Malignant Catarrhal Fever

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SUMMARY

Malignant catarrhal fever (MCF) is an acute, generalised, usually fatal disease affecting many species of Artiodactyla. The disease has been most often described as affecting species of the subfamily Bovinae (cattle and buffalo) and Cervidae (deer), but also affects domestic pigs, giraffe and antelope of the subfamily Tragelaphine.

MCF is defined by the recognition of characteristic lymphoid cell accumulations in non-lymphoid organs, vasculitis, and T-lymphocyte hyperplasia in lymphoid organs, and can be caused by either of two gamma-herpesviruses. The alcelaphine herpesvirus-1 (AIHV-1), the natural host of which, the wildebeest, is infected inapparently, causes the disease in cattle in regions of Africa and various ruminant species in zoos worldwide. Ovine herpesvirus-2 (OvHV-2), which is prevalent in all domestic sheep as a subclinical infection, is the cause of MCF in other ruminants in most regions of the world. This form of the disease has formerly been referred to as sheep-associated MCF. In both forms of the disease, animals with clinical disease are not a source of infection because virus is excreted only by the natural hosts, wildebeest and sheep.

MCF usually appears sporadically and affects few animals, though both viruses 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 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 signs, ranging from the acute form, when minimal changes are seen before death, to the more florid cases with high fever, bilateral corneal opacity, profuse catarrhal discharges from the eyes and nares, necrosis of the muzzle and erosion of the buccal epithelium. AIHV-1 can be recovered from animals with MCF that are infected with that virus only by using methods that don't kill host cells. OvHV-2 has never been recovered from affected animals. Diagnosis is usually made only by seeing 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 live peripheral leukocytes or cell suspensions prepared from lymph nodes and spleen; live virus cannot be recovered from dead cells. AIHV-1 may be recovered from a greater range of live cells from wildebeest.

Most monolayer cultures of cells from ruminants are probably susceptible and develop cytopathic effects (cpe), although bovine thyroid cell cultures have been used extensively for recovery of virus. Primary isolates typically produce multinucleated cells 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.

Viral DNA has been detected in lymphoid cells from animals with MCF caused by both AIHV-1 and OvHV-2 using the polymerase chain reaction, which is becoming the method of choice for diagnosing MCF caused by OvHV-2.

Serological tests

Infected wildebeest, the natural host, consistently develop antibodies to AIHV-1, which can be detected in various assays including virus neutralisation, immunoblotting, enzyme-linked immunosorbent assay, immunofluorescence, and immunocytochemistry. However, the antibody response of clinically affected animals is limited, with no neutralising antibodies developing, so that detection relies on the use of immunofluorescence or immunoblotting. Antibodies to OvHV-2 have

been detected only by using AIHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence or immunoblotting. Whereas antibodies can often be detected in cattle with MCF by immunofluorescence, in the more acutely affected animals such as deer, antibodies are frequently not detectable.

Currently no serological test is recognised for international trade.

Status of Australia and New Zealand

MCF caused by OvHV-2 has been reported in cattle, deer, buffalo and bison in Australia and New Zealand. The disease is widespread and sporadic, although occasional outbreaks have been reported.

MCF is a major cause of mortality in intensively farmed red deer. Other species of deer vary in their susceptibility to MCF. White-tailed deer, sika deer and Père David's deer are highly susceptible whereas fallow deer are resistant. In Indonesia, MCF is an important disease of Bali cattle and swamp buffalo.

Disease caused by AIHV-1 has not been reported although it potentially could occur in zoological collections that include wildebeest.

Introduction

Aetiology

Malignant catarrhal fever (MCF) is a generally fatal disease of cattle and many other species of Artiodactyla, which occurs after infection with either alcelaphine herpesvirus-1 (AIHV-1) or ovine herpesvirus-2 (OvHV-2). Wildebeest (*Connochaetes* spp of the subfamily Alcelaphinae), the natural host of AIHV-1, experience no clinical disease after infection. Likewise, the infection of domestic sheep, the natural host of OvHV-2, has not been associated with any signs of disease. Disease caused by AIHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and has formerly been referred to as wildebeest-derived MCF. The OvHV-2 form of the disease occurs worldwide 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.

