Chapter 2.4.13.

Infectious Bovine Rhinotracheitis/
Infectious Pustular Vulvovaginitis

Summary

Definition of the disease: Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis, caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. The virus is distributed worldwide, but has been eradicated from Austria, Denmark, Finland, Sweden, Italy (Province of Bolzano), Switzerland and Norway and control programmes are running in some other countries.

Description of disease: The disease is characterised by clinical signs of the upper respiratory tract, such as a (muco)purulent nasal discharge, and by conjunctivitis. Signs of general illness are fever, depression, inappetance, abortions and reduced milk yield. The virus can also infect the genital tract and cause pustular vulvovaginitis and balanoposthitis. Post-mortem examinations reveal rhinitis, laryngitis and tracheitis. Mortality is low. Many infections run a subclinical course. Secondary bacterial infections can lead to more severe respiratory disease.

Identification of the agent: The virus can be isolated from nasal swabs or genital swabs, from animals with vulvovaginitis or balanoposthitis, taken during the acute phase of the infection, and from various organs collected at post-mortem.

For virus isolation, various cell cultures of bovine origin are used, for example, secondary lung or kidney cells or the Madin–Darby bovine kidney cell line. The virus produces a cytopathic effect in 2–4 days. It is identified by neutralisation or antigen detection methods using monospecific antisera or monoclonal antibodies. The BoHV-1 isolates can be further subtyped by DNA restriction enzyme analysis into subtypes 1.1, 1.2 and 1.3. BHV 1.2 isolates can be further differentiated into 2a and 2b. The virus previously referred to as BHV 1.3, a neuropathogenic agent, is now classified at BHV-5.

Viral DNA detection methods have been developed, and the polymerase chain reaction technique is increasingly used in routine diagnosis.

Serological tests: The virus neutralisation test and various enzyme-linked immunosorbent assays (ELISA) are most widely used for antibody detection. With an ELISA antibodies can be detected in serum and with lower sensitivity in milk.

Requirements for vaccines and diagnostic biologicals: Attenuated and killed vaccines are available. The vaccines must protect cattle clinically in case of infection and markedly reduce the subsequent shedding of field virus. The vaccines must not induce disease, abortion, or any local or systemic reaction, and must be genetically stable. BoHV-1 glycoprotein E deleted mutant marker vaccines are now generally available. The use of a gE ELISA makes it possible to distinguish infected cattle from cattle vaccinated with such a marker vaccine.

A. Introduction

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. BoHV-1 is a member of the genus Varicellovirus in the subfamily alphaherpesvirinae, which belongs to the Herpesviridae family. The viral genome consists of double-stranded DNA that codes for about 70 proteins, of which 33 structural and up to 15 nonstructural proteins have been demonstrated. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity. BoHV-1 can be differentiated into subtypes 1.1, 1.2a and 1.2b, and
1.3 (25). BoHV-1.3, which is a neuropathogenic agent, has been newly classified as BoHV-5 (21). The BoHV-1.2 subtypes may be less virulent than subtype 1.1 (11).

After an incubation period of 2–4 days, a serous nasal discharge, salivation, fever, inappetance, and depression become evident. Within a few days the nasal and ocular discharges change to mucopurulent. Where natural mating is practised, genital infection can lead to pustular vulvovaginitis or balanoposthitis. Many infections run a subclinical course (41).

Uncomplicated cases of respiratory or genital disease caused by BoHV-1 last 5–10 days.

The virus enters the animal via the nose and replicates to high titres in mucous membranes of the upper respiratory tract and in the tonsils. It subsequently disseminates to conjunctivae and by neuronal axonal transport reaches the trigeminal ganglion. After genital infection, BoHV-1 replicates in mucous membranes of the vagina or prepuce, and becomes latent in sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host. Stress, such as transport and parturition, can induce reactivation of the latent infection. Consequently, the virus may be shed intermittently into the environment.

An infection normally elicits an antibody response and a cell-mediated immune response within 7–10 days. The immune response is presumed to persist for life, although it may fall below the detection limit of some tests. Maternal antibodies are transferred via colostrum to the young calf, which is consequently protected against BoHV-1-induced disease (24). Maternal antibodies have a biological half-life of about 3 weeks, but may be detected occasionally in animals up to 9 months old, and rarely in animals over this age.

The virus is distributed world-wide, parallelling the distribution of domestic cattle. Other ruminants may be infected with BoHV-1. After infection, nasal viral shedding is detected for 10–14 days, with peak titres of $10^8–10^{10}$ TCID$_{50}$ (50% tissue culture infective doses) per ml of nasal secretion. The semen of an infected bull may contain BoHV-1 and the virus can thus be transmitted by natural mating and artificial insemination (28).

The control of BoHV-1 is based on the normal hygienic measures taken on a farm. Ideally, a 2–3-week quarantine period is imposed for newly introduced cattle. Only cattle that are BoHV-1-seronegative are then admitted to the herd. Vaccines usually prevent the development of severe clinical signs and reduce the shedding of virus after infection, but do not prevent infection. Several eradication campaigns have been or are running in different countries including test-and-removal programmes and/or vaccination campaigns (see Section C).

BoHV-1 infection may be suspected as the cause of disease on the basis of clinical, pathological and epidemiological signs. To make a definite diagnosis, however, laboratory examinations are required. A complete diagnostic procedure in the laboratory is aimed at detecting the causative virus (or viral components) and the specific antibodies they induce.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Collection and processing of samples

Nasal swabs are collected from several (from five to ten) affected cattle in the early phase of the infection. These cattle still have serous rather than mucopurulent nasal discharge. In cases of vulvovaginitis or balanoposthitis, swabs are taken from the genitals. The swabs should be vigorously rubbed against the mucosal surfaces. The prepuce can also be washed with saline; the washing fluid is then collected. The specimens are suspended in transport medium (cell culture medium containing antibiotics and 2–10% fetal bovine serum to protect the virus from inactivation), cooled at 4°C, and rapidly submitted to the laboratory.

