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Establishment of a multiplex real-time PCR assay for the detection and differentiation of wild-type and glycoprotein E-deleted vaccine strains of bovine herpesvirus type 1

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„Nur wer nicht sucht, ist vor Irrtum sicher."

(Albert Einstein)

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I. INTRODUCTION

Real-time polymerase chain reaction (qPCR) assays, based on detection of fluorescence signal increase during amplification (Higuchi et al., 1992), have become a method widely used for genome detection of pathogens in the past years (Mackay, Arden, and Nitsche, 2002). Due to the combination of high sensitivity, a reduced risk of cross-contamination through the absence of post-amplification handling and the possibility of quantitative analysis (Belak and Thoren, 2001; Espy et al., 2006; Niesters, 2004), qPCR assays are an excellent tool in molecular diagnostics. The simultaneous detection of multiple target sequences within a single reaction, referred to as "multiplex PCR", is permitted by uniquely labelled probes and the separate detection of the spectra of their individual dyes (Wittwer et al., 2001). Possible applications of multiplex assays include the co-amplification of internal controls, the detection of different pathogens related to a disease complex or wild-type and vaccine strains of a virus for instance.

Bovine herpesvirus type 1 (BoHV-1) associated diseases are distributed globally. They are characterized by rhinotracheitis (IBR), pustular balanoposthitis (IBP) and vulvovaginitis (IPV), abortion, conjunctivitis and occasional encephalitis (Gibbs and Rweyemamu, 1977). After initial replication in either respiratory, prepuce or vagina mucosal epithelium, the virus can enter neural cells and establish a life-long latent infection in the sensory ganglia of the nervous system (Engels and Ackermann, 1996; Muylkens et al., 2007). The latent virus, representing a long-term reservoir in hosts, may be reactivated by diverse stimuli resulting in recurred disease and shedding of the virus (Jones, 2003; Pastoret et al., 1979; Thiry et al., 1987). Latency and re-activation are responsible for the maintenance of BoHV-1 within cattle herds as well as for the introduction into free holdings (Winkler, Doster, and Jones, 2000).

Extensive control programs have been initiated in several European countries including Germany. Major components of these programs are the detection of outbreaks and the vaccination with BoHV-1 mutants with deleted glycoprotein E (gE) coding regions. These marker vaccine viruses allow the serological discrimination between vaccination and field infection. As the deletion of gE in vaccine strains does not abolish the ability to establish latent infection and reactivation in the host (Lemaire et al., 2001; Straub, 1996; van Engelenburg et

al., 1995a), it is important to monitor the spread and shedding of wild-type BHV-1 and differentiate them from live marker vaccine viruses.

To allow the rapid nucleic-acid-based detection of gE-positive wild-type strains and their differentiation from gE-negative vaccine viruses within a single reaction, a multiplex PCR assay was developed and validated.

II.   LITERATURE REVIEW

1.   Polymerase chain reaction

1.1.   Basic principle

Developed by Mullis, acknowledged with the Nobel Prize in chemistry (http://nobelprizes.com/nobel/chemistry/), polymerase chain reaction (PCR) has become one of the most widely used methods for the amplification and detection of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

Besides the DNA template, PCR reactions contain oligonucleotide primers, four deoxyribonucleotide triphosphates (dNTP), a buffer with magnesium ions and the DNA polymerase enzyme. The primers are two oligonucleotides that flank the DNA sequence to be amplified. They are complementary to the sense and antisense strands of the template, respectively. Repetitive cycles of heating and cooling are carried out resulting in billion-fold amplification of the nucleic acid fragment between the primer binding sites by the DNA polymerase. Each cycle comprises a denaturation, an annealing and an elongation step (Kubista et al., 2006; Mullis et al., 1986). Nested PCR assays consist of two consecutive amplification reactions, the first using two external primers, followed by a reaction containing two internal primers targeting sequences within the first amplicon.

For analysis of RNA targets the initial conversion into complementary deoxyribonucleic acid (cDNA) by a RNA-dependent DNA polymerase, the reverse transcription (RT), is required (Bustin and Mueller, 2005).

The obtained PCR products of defined length can be visualized by electrophoresis using ethidiumbromide-stained agarose gels and a size marker for comparison.

1.2.   Real-time polymerase chain reaction

In the real-time format, the increase of PCR products is detected directly during amplification (Higuchi et al., 1992). The two general formats for amplicon detection comprise fluorescent dyes intercalating with double-stranded DNA (dsDNA) and fluorogenic probes allowing specific detection of target sequences.

Once bound to dsDNA, intercalating dyes give a fluorescence signal measurable

by qPCR cyclers (Figure 1). The accumulating amount of dsDNA over the course of PCR causes an increase in fluorescence from cycle to cycle. Besides ethidium bromide (Higuchi et al., 1992; Higuchi et al., 1993) and SYBR Green I (Ririe, Rasmussen, and Wittwer, 1997; Zipper et al., 2004), the currently most widely used intercalating dye, a variety of dyes such as ResoLight, Eva-Green or SYTO-82 are applied to monitor the accumulation of dsDNA during PCR (Li et al., 2010a; Mao, Leung, and Xin, 2007; Tajiri-Utagawa et al., 2009). Despite differences in the efficiency and level of PCR inhibition (Gudnason et al., 2007), all these dyes intercalate with any dsDNA including non-specific PCR products and primer dimers. As the dissociation temperature of the PCR product depends on its GC/AT-ratio, nucleotide sequence and length, discrimination of specific from unspecific PCR products is permitted by melting curve analysis, which is the evaluation of dsDNA dissociation and associated decrease of fluorescence during heating (Helps et al., 2002; Ririe et al., 1997).
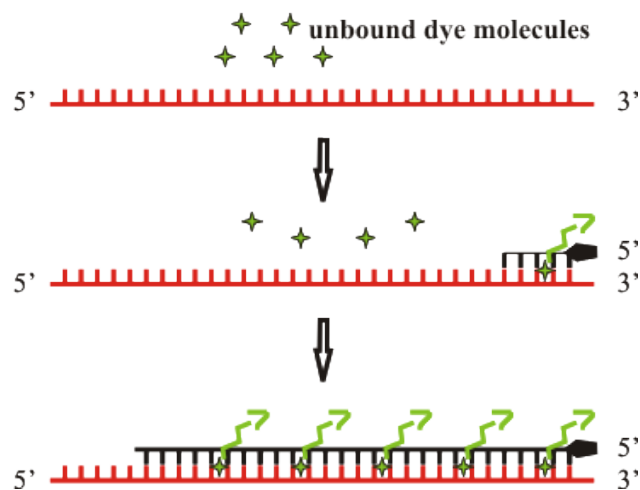

Fig. 1: Basic principle of intercalating fluorescent dyes. Showing negligible fluorescence when distributed unbound in reaction solution, dye molecules intercalate with emerging double-stranded DNA during annealing and elongation steps of qPCR resulting in an increasing fluorescence signal.

In contrast to intercalating dyes, the application of oligonucleotide probes labelled with a fluorophore permits a more specific detection of amplicon sequences. Common chemistries comprise hydrolysis probes, minor groove binder (MGB), locked nucleic acid (LNA), hybridisation probes and molecular beacons.

Hydrolysis probes, designed to hybridize within the target sequence between the primers, are oligonucleotides labelled with a reporter fluorophore at their 5' end and a quencher at their 3' end (Figure 2A). When exposed to light of a specific wavelength that varies with the used fluorophore, absorption of energy promotes electrons in the fluorophore from their ground state to an excited state. With the quencher in close proximity, this energy is transmitted non-radiatively to the quencher, returning the dye to its ground state. The quencher in turn releases the energy as light of a higher wavelength or, in case of non-fluorescent black hole quencher, as heat (Bustin, 2002; May et al., 2005; Morrison, 2010). Due to the $5' \rightarrow 3'$ exonuclease activity of the DNA polymerase, the probe is degraded into smaller fragments during amplification (Holland et al., 1991). The reporter becomes separated from the quencher resulting in an increasing, measurable fluorescence signal of a wavelength specific to the used reporter dye (Gibson, Heid, and Williams, 1996).

An identical mechanism is used by probes conjugated with MGB groups (Figure 2B). Because of forming stable duplexes with single-stranded DNA, MGB groups allow the design of shorter probes for hybridization based assays (Kumar et al., 1998; Kutyavin et al., 2000).

The incorporation of LNA, a nucleic acid analogue with unequalled hybridization affinity towards complementary DNA and RNA (Karkare and Bhatnagar, 2006), into probes increases the melting temperature which enables the use of significantly shorter probes (Alonso, Amoros, and Cuesta, 2010; Costa et al., 2004).

Hybridisation probes use a pair of oligonucleotides complementary to distinct but closely spaced sequences, one of them labelled 3'-terminally with a donor fluorophore, the other at the 5' end with an acceptor (Figure 2C). After excitation, the donor fluorophore passes energy to the nearby acceptor through Förster resonance energy transfer (FRET), evoking an increasing, detectable fluorescence signal from the latter (Cardullo et al., 1988).

Molecular beacons contain a hairpin loop structure terminally labelled with reporter and quencher (Figure 2D). Their close contact is causing the quenching (collisional quenching) and the energy is dissipated as heat (Bustin, 2002). When hybridizing to the target sequence, the probe undergoes a conformational change separating the reporter fluorophore from the quencher.
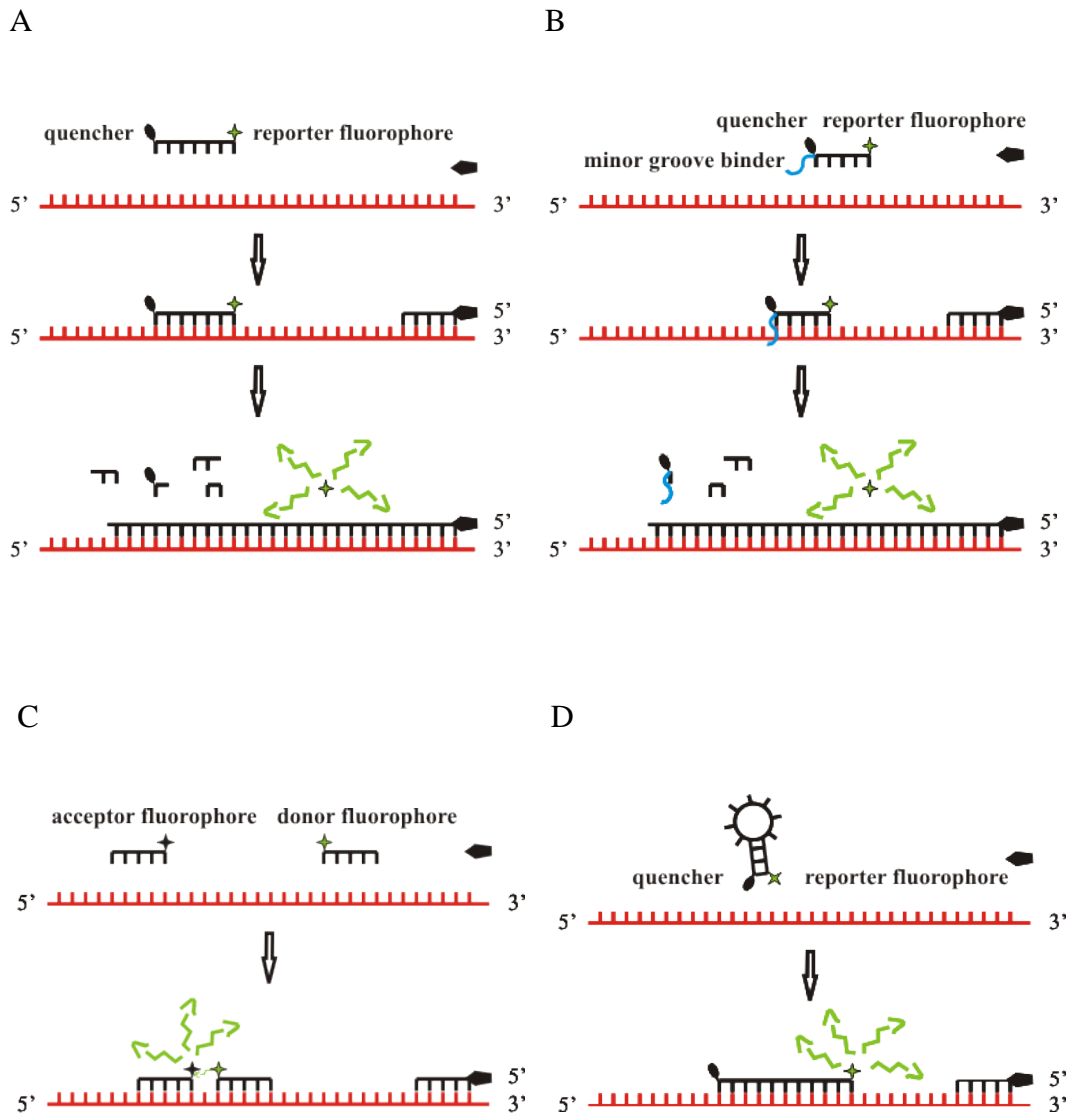

Fig. 2: Behaviour of different probe types over the course of a qPCR reaction. A. Hydrolysis probe. B. Minor groove binder probe. C. Hybridisation probe. D. Molecular beacon. In each case the first image depicts the denaturation step, followed by the annealing and elongation steps.

In both cases, intercalating dyes and fluorogenic probes, the measurement of the fluorescence signal is performed through the transparent lid of the reaction vessel thereby avoiding post-PCR handling (Belak and Thoren, 2001). As a result, the risk of cross-contamination and the time required for the generation of results is reduced considerably compared to conventional PCR evaluated by agarose gel electrophoresis (Espy et al., 2006; Mackay, 2004; Niesters, 2004). In addition, the 96-well plate format is common in real time machines, permitting a combination with timesaving automated nucleic acid extraction.