Epidemiology

Alcelaphine herpesvirus-1

AIHV-1 causes disease in cattle in those areas of eastern and southern Africa where wildebeest and cattle are grazed together. The disease, however, can also affect other ruminant species in zoological collections worldwide, and, apart from antelope of the subfamilies Alcelaphinae and Hippotraginae, it is advisable to regard all ruminants as susceptible. Most studies have used the single attenuated isolate, WC11, which has been subjected to many laboratory passages, as a source of viral antigen and DNA.[10] The recent publication of the full nucleotide sequence of the virulent low passage virus – C500 – will form the basis of further studies of this virus.[5]

Ovine herpesvirus-2

OvHV-2 causes disease in cattle worldwide, usually appearing sporadically and affecting only one or several animals. The latter case appears to be associated with certain sheep flocks that may excrete OvHV-2 for some years. OvHV-2 can spread more readily to red deer (*Cervus elephus*) and other deer and cattle species, and to water buffalo (*Bubalus bubalis*). Père David's deer (*Elaphurus davidianus*), sika deer (*Cervus nippon*) and Bali cattle (*Bos javanicus)* are especially susceptible. OvHV-2 causes MCF in giraffe and other species in zoos. Disease in pigs has been reported from several countries, most frequently in Norway where incidents involving several animals regularly occur.

Natural Hosts

The domestic sheep is the natural host of OvHV-2 and all sheep tested have been shown to possess antibodies that reacted with AIHV-1. Furthermore, it seems that at least some lambs become infected in utero, making identification of uninfected animals very difficult. Neonatal lambs are apparently the principal source of infection, though sheep of all ages can transmit the virus. OvHV-2 has never been recovered from sheep. However, the polymerase chain reaction (PCR), which was developed to detect OvHV-2 DNA in clinical cases of MCF, has been used to detect virus in the peripheral blood leukocytes of normal adult sheep and in nasal secretions and oropharynx of lambs during the first months of life. Thus it would appear that, as with AIHV-1 infection in wildebeest, sheep become infected with OHV-2 when young and remain latently infected for life.

Factors that predispose to virus shedding and transmission to MCF-susceptible hosts are unknown.

In addition to domestic sheep, sera from domestic goats and other members of the subfamily Caprinae have antibodies that react with AIHV-1 in a similar pattern to sheep serum. This implies that these species are infected with viruses similar to OvHV-2, though their potential role in causing MCF is not known.

Clinical Signs

The clinical signs of MCF are highly variable and range through peracute to chronic with, in general, the most obvious signs developing in the more protracted cases. In the peracute form, either no clinical signs may be detected, or depression followed by diarrhoea and dysentery may be seen for 12-24 hours before death. In general, the first signs are fever, increased serous lachrymation and nasal exudate, which progresses to profuse mucopurulent discharges. Animals may be inappetent, and milk yields may decrease. Characteristically, progressive bilateral corneal opacity develops, starting at the limbus. 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 OHV-2-induced disease, ranging from *Bos taurus* and *B indicus*, which are relatively resistant, through most species of deer and water buffalo (*Bubalus*

bubalis), which are much more susceptible, to the extremely susceptible Bali cattle (*B javanicus*) and Père David's deer (*Elaphurus davidianus*). The more resistant species tend to have a longer clinical course and florid lesions, whereas in the more susceptible species, the disease course tends to be shorter and the clinical signs less pronounced.

The report of a mild form of the disease in 1930 was regarded with some scepticism because definitive diagnosis relied on the presence of characteristic histological lesions at necropsy. However, investigations using molecular and serological methods suggest that a few infected animals may recover after mild or even quite severe clinical reactions.

Gross Pathology

Gross pathological changes, which reflect the severity of clinical signs, are generally widespread and may involve most organ systems. Erosions and haemorrhages may be present throughout the gastro-intestinal tract, and in the more acute cases can be associated with blood-stained intestinal contents. Some or many lymph nodes are enlarged. The cut surface of lymph nodes may be firm and white but some, in particular the submandibular and retropharyngeal nodes, may be haemorrhagic and even necrotic. Catarrhal accumulations, erosions, and the formation of a diphtheritic membrane may be seen in the respiratory tract.

