

CHAPTER 2.4.13.

INFECTIOUS BOVINE RHINOTRACHEITIS/ INFECTIOUS PUSTULAR VULVOVAGINITIS

SUMMARY

Definition of the disease: *Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. The virus is distributed world-wide, but has been eradicated from Austria, Denmark, Finland, Sweden, Italy (Province of Bolzano), Switzerland, Norway and parts of Germany (the 'Oberfranken' and 'Oberpfalz' districts of Bavaria). Control programmes are running in several other countries, for example in Germany and Italy.*

Description of disease: *The disease is characterised by clinical signs of the upper respiratory tract, such as a (muco)purulent nasal discharge, hyperaemia of the muzzle (red nose disease) and by conjunctivitis. Signs of general illness are fever, depression, inappetence, 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, and most infections run a subclinical course. Secondary bacterial infections can lead to more severe respiratory disease, and BoHV-1 could play a role in multifactor diseases such as 'shipping fever'.*

Identification of the agent: *The virus can be isolated from nasal or genital swabs from animals with respiratory signs, vulvovaginitis or balanoposthitis, taken during the acute phase of the infection, and, in severe cases, from various organs collected at post-mortem. Following infection, BoHV-1 may persist in infected animals in a latent state in sensory neurons, e.g. in the trigeminal or sacral ganglia. The virus can be reactivated and this results in virus shedding (re-excretion) without exhibition of clinical disease. Therefore, antibody-positive animals have to be classified as infected with BoHV-1 (with two exceptions: serological responses induced by vaccination with an inactivated vaccine or by colostral antibodies).*

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 (MDBK). 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. BoHV-1 isolates can be further subtyped by DNA restriction enzyme analysis (RFLP) into subtypes 1.1 and 1.2. BoHV-1.2 isolates can be further differentiated into 2a and 2b. Development of rhinotracheitis or vulvovaginitis/balanoposthitis depends more on the route of infection than on the subtype of the virus. The virus previously referred to as BoHV-1.3, a neuropathogenic agent, is now classified as BoHV-5.

Viral DNA detection methods have been developed, and the polymerase chain reaction technique is increasingly used in routine diagnosis including real-time polymerase chain reaction (PCR).

Serological tests: *The virus neutralisation test and various enzyme-linked immunosorbent assays (ELISA; indirect or gB-blocking) are most widely used for antibody detection. With the ELISAs, antibodies can be detected in serum or plasma, and with lower sensitivity in milk or bulk milk samples.*

Requirements for vaccines and diagnostic biologicals: *Inactivated and attenuated live vaccines are available. The vaccines protect cattle clinically in case of infection and markedly reduce the subsequent shedding of field virus. Although vaccination may not prevent field virus infection of individual animals, spreading of wild-type virus in infected herds is efficiently reduced. 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 (live or inactivated). The use of a gE-antibody-ELISA makes it possible to distinguish field virus infected cattle from cattle vaccinated with such a marker vaccine (DIVA strategy).

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, order *Herpesvirales*. The viral genome consists of double-stranded DNA that encodes for about 70 proteins, of which 33 structural and more than 15 nonstructural proteins have been identified. 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 (Metzler *et al.*, 1985). The BoHV-1.2 subtypes may be less virulent than subtype 1.1 (Edwards *et al.*, 1990). The former BoHV-1.3, which may act as a neuropathogenic agent in calves, has been reclassified as BoHV-5 (Magyar *et al.*, 1993). BoHV-1 shares antigenic and genetic close relationships with other ruminant alphaherpesviruses: BoHV-5, caprine herpesvirus 1, cervid herpesvirus 1 (red deer), cervid herpesvirus 2 (reindeer), bubaline herpesvirus 1 and elk herpesvirus 1 (Thiry *et al.*, 2006).

After an incubation period of 2–4 days, serous nasal discharge, salivation, fever, inappetence, 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. However, most infections run a very mild or subclinical course (Van Oirschot *et al.*, 1993). Uncomplicated cases of respiratory or genital disease caused by BoHV-1 last about 5–10 days. Secondary bacterial or viral agents may contribute to a multifactor disease complex resulting in severe respiratory disease of young animals ('shipping' or 'crowding fever').