The observed increase in fluorescence during qPCR is proportional to the increase in concentration of the accumulating product, thus dependent on the starting amount of the target sequence (Higuchi et al., 1993). The amplification cycle in which the fluorescence signal first exceeds the background, referred to as quantification cycle (Cq) (Bustin et al., 2009), is used for quantification of the target (Gibson et al., 1996). The amount of target sequences is quantified either absolutely or relatively (Mackay, 2004).

Absolute quantification requires a standard curve, generated by plotting the Cq-value against the logarithm of the starting copy number, after analysing a serial dilution of a known sample, for example a plasmid containing the sequence of the entire amplicon or a synthetic single-stranded positive sense oligonucleotide. The copy number of the sequence of interest in the unknown sample is calculated from the linear regression of the standard curve (Arya et al., 2005; Bustin, 2000).

In relative quantification, also referred to as comparative threshold method, the ratio between a reference control and the amounts of target sequence, normalized to one or more co-amplified housekeeping genes, is determined without the need of standard curve generation. For example untreated samples or samples from the starting point in time-course studies are used as reference control. To obtain reliable results it is essential that the amplification efficiencies of housekeeping gene and target sequences are approximately equal (Arya et al., 2005; Bustin, 2002).

1.3. Multiplex polymerase chain reaction

The amplification of multiple templates using multiple primers within a single reaction is called multiplex PCR, an opportunity that is provided by conventional as well as qPCR.

In conventional PCR assays, multiplexing is permitted by differences in base pair (bp) length of the amplicons generated by the diverse primer pairs. An adequate resolution of the subsequent visualization by gel electrophoresis is necessary for discrimination (Chamberlain et al., 1988).

Differences in the melting point of the amplicons as well as oligonucleotide probes labelled with fluorophores that have unique emission spectra enable multiplexing in the qPCR format (Wittwer et al., 2001).

As mentioned above, dyes like SYBR Green I intercalate with any dsDNA. Nevertheless, differentiation of PCR products is possible by melting curve analysis, were the fluorescence is measured continuously while the temperature is raised. The strands of dsDNA become separated at a specific temperature resulting in a sudden decrease of fluorescence to the background level (Figure 3A-B). This melting temperature (T_m), determined as the maximum of the negative first derivative of the melting curve (Kubista et al., 2006), depends on GC/AT ratio, length and nucleotide sequence of the dsDNA fragment. Primer dimers are distinguishable due to significant differences in length compared to specific products, resulting in a lower T_m (Ririe et al., 1997). Differences in T_m of reaction products up to 2°C are resolvable, a change of 1°C is expected with a variances of 2.4% in GC content for instance (Wittwer et al., 2001). In contrast to gel electrophoresis, qPCR using intercalating dyes and subsequent melting curve analyses enables the discrimination of sequences of equal length but different GC/AT ratio (Ririe et al., 1997).

Contrary to differentiation by melting curves, colour multiplexing uses uniquely labelled probes (Figure 3C). A large number of fluorescent dyes are available currently; a selection of dyes used commonly and their optimal excitation and emission wavelengths is shown in Table 1.

A

B

C
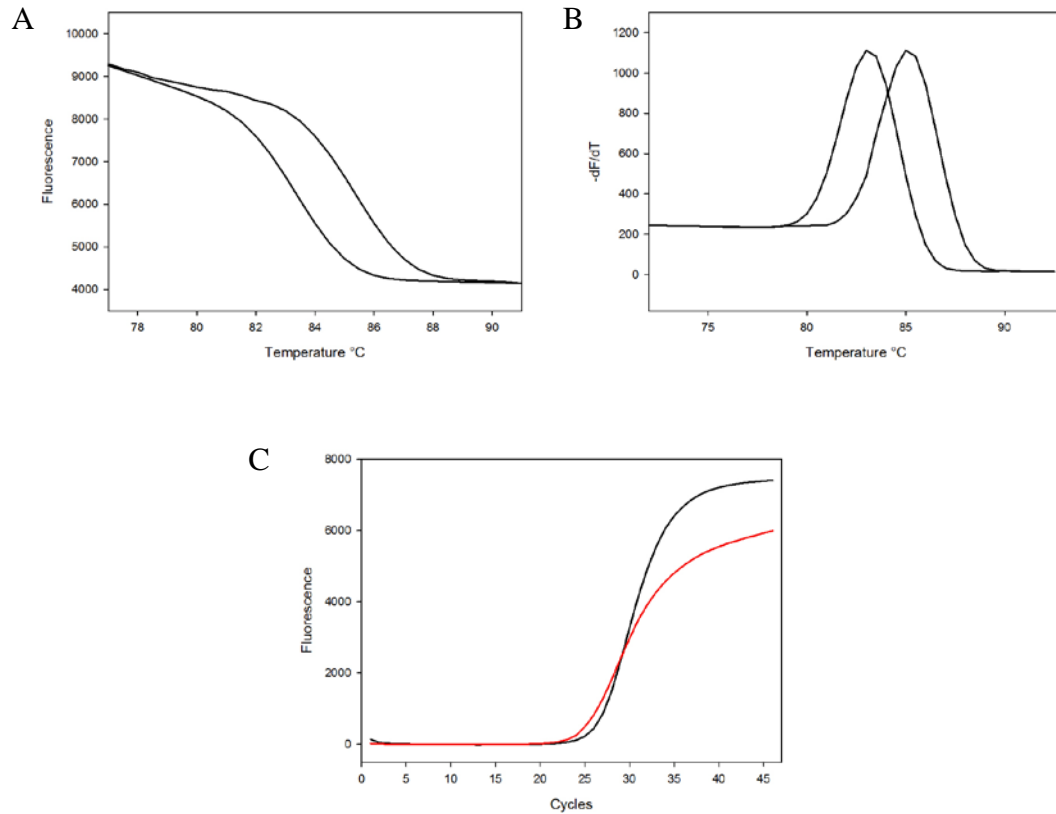
Fig. 3: Differentiation of two qPCR products in one reaction by melting
temperature (A-B) and colour (C). A depicts the melting curves and B the
negative first derivative of the melting curve.

Dye	Excitation maximum (nm)	Emission maximum (nm)
Alexa Fluor 350	346	442
ResoLight	487	503
6-carboxy-Fluorescein (**FAM**)	492	518
SYBR Green	497	520
EvaGreen	503	527
Tetrachloro-6-carboxy-fluorescein (**TET**)	521	538
2,7-Dimethoxy-4,5-dichloro-6-carboxyfluorescein (**JOE**)	520	548
Yakima Yellow	530	549
Hexachloro-6-carboxy-fluorescein (**HEX**)	535	556
Carbocyanin 3 (**Cy3**)	550	570
6-carboxy-tetramethyl-rhodamine (**TAMRA**)	544	576
6-carboxy-X-Rhodamin (**ROX**)	575	602
Sulforhodamine-101-acid-chloride (**Texas Red**)	589	615
Carbocyanin 5 (**Cy5**)	635	665

Table 1: Selection of fluorescent dyes used in qPCR. Dyes intercalating with
double-stranded DNA are printed in italics.

However, the spectra of individual dyes overlap thereby reducing the number of possible combinations. In multiplex assays dyes with excitation and emission wavelengths different enough to detect them separately have to be selected. Otherwise the increasing fluorescent signal in one detection channel can spread to a channel used to detect another dye, referred to as crosstalk, which results in a falsely positive result in the second channel (Gunson et al., 2008). Crosstalk is preventable by appropriate choice of dyes to be combined in a multiplex assay and proper PCR machine calibration including spectral compensation (Ishii, Sootome, and Yamashita, 2007; Wittwer et al., 2001). On the other hand, the number of detection channels in real-time PCR machines, currently four to five in most cases, limits the number of target sequences discriminable in a single reaction by colour multiplexing (Gunson, Collins, and Carman, 2006). Some wavelengths previously occupied by the emissions from the early quenchers themselves, have been made available by the development and application of non-fluorescent black hole quenchers (Mackay, 2004).

Multiplex PCR and qPCR offer a variety of possible applications. A common implementation for duplex PCR, the amplification of two targets, is the co-amplification of an internal control (IC) along with a pathogen-specific target. The usage of ICs is essential to verify efficient extraction of nucleic acids and to ensure the absence of PCR-inhibiting factors, especially when investigating potentially difficult biological matrices (Belak and Thoren, 2001; Hoffmann et al., 2009). Either heterologous internal controls, meaning nucleic acid of non-sample origin added to the sample during nucleic acid extraction (Hartman, Coyne, and Norwood, 2005; Hoffmann et al., 2005; Hoffmann et al., 2006; Niesters, 2004; Stranska et al., 2004), or ICs based on housekeeping genes are used. The latter are genes that are theoretically expressed steadily in different tissues and at all stages of development (Arya et al., 2005). Examples for housekeeping genes used in PCR diagnostics are beta-actin, a cytoskeletal structural protein, glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme, ubiquitin C, involved in protein degradation, beta-2-microglobulin, the beta-chain of major histocompatibility complex class I molecules, or hypoxanthine phosphoribosyl-transferase 1, an enzyme in purine nucleotide synthesis (Li et al., 2010b; Moniwa et al., 2007; Oleksiewicz, Donaldson, and Alexandersen, 2001; Toussaint et al., 2007;

Vandesompele et al., 2002; Wakeley et al., 2005). However, even housekeeping genes have variable messenger RNA (mRNA) concentrations, depending on the quality of the clinical sample.

1.4.   Optimization and validation

Optimization, the process by which the most important parameters of an assay are evaluated and adjusted to ensure that the performance characteristics of the assay are best suited to the intended application, and validation, the process that determines the fitness of an assay for an intended purpose (OIE, 2011; OIE - World Organisation for Animal Health), are crucial in the development of PCR assays. Already difficult in the development of single-target approaches, both processes are a huge challenge in the design of multiplex assays. Every primer pair and probe included in a multiplex PCR has to be optimized for identical reaction conditions, including temperature, concentration of dNTPs, magnesium chloride and template nucleic acid.

Major critical factors of influence are the sequence and concentration of primers. Designed to detect as many strains or types as possible of the target pathogen, false-positive results caused by closely related species have to be avoided. Initially optimized in a single-target reaction, unpredictable interactions such as reduced sensitivity or the formation of non-specific PCR products may occur when adding another primer pair (Gunson et al., 2008). The likelihood of obtaining non-specific products, primarily primer dimers, increases noticeably when using more than one primer pair per PCR reaction (Brownie et al., 1997). In addition, the preferential amplification of one of the targets in multiplex assays is a well known phenomenon (Mutter and Boynton, 1995; Polz and Cavanaugh, 1998). As PCR reactions have a limited supply of enzyme and dNTPs for which all products compete, the target which is amplified more efficiently negatively influences the yield of further products (Henegariu et al., 1997; Markoulatos, Siafakas, and Moncany, 2002). Possible explanations for the bias towards one template are differences in the GC content, causing preferential denaturation, and secondary structures in the target, leading to varying accessibility of the target region within the genome (Elnifro et al., 2000). Differences in GC/AT ratio between the primers cause unequal binding efficiencies. Consequently, the GC

contents of the primers combined in one assay, as well as their lengths, have to be dovetailed to result in nearly identical annealing temperatures. To reduce the amplification bias, the application of a reduced concentration of primers specific for the more efficient target is useful (Henegariu et al., 1997; Markoulatos et al., 2002). Additionally, the lower primer concentration reduces the likelihood of non-specific products. An increase of the length of primers, allowing higher annealing temperature, yields fewer non-specific products as well (Henegariu et al., 1997). In conclusion, the adjustment of the sequence and concentration of each primer specific for every target in the multiplex PCR is essential.

Apart from primers, the amount of DNA polymerase is critical in multiplex PCR assays. The application of higher enzyme concentrations compared to single-target assays significantly improves the reliability of multiplex systems (Persson, Hamby, and Ugozzoli, 2005). On the other hand an unbalanced amplification of the targets and an increase in background is observed when using too much enzyme, possibly caused by the high glycerol concentration in the stock solution (Henegariu et al., 1997; Markoulatos et al., 2002).

As the enzymatic activity of the DNA polymerase is magnesium-dependent an optimal amount within the reaction is required. Too much magnesium stabilizes dsDNA, preventing complete denaturation, and inadequately low concentrations result in a reduced quantity of the PCR product (Markoulatos et al., 2002). Due to magnesium binding, an excessive increase of dNTP concentrations inhibits PCR, however extremely low concentration results in a reduced amount of product. A balanced ratio between magnesium and dNTP is required (Henegariu et al., 1997). In general, variation in the concentration of each PCR component is greater in multiplex compared to single-target assays. However, the effects of individual PCR conditions have to be determined empirically (Gunson et al., 2006; Wittwer et al., 2001).

After development and optimization, the subsequent validation of a diagnostic assay for infectious diseases includes an estimation of its performance, comprising analytical and diagnostic sensitivity and specificity, repeatability and reproducibility among others (OIE, 2011).

2.   Bovine herpesvirus type 1

2.1.   Taxonomy and molecular characteristics

Cattle can be infected by various members of the family *Herpesviridae* (Trapp, Beer, and Mettenleiter, 2003), which is divided into the subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* by the International Committee on the Taxonomy of Viruses (Davison et al., 2009; Fauquet et al., 2005). Currently described herpesviruses are assigned to two subfamilies. BoHV-4 and BoHV-6 are regarded as *Gammaherpesvirinae* and BoHV-1, BoHV-2 and BoHV-5 as *Alphaherpesvirinae*. Characterized by a short reproductive cycle, rapid spread in culture, a wide host range and the ability to establish lifelong latency (Roizman et al., 1981; Roizmann et al., 1992), *Alphaherpesvirinae* are subdivided into the genera *Itovirus*, *Mardivirus*, *Simplexvirus* and *Varicellovirus*. Based on biological and genomic properties, BoHV-1, the prototype member of ruminant herpesviruses (Schwyzer and Ackermann, 1996), is classified as belonging to the genus *Varicellovirus*, together with equine herpesvirus 1 and 4 (EHV-1, EHV-4), suid herpesvirus 1 (pseudorabies virus, SuHV-1) and the ruminant herpesviruses BoHV-5, bubaline herpesvirus (BuHV), cervine herpesvirus 1 and 2 (CvHV-1, -2) and caprine herpesvirus 1 (CapHV-1), among others. BoHV-2 is assigned to the genus *Simplexvirus* (Davison et al., 2009; Fauquet et al., 2005).

