CHAPTER 3.4.13.

MALIGNANT CATARRHAL FEVER

SUMMARY

Description and importance of the disease: *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 is also recognised in domestic pigs as well as giraffe and species of antelope belonging to the subfamily* Tragelaphinae*. MCF is characterised by subepithelial lymphoid cell accumulations and infiltrations, vasculitis and generalised lymphoid proliferation and necrosis. At least ten MCF viruses have been reported, including two well characterised viruses:* Alcelaphine gammaherpesvirus-1 (*AlHV-1*) *and* Ovine gammaherpesvirus-2 (*OvHV-2*)*. AlHV-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. OvHV-2, which is prevalent in domestic sheep as a subclinical infection, is the cause of MCF in most regions of the world. 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 AlHV-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. 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: *AlHV-1 may be recovered from clinically affected animals using peripheral blood leukocytes or lymphoid cell suspensions, 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 to AlHV-1 and develop cytopathic effect (CPE). 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 isolated in culture, 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 AlHV-1 and OvHV-2 using PCR, and this is becoming the method of choice for diagnosing both forms of the disease.

Serological tests: *Infected wildebeest, the natural host, consistently develop antibody to AlHV-1, which can be detected in a variety of assays including virus neutralisation, immunoblotting, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. Antibody to OvHV-2 can be detected by using AlHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence, ELISA or immunoblotting. For both viruses the antibody response in MCF-affected animals is limited, with no neutralising antibody developing.*

Requirements for vaccines: *No vaccine is currently available for this disease.*

A. INTRODUCTION

1. Description and impact of the disease

Malignant catarrhal fever (MCF) is a generally fatal disease of cattle and many other species of *Artiodactyla* that occurs following infection with certain herpesviruses of the genus *Macavirus*. At least six herpesviruses can cause MCF, the best characterised being *Alcelaphine gammaherpesvirus-1* (AlHV-1) and *Ovine gammaherpesvirus-2* (OvHV-2). MCF is characterised by systemic lymphoproliferation and is usually fatal, with infected cells being detectable in blood and most tissues at necropsy by PCR. Natural hosts of these viruses, including wildebeest (*Connochaetes* spp. of the subfamily *Alcelaphinae*) for AlHV-1 and domestic sheep for OvHV-2, experience no clinical disease following infection.

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. Nervous signs may be present. 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.

In addition, a number of cases of MCF with dermatological presentation have been described in sika deer infected with caprine herpesvirus 2 (CpHV-2; Foyle *et al*., 2009 and references cited therein). These cases exhibited cutaneous lesions combined with lymphocytic vasculitis characteristic of MCF, with CpHV-2 being detected by PCR and DNA sequencing.

Wildebeest-associated (WA-) MCF occurs in the cattle-raising regions of eastern and southern Africa 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.

Sheep-associated (SA-) MCF 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 multiple animals become affected. The disease can also infect 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 in 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.

Reports from several countries, and in particular from Norway, that MCF affects domestic pigs have been confirmed by the detection of virus DNA in affected animals (Loken *et al*., 1998). Experimental infection of pigs with OvHV-2 has also been documented (Li *et al.*, 2012). Signs are very similar to those seen in acutely affected cattle.

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. Some studies also suggest that substantial numbers of animals may become infected without developing clinical disease (for an example see Lankester *et al.,* 2016).

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 lymph nodes are enlarged, although the extent of lymph node involvement varies within an animal. 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, especially in bison.

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. 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. The brain may also show a nonsuppurative meningoencephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid. Lymph-node hyperplasia is characterised by an expansion of lymphoblastoid cells in the paracortex, while degenerative lesions are generally associated with the follicles. 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 with development of multiple raised white foci, each 1–5 mm in diameter.

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 tend to be virus-specific and are indicated below for each virus.

2. Nature and classification of the pathogen

MCF is caused by viruses of the genus *Macavirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae*, which share features of sequence and antigenicity and infect three subfamilies of *Bovidae* (*Alcelaphinae*, *Hippotraginae* and *Caprinae*). Disease caused by AlHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and has been referred to as WA-MCF. The OvHV-2 form of the disease occurs world-wide wherever sheep husbandry is practised and has been described as 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. On rare occasions macaviruses other than AlHV-1 and OvHV-2 have been identified as a cause of MCF.