After infection via the airborne route, BoHV-1 replicates to high titres in mucous membranes of the upper respiratory tract and in the tonsils. Subsequently, the virus disseminates to conjunctivae and reaches the trigeminal ganglia by neuronal axonal transport. After genital infection, BoHV-1 replicates in the mucous membranes of the vagina or prepuce, and becomes latent in the sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host (status of latency). Stress, such as transport and parturition, but also the application of corticosteroids can induce reactivation of the latent infection. Consequently, the virus may switch between latent and lytic infection and may be shed intermittently into the environment and spread to contact animals.

BoHV-1 infection elicits an antibody response and a cell-mediated immune response within 7–14 days. The immune response is presumed to persist life-long, although it may fall below the detection limit of some tests after a number of years. Maternal antibodies are transferred via colostrum to the young calf, which is consequently protected against BoHV-1-induced clinical disease (Mechor *et al.*, 1987). 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, with the exception of the BoHV-1-free countries, paralleling the distribution of domestic cattle. Other *Artiodactyla* (e.g. goats, sheep, water buffaloes, camelids) may be infected with BoHV-1. After infection, nasal viral shedding is detected for 5–14 days, with peak titres of 10^8 – 10^{10} TCID₅₀ (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 (Parsonson & Snowdon, 1975).

Prevention and control of BoHV-1 infections are based on thorough farm management including hygienic measures, vaccination schedules and removal of infected animals. Ideally, a 4-week quarantine period is imposed for newly introduced cattle, if the cattle are not from certified BoHV-1-free farms. Only cattle that are BoHV-1-seronegative are then admitted to a free herd. Natural mating should be avoided and only semen from BoHV-1-negative bulls should be used.

Vaccines usually prevent the development of clinical signs and markedly reduce the shedding of virus after infection, but do not completely prevent infection. Several eradication campaigns have been carried out or are currently running in different countries including test-and-removal programmes and/or vaccination campaigns (see Section C).

BoHV-1 infection may be suspected on the basis of clinical, pathological and epidemiological findings. However, to make a definite diagnosis, laboratory examinations (serology and/or virus detection) 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. Nevertheless, because of latent infection induced by BoHV-1, detection of antibodies could be sufficient for the determination of the BoHV-1 status of individual animals.

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% BoHV-1-free 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 samples of the tonsil, lung and bronchial lymph nodes are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and placental cotyledons are examined. Samples should be kept on ice and sent to the laboratory as quickly as possible.

After arrival at the laboratory, swabs are agitated at room temperature for 30 minutes in the transport medium to elute virus. 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, bovine cells of various origins 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 (MDBK), are suitable for BoHV-1 propagation. 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 (Brunner *et al.*, 1988; Edwards *et al.*, 1983).

To identify the recovered virus 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 log₁₀) 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 pre-incubated 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, although definitive confirmation would require molecular characterisation to distinguish it from related ruminant alphaherpesviruses.

An alternative method of virus identification is the direct verification of BoHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (Kaashoek *et al.*, 1994) with conjugated monospecific antiserum or MAb. Furthermore, the supernatant can be used as template for restriction endonuclease fragment length polymorphism (RFLP) (see Section B.1.e) and polymerase chain reaction (PCR) (see Section B.1.c) analyses.

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

0.05 to 0.1 ml of raw semen should be tested with two passages in cell culture. Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10) before being added to cell cultures. A similar problem may

sometimes arise with extended semen. For extended semen, an approximation should be made to ensure that the equivalent of a minimum of 0.1 ml raw semen is examined (e.g. a minimum of 0.5 ml extended serum). Multiple diluted samples may need to be tested with this procedure to reach a volume equivalent to 0.1 ml raw semen (e.g. 5 × 1 ml of a 1/10 diluted sample of extended semen). A suitable test procedure is given below. See also Brunner *et al.* (1988).