Based on antigenetic properties and restriction enzyme digestion patterns, BoHV-1 isolates are classified into subtypes 1.1 and 1.2, the latter is further subdivided into BoHV-1.2a and BoHV-1.2b (Miller, Whetstone, and Van der Maaten, 1991). The former subtype 1.3, responsible for fatal meningoencephalitis in calves (Meyer et al., 2001), has been reclassified as distinct herpesvirus named BoHV-5 (Roizmann et al., 1992; Smith, Young, and Reed, 1995).

BoHV-1 particles, like typical herpesvirus virions, are approximately 150 to 200 nm in size and consist of four components, namely core, capsid, tegument and envelope (Roizman and Baines, 1991; Roizmann et al., 1992) (Figure 4).
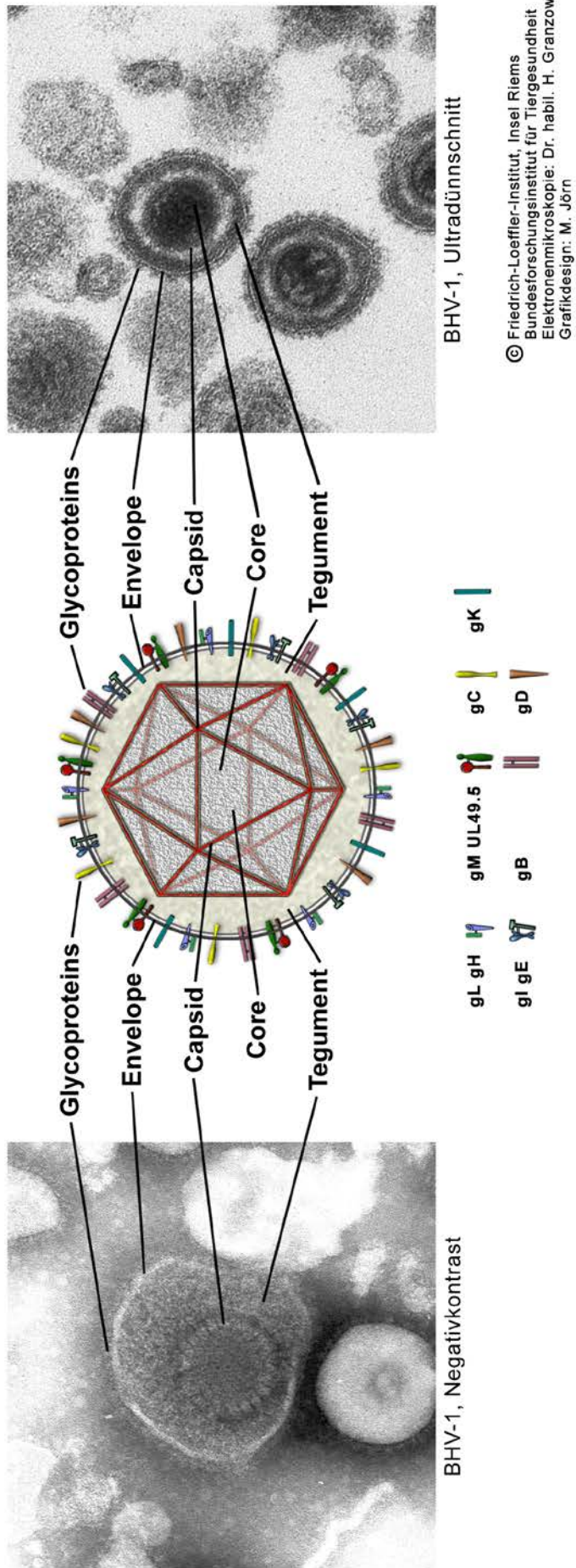.

Fig. 4: Virion morphology of BoHV-1, kindly provided by Dr. H. Granzow, Friedrich-Loeffler-Institut, Insel Riems, Germany.

The core, associated with the linear dsDNA genome, is included in an icosahedral capsid constructed of 162 capsomeres. Core and capsid form the nucleocapsid, which is surrounded by a layer of proteinaceous material, referred to as tegument. The latter, in turn, is enclosed in the outer lipid bilayer membrane of the virion, the envelope, which contains viral glycoproteins (de Oliveira et al., 2008; Mettenleiter, 2002).

The dsDNA genome of BoHV-1 is approximately 136,000 bp of size (Schwyzer and Ackermann, 1996) and contains a unique long (U_L) and a unique short segment (U_S). The latter is flanked by two inverted repetitive sequences, referred to as internal repeat (IR) and terminal repeat (TR). This genome organisation corresponds to class D of herpesvirus genomes, in which the U_L segment is predominantly arranged in one orientation, while the U_S region inverts its orientation relative to U_L resulting in two isomeric forms of virion DNA in equimolar amounts (Muylkens et al., 2007; Pellett and Roizman, 2007; Roizman, 1996; Wirth, Vogt, and Schwyzer, 1991) (Figure 5).

The majority of the BoHV-1 genome consists of genes homologous to other alphaherpesviruses and genes are generally labelled in accordance with the related genes of the prototype herpes simplex virus 1 (Muylkens et al., 2007). The BoHV-1 genome, comprising a GC content of 71.5% (Meyer et al., 1997), codes for at least 70 proteins, among them a number of enzymes, a group of regulatory proteins, major and additional glycoproteins (Muylkens et al., 2007; Nandi et al., 2009).
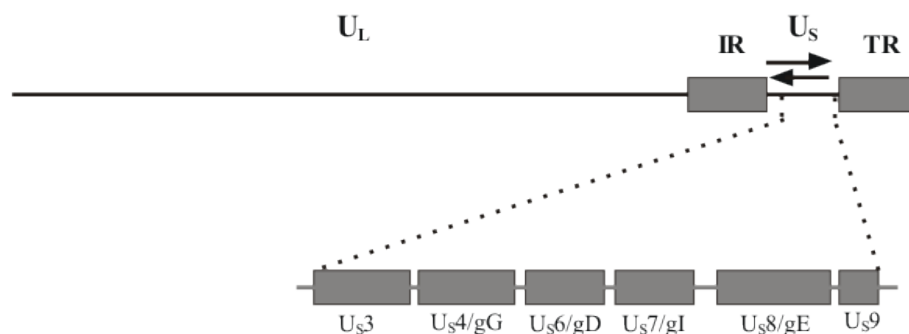

Fig. 5: Scheme of BoHV-1 genome, which is divided into a unique long segment (U_L) and a unique short segment (U_S) flanked by internal (IR) and terminal (TR) inverted repeats. Arrows indicate inversion of the U_S region resulting in two equimolar isomeric forms. (Beer et al., 2010a, modified)

2.2.   Glycoprotein E

Based on the impact of its deletion on the ability to grow in cell culture, every BoHV-1 gene is classified as either essential or non-essential. The deletion of essential genes results in a lethal non-replicative mutant, while the absence of non-essential genes allows further, but possibly stunted, growth *in vitro* (Muylkens et al., 2007). In BoHV-1, the glycoprotein E (gE = U_S8), located in the U_S region (Figure 2) and encoding a 575 amino acid (aa) polypeptide with a predicted molecular mass of 65 kiloDalton (Rebordosa et al., 1994), is known to be non-essential (Baranowski et al., 1996; Muylkens et al., 2006).

Due to the involvement of gE in direct cell-to-cell spread, gE-deleted mutants can only spread to neighboring cells via the extracellular fluid. This mechanism is less efficient and results in formation of smaller plaques (Rebordosa et al., 1996). Correlated with this observation, gE-deleted BoHV-1 strains are significantly less virulent in cattle compared to the parental wild-type strains (Kaashoek et al., 1998; van Engelenburg et al., 1994), due to a highly impaired neuronal spread.

2.3.   Pathogenesis, clinical disease, immunology

The majority of herpesviruses is associated closely with a single host species (Davison, 2002), BoHV-1 predominantly infects cattle. Nevertheless, other species of domestic or wild ruminants, such as goat, sheep, buffalo, bison, red deer and reindeer can be infected by BoHV-1 as well (Kalman and Egyed, 2005; Sausker and Dyer, 2002; Scicluna et al., 2010; Thiry et al., 2006).

Most commonly, BoHV-1 is transmitted directly by aerosol or by close contact between infected animals. The main sources of infection are nasal exudates, genital secretions, semen and foetal tissues. Indirect transmission by contaminated food or water may occur likewise (Beer, 2010a; Engels and Ackermann, 1996; Nandi et al., 2009). Portals of entry are the respiratory tract, the eyes and the genital tract.

After an incubation period of 2–7 days, a variety of syndromes may be caused by BoHV-1, namely infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) in cows and infectious balanoposthitis (IPB) in bulls, while a subclinical course may occur as well (Gibbs and Rweyemamu, 1977).

IBR is characterized by acute inflammation of the upper respiratory tract resulting

in fever up to 42°C, inappetence, depression, dyspnoea, coughing, sneezing, hyperaemia of the nasal mucosa and nasal discharge, which is initially serous and later muco-purulent. The mucosal membranes of eyes, mouth, or genital tract may be affected as well. Usually, animals recover within 2 weeks. The morbidity may amount to 100%. The low mortality rate of 2-10% may be increased by secondary infections, either of viral or bacterial origin (Gibbs and Rweyemamu, 1977; Nandi et al., 2009; Turin, Russo, and Poli, 1999; Yates, 1982).

Symptoms of the genital forms include fever, depression and painful urination. In cows, the vulva is swollen and hyperaemic with pustules and discharge. In bulls, the lesions on the mucosa of penis and prepuce are similar to those of IPV and may be associated with a decrease in semen quality. Usually, lesions resolve within two weeks in both cases, IPV and IBP (Turin et al., 1999).

After emerging in cattle holdings that include pregnant cows, abortions may occur from 3 to 6 weeks post infection, mainly between the fifth and eight month of pregnancy (Muylkens et al., 2007; Nandi et al., 2009; Turin et al., 1999). Furthermore, BoHV-1 may cause conjunctivitis associated with profuse lacrimation and, extremely rarely, meningoencephalitis. In those cases, clinical signs in affected animals comprise ataxia from stumbling to falling, depression, tremor, opisthotonus and clonic spasms among others. Coma and death often occur within four days after the onset of neurological symptoms (Gibbs and Rweyemamu, 1977).

Besides bovine viral diarrhoea virus and bovine parainfluenza virus 3, BoHV-1 is one of the pathogens involved in the multi-factorial bovine respiratory disease complex (BRDC), also referred to as shipping fever. The immunosuppression induced by BoHV-1 leads frequently to secondary bacterial infections with *Pasteurella haemolytica*, *Pasteurella multocida* and *Haemophilus somnus* for instance. These superinfections may even cause severe pneumonia with resulting casualties (Jones and Chowdhury, 2007).

In the hosts, BoHV-1 infection induces a specific immune response mediated by B- and T-cells, as well as a non-specific response, which is mediated by macrophages, polymorphonuclear neutrophils, natural killer cells, interferon and complement (Campos et al., 1989; Denis et al., 1993). The humoral immunity is becoming detectable from 8 days after infection. Specific antibodies against the

glycoproteins B and D are measurable starting 8-14 days post infection, while gE-specific antibodies are detectable for the first time 14-35 days after infection. This discrepancy is attributed to the reduced immunogenicity of gE (Beer, 2010a; Beer et al., 2003). Colostrum-derived maternal antibodies persist up to 9, in rare cases up to 12, months. Although protecting against clinical disease, maternal antibodies may inhibit the specific antibody production after vaccination (Lemaire et al., 2000; Menanteau-Horta et al., 1985). Immunity to BoHV-1 protects against clinical disease, not from infection and virus replication and shedding. Elimination of the virus is not achieved, because vaccination can not prevent lifelong latency in the sensory ganglia (Ackermann and Engels, 2006; Babiuk, van Drunen Littel-van den Hurk, and Tikoo, 1996).

2.4. Latency and reactivation

According to other alphaherpesviruses, BoHV-1 establishes a lifelong latent infection subsequent to an acute infection (Muylkens et al., 2007; Pastoret and Thiry, 1985). Latency is defined as a period without successful virus detection by standard virus isolation procedures and may last for whole life of the host. The latency-reactivation cycle may be divided into the major steps establishment, maintenance, and reactivation (Jones, 2003). After initial replication in either respiratory, prepuce or vagina mucosal epithelium the virus can enter the axons of local nerve cells. By subsequent intra-axonal transport, the viruses reach the neuron bodies in the regional ganglia, where latency can be established (Engels and Ackermann, 1996). Viral genomic DNA can be detected in the sensory ganglia of the trigeminal nerve or, in case of IPV/IPB, in sacral spinal ganglia (Ackermann and Wyler, 1984; Nandi et al., 2009). Latency may also occur in tonsillar lymphoid cells and peripheral blood lymphocytes (Mweene, Okazaki, and Kida, 1996). During latency, viral antigens are not synthesized, only one viral transcript is known to be expressed abundantly, the latency-related RNA (LR RNA), which inhibits apoptosis in transiently transfected cells (Ciacci-Zanella et al., 1999).

The latent virus, representing a long-term reservoir in hosts, can be reactivated by several stimuli such as stress, parturition, transport, superinfection with other pathogens or immunosuppression by glucocorticoids (Msolla et al., 1983; Nandi

et al., 2009; Pastoret et al., 1979; Thiry et al., 1987). The reactivated virus is transported intra-axonally to the original portal of entry resulting in recurrent disease and the possible transmission to other susceptible hosts (Engels and Ackermann, 1996; Jones, 2003).