Frequently there are ecchymotic haemorrhages on the epithelial lining of the bladder and multiple, raised, white foci on the renal cortex. These foci, each 1-5 mm in diameter, are sometimes surrounded by a thin zone of haemorrhage.

Diagnostic Tests

Diagnosis based on clinical signs and gross pathological findings 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 antibodies to the virus and/or viral DNA can now also be attempted and the latter is rapidly becoming the method of choice.

Histopathology

Histological changes have been the basis for confirming a diagnosis of MCF. The changes seen are epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs, and widespread interstitial accumulations of lymphoid cells in non-lymphoid organs.

Epithelial lesions, which may be present at all epithelial surfaces, are erosion and ulceration, frequently with subepithelial and intra-epithelial 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 seen as lymphoid cell infiltration of the tunica adventitia and tunica media, often associated with fibrinoid degeneration. In the lumen, there may be 'pavementing' by lymphoid cells. In severe cases, endothelial damage and subendothelial accumulations of lymphoid cells can lead to occlusion.

Lymph node hyperplasia is seen as an increase of lymphoblastoid cells in the paracortex, whereas degenerative lesions are generally associated with the follicles. Oedema with lymphoid inflammation is often seen in the perinodal tissue.

Interstitial accumulation of lymphoid cells in non-lymphoid 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. A nonsuppurative meningo-encephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid may be seen in the brain.

The histological appearance of gross lesions seen in the cornea is lymphoid cell infiltration originating in the limbus and progressing centrally, with oedema and erosion developing in the more advanced cases. Vasculitis, hypopyon and irido-cyclitis may also be present.

The pathological features of MCF caused by either virus are essentially similar. However, apart from histological examination, the diagnostic features of AIHV-1- and OvHV-2-induced disease are very different and are thus considered separately.

AIHV-1

Since few laboratories in Australia and New Zealand hold the WC11 isolate, the laboratory techniques using this virus will not be described in detail in this text. These are well described in the OIE Manual of Standards and include virus isolation, PCR and the following serological tests: indirect fluorescent antibody (IFA) test, immunoperoxidase (IP) test and virus neutralisation (VN) test.[12] A restriction map of WC 11 has been published.[2] Sequence data and primers suitable for use have also been identified.[7]

A PCR for AIHV-1, which will also detect OvHV-2, albeit more weakly, has been developed in New Zealand.[13]

OvHV-2

It must be emphasised that the viral cause of SA-MCF has not been isolated and evidence for OvHV-2 relies on: (a) the presence in sera of all

domestic sheep of antibodies that cross-react with AIHV-1 antigens in the IFA test and immunoblots[6], but not in VN assays; (b) the development of antibodies that cross-react with AIHV-1 in the IFA test in a proportion of cattle with SA-MCF and in all experimentally infected hamsters; and (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.

All attempts to recover the causal virus OvHV-2 from clinical cases have failed. There are, however, several reports of the recovery of different viruses from clinical cases, none of which has established any causal relationship; their isolation is either fortuitous or due to laboratory contamination. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which cell lines transmit disease after inoculation into experimental animals.[11] Such cell lines contain viral sequences that hybridise with clones of AIHV-1 DNA.[3] Several viral fragments have been cloned from a genomic library of one such line and have been characterised. A subclone, which did not hybridise with AIHV-1, was chosen for sequencing and found to code for a protein very similar to the tegument protein of Epstein-Barr virus. Primers that were identified within this sequence were suitable for use in a PCR. A protocol was designed in which a fragment of 422 bp is amplified initially, followed by amplification of a truncated fragment of 310 bp. This test is able to detect as few as 35 viral genome equivalents and 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 used worldwide in studies of the disease in clinically affected animals and the natural host. This robust test may be used to detect viral DNA in peripheral blood leukocytes of clinically affected animals as well as in fresh tissues and paraffin-embedded samples collected post mortem.[14] The use of magnetic particles to purify DNA before amplification has been reported to be an additional improvement to the test.[4]