3. Differential diagnosis

The clinical signs of the 'head and eye' form of MCF resemble those of other diseases that cause oral lesions (Holliman, 2005). Thus BVD/mucosal disease, rinderpest, foot and mouth disease, bluetongue and vesicular stomatitis may be considered as potential differential diagnoses where MCF is suspected. A clear diagnosis of MCF may be supported by additional evidence such as detection of MCF virus DNA, virus-specific antibody response and/or histopathology consistent with MCF.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of malignant catarrhal fever (AlHV-1 and OHV-2) and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
PCR	+	+	+	+++	++	n/a
Virus isolation	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	n/a
Detection of immune response						
C-ELISA	+++	+++	+++	++	+++	++
Virus neutralisation	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	n/a	n/a	+(AlHV-1)
IFAT	+	+	–	+	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Immuno-peroxidase test	+	+	–	+	–	–

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method but may need further validation;
+ = may be used in some situations, but cost, reliability, or other factors severely limits its application;
– = not appropriate for this purpose; n/a = purpose not applicable.
PCR = polymerase chain reaction; C-ELISA = competitive inhibition enzyme-linked immunosorbent assay;
IFAT = indirect fluorescent antibody test.
Note that virus isolation and virus neutralisation have only been documented for AlHV-1.

It must be emphasised that the viral cause of SA-MCF cannot be isolated reliably and evidence for OvHV-2 as a cause of MCF relies on: (a) the presence of antibody in sera of all domestic sheep that cross-reacts with AlHV-1 antigens (Hart *et al*., 2007, Li *et al.*, 2001); (b) the development of antibody that cross-reacts with AlHV-1 antigens in most cattle with SA-MCF and in experimentally infected animal models; (c) the detection of OvHV-2 sequences by PCR of DNA from peripheral blood or affected tissues of animals with SA-MCF; (d) the sequencing of OvHV-2 from sheep nasal secretions and their use to induce MCF with characteristic clinical signs and histopathology in rabbits, cattle and bison (Li *et al*., 2011; Taus *et al*., 2006; 2007).

Diagnosis of MCF 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, are necessary for reaching a more certain diagnosis. However, detection of antibody to the virus and/or viral DNA can now also be attempted and these are rapidly becoming the methods of choice.

Most laboratory-based tests to detect virus-specific antibody have relied on one attenuated isolate of AlHV-1 (WC11) that has been subjected to many laboratory passages as a source of viral antigen and DNA (Plowright *et al*., 1960). 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 (Ensser *et al*., 1997). Laboratory passage of the AlHV-1 C500 strain leads to attenuation of virulence and the ability to propagate in a cell-free manner, accompanied by genomic changes (Wright *et al*., 2003). This high passage derivative of AlHV-1 C500 has been used as a candidate vaccine for wildebeest-associated MCF and as a source of antigen for serological analysis (Haig *et al*., 2008; Russell *et al*., 2012). Individual MCF virus antigens, expressed either in bacteria or in mammalian cell culture, have recently been shown to be recognised by sera from MCF-affected animals and, in the case of OvHV-2 glycoproteins, induce virus-specific antibodies in hyperimmune rabbits (Bartley *et al.,* 2014; Cunha *et al.,* 2015; Dry *et al.,* 2016).

1. Identification of the agent

1.1. Clinically affected animals

1.1.1. Cell culture or Isolation

A striking feature of MCF is the lack of detectable viral antigen or herpesvirus-specific cytology within lesions. Confirmation of infection by virus recovery can only be performed for AlHV-1 to date, while attempts to recover the disease-causing virus from clinical cases of SA-MCF have failed consistently. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which transmit MCF following inoculation into experimental animals (Reid *et al*., 1989).

Generally, AlHV-1 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 × 10_6 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 AlHV-1. 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. Identification of the isolate should be determined by PCR analysis or MCF-specific antibodies in fluorescence or immunocytochemical tests.

1.1.2. Molecular methods – detection of viral nucleic acids

Characteristically, little viral DNA can be detected within affected tissues, hence it is necessary to amplify the viral genome either by conventional culture (in the case of AlHV-1) or by polymerase chain reaction (PCR).

The full sequence of the C500 isolate of AlHV-1 and of two isolates of OvHV-2 have been published, permitting the design of primers for PCR reactions from conserved regions of the genome (Ensser *et al*. 1997; Hart *et al.,* 2007; Taus *et al*., 2007). Nested and real-time PCR assays have been developed for AlHV-1 and OvHV-2 (Baxter *et al*., 1993; Flach *et al*., 2002; Hussy *et al*., 2001; Traul *et al*., 2005) while a pan-herpesvirus PCR (VanDevanter *et al*., 1996) has been used to identify CpHV-2 in Sika deer with MCF (Foyle *et al*., 2009) and a virus associated with MCF in white-tailed deer (Li *et al*., 2000). This assay targets the viral DNA polymerase gene sequence and has been employed for phylogenetic comparison of AlHV-1 and related viruses (Li *et al*., 2005).

