MALIGNANT CATARRHAL FEVER

SUMMARY

Malignant catarrhal fever (MCF) is an acute, generalised and usually fatal disease affecting many species of Artiodactyla. The disease has been most often described as affecting species of the subfamily Bovinae and family Cervidae, but it is also recognised in domestic pigs as well as giraffe and species of antelope belonging to the subfamily Tragelaphinae. MCF is defined by the recognition of characteristic lymphoid cell accumulations in nonlymphoid organs, vasculitis and T-lymphocyte hyperplasia in lymphoid organs, the main cause of which is either of two gammaherpesviruses. The alcelaphine herpesvirus-1 (AIHV-1), which is maintained by inapparently infected wildebeest, causes the disease in cattle in regions of Africa and in a variety of ruminant species in zoological collections world-wide. Ovine herpesvirus-2 (OvHV-2), which is prevalent in all varieties of domestic sheep as a subclinical infection, is the cause of MCF in most regions of the world. This form of the disease is also known as sheep-associated MCF. In both forms of the disease, animals with clinical disease are not a source of infection as virus is only excreted by the natural hosts – wildebeest and sheep, respectively.

MCF usually appears sporadically and affects few animals, though both AIHV-1 and OvHV-2 can give rise to epizootics. There is a marked gradation in susceptibility to the OvHV-2 form of MCF ranging from the relatively resistant Bos taurus and B. indicus, through water buffalo, North American bison and many species of deer, to the extremely susceptible Père David’s deer, and Bali cattle. The disease may present a wide spectrum of clinical manifestations ranging from the acute form, when minimal changes are observed prior to death, to the more florid cases characterised by high fever, bilateral corneal opacity, profuse catarrhal discharges from the eye and nares, necrosis of the muzzle and erosion of the buccal epithelium. Infectivity from animals with the AIHV-1 form of MCF can be recovered only by employing techniques that retain the viability of host cells, while OvHV-2 has never been recovered from affected animals. Diagnosis is normally achieved by observing the characteristic histopathological changes, though detection of viral DNA in either form of the disease has become the preferred option.

Identification of the agent: AIHV-1 may be recovered from clinically affected animals using peripheral blood leukocytes or cell suspensions prepared from lymph nodes and spleen, but cell viability must be preserved during processing, as infectivity cannot be recovered from dead cells. Virus can also be recovered from wildebeest, either from peripheral blood leukocytes or from cell suspensions of other organs. Most monolayer cultures of ruminant origin are probably susceptible and develop cytopathic effect (CPE), although bovine thyroid cell cultures have been used extensively for recovery of virus. Primary isolates typically produce multinucleated CPE in which viral antigen can be identified by immunofluorescence or immunocytochemistry using suitable antisera or monoclonal antibodies. The OvHV-2 agent has never been identified formally, although lymphoblastoid cell lines propagated from affected animals contain OvHV-2-specific DNA. Both agents have been transmitted experimentally to rabbits and hamsters, which develop lesions characteristic of MCF.

Viral DNA has been detected in clinical material from cases of MCF caused by both AIHV-1 and OvHV-2 using the polymerase chain reaction, and this is becoming the method of choice for diagnosing the OvHV-2 form of the disease.

Serological tests: Infected wildebeest, the natural host, consistently develop antibody to AIHV-1, which can be detected in a variety of assays including virus neutralisation, immunoblotting, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. However, the antibody response of clinically affected animals is limited, with no neutralising antibody developing, so that detection relies on the use of immunofluorescence, ELISA or immunoblotting. Antibody to OvHV-2 has only
been detected by using AIHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence, ELISA or immunoblotting. While antibody often can be detected by immunofluorescence and ELISA in cattle with MCF, in more acutely affected animals, such as deer, antibody is not always present. The competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) appears to have a sensitivity and specificity that are equal to or better than the other tests, although a recently described ELISA gave good concordance with this test.

Requirements for vaccines and diagnostic biologicals: No vaccine has been developed for this disease.