During necropsy, mucous membranes of the respiratory tract, and portions of the tonsil, lung and bronchial lymph nodes are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and a placental cotyledon are examined. Samples should be sent to the laboratory as quickly as possible, on ice.

After arrival at the laboratory, swabs are agitated in the transport medium to elute virus and left at room temperature for 30 minutes. Following removal of the swabs, the transport medium is clarified by centrifugation at 1500 g for 10 minutes. Tissues are homogenised to a 10–20% (w/v) suspension in cell culture medium before centrifugation at 1500 g for 10 minutes. The supernatants of these specimens are filtered through 0.45 µm filters and used for virus isolation.

The isolation of virus from semen needs some special adaptations, because the seminal fluid contains enzymes and other factors that are toxic to the cells and inhibit viral replication (see below).
b) **Virus isolation**

For virus isolation, various cell cultures can be used. Primary or secondary bovine kidney, lung or testis cells, cell strains derived from bovine fetal lung, turbinate or trachea, and established cell lines, such as the Madin–Darby bovine kidney cell line, are all suitable. Cell cultures can be grown in glass or plastic tubes, plates or dishes. When 24-well plastic plates are used, a 100–200 µl volume of the supernatants described above is inoculated into these cell cultures. After a 1-hour adsorption period, the cultures are rinsed and maintenance medium is added. The serum used as a medium supplement in the maintenance medium should be free of antibodies against BoHV-1. The cell cultures are observed daily for CPE, which usually appears within 3 days after inoculation. It is characterised by grape-like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei may be observed. Experience is needed to recognise this characteristic appearance. When, after 7 days, no CPE has appeared, a blind passage must be made. The cell culture is freeze–thawed and clarified by centrifugation, and the supernatant is used for inoculation of fresh monolayers (6, 9).

To identify the virus that produces the CPE as BoHV-1, the supernatant of the culture should be neutralised with a monospecific BoHV-1 antiserum or neutralising monoclonal antibody (MAb). For this purpose, serial tenfold dilutions of the test supernatant are made, and to each dilution monospecific BoHV-1 antiserum or negative control serum is added. Following incubation at 37°C for 1 hour, the mixtures are inoculated into cell cultures; 3–5 days later, the neutralisation index is calculated. The neutralisation index is the virus titre (in log_{10}) in the presence of negative control serum minus the virus titre in the presence of specific antiserum. If the neutralisation index is greater than 1.5, the isolate may be considered to be BoHV-1. To shorten the virus isolation procedure, two specimens may be inoculated into cell culture: one that has been preincubated with monospecific antiserum and another that has been preincubated with negative control serum. If the CPE is inhibited by the monospecific antiserum, the isolate can be considered to be BoHV-1.

An alternative method of virus identification is by direct demonstration of BoHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (16) with conjugated monospecific antiserum or MAb.

o **Virus isolation from semen (a prescribed test for international trade)**

One straw, 0.5 ml, of extended semen or 0.02 ml of raw semen, should be tested, with two passages in cell culture. For extended semen, an approximation should be made to ensure that the equivalent of 0.05 ml raw semen is examined. Raw semen is generally cytotoxic and should be prediluted before being added to cell cultures. A similar problem may sometimes arise with extended semen. A suitable test procedure is given below.

o **Test procedure**

i) Dilute 200 µl fresh semen in 2 ml fetal bovine serum (free from antibody against BoHV-1) with added antibiotics.

ii) Mix vigorously and leave for 30 minutes at room temperature.

iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation above) in a six-well tissue culture plate.

iv) Incubate the plates for 1 hour at 37°C.

v) Remove the mixture, wash the monolayer twice with 5 ml maintenance medium, and add 5 ml maintenance medium to each well.

vi) Include BoHV-1 negative and positive controls in the test. Extreme caution must be taken to avoid accidental contamination of test wells by the positive control, for example always handling the control last, and using separate plates.

vii) Observe plates under a microscope daily for the appearance of a CPE. If a CPE appears, confirmatory tests for BoHV-1 are made by specific neutralisation or immunolabelling methods (see above).

viii) If there is no CPE after 7 days, the cultures are frozen and thawed, clarified by centrifugation, and the supernatant is used to inoculate fresh monolayers.

ix) The sample is considered to be negative if there is no evidence of a CPE after 7 days' incubation of the passaged cultures.

c) **Viral antigen detection**

Nasal, ocular or genital swabs can be directly smeared on to glass cover-slips, or, following centrifugation, the cell deposit (see Section B.1.a) may be spotted on to cover-slips. These cover-slips are subjected to a standard direct or indirect fluorescent antibody test. In a direct immunofluorescence test, the monospecific
antiserum is conjugated to fluorescein isothiocyanate, whereas in the indirect procedure it is the anti-species immunoglobulin second antibody that is conjugated to fluorescein isothiocyanate. To obtain the best results, it is necessary to sample several animals in a herd that have fever and a slight, serous nasal discharge. Smears should be air-dried and fixed in acetone within 24 hours. Smears from nasal swabs from cattle with a purulent or haemorrhagic nasal discharge are often negative (37). The advantage of this antigen-detection technique is that it can lead to a same-day diagnosis. However, the sensitivity of this procedure is lower than that of virus isolation (9). Positive and negative controls must be included in each test.