2.5.   Diagnosis

Acute BoHV-1 infection may be suspected based on clinical and epidemiological findings. Nevertheless, a laboratory examination, using serological tests and/or virus detection, is required to reach a definite diagnosis (OIE, 2010). Serological tests include the virus neutralisation test and indirect or blocking enzyme-linked immunosorbent assays (ELISA). The various ELISAs permit the detection of antibodies in serum or plasma and, with lower sensitivity, in milk or bulk milk samples. During the acute phase of the infection virus may be isolated from nasal or genital swabs. In various cell cultures of bovine origin, the virus induces a cytopathic effect within 2–4 days. BoHV-1 is subsequently identified by neutralisation or antigen detection methods using monospecific antisera or monoclonal antibodies (OIE, 2010). Several conventional PCR assays have been developed for the detection of viral genome sequences in clinical samples such as nasal swabs, semen, blood, ganglia and organ specimens including lung and lymph nodes (de Gee et al, 1996; Santurde et al., 1996; van Engelenburg et al., 1993; van Engelenburg et al., 1995b; Wagter et al., 1996). Furthermore, an OIE-validated real-time PCR for BoHV-1 genome detection in extended bovine semen has been published (Wang et al., 2007). The diverse PCR assays enable the detection of BHV-1 prior to seroconversion. However, none of them, neither conventional nor real-time, enable the differentiation of vaccine strains and wild-type strains.

2.6.   Vaccination and control programs

BoHV-1 is distributed globally; however, significant differences in regional incidence and prevalence were found (Ackermann and Engels, 2006; Straub, 1991). Subtype 1 strains are present in Europe, North and South America. Subtype 2a has been isolated in Brazil and prior to the 1970s in Europe, while 2b strains are prevalent in Australia and Europe, but not Brazil (Edwards, White, and

Nixon, 1990; Jones and Chowdhury, 2007; Takiuchi et al., 2005; van Oirschot, 1995).

In several European countries control programs have been initiated with the aim of BoHV-1 elimination. As a result, the disease has been eradicated from Austria, Denmark, Finland, Sweden, Italy (Province of Bolzano), Switzerland, Norway and parts of Germany (Oberpfalz, Oberfranken, Unterfranken and Mittelfranken districts of Bavaria) according to OIE. Within the German control program, BoHV-1 infections are regulated by law since 1997 (Verordnung zum Schutz der Rinder vor einer Infektion mit dem Bovinen Herpesvirus Typ 1, BHV1-Verordnung; geänderte Fassung vom 20.12.2005). Long-term objectives are the elimination of the disease and prevention of re-emergence in cattle holdings free of BoHV-1. However, the starting situation differed widely between the federal states, in Bavaria for example more than 80% of herds were free of BoHV-1, while in Brandenburg or Saxony-Anhalt the amount of cattle holdings positive for BoHV-1 exceeded 50% (Anonymous, 2001). Depending on the prevalence, the initiated control programs were either based on detection and culling of animals tested positive or on vaccination (Beer, 2010c). The former is used in regions with low prevalence, e.g. Bavaria; the latter in federal states in which BoHV-1 infection is distributed widespread.

Apart from safety and an efficacious induction of protective immunity, the possibility to differentiate animals infected naturally from vaccinated animals is an important feature of vaccines, especially in the context of areas free of BoHV-1 and imposed trade restrictions in Europe (van Oirschot, 2001; Vannie et al., 2007). Vaccine candidates with deletions of the non-essential glycoproteins gC, gE, gI and gG were evaluated, gC-deleted strains remained too virulent, while the immunogenicity of gI-null mutants was not adequate (Kaashoek et al., 1998; van Engelenburg et al., 1994). In analogy to the effective DIVA (<u>d</u>ifferentiating <u>i</u>nfected form <u>v</u>accinated <u>a</u>nimals) strategy for eradication of SuHV-1, gE-deleted strains were eventually selected for use as marker vaccines (Strube et al., 1996; van Oirschot et al., 1996). Exhibiting little residual virulence, the relevant immune response against BoHV-1 are induced by these vaccines strains, with the exception of gE-specific antibodies (Strube et al., 1996; van Engelenburg et al., 1994). The vast majority of wild-type strains have shown to express gE (Rijsewijk

et al., 2000) and, as a consequence, induce a BoHV-1-specific immune reaction that includes antibodies against gE, which may be used for the serological differentiation of infected and vaccinated animals (Beer, 2010b). The DIVA strategy requires reliable test systems to identify vaccinated animals. In the case of BoHV-1, a gE-specific blocking-ELISA is widely used. The reduced sensitivity and specificity of the available gE-specific ELISA compared to the gB-specific ELISA (Beer et al., 2003; Geraghty et al., 2011; Kramps et al., 2004), is acceptable in early stages of vaccination-based control programs. In herds free of BoHV-1, a combination of serological tests may be necessary (Beer, 2010b; Vannie et al., 2007).

As far as replicating virus is concerned, the detection of gE-deleted strains from live marker vaccines and their reliable distinction from wild-type strains with conventional methods is based on isolation in cell culture and subsequent immunofluorescence staining or restriction enzyme fragment length polymorphism analysis. For genome detection of gE-deleted BoHV-1 strains conventional PCR assay have been developed (Fuchs et al., 1999; Schynts et al., 1999).

Due to consequent implementation of marker vaccination, good progress in BoHV-1 eradication was achieved in several federal states. In Brandenburg or Saxony-Anhalt the share of cattle holdings free of wild-type virus now exceeds 95% (Anonymous, 2011).

However, the disadvantages of gE-deleted BoHV-1 vaccine strains have to be considered. They prevent the development of clinical signs and markedly reduce the shedding of virus after infection with a wild-type strain, but the infection itself is not avoided completely (OIE, 2010). In addition, the intranasal inoculation of live-attenuated vaccine strains can also lead to latent infection (Kalthoff et al., 2010; Lemaire et al., 2001; van Engelenburg et al., 1995a). Furthermore, reactivation from latency and re-excretion of vaccine virus from the upper respiratory tract following experimental stimuli and in field conditions has been demonstrated (Dispas et al., 2003; Schynts et al., 2003; Straub, 1996). Hence, the monitoring of shedding and spread of wild-type BoHV-1 and the differentiation from live marker vaccine virus is an important measure within eradication programs. Furthermore, a reliable real-time PCR method is very useful e.g. in diagnostics of acute respiratory symptoms in cattle.

For the detection of recently applied or re-excreted gE-deleted BoHV-1 vaccine strains and the simultaneous detection and differentiation of wild-type strains no qPCR assay has been available. For this reason, a multiplex qPCR system, combining BoHV-1 gE- and gD-specific assays as well as an internal control based on the bovine beta-actin gene, was newly developed and fully validated following the OIE recommendations.

III.   RESULTS

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Development and validation of a triplex real-time PCR assay for the rapid detection and differentiation of wild-type and glycoprotein E-deleted vaccine strains of Bovine herpesvirus type 1

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Abstract

Two important components of control programs for Bovine herpesvirus type 1 (BoHV-1), a major pathogen of cattle, are the detection of outbreaks and vaccination with glycoprotein E (gE)-deleted marker vaccines. In addition to serology, rapid and accurate investigation of BoHV-1 and genetic differentiation of vaccine and wild-type strains are also important methods. Therefore, a triplex quantitative real-time polymerase chain reaction (qPCR) for testing BoHV-1 was developed. Apart from a BoHV-1 specific glycoproteinD (gD) assay, a gE-specific system for differentiation between wild-type BoHV-1 and gE-deleted vaccine strains was established. Finally, an internal control, based on the beta-actin gene was introduced successfully completing the multiplex system.

The triplex BoHV-1-qPCR has an analytical sensitivity of less than 10 genome copies per reaction, and the diagnostic sensitivity was equal to or even greater than that of the 'gold standard' method of virus isolation in cell culture. A series of reference strains, including gE-deleted BoHV-1 and field isolates were detected reliably. For validation of the specificity of the test, nasal swabs, semen and different organ material from cattle, negative for BoHV-1, and genetically related herpesvirus strains were examined.

The new multiplex BoHV-1-specific qPCR system allows highly sensitive and rapid genetic detection and differentiation of BoHV-1 and will be a valuable method for the control of BoHV-1 infection.

1. Introduction

Bovine herpesvirus 1 (BoHV-1), an *Alphaherpesvirus*, is a major pathogen of cattle causing a variety of syndromes such as Infectious Bovine Rhinotracheitis (IBR), Infectious Pustular Vulvovaginitis (IPV) in cows, and Infectious Balanoposthitis (IBP) in bulls (Gibbs and Rweyemamu, 1977).

After acute infection, the virus can enter neural cells and establish a life-long latent infection in the sensory ganglia of the nervous system (Muylkens et al., 2007). The latent virus can be reactivated by several stimuli such as stressful conditions (Thiry et al., 1987) or immunosuppression with glucocorticoids (Pastoret et al., 1979). The resumption of lytic BoHV-1 replication in animals infected latently is responsible for the maintenance of BoHV-1 within cattle herds and for the introduction of the infection into free holdings (Winkler et al., 2000).

Control programs for BoHV-1 include vaccination with inactivated or modified live gE-deleted marker vaccines. These vaccines allow differentiation between cattle infected naturally and vaccinated cattle (van Oirschot et al., 1996) in analogy to the DIVA (differentiating infected from vaccinated animals) strategy for eradication of pseudorabies virus (SuHV) (Stegeman, 1995). Nevertheless, gE-deleted BoHV-1 strains are also shed, e.g. following intranasal inoculation, and can establish latent infections (Lemaire et al., 2001; van Engelenburg et al., 1995a; Kalthoff et al., 2010). It has also been demonstrated that gE-negative vaccine viruses can be reactivated from latency and re-excreted from the upper respiratory tract (Straub, 1996). Therefore, it is important to develop methods for monitoring the shedding and spread of live marker vaccine viruses. Especially for diseased cattle or animals succumbed to the disease, a diagnostic instrument for rapid differentiation between injected recently or reactivated marker vaccine virus and infection with a BoHV-1 field virus is crucial for determination of the cause of the disease and for the implementation of protective measures against wild-type BoHV-1 infection.

Various PCR assays for detection of BoHV-1 have been described (de Gee et al., 1996; van Engelenburg et al., 1993, 1995b; Wagter et al., 1996). Compared to conventional PCR, real-time quantitative PCR assays (qPCR) have several advantages, such as a reduced risk of cross-contamination in the absence of

post-amplification handling of samples, semi-quantitative analysis, and simultaneous detection of several target sequences using multichannel analysis (Mackay, 2004; Mackay et al., 2002; Wittwer et al., 2001). An OIE-validated qPCR for detection of BoHV-1 glycoprotein B-specific sequences in extended bovine semen has been developed by Wang et al. (2007).

The aim of this study was to develop a multiplex qPCR method for simultaneous detection of BoHV-1 gD- and gE-specific sequences combined with an internal control system. The new triplex BoHV-1 qPCR assay allows BoHV-1-specific detection and differentiation between wild-type BoHV-1 strains (gE-positive) and gE-negative vaccine viruses in clinical samples such as nasal swabs and bovine semen. In addition, an internal control (IC) based on the beta-actin gene was included to verify efficient DNA extraction and PCR amplification.

2. Materials and methods

2.1. Virus strains and samples for diagnosis

A series of bovine herpesviruses including both BoHV-1.1 and BoHV-1.2 reference strains, gE-deleted BoHV-1 vaccine virus strains, BoHV-1 field isolates, BoHV-2, BoHV-4 and BoHV-5 were used in this study. In addition, cervid (CvHV-1, CvHV-2), bubaline (BuHV-1), caprine (CapHV-1), equine (EHV-1), and porcine (SuHV-1) herpesviruses were tested. All strains were provided by the virus collection of the Friedrich-Loeffler-Institut, Insel Riems, Germany or kindly provided by Günther Keil (Friedrich-Loeffler-Institut, Insel Riems, Germany). BoHV-1 positive nasal swabs, serum, tissue samples and spiked negative semen were tested as well as samples from BoHV-1 negative cattle.

2.2. Virus isolation

Virus isolation was carried out using MDBK cells (Rie261, Collection of cell lines in veterinary medicine, CCLV, Friedrich-Loeffler-Institut, Insel Riems, Germany) according to the OIE manual (OIE, 2010).

2.3. DNA extraction

DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics Deutschland, Mannheim, Germany) according to the manufacturer's recommendations. Nasal swab samples were obtained from an animal experiment, where marker vaccinated and mock vaccinated animals were challenged with a BoHV 1.1 wild-type virus (Kalthoff et al., 2010). The animal experiment was reviewed and approved by the responsible State ethics committee. DNA was extracted with the NucleoSpin® 96 Virus Core Kit for automated extraction (Macherey-Nagel, Düren, Germany) and eluted in 100µl buffer finally.

2.4. Primers, probes and qPCR

For the selection of primers and probes, published sequences of BoHV-1 (NCBI database) were used and alignment-based primer and probe selection was supported by the software package Beacon Designer 2.06 (Premier Biosoft International, Palo Alto, CA, USA). The selected gD-sequences are highly conserved for BoHV-1, but to a lesser extent within the closely related ruminant alphaherpesviruses. The design of the gE-specific assay was optimized according to the respective gE-gene deletions in the different commercial BoHV-1 marker vaccines. The sequences of primers and probes are summarized in Table 1.

Name	Sequence 5'- 3'	Genome position[a]	Reference
BHV1-gD5595-F	CCGCCGTATTTTGAGGAGTCG	119658-119678	this study
BHV1-gD5704-R	TCGGTCTCCCCTTCRTCCTC	119767-119748	
BHV1-gD-TEX	Texas Red-TACGAGCCGCCGCCTGCCGC-BHQ2	119685-119704	
BHV1-gE3-883F	CAATAACAGCGTAGACCTGGTC	122739-122760	this study
BHV1-gE3-989R	GCTGTAGTCCCAAGCTTCCAC	122844-122824	
BHV1-gE3-FAM	FAM-TGCGGCCTCCGGGCTTTACGTCT-BHQ1	122778-122800	
[b]ACT2-1030-F	AGCGCAAGTACTCCGTGTG	2679-2697	Modified
ACT-1135-R	CGGACTCATCGTACTCCTGCTT	2753-2774	Toussaint et al.(2007)
ACT-1081-HEX	HEX-TCGCTGTCCACCTTCCAGCAGATGT-BHQ1	2720-2744	
gB-F (forward)	TGTGGACCTAAACCTCACGGT	57499–57519	Wang et al. (2007)
gB-R (reverse)	GTAGTCGAGCAGACCCGTGTC	57595–57575	
Probe (BoHV-1)	FAM-AGGACCGCGAGTTCTTGCCGC-TAMRA	57525–57545	

Table 1: Sequences of primers and probes used in the study.
  [a] BoHV-1: genome position according to Bovine herpesvirus type 1.1 complete genome (GenBank accession number AJ 004801).
  [b] ACT: genome position according to Bos taurus chromosome 25 (GenBank accession number NC 007326).