### A. INTRODUCTION

Malignant catarrhal fever (MCF) is a generally fatal disease of cattle and many other species of Artiodactyla, which occurs following infection with either alcelaphine herpesvirus-1 (AIHV-1) or ovine herpesvirus-2 (OvHV-2). Wildebeest (Connochaetes spp. of the subfamily Alcelaphinae), the natural hosts of AIHV-1, experience no clinical disease following infection. Likewise, infection of domestic sheep, the natural host of OvHV-2, has not been associated with any clinical reaction following natural infection, although experimentally, large doses of virus produced clinical signs of MCF, when inoculated into susceptible sheep (11). Disease caused by AIHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and has been referred to as wildebeest-associated MCF. The OvHV-2 form of the disease occurs world-wide wherever sheep husbandry is practised and has been described as sheep-associated (SA) MCF. Both forms of the disease may present a wide spectrum of clinical entities, though the characteristic histopathological changes are very similar in all cases. These two viruses belong to a subgroup of closely related ruminant rhadinoviruses that infect three subfamilies of Bovidae (Alcelaphinae, Hippotraginae and Caprinae); all probably have a potential to cause typical MCF. On rare occasions members of this group of viruses other than AIHV-1 and OvHV-2 have been identified as a cause of MCF.

- **Clinical and pathological changes**

The clinical signs of MCF are highly variable and range from peracute to chronic with, in general, the most obvious manifestations developing in the more protracted cases. In the peracute form, either no clinical signs are detected, or depression followed by diarrhoea and dysentery may develop for 12–24 hours prior to death. In general, the onset of signs is associated with the development of a high fever, increased serous lachrymation and nasal exudate, which progresses to profuse mucopurulent discharges. Animals may be inappetent and milk yields may drop. Characteristically, progressive bilateral corneal opacity develops, starting at the periphery. In some cases skin lesions appear (characterised by ulceration and exudation), which may form hardened scabs associated with necrosis of the epidermis, and are often restricted to the perineum, udder and teats. Salivation associated with hyperaemia may be an early sign, progressing to erosions of the tongue, hard palate, gums and, characteristically, the tips of the buccal papillae. Superficial lymph nodes may be enlarged and limb joints may be swollen.

Nervous signs such as hyperaesthesia, incoordination, nystagmus and head pressing may be present in the absence of other clinical signs or as part of a broader more characteristic syndrome.

There is a wide spectrum of susceptibility to OvHV-2-induced disease, ranging from *Bos taurus* and *B. indicus*, which are relatively resistant, through most species of deer, bison (*Bison bison*) and water buffalo (*Bubalus bubalis*), which are much more susceptible, to the extremely susceptible Bali cattle (*Bos javanicus*) and Père David’s deer (*Elaphurus davidianus*). The more resistant species tend to experience a more protracted infection and florid lesions, while in the more susceptible species the disease course tends to be shorter and the clinical signs less dramatic.

Reports from several countries, and in particular from Norway, that the disease affects domestic pigs have recently been confirmed (14). Signs are very similar to those seen in acutely affected cattle.

A mild form of the disease described in 1930 was regarded with some scepticism because the disease could be confirmed only by histological changes observed at post-mortem. However, recent investigations using molecular and serological methods would appear to confirm that a few infected animals may recover following mild or even quite severe clinical reactions (15). Some studies indicate that substantial numbers of animals may become infected without developing clinical disease.
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**Pathology**

Gross pathological changes reflect the severity of clinical signs, but are generally widespread and may involve most organ systems. Erosions and haemorrhages may be present throughout the gastrointestinal tract, and in the more acute cases can be associated with haemorrhagic intestinal contents. In general, lymph nodes are enlarged, although the extent of lymph node involvement varies within an animal. Lymph nodes can often be firm and white when cross-sectioned, while others, in particular submandibular and retropharyngeal, may be haemorrhagic and even necrotic. Catarrhal accumulations, erosions and the formation of a diphtheritic membrane are often observed in the respiratory tract.

Within the urinary tract characteristic echymotic haemorrhages of the epithelial lining of the bladder are often present, while the renal cortex may be affected with multiple raised white foci, each 1–5 mm in diameter and sometimes surrounded by a thin zone of haemorrhage.

Histological changes have been the basis for confirming cases of MCF and are characterised by epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs, and widespread interstitial accumulations of lymphoid cells in nonlymphoid organs. Epithelial lesions may be present at all epithelial surfaces and are characterised by erosion and ulceration, frequently with subepithelial and intraepithelial lymphoid cell infiltration, which may be associated with vasculitis and haemorrhages.

Vasculitis is generally present and may be pronounced in the brain, affecting veins, arteries, arterioles and venules. It is characterised by lymphoid cell infiltration of the tunica adventitia and media, often associated with fibrinoid degeneration. In the lumen there may be ‘pavementing’ by lymphoid cells, and in severe cases, endothelial damage and subendothelial accumulations by lymphoid cells can sometimes lead to occlusion.