- **Test procedure**

- i) Dilute 200 µl fresh semen in 2 ml fetal bovine serum (free from antibodies against BoHV-1) with 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. Special 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) **Nucleic acid detection**

During the past decade, various methods for the detection of BoHV-1 DNA in clinical samples have been described, including DNA–DNA hybridisation and the PCR. The PCR is also increasingly used in routine diagnostic submissions (Moore *et al.*, 2000). 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 is also possible to detect episomal DNA of non-replicating virus in sensory ganglia (Van Engelenburg *et al.*, 1993), such as the trigeminal ganglion, in the latent phase of infection. The disadvantage is that PCR analyses are 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 real-time or quantitative PCR (see below) (Abril *et al.*, 2004; Lovato *et al.*, 2003).

So far PCR has been used mainly to detect BoHV-1 DNA in artificially (Kramps *et al.*, 1993) or naturally (Van Engelenburg *et al.*, 1993) infected semen samples. It is important to thoroughly optimise the PCR conditions, including the preparation of the samples, the concentration of Mg²⁺, primers and 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. In addition, PCRs based on detection of gE sequences can be used to differentiate between wild-type virus and gE-deleted vaccine strains (Fuchs *et al.*, 1999; Schynts *et al.*, 1999). Discrimination between infection with virulent IBR strains and infection with live attenuated strains is not possible with the PCR technique, and RFLP is used for this purpose. Specific PCRs have been developed that are able to discriminate between BoHV-1, BoHV-5 and other related alphaherpesviruses (Ashbaugh *et al.*, 1997; Ros *et al.*, 1999).

Experimentally, 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 (Van Engelenburg *et al.*, 1995). The detection limit of validated PCR assays amounts to only a few genome copies per PCR reaction. 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 (Ros *et al.*, 1999; Van Engelenburg *et al.*, 1993). 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 PCR methods used for the diagnosis of infectious diseases). DNA extraction and quality of the DNA preparations can also be controlled by amplification of cellular sequences (housekeeping genes) or by addition of 'artificial' DNA sequences prior to extraction

procedures (e.g. green fluorescent protein, non-BoHV-related viruses) as internal controls. To enhance the sensitivity and specificity of the BoHV-1 PCR, real-time PCR systems are the methods of choice.

- **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 destined 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 (Wang *et al.*, 2008).

A number of studies have shown that PCR assays are more sensitive than virus isolation (Smits *et al.*, 2000; Van Engelenburg *et al.*, 1995; Vilcek *et al.*, 1994; Wang *et al.*, 2008; Wiedmann *et al.*, 1993). Real-time PCR has been used for the detection of BoHV-1 and BoHV-5 in experimentally infected cattle and mice (Abril *et al.*, 2004; Lovato *et al.*, 2003) and a series of conventional PCR assays have been used for the detection of BoHV-1 DNA in artificially or naturally infected bovine semen samples (Deka *et al.*, 2005; Grom *et al.*, 2006; Masri *et al.*, 1996; Van Engelenburg *et al.*, 1993; Weiblen *et al.*, 1992; Wiedmann *et al.*, 1993; Xia *et al.*, 1995). Conventional detection of amplified PCR products relies on gel electrophoresis analysis (Rola *et al.*, 2003). Sequence-specific primers have been selected to amplify different parts of conserved glycoprotein genes of the BoHV-1 genome, including glycoprotein B (gB) gene (Grom *et al.*, 2006; Santurde *et al.*, 1996), gC gene (Smits *et al.*, 2000; Van Engelenburg *et al.*, 1995), gD gene (Smits *et al.*, 2000; Wiedmann *et al.*, 1993), gE gene (Grom *et al.*, 2006), and the thymidine kinase (tk) gene (Moore *et al.*, 2000; Yason *et al.*, 1995).

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 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, and tubes have not to be opened for product analysis on agarose gels. There is no post-amplification PCR product handling, which significantly reduces the risk of contamination, and it is possible to perform quantitative analysis.

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 BoHV-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 affect PCR test result.
- ii) Three straws from each batch of semen 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 manufacturers. In the procedure described below, a RotorGene 3000, Corbett Research Ltd, Australia, was used. Other real-time PCR detection systems can also be applied. Other equipment required for the test includes a micro-centrifuge, a heating block, a boiling water bath, a micro-vortex, magnetic stirrer and micropipettes. Real-time PCR assays are able to detect very small amounts of target nucleic acid molecules therefore appropriate measures are required to avoid contamination¹. Furthermore, a minimum of one negative sample should be processed in parallel to estimate the risk of low level contamination.
- 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 PCR analysis of the extracted DNA template in a real-time PCR

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.