All oligonucleotides were synthesized by Metabion International (Planegg-Martinsried, Germany). The primer ACT-1135-R and the probe ACT-1081-HEX for actin detection were used as described by Toussaint et al. (2007). The primer ACT2-1030F was designed with the Beacon Designer 2.06 software package using published sequence information of the bovine ACTB-gene. In contrast to the mRNA-specific forward primer by Toussaint, ACT2-1030F also amplifies genomic β-actin DNA.

The triplex BoHV-1 qPCR was carried out using the Quanti-Tect Multiplex PCR NoROX Kit (Qiagen, Hilden, Germany). The triplex qPCR assay was optimized using a total reaction volume of 25 µl. For a single reaction, 1.5 µl RNase-free water, 12.5 µl 2× QuantiTect Multiplex PCR noRox Master Mix, 2 µl gD-specific Texas Red-labelled primer-probe-mix (10 pmol/µl BHV1-gD5595-F, 10 pmol/µl BHV1-gD5704-R, 1.25 pmol/µl BHV1-gD-TEX), 2 µl gE-specific FAM-labelled primer-probe-mix (10 pmol/µl BHV1-gE3-883F, 10 pmol/µl BHV1-gE3-989R, 1.875 pmol/µl BHV1-gE3-FAM) and 2µl beta-actin-specific HEX-labelled primer-probe-mix (2.5 pmol/µl ACT2-1030-F, 2.5 pmol/µl ACT-1135-R, 1.25 pmol/µl ACT-1081-HEX) were merged as master mix. Finally, 5 µl DNA template was added and qPCR was carried out using a Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad, Hercules, CA, USA). The following thermal profile was used:

PCR initial activation step at 95 °C for 15 min; 42 cycles of three-step cycling consisting of denaturation at 95 °C for 1min, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s.

To compare the results with an established method, a BoHV-1 gB-specific qPCR was performed as described by Wang et al. (2007) using the identical DNA template. However, Wang et al. (2007) used DNA extracted by the Chelex method, therefore the qPCR has been modified regarding the extraction method. For a single reaction, 5.5 µl RNase-free water, 12.5 µl 2× Platinum Quantitative PCR SuperMix-UDG master mixture (Invitrogen, Carlsbad, CA, USA) and 2 µl gB-specific primer-probe-mix (4.5 pmol/µl of each primer, 3.0 pmol/µl probe) were pooled as master mix and 5 µl DNA template were added.

All samples were tested as duplicates or triplicates, while all standard dilution series were tested in quadruplicates. Samples were considered positive when the Cq value was equal or less than 40.

2.5. Positive BoHV-1 standard DNA

Supernatant of BoHV-1-infected cell culture was harvested and cleared by centrifugation (1500 x g, 4°C, 15 min). Subsequently the remaining supernatant was weighed and PEG6000 (Sigma, Germany) was filled up to 8% of the total weight. The solution was stirred at room temperature until PEG6000 was dissolved. Another stirring step at 4°C overnight was performed and after that the solution was cleared by centrifugation (3650 x g, 4°C, 30 min). Pelleted material was resuspended in an appropriate volume of NTE-buffer (150mM NaCl, 50mM Tris.HCl, 1mM EDTA). Purified DNA was extracted from this starting material by High Pure PCR Template Preparation Kit (Roche Diagnostics Deutschland, Mannheim, Germany), the concentration was determined by spectrophotometry, and the exact number of DNA molecules was calculated supported by an online program provided at http://www.molbiol.edu.ru/eng/scripts/01_07.html.

2.6. Synthetic positive control DNA

In order to generate quantifiable, stable and consistent standards, synthetic oligonucleotides were designed. To exclude potential cross-contaminations, the peak fluorescent signal for the positive control should be markedly lower than for positive samples. This was achieved by the artificial introduction of mismatches into the synthetic oligonucleotides. A variable number of mismatches in different positions of the probe-binding region were tested.

3. Results

3.1. Sensitivity

The analytical sensitivity of the triplex BoHV-1 qPCR was determined using a series of 10-fold dilutions of the standard BoHV- 1.2-DNA. The assay amplified the DNA in a linear fashion from 10^6 copies down to 10^1 copies per reaction with an efficiency of more than 97% (Fig. 1).
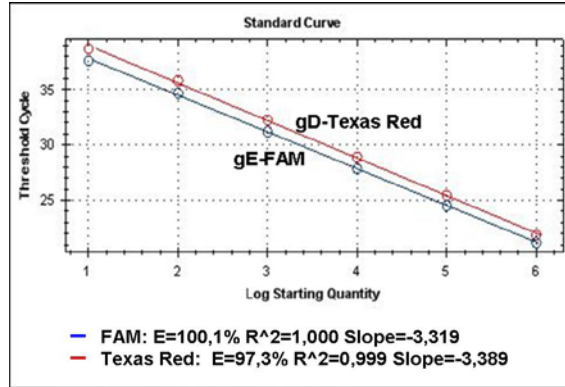
Fig. 1: Analytical sensitivity of the BoHV-1 triplex assay based on 10-fold dilution series of the purified BoHV-1 strain "Schoenboeken" standard DNA. A regression curve was estimated by Bio-Rad CFX Manager™ Software (Bio-Rad, Hercules, CA, USA). Samples were tested in quadruplicates.

Comparison of the amplification efficiencies of the gE- and gD-target sequences revealed comparable quantification cycle (Cq; Bustin et al., 2009) values with only a marginal preference for the gE-amplification (Table 2). An identical analytical sensitivity was achieved using gD and gE plasmid standards (data not shown). The sensitivity of the new triplex-BoHV-1 qPCR was compared to virus isolation and the published gB-specific PCR (Wang et al., 2007). In addition, 10-fold dilution series of BoHV-1 in cell culture medium and in extended semen were analysed by virus isolation, the gB-specific qPCR, and the triplex BoHV-1 qPCR system. For all preparations, the sensitivity of the qPCR was equal to or greater compared to the 'gold standard' method of virus isolation. In addition, both PCR-systems, the gB-specific assay and the triplex BoHV-1 PCR, showed comparable results (Table 2).

The diagnostic sensitivity was evaluated by testing different sample types including diagnostic submissions to the OIE and National Reference Laboratory for BoHV-1, Friedrich-Loeffler-Institut Insel Riems, Germany (cell culture material, nasal swabs; see Table 3), and nasal swabs obtained from an experimental BoHV-1 infection (Kalthoff et al., 2010).

The nasal swabs from BoHV-1-infected calves showed comparable results when tested with the triplex BoHV-1 qPCR and the gB-specific assay by Wang et al. (2007). However, three very weak positive samples scored negative by the

gD-specific assay, whereas the gE-specific assay gave a positive result (Table 4). In one of these samples the gB-specific PCR showed a positive result. Infected calve 587 was negative by the gD-, gE- and gB-specific PCR on 7 dpi and again positive on 5 and 9 dpi, calve 605 gave a negative result on 5 dpi, but was positive on 3 and 7 dpi.

Dilution of BoHV 1	Virus isolation /positive isolations/ number of replicates)		Mean [a]Cq of two extractions			
			Multiplex BoHV‑1 assay			gB
			gD	gE	β-Actin	
In medium						
10^{-2}	2/2	2/2	24.59	23.92	27.66	24.91
10^{-3}	2/2	2/2	27.74	27.01	30.15	28.00
10^{-4}	2/2	2/2	31.03	30.10	32.43	31.16
10^{-5}	2/2	2/2	34.32	33.21	33.61	34.75
10^{-6}	2/2	2/2	38.11	36.99	34.34	37.39
10^{-7}	0/2	0/2	>40	39.59	35.02	39.64
10^{-8}	0/2	0/2	>40	>40	35.29	>40
10^{-9}	0/2	0/2	>40	>40	34.91	>40
In negative semen						
10^{-1}	2/2	2/2	24.60	24.94	32.76	27.24
10^{-2}	2/2	2/2	28.20	28.22	32.38	30.72
10^{-3}	2/2	2/2	31.41	31.09	33.24	33.53
10^{-4}	2/2	2/2	34.43	33.70	33.87	36.71
10^{-5}	2/2	2/2	39.40	37.69	33.15	>40
10^{-6}	0/2	0/2	>40	>40	33.88	>40
10^{-7}	0/2	0/2	>40	>40	34.00	>40
10^{-8}	0/2	0/2	>40	>40	33.56	>40

Table 2: Sensitivity of the triplex BoHV-1 qPCR assay compared to virus isolation or the BoHV 1-gB assay (Wang et al., 2007).
[a] Cq = quantification cycle.

3.2. Specificity

All BoHV-1 field virus strains tested were positive by the gD-specific PCR as well as by the gE-specific system with comparable Cq values, whereas all different gE-deleted vaccine strains scored negative by the gE-specific assay. The BoHV-1 strains were positive with similar Cq-values compared to the published gB-specific assay (Table 3).

For validation of the diagnostic specificity of the BoHV-1 triplex qPCR assay, 41 nasal swabs, 24 serum samples, 10 semen samples and 17 different tissue samples from BoHV-1 negative cattle were tested. All control samples were clearly negative by the gD-specific system as well as by the gE-specific assay with unequivocal positive results by the beta-actin PCR.

Virus	Strain/isolate	Multiplex BoHV‑1 assay			gB
		gD	gE	β-actin	
		[a]Cq	Cq	Cq	Cq
BoHV‑1	Schoenboeken 998	25.33	24.05	31.88	24.46
	Wangen	28.56	28.60	30.96	29.87
	California	26.01	25.49	30.60	27.02
	Würzburg	26.33	25.79	31.11	27.10
	Calw	27.34	26.92	31.47	28.23
	Schleswig Holstein	26.62	26.01	31.42	26.63
	Intervet Lebendvacc	26.99	26.46	31.68	27.76
	Lam	26.07	25.22	31.06	25.76
	B30/01	27.10	26.41	31.18	27.34
	RIAM (conventinal vaccine strain)	26.05	24.53	30.49	24.74
	Cooper	27.55	26.74	32.35	27.65
	2204	26.29	25.85	30.75	26.50
	AUS12	27.83	25.83	30.49	26.18
	Schoenboeken	27.89	26.91	30.88	27.66
	RVB 008 SV 0990	27.07	25.99	29.24	27.01
	RVB 091 33/81 II	28.07	27.57	32.22	28.31
	V RVB 095 35097	27.77	27.10	31.28	28.25
	RVB 097 Elö-Vakzinevirus	26.66	26.30	31.07	27.31
	RVB x128 HW 2/80	27.07	26.38	30.41	27.10
	RVB x129 IBR 9/87	30.01	29.44	33.89	30.41
	RVB 191 TV5/84	28.59	28.06	32.84	28.90
	RVB 366 Los Angeles	26.78	26.36	31.11	27.34
	40F2	29.00	28.08	33.97	28.90
	4393	27.68	27.15	31.95	28.09
	11456	28.01	27.19	32.75	27.97
	994	27.38	26.89	32.77	27.15
	10295	29.56	29.03	32.71	29.37
	10678	27.82	27.18	32.57	27.91
	11850/1	27.39	26.91	32.83	27.03
	3935	28.33	27.43	32.71	28.23
	4073	28.48	27.52	33.32	28.19
	SD 23/01	28.88	27.96	31.71	28.24
	RD 1824 I	29.54	28.70	32.05	29.36
	NB 441/01 I	27.28	26.72	32.35	27.09
	RI 892/01	30.68	29.78	32.53	30.94
	SD 8	28.09	27.46	32.53	28.03
	ND 653	27.15	25.05	34.60	25.01
	RI 1129/00	28.24	25.66	35.39	25.63
	NB 394	25.94	24.51	33.64	24.00
BoHV‑1ΔgE	BAYOVAC® IBR-MARKER VIVUM, Bayer	26.71	>40	31.93	27.76
	Bovilis® IBR, Intervet	22.77	>40	35.53	26.06
	Rispoval® IBR-Marker vivum, Pfizer	16.98	>40	25.05	20.32
BoHV-2		>40	>40	30.61	>40
BoHV-4		>40	>40	24.93	>40
BoHV-5		>40	>40	31.41	25.07
BuHV-1		>40	26.75	29.97	23.48
CvHV-1		>40	33.67	33.02	25.74
CvHV-2		>40	33,12	29.80	25.32
CapHV-1		>40	>40	31.01	34.18
EHV-1		>40	>40	29.90	>40
SuHV-1		>40	>40	28.13	>40

Table 3: Virus strains and isolates analysed by the triplex qPCR and by the
gB-specific assay designed by Wang et al. (2007).
[a] Cq = quantification cycle.

