Detection of EHV-1 neuropathogenic strains using real-time PCR in the neural tissue of horses with myeloencephalopathy

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EQUINE herpesvirus type 1 (EHV-1) infection is widespread in horse populations throughout the world and produces well-documented syndromes of respiratory disease, abortion, neonatal foal death and myeloencephalopathy (van Maanen 2002). The epidemiology of EHV-1 is characterised by lifelong latent infection after primary exposure. The reactivation of a latent stage in a subclinically affected animal often leads to viral shedding via nasal secretions and horizontal transmission (Edington and others 1985). PCR assays that are both more sensitive and rapid have largely replaced the time-consuming procedure of virus isolation (Wagner and others 1992, Kirisawa and others 1993, Lawrence and others 1994). Novel molecular platforms such as real-time PCR have allowed the study of EHV-1 viral kinetics by quantitating viral load (Allen and Breathnach 2006, Hussey and others 2006, Pusterla and others 2006). Molecular analysis of EHV-1 strains has recently shown a single-point mutation in the open reading frame 30 (ORF30) in 86 per cent of neuropathogenic EHV-1 strains (Nugent and others 2006). The sequence variation occurs in the DNA polymerase gene of the virus, which is involved in initial viral replication within infected cells and may also be involved in the establishment of latency and reactivation.

The need to distinguish between neuropathogenic and non-neuropathogenic EHV-1 strains is crucial for implementation of management practices that decrease the risk of exposure of susceptible horses, as well as for the study of the pathogenesis of EHV-1. So far, strain differentiation has only been described by nucleotide sequence determination or sequence capture PCR coupled with restriction fragment length polymorphism (RFLP) (Allen 2006). Real-time TaqMan PCR is a unique and proven molecular diagnostic tool that allows for the differentiation of a single nucleotide polymorphism (SNP) as exemplified by the commercial availability of more than four million assays for human SNPs (Applied Biosystems). DNA TaqMan probes with conjugated minor groove binder (MGB) groups form extremely stable duplexes with single-stranded DNA targets, allowing shorter probes to be used for hybridisation-based assays. The goal of the study reported here was to design and validate real-time TaqMan PCR assays based on the MGB probe chemistry able to differentiate between non-neuropathogenic and neuropathogenic EHV-1 field strains based on the $A_{2254}G$ SNP in the ORF30 of the DNA polymerase gene.

