

## Sequence analysis of selected nucleotide sequences of abortogenic isolate of Equine Herpesvirus 1 and changes caused by serial passage *in vitro*

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### Abstract

The aim of this work was to isolate the abortogenic virus strain of Equine Herpesvirus 1 representing the current infection situation in the Czech Republic, describe it at the molecular level with accent on genes coding viral glycoproteins and observe the changes caused by the passaging of the virus on a cell culture. In 2009, an isolate of equine herpesvirus 1 was obtained from an abortion case in a mare from a herd affected by abortion storm. The virus identification was performed using the PCR method. The virus was isolated on the RK 13 cell line and after 6 passages *in vitro* the stability of sequences of selected sections of the virus genome was assessed and compared with the original field isolate. The virus sequences were also compared with known sequences of the abortogenic reference virus strain (V592) and with other known viral strains. One point mutation in a nucleotide sequence coding glycoprotein G was found, distinguishing the field isolate from V592. One point mutation in the gene for glycoprotein C was passage-induced. It was noted that the virus during the passage on RK13 cell line in the monitored sections was stable and is a suitable starting material for next experiments.

*EHV-1, viral abortion in mares, glycoproteins, genom*

Up to now a total of five herpesviruses have been isolated in horses. Only three of them cause clinically significant disease. They are especially the Equine Herpesvirus 1, Equine Herpesvirus 3 and Equine Herpesvirus 4 (EHV-1, EHV-3 and EHV-4). Diseases caused by EHV-1 and EHV-4 have the greatest impacts on health. EHV-1 is currently known as the etiologic agent of herpesviral abortion in mares (Studdert et al. 1981).

In pregnant mares the EHV-1 infection may result in the abortion of the foetus (Studdert and Blackney 1979). Crucial to the clinical course of disease is the stage of gestation. Abortions which are due to EHV-1 infection occur during the last three months of pregnancy (Awan et al. 1995). The number of aborting mares in the herd cannot be predicted. The incidence varies from isolated cases up to 70%.

The size of the EHV-1 genome is approximately 150,000 bp. The EHV-1 genome is composed of two subunits. The smaller subunit (unique short -  $U_s$ ) is bounded by repeated stretches called “terminal repeats” ( $TR_s$ ) and “internal repeats” ( $IR_s$ ). The larger subunit (unique long -  $U_l$ ) is bounded by repeated stretches of  $TR_l$  and  $IR_l$ , which are smaller than the corresponding sequences of  $U_s$  (Telford et al. 1998).

Viral glycoproteins play an important role in the disease pathogenesis. They are responsible for the virus pathogenicity (Manservigi and Cassai 1991) and the immune response of an infected organism is directed against them (Whalley et al. 1995). They are glycosylated proteins integrated into the virus lipid envelope. In total, 11 glycoproteins were identified in EHV-1, referred to as glycoprotein B (gB), gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM.

Surface glycoproteins are conserved within the *Herpesviridae* family to some extent. gB and gH are considered the most conserved glycoproteins. Glycoproteins E and I are conserved especially in neurotropic strains of herpesvirus (Frampton et al. 2004).

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Glycoproteins are divided into those that are essential for virus replication (gB, gD, gH / L, gK) and those that are not (gC, gE, gI, gG, gM) (Cairns et al. 2003).

Due to the localization of viral glycoproteins on the virus particles surface they are molecules coming into direct contact with the host immune system. In essential glycoproteins, therefore, we encounter the presence of neutralizing epitopes.

The aim of the study was to describe the field isolate of highly abortogenic EHV-1 at the genome level, especially in genes that encode proteins essential for the immune response of the body, i.e. for viral glycoproteins. Nucleotide sequences observed in this study encode glycoproteins C, D, E, G and I. In the genome of EHV-1 they are located inside U<sub>1</sub> (gC: CDS16), but mostly within the U<sub>8</sub> (gG: CDS70, gD: CDS72, gI: CDS73 and gE: CDS74). This virus was intended to isolate on the cell culture, and then test for the stability of the observed sequences after the 6<sup>th</sup> passage. The obtained sequences were compared with the known abortogenic strain EHV-1 labelled V592 (GenBank AY464052), with a representative of attenuated strains of the virus Kentucky A (KyA) (GenBank S57839.1 for gC and M62923.1 for gD) and with a partial sequence for gD of isolate Lucina, obtained from the Czech Republic in 2003 (Molinkova et al. 2004).

### Materials and Methods

Ten-percent suspension of liver and placental tissue of aborted foal in Hank's balanced salt solution was used for isolating the virus. The suspension was centrifuged for 10 min at 6,000 g and filtered through a 0.22 µm filter (Sigma, USA). The material was stored at -80 °C.