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 particular kits selected need 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 supplied by Sigma-Genosys.

- **Extraction of DNA**

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

Chelex 100 sodium (Sigma) (10% w/v in distilled deionised water)	100 µl.
Proteinase K (10 mg/ml, Sigma)	11.5 µl
DL-Dithiothreitol (1 M, Sigma)	7.5 µl
Nuclease-free water	90 µl
Semen sample	10 µl

 Mix gently by pipetting².
- ii) The samples are incubated at 56°C for 30 minutes and then vortexed at high speed for 10 seconds.
- iii) Subsequently, the tubes are 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.

- **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 and probe are made with nuclease-free water at the concentration of 4.5 µM and 3 µM, respectively. The stock solutions 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 separate laboratory room. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC, reagents only), appropriate negative controls, i.e. 1 per 10 test samples, and two positive controls (moderate and weak positive) 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

2 It is important that the Chelex 100 solution is homogeneous while pipetting, as Chelex 100 sodium is not soluble. This can be achieved 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.

- 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 to the PCR mix 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

One cycle:	Hold 50°C	2 minutes
One cycle:	Hold 95°C	2 minutes ⁵
45 cycles:	Hold 95°C	15 seconds
	Hold 60°C	45 seconds

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.

• **Interpretation of results**

• **Test controls**

Positive and negative controls, as well as reagent controls, should be included in each PCR test. Negative semen, from 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 difficult to obtain. Alternatively, positive controls can be derived from negative semen spiked with defined quantities of BoHV-1 virus.

• **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. To minimise the risk of contamination by the positive control, a dilution resulting in a Ct value of about 30 to 33 should be used.

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

d) **Viral antigen detection**

Nasal, ocular or genital swabs can be directly smeared onto glass cover-slips, or, following centrifugation, the cell deposit (see Section B.1.a) may be spotted onto 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 a fluorescent dye e.g. fluorescein isothiocyanate (FITC), whereas in the indirect procedure, the anti-species immunoglobulin secondary antibody is conjugated to a fluorochrome. To obtain reliable 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. Smears from nasal swabs from cattle with a purulent or haemorrhagic nasal discharge are often negative (Terpstra, 1979). 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 (Edwards *et al.*, 1983) or PCR. 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 immunofluorescence analyses of frozen sections. Immunohistochemistry may also be applied for BoHV-1 detection and determination of the antigen location in the tissues. 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

4 These PCR parameters are adapted to the RotorGene 3000, Corbett Research Ltd, Australia, and may vary with different PCR platforms.

5 PCR Taq polymerase systems from different commercial sources may require a prolonged initial denaturation (95°C) time up to 10 minutes. Please follow the manufacturer's instructions.

on microplates. Amounts of antigen equivalent to 10^4 – 10^5 TCID₅₀ of BoHV-1 are required in order to obtain reliable positive results (Collins *et al.*, 1988). This may not be unrealistically high, because titres of 10^8 – 10^9 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 Edwards & Gitao, 1987).

In contrast to virus isolation, no cell culture facilities are required for direct antigen detection techniques and a laboratory diagnosis can be made within 1 day. The disadvantages are the lower sensitivity of direct antigen detection and the extra requirement to perform additional virus isolation, if the isolate is required for further studies.

e) Differentiation of bovine herpesvirus 1 subtypes and of ruminant alphaherpesviruses related to bovine herpesvirus 1

By using appropriate MAbs for immunofluorescence, radioimmunoprecipitation, immunoperoxidase or immunoblot assays, BoHV-1 subtype 1 and subtype 2b can be differentiated (Rijsewijk *et al.*, 1999; Wyler *et al.*, 1989). Restriction endonuclease digestion of viral DNA enables differentiation between BoHV-1 subtypes. RFLP 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. Differentiation of the BoHV-1 subtypes 1, 2a and 2b by *HindIII* endonuclease digestion is based on the molecular weight of three relevant DNA fragments (*I*, *K* and *L*) (Metzler *et al.*, 1985). RFLP techniques are of limited diagnostic value, but may be useful in epidemiological studies. Furthermore, RFLP pattern of virus isolates can be compared with that of live vaccine strains.