Tissues collected at post-mortem can be examined for the presence of BoHV-1 antigen by the immunofluorescence test on frozen sections. Immunohistochemistry may also be used. The advantage is that the location of the antigen can be determined. MAbs are increasingly being used for detecting BoHV-1 antigen, leading to enhanced specificity of the test. However, such MAbs must be carefully selected, because they must be directed against conserved epitopes that are present on all isolates of BoHV-1.

Another possibility for direct rapid detection of viral antigen is the use of an enzyme-linked immunosorbent assay (ELISA). Antigen can be captured by MAbs or polyclonal antibodies coated on a solid phase, usually the well of a microplate. Amounts of antigen equivalent to 10⁴–10⁵ TCID₅₀ of BoHV-1 are required in order to have a high rate of positive results (7). This may not be unrealistically high, because titres of 10⁸–10⁹ TCID₅₀/ml of nasal fluid can be excreted by cattle 3–5 days after infection with BoHV-1. Sensitivity can be increased by amplification systems (see ref. 10 for an example).

The advantages of antigen-detection methods versus virus isolation are that no cell culture facilities are required and a laboratory diagnosis can be made in 1 day. The disadvantages are the lower sensitivity of direct antigen detection and the extra requirement to perform virus isolation, if the isolate is required for further study.

d) Nucleic acid detection

During the past decade, various methods of demonstrating BoHV-1 DNA in clinical samples have been described, including DNA–DNA hybridisation and the polymerase chain reaction (PCR). The PCR is also increasingly used in routine diagnostic submissions (26). Compared with virus isolation, the PCR has the primary advantages of being more sensitive and more rapid: it can be performed in 1–2 days. It can also detect DNA in latently infected sensory ganglia (38). The disadvantage is that it is prone to contamination and therefore precautions have to be taken to prevent false-positive results. Risk of contamination is markedly reduced by new PCR techniques, such as the so-called real-time or quantitative PCR (QPCR) (1, 20).

So far the PCR has been used mainly to detect BoHV-1 DNA in artificially (19) or naturally (38) infected semen samples. These workers found that it was important to thoroughly optimise the PCR conditions, including the preparation of the samples, the concentration of Mg²⁺, primers and Taq polymerase, and the cycle programmes. The target region for amplification must be present in all BoHV-1 strains, and its nucleotide sequence must be conserved. The TK, gB, gC, gD and gE genes have been used as targets for PCR amplification. PCRs based on detection of gE sequences can be used to differentiate between wild-type virus and gE-deleted vaccine strains (14, 35). Discrimination between infection with virulent IBR strains and infection with other live attenuated strains is not possible with the PCR technique. PCRs have been developed that discriminate between BoHV-1 and BoHV-5 (2, 33).

Experimentally, the PCR was found to be more sensitive than virus isolation: in egg yolk-extended semen samples obtained from experimentally infected bulls, PCR detected five times as many positives as did virus isolation (39). In addition, it had a detection limit of only three molecules. Nevertheless, false-negative results cannot be excluded. To identify possible false-negative results, it is recommended to spike an internal control template into the reaction tube of the semen sample to be amplified by the same primers. Such a control template may be constructed by inserting, for example, a 100 base-pair fragment into the target region. This control template also makes it possible to semi-quantify the amount of DNA that is detected (33, 38). When using an internal control, extensive testing is necessary to ensure that PCR amplification of the added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see also Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases).

c) Real-time polymerase chain reaction (a prescribed test for international trade)

The following real-time PCR test method has been developed to detect BoHV-1 in extended bovine semen intended for trade. The method has been validated according to Chapter 1.1.5, and includes a comprehensive international inter-laboratory comparison involving six collaborating laboratories with specialist status in IBR testing.
A number of studies has shown that PCR assays are more sensitive than virus isolation (36, 39, 42, 47). Real-time PCR has been used for detection of BoHV-1 and BoHV-5 in experimentally infected cattle and mice (1, 20) and a number of conventional PCR assays have been used for the detection of BoHV-1 DNA in artificially or naturally infected bovine semen samples (8, 15, 23, 38, 44, 45, 47, 49). Conventional detection of amplified PCR products relies on gel electrophoresis analysis (32). Sequence-specific primers have been selected to amplify different parts of conserved glycoprotein gene of BHV-1 genome, including glycoprotein B (gB) gene (15, 34), gC gene (36, 39), gD gene (36, 47), gE gene (15), and the thymidine kinase (tk) gene (26, 50).

Real-time PCR differs from standard PCR in that the amplified PCR products are detected directly during the amplification cycle using a hybridisation probe, which enhances assay specificity. Real-time PCR assays have several advantages over the conventional PCR methods. Real-time PCR assays using only one pair of primers are able to provide sensitivity close or equal to nested PCR methods with a much lower risk of contamination. The amplification and detection of target is conducted simultaneously. There is no post-amplification PCR product handling, which significantly reduces the risk of contamination, and it is possible to perform quantitative analysis with real-time PCR systems.

The real-time PCR described here uses a pair of sequence-specific primers for amplification of target DNA and a 5'-nuclease oligoprobe (TaqMan) for detection of amplified products. The oligoprobe is a single, sequence-specific oligonucleotide labelled with two different fluorophores, the reporter/donor, 5-carboxyfluorescein (FAM) at the 5’ end, and the acceptor/quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end. This real-time PCR assay is designed to detect viral DNA of all BHV-1 strains, including subtype 1 and 2, from extended bovine semen. The assay selectively amplifies a 97 basepair sequence of the glycoprotein B (gB) gene. Details of the primers and probes are given in the protocol outlined below.

