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*Original article*

# Replication kinetics of neuropathogenic and non-neuropathogenic equine herpesvirus type 1 (EHV-1) strains in primary murine neurons and ED cell line

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## Abstract

Equine herpesvirus type 1 (EHV-1) causes respiratory infections, abortion and neurological disorders in horses. Molecular epidemiology studies have demonstrated that a single-point mutation in DNA polymerase gene, resulting in an amino acid variation (N752/D752), is significantly associated with the neuropathogenic potential of EHV-1 strains. The aim of the study was to elucidate if there are any differences between neuropathogenic (EHV-1 26) and non-neuropathogenic (Jan-E and Rac-H) EHV-1 strains in their ability to infect neuronal cells. For the tested EHV-1 strains, cytopathic effect (CPE) was manifested by changed morphology of cells, destruction of actin cytoskeleton and nuclei degeneration, which led to focal degeneration. Moreover, EHV-1 26 strain caused fusion of the infected cells to form syncytia in culture. Real-time PCR analysis demonstrated that both neuropathogenic and non-neuropathogenic EHV-1 strains replicated in neurons and ED cells (equine dermal cell line) at a similar level. We can assume that a point mutation in the EHV-1 polymerase does not affect viral replication in this cell type.

**Key words:** EHV-1, primary murine neurons, ED cells, neurotropism, neuropathogenicity

## Introduction

Equine herpesvirus type 1 (EHV-1), a member of the subfamily *Alphaherpesvirinae* of the family *Herpesviridae*, is a major cause of abortion, respiratory disease and neurological disorders in horses worldwide (Bryans and Allen 1989). As other alpha-herpesviruses, EHV-1 is neurotropic and establishes lifelong latency in the nervous system on the host. On the other hand, its neuropathogenicity appears to be associated rather with the ability to infect endothelial cells within the CNS, causing vasculitis and damage to surrounding tissue, than with the ability of the virus to replicate in the neural cells. Nugent et al. (2006) identified non-synonymous point mutation significantly associated with the neuropathogenicity of EHV-1 strains. This nucleotide polymorphism is a substitution of adenine (A) for guanine (G) at 2254 position within the gene encoding catalytic subunit of the viral DNA polymerase (ORF30) and it is used as a marker to identify strains with a tendency to cause neurological disorders (Goodman et al. 2007).

Until recently, the most serious consequence of EHV-1 infection in mares was abortion. Though it has been observed, that the incidence and severity of neurological disorders caused by EHV-1 has significantly increased in recent years (Mori et al. 2015). The first isolation of neuropathogenic EHV-1 strain has been described by Saxegaard in 1966 (Vissani et al. 2009). In Poland, studies on the prevalence of neuropathogenic EHV-1 have been also conducted. In 2015 the first neuropathogenic strains of EHV-1 in the Polish horse population were isolated. It was shown that of twenty clinical samples containing EHV-1, two were neuropathogenic (Stasiak et al. 2015).

Presently, increased attention is paid to the molecular aspects of neuropathogenicity of EHV-1 strains, therefore determination of the differences between the non-neuropathogenic and neuropathogenic strains may be important for better understanding of the molecular mechanisms involved in EHV-1 neurotropism and neuropathogenicity. Although it has been demonstrated that a single-nucleotide polymorphism in the EHV-1 DNA polymerase gene was associated with the neurological disease in horses, it is not clear whether this mutation affects, in any way, neurotropism of the virus.

The aim of the study was to elucidate if there are any differences between neuropathogenic and non-neuropathogenic EHV-1 strains in their ability to infect neural cells by comparison of the replication kinetics of neuropathogenic and non-neuropathogenic EHV-1 strains in primary murine neuronal cells and ED cells.

## Materials and Methods

### Ethics statement

All animals were handled and cared for according to Polish and European Animal Care and Use guidelines and regulations. All procedures involving live animals were approved by a Local Ethics Committee and confirmed to applicable international standards agreement No. 24/2012.

### Neuronal Cells Culture

To address the question of EHV-1 neuropathogenicity we have established an *in vitro* culture system of embryonal murine neuronal cells, as described before (Cymerys et al. 2010). Neuronal cells were suspended in B-27 Neuron Plating Medium consisting of neurobasal medium, B27 supplement, glutamine (200 mM), glutamate (10 mM), antibiotics (penicillin and streptomycin) with 10% supplement of fetal and equine serum (Gibco) and plated onto coverslips coated with poly-L-lysine, or poly-D-lysine with laminin ( $10^4 - 10^5$  neurons per well) and incubated at 37°C with 5% CO<sub>2</sub>. Four days after plating the medium was removed and replaced with Neuron Feeding Medium (B-27 Neuron Plating Medium without glutamate). Then the culture of neurons was maintained for the next 6 days prior to further manipulations.