Lymph-node hyperplasia is characterised by an expansion of lymphoblastoid cells in the paracortex, while degenerative lesions are generally associated with the follicles. Oedema with lymphoid inflammation often affects the perinodal tissue.

The interstitial accumulation of lymphoid cells in nonlymphoid organs, in particular the renal cortex and periportal areas of the liver, is typical, and in the case of the kidney may be very extensive. In the brain there may be a nonsuppurative meningoencephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid.

The macroscopic lesions observed in the cornea are reflected histologically by lymphoid cell infiltration originating in the limbus and progressing centrally, with oedema and erosion developing in the more advanced cases. Vasculitis, hydrops and iridocyclitis also may be present.

The pathological features of MCF irrespective of the agent involved are essentially similar. However, apart from histological examination, the methods available for diagnosing AlHV-1- and OvHV-2-induced disease are very different and are thus considered separately.

**B. DIAGNOSTIC TECHNIQUES**

**B1. Alcelaphine herpesvirus-1**

This form of the disease occurs in the cattle-raising regions of eastern Africa where pastoralists use areas grazed by wildebeest, and in southern Africa in areas where wildebeest and cattle are grazed together. The disease, however, can also affect a variety of other ruminant species in zoological collections world-wide and so, apart from antelope of the subfamilies Alcelaphinae and Hippotraginae, it is advisable to regard all ruminants as susceptible.

Most laboratory-based tests have relied on one attenuated isolate (WC11) that has been subjected to many laboratory passages as a source of viral antigen and DNA (17). The full nucleotide sequence of the virulent low passage virus (C500) is now available and will form the basis of further studies of this virus (4).

1. Identification of the agent

   **Clinically affected animals**

   a) Isolation

   The striking feature of AlHV-1-induced MCF is the lack of detectable viral antigen or herpesvirus-specific cytology within lesions. Confirmation of infection thus relies on virus recovery. Generally, infectivity is strictly cell associated and thus isolation can be achieved only from cell suspensions either of peripheral blood leukocytes, lymph nodes or other affected tissues. Cell suspensions are prepared in tissue culture fluid, approximately $5 \times 10^8$ cells/ml, and inoculated into preformed monolayer cell cultures. Bovine thyroid cells have been used extensively, but most primary and low passage monolayer cell cultures of ruminant origin
will probably provide a suitable cell substrate for isolating the virus. Following 36–48 hours’ incubation, culture medium should be changed and monolayers should be examined microscopically (×40) for evidence of cytopathic effects (CPE). These appear characteristically as multinucleate foci within the monolayers, which then progressively retract forming dense bodies with cytoplasmic processes that may detach. This is followed by regrowth of normal monolayers. A CPE may take up to 21 days to become visible and is seldom present before day 7. Infectivity at this stage tends to be largely cell associated and thus any further passage or storage must employ methods that ensure that cell viability is retained. Specificity of the isolate should be determined using specific antisera or monoclonal antibodies (MAbs) in fluorescence or immunocytochemical tests.

b) Viral DNA

Characteristically, very little viral DNA can be detected within affected tissues, hence it is necessary to amplify the viral genome either by conventional culture or the polymerase chain reaction (PCR).

The full sequence of the C500 isolate has been published permitting the design of primers for PCR reactions from conserved regions of the genome. The polymerase gene sequence has been employed for phylogenetic comparison of AlHV-1 and related viruses (9).

• Natural hosts

It is almost certain that all free-living wildebeest are infected with AlHV-1 by 6 months of age, virus having been spread as an intense epizootic during the perinatal period. The species Connochaetes taurinus, C. t. albajubatus and C. gnu are all assumed to be infected with the same virus. Infection also appears to persist in most groups of wildebeest held in zoological collections. However, it is possible that infection may be absent in animals that have been isolated during calf-hood or that live in small groups. Natural infection has been successfully demonstrated by in-situ hybridisation on lung sections from C. t. taurinus calves in South Africa (16).

Following infection there is a brief period when virus is excreted in a cell-free form and can be isolated from nasal swabs. Virus can also be isolated from blood leukocytes at this time, but in older animals this is less likely to be successful unless the animal is immunosuppressed either through stress or pharmacological intervention. In addition, virus may be isolated by establishing cultures of tissues from apparently normal animals, and this has been achieved in monolayer cultures of both kidney and thyroid cells from adult animals.