**Sample preparation, equipment and reagents**

i) The samples used for the test are, typically, extended bovine semen stored in liquid nitrogen. The semen samples can be transported to the laboratory in liquid nitrogen, or shipped at 4°C, and stored in liquid nitrogen or at −70°C (for long-term storage) or 4°C (for short-term storage). Storing semen at 4°C for a short period (up to 7 days) does not appear to affect PCR test result.

ii) Three straws from each batch of semen to be tested should be processed. Duplicate PCR amplifications should be carried out for each DNA preparation (six amplifications in total) to ensure the detection of DNA in samples containing low levels of virus.

iii) A real-time PCR detection system, and the associated data analysis software, is required to perform the assay. A number of real-time PCR detection systems are available from various sources. In the procedure described below, a RotorGene 3000, Corbett Research Ltd, Australia, was used. Other real-time PCR detection systems can also be used. Other equipment required for the test includes a micro-centrifuge, a heating block, a boiling water bath, a micro-vortex, magnetic stirrer and micropipettes.

iv) The real-time PCR assay described here involves two separate procedures. Firstly, BoHV-1 DNA is extracted from semen using Chelex-100 chelating resin, along with proteinase K and DL-Dithiothreitol (DTT). The second procedure is the amplification and detection of the extracted DNA template by a real-time PCR detection system using a PCR reaction mixture: Platinum Quantitative PCR SuperMix-UDG, Invitrogen Technologies (note that there are a number of other commercial real-time PCR amplification kits available from various sources and the kit selected needs to be compatible with the real-time PCR platform selected). The required primers and probes can be synthesised by various commercial companies. In this protocol, all the primers and probes used were synthesised by Sigma-Genosys.

**Extraction of DNA**

i) In a screw top 1.5 ml tube, add:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chelex 100 sodium (Sigma)</td>
<td>100 µl</td>
</tr>
<tr>
<td>Proteinase K (10 mg/ml, Sigma)</td>
<td>11.5 µl</td>
</tr>
<tr>
<td>DL-Dithiothreitol (1 M, Sigma)</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>90 µl</td>
</tr>
<tr>
<td>Semen sample</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

1 Sources of contamination may include product carry-over from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments. Samples and reagents should be handled in separate areas, with separate equipment for reagent and sample preparation and amplification/detection.
Mix gently by pipetting².

ii) The sample tubes are incubated at 56°C for 30 minutes and then vortexed at high speed for 10 seconds.

iii) The tubes are then incubated in a boiling water bath for 8 minutes and then vortexed at high speed for 10 seconds.

iv) The tubes are centrifuged at 10,000 g for 3 minutes.

v) The supernatant³ is transferred into a new microtube and can be used directly for PCR, or stored at −20°C for testing at a later date.

○ Preparation of reagents

The real-time PCR reaction mixture (Platinum Quantitative PCR SuperMix-UDG, or other reaction mixture) is normally provided as a 2 × concentration ready for use. The manufacturer’s instructions should be followed for application and storage.

Working stock solutions for primers are made with nuclease-free water at the concentration of 4.5 µM and 3 µM, respectively. The stock solution of primers and probe are stored at −20°C and the probe solution should be kept in the dark. Single-use aliquots can be prepared to limit freeze-thawing of primers and probes and extend their shelf life.

○ Real-time PCR test procedure

i) Primers and probe sequences

Selection of the primers and probe are outlined in Abril et al. (2004) and described below.

Primer gB-F: 5’-TGT-GGA-CCT-AAA-CCT-CAC-GGT-3’ (position 57499–57519 GenBank®, accession AJ004801)

Primer gB-R: 5’-GTA-GTC-GAG-CAG-ACC-CGT-GTC-3’ (position 57595–57575 GenBank®, accession AJ004801)

TaqMan Probe: 5’-FAM-AGG-ACC-GCG-AGT-TCT-TGC-CGC-TAMRA-3’ (position 57525–57545 GenBank®, accession AJ004801)

ii) Preparation of reaction mixtures

The PCR reaction mixtures are prepared in a clean laboratory room. All the reagents except the test samples are mixed before distribution to each individual reaction tube. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC, reagent only), appropriate negative controls, i.e. 1 per 10 test samples, and two positive controls (strong and weak) should be included. Each test sample and control is tested in duplicate. The PCR amplifications are carried out in a volume of 25 µl.

a) PCR reagent mixtures are added in a clean room (no viral cultures, DNA extracts or post-amplification products should be handled here)

   2 × Platinum Quantitative PCR SuperMix-UDG  12.5 µl
   ROX reference dye (optional)  0.5 µl
   Forward primer (gB-F, 4.5 µM)  1 µl
   Reverse primer (gB-R, 4.5 µM)  1 µl
   Probe (3 µM)  1 µl
   Nuclease free water  4 µl

b) 5 µl of the DNA template are added to the PCR reagent mixture to a final volume of 25 µl. DNA samples are prepared and added in a separate room.

iii) Real-time (TaqMan) polymerase chain reaction

The PCR tubes are placed in the real-time PCR detection system in a separate, designated PCR room.

The PCR detection system is programmed for the test as follows:

PCR Reaction Parameters⁴

2 It is important that Chelex 100 sodium be distributed evenly in the solution while pipetting, as Chelex 100 sodium is not soluble. This can be done by putting the vessel containing Chelex-100 solution on a magnetic stirrer while pipetting.