Animal	dpi	[a]Cq values multiplex BoHV 1 assay			
		gD	gE	β-actin	gB
Vaccinated animals post infection with BoHV-1					
587	0	>40	>40	26.55	>40
	1	31.65	31.06	28.99	32.14
	3	25.34	25.25	27.57	25.92
	5	22.90	23.07	25.66	24.16
	7	>40	>40	26.20	>40
	9	31.96	30.99	27.82	32.01
	11	>40	>40	28.39	>40
	13	>40	>40	27.58	>40
	15	>40	>40	27.90	>40
590	0	>40	>40	28.33	>40
	1	>40	>40	29.88	>40
	3	26.84	26.78	29.17	27.73
	5	27.25	27.10	28.22	28.09
	7	>40	35.14[b]	30.41	>40
	9	27.14	26.54	26.92	27.18
	11	>40	>40	28.74	>40
	13	>40	>40	30.45	>40
	15	>40	>40	28.12	>40
600	0	>40	>40	22.32	>40
	1	28.60	27.93	25.43	29.13
	3	>40	34.20	27.31	35.47
	5	33.94	33.71	28.13	35.00
	7	>40	>40	23.88	>40
	9	>40	>40	28.06	>40
	11	>40	>40	29.92	>40
	13	>40	>40	29.38	>40
	15	>40	>40	30.39	>40
605	0	>40	>40	28.40	>40
	1	29.24	28.95	28.86	29.72
	3	35.36	33.91	31.00	34.45
	5	>40	>40	31.01	>40
	7	31.08	29.67	29.68	30.88
	9	>40	>40	29.85	>40
	11	>40	>40	30.17	>40
	13	>40	>40	30.32	>40
	15	>40	>40	29.79	>40
Mock-vaccinated animals post infection with BoHV-1					
117	0	>40	>40	30.34	>40
	1	30.09	29.47	30.03	30.77
	3	25.05	25.35	27.57	26.06
	5	24.77	24.70	27.52	25.70
	7	22.88	22.63	25.55	23.52
	9	27.08	26.37	27.58	29.04
	11	32.47	31.45	30.22	32.82
	13	>40	>40	29.76	>40
	15	>40	>40	29.79	>40
220	0	>40	>40	31.77	>40
	1	26.32	26.42	31.17	27.26
	3	28.29	28.06	31.04	28.70
	5	21.07	21.20	24.61	21.75
	7	24.17	23.92	26.52	24.50
	9	29.40	28.59	27.28	29.42
	11	29.84	29.09	27.82	29.96
	13	>40	35.57[b]	30.24	>40
	15	>40	>40	31.17	>40

581	0	>40	>40	31.74	>40
	1	26.13	26.23	29.31	27.15
	3	25.07	25.00	28.62	25.43
	5	23.88	23.86	26.97	24.77
	7	23.19	22.96	25.53	23.63
	9	29.11	29.11	28.52	29.91
	11	35.69	33.78	29.27	35.55
	13	>40	>40	29.61	>40
	15	>40	>40	30.14	>40
593	0	>40	>40	30.05	>40
	1	28.89	28.34	32.23	27.53
	3	22.69	22.62	25.97	23.21
	5	21.41	21.29	24.51	21.83
	7	23.58	23.11	24.94	23.84
	9	28.93	28.01	27.96	29.11
	11	30.31	29.23	27.53	29.92
	13	34.31	32.33	28.89	34.08
	15	>40	>40	27.97	>40

Table 4: Analyses of nasal swabs from calves infected with BoHV-1 by the triplex qPCR and by the gB-specific assay described by Wang et al. (2007).
[a] Cq = quantification cycle.
[b] Only one of the duplicates scored positive.

In addition, the triplex qPCR assay was carried out with DNA from a series of further herpesviruses, namely CvHV-1, CvHV-2, BuHV-1, CapHV-1, EHV-1 and SuHV-1, with a special emphasis on closely related ruminant alphaherpesviruses. All non-BoHV-1 herpesviruses were negative by the gD-specific qPCR and most of the non-BoHV-1 herpesviruses tested negative by the gE-specific assay. Interestingly, *Bubaline herpesvirus 1* and *Cervid herpesvirus 1* and *2* were positive with the gE-specific assay. However, in the published gB-specific assay BoHV-5, CapHV-1, BuHV-1, CvHV-1, and CvHV-2 were positive, demonstrating a much greater BoHV-1-specificity of the triplex qPCR. In addition, other common bovine viruses such as bovine viral diarrhea virus, bovine respiratory syncytial virus, parainfluenza virus and bluetongue virus were tested and both the gD- and gE-specific assay showed no nonspecific reactivity.

3.3. Reproducibility and repeatability

Intra-assay reproducibility of the new triplex qPCR was assessed with four replicates, of a 10-fold dilution series of standard BoHV-1.2 DNA.
The inter-assay repeatability was determined with 10-fold dilution series on four separate days (Fig. 2). It was shown that 10 copies per reaction were detected reliably in the gD- as well as in the gE- specific assay. Mean values, standard deviations and coefficients of variation are shown in Fig. 2.
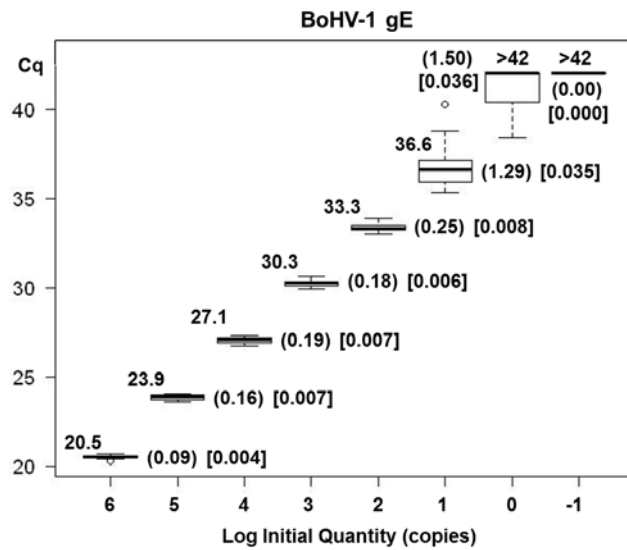
a
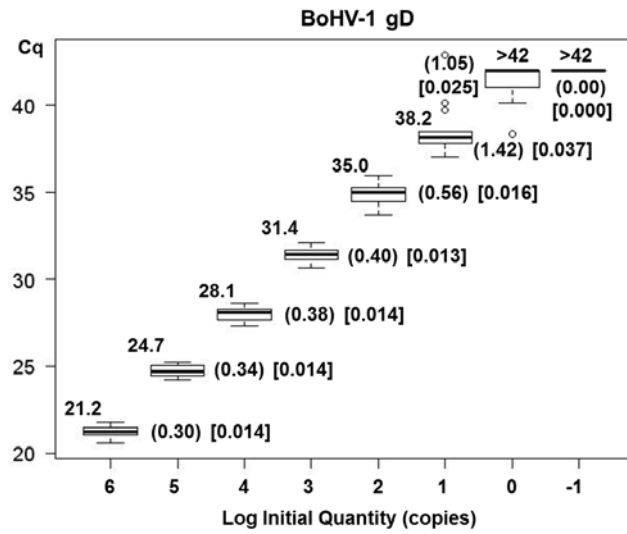
b

Fig. 2: Reproducibility and repeatability of the triplex BoHV-1 qPCR. The Cq values (y-axis) of 16 replicates of the BoHV-1 gD-assay (a) and of the BoHV-1 gE-assay (b) using a 10-fold dilution series of the BoHV-1 "Schoenboeken" standard DNA (x-axis: log 10 initial quantity) are shown. Mean values are indicated alongside of each boxplot, standard deviations are depicted in parentheses and coefficients of variation in square brackets. Boxplots were designed by the R software (R Development Core Team, 2009)

3.4. Synthetic positive Control

In order to design a positive control differentiable from positive samples by a lower peak fluorescent signal, a variable number of mismatches in different positions of the probe-binding region were introduced into synthetic oligonucleotides (sequences are shown in Table 5). As shown in Fig. 3, the selected synthetic controls indeed allowed a differentiation of control DNA from natural BoHV-1 DNA due to the markedly lower fluorescence signals.

Name	Sequence 5'-3'
oLPC12-BHV1-gD	gaattcga**CCGCCGTATTTTGAGGAGTCG**tacagc
	TACGAGCCGCGGCCTGCCGG
	gatga**GAGGATGAAGGGGAGACCGA**atttcacg
oLPC3-BHV1-gE	gaattcga**CAATAACAGCGTAGACCTGGTC**tacagc
	TGCGGCCTCCGCGCTTTAGGTCT
	gatga**GTGGAAGCTTGGGACTACAGC**atttcacg

Table 5: Sequences of synthetic oligonucleotides used as positive controls. Oligo binding regions are depicted in capitals, probe binding sites are underlined. Mismatches in the probe region are highlighted in grey.

3.5. Internal Control (IC)

For validation of the IC-PCR, a comprehensive panel of tissue samples and body fluids such as nasal swabs, semen, and serum from BoHV-1 negative cattle were examined and all were positive by the β-actin specific qPCR assay (Table 5). In addition, reproducible unaltered positive reactions were obtained with all herpesvirus DNA preparations tested (Table 3) and gE- and gD- positive nasal swabs from BoHV-1 infected calves (Table 4). The amplification efficacy of the internal control-DNA was not affected by simultaneous co-amplification of BoHV-1 specific sequences. Reproducibility and repeatability were assessed with four replicates of cattle DNA extracted from blood on four separate days and the mean value was 33.08 with a standard deviation of 0.57.
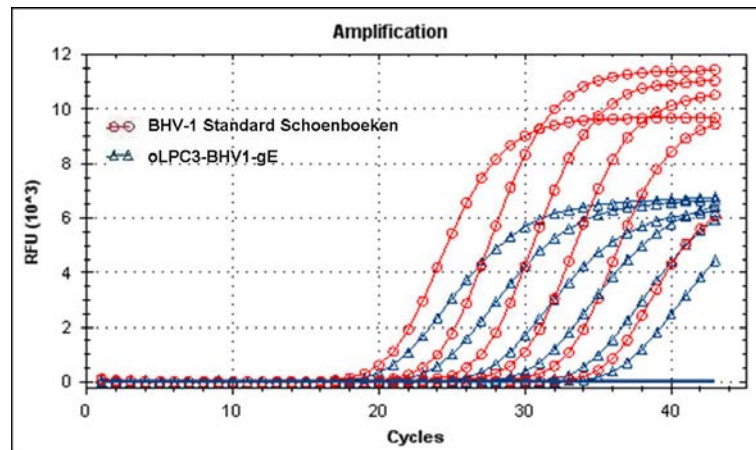
a
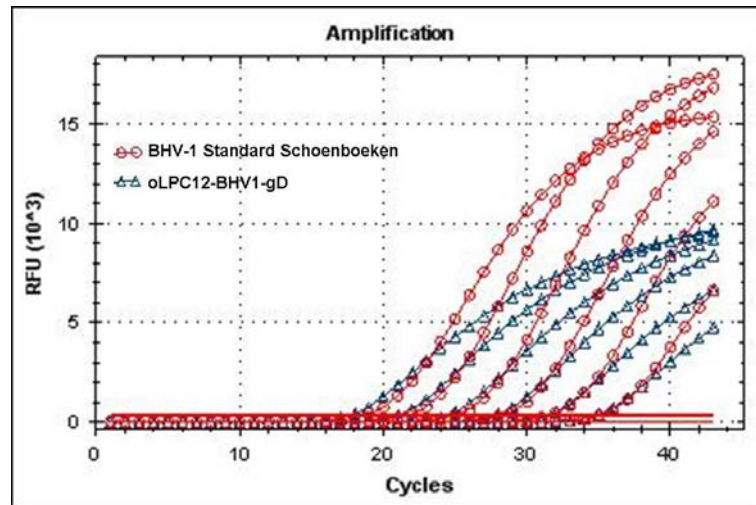
b

Fig.3: Amplification plots of 10-fold dilution series of standard BoHV-1-DNA and BoHV-1 gD- (a) and BoHV-1 gE- (b) positive synthetic controls tested with the triplex-BoHV-1 qPCR assay.

4. Discussion

In this study, a triplex qPCR-assay for simultaneous detection of BoHV-1 gD- and gE-specific sequences was developed and validated. The new assay was designed to enable differentiation of BoHV-1 wild-type viruses and gE-deleted vaccine strains, in combination with an internal control based on the amplification of the bovine β-actin gene (Toussaint et al., 2007; Hoffmann et al., 2009a).

Various conventional PCR assays have been described for the detection of BoHV-1 genomes in clinical samples such as bovine semen (van Engelenburg et al., 1993; Wagter et al., 1996), nasal swabs (de Gee et al., 1996), blood, ganglia (van Engelenburg et al., 1995a), and organ specimens such as lung and lymph nodes (Santurde et al., 1996). None of these assays, however, differentiate between infected animals and animals shedding an attenuated gE-deleted vaccine strain. A gE-specific conventional PCR-assay, which detects up to 3000 molecules of viral genomes per reaction has been developed by Schynts et al. (1999). Nevertheless, BuHV-1, CapHV, CvHV-1 and -2 could be diagnosed falsely as BoHV-1 gE-negative strains by this PCR assay.

Compared to conventional PCR, qPCR assays have several advantages, such as a reduced risk of cross-contamination based on the absence of post-amplification handling of samples and semiquantitative analysis. The possibility of simultaneous detection of several target sequences using multichannel analysis permits multiplex assays. These test systems can be used to detect different target sequences as well as for co-amplification of internal controls (Hoffmann et al., 2009a, b, 2010).

With the new assay, gD- and gE-specific sequences within the BoHV-1 genome can be detected, allowing differentiation between wild-type BoHV-1 and gE-deleted live vaccines simultaneously. As gE-deletion does not affect the ability of BoHV-1 to be shed or to establish a latent infection (Lemaire et al., 2001; van Engelenburg et al., 1995a; Straub, 1996), it is important to monitor the spread of gE-deleted vaccine viruses. Indeed, a gE-deleted virus was isolated from two cows in a field study (Dispas et al., 2003). In addition, the OIE and National Reference Laboratory for BHV1 of the Friedrich-Loeffler-Institut, Insel Riems,

Germany, received clinical samples which were positive for BoHV-1 repeatedly, which turned out to harbour gE-deleted marker vaccine virus. Reliable discrimination with conventional methods is associated with time consuming growth of the virus isolates in cell culture and subsequent immunofluorescence staining or restriction enzyme fragment length polymorphism (RFLP) analyses. In contrast, the new triplex qPCR enables rapid differentiation even without isolation in cell culture and contemporary implementation or relaxation of disease control measures.