Serological Tests

Clinically affected animals

Antibodies to AIHV-1 can be detected in 70-80% of affected cattle by IFA or IP tests, but generally are not present in affected deer or animals that develop acute or peracute disease. Antibodies are detected by IFA using tissue culture cells infected with AIHV-1. Cell monolayers grown on cover-slips showing 10-50% CPE are harvested, washed, fixed in acetone and used in the test. Cover-slips are mounted with DPX, the side containing the cells facing uppermost, on microscope slides and treated with 10% normal horse serum before continuing with a conventional IFA test. The IP test can be done as for AIHV-1. Bovine herpesvirus-4 (BHV-4) is the only virus of cattle that cross-reacts with AIHV-1. Thus the negative control for this test should be similarly infected monolayers of BHV-4. Sera are considered to be positive when foci showing characteristic intranuclear distribution of antigen with little or no cytoplasmic staining are seen in AIHV-1-infected cells but not in BHV-4-infected cells. Sera that react to antigens of both viruses are considered to be inconclusive. A competitive enzyme-linked immunosorbent assay (Cl-ELISA) has been developed for detecting antibodies to OvHV-2[8] using a Mab (15-A) raised against the so-called Minnesota isolate of the virus, which is indistinguishable from AIHV-1. The test has been used to detect antibodies in the sera of wild and domestic ruminants in North America and appears to have some merit. However, there was poor correlation between the development of antibodies detected in sera of lambs and the acquisition of infection with OvHV-2 as indicated be the presence of viral DNA detected by PCR. Moreover, the sera of only some sheep were found to be positive. In contrast, the results of IFA tests show that most, if not all, domestic sheep are serologically positive. 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. Thus it is unlikely that some of the negative results obtained by CI-ELISA are due to the extreme epitope specificity of such a Mab-based test that fails to detect antibodies in a proportion of sera. Results from such a test should therefore be interpreted with caution.

An improved, more sensitive, indirect CI-ELISA has recently been described which detected 100% of PCR-positive MCF cases (37 cattle) compared with 62% when using the earlier CI-ELISA.[9]

Control

Control at present relies on segregating natural hosts from susceptible species; the extent to which this is enforced depends on the species involved. With AIHV-1, it seems 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 appear to transmit infection efficiently to most other ruminant species, hence their segregation in zoos is important. Likewise, pastoralists must ensure that cattle are segregated from wildebeest and pastures recently grazed by them, particularly when wildebeest calve.

With OvHV-2, the requirement to segregate sheep depends on the susceptibility of the species involved. Thus Père David's deer and Bali cattle should be segregated and avoid contact via fomites. Similarly, sheep and farmed deer should be segregated, although fallow deer (*Dama dama*) appear to be resistant to MCF. Cattle rarely develop

SA-MCF so sheep and cattle are not usually segregated. Segregation is needed when multiple cases occur in cattle. As the sheep flocks may excrete virus for some years, their disposal for slaughter should be considered.

As the period of incubation can be 9 months, prognosis should be guarded when advising on the control of such outbreaks.

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APPENDIX

Procedure for OvHV-2 PCR

1. Preparation of samples for DNA extraction

- On receipt of sample, identify each clinical sample and label all tubes and submission forms.
- Store samples at 4°C until ready for DNA extraction.

a) Unclotted blood samples

- Blood is collected in a tube containing an anticoagulant.
- Centrifuge the tube at 4°C at 3000 rpm for 20 min.
- Draw off the buffy coat with a sterile Pasteur pipette and place in a sterile 1.5 mL tube. Contamination with some red blood cells does not matter. [If using a magnetic beads DNA extraction kit, 2 – 3 µL of buffy coat may be transferred to a fresh, labelled 1.5 mL tube for immediate extraction – see below]
- Add 10 mL of ice-cold 0.17 M NH_4Cl and shake to suspend cells. Stand at room temperature for 10 min to lyse any red blood cell.
- Centrifuge at 4°C at 3000 rpm for 10 min.
- Discard supernatant and add 15 mL ice-cold PBS and mix.
- Centrifuge at 4°C at 2000 rpm for 10 min.
- Discard supernatant and resuspend cell pellet in 1 mL PBS.
- Alternatively, after drawing off the buffy coat, 2-3 µL may be transferred to a fresh, labelled 1.5 mL tube for extraction with the magnetic beads DNA kit.

For DNA Extraction: Pipette 200 µL into a fresh, labelled 1.5 mL tube and proceed, using a mincolumn DNA extraction kit.