Cell line RK-13 (Rabbit kidney) was used for isolating the virus. Cells were cultured in Dulbecco's modified Eagle's medium with an addition of 10% bovine foetal serum to achieve a 70-80% monolayer. Then they were inoculated with the tested material. The daily emergence of cythopathic effect was monitored in the infected cell culture. When it reached 70%, the culture was frozen at -80 °C. The following passages of the virus were carried out repeatedly with deep-frozen and defrosted material.

Total DNA was extracted from an aborted foetus placental tissue using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions. DNA from the 6<sup>th</sup> passage of the virus on RK13 was isolated using the Kit NucleoSpin Blood (Macherey-Nagel, Germany) following the manufacturer's instructions. The obtained DNA was labelled and stored at -80 °C.

The presence of EHV-1 DNA was confirmed both in the material obtained directly from the aborted foal placenta as well as in the material from the 6<sup>th</sup> passage of the obtained virus isolate on the RK13 cell line using the species-specific polymerase chain reaction (PCR) as described by Lawrence et al. (1994).

Selected sections of DNA designated for the monitoring of nucleotide sequences were amplified in 100 µl PCR using reaction mixture Combi PPP MasterMix (TopBIO, Czech Republic) with the addition of appropriate pairs of oligonucleotide primers (Table 1). Reaction conditions: after 4 min at 94 °C of initial denaturation, 35 cycles followed: denaturation (45 s/94 °C), annealing (45 s/52 °C), extension (1 min/72 °C).

Table 1. Sequence and position of primers used

	Sequence	Position in V592 genome
gE-r	AATACAGCCCCACACCACC	135374
gE-f	CATCAGAGCGACCAAGATCC	134921
gI-r	CGACCAGTAGAACATCTTCC	133415
gI-f	TTCGCGTAAAACTTGACC	133014
gC-1,4	GAGACGGTAACGCTGGTACTGTAA <sup>1</sup>	22200
gC-4	AGCCACGAACAACTCAACCGATGT <sup>1</sup>	specif. EHV-4
gC-1	GCGAGATGTGGTTGCCTAATCTCG <sup>1</sup>	22848
gG-r	TTAAGCAACGTACTCAA	128391
gG-f	TTGACTGTCTTAGCA	127159
gD-r	TCCTTCTCCGACGCAAGCA	132397
gD-f	CACCACCTCAAGCTTATGATGGA	131271

<sup>1</sup> Lawrence et al. 1994

The sequencing reaction took place in 20 µl of reaction mixture composed of 2 µl buffer, 2 µl 2 × concentrated sequencing mixture Big Dye Terminator v3.1 cycle Sequencing Kit (Applied Biosystems, USA), 3 µl of DNA, 3.5 µl of the appropriate primer (10 pmol/µl), 9.5 µl H<sub>2</sub>O; in the following conditions: 30 cycles of denaturation (20 s/96 °C), annealing (20 s/50 °C) and elongation (4 min/60 °C).

Data obtained through sequencing were processed and analysed using the Chromas Lite (Technelysium Pty Ltd., Austria) and Vektor NTI (Invitrogen, USA) software.

## Results

In our study we isolated the abortogenic strain of EHV-1 which was named “Prostejov” after the location of the affected farm.

The total length of nucleotide sequences obtained both in the field isolate and in the virus obtained in the cell culture by passaging was 3045 bp. We obtained partial sequences of genes encoding glycoproteins C (GenBank JN39738), D (GenBank JN397384), E (GenBank JN397385), G (GenBank JN397386) and I (GenBank JN397387). The lengths of these sequences were: 511bp for gC, 1029 for gD, 441 for gE, 1118 for gG and 387 for gI.

Comparison of sequences of Prostejov field isolate demonstrated a high compliance with the reference abortogenic strain EHV-1 known as V592 (GenBank AY464052). In the monitored sections of DNA we found only one point mutation at position 127679 of the V592 genom. The change can be identified in the gene for glycoprotein G. While in the V592 genome there is guanine at this position, in Prostejov and even in the virus attenuated at the cell culture, adenine was found in this position. For the encoded sequence of amino acids this means a change from asparagine to aspartic acid (Fig. 1).

Fig. 1. Comparison of the sequences encoding part of glycoprotein G. Change of amino acid from D (aspartic acid) to N (asparagine) caused by point mutation distinguishing V592 from the Prostejov isolate and the passaged virus.