Other large antelope of the subfamilies Alcelaphinae and Hippotraginae are also infected with antigenically closely related gammaherpesviruses, but there is no evidence that they can spread to other species and cause MCF, except rarely in captive populations.

2. Serological tests

• Clinically affected animals

The antibody response of clinically affected animals is limited, with no neutralising antibody developing. Antibody in clinical cases can be demonstrated consistently by immunofluorescence or the immunoperoxidase test (IPT) using WC11-infected cell cultures as substrate. A competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) was first developed for detecting antibody to OvHV-2 (12) using an MAb (15-A) that targets an epitope that appears to be conserved among all MCF viruses and is probably also applicable to AlHV-1 infected animals.

• Natural hosts

Antibody appears to develop consistently in wildebeest following infection and can be identified by neutralisation assays using the cell-free isolate WC11, or by immunofluorescence, again using the WC11 isolate and anti-bovine IgG, which has been shown to react with wildebeest IgG. The Minnesota MCF virus strain, which is indistinguishable from the WC11 strain of AlHV-1, is used for CI-ELISA antigen production.

There has been no attempt so far to standardise the indirect fluorescent antibody (IFA) test and the IPT, but the two methods below are given as examples. The CI-ELISA is available as a commercial kit

a) Indirect fluorescent antibody test

The IFA is less specific than virus neutralisation (VN); it can be used to demonstrate several varieties of ‘early’ and ‘late’ antigens in AlHV-1-infected cell monolayers. Antibodies reacting in the IFA test or the IPT develop in cattle and experimentally infected rabbits during the incubation period, and later in the clinical course of the disease, though cross-reactions with some other bovine herpesviruses, as well as OvHV-2, reduce the differential diagnostic value. Detection of such cross-reacting antibodies can sometimes be useful in supporting a diagnosis of SA-MCF.
• Preparation of fixed slides

Inoculate nearly or newly confluent cell cultures (see Section B1.2.c) with AIHV-1 (strain WC11). Uninoculated control cultures should be processed in parallel. At about 4 days – when the first signs of CPE are expected to appear but before overt CPE is visible – treat the cultures as follows: discard the medium, wash with PBS, remove the cells with trypsin–verosene solution, spin down cells at approximate 800 g for 5 minutes, discard the supernatant fluid, and resuspend the cells in 10 ml of phosphate buffered saline (PBS) for each 800 ml plastic bottle of cell culture.

Make test spots of the cell suspension on two wells of a polytetrafluoroethylene-coated multiwell slide; air-dry and fix in acetone. Stain the spots with positive standard serum and conjugated anti-IgG to the appropriate species. Examine the incidence of positive and negative cells under a fluorescent microscope. Adjust the cell suspension by adding noninfected cells and/or PBS to give a suitable concentration that will form a single layer of cells when spotted on to the slide, with clearly defined positive cells among a background of negative cells.

Spot the adjusted positive cell suspension and the control negative suspensions on to multiwell slides in the desired pattern, and air-dry. Fix in acetone for 10 minutes. Rinse, dry and store over silica gel in a sealed container at –70°C.

An alternative procedure, which is easier to evaluate, is to prepare monolayers of infected and noninfected cells in Leighton tubes or chamber slides. The cell monolayers are infected with from 150 to 200 TCID₅₀ (50% tissue culture infective dose) of virus that has been diluted in cell culture medium. The infected and noninfected slides are fixed in acetone and stored, as above, at –70°C.

• Test procedure

i) Rehydrate the slides for 5 minutes with PBS, rinse in distilled water and air-dry.

ii) Dilute sera 1/20 in PBS. Samples that give high background staining may be retested at higher dilutions. Apply diluted fluids to one MCF virus-positive cell spot and one negative control spot for each sample. Include positive and negative serum controls. Ideally, the test should be validated by titrating the control positive to determine its end-point.

iii) Incubate at 37°C for 30 minutes in a humid chamber.

iv) Drain the fluids from the spots. Wash the slides in two changes of PBS, for 5 minutes each.

v) Wash in PBS for 1 hour with stirring, and then air-dry the slides.

vi) Apply rabbit anti-bovine IgG fluorescein isothiocyanate (FITC) conjugate at a predetermined working dilution.

vii) Incubate at 37°C for 20 minutes, drain the slides, and wash twice in PBS for 10 minutes each.

viii) Counterstain in Evans blue 1/10⁴ for 30 seconds, and wash with PBS for 2 minutes. Dip in distilled water, dry and mount in PBS/glycerol (50/50).

ix) Examine by fluorescence microscopy for specific binding of antibody to the infected cells.

b) Immunoperoxidase test

A dilution of bovine turbinate (BT) cell-cultured AIHV-1 containing approximately 10³ TCID₅₀ is made in a freshly trypsinised suspension of BT cells and seeded into Leighton tubes containing glass cover-slips, 1.6 ml per tube, or four-chambered slides, 1.0 ml per chamber.