When using a magnetic beads DNA extraction kit, use 2 – 3 µL of unprocessed buffy coat and proceed.

The remaining suspension can be stored for future extractions if required.

b) Clotted blood samples

- Take ~1 cm^3 clot and break up manually in a sterile petri dish with forceps and scissors.
- Place the minced clot in a 15 mL tube and add an equal volume of Tris EDTA (TE) buffer containing 2% v/v Tween-20.
- Shake vigorously to homogenise clot.
- Stand at room temperature for at least 30 min and shake vigorously again.
- Alternatively, resuspend the minced clot in an equal volume of sterile deionised water and mix by inversion to lyse the red cells. Restore isotonicity immediately by adding the same volume of 2x (double concentration) phosphate buffered saline and mix by inversion.

For DNA Extraction: Dispense 200 µL in a fresh, labelled 1.5 mL tube and proceed, using a minicolumn DNA extraction kit.

In the alternative procedure, allow fragments of clot to sediment and use 10 µL of the supernatant in a magnetic beads extraction procedure.

The remaining homogenate can be stored for future extractions if required.

c) Serum, plasma and other body fluids

Serum and other body fluids often contain very low numbers of cells, bacteria or viruses. Optional: Samples can be concentrated from up to 3.5 mL to a final volume of 200 µL using a vacuum concentrator. Centrifugation for up to 6 h may be necessary.

- Dispense 200 µL of serum in a fresh, labelled 1.5 mL tube.

- Alternatively, serum may be ultracentrifuged to concentrate the virus as follows: Transfer 5 – 6 mL serum to an ultraclear ultracentrifuge tube. Add sterile deionised water until the volume is 2 – 3 mm from the top of the tube. Cap with parafilm and mix by inversion. Prepare negative control serum (5 mL negative serum and 6 mL sterile deionised water) and positive control (5 mL negative serum + 100 µL sonicated, high titre positive sample + 5.9 mL sterile deionised water). Remove parafilm and balance pairs of tubes to within 0.002 g of each other. Centrifuge in a swing-out head at 30,000 rpm (average >110,000 x g) for 2 h. Pour off the supernatant to a 10 mL disposable tube (and store at 4°C), leaving ~200 µL with the pellet. Digest the pellet and remainder of supernatant in the ultraclear tube with Proteinase K as follows: Add 500 µL 0.01M Tris / 0.001 M EDTA pH 8.0 containing 11.6 µL of 6M NaCl, 14 µL of 10% SDS and 17 µL Proteinase K (15.6 mg/mL) to make a final volume of ~700 µL. Incubate at 56°C for 72 h.
  Note: for a positive control, use a positive tissue or buffy coat sample. Dilute this 1 in 5 in deionised water. Sonicate for 20 s (4 bursts of 5 s). Centrifuge at 4°C at 30,000 x g for 10 min before storing the supernatant in aliquots at -20°C.

- For DNA Extraction: Add three volumes of Digestion Buffer (100 mM NaCl, 10 mM Tris HCl pH 8, 25 mM EDTA pH 8, 0.5% SDS) and proceed with extraction using Phenol:Chloroform:Isoamyl alcohol (PCIA) method. In the alternative procedure after ultracentrifugation and Proteinase K digestion, transfer the digest to a 1.5 mL tube and proceed with extraction using the PCIA method.

- Alternatively, using a minicolumn DNA extraction kit, add 200µL of lysis buffer (provided with kit) and proceed.

The remaining serum can be stored for future extractions if required.

d) Animal tissue samples

Take a 200 mg to 1 g (up to about 1 cm^3) section of tissue using a sterile scalpel (or as much as is available). The remaining tissue is stored at -70°C for future extractions.

- Mince the tissue as fine as possible using either sterile scissors or a scalpel in a petri dish, a mortar and pestle, a glass grinder or a microfuge grinder (maximum 25 to 30 mg of tissue).Sterile glass beads will aid in homogenisation

For DNA Extraction: Place homogenate in a sterile tube and add 1.2 mL of Digestion Buffer per 100 mg of tissue and proceed with an extraction using the PCIA Method. Alternatively, place 25 to 50 mg of the tissue homogenate in a sterile microfuge tube and proceed with an extraction using a PCR Template Purification Kit.