Formalin-fixed and paraffin-embedded brain tissues from eight horses with EHV-1 myeloencephalopathy confirmed by immunoperoxidase staining were used for this study. Formalin-fixed and paraffin-embedded placentas from five mares with confirmed EHV-1 abortion by immunoperoxidase staining served as non-neurological EHV-1 controls. Additionally, 86 field samples (whole blood and nasopharyngeal secretions) collected from 52 adult horses known to have been exposed to an EHV-1 myeloencephalopathic strain during an outbreak were also analysed. DNA was extracted from the samples using the NucPrep chemistry on a semi-automated nucleic acid handling system (6100 Sample PrepStation; Applied Biosystems) according to the manufacturer's guidelines. The genomic DNA (gDNA) was eluted in a volume of 200 µl. Each gDNA sample was run with a universal 18S rRNA (ssrRNA; Applied Biosystems) for quality control purposes. The following real-time TaqMan PCR assays were used: a glycoprotein B-based EHV-1 real-time TaqMan PCR (ORF33; NC_001491) (Pusterla and others 2006); a DNA polymerase-based EHV-1 with a TaqMan MGB probe (5′ label 6FAM; Applied Biosystems) specific for the neuropathogenic EHV-1 strain (ORF30; reference strain Ab4, AY665713); and a DNA polymerase-based EHV-1 assay recognising the EHV-1 strain (same PCR primers, TaqMan MGB probe [5′ label VIC]) specific for the non-neuropathogenic reference strain V592 (AY464052; Table 1). Surrounding the real-time TaqMan PCR product, two sequencing primers were designed for the generation of a 141 base pair (bp) PCR product to re-sequence the TaqMan PCR target reaction. The PCR primers and TaqMan probes were tested in silico by BLAST analysis for the presence of the correct SNP at 2254 and absence of additional sequence variations within the primer region. Real-time TaqMan PCR reactions contained 400nM of each primer, 80nM of the TaqMan probe and mastermix (Universal TaqMan Mastermix with AmpErase UNG; Applied Biosystems) and 5 µl of the gDNA sample in a final volume of 12 µl. The samples were amplified in a combined thermocycler/fluorometer (7900 HTA; Applied Biosystems) with the standard thermal cycling protocol: two minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The DNA polymerase EHV-1 real-time TaqMan PCR assays were validated for their discriminatory ability of the SNP at nucleotide position 2254 (A to G transition) using synthetic oligonucleotides (EHV-1 neuropathogenic control: TGAAGTGGATCTGGCCGGGCTTCAACCATCCGTCGACTACTCGCGACG TTCGAGGTGGGTGACCAAAAGTTA; EHV-1 non-neuropathogenic control: TGAAGTGGATCTGGCCGGGCTTCAACCATCCGTCAACTACT CGACGTTCGAGGTGGGTGACCAAAAGTTA) of the DNA polymerase target region for both isolates. In addition, standard curves were run for both assays and the amplification efficiency calculated from the slope using the formula $E=10^{-1/s}-1$. The discriminatory ability of the two DNA polymerase-based real-time TaqMan PCR assays was shown using large numbers of synthetic oligonucleotides (approximately 10^7 molecules per reaction) and analysed in triplicate. This high signal strength is not observed in naturally infected animals and was used solely for the purpose of proving the high discrimination power of the two MGB TaqMan probes. The same synthetic oligonucleotides were used to determine the analytical sensitivity of both real-time PCR assays.

Each TaqMan probe only recognised its own synthetic target oligonucleotide without detecting the synthetic oligonucleotide with the nucleotide polymorphism, underlining the high analytical specificity of the two TaqMan PCR assays. The detection limit of both real-time PCR assays was 10 molecules of the corresponding synthetic oligonucleotides. Additionally, the amplification efficiencies were each at 98·6 per cent, indicating a very high analytical sensitivity. All eight neuronal tissues were positive for the presence of both the glycoprotein B gene and the neuropathogenic ORF30 gene, and negative for the non-neuropathogenic ORF30 gene (Table 2). The five placentas were positive for the presence of both the glycoprotein B gene and the non-neuropathogenic ORF30 gene, and negative for the neuropathogenic ORF30 gene. Nine field specimens, three blood samples and six nasopharyngeal swabs tested positive for the presence of the glycoprotein B gene and the neuropathogenic ORF30 gene, and negative for the non-neuropathogenic ORF30 gene. Coefficients of variation (CV) on repetitive testing (triplicate) during the same or different PCR runs were in the expected range (0·22 to 1·32 per cent for CV calculated with cycle threshold values and 2·36 to 9·73 per cent

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TABLE 1: Sequence of PCR primers and TaqMan probes used in the real-time PCR assays

Target gene	GenBank accession number	Primer designation	Sequence (5'-3')	Product size	Probe designation (label)	Probe sequence (5'-3')
Glycoprotein B (ORF 33)	NC_001491	EHV1-387f EHV1-476r	TATACTCGCTGAGGATGGAGACTTT TTGGGGCAAGTTCTAGGTGGTT	90	EHV1-413P (6FAM)	ACACCTGCCCACCGCCTACCG
DNA polymerase non-neuropathogenic (ORF 30)	AY464052	EHV1-29f EHV1-82r	ATCTGGCCGGGCTTCAAC GGTCACCCACCTCGAACGT	54	EHV1NNP-(VIC)	ATCCGTCAACTACTCG
DNA polymerase neuropathogenic (ORF 30)	AY665713	EHV1-29f EHV1-82r	ATCTGGCCGGGCTTCAAC GGTCACCCACCTCGAACGT	54	EHV1NP-(6FAM)	ATCCGTCGACTACTCG
DNA polymerase sequencing primers	AY464052 AY665713	EHV1DP-6f EHV1N-146r	CACCCTGGCGCTCGATG CAGTCGCGCAGCAAGATG	141	Not applicable	Not applicable