When differentiation is required between antigenically and genetically related alphaherpesviruses (BoHV-1, BoHV-5) caprine herpesvirus 1, cervid herpesvirus 1 and 2, elk herpesvirus 1, bubaline herpesvirus 1), improved methods are available using monoclonal antibodies (Keuser *et al.*, 2004) or PCR amplification and sequencing (Ros *et al.*, 1999).

f) Interpretation of results

The isolation of BoHV-1 from a diseased animal does not unequivocally mean that this virus is the cause of the illness. 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 increase in BoHV-1-specific antibody titres. Paired serum samples collected 3–4 weeks apart are examined in a serological test for the presence of specific antibodies (see Section B.2).

2. Serological tests

Serological tests can be used for several purposes:

- i) To diagnose an acute infection: paired serum samples from the acute and convalescent stages of infection of the same animals are examined in one test. A seroconversion from negative to positive or a four-fold or higher increase in antibody titres is considered to prove an acute infection.
- ii) To demonstrate absence of infection, for instance, for international trade purposes.
- iii) To determine the prevalence of infection in sero-epidemiological studies.
- iv) To support eradication programmes and subsequent surveillance.
- v) For research purposes, for instance, the evaluation of the antibody response after vaccination and challenge infection.

Virus neutralisation (VN) tests (Bitsch, 1978) and various ELISAs (Kramps *et al.*, 1993) 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 antibodies to the virus is considered to be a carrier and potential intermittent excretor of the virus. The only exceptions are calves that have acquired passive colostral antibodies from their dam, and noninfected cattle vaccinated with inactivated vaccines. There is also a risk that calves infected under cover of maternal immunity may become serologically negative while carrying a reactivatable latent infection (Lemaire *et al.*, 2000).

In general, BoHV-1 serological tests can be divided into conventional and marker tests. Up to now, the only serological marker tests available are the BoHV-1 gE-antibody blocking ELISAs (Van Oirschot *et al.*, 1997). Animals vaccinated with gE-deleted marker vaccines can be discriminated from field-virus infected animals by a negative serological reaction for gE. For conventional serology, VNT, BoHV1-antibody blocking ELISAs indirect ELISAs may be used.

ELISAs, including the gE-ELISA, are increasingly used for the detection of antibodies in (bulk) milk samples (Wellenberg *et al.*, 1998a), but have some limitations. By testing bulk milk, a positive gB-specific test indicates that the infection has already spread in the herd (Frankena *et al.*, 1997). With the gE-blocking ELISA, bulk milk gives a positive reaction when more than 10–15% of the herd is infected ((Wellenberg *et al.*, 1998b). Consequently, it is not possible to declare a herd to be free from BoHV-1 infection with these tests on the basis of bulk or pooled milk samples, and a negative gE- or gB-ELISA bulk milk test should be followed up with individual blood samples from all cattle in the herd. However, indirect ELISAs optimised for use with bulk milk samples of up to 50 individual cows can indicate reliably the BoHV-1 status of these animals. For general surveillance purposes, bulk milk tank tests can give an estimate of BoHV-1 prevalence in a herd, an area or country (Nylín *et al.*, 2000). These should be supplemented by serum testing (individual or pooled) from non-milking herds. For monitoring a BoHV-1 status in dairy herds, bulk milk samples of up to 50 animals should be tested 3–4 times per year with a suitable indirect ELISA. In herds of more than 50 cows, several bulks of milk from up to 50 animals should be tested. Positive bulk milk results have to be confirmed by testing individual blood samples from all animals included in a positive bulk milk sample.