Alternatively, thoroughly mix the homogenised tissue with an equal volume of sterile distilled water, transfer 2 – 3 µL to a sterile 1.5 mL tube and extract with a magnetic beads DNA extraction kit.

e) Embedded tissue samples

- Tissue samples are fixed in 10% formalin then embedded in paraffin. From this, 10 x 5 µm slices are taken and placed into a sterile polypropylene microfuge tube (xylene degrades polystyrene tubes).

- Add 1 mL xylene to 1.5 mL microfuge tube, shake lightly and stand at room temperature for 30 min.

- Centrifuge at 13,000 rpm for 5 min in a microcentrifuge.

- Decant xylene into the xylene waste discard in a fume cupboard.

- Add 1 mL xylene, shake lightly and stand at room temperature for 30 min.

- Centrifuge at 13,000 rpm for 5 min in a microcentrifuge.

- Decant xylene into xylene waste discard.

- Add 1 mL absolute ethanol and shake lightly to mix.
- Centrifuge at 13,000 rpm for 5 min and discard the supernatant.
- Add 500 µL of 95% ethanol and shake lightly to mix.
- Centrifuge at 13,000 rpm for 5 min.
- Decant ethanol and dry in air or using a vacuum concentrator, thus leaving dry tissue pellet.

For DNA Extraction: Suspend the pellet in 200 µL Tissue Lysis Buffer and proceed with an extraction using a PCR Template Purification Kit

f) Swabs

- Add 1 mL of TE containing 2% v/v Tween-20 to the bottle containing the swab.
- Shake vigorously.
- Stand at room temperature for at least 30 min and shake again.
- Squeeze out all liquid from swab using sterile forceps and discard the swab.

For DNA Extraction: In a microfuge tube take 200 µL, and add equal volume of Tissue Lysis Buffer. Proceed with an extraction using a PCR Template Purification Kit.

The remaining suspension is stored for future extractions.

g) Tissue culture

- Scrape the tissue culture flask with a cell scraper and pour the detached cells (bovine lung or lymphoblastoid cells) into a sterile 50 mL tube.
- Rinse the flask with ~10 mL sterile PBS and pour into the same tube.
- Centrifuge at 4^{o}C at 5000 rpm for 5 min. Decant and discard the supernatant.

For DNA Extraction: Resuspend the cell pellet in 200 µL Tissue Lysis Buffer and proceed with an extraction using a PCR Template Purification Kit

2. DNA Extraction

DNA is extracted from clinical samples using either a minicolumn DNA extraction kit or a PCR Template Purification Kit or a magnetic beads DNA extraction kit. (see above.)

Bovine Lung (BL) cells are extracted alongside clinical samples as a negative control.

Ensure all tubes are clearly identified with a sample number and date of extraction.

Note the DNA yield of each sample extracted using the spectrophotometer.

3. Polymerase Chain Reaction

- MCF Primers B9.1/Bax 755 and Bax555/Bax556 are reconstituted to 1 µg/µL with TE buffer (from which sub-samples of 200 ng/µL are made).
- Write up the PCR assay and identify each reaction tube with a PCR number and tube number.
- Include the negative control BL extract and water blank (sterile distilled water in place of template) in the assay.

- Include strong and weak positive control MCF + DNA extracts alongside clinical samples.

a) First Round/Primary Amplification Reaction

Reagent	**Volume** per reaction	**Final concentration** per reaction
Sterile dist. water	31.6 µL	
10x High Buffer*	5.0	1 x
25mM $MgCl_2$	1.0	2 mM
2mM dNTPs	5.0	200 µM
200 ng/ul Primer, forward	1.0	1 µM
200 ng/ul Primer, reverse	1.0	1 µM
5 U/ µLTaq Polymerase	0.4	2 units

* Supplied with Taq Polymerase

Calculate the master-mix for n + 1 tubes where n = number of clinical and control samples.

- Dispense 45 µL of the master-mix into each 0.5 mL thin-walled PCR tube.

Primary PCR reaction using B9.1 / Bax755

Add 5 µL of template DNA to the appropriate tube (5 µL of sterile distilled water for the negative blank). Total volume = 50 µL.