For detection of BoHV-1 in extended bovine semen, an important measure to ensure BoHV-1-free semen batches, an OIE-validated qPCR has been reported (Wang et al., 2007). Using the identical DNA template, the new triplex BoHV-1 qPCR showed an equivalent performance compared to the mentioned gB-specific PCR. It has also been demonstrated that the new triplex PCR detects BoHV-1 in other material such as nasal swabs.

An equal or even greater sensitivity was attained by the triplex qPCR in comparison to the reference method (virus isolation according to the OIE manual), demonstrating the equivalence of the time-saving qPCR approach. By testing dilution series of the standard DNA from BoHV-1.2 strain "Schoenboeken", it was demonstrated that the new triplex system is a highly sensitive method for detection and differentiation of BoHV-1 genomes.

A variety of different wild-type BoHV-1 strains was tested. All were found to be positive by the gD-specific PCR as well as by the gE-specific system, whereas all gE-deleted vaccine strains were negative by the gE-specific assay. The sensitivity of the gE-specific system was slightly greater compared to the gD assay, when testing diverse virus strains and the BoHV-1 dilution series, which reduces subsequently the likelihood of false results in an infected animal as a marker of vaccination. However, due to the marginal preference of the amplification of the gE-specific sequences by the triplex qPCR assay, the presence of BoHV-1 genome in very weak gE-positive and gD-negative samples cannot be excluded.

Testing nasal swabs collected from an animal experiment, samples of two individual calves were negative, while samples from the subsequent day were

positive. However, it should be noted that nasal swab material could show a greater variation since the amount of material is less standardised.

BoHV-1 is closely related genetically and serologically to several ruminant alphaherpesviruses, namely BoHV-5, BuHV-1, CapHV-1, CvHV-1 and 2 (Ros and Belak, 1999, 2002; Thiry et al., 2006). BoHV-5 can cause fatal meningoencephalitis in calves (Meyer et al., 2001), and a PCR assay for differentiation of BoHV-1 and BoHV-5 has been developed by Claus et al. (2005). However, related non-BoHV-1 ruminant herpesviruses are able to cross the species barrier to infect cattle. Seroconversion was observed in cows infected with CapHV-1 (Engels et al., 1992) or with BuHV-1. Buffaloes can *vice versa* be infected successfully with BoHV-1 (Scicluna et al., 2010). The BoHV-1 genome has also been detected in goats, sheep and several deer species (Kalman and Egyed, 2005). Because of the high antigenetic and genetic relationship among these viruses, serological cross-reactivity occurs (Kramps et al., 2004; Lyaku et al., 1996) and false positive results by different PCR-assays were observed (Santurde et al., 1996; Schynts et al., 1999; Wang et al., 2007). In this way, BoHV-1-related ruminant herpesviruses may interfere with BoHV-1 eradication and control programs, and therefore BoHV-1-specific assays are also needed. Nucleotide homologies of parts of the highly conserved glycoprotein B gene from the five ruminant alphaherpesviruses, BoHV-1, BoHV-5, CapHV-1, CvHV 1 and CvHV 2, range between 87.2 and 99.6% (Ros and Belak, 1999). The phylogenetic analysis of the more variable gD-gene revealed homologies ranging between 71.3 and 98.9%. As a consequence, all tested non-BoHV-1 herpesviruses were clearly negative by the BoHV-1 gD-specific qPCR in contrast to the gB-specific system. Only ElkHV-1 was not tested by the BoHV-1 triplex qPCR. However, due to the fact that the more closely related ruminant herpesviruses BoHV-5, CvHV-1 and -2 were negative by the BoHV-1 gD qPCR, false positive results for ElkHV-1 were not expected. Furthermore, BuHV-1, CvHV-1 and CvHV-2 were positive by the BoHV-1 gE-specific assay, offering the possibility to detect infections with these viruses and differentiate them from BoHV-1 genome with a qPCR assay.

In addition to the BoHV-1 gD- and gE-specific assays, an internal control (IC) PCR based on amplification of the bovine β-actin gene sequences was introduced.

To verify efficient DNA extraction and uninhibited amplification, heterologous internal controls (Hoffmann et al., 2005, 2006, 2009a) or IC based on housekeeping genes (Oleksiewicz et al., 2001) were used. The β-actin gene has been shown to be a suitable target for IC in several species (Oleksiewicz et al., 2001; Wakeley et al., 2005). A large number of different tissue samples from BoHV-1 negative cattle were negative by the BoHV-1 gD- and gE-systems, whereas they were positive by the β-actin-specific qPCR in an equivalent quantity. Successful amplification of gD- and gE-specific sequences in samples from animals infected with BoHV-1 also did not interfere with amplification of the β-actin-IC.

In conclusion, the new triplex qPCR is a sensitive and specific method for confirming the presence of BoHV-1 genome in field samples. In addition, this assay can differentiate between wild-type viruses and gE-deleted vaccine strains by a single qPCR reaction.

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IV. DISCUSSION

1. Multiplex polymerase chain reaction

In this study, a three-colour multiplex qPCR based on hydrolysis probes was developed and validated. Owing to its diverse advantages, including the reduced risk of cross-contamination, a high sensitivity and the possibility of quantitative analysis, the real-time format was preferred to conventional PCR.

The use of probes is associated with an increase in specificity compared to intercalating dyes like SYBR Green I. However, the choice of a genome region and probe sequence is challenging, since as many strains or types as possible of the target pathogen should be detected. The nucleotide mismatches between the probe and the target sequence, two in the case of the synthetic BoHV-1 positive controls, leads to a markedly lower florescence signal. Probes with incorporated LNA or MGB, which results in an increase in melting temperature and thereby a significant reduction of probe length, are even sensitive to single nucleotide mismatches, which is used in allele-specific assays for genotyping for instance (Alonso et al., 2010; Kutyavin et al., 2000). The effect of various primer and probe mismatches is complicated. Nevertheless, known consequences are the reduction of PCR efficiency with an increasing number of primer and/or probe mismatches, the even stronger effects of mismatches in both, primers *and* probe, on efficiency and the great effect of mismatches in the probe binding region (Smith, Vigilant, and Morin, 2002). It has been demonstrated that single point mutations in primer or probe binding regions in hydrolysis assays decrease PCR efficiency without fully abolishing the quantification, whereas mismatches of three or four nucleotides lead to complete inhibition of qPCR detection (Klein et al., 1999).

In this study, the selected gD-sequences are highly conserved for BoHV-1, but to a lesser extent within the closely related alphaherpesviruses. As a result, the diverse tested BoHV-1 strains scored clearly positive in the gD-assay, whereas the closely related ruminant alphaherpesviruses were not picked up.

Multiplexing, the combination of two or more assays in one reaction, offers a variety of advantages including savings in time, sample material and total costs. The development of multi-colour florescence-based PCR instruments allows

multiplexing by uniquely labelled probes. The spectra of the fluorescent dyes selected for probe labelling in this study are different enough to detect them separately. The described phenomenon of crosstalk in multiplex assays (Gunson et al., 2008) was not observed in any case.

The critical optimization of multiplex assays was simplified in recent years by the provision of PCR reagents that are developed specifically for multiplex PCR, by some manufacturers. As an example, the ready-to-use master mix supplied by Qiagen, which has been used in this study, enables the reliable detection of up to five genomic DNA or cDNA targets in a single reaction (www.qiagen.com). However, minor differences observed in reagent lots of a SYBRGreen-based kit (Burgos et al., 2002) may also occur in other chemistries. For high amounts of template an adequate lot-to-lot reproducibility was observed, whereas the sensitivity varied for low copy numbers of the target (Burgos et al., 2002). Variation between different batches of primers and probes resulting in reduced PCR sensitivity have been described as well (Gunson et al., 2006). The comparative testing of new and old batches within the same PCR run using identical samples is recommended to avoid unnoticed lot-to-lot variations on sensitivity (Gunson et al., 2006).

Another potential difficulty in multiplex reactions is the preferential amplification of one target (Mutter and Boynton, 1995; Polz and Cavanaugh, 1998). Therefore, in the PCR assay described in this study, a minimal concentration of the IC specific primers and probe was used in order to avoid competition with the pathogen-specific target. When analysing BoHV-1 genome, equal quantities of gE- and gD-specific sequences are expected. Consequently, a bias towards a higher concentrated target, which may be observed during the amplification of independent targets, is not expected in the BoHV-1 multiplex qPCR.

Apart from the IC, the presence of several or even all significant pathogens in a specific disease complex, like respiratory, enteric, vesicular or reproductive, can be detected simultaneously with multiplex PCR. Diseases that are hard to distinguish by clinical signs are discernible in a single reaction. Thereby the quantity of required sample volume is reduced, which is especially important when sample material is rare (Persson et al., 2005). Furthermore, total testing costs and time are shortened (Gunson et al., 2008; Gunson et al., 2006).

A multiplex qPCR for the detection and differentiation of the three major viruses

associated with the multi-factorial BRDC, namely BoHV-1, bovine viral diarrhoea virus and bovine parainfluenza virus 3, has been developed (Horwood and Mahony, 2011). The simultaneous detection of the pathogens potentially involved in an outbreak of bovine respiratory disease complex enables the appropriate treatment or management in a timely manner with minimal morbidity and mortality (Horwood and Mahony, 2011). To identify the causative agent of mucosal lesions in cattle, sheep and goats an assay for the detection of Rift Valley fever, bluetongue, rinderpest, and peste des petits ruminants viruses also described recently (Yeh et al., 2011).

For other animal species, multiplex PCR assays have been developed as well. As an example different viruses inducing reproductive and respiratory failure in swine, namely porcine circovirus type 2, classical swine fever virus, porcine parvovirus and porcine reproductive and respiratory syndrome virus, are discernible within a single reaction (Jiang et al., 2010). Classical and African swine fever are indistinguishable by either clinical or post-mortem examination (Kleiboeker, 2002), however multiplex PCR permits the fast genetic differentiation of the disease-causing virus (Aguero et al., 2004). The causative agent of indistinguishable vesicular lesions in epithelial tissues, be it foot-and-mouth disease virus, swine vesicular disease virus or vesicular stomatitis virus can be identified reliably in a timesaving multiplex approach (Fernandez et al., 2008). The rapid and specific diagnosis, especially after the unexpected re-emergence of a disease in free countries, is essential to prevent the spread into larger geographic areas affecting large animal populations (Belak, 2007).

Furthermore, multiplex PCR is used to distinguish between viruses related genetically like BoHV-1 and 5 (Alegre, Nanni, and Fondevila, 2001; Claus et al., 2005; Diallo, Corney, and Rodwell, 2011) or to differentiate genotypes of viruses like European and North American porcine reproductive and respiratory syndrome viruses (Egli et al., 2001; Kleiboeker et al., 2005).

Another possible application for multiplex PCR assays is the genetic differentiation between vaccine and wild-type strains of a pathogen, used in the diagnosis of classical swine fever (Leifer et al., 2009) or EHV-1 infection (Osterrieder et al., 1994) for instance. This kind of differentiating on the basis of pathogen genomes is also called "genetic DIVA" (Beer et al., 2007)

2.   Detection of Bovine herpesvirus type 1 genomes

Control programs for BoHV-1 include vaccination with inactivated or attenuated gE-deleted marker vaccines. Although a number of BoHV-1-specific PCR assays, conventional as well as in real-time, have been described for different clinical samples, none of them enable the differentiation of vaccine strains and wild-type strains (de Gee et al., 1996; Santurde et al., 1996; van Engelenburg et al., 1993; van Engelenburg et al., 1995b; Wagter et al., 1996; Wang et al., 2007; Wiedmann et al., 1993). Considering the abundant nasal shedding of gE-deleted vaccine strains after intranasal application and their ability to establish latent infections with the possibility of reactivation and subsequent shedding, the monitoring of vaccine strains and their distinction from wild-type strains are important features in BoHV-1 diagnostics.

In vaccination trials significant titres of live gE-negative vaccine strains were shown to be excreted after intranasal, but not after intramuscular application (Mars, de Jong, and van Oirschot, 2000; Strube et al., 1996). Since any excreted live virus could in principle infect other individuals, transmission experiments have been performed by Mars and colleagues (2000) to evaluate the average number of contact infections per infected individual. Although high amounts of BoHV-1 were observed in the nasal fluids of vaccinated animals, the gE-negative virus was transmitted to only one out of 15 co-housed sentinel cattle. It has been concluded that perpetuation of vaccine strains within cattle population is highly unlikely (Mars et al., 2000). Nevertheless, cases of gE-negative strains in clinical samples occur sporadically. In a field study, gE-deleted virus was isolated from two cows (Dispas et al., 2003). Therefore, it is important to monitor the spread of vaccine virus and to enable a rapid differentiation between vaccine virus replication and introduction of BoHV-1 wild-type virus into cattle holdings.

To detect gE-deleted strains a conventional PCR assay has been described (Fuchs et al., 1999; Schynts et al., 1999). Noting the disadvantages of post-amplification handling, in this study a qPCR assay specific for gE-deleted BoHV-1 strains was developed, standardized and subsequently combined with an assay specific for BoHV-1 gD and an IC based on the bovine beta-actin gene (Toussaint et al., 2007). In the gD-specific qPCR system wild-type as well as vaccine strains are picked up. As a consequence, samples positive in both BoHV-1 assays contain a

wild-type strain, whereas gE-deleted vaccine strains score positive in the gD-specific assay only. The IC-specific qPCR assay, included to verify efficient DNA extraction and uninhibited amplification, should give a positive result in case of both wild-type and vaccine strains as well as in samples containing neither.

