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Molecular double-check strategy for the identification and characterization of Suid herpesvirus 1

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ABSTRACT

Large scale vaccination with glycoprotein E (gE)-deleted marker vaccines and the rapid and reliable differentiation of wild-type and marker vaccine strains are important aspects in eradication programs for Suid herpesvirus 1 [SuHV-1, syn. Aujeszky's disease virus (ADV) or pseudorabies virus (PrV)]. Therefore, two multiplex real-time PCR (qPCR) assays for the genetic differentiation of wild-type and gE-deleted vaccine SuHV-1 strains have been developed. In the first multiplex qPCR SuHV-1 gB-gene specific detection was combined with a gE-gene specific assay and an internal control based on heterologous DNA. In the second system, a SuHV-1 UL19 (major capsid protein gene) assay, a different gE-gene specific assay and an internal control based on the beta-actin gene were combined.

The gB-gene, UL19 as well as both gE-gene specific assays had an analytical sensitivity of less than 10 genome copies per reaction in the respective multiplex approaches. A series of reference strains including field isolates obtained from domestic and wild animals, and gE-deleted SuHV-1 were reliably detected, while genetically related non-SuHV-1 herpesviruses tested negative.

Both newly developed triplex SuHV-1-specific qPCR assays are specific and sensitive methods for the rapid genetic differentiation of wild-type viruses and gE-deleted vaccine strains in a single reaction.
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1. Introduction

Aujeszky's disease (AD, syn. pseudorabies) is caused by Suid herpesvirus 1 [SuHV-1, syn. Aujeszky's disease virus (ADV) or pseudorabies virus (PrV)], which belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Mettenleiter et al., 2012). Since AD can cause substantial economic losses in swine production it is declared a notifiable disease by OIE (OIE, 2012; Moynagh, 1997). Despite the presence of SuHV-1 in wild swine (Müller et al., 2011), increased control efforts and the strict implementation of national eradication programs which included large scale vaccination with glycoprotein E (gE)-deleted vaccines and the so-called DIVA ("differentiating infected from vaccinated animals") strategy have led to a virtual disappearance of SuHV-1 from domestic pigs in several parts of Europe, North America and New Zealand. Elsewhere, SuHV-1 remains one of the most important diseases of domestic pigs particularly in regions with dense pig populations (Mettenleiter et al., 2012). Following oro-nasal infection and primary replication in epithelial cells of the upper respiratory tract, the virus establishes a life-long latent infection by persistence in trigeminal ganglia, sacral ganglia and tonsils. Latent virus can be reactivated by several stimuli such as stressful conditions or immunosuppression with glucocorticoids (Mettenleiter et al., 2012).

The diagnostic identification of the agent is done by virus isolation in cell culture. However, because of the higher sensitivity and speed, amplification of viral DNA using polymerase chain reaction (PCR) is frequently used. Numerous conventional PCR assays have been described that usually target a sequence conserved among SuHV-1 strains, e.g. parts of the genes encoding envelope glycoproteins gE, gB, or gD. Amplification is measured following a single round PCR (Belak et al., 1989; Jestin et al., 1990) or after a second round using a nested PCR approach (Yoon et al., 2005). Because of the need to differentiate between marker vaccine and wild type viruses during vaccination campaigns, the utility of PCRs for DIVA was proven (Fonseca et al., 2010; Hasebe et al., 1993; Ishikawa et al., 1995; Scherba et al., 1992). However, compared to those conventional PCR assays, real-time PCR (qPCR) systems have

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several advantages such as semi-quantitative analysis or a reduced risk of cross-contamination in the absence of post-amplification handling of samples (Mackay et al., 2002; Mackay, 2004). Additionally, qPCR enables the simultaneous detection of several targets by using multichannel analysis which has been previously used for the simultaneous detection of different viral pathogens of swine including SuHV-1 (Cao et al., 2005; Huang et al., 2004; Lee et al., 2007; Liu et al., 2013; Perez et al., 2012; Sami et al., 2007; van Rijn et al., 2004; Wernike et al., 2013; Xu et al., 2012; Yin et al., 2012; Yue et al., 2009). In the context of eradication programs, which are primarily based on vaccination with gE-deleted vaccines, the molecular differentiation between wild-type viruses and gE-deleted vaccine strains in clinical samples is crucial. Recently, a set of two real-time PCR systems as a differential test for SuHV-1 was developed (Ma et al., 2008). However, no internal control system was used to verify efficient DNA extraction and PCR amplification, which is highly recommended by the World Organization for Animal Health (OIE) for the use of PCR precautions and quality control measures (OIE, 2012). Therefore, two independent triplex qPCR assays for the simultaneous detection of SuHV-1 gB-gene or UL19 and gE-gene specific sequences combined with two different internal control systems were developed and validated.