Cycling details are as shown below:

Primer:*	**B9.1**
Sequence:	5'-AAG CTT CAG CTT ACT CCC TTT ACT CT
Nucleotide Location:	1-26
Primer:*	**Bax755**
Sequence:	5'-AAG ATA AGC ACC AGT TAT GCA TCT GAT AAA
Nucleotide Location:	460-431

* Custom oligonucleotides supplied commercially

B9.1 / Bax755 will yield a 460 base pair (bp) PCR product in a positive sample.

Alternatively, the first round of amplification may be done with primers **Bax556 / Bax755**, as originally described by Baxter et al.[1] This primer pair will yield a 422 base pair (bp) product in a positive sample.

b) Second Round/Nested Amplification Reaction

Nested PCR reactions using Primers Bax556G / Bax555

A 2% portion of the primary PCR amplification product is transferred directly to a second reaction mixture and run under the same cycling conditions. The total volume of 50 µL is made up with an additional 4 µL of sterile distilled water.

Reagent:	**Volume**	**Final concentration**
	per reaction	per reaction
Sterile dist.water	35.6 µL	
10x High Buffer	5.0	1 x
25mM $MgCl_2$	1.0	2 mM
2mM dNTPs	5.0	200 µM
200 ng/ µL Primer, forward	1.0	1 µM
200 ng/ µL Primer, reverse	1.0	1 µM
5U/ µL Taq Polymerase	0.4	2 units

Calculate the master-mix for n + 1 tubes where n = number of clinical and control samples.

- Dispense 49 µL of the master-mix into each 0.5 mL thin-walled PCR tube.
- Add 1 µL of template DNA to the appropriate tube (1 µL of sterile distilled water or the negative blank). Total volume = 50 µL.

Cycling details for both primary and nested reactions are as shown below:

Primer:*	**Bax556G**
Sequence:	5'-AGT CTG GGG TAT ATG AAT CCA GAT GGC TCT C
Nucleotide Location:	38-66
Primer:*	**Bax555**
Sequence:	5'-TTC TGG GGT AGT GGC GAG CGA AGG CTT C
Nucleotide Location:	274-247

* Custom oligonucleotides supplied commercially

Bax555 / Bax556G will yield a **238** bp PCR product which will confirm the presence of OHV-2.

Thermocycle:

Program: MCF		
	Cycling Conditions:	99°C for 3 min
	Denaturation:	94°C for 20 sec
	Annealing:	60°C for 30 sec – 30 cycles
	Extension:	72°C for 30 seconds
	72°C for	5 min
	4°C	Hold.

4. Agarose gel electrophoresis

To evaluate a PCR reaction, products are separated by agarose gel electrophoresis and visualised by staining with ethidium bromide and illumination under ultraviolet light. Make up a 1.5-3% agarose gel in the appropriate electrophoresis tank to run all PCR products and molecular weight markers.

Run 10 µL of primary and nested PCR products alongside Molecular Weight Marker VIII and DIG-labelled Molecular Weight Marker VIII, or a "100 bp Ladder".

5. Southern Blot Transfer of DNA by vacuum blotting

DNA is transferred by vacuum suction from the gel to a positively charged nylon membrane using a vacuum blotter or a pressure blotter.

Ensure the well edge of the membrane is labelled with the PCR number as this orientates the membrane at the colourmetric stage. Complete transfer takes about 1 h.

6. Non-radioactive detection of DNA using the DIG-system

Where no bands are visible in the agarose for clinical samples, Southern blot hybridisation analysis is carried out using a digoxigenin (DIG)-labelled oligonucleotide probe, homologous to an internal region of the target PCR product.

	Probe:*:	**B9.2**
	Sequence:	5'-CCA GTA TCA TGC TGA CCC CTT GCA GA
	Nucleotide Location:	92 – 117

***** Custom oligonucleotides supplied commercially

Hybridisation Temperature: B9.2 = 50°C.

Hybridisation of the DIG-labelled probe is carried out overnight in the hybridisation oven at the appropriate temperature.

Colourmetric detection is carried out using Anti-Digoxigenin-AP and NBT/BCIP tablets.

Once the NBT/BCIP tablet is added, the membrane is stored in the dark for 1 h or until the signal is detected. The membrane is then photographed using the gel documentation system or on the photocopier.