In an extensive study, tests for the detection of antibodies as routinely used by national reference laboratories in Europe were evaluated (Kramps *et al.*, 2004). 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) (Perrin *et al.*, 1994). It was concluded that VNT and gB-specific ELISAs are the most sensitive tests for the detection of antibodies in serum. Owing to the very high sensitivity of the gB-blocking ELISAs, gB-antibody weak positive results can often not be confirmed by alternative test systems (indirect ELISA, VNT). 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 (Beer *et al.*, 2003).

gE-ELISAs are less sensitive and specific than the conventional test systems. In addition, sero-conversion against gE can be delayed, especially in vaccinated animals, and is often not detectable before day 21 to 35 post-infection. Furthermore, second generation indirect ELISAs were found to be the most sensitive tests for the detection of BoHV-1-specific antibodies in milk. Moreover, it has been observed that commercial ELISAs perform better than home-made ELISAs.

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%) (Perrin *et al.*, 1993). Among these variables, the virus/serum incubation period has the most profound effect on the sensitivity of the VNT. A 24-hour incubation period may score up to 16-fold higher antibody titres than a 1-hour incubation period (Bitsch, 1978), 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 (MDBK) 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 three 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 TCID₅₀ 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 × 10⁴ 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 TCID₅₀ with a permissible range of 30–300 TCID₅₀), the control sera

and the cell control wells. The positive control serum should give a titre of ± 1 twofold dilution ($\pm 0.3 \log_{10}$ units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation (equivalent to a final dilution of 1/2 at the neutralisation stage). In the cell control wells, the monolayers 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 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 cytotoxicity of a serum interferes with the interpretation of the neutralising activity of the sample, changing the medium in the wells of the lowest two or three dilutions 16–24 hours after the addition of cells may remove the cytotoxic effects.

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 (Kramps *et al.*, 2004). 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 the 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 comprehensive panel of well defined (e.g. by VN test) strong positive, weak positive and negative sera has to be tested. However, it is recommended to use commercially available ELISAs that have been shown to perform better than home-made assays (Kramps *et al.*, 2004).

- **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.

- **Blocking enzyme-linked immunosorbent assay**

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

- 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 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 are described in the published literature.
- 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 .
- 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 nonspecific responses.
- Wash the plates thoroughly and add 100 μl of an anti-BoHV-1-gB-monoclonal 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 BoVH-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 H₂O₂ [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 $[(OD_{FCS} - OD_{test\ sample})/OD_{FCS} \times 100\%]$
- vii) A test sample is considered to be positive if it has a blocking percentage of e.g. 50% or higher. The test is valid if the positive and weak positive control sera are positive and the negative control serum reacts negatively. 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 (Perrin *et al.*, 1994). 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/IPV⁶. 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. Additional reference sera are available in limited quantities from the OIE Reference Laboratories (e.g. R1, R2 and R3 as positive, weak positive and very weak positive standard sera from the OIE Reference Laboratory in Germany).

d) Nonspecific reactivity in BoHV-1-serology and 'pseudo-vaccines'

Nonspecific reactivity of sera in the BoHV-1-ELISAs should be taken into consideration, and is more often seen for the marker test than for the conventional serology. There are several reasons for nonspecific reactions:

- batch variation of the ELISA used;
- samples were tested very early after collection (freshness phenomenon);
- samples were collected within 4 weeks after vaccination (vaccination phenomenon);
- bad sample quality (e.g. haemolysed samples).

Therefore the following measures should be considered:

- validation of each test batch, and batch release tests have to be implemented;
- samples should be stored at 4°C and should not be tested before 24–48 hours after sample collection;
- samples should be subjected to a freeze–thaw cycle (–20°C) and to subsequent heat inactivation (30 minutes/56°C);
- Cattle should not be serologically tested for BoHV-1 prior to 4 weeks after any vaccination;
- gE-ELISAs should not be used for classification of unvaccinated animals.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Background

a) Rationale and intended use of the product

Several attenuated and inactivated BoHV-1 vaccines are currently available. The vaccine strains have usually undergone multiple passages in cell culture. Some of the vaccine virus strains have a temperature-

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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. Vaccination against BoHV-1 is used to protect animals from the clinical outcome of infection, and as an aid in control and eradication programmes.