* Differences in detection probe are underlined
ORF Open reading frame

CV calculated with absolute values after transformation onto the respective standard curves). To test for analytical specificity, four TaqMan PCR products of each assay were sequenced using standard sequencing procedures (BigDye Terminator chemistry, ABI 3730; Applied Biosystems) and the correct base at nucleotide position 2254 confirmed.

In addition to previously described conventional PCR assays or the sequence capture nested PCR, real-time PCR allows the accurate quantitation and quantitative comparison of EHV-1 strains present within the same sample. This will be helpful to associate the viral load in different tissues with disease progression, induction and severity, as was attempted in a previous study (Allen and Breathnach 2006). In neuronal tissues from eight symptomatic horses with neurological disease, all tissue sections only harboured neuropathogenic strains, while placentas only tested positive for the non-neuropathogenic assay. The molecular detection of neuropathogenic EHV-1 in field samples confirmed prior exposure of these horses to a myeloencephalopathic strain of EHV-1. These results strongly support the DNA polymerase mutation $A_{2254}G$ being associated with clinical neurological disease.

The real-time TaqMan PCR assay described here targets a SNP in the catalytic domain of the DNA polymerase gene (ORF30) believed to be strongly associated with EHV-1 isolates inducing neuropathogenic properties. The wild type nucleotide A_{2254} is present in 95 per cent of non-neuropathogenic isolates, whereas 86 per cent of the neuropathogenic isolates encoded G_{2254}, leading to an amino acid change at $N_{752}D$. It seems obvious, therefore, that additional SNPs, alone or more likely in combination, may increase the accuracy of future MGB probe-based diagnostic TaqMan assays.

TABLE 2: Sample description and TaqMan PCR results for equine herpesvirus type 1 (EHV-1) glycoprotein B (gB), neuropathogenic (NP) and non-neuropathogenic (NNP) assays

Sample description	Sample (n)	EHV-1 gB	EHV-1 NP	EHV-1 NNP
Synthetic oligonucleotide	NP control	Not applicable	+	–
	NNP control	Not applicable	–	+
Neuronal tissue (eight horses)	Horse 1	+	+	–
	Horse 2	+	+	–
	Horse 3	+	+	–
	Horse 4	+	+	–
	Horse 5	+	+	–
	Horse 6	+	+	–
	Horse 7	+	+	–
	Horse 8	+	+	–
Placenta (five mares)	Mare 1	+	–	+
	Mare 2	+	–	+
	Mare 3	+	–	+
	Mare 4	+	–	+
	Mare 5	+	–	+
Field samples (52 horses)	Whole blood (36)	+ (3)*	+(3)*	–
	Nasal secretions (50)	+(6)*	+(6)*	–

* Number of PCR positive samples in parentheses
+ Positive TaqMan PCR result, – Negative TaqMan PCR result

Sensitive and specific assays are required to monitor the different isolates during EHV-1 outbreaks and disease. The assay described here is able to detect neuropathogenic EHV-1 strains with high amplification efficiencies and analytical sensitivity in a high throughput format within five hours of receipt of the samples. This is a significant improvement in assay performance compared with previous methods, including direct PCR product sequencing to identify the SNP, or sequence capture PCR coupled with RFLP protocols. Since not all investigated neuropathogenic isolates harbour the mutation, assays able to differentiate between neuropathogenic and non-neuropathogenic EHV-1 must be used judiciously for clinical purposes and the results should always be interpreted in the context of clinical presentation. Results pertaining to the type of isolate are important in order to determine the risk of developing neurological signs in exposed/infected horses, limiting and preventing spread by instituting appropriate biosecurity measures during outbreaks, and to study viral kinetics in horse populations.

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