### ED cells culture

ED cell line (equine dermal; ATCC CCL57), a homologous cell line, was suspended in Eagle's minimum essential medium (MEM) with Earle's salts, 5% of inactivated fetal calf serum and 40 mg/ml of gentamicin (Gibco Life Technologies). ED cells were maintained at 37°C with 5% CO<sub>2</sub>.

### Virus strains

In the current study three EHV-1 strains were used. Two neurotropic, non-neuropathogenic strains of EHV-1 from the collection of the Virology Laboratory at Warsaw University of Life Sciences-SGGW: (i) Jan-E EHV-1 isolated from aborted fetus (12<sup>th</sup> passage in ED cells; lack of neuropathogenicity as confirmed by PCR-RFLP neuropathogenic/non-neuropathogenic discrimination test) (Stasiak et al. 2015) and (ii) Rac-H EHV-1, which has been passaged through a series of cell cultures and is defined as

„pantropic, non-pathogenic” (Nugent et al. 2006). Neuropathogenic strain of EHV-1 (EHV-1 26) was isolated from aborted fetus in Hungary in 2004 (neuropathogenicity confirmed by PriProET technique) (Malik et al. 2010).

### Cell Cultures infection

Prepared as shown above, primary murine neuronal cells and ED cells were infected with Rac-H, Jan-E or EHV-1 26 strains of EHV-1 (MOI 1.0) for 60 min at 37°C. After incubation the inoculum was removed and a fresh culture medium was added. Subsequently, infected cells were incubated for 24, 48 and 72 hours at 37°C with 5% CO<sub>2</sub>.

### Immunofluorescent staining procedures

After washing in PBS, cells were fixed in 3.7% paraformaldehyde/PBS (Sigma-Aldrich) for 10 min at room temperature and suspended in cold acetone (-20°C) for 5 min. Before staining, fixed cells were blocked with PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich) for 30 min at room temperature. Filament structures of actin were visualised by TRITC-phalloidin conjugate (500 ng/ml; Sigma-Aldrich). The presence of viral antigen was detected by means of direct immunofluorescence, using polyclonal antiserum EHV-1/ERV conjugated to FITC (VMRD, Inc.). Cell nuclei were stained with Bisbenzimidine/Hoechst 33258, in compliance with manufacturer's recommendations. Afterwards, coverslips were mounted on microscope slides using anti-fade mounting medium (Sigma-Aldrich). Confocal microscope images were obtained using Leica SP8-WLL white-light laser confocal microscope under 63x oil-immersion lens and analysed with LAS AF Lite 3.2.0 and Adobe Photoshop software.

### Quantitative real-time PCR

The quantity of the viral DNA in primary murine neurons and ED cells infected with Jan-E, Rac-H or EHV-1 26 strains of EHV-1 was estimated using quantitative real-time PCR (qPCR). Samples were collected 24, 48 or 72 hours post infection (h p.i.). Tests were run on the LightCycler 2.0 instrument (Roche Diagnostics) with fluorescent TaqMan probes, complementary to the sequence within the amplified product, according to the in-house quantitative method (Dzieciatkowski et al. 2009). Jan-E EHV-1 strain serial dilutions (from CCID<sub>50</sub> = 10<sup>6</sup> to

CCID<sub>50</sub> = 10<sup>2</sup>) were used as reaction standards, whereas non-infected ED cells or neuronal cells served as negative control. Each sample was amplified with internal control (positive control of the amplification process) and tested in independent triplicates.

### Statistical evaluation

The results were statistically evaluated by one-way analysis of variation (ANOVA) using Student-Newman-Keuls multiple comparisons. This analysis was performed using GraphPad Prism™ version 4.03 software (GraphPad Software Inc., San Diego, CA, USA). Statistical differences were interpreted as significant at p<0.05 (\*), highly significant at p<0.01 (\*\*), extremely significant at p=0.0001 to 0.001 (\*\*\*) and not significant at p>0.05.

### Results

Irrespective of the EHV-1 strain used, in both cultured murine neuronal cells and ED cells (equine dermal cell line), cytopathic effect (CPE) was observed after 24 h p.i. (Fig. 1 and 2). At this time the organization of the cytoskeleton in cells infected with EHV-1 was completely changed as compared with control. In mock-infected ED cells, actin filaments formed a network of fibers within the cytoplasm, as well as in the cortical layer of the cytoplasm. In Rac-H and Jan-E EHV-1-infected cells (24 and 48 h p.i.) microfilaments were disrupted, whereas CPE was mainly manifested by destruction and focal degeneration of cells. EHV-1 26 strain caused more severe changes in ED cells morphology. CPE was manifested by destruction of actin cytoskeleton, disintegration and nucleus degradation which led to cell degeneration. Moreover, fusion of the adjacent infected cells forming syncytia in culture was observed (Fig. 1, arrows).