Observe the cell cultures at 4–6 days for CPE and fix the cultures with acetone when signs of CPE begin. Remove the plastic chambers, but not the gaskets, from the slide chambers before fixation, and use acetone (e.g. UltimAR) that will not degrade the gasket. Store the fixed cells at –70°C.

• Test procedure

i) Prepare IPT diluent (21.0 g NaCl and 0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2) and washing fluid (0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2).

ii) Dilute the serum to be tested 1/20 in IPT diluent and overlay 150–200 µl on to a fixed virus-infected cover-slip or slide chamber.

iii) Incubate the cover-slip in a humid chamber at 37°C for 30 minutes.

iv) Dip the cover-slip three times in washing fluid.

v) Overlay 150–200 µl of diluted (1/5000 in IPT diluent) peroxidase-labelled anti-bovine IgG on to the cover-slip or slide chamber.
vi) Incubate the cover-slip or slide chamber in a humid chamber at 37°C for 30 minutes.

vii) Dip the cover-slip three times in washing fluid.

viii) Dilute the AEC substrate (3-amino-9-ethylcarbazole) in distilled water (5 ml of distilled water, 2 drops buffer, 2 drops hydrogen peroxide, and 3 drops AEC) and apply to the cover-slip or slide chamber.

ix) Incubate in a humid chamber at 37°C for 8–10 minutes.

x) Dip the cover-slip in distilled water, air-dry, and mount on a glass slide. Slide chambers are read dry.

xi) The slide is read on a light microscope. The presence of a reddish-brown colour in the nuclei of the infected cells indicates a positive reaction.

c) Virus neutralisation

Tests have been developed for detecting antibodies to AlHV-1 in both naturally infected reservoir and indicator hosts. The first of these is a VN test using cell-free virus of the WC11 strain, and another uses a hartebeest isolate (AlHV-2). AlHV-1 and AlHV-2 have cross-reactive antigens and therefore either strain can be used in the test. The test is laborious, but can be performed in microtitre plates using low passage cells or cell lines. The main applications have been in studying the range and extent of natural gammaherpes viruses infection in wildlife, captive species in zoos and, to a lesser extent, sheep populations. It has also been useful in attempts to develop vaccines, all of which have had limited success. The VN test is of no value as a diagnostic test in clinically affected animals as no VN antibody develops in clinically susceptible species.

AIHV-1 stock (strain WC11) is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, low passage bovine testis, or another permissive cell type. The virus is stored in aliquots at –70°C. The stock is titrated to determine the dilution that will give 100 TCID₅₀ in 25 µl under the conditions of the test.

• Test procedure

i) Inactivate the sera for 30 minutes in a water bath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium from 1/2 to 1/16 using a 96-well flat-bottomed cell-culture grade microtitre plate, four wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.

iii) Add 25 µl per well of WC11 virus stock at a dilution in culture medium calculated to provide 100 TCID₅₀ per well.

iv) Incubate for 1 hour at 37°C. The residual virus stock is also incubated.

v) Back titrate the residual virus in four tenfold dilution steps, using 25 µl per well and at least four wells per dilution.

vi) Add 50 µl per well of bovine kidney cell suspension at 3 × 10⁵ cells/ml.

vii) Incubate the plates in a humidified CO₂ atmosphere at 37°C for 7–10 days.

viii) Read the plates microscopically for CPE. Validate the test by checking the back titration of virus (which should give a value of 100 TCID₅₀ with a permissible range 30–300) and the control sera. The standard positive serum should give a titre within 0.3 log₁₀ units of its predetermined mean.

ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.

x) A negative serum should give no neutralisation at the lowest dilution tested (1/2 equivalent to a dilution of 1/4 at the neutralisation stage).

d) Competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA)

A CI-ELISA was first developed for detecting antibody to OvHV-2 (12) using a MAb (15-A) that targets an epitope on a complex of glycoproteins that appears to be conserved among all MCF viruses. The MAb was raised against the Minnesota isolate of virus, which is indistinguishable from the WC11 strain of AlHV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and antibody to the following pathogenic viruses has been detected: AlHV-1, AlHV-2, OvHV-2, CpHV-2 and the herpesvirus of unknown origin observed to cause classic MCF in white-tailed deer, as well as the MCF-group viruses not yet reported to be pathogenic, such as those carried by the oryx, muskox, and others. The test has recently been reformatted to increase sensitivity (8). This change was made to enable the detection antibody in newly infected lambs and animals in the acute stage of the disease, which were sometimes not detected in the previous format. The CI-ELISA has the advantage of being faster and more efficient than the IFA or IPT. Additional validation data will become available as its use is expanded to more laboratories in other parts of the world.
The complete reagent set for the CI-ELISA, including pre-coated plates, labelled MAb and control sera, is commercially available. For laboratories wishing to prepare their own antigen-coated plates, the following protocol is provided. Immuno 4 ELISA plates (Dynatech Lab, Chantilly, Virginia) are coated at 4°C (39°F) for 18–20 hours with 50 µl of a solution containing 0.2 µg per well of semi-purified MCF viral antigens (Minnesota or WC11 isolates of AlHV-1) in 50 mM carbonate/bicarbonate buffer (pH 9.0). The coated plates are blocked at room temperature (21–25°C, 70–77°F) for 2 hours with 0.05 M PBS containing 2% sucrose, 0.1 M glycine, 0.5% bovine serum albumin and 0.44% NaCl (pH 7.2). After blocking, wells are emptied and the plates are then dried in a low humidity environment at 37°C for 18 hours, sealed in plastic bags with desiccant, and stored at 4°C (39°F) (10). MAb 15-A is conjugated with horseradish peroxidase by the VMRD, Inc. using a standard periodate method.

• **Test procedure**
  i) Dilute positive and negative controls and test samples (either serum or plasma) 1/5 with dilution buffer (PBS containing 0.1% Tween 20, pH 7.2).
  ii) Add 50 µl of diluted test or control samples to the antigen-coated plate (four wells for negative control and two wells for positive control). Leave well A1 empty and for use as a blank for the plate reader.
  iii) Cover the plate with parafilm and incubate for 60 minutes at room temperature, (21–25°C, 70–77°F).
  iv) Using a wash bottle, wash the plate three times with wash buffer (same as dilution buffer: PBS containing 0.1% Tween 20, pH 7.2).
  v) Prepare fresh 1 × antibody-peroxidase conjugate by diluting one part of the 100 × conjugate with 99 parts of dilution buffer.
  vi) Add 50 µl of diluted antibody-peroxidase conjugate to each sample well. Cover the plate with parafilm and incubate for 60 minutes at room temperature (21–25°C, 70–77°F).
  vii) Wash the plate with wash buffer three times.
  viii) Add 100 µl of substrate solution (TMB Microwell, BioFX Laboratories, Owings Mills, Maryland) to each sample well. Incubate for 60 minutes at room temperature (21–25°C; 70–77°F). Do not remove the solution from the wells.
  ix) Add 100 µl of stop solution (0.18 M sulphuric acid) to each well. Do not remove the solution from the wells.
  x) Read the optical densities (OD) on an ELISA plate reader at 450 nm.
  xi) Calculating % inhibition:

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100 - \frac{\text{Sample OD (Average)}}{\text{Mean negative control OD}} \times 100 = \% \text{Inhibition}
\]

xii) **Interpreting the results:** If a test sample yields equal to or greater than 25% inhibition, it is considered positive. If a test sample yields less than 25% inhibition, it is considered negative.

xiii) **Test validation:** The mean OD of the negative control must fall between 0.40 and 2.10. The mean of the positive control must yield greater than 25% inhibition.

**B2. Ovine herpesvirus-2**

This form of the disease occurs world-wide in cattle and other species, normally appearing sporadically and affecting only one or a few animals. However, on occasion, incidents occur in which several animals become affected, and this appears to be associated with certain sheep flocks that may continue to transmit disease for a number of years. The disease can also spread and cause substantial losses in North American Bison (*Bison bison*), red deer (*Cervus elaphus*), other deer species and water buffalo (*Bubalus bubalis*) and even more readily to Père David’s deer (*Elaphurus davidianus*) and Bali cattle (*Bos javanicus*). OvHV-2 is also responsible for causing MCF in zoological collections, where disease has been reported in a variety of species including giraffe. Disease in pigs has been reported from several countries, but is most frequently recognised in Norway where incidents involving several animals regularly occur.