Nucleotide sequences gG			Aminoacid sequences
		127666	127689
V592 AY464052	(127666)	GAAGTGGAAAAG <u>G</u> ACACAAACTAT	RVTLEVEK <u>D</u> TNYPGMIH
gG Prostejov	(460)	GAAGTGGAAAAG <u>A</u> ACACAAACTAT	RVTLEVEK <u>N</u> TNYPGMIH
gG cell culture	(460)	GAAGTGGAAAAG <u>A</u> ACACAAACTAT	RVTLEVEK <u>N</u> TNYPGMIH

Comparison of sequences of attenuated virus with sequences of the original field isolate also showed a solid match. Only one point mutation was found again in position 22412 of the V592 genom, which occurred in the stretch encoding gC for a change of cytosine to thymine. For the amino acid sequence this means a change: glutamic acid - lysine (Fig. 2). When compared with sequences of the known Kentucky A strain (GenBank S57839) which was passaged *in vitro* many times (Colle et al. 1996), this mutation was found also in this representative of laboratory strains. However, our trial

Fig. 2. Comparison of the sequences encoding glycoprotein C. Changing the amino acid E (glutamic acid) to K (lysine) at the point mutation, resulting from the passaging of the Prostejov virus in the cell culture.

Nucleotide sequences gC			Aminoacid sequences
		22400	22422
V592 AY464052	(22400)	TTTTTTCCTCT <u>C</u> TGCCGTTGAGA	LLLFTA <u>E</u> RRKKSRRGGQL
gC Prostejov	(113)	TTTTTTCCTCT <u>C</u> TGCCGTTGAGA	LLLFTA <u>E</u> RRKKSRRGGQL
gC cell culture	(133)	TTTTTTCCTCT <u>T</u> TGCCGTTGAGA	LLLFTA <u>K</u> RRKKSRRGGQL
gC Kentucky A	(2183)	TTTTTTCCTCT <u>T</u> TGCCGTTGAGA	LLLFTA <u>K</u> RRKKSRRGGQL

does not prove another difference between the V592 and KyA (position 22526) that occurs in the monitored section.

Other differences were found between the sequences of gD Prostejov and Lucina isolates. Both isolates come from the Czech Republic, but they were obtained at different times (Lucina in 2003, Prostejov in 2009). In these sequences the Prostejov isolate and the passaged virus correspond in 100% with V592 sequences, but differ from Lucina in three loci. No passage-induced point mutations were found in gD sequences of our passaged virus in contrast to KyA (GenBank M62923.1) (data not shown).

The sequence of gene sections for gE and gI are fully consistent with the relevant sequences of the reference virus strain V592.

## Discussion

Sections of the genome of the field isolate coding for the main surface glycoproteins of EHV-1 were selected for surveying the Prostejov field isolate and for determining the stability of the isolated virus during the passaging in the cell culture. These glycoproteins are crucial both for the properties of the virus during infection of host cells (gC, gD, gI and gE [Damiani et al. 2000; Schimmer and Neubauer 2003; Spear and Longnecker 2003]) and for a specific immune reaction of the host organism (gC, gD and gE gI [Manservigi and Cassai 1991; Damiani et al. 2000]). The gene for glycoprotein G was observed due to the possible use in controlling post-vaccination antibody titres of species-specific serological ELISA test distinguishing the closely related EHV-4 (Crabb and Studdert 1993).

Prostejov isolate and the virus cultured in cell culture differed from the reference abortogenic strain V592 only in the sequence of the gene for gG. This point mutation does not affect the species-specific immunogenic epitopes located in the C-terminal part of glycoprotein G (Crabb and Studdert 1993). In other genome sections under observation the Prostejov isolate does not vary from the reference strain V592 which proves the conservation of the genome of the EHV-1 virus; similarly to the absolute match in the sequences of parts of genes for gE and gI (Ghanem et al. 2007).

In monitoring the stability of our isolate during the *in vitro* passage on cell line RK13 (6 passages) we discovered a single point mutation which differentiates the sequence of the passaged virus from the sequence of the field isolate in the section encoding gC. This mutation occurs in the Kentucky A strain which was subjected to passage in heterologous hosts and in cell cultures (Colle et al. 1996). It appears that this is the locus of the virus genome passage prone to change. Unfortunately, we did not obtain the sequence encoding the main immunodominant epitopes of gC (Crabb and Studdert 1995). More work should be done in this field. Other changes that we could observe both in sequences gC and gD in Kentucky A did not occur in the sequences of passaged virus from our experiment.

It was interesting to compare the Prostejov isolate sequences with the other Czech isolate called Lucina obtained in 2003. Unfortunately, only a partial gene sequence for gD is available in this isolate. In the monitored gD section the sequences of both viruses differ in three loci. This may suggest the occurrence of an entirely different abortogenic strain in the Czech Republic in the span of six years.

Although the point mutation in the gene for a functional protein EHV-1 can mean, for example, an increase in the virus neuropathogenicity as was demonstrated in the gene for DNA polymerase (Nugent et al. 2006), we suspect that the nucleotide variation in the gene for glycoprotein G in the Prostejov isolate observed in this work will not have any importance for the pathogenesis of the disease. Similarly, the mutation in the gene for gC in the passaged virus will not have any effect on the construction of key neutralizing epitopes of this virus.

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