3 Some DNA samples can become cloudy and a thin white membrane may form occasionally after freezing and thawing. This appears to have no influence on the PCR performance. No heating or re-centrifuging of the samples is necessary.
iv) Analysis of real-time PCR data

The threshold level is usually set according to the manufacturers instructions for the selected analysis software used. Alternatively, virus isolation negative semen samples, from sero-negative animals, can be run exhaustively (e.g. up to 60 amplification cycles) to determine the background reaction associated with the detection system used.

o Interpretation of results

o Test controls

Positive and negative controls, as well as reagent controls, should be included in each PCR test. Negative semen, from virus isolation negative sero-negative bulls, can be used as a negative control. Positive semen from naturally infected bulls is preferable as a positive control. However, this might be hard to obtain. Alternatively, positive controls can be derived from negative semen spiked with known quantities of BoHV-1 virus.

o Test results

Positive result: Any sample that has a cycle threshold (Ct) value equal or less than 45 is regarded as positive. The positive control should have a Ct value within an acceptable range (± 3 Ct values) as previously determined by repeatability testing.

Negative result: Any sample that shows no Ct value is regarded as negative. Negative control and no template control should have no Ct values.

f) Differentiation of bovine herpesvirus 1 subtypes and of viruses related to bovine herpesvirus 1

By using MAbs and immunofluorescence, radioimmuno precipitation, immunoperoxidase or immunoblot assays, BoHV-1 subtype 1 and subtype 2b can be differentiated (31, 48). Restriction HindIII endonuclease analysis makes it possible to differentiate among all the recognised BoHV-1 subtypes 1, 2a and 2b. This differentiation is based on the molecular weight of three relevant DNA fragments (I, K and L) (25). Restriction endonuclease analysis includes extraction of the DNA from virions or from infected cells, digestion of the isolated DNA by restriction endonucleases, and separation of the resulting fragments by agarose gel electrophoresis. Such techniques are of limited diagnostic value, but may be useful in epidemiological studies.

When differentiation between antigenically and genetically related alphaherpesviruses (BoHV-1, BoHV-5), caprine herpesvirus (CpHV-1), and cervine herpesvirus 1 (CvHV-1 and CvHV-2) is needed, improved methods are available using monoclonal antibodies (17).

g) Interpretation of results

The isolation of BoHV-1 from an animal does not unequivocally mean that this virus is the cause of the disease outbreak. It may, for instance, be a latent virus that has been reactivated due to stressful conditions. A confirmatory laboratory diagnosis must be made from a group of animals and must be accompanied by seroconversion from negative to positive, or a four-fold or higher titre rise in antibodies to BoHV-1. Cattle from which the nasal swabs are to be collected must be bled twice, 2–3 weeks apart. These paired serum samples are examined together in a serological test for the presence of specific antibody (see Section B.2).

2. Serological tests

Serological tests can be used for several purposes:

i) To diagnose an acute infection: serum samples from the acute and convalescent stages of infection in the same animals are examined in one test. A seroconversion from negative to positive or a four-fold or higher increase in antibody titre is considered to indicate infection.
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To demonstrate the absence of infection, for instance, for international trade purposes.

To determine the prevalence of infection in seroepidemiological studies.

To support eradication programmes and subsequent surveillance.

For research purposes, for instance, the evaluation of the antibody response after vaccination and challenge infection.

Virus neutralisation (VN) tests (4) and various ELISAs (19) are usually used for detecting antibodies against BoHV-1 in serum. Because virus latency is a normal sequel to BoHV-1 infection, the identification of serologically positive animals provides a useful and reliable indicator of infection status. Any animal with antibody to the virus is considered to be a carrier and potential intermittent excretor of the virus. The only exceptions to this are young calves that have acquired passive colostral antibody from their dam, and noninfected cattle vaccinated with inactivated vaccines.

In general, BoHV-1 serological tests can be divided into conventional and marker tests. The only marker serological test available at this time is the BoHV-1 gE antibody blocking ELISAs (40). For conventional serology, VN, BoHV1 antibody blocking ELISAs as well as indirect ELISAs are used.

ELISAs, including the gE-ELISA, are increasingly used for the detection of antibodies in (bulk) milk samples (45), but have some limitations. By testing bulk milk, a positive gB-specific test indicates that the infection has already spread in the herd (13). With the gE blocking ELISA, bulk milk gives a positive reaction when more than 10–15% of the herd is infected (46). Consequently, it is not possible to declare a herd to be free from BoHV-1 infection on the basis of bulk or pooled milk tests, and a negative bulk milk test should be followed up with individual serum samples from all cattle in the herd. For general surveillance purposes, bulk milk tank tests can give an estimate of BoHV-1 prevalence in a herd, an area or country (27). These should be supplemented by serum testing (individual or pooled) from non-milking herds.

In a recent extensive study, tests for the detection of antibodies as routinely used by national reference laboratories in Europe were evaluated (18). Twelve reference laboratories from 12 European countries participated in this study. Fifty three serum samples and 13 milk samples, originating from several countries, were sent in duplicate under code to the participating laboratories. The serum samples included the three European reference sera EU1 (antibody positive), EU2 (antibody weak positive and defined as borderline sample) and EU3 (antibody negative) (30). It was concluded that VNT and gB-specific ELISAs are the most sensitive tests for the detection of antibodies in serum. In contrast, indirect ELISAs were found to be the most sensitive for the detection of antibodies in milk. Moreover, it was observed that commercially obtained ELISAs performed better than home-made ELISAs.