Diagnosis based on clinical signs and gross pathological examination cannot be relied on as these can be extremely variable. Histological examination of a variety of tissues including, by preference, kidney, liver, urinary bladder, buccal epithelium, cornea/conjunctiva and brain, has been the only method of reaching a more certain diagnosis. However, detection of antibody to the virus and/or viral DNA can now also be attempted and are rapidly becoming the methods of choice.

It must be emphasised that the viral cause of SA-MCF cannot be reliably isolated and evidence for OvHV-2 relies on: (a) the presence of antibody in sera of all domestic sheep that cross-reacts with AlHV-1 antigens in the IFA.
test and immunoblots (5), but not in neutralisation assays; (b) the development of antibody that cross-reacts with AIHV-1 in the IFA test and CI ELISA in most cattle with SA-MCF and in all experimentally infected hamsters; (c) the detection and cloning of DNA from lymphoblastoid cell lines derived from natural cases of SA-MCF that cross-hybridises with, but is distinct from, AIHV-1 DNA; (d) the detection by PCR of amplicons unique to OvHV-2 in peripheral blood and affected tissues.

1. Identification of the agent

- Clinically affected animals

Attempts to recover the disease-causing virus from clinical cases have failed consistently. There are, however, several reports of the recovery of different viral agents from clinical cases, none of which has established any causal relationship; their isolation is certainly fortuitous or due to laboratory contamination. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which transmit MCF following inoculation into experimental animals (18). Such cell lines contain viral sequences that hybridise with clones of AIHV-1 DNA (3). A virus sequence was cloned from such a cell line that coded for a tegument protein that was distinct from AlHV-1. Subsequently the whole length of the viral genome has been cloned and the nucleotide sequence determined (5). Primers were identified within this sequence that were suitable for use in the PCR, and a sensitive protocol was designed in which a fragment of 422 base pairs (bp) is amplified initially, followed by amplification of a truncated internal fragment of 238 bp. It has been proven that this test is able to detect as few as 35 viral genome equivalents and that no product is amplified from AIHV-1 or other bovid herpesviruses (1). This PCR is thus both highly specific and sensitive for OvHV-2 and has been employed worldwide in studies of the disease in clinically affected animals and the natural host. It is emerging as a robust test that can be employed to detect viral DNA in peripheral blood leukocytes of clinically affected animals as well as fresh tissues and paraffin-embedded samples collected at post-mortem. The use of magnetic particles to purify DNA prior to amplification has been reported to be an additional improvement to the test, but is yet to be evaluated. A quantitative fluorogenic PCR assay for OvHV-2 has also been established and validated using the semi-nested PCR (1) as a gold standard (7) and is likely to have valuable future application.

While early studies indicated that infection of MCF-susceptible species would normally result in death, some prospective studies in high incidence herds of animals suggest that animals may become infected without developing a clinical response. Factors that predispose animals to infection and development of disease are not understood and it is likely to be a complex interaction of environmental, host factors and the infecting virus. That MHC class 11a polymorphism may contribute to resistance of American bison as suggested in one study (19) is of interest and should be further examined.

- Polymerase chain reaction

Extraction of DNA from clinical material is performed according to the protocol defined in an appropriate extraction kit (e.g. Quiagen DNeasy Tissue Kit). Amplification reactions are performed in 50 µl volumes containing not more than 2 µg test DNA in 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.01% (v/v) gelatine, 10% (v/v) dimethyl sulfoxide (DMSO), 200 µm dATP, dCTP, dGTP and dTTP (Pharmacia), 1 µM of each primer and 2 units Taq DNA-polymerase overlaid with 50 µl mineral oil (Sigma) to prevent evaporation.

The programme consists of a precycle at 99°C for 3 minutes, after which dNTP and enzyme mix are added. This is followed by 25 cycles of 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A 2 µl aliquot of the primary amplification product, specified by the primer pair 556/755, is transferred directly to a new reaction mixture and amplified using the primer pairs 556/555 under identical conditions for a further 25 cycles with a final extension at 72°C for 5 minutes.

Final amplification products (10 µl) are analysed directly by 1.8% agarose gel electrophoresis and ethidium bromide fluorescence. With each batch of test samples a known positive control and distilled water are also amplified and analysed.