1.1.2.1. PCR protocols

These protocols are based on published nested PCR assays designed to detect OvHV-2 (Baxter *et al*., 1993) or to distinguish AlHV-1 and OvHV-2 (Flach *et al*., 2002) in DNA samples from natural hosts or MCF-affected species. Silica-based genomic DNA extraction methods have been extensively used and appear reliable. Methods for extraction of DNA from fixed tissue samples should be validated before use in these assays. An example protocol is given below but optimal reaction conditions should be validated for each system of enzymes and buffers. Protocols for real-time PCR to detect MCF virus DNA (Hussy *et al*., 2001; Traul *et al*., 2005) are not given as these should be optimised for each reagent set and analysis system used.

1.1.2.2. Protocol 1: Hemi-nested PCR to detect OvHV-2 DNA (Baxter *et al.,* 1993)

i) Primers

Name	Length	Sequence
556	30 mer	5’-AGT CTG GGT ATA TGA ATC CAG ATG GCT CTC-3’
555	28 mer	5’-TTC TGG GGT AGT GGC GAG CGA AGG CTTC-3’
755	30 mer	5’-AAG ATA AGC ACC AGT TAT GCA TCT GAT AAA-3’

ii) Primary amplification (product size 422 bp)

Prior to PCR, a master mix is made up, comprising all components except template DNA. This is then dispensed into PCR tubes containing test or control DNA. This approach minimises pipetting errors when assaying large numbers of samples. The master mix comprises (per reaction): 10× PCR buffer, 5 µl; $MgCl_2$ (25 mM), 1 µl; dNTP mix (1 mM), 5 µl; primer 556 (10 µM), 1 µl; primer 755 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; and nuclease-free water, 31.875 µl; making a total of 45 µl per reaction. Samples of 5 µl, containing up to 1 µg of test or control DNA, are placed in PCR tubes and 45 µl of master mix are added to each tube. The tubes are then used for PCR according to the following protocol, using a thermal cycler with heated lid. To use a thermal cycler without a heated lid, mineral oil should be overlaid on each PCR reaction to prevent evaporation.

Suggested cycling conditions are: Hot-start activation or denaturation at 95°C for up to 15 minutes; followed by 15 cycles of 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds; with a final extension at 72°C for 10 minutes. The conditions should be adjusted according to the Taq polymerase and the thermal cycler used.

iii) Secondary amplification (product size 238 bp)

The master mix for the secondary amplification comprises (per reaction): 10× PCR buffer, 5 µl; $MgCl_2$ (25 mM), 1 µl; dNTP mix (1 mM), 5 µl; primer 556 (10 µM), 1 µl; primer 555

(10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; and nuclease-free water, 33.875 µl; making a total of 48 µl. Samples of 2 µl of each primary amplification product are placed in PCR tubes and 48 µl of master mix are added to each tube. Cycling conditions for the secondary PCR are the same as for the primary PCR, except that 30 cycles of amplification are used. After amplification approximately 10 µl of each secondary PCR reaction should be run on a 1.8 % agarose gel to visualise the PCR products.

1.1.2.3. Protocol 2: Hemi-nested PCR to distinguish AlHV-1 and OvHV-2 DNA (Flach *et al.,* 2002)

i) Primers

Name	Length	Sequence*
Primer POL1	24-mer	5'-GGC (CT)CA (CT)AA (CT)CT ATG CTA CTC CAC-3'
Primer POL2	21-mer	5'-ATT (AG)TC CAC AAA CTG TTT TGT-3'
Primer OHVPol	20-mer	5'-AAA AAC TCA GGG CCA TTC TG-3'
Primer AHVPol	20-mer	5'-CCA AAA TGA AGA CCA TCT TA-3'

*base positions in parentheses are degenerate – the oligonucleotide will contain either of the two indicated bases at these positions.