Recently, new indirect BoHV-1 ELISAs have been developed that are highly sensitive and specific. The results of these ELISAs are comparable with those obtained using gB blocking ELISAs or VNT (3).

a) Virus neutralisation (a prescribed test for international trade)

VN tests are performed with various modifications. Tests vary with regard to the virus strain used in the test protocol, the starting dilution of the serum, the virus/serum incubation period (1–24 hours), the type of cells used, the day of final reading and the reading of the end-point (50% versus 100%) (29). Of these variables, the virus/serum incubation period has the most profound effect on the antibody titre. A 24-hour incubation period may score up to 16-fold higher antibody titres than a 1-hour incubation period (4), and is recommended where maximum sensitivity is required (e.g. for international trade purposes). Various bovine cells or cell lines are suitable for use in the VN test, including secondary bovine kidney or testis cells, cell strains of bovine lung or tracheal cells, or the established Madin–Darby bovine kidney cell line.

A suitable protocol for a VN test is shown below.

i) Inactivate sera, including control standard sera, for 30 minutes in a water bath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium. Start with undiluted serum and continue to 1/1024 horizontally in a 96-well flat-bottomed cell-culture grade microtitre plate, at least two wells per dilution and 50 µl volumes per well. Dilutions of a positive control serum, and of weak positive and negative internal control sera, are also included in the test. An extra well with undiluted test serum is used for toxicity control of sera.

iii) Add 50 µl per well of BoHV-1 stock at a dilution in culture medium calculated to provide 100–200 TCID₉₀ per well. In the toxicity control wells, add 50 µl of culture medium in place of virus. Add 100 µl of culture medium to ten empty wells for cell controls.

iv) Make at least four tenfold dilutions of the residual virus stock (back titration) in culture medium, using 50 µl per well and at least four wells per dilution.
v) Incubate the plates for 24 hours at 37°C.

vi) Add 100 µl per well of the cell suspension at 3 × 10^4 cells per well.

vii) Incubate the plates for 3–5 days at 37°C.

viii) Read the plates microscopically for CPEs. Validate the test by checking the back titration of virus (which should give a value of 100 TCID_{50} with a permissible range of 30–300 TCID_{50}), the control sera and the cell control wells. The positive control serum should give a titre of ± 1 twofold dilution (±0.3 log_{10} units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation when tested undiluted (equivalent to a final dilution of 1/2 at the neutralisation stage). In the cell control wells, the monolayer should be intact.

ix) The test serum results are expressed as the reciprocal of the dilution of serum that neutralised the virus in 50% of the wells. If 50% of the wells with undiluted serum neutralised the virus, the (initial dilution) titre is read as 1 (1/2 using the final dilution convention). If all the undiluted and 50% of the wells with 1/2 diluted serum neutralised the virus, the (initial dilution) titre is 2 (final dilution 1/4). For qualitative results, any neutralisation at a titre of 1 or above (initial dilution convention) is considered to be positive. If cytotoxicity is observed in the serum toxicity control wells, the sample is reported to be toxic (no result) unless neutralisation of the virus without cytotoxicity is observed at higher dilutions and a titre can be read without ambiguity. Where there is cytotoxicity with a serum from which it is critical to obtain a result, changing the medium in the wells of the lowest two or three dilutions 16–24 hours after the addition of cells will remove the cytotoxic effect with many problem sera.

b) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

ELISAs for the detection of antibody against BoHV-1 appear to be gradually replacing VN tests. A standard procedure for ELISA has not been established. Several types of ELISA are commercially available, including indirect and blocking ELISAs, some of which are also suitable for detecting antibodies in milk (18). For reasons of standardisation in a country or state, it may be desirable to compare the quality of the kits and to perform batch release tests by previously defined criteria in one national reference laboratory, before it is used by other laboratories in the country.

There are a number of variations in the ELISA procedures. The most common are: antigen preparation and coating, the dilution of the test sample, the incubation period of antigen and test sample, and the substrate/chromogen solution. Before being used routinely, an ELISA should be validated with respect to sensitivity, specificity and reproducibility (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases). For this purpose, a panel of well defined (e.g. by VN test) strong positive, weak positive and negative sera should be tested.

i) Indirect enzyme-linked immunosorbent assay

The principle of an indirect ELISA is based on the binding of BoHV-1-specific antibodies present in the test sample to immobilised BoHV-1 antigen. The bound antibodies are detected using enzyme-labelled anti-bovine immunoglobulin antiserum. The presence of antibodies in the test sample will result in colour development after addition of the substrate/chromogen solution.

ii) Blocking enzyme-linked immunosorbent assay

The principle of a blocking or competitive ELISA is based on blocking the binding of antigen to an enzyme-labelled BoHV-1 antiserum or anti-BoHV-1 MAb by antibodies in the test sample. The presence of antibodies in the test sample will block binding, resulting in reduced colour development after addition of the substrate/chromogen solution. An example of a gB blocking ELISA procedure is given below:

i) Prepare the antigen by growing BoHV-1 in cell cultures. When extensive CPE is observed, cells and medium are frozen at −20°C. After thawing, the resulting cellular lysate is centrifuged for 4 hours at 8500 g. The virus-containing pellet is suspended in a small volume of phosphate buffered saline (PBS), cooled on ice and disrupted using an ultrasonic disintegrator. The antigen preparation is then centrifuged for 10 minutes at 800 g, and inactivated if needed by adding detergent (final concentration of 0.5% Nonidet P 40). The antigen preparation is used at an appropriate dilution to coat the microtitre plates. Many alternative methods of antigen production may be found in the published literature.

ii) Coat the microtitre plates with antigen by adding 100 µl of diluted antigen (in 0.05 M carbonate buffer, pH 9.6) to each well. Seal the plates with tape, incubate at 37°C overnight, and store at −20°C.

