explants (see 6.1.) prepared from a variety of tissues. Synovial membranes from joints, tendon sheaths or bursae; lung; choroid plexus will all grow successfully. Joint synovial membrane or lungs are probably the most convenient. Syncytia appear within the periphery of the explant outgrowth from 10 days onwards. If no syncytia are observed after 30 days incubation, cells are trypsinised from the flask and cocultivated with fresh GSM cell cultures for an additional 30 days.

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6. Appendixes

6.1. Appendix 1 — Explant Cultures

6.1.1. Preparation of Explant Cultures

6.1.1.1. Preparation of tissues

Observe an aseptic procedure using sterile instruments. Tissues commonly used are:

6.1.1.1.1. Synovial membranes. Skin the joint - carpus or shoulder; flame briefly; carefully dissect away any superficial fascia, muscle, tendon insertions or fatty tissue, leaving either the joint capsule (in the case of the shoulder) or the tendon sheath/bursa. Separate this from the bone/cartilage of the joint and place in PBS in a Petri dish. Remove any obvious fragments of fat or muscle.

6.1.1.1.2. Choroid plexus. With the brain removed from the cranium and on a flat sterile surface, cut in the horizontal plane opening the two lateral ventricles and exposing the third ventricle. Carefully pick up the lining (choroid plexus) with forceps. The whole is extracted usually in one piece and placed in PBS.

6.1.1.1.3. Lung or foetal lung. Cut a strip from the edge of the lung and place in PBS.

6.1.1.1.4. Other organs. These may be treated in the same way removing any fat, capsule or connective tissue before processing.

6.1.2. Treatment of Explant Tissues

(a) With crossed scalpels, finely mince the tissues in a small quantity of PBS in a Petri dish; by gently swirling the contents, larger pieces collect in the centre allowing the smaller fragments to remain in the periphery.

(b) Fragments of 1 mm^3 can be picked up in a pasteur pipette and placed in a container.

(c) Wash once by centrifugation, discarding the supernatant (with healthy adult lungs, fragments tend to float in a solid plug, allowing the fluid to be removed from beneath with a pipette).
(d) Add a small quantity of medium and disperse the fragments. Medium 199 with 10% FCS gives good growth.
(e) Place a small quantity of fragments of tissue into a flask. It will sometimes help to previously moisten the inside of the flask with fresh medium. Gently agitate to disperse, aiming at about 2–3 fragments per 10 mm^2. Surface tension should hold the fragments to the bottom surface of the flask.
(f) The flask is then placed at 37°C and left undisturbed for 3–4 days.
(g) Carefully examine and add medium as necessary. 'Islands' of cells should grow out of some fragments by three to seven days and confluent monolayers should be obtained by 14–21 days.

6.2. *Appendix 2 — Procedure for Caprine Arthritis–Encephalomyelitis Virus Enzyme-linked Immunosorbent Assay*

(a) Coat a 96-well ELISA microplate with 50 µL of antigen per well optimally diluted in carbonate–bicarbonate buffer (pH 9.6) and leave at room temperature overnight in a humidified container.
(b) Wash the plates the next day three times with washing solution (distilled water, 0.05% Tween 20).
(c) Add reference and test sera appropriately diluted at the rate of 50 µL per well. Reference sera should be diluted at 1:10 and at two further dilutions to give intermediate and weak positive reactions. The weak positive value should be just higher than the established 'cut off' value for the test. The actual dilutions for different reference sera will be provided when they are supplied. Test sera are diluted at 1:10. All serum dilutions are made in high salt diluent (0.5 mol/L sodium chloride, 0.5% Tween 20, 10% FCS).
Incubate plates for two hours at 37°C in a humidified container.
(d) Wash plates six times with washing solution.
(e) Peroxidase conjugated antigoat IgG is added to all wells at the rate of 50 µL per well at the appropriate dilution of conjugate in high salt diluent.
Plates are incubated for two hours at 37°C in a humidified container.
(f) Plates are washed ten times with washing solution and twice with distilled water.
(g) Add substrate (100 µL) to each well and plates are incubated at room temperature in the dark for the optimum time period for the substrate (see 4.2.5. and 4.2.6.).
(h) Plates are agitated for one minute on a plate shaker before reading the OD at the appropriate setting for the substrate. For TMB substrate the reaction is stoppped by the addition of 100 µL of 1 mol/L sulfuric acid (H_2SO_4) before reading the OD at the dual wavelengths of 450 and 620 nm. For the ABTS substrate the OD is read at 405 nm.
(i) For the test to be accepted as valid the performance of the reference sera should be checked to confirm that ODs within a predetermined range have been achieved. If the result for the negative control serum is too high or the weak positive serum too low, the test should be repeated. The 'cut-off' point for the test has been determined for a standard set of reagents in combination (i.e. both antigen and reference sera) and will be provided for those reagents when they are supplied. Reagents produced 'in-house' should be standardised to the National Reference reagents (NSW Agriculture, Elizabeth Macarthur Agricultural Institute, PMB 8, Camden, NSW 2570, Australia. Tel. (046) 293 333; Fax (046) 293 400.