- Natural hosts

The domestic sheep is the natural host of OvHV-2 and probably all sheep populations are infected with the virus in the absence of any clinical response. Studies of the dynamics of infection within sheep flocks have however, generated conflicting results with some suggesting productive infection occurs in the first weeks of a lamb's life while others suggest infection of most lambs does not occur until 3 months of age with excretion of infectious virus occurring between 5 and 6 months (13). There is also evidence that some lambs may become infected in utero while other studies suggest that removal of lambs from their dams during the first week permits the establishment of virus-free animals. There may therefore be considerable variation in the dynamics of infection in different flocks. However, circumstantial evidence of the occurrence of MCF in susceptible species does suggest that the perinatal
sheep flock is the principal source of infection, but that periodic recrudescence of infection may occur in sheep of all ages.

Factors that predispose to virus shedding and transmission to MCF-susceptible hosts remain speculative.

In addition to domestic sheep, domestic goats and other members of the subfamily Caprinae have antibody that reacts with AIHV-1 in a similar pattern to sheep serum. This implies that these species are infected with viruses similar to OvHV-2, and some goats have been found to be positive to an OvHV-2 PCR, though their potential role in causing MCF would appear to be very limited.

2. Serological tests

Antibody to OvHV-2 has only been detected using AIHV-1 as the source of antigen. Antibody to AIHV-1 can be detected in 70–80% of clinically affected cattle by IFA or IPT procedures, but may not be present in affected deer or animals that develop acute or peracute disease. Antibody is detected by IFA using tissue culture cells infected with AIHV-1. Cell monolayers grown on cover-slips exhibiting 10–50% CPE are harvested, washed, fixed in acetone and used in the assay. Cover-slips are mounted with DPX, the side containing the cells facing uppermost, on microscope slides and treated with 10% normal horse serum before progressing with a conventional IFA test. The IPT procedure can be carried out as for AIHV-1. The only virus of cattle that has been reported to cross-react with AIHV-1 is bovine herpesvirus-4 (BHV-4). Thus the negative control for this test should be similarly infected monolayers of BHV-4. Sera are only considered to be positive when foci show characteristic intranuclear distribution of antigen with little or no cytoplasmic staining being detected in the AIHV-1-infected cells and no reaction in the BHV-4-infected cells. Sera that react to antigens of both viruses are considered to be inconclusive.

A CI-ELISA has been developed for detecting antibody to OvHV-2 (12) using a MAb (15-A) raised against the so-called Minnesota isolate of virus, which is indistinguishable from AIHV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and appears to have some merit (Section B1.2.d) (10). In a study on the reaction of sheep serum to the structural proteins of AIHV-1 in immunoblots, the reactivity of different sera varied strikingly, indicating that individual sheep responded differently with regard to antibody recognition of cross-reacting epitopes of AIHV-1.

B3. Control

Control at present relies on segregating natural hosts from susceptible species, the extent to which this is enforced depending on the species involved. With AIHV-1, it would appear that MCF-affected animals never or rarely transmit infection, hence it is only the natural hosts that can act as a source of infection. Wildebeest would appear to be relatively efficient transmitters of infection to most other categories of ruminant, and hence their segregation in mixed collections is important. Likewise, pastoralists must ensure that cattle are entirely segregated from the vicinity of wildebeest and pastures recently grazed by them, particularly around the time of wildebeest calving.

With OvHV-2, the requirement to segregate sheep depends on the susceptibility of the species involved. Thus with Père David’s deer and Bali cattle, strict separation and avoidance of contact through fomites must be ensured. Equally, with bison and farmed deer every reasonable effort must be taken to segregate the management of sheep, although fallow deer (Dama dama) appear to be more resistant to MCF. Cattle only rarely develop SA-MCF, and thus are generally managed with sheep without taking precautions to guard against disease transmission. However, if multiple cases do occur, it is essential to segregate the sheep flock as far as possible from cattle. As such flocks may continue to be sources of infection for some years, disposal of these flocks for slaughter should be considered.

Virus also appears to have been transmitted over substantial distances thus it is not possible to define the distance that sheep should be segregated.

The possibility that very long incubation periods may occur, up to 9 months, further necessitates a guarded prognosis when advising on the control of such outbreaks.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Numerous attempts to produce a protective vaccine against the AIHV-1 form of the disease have met with disappointing results. However, recent trials which have focussed on stimulating high titres of neutralising antibody in nasal secretions of cattle have produced encouraging results and should be the target for further research.

As OvHV-2 cannot be successfully propagated in the laboratory no attempts at developing a vaccine have been attempted.
REFERENCES


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