

**Szent István University**  
**Postgraduate School of Veterinary Science**

**Molecular characterisation of equid herpesvirus type 1  
strains isolated in Hungary between 1977 and 2008**

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PhD dissertation theses

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## INTRODUCTION

The equid herpesvirus-1 (EHV-1) is a worldwide spread pathogen of horses, which can be associated with respiratory, abortogenic and neurological diseases. The economical losses connected with the outbreaks of the virus have not decreased in the last decades, despite the vaccination of horses. The occurrence of neurological signs among valuable racing and breeding horses can cause huge losses for the owner of a herd. Several studies provided data about the distribution of EHV-1 in Hungary; according to the results of earlier studies this virus was the causative agent in 10-15% of the abortions and perinatal foal losses in the country. Unfortunately about the occurrence of neuropathogenic strains in Hungarian horses we do not have exact data.

The virus is spread by saliva and nasal discharge, as well as by aborted fetuses, placentas or placental fluid. EHV-1 can cause primary infection, but as in the case of other herpesviruses, reinfection or reactivation also can occur. The clinical signs depend on several factors including the pathogenic potential of the virus strains, the health status of the host animals and stress that affects the animals. Latent infection as a herpesvirus-specific type of infection can also occur. The latently infected animals in a herd form the reservoir of the virus. The data of previous studies prove that the significance of EHV-1 cannot be underrated even when vaccines are regularly used in the herd. Vaccines only reduce the amount the virus shedding but cannot prevent the reactivation and reinfection, only reduce the chance of the infection among horses.

EHV-1 is a member of the subfamily *Alphaherpesvirinae* within the family *Herpesviridae*. Its genome is a linear double-stranded DNA, which contains 76 open reading frames (ORFs). In a former study, two EHV-1 strains (Ab4, V592) were compared, and the results showed that two regions (ORF30, ORF68) are suitable for the distinction of EHV-1 strains. A single nucleotide polymorphism (SNP) in the 2254 nucleotide position of the ORF30 region resulting in an amino acid variation (N/D752) is significantly associated with the neuropathogenic potential of the EHV-1 strain. According to the nucleotide substitutions of the ORF68 region several genetic groups can be set up. The alterations of the ORF68 regions cannot influence the pathogenicity of the EHV-1 strains, but they showed some correlations with the geographic origin.

In previous studies we cannot find data about the genetic variability of Hungarian EHV-1 strains and the distribution of strains with distinct pathogenicity. The aim of my examinations was to analyse and classify 35 EHV-1 field isolates originated from aborted horse fetuses, sent as clinical samples to the Central Veterinary Institute between 1977 and 2008.

## THE AIMS OF MY STUDY

With the genetic analysis of the 35 Hungarian EHV-1 isolates, we hoped that we can provide data on the genetic variability of our isolates and the following questions could be answered:

1. How many isolates code the neuropathogenic genotype-specific single nucleotide polymorphism (G2254) among the 35 Hungarian samples?
2. The isolate (04\_04), which could be associated with neurological signs during the infection, also has the genetic marker of the neuropathogenic genotype?
3. Is it possible to develop a new, faster but simple and robust method for the differentiation of the two (neuropathogenic and non neuropathogenic) genotypes?
4. After the analysis of the ORF68 region how many genetic groups can be set up from the 35 Hungarian isolates? Are there any unidentified nucleotide substitutions in this region? If the answer is yes, how many newly formed groups should be set up?
5. Is there any kind of correlation between the area of distribution and the group specific SNPs of the EHV-1 isolates?
6. How can the veterinary practice utilize the data of the analysis of these two open reading frames?
7. Are there any kind of vaccine specific genetic markers in the analysed regions ORF30 and ORF68 of *RacH*, which can be used for the distinction of field isolates and vaccine strains?

## **MATERIALS AND METHODS**

The 35 field isolates used in this study were isolated from organs (liver, spleen) of aborted horse foetuses, sent as clinical samples between 1977 and 2008. The codes of the isolates were created from the year of isolation and the order of samples within a year. Some samples originated from the same outbreak, and some derived from the same stud but from different outbreaks. Beside the field isolates a vaccine strain (RachH) and a known abortigenic strain (Army 183) were also included in the studies.

### Virus isolation

All strains were cultured on rabbit kidney cells (RK-13). After cytopathic effect (CPE) reached a level of 90-95% the flasks were frozen and thawed and the supernatant was clarified by centrifugation.

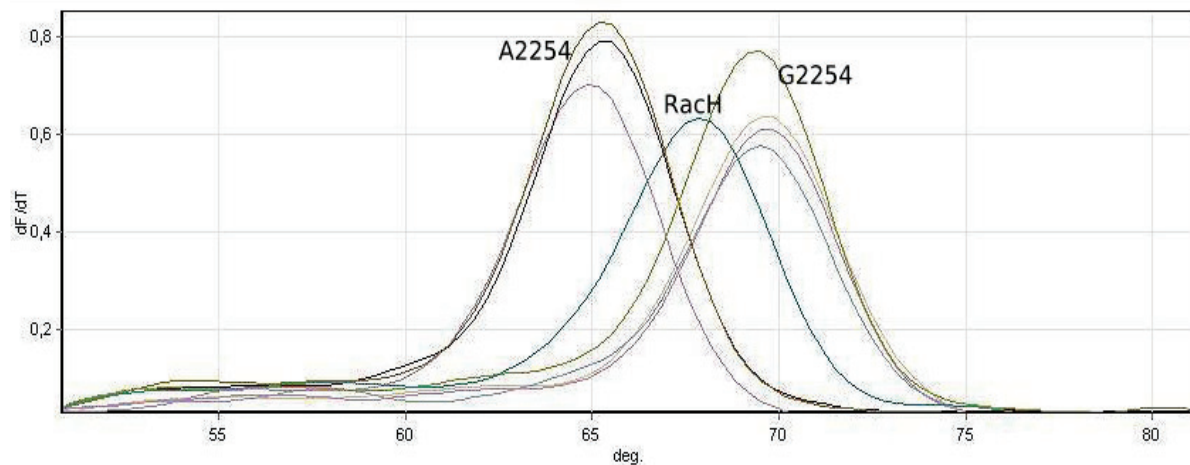
### Identification of EHV-1 with a multiplex PCR method (Carvalho et al., 2000)

After the centrifugation of frozen and thawed supernatant, the viral DNA was extracted. Before the genetic analysis every isolates were checked with the multiplex PCR, so all isolates that were included in our examinations proved to be EHV-1.