The primers POL1 and POL2 target a segment of the DNA polymerase gene which is conserved in both OvHV-2 and AlHV-1, amplifying a fragment of 386bp. OHVPol and AHVPol are specific primers for OvHV-2 and AlHV-1 respectively, which amplify 172bp products.

ii) Primary amplification

Master mix, per reaction: 10× buffer, 2.5 µl; $MgCl_2$ (25 mM), 0.5 µl; dNTP mix (1 mM), 2.5 µl; primer POL1 (10 µM), 1 µl; primer POL2 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; nuclease-free water, 12.375 µl (to 25 µl). Samples of 5 µl, containing up to 1 µg of test or control DNA, are placed in PCR tubes and 20 µl of master mix is added to each tube. The tubes are then used for PCR according to the following protocol: Hot-start activation or denaturation at 95°C for up to 15 minutes; followed by 25 cycles of 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds; with a final extension at 72°C for 10 minutes. The conditions should be adjusted according to the Taq polymerase and the thermal cycler used.

iii) Secondary amplification

Master mix, per reaction: 10× buffer, 2.5 µl; $MgCl_2$ (25 mM), 0.5 µl; dNTP mix (1 mM), 2.5 µl; primer AHVpol or OHVpol (10 µM), 1 µl; primer POL2 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; nuclease-free water, 12.375 µl (to 25 µl). Samples of 2 µl of each primary amplification product are placed in PCR tubes and 23 µl of master mix are added to each tube. Cycling conditions for the secondary PCR are the same as for the primary PCR, except that 30 cycles of amplification are used. After amplification approximately 10 µl of each secondary PCR reaction should be run on a 1.8% agarose gel.

1.2. 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 intensively during the perinatal period (Lankester *et al.,* 2015). The species *Connochaetes taurinus taurinus*, *C.t. albojubatus* 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.

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.

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 (Li *et al*., 2004). 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.

In latently infected adult sheep or wildebeest, the very low circulating viral load may reduce the reliability of PCR tests. However in these animals, a clear virus-specific antibody response should be detectable.

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. Some goats have been found to be OvHV-2 positive by PCR, while a few cases of MCF caused by CpHV-2 have been reported (Foyle *et al*., 2009). Other large antelope of the subfamilies *Alcelaphinae* and *Hippotraginae* are also infected with antigenically closely related gammaherpesviruses (Li *et al*., 2005). These similarities have been supported by recent analysis of cross-neutralisation of AlHV-1 and OvHV-2 viruses by sera from ovine, caprine, hippotragine or alcelaphine reservoir host species, which suggests that AlHV-1 may be neutralised by hippotragine or alcelaphine sera while OvHV-2 may be neutralised by ovine or caprine sera (Taus *et al.,* 2015). This implies a hierarchy of relatedness among the MCF viruses that may influence the reliability of serological tests.

2. Serological tests

2.1. Clinically affected animals

The antibody response of clinically affected animals is limited, with no neutralising antibody developing. Antibody to OvHV-2 has historically been detected using AIHV-1 as the source of antigen, although detection of recombinant OvHV-2 proteins by sera from MCF-affected cattle has been reported (Bartley *et al.,* 2014). Antibody to AIHV-1 can be detected in 70–80% of clinically affected cattle by indirect fluorescent antibody (IFA) or immunoperoxidase test (IPT) procedures, but may not be present in affected deer or animals that develop acute or peracute disease. A competitive inhibition enzyme-linked immunosorbent assay (C-ELISA) using a monoclonal antibody (MAb) (15-A) that targets an epitope conserved among MCF viruses has been employed to detect antibody in serum of wild and domestic ruminants in North America (Section B.2.2.2.) (Li *et al*., 2001). A comparative study of MCF diagnosis by histopathology, C-ELISA and OvHV-2-specific PCR showed that most cattle classified as MCF-positive by histopathology also had detectable MCF virus-specific antibodies and OvHV-2 DNA in the blood (Muller-Doblies *et al*., 1998). Direct ELISAs based on antigens from AlHV-1 strains WC11 or C500 high-passage (HP) have also been reported (Fraser *et al*. 2006; Russell *et al.,* 2012).

2.2. Natural hosts

Antibody appears to develop consistently in wildebeest following infection and can be identified by neutralisation assays using the cell-free isolates WC11 or C500 HP, or by immunofluorescence, using 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 C-ELISA antigen production.

There has been no attempt so far to standardise the IFA test and the IPT, but the two methods below are given as examples. The C-ELISA may be available as a commercial kit.