2. Materials and methods

2.1. Virus strains and samples for diagnosis

A series of SuHV-1 wild-type strains and gE-deleted vaccine viruses (Akipor 6.3®, Merial, strain Bartha; Suvaxyn Aujeszky 783, Fort Dodge Animal Health, strain NIA3-783) were used in this study. In addition, bovine (BoHV-1, BoHV-5), cervid (CvHV-1, CvHV-2), bubaline (BuHV-1), caprine (CapHV-1) and equine (EHV-1) herpesviruses were tested; all strains were provided by the virus collection of the Friedrich-Loeffler-Institut, Insel Riems, Germany. Furthermore, SuHV-1 positive tissue samples obtained from experimental or natural SuHV-1 infections, and serum and blood samples from SuHV-1 negative swine were tested.

2.2. DNA extraction

DNA was extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit for automated extraction (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) or DNA-Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, modified by addition of an internal control DNA (IC2) as described by Hoffmann et al. (2006), and finally eluted in 100 μl of buffer.

2.3. Primers, probes and qPCR

Two distinct triplex qPCR-assays have been developed. In the first multiplex PCR a SuHV-1 gB-gene specific system was combined with a gE-gene specific assay (gE1) and an internal control (IC) based on heterologous DNA (Hoffmann et al., 2006). In the second triplex qPCR, a SuHV-1 UL19 assay (Wernike et al., 2013), a further gE-gene specific assay (gE2) and an IC based on the beta-actin gene (Wernike et al., 2011) were integrated. To select the primers and probe in the SuHV-1 gB gene, published sequence information (NCBI database) was 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 design of the gE-gene-specific assays was optimized according to the respective gE-gene deletions in the different commercial SuHV-1 marker vaccines. The sequences of primers and probes are summarized in Table 1. All oligonucleotides were synthesized by Metabion International (Planegg-Martinsried, Germany).

Both triplex SuHV-1 qPCRs were carried out using the QuantiTect Multiplex PCR NoROX Kit (Qiagen, Hilden, Germany) in 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 and primers and probes in concentrations given in Table 1 were merged as a 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 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. All samples were tested in duplicates.

3. Results

3.1. Sensitivity

The analytical sensitivity of the SuHV-1 triplex qPCR assays was determined using a series of 10-fold dilutions of a plasmid standard, which contained primer and probe sequences of each SuHV-1-specific qPCR. The DNA was amplified in a linear fashion from 10^6 copies down to 10^1 copies per reaction in both triplex qPCR assays (Fig. 1). Comparison of the amplification efficiencies

Table 1
Primers and probes used in this study.

Name	Sequence 5′–3′	Concentration (pmol/reaction)	Reference
Triplex qPCR 1			
PrV-gB-778F	ACC AAC GAC ACC TAC ACC AAG	20	This study
PrV-gB-856R	CCT CCT CGA CGA TGC AGT TG	20	
PrV-gB-809TEX	Texas Red-CGG GCT TCT ACC ACA CGG GCA CCT-BHQ2	2.5	
SuHV1-dgE-689F	CTG TAC GTG CTC GTG ATG AC	20	This study
SuHV1-dgE-781R	CTC CTT GRT GAC CGT GAC G	20	
SuHV1-dgE-723FAM	FAM-TCG CCA CCT GGG ACT ACA CGC TCG-BHQ1	2.5	
EGFP-11-F	CAG CCA CAA CGT CTA TAT CAT G	5	Hoffmann et al. (2006)
EGFP-10-R	CTT GTA CAG CTC GTC CAT GC	5	
EGFP-HEX	HEX-AGC ACC CAG TCC GCC CTG AGC A-BHQ1	2.5	
Triplex qPCR 2			
PrV-UL19-1871F	CGC AGT GCA TCC AGA GCT AC	20	Wernike et al. (2013)
PrV-UL19-1966R	CGT TGC CCA GGT AGG TGT TG	20	
PrV-UL19-1894TEX	Texas Red-CGC AAC ACG CAC AAC GCC GCC-BHQ2	2.5	
SuHV1-dgE-328F	CTTCCACTCGCAGCTCTTCTC	20	This study
SuHV1-dgE-401R	AGT CGC CCA TGT YCG AGA C	20	
SuHV1-dgE-357FAM	FAM-ACA CGT TCG ACC TGA TGC CGC GC-BHQ1	2.5	
ACT2-1030-F	AGC GCA AGT ACT CCG TGT G	5	Wernike et al. (2011)
ACT-1135-R	CGG ACT CAT CGT ACT CCT GCT T	5	
ACT-1081-HEX	HEX-TCG CTG TCC ACC TTC CAG CAG ATG T-BHQ1	2.5	

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