## RESULTS AND CONCLUSIONS

### Results of the analysis of ORF30 region

Our newly developed PCR is based on primer-probe energy transfer (PriProET) method. Contrary to the real-time PCRs, which use duplex Taqman probes, this method needs only one probe, and the alterations of the specific sequences resulted in different melting temperatures. Therefore the two genotypes can easily be distinguished with the melting curve analysis. The melting curve analysis of the two genotypes and the RachH strain is shown in Figure 1.



*Figure 1. The melting curves of isolates coding A2254 (non neuropathogenic) and G2254 (neuropathogenic) and RachH vaccine strain*

Among the 35 Hungarian isolates 5 coded G at the 2254 nucleotide position (nt), so the 14% of the isolates represented the neuropathogenic genotype of the EHV-1. The only isolate (04\_04) that could be associated with neurological signs also was the representative of the neuropathogenic genotype. The two laboratory strains also coded G in this specific position but RachH strain has a very unique SNP at nt 2259 (C2259). The changes of ORF30 in RachH can explain the separate peak visible on the melting point graph as well as the intermediate melting temperature.

### Results of the analysis of ORF68 region

A region of ~600 bp region of ORF68 was particularly polymorphic so it can be adopted as a marker system for the grouping of EHV-1 isolates. ORF68 does not provide information on the neuropathogenic potential of EHV-1 isolates but it can be correlated with the geographic origin of the isolates. In a previous study six groups could be created from the 131 analysed isolates. Because we couldn't get consequent results with the usage of the recommended PCR method of the original study, we designed new PCR primes for the analysis of this

region. On the basis of the grouping criteria of the original study 23 Hungarian EHV-1 isolates (66%) could be placed into three of the six previously formed groups. Based on the nucleotide substitutions of the remaining 12 Hungarian EHV-1 isolates, four new groups could be set up. So our study revealed that the polymorphism of the ORF68 region could be higher than previously suspected. Restrictions in the yearly occurrence and limitations of the geographic distribution belonging to certain groups could be observed. So in these cases we found correlations between the group specific SNPs and the geographic distribution and the yearly occurrence.

Our data suggest that the usage of these two genetic markers can be a useful tool in epidemiological studies, it can provide information about the origin of the strain. The analysis of the ORF68 in the strains from this region of Europe can reveal more group-specific SNPs, and the number of groups can probably increase.

## NEW SCIENTIFIC RESULTS

The data obtained during our study provided us with answers to our initial questions:

1. Five (14%) out of 35 Hungarian strains were shown to carry the unique nucleotide polymorphism characteristic of neuropathogenic strains.
2. The isolate marked 04\_04, which was documented to cause neurologic symptoms, had a guanine base at position 2254 of ORF30, thus belongs to the neuropathogenic genotype.
3. We developed a primer-probe energy transfer (PriProET) system, a quick and specific method to distinguish between neuropathogenic and non-neuropathogenic strains.
4. We developed a new method to study point mutations occurring in the ORF68 region of the EHV-1 genome. Using this method we determined the frequencies of the previously defined groups among Hungarian EHV-1 isolates. Twelve of the 35 isolates carried point mutations not described previously. Based on these results four new groups could be set up in addition to the existing 6.
5. Since not all EHV-1 strains isolated in Hungary during the past 30 years were included in our study, the place of origin of each isolate can only be considered a temporary stage in the circulation of the strains. The studied genetic markers do not provide information on the geographic distribution of the groups, only the strains circulating in a certain area may be identified and followed up.
6. The detection and documentation of SNPs within ORF30 and ORF68 may be especially important in the epidemiological investigation of EHV-1 outbreaks. More genetic markers determined in genome of an EHV-1 isolate allows easier identification of the place of origin of the strain causing the infection.



## LIST OF PUBLICATIONS

### Studies published in referred Hungarian or international scientific periodicals:

Malik, P., Palfi, V., Balint, A., 2010. Development of a new primer–probe energy transfer method for the differentiation of neuropathogenic and non-neuropathogenic strains of equine herpesvirus-1. J. Virol. Methods. 169, 425-427.

Malik, P., Bálint, Á., Dán, Á., Pálfi, V. 2012. Molecular characterisation of the ORF68 region of equine herpesvirus-1 strains isolated from aborted fetuses in Hungary between 1977 and 2008. Acta Vet. Hung. 60, 175-187.

Malik Péter, Pálfi Vilmos 2012. Az EHV-1 és EHV-4 diagnosztikájának gyakorlati kérdései Magyar Állatorvosok Lapja 134, 67-75.

### Presentations of international conferences:

P. Malik, Á. Bálint, Á. Dán, V. Pálfi (poster): Molecular analysis of equine herpesvirus 1 strains isolated in the last 30 years in Hungary. 8th International Congress of Veterinary Virology, Budapest, Hungary, 23–26 August 2009

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