2.2.1. 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 virus neutralisation (VN) test using cell-free virus of the WC11 strain, and another uses a hartebeest isolate (AlHV-2). The attenuated (HP) strain of AlHV-1 C500 may also be used. These viruses have cross-reactive antigens and therefore either strain can be used in the test. The test 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 macavirus infection in wildlife, captive species in zoos and, to a lesser extent, sheep populations. It has also been useful in attempts to develop vaccines, including the recent AlHV-1 vaccine that induced neutralising antibodies in cattle blood plasma and nasal secretions (Haig *et al*., 2008). The VN test is of no value as a diagnostic test in clinically affected animals as no VN antibody develops in clinically susceptible species.

AlHV-1 stock virus is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, bovine turbinate, 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 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl under the conditions of the test.

2.2.1.1. Test procedure

i) Inactivate the test 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 AlHV-1 virus stock at a dilution in culture medium calculated to provide 100 $TCID_{50}$ 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 permissive cell suspension at $3 \times 10_5$ cells/ml.

vii) Incubate the plates in a humidified CO_2 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 $TCID_{50}$ with a permissible range 30–300) and the control sera. The standard positive serum should give a titre within 0.3 $\log_{10}$ 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).

2.2.2. Competitive inhibition enzyme-linked immunosorbent assay (C-ELISA)

The C-ELISA targets an epitope on the major surface glycoprotein that appears to be conserved among all MCF viruses. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and elsewhere. The test has detected antibody to the following MCF viruses: 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 (Li *et al*. 2005). The C-ELISA has the advantage of being faster and more efficient than the IFA or IPT. Comparison between C-ELISA, PCR and histopathological diagnosis of SA-MCF (Muller-Doblies *et al*., 1998) suggested that the three approaches had good concordance.

The complete reagent set for the C-ELISA, including pre-coated plates, labelled MAb and control sera, may be commercially available. For laboratories wishing to prepare their own antigen-coated plates, the following protocol is provided. ELISA plates 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 phosphate buffered saline (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) (Li *et al*., 2001). MAb 15-A is conjugated with horseradish peroxidase using a standard periodate method.

2.2.2.1. 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 in dilution buffer according to previous titration/optimisation for each conjugate preparation, or to the manufacturer's instructions.

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 tetramethylbenzidine substrate solution 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:

$$100 \quad - \quad \frac{\text{Sample OD (Average)} \times 100}{\text{Mean negative control OD}} \quad = \quad \text{\%inhibition}$$

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.

2.2.3 Immunofluorescence

The IFA test 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.

2.2.3.1. Preparation of fixed slides

Inoculate nearly or newly confluent cell cultures with AlHV-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–versene solution, spin down cells at approximate 800 ***g*** for 5 minutes, discard the supernatant fluid, and resuspend the cells in 10 ml of 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 fluorescence 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 TCID$_{50}$ 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.

2.2.3.2. 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 1/100,000 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.

2.2.4. Immunoperoxidase test

A dilution of bovine turbinate (BT) cell-cultured AlHV-1 containing approximately 10_3 TCID$_{50}$ 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 grade) that will not degrade the gasket. Store the fixed cells at –70°C.

2.2.4.1. 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, 20 mg/ml in dimethyl formamide) in distilled water (5 ml of distilled water, 2 drops 50 mM sodium acetate buffer pH 5.0, 2 drops hydrogen peroxide (30%), 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. REQUIREMENTS FOR VACCINES

At present no vaccine has been licensed for this disease.

Vaccination against MCF could be considered for use in those farmed species that have higher exposure or susceptibility to MCF, such as cattle in regions of East and South Africa where breeding wildebeest are prevalent, Bali cattle, bison in North America, farmed deer worldwide and susceptible species in zoological collections. Vaccination of reservoir hosts, such as wildebeest or sheep, is unlikely to be commercially viable and this is also the case for the majority of cattle herds that are at risk of sporadic SA-MCF. Numerous attempts to produce a protective vaccine against the AlHV-1 form of the disease have met with disappointing results. However, recent trials that focussed on stimulating high titres of neutralising antibody in nasal secretions of cattle have produced encouraging results (Haig *et al*., 2008). This live attenuated vaccine induced protection against intranasal experimental challenge with pathogenic AlHV-1. Protection was also found to persist for at least 6 months (Russell *et al*., 2012). This approach is likely to be the target for further research, including field trials.

As OvHV-2 cannot be successfully propagated in the laboratory no attempts have been made to develop a vaccine. However, recent work has developed a challenge system for OvHV-2 using virus from sheep nasal secretions (Taus *et al.,* 2006), which makes the testing of OvHV-2 vaccine candidates a possibility.

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NB: FIRST ADOPTED IN 1990; MOST RECENT UPDATES ADOPTED IN 2018.