The control programs initiated in several European counties see only *Bovinae* as crucial in maintenance and spreading of BoHV-1. However, further species of domestic and wild ruminants may be also infected. BoHV-1 genome has been detected in goats, sheep, buffaloes, bison and several deer species (Kalman and Egyed, 2005; Sausker and Dyer, 2002; Scicluna et al., 2010; Thiry et al., 2006). Seropositive animals of other species could be considered as potential reservoir, though the epidemiological impact is still unknown (Kalman and Egyed, 2005; Scicluna et al., 2010). Cattle in turn may be infected with related non-BoHV-1 ruminant herpesviruses (Engels et al., 1992). These genetically and serologically closely related pathogens include bubaline (BuHV-1), caprine (CapHV-1) and cervid (CvHV-1, CvHV-2) herpesviruses (Ros and Belak, 1999; Ros and Belak, 2002). A closely related bovine herpesvirus possibly interfering with BoHV-1 diagnosis is BoHV-5, formerly classified as BoHV-1 subtype 1.3. BoHV-5, which has never been reported in Germany (Beer, 2010a), can cause fatal meningoencephalitis in calves (Meyer et al., 2001). Several multiplex PCR assays, conventional (Alegre et al., 2001; Claus et al., 2005) as well as in real-time format (Diallo et al., 2011), for the differentiation of both bovine herpesviruses have been developed.

The close genetic and serological relationship between ruminant herpesviruses and their ability to cross species barriers may interfere with diagnostics resulting in false-positive results. Indeed, significant serological cross-reactivity was observed, caprine and cervine herpesvirus 1 were shown to be more closely related to BoHV-1 than they are to each other (Lyaku, Nettleton, and Marsden, 1992). False-positive results were likewise obtained in different PCR assays (Santurde et al., 1996; Wang et al., 2007), using the gE-specific conventional PCR described by Schynts et al. (1999). BuHV-1, CapHV-1, CvHV-1 and CvHV-2 may be falsely classified as gE-deleted BoHV-1 strains. Within the gE-specific assay included in the multiplex qPCR of this study, BuHV-1, CvHV-1 and -2

were likewise tested positive. However, none of the tested non-BoHV-1 ruminant herpesviruses gave positive results in the gD-specific assay. In addition to the differentiation of wild-type and vaccine BoHV-1 strains, the newly developed multiplex qPCR offers the possibility to detect infections with the ruminant herpesviruses BuHV-1, CvHV-1 and 2 and simultaneously distinguish them from BoHV-1. The negative results in the gD-specific assay for the related viruses that the OIE-validated gB-specific assay described by Wang (2007) had picked up (BoHV-5, BuHV-1, CvHV-1, -2 and CapHV-1) may be explained by the different sequence homology of the gB and gD genes. Nucleotide homology between both BoHV-1 subtypes and non-BoHV-1 viruses is greater in the highly conserved gB-gene than within the gD-gene (Figure 6).

gB		Nucleotide similarity						
	1	2	3	4	5	6		
1	■	99.6	95.5	94.4	87.4	70.0	1	BoHV 1.1
2	100.0	■	95.9	94.9	87.2	70.4	2	BoHV 1.2
3	98.1	98.1	■	96.6	88.2	71.9	3	BoHV-5
4	94.8	94.8	95.5	■	87.8	71.7	4	CvHV-1
5	89.0	89.0	89.0	88.4	■	72.7	5	CapHV-1
6	69.7	69.7	69.0	68.4	70.0	■	6	SuHV-1
	1	2	3	4	5	6		

Amino acid similarity (left vertical axis label for gB table)

gD		Nucleotide similarity						
	1	2	3	4	5	6		
1	■	98.9	90.7	88.8	72.1	53.1	1	BoHV 1.1
2	97.8	■	90.3	88.6	71.3	52.9	2	BoHV 1.2
3	90.5	88.8	■	91.1	73.9	53.5	3	BoHV-5
4	86.6	86.0	84.4	■	76.2	56.7	4	CvHV-1
5	68.7	68.2	68.7	71.5	■	51.0	5	CapHV-1
6	40.7	40.1	40.1	43.5	38.4	■	6	SuHV-1
	1	2	3	4	5	6		

Amino acid similarity (left vertical axis label for gD table)

Fig. 6: Nucleotide and amino acid sequence similarities (%) for the gB and gD regions of ruminant *Alphaherpesvirinae*, SuHV-1 was used as the outgroup. (Ros et al. 1999, modified)

BoHV-2 and -4 are not assigned to the genus *Varicellovirus* within family *Alphaherpesvirinae*, but in genus *Simplexvirus* and the family *Gamma-herpesvirinae,* respectively (Davison et al., 2009; Fauquet et al., 2005). Both viruses were tested negative by BoHV-1 gD-, gE- as well as gB-specific PCR which is due to the more distant relationship of these viruses.

The diagnostic sensitivity and specificity was evaluated by testing different sample types, including cell culture supernatant, serum, nasal swabs and bovine semen. The main routes of excretion during primary infection are nasal exudates and genital secretions. After possible reactivation the virus is once again transported to the original portal of entry (Engels and Ackermann, 1996; Jones, 2003). Consequently, the most important diagnostic samples are nasal swabs and semen. In addition, virus isolation as well as viral genome detection are important to verify the absence of BoHV-1 in extended bovine semen intended for international trade (OIE, 2010). When investigating serial 10-fold dilutions of BoHV-1 in extended semen, the sensitivity of the qPCR was equivalent to that of the "gold standard" method virus isolation in cell culture.

Taking the saving of time and effort into consideration, qPCR is a suitable alternative to virus isolation, especially when screening semen samples prior to artificial insemination. Because a single ejaculate is diluted and inseminated into many females, semen should be screened for BoHV-1 to prevent virus transmission (Grom et al., 2006). BoHV-1 has been detected in semen of healthy, sero-negative bulls by virus isolation as well as by PCR (Deka et al., 2005; Kupferschmied et al., 1986; Rocha et al., 1998). Consequently, a serological negative status does not eliminate the risk of virus transmission through semen (Deka et al., 2005). However, in latently infected animals BoHV-1 diagnosis is limited to serological methods. During latency, defined as a period without virus detectable by standard virus isolation procedures, viral genome is not detectable by PCR in common diagnostic samples such as serum, nasal swabs or semen. Nevertheless, for diseased animals or animals shedding the virus without showing clinical signs, the newly developed qPCR is a rapid, sensitive and reliable tool to identify the disease-causing agent.

3.   Concluding remarks

Real-time polymerase chain reaction assays are widely used for genome detection of pathogens. Probes labelled with unique dyes in combination with the separate detection of their individual emission spectra in appropriate qPCR machines permits multiplex assays, which have recently been developed for several applications. Often internal controls were co-amplified in duplex assays. The main advantages of the amplification of multiple targets per reaction include a reduction of the required sample material, required time and total testing costs. The availability of ready-to-use reagents developed especially for multiplex assays has simplified the optimization of multi-target PCR systems and may lead to a further intensification of multiplexing.

In this study, a three-colour multiplex qPCR assay for the detection and differentiation of BoHV-1 wild-type and gE-negative vaccine strains in a single reaction was developed and validated. In addition, it could be demonstrated that the ruminant herpesviruses BuHV-1, CvHV-1 and -2, which may infect cattle and interfere with control programs, are detected and distinguished from BoHV-1 by the multiplex qPCR. Especially for diseased animals, the newly developed multiplex qPCR is therefore a valuable diagnostic instrument for rapid, sensitive and reliable differentiation between recently acquired or reactivated gE-negative vaccine virus and infection with BoHV-1 wild-type strains. Furthermore, the qPCR may be used to monitor the spread of vaccine virus in cattle population, which has been reported sporadically, during screening tests for wild-type BoHV-1 without any additional expenditure.

V.   SUMMARY

Bovine herpesvirus type 1 (BoHV-1), an alphaherpesvirus, is a major pathogen of cattle causing different syndromes such as infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious balanoposthitis (IBP). BoHV-1 control programs have been initiated in several European countries including Germany. One of the major components is the vaccination with inactivated or attenuated glycoprotein E (gE)-deleted live marker vaccines.

The aim of this study was the development of a triplex real-time polymerase chain reaction (qPCR) assay for the sensitive, specific and reliable BoHV-1 detection. A BoHV-1-specific glycoprotein D (gD) assay was combined with a gE-specific qPCR system for differentiation between wild-type strains and gE-negative vaccine virus strains. Finally, an internal control based on amplification of the bovine beta-actin gene was introduced to verify efficient DNA extraction and PCR amplification.

The analytical sensitivity of the triplex BoHV-1 qPCR enables the detection of 10 genome copies per reaction. Furthermore, the sensitivity of the newly developed qPCR assay was compared to an OIE-validated qPCR and the "gold standard" method of virus isolation in cell culture using 10-fold dilution series of BoHV-1 in extended semen as well as in cell culture medium. For all preparations, the tested qPCR assays showed comparable results and the sensitivity of the triplex qPCR was equal or even greater than that of virus isolation. A broad spectrum of reference strains and field isolates was detected reliably. The specificity of the test was confirmed using nasal swabs, semen and different organ materials of BoHV-1-negative cattle.

Bovine herpesviruses type 2, 4 and 5 and further ruminant herpesviruses, namely bubaline herpesvirus (BuHV-1), caprine herpesvirus type 1 and cervine herpesvirus type 1 and 2 (CvHV-1, -2) were tested as well. The close genetic and serological relationship between these viruses combined with their ability to infect bovines may interfere with diagnostics resulting in false-positive results. All non-BoHV-1 herpesviruses were negative in the gD-specific assay, while BuHV-1, CvHV-1 and -2 were tested positive by the gE-specific qPCR. Consequently, the triplex qPCR offers for the first time the possibility to detect some related herpesviruses and distinguish them from BoHV-1 in addition to the simultaneous differentiation of BoHV-1 wild-type and gE-deleted vaccine strains.

VI.   ZUSAMMENFASSUNG

Das Bovine Herpesvirus vom Typ 1 (BoHV-1), ein *Alphaherpesvirus*, verursacht die wirtschaftlich bedeutenden Krankheitsbilder der infektiösen bovinen Rhinotracheitis (IBR), infektiösen pustulären Vulvovaginitis (IPV) und infektiösen Balanoposthitis (IBP). Umfassende Bekämpfungsprogramme wurden in mehreren europäischen Ländern, so auch in Deutschland, gestartet. Ein wichtiger Bestandteil dieser Programme ist die Vakzinierung mit inaktivierten oder attenuierten Glykoprotein E (gE)-deletierten Markerimpfstoffen.

Ziel dieser Arbeit war es, eine Triplex Real-Time Polymerase-Kettenreaktion (qPCR) zum BoHV-1 Genomnachweis zu entwickeln. Ein Glykoprotein D (gD) spezifischer Assay wurde mit einem zweiten, gE-spezifischen qPCR System kombiniert, was die Differenzierung zwischen Stämmen vom Wildtyp und gE-deletierten Impfstoffen ermöglichte. Als Extraktions- und Amplifikationskontrolle wurde zudem der Nachweis von bovinem beta-Aktin integriert.

Die analytische Sensitivität der Triplex BoHV-1 qPCR beträgt 10 Genomkopien pro Reaktion. Weiterhin wurde die Sensitivität der neuen Triplex-PCR mit der einer OIE-validierten qPCR und der „Goldstandard-Methode" Virusisolierung in Zellkultur verglichen. Es wurden Verdünnungsreihen in Sperma und Zellkulturmedium untersucht, wobei beide qPCR-Systeme vergleichbare Ergebnisse erzielten und die neu entwickelte Triplex BoHV-1 qPCR eine der Virusisolierung mindestens gleichwertige Sensitivität zeigte. Eine Vielzahl von Referenzstämmen und Feldisolaten wurde zuverlässig erkannt. Die Spezifität wurde anhand von Nasentupfern, Sperma und verschiedenen Organmaterialien BoHV-1-negativer Rinder getestet.

Weiterhin wurden die Bovinen Herpesviren vom Typ 2, 4 und 5, sowie die dem BoHV-1 nah verwandten Herpesviren der Büffelartigen (BuHV-1), Ziegen (CapHV-1), Hirsche und Rentiere (CvHV-1, -2), die auch bei Rindern eine Infektion hervorrufen können, getestet. Die enge genetische und antigenetische Verwandtschaft kann zu einer Beeinflussung der BoHV-1-Diagnostik in Form von falsch-positiven Ergebnissen führen. Sämtliche nicht-BoHV-1 Herpesviren wurden in der gD-spezifischen qPCR korrekt als negativ bewertet, während

BuHV-1, CvHV-1 and -2 positive Ergebnisse im gE-spezifischen Assay lieferten. Damit ermöglicht die Triplex qPCR, zusätzlich zur Unterscheidung von BoHV-1-Stämmen vom Wildtyp und gE-deletierten Impfviren, die zeitgleiche Detektion einiger nah verwandter Herpesviren und deren Differenzierung von BoHV-1.

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VIII. ABBREVIATIONS

aa	Amino acids
bp	Base pair
BoHV	Bovine herpesvirus
BuHV	Bubaline herpesvirus
BRDC	Bovine respiratory disease complex
CapHV	Caprine herpesvirus
cDNA	Complementary desoxyribonucleic acid
Cq	Quantification cycle
CvHV	Cervid herpesvirus
DIVA	Differentiating infected from vaccinated animals
dNTP	Deoxyribonucleotide triphosphates
(ds)DNA	(Double-stranded) deoxyribonucleic acid
EHV	Equine herpesvirus
ELISA	Enzyme-linked immunosorbent assay
FRET	Förster resonance energy transfer
gE	Glycoprotein E, designations of glycoproteins appear with the prefix "g"
IC	Internal control
IBR	Infectious bovine rhinotracheitis
IPB	Infectious balanoposthitis
IPV	Infectious pustular vulvovaginitis
IR	Internal repeat
LR RNA	Latency-related RNA
LNA	Locked nucleic acid
MGB	Minor groove binder
OIE	World Organisation for Animal Health
(m)RNA	(Messenger) Ribonucleic acid
(RT)-PCR	(Reverse transcription) Polymerase chain reaction
(RT)-qPCR	Real-time (reverse transcription) polymerase chain reaction
SuHV	Suid herpesvirus
T_m	Melting temperature
TR	Terminal repeat
U_L	Unique long
U_S	Unique short

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