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 (deletion of gE) or on a subunit of the virion, for instance glycoprotein D. The use of such marker vaccines in conjunction with companion diagnostic tests allows the distinction between infected and vaccinated cattle (DIVA principle), and provides the basis for BoHV-1 eradication programmes in countries or regions with a high prevalence of field-virus infected animals. Intensive vaccination programmes can reduce the prevalence of infected animals (Bosch *et al.*, 1998; Mars *et al.*, 2001), which could be monitored by using an appropriate diagnostic test. In situations where it is economically justifiable, the residual infected animals could be slaughtered, 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. To date, in the European Union, only gE-deleted DIVA vaccines (live as well as killed) have been marketed and used for control or eradication programmes. As there is no proven advantage of conventional non-marker BoHV-1 vaccines, gE-deleted marker vaccines (live or inactivated) should be the vaccines of choice.

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 of general nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

The vaccine is prepared using a seed-lot system. 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 have to be 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.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

The seed lot is tested for absence of extraneous viruses and absence from contamination with bacteria, fungi or mycoplasma. 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 and atypical pestiviruses, bovine rotavirus, vaccinia virus, and the viruses of Aujeszky's disease, bluetongue, bovine ephemeral fever, bovine leukaemia, bovine papilloma, bovine papular stomatitis, cowpox, foot and mouth disease, lumpy skin disease, malignant catarrhal 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 the absence of BVDV. In addition, new atypical pestiviruses (HoBi or HoBi-like) have to be taken into consideration as possible contaminants.

b) Method of manufacture

i) Procedure

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 (MDBK) 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.

ii) Requirements for substrates and media

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 ensuring complete inactivation of the virus is carried out. The test should include 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).

iii) In-process controls.

Working cell seed and working virus seed must have been shown to be free from contaminants. The cells must show inconspicuous morphology before being inoculated with virus. The CPE is checked 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 must be tested for freedom from contaminants.

iii) Final product batch tests

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.

Sterility/purity

Bacteria, fungi, mycoplasma 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.

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.

Batch potency

It is sufficient to test one representative batch for efficacy, as described in Section C.1.c.ii. In the case of live vaccines, 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.

c) Requirements for authorisation

i) Safety requirements

Target and non-target animal 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.

Reversion-to-virulence for attenuated/live vaccines

The selected final vaccine strain should not revert to virulence during a minimum of five serial passages in calves.

Environmental consideration

Attenuated vaccine strains should not be able to perpetuate autonomously in a cattle population ($R_0 < 1$).

ii) Efficacy requirements

For animal production

This must be shown in vaccination challenge experiments under laboratory conditions. Example guidelines are given in a monograph of the European Pharmacopoeia (Third Edition (1997)). Briefly, the vaccine is administered to ten 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 clinical signs of a BoHV-1 infection. The vaccinated calves should show no or only very mild signs. The maximum (peak) virus titre in the nasal mucus of vaccinated calves should be at least 100 times lower than that in control calves. The virus excretion period should be at least 3 days shorter in vaccinated than in control calves.

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.

For control and eradication

In addition to the above-mentioned criteria, BoHV-1-vaccines for control and eradication should be marker vaccines (e.g. gE-deleted vaccines) allowing the differentiation of infected from vaccinated animals (DIVA-strategy). Several gE-deleted vaccines (inactivated preparations as well as modified live vaccines) are commercially available.

iii) 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.

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.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

There is a glycoprotein E (gE)-deleted inactivated vaccine available which is based on a recombinant strain. The vaccine is comparable to other gE-deleted vaccines and is licensed by European Medicines Agency (EMA) for use in the European Union.

Additional recombinant vaccines like gD-subunits or genetically engineered deletion mutants of BoHV-1 (e.g. with deletions of gE and/or gG) are described and available as prototypes.

Advantages of BoHV-1 vaccines based on biotechnology could be the possibility of additional marker features for the differentiation of infected from vaccinated animals (DIVA; e.g. gB-antibody-ELISAs for gD-subunit vaccines or gG-antibody-ELISAs for the respective deletion mutants).

b) Special requirements for biotechnological vaccines, if any

Recombinant vaccines, which are destined for use in the European Union have to be licensed by EMA.

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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).