iii) Before the test is performed, wash the plates with 0.05% Tween 80. Add 100 µl negative serum (fetal calf serum, FCS), 100 µl of each of the serum test samples and 100 µl of positive, weak positive and negative control sera. Usually, serum samples are tested undiluted. Shake, seal the plates and
incubate overnight at 37°C. With some ELISAs, it is necessary to heat sera for 30 minutes at 56°C before testing in order to avoid weak nonspecific responses.

iv) Wash the plates thoroughly and add 100 µl of an anti-BoHV-1-gB-monoconal antibody/horseradish peroxidase conjugate at a predetermined dilution, and incubate again for 1 hour at 37°C. The monoclonal antibody must be selected carefully for its specificity to gB of BoHV-1.

v) Wash the plates and add freshly prepared substrate/chromogen solution (e.g. 0.05 M citric acid buffer, pH 4.5, containing 2,2’-azino-bis-[3-ethylbenzothiazoline]-6-sulphonic acid [ABTS; 0.55 mg/ml] and a 3% solution of freshly added H$_2$O$_2$ [5 µl/ml]), and incubate for the appropriate time (1–2 hours at room temperature).

vi) Measure the absorbance of the plates on a microplate photometer at 405 nm.

vii) Calculate for each test sample the blocking percentage [(OD$_{FCS}$ – OD$_{test\ sample}$)/OD$_{FCS}$ × 100%]

vii) A test sample is considered to be positive if it has a blocking percentage of e.g. 50% of higher. The test is valid if the positive and weak positive control sera are positive and the negative control serum is negative. The acceptable limits for control and cut-off values must be determined for the individual assay.

c) Standardisation

In each serological test, appropriate controls of strong positive, weak positive and negative serum should be included. A scientific group in Europe, initiated by the group of artificial insemination veterinarians of the European Union (EU), has agreed on the use of a strong positive (EU1), a weak positive (EU2) and negative serum (EU3) for standardisation of BoHV-1 tests in laboratories that routinely examine samples from artificial insemination centres (30). These sera have been adopted as OIE international standards for BoHV-1 tests and are available in limited quantities at the OIE Reference Laboratories for IBR/IPV. Prescribed tests for international trade purposes (VN or ELISA) must be capable of scoring both the strong and weak positive standards (or secondary national standards of equivalent potency) as positive. Because of the limited availability of the international standard sera, there is a need to prepare a new extended panel of reference lyophilised serum (and milk) samples taken from infected as well as from vaccinated animals. This panel should be used to validate newly developed tests and to harmonise tests between laboratories.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Several attenuated and inactivated BoHV-1 vaccines are available currently. The vaccines contain virus strains that have usually undergone multiple passages in cell culture. Some of the vaccine virus strains have a temperature-sensitive phenotype, i.e. they do not replicate at temperatures of 39°C or higher. Attenuated vaccines are administered intranasally or intramuscularly. Inactivated vaccines contain high levels of inactivated virus or portions of the virus particle (glycoproteins) supplemented with an adjuvant to stimulate an adequate immune response. Inactivated vaccines are given intramuscularly or subcutaneously.

Marker or DIVA (differentiation of infected from vaccinated animals) vaccines are now available in various countries. These attenuated or inactivated marker vaccines are based on deletion mutants or on a subunit of the virion, for instance glycoprotein D. The use of such marker vaccines in conjunction with companion diagnostic tests makes possible the distinction between infected and vaccinated cattle, and may thus provide the basis for eradication programmes of BoHV-1. Intensive vaccination programmes can reduce the prevalence of infected animals (5, 22), which could be monitored by using a companion diagnostic test. In situations where it is economically justifiable, the residual infected animals could then be culled, if necessary, resulting in a region free from BoHV-1. Control and eradication of BoHV-1 was started in some countries in the early 1980s. Different policies have been used due to differences in herd prevalence, breeding practices and disease eradication strategies. In the European Union at this time, only gE-deleted DIVA vaccines (live as well as killed) have been marketed and used for these control or eradication programmes.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

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6 Obtainable from the Central Institute for Animal Disease Control, Division of Virology, P.O. Box 2004, 8203 AA Lelystad, The Netherlands, and AFSSA Lyon, Laboratoire de pathologie bovine, 31 avenue Tony Garnier, BP 7033, 69342 Lyon Cedex 07, France.
The vaccine is prepared using a seed-lot system. The origin, passage history and storage conditions of the master seed virus (MSV) must be recorded. A virus identity test must be performed on the MSV. The seed lot contains BoHV-1 strains that have been attenuated to yield a live vaccine strain. The strains can be attenuated by multiple passages in cell cultures, by adapting virus to grow at low temperatures (temperature-sensitive mutants), or by genetic engineering, for example, by deleting one or more viral genes (e.g., the BoHV-1 glycoprotein E) that are nonessential for replication. There should be some means of distinguishing the live vaccine virus from field viruses (for example temperature-specific growth patterns or restriction fragment length polymorphisms). Strains used for the preparation of inactivated vaccines need not be attenuated. The seed lot must be free from contaminants.

b) Method of culture

The cells used for vaccine production are prepared using a seed-lot system. The virus should be cultured on established cell lines that have been shown to be suitable for vaccine production, for example the Madin–Darby bovine kidney cell line. The history of the cell line must be known. The cell line must be free from extraneous agents and may be tested for tumorigenicity.

c) Validation as a vaccine

Irrespective of the method of preparation of the seed-lot vaccine virus, the seed-lot virus destined for incorporation in a live vaccine must be shown to be efficacious, safe and pure.

i) Efficacy

This must be shown in a vaccination challenge experiment under laboratory conditions. Example guidelines are given in a monograph of the European Pharmacopoeia (12). Briefly, the vaccine is administered to five 2–3-month-old BoHV-1 seronegative calves. Two calves are kept as controls. All the calves are challenged intranasally 3 weeks later with a virulent strain of BoHV-1 that gives rise to typical signs of a BoHV-1 infection. The vaccinated calves should show no or only mild signs. The maximum virus titre found in the nasal mucus of vaccinated calves should be at least 100 times lower than that found in control calves. The virus excretion period should be at least 3 days fewer in vaccinated than in control calves.

ii) Safety

A quantity of virus equivalent to ten doses of vaccine should (a) not induce significant local or systemic reactions in young calves; (b) not cause fetal infection or abortion, and (c) not revert to virulence during five serial passages in calves. For inactivated vaccine, a double dose is usually administered. The reversion to virulence test is not applicable to inactivated vaccines.

iii) Purity

The seed lot is tested for absence of extraneous viruses and absence from contamination with bacteria, fungi or mycoplasmas. The following extraneous viruses should be specifically excluded in BoHV-1 vaccines: adenovirus, Akabane virus, bovine coronavirus, bovine herpesviruses 2, 4 and 5, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrhoea virus, bovine rotavirus, vaccinia virus, and the viruses of Aujeszky’s disease, bluetongue, bovine ephemeral fever, bovine leukoaemia, bovine papilloma, bovine papular stomatitis, cowpox, foot and mouth disease, lumpy skin disease, malignant catarhral fever, parainfluenza 3, rabies, rinderpest, and vesicular stomatitis. As bovine viral diarrhoea virus (either CPE and/or non-CPE) has regularly been found to be a contaminant of vaccines, special attention should be paid to ensure that it is absent.

2. Method of manufacture

All substances used for the manufacture of vaccines must be free from contaminants. Cells should be used that are not further than 20 passages from the master cell seed. The seed virus should not be more than five passages from the MSV. Genetically engineered vaccine virus strains are treated in the same way as conventionally attenuated vaccine virus strains. When sufficient cells are grown, infection of the cell line with the vaccine virus takes place. The addition of antibiotics is normally restricted to cell culture fluids. The supernatant fluid is harvested at times when the virus (antigen) production peaks. For live vaccines, the supernatant is clarified, mixed with a stabiliser, freeze-dried and bottled. For the production of classical inactivated vaccines, the supernatant is homogenised before the inactivating agent is added in order to ensure proper inactivation. After the inactivation procedure, a test for detecting complete inactivation of the virus is carried out. The test should consist of at least two passages in cells. The inactivated virus suspension is then mixed with an adjuvant and bottled. The manufacture of vaccines must comply with guidelines for Good Manufacturing Practice (GMP).

3. In-process control
Working cell seed and working virus seed must have been shown to be free from contaminants. The cells must have their normal morphology before being inoculated with virus. They are checked for CPE during cultivation. Uninoculated control cells must have retained their morphology until the time of harvesting. A virus titration is performed on the harvested supernatant. During the production of inactivated vaccines, tests are performed to ensure inactivation. The final bulk should be tested for freedom from contaminants.

4. Batch control

The following tests must normally be performed on each batch. Example guidelines for performing batch control can be found in EU directives, the European Pharmacopoeia and the United States Department of Agriculture’s Code of Federal Regulations.

a) Sterility

Bacteria, fungi, mycoplasmas and extraneous viruses must not be present. Tests for sterility and freedom from contamination of biological material may be found in Chapter 1.1.9.

b) Safety

For inactivated vaccines, a twofold dose of vaccine, and for live vaccines, a tenfold dose of vaccine, must not produce adverse effects in young BoHV-1 seronegative calves.

c) Potency

It is sufficient to test one representative batch for efficacy, as described in Section C.1.c.i. In the case of live vaccine, the virus titre of each batch must be determined and must be not higher than 1/10 of the dose at which the vaccine has been shown to be safe, and no lower than the minimum release titre. In the case of inactivated vaccines, the potency is tested using another validated method, for instance, efficacy assessment in calves.

d) Duration of immunity

It is sufficient to test this on the seed lot of vaccine virus. An efficacious BoHV-1 vaccine should induce protective immunity for at least 1 year, although many existing vaccines have not been tested to this standard.

e) Stability

For live vaccines, virus titrations should be carried out 3 months beyond the indicated shelf life. In addition, tests for determining moisture content, concentrations of preservatives, and pH are performed. For inactivated vaccines, the viscosity and stability of the emulsion are also tested.

f) Preservatives

The efficacy of preservatives should be demonstrated. The concentration of the preservative and its persistence throughout shelf life should be checked. The concentration must be in conformity with the limits set for the preservative.

g) Precautions (hazards)

No special precautions need to be taken. BoHV-1 is not pathogenic for humans.

5. Tests on the final product

a) Safety

Each product must be shown to be safe in at least two susceptible calves that receive a twofold (inactivated vaccine) or a tenfold (live vaccine) dose of vaccine.

b) Potency

See Section C.4.c.
REFERENCES


Chapter 2.4.13. -- Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis


20. LOVATO L., INMAN M., HENDERSON G., DOSTER A. & JONES C. (2003). Infection of cattle with a bovine Herpesvirus 1 strain that contains a mutation in the latency-related gene leads to increased apoptosis in trigeminal ganglia during the transition from acute infection to latency. J. Virol., 77, 4848–4857.


Chapter 2.4.13. - Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis


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**NB:** There are OIE Reference Laboratories for Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).