In mock-infected primary murine neurons the densest distribution of microfilaments was observed within the cytoplasm and in the peripheral region of the plasma membrane. In neurons infected with Rac-H EHV-1 strain disruption of the microfilaments system and general depolymerization of actin filaments occurred, whereas Jan-E EHV-1 infection stimulated actin polymerization in the peripheral region of the neurons and induced formation of long actin-containing projections, which stretched from cell to cell. In EHV-1 26-infected neurons, long actin-containing projections and accumulation of viral antigen inside these structures were also detected (Fig. 2, arrows).

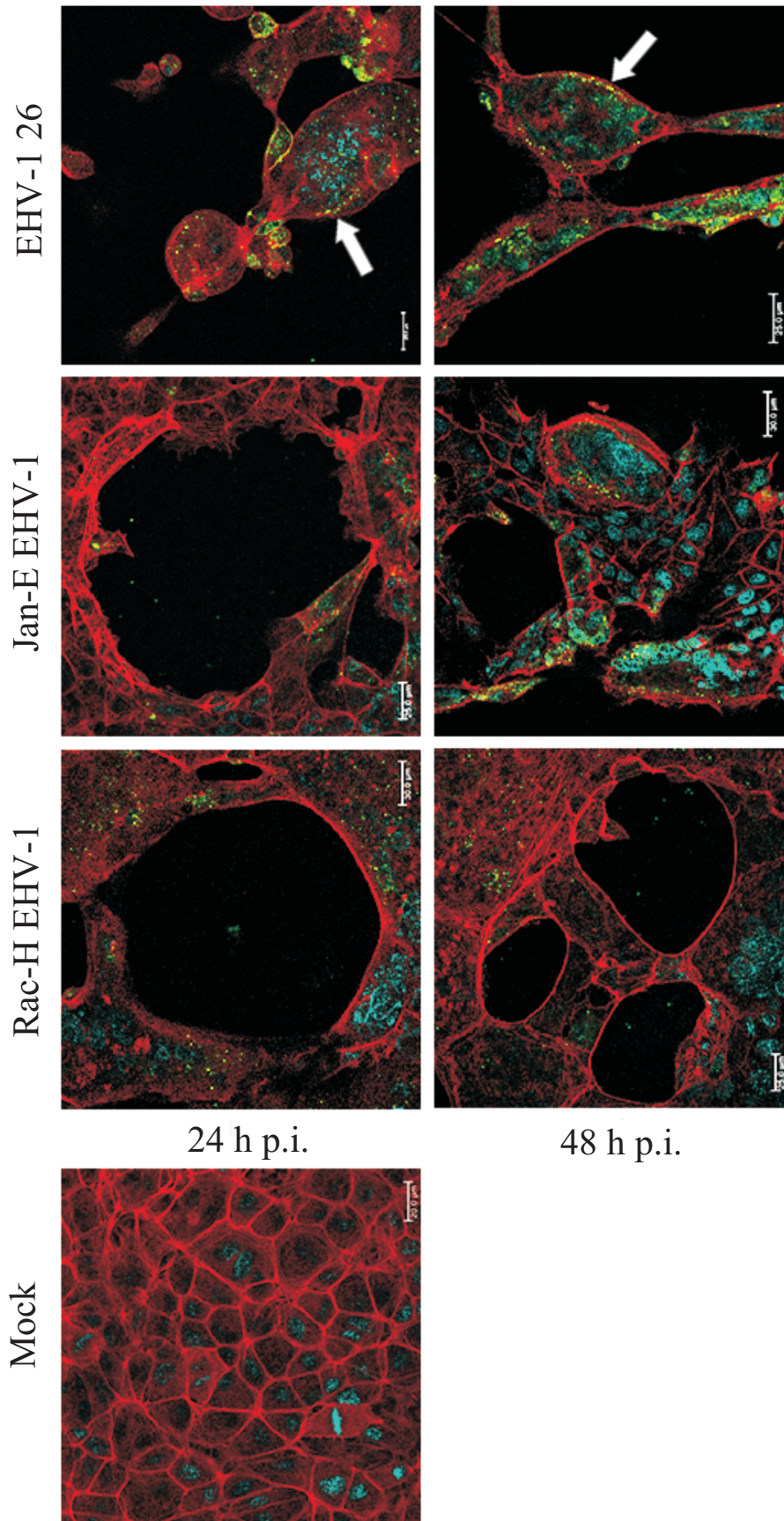


Fig. 1. The effect of EHV-1 infection on the cell morphology and actin filaments in ED cells. Confocal images of mock-treated, Rac-H EHV-1-infected, Jan-E EHV-1-infected and EHV-1 26-infected ED cells (24 and 48 h p.i.). Actin filaments are shown in red, EHV-1 antigens in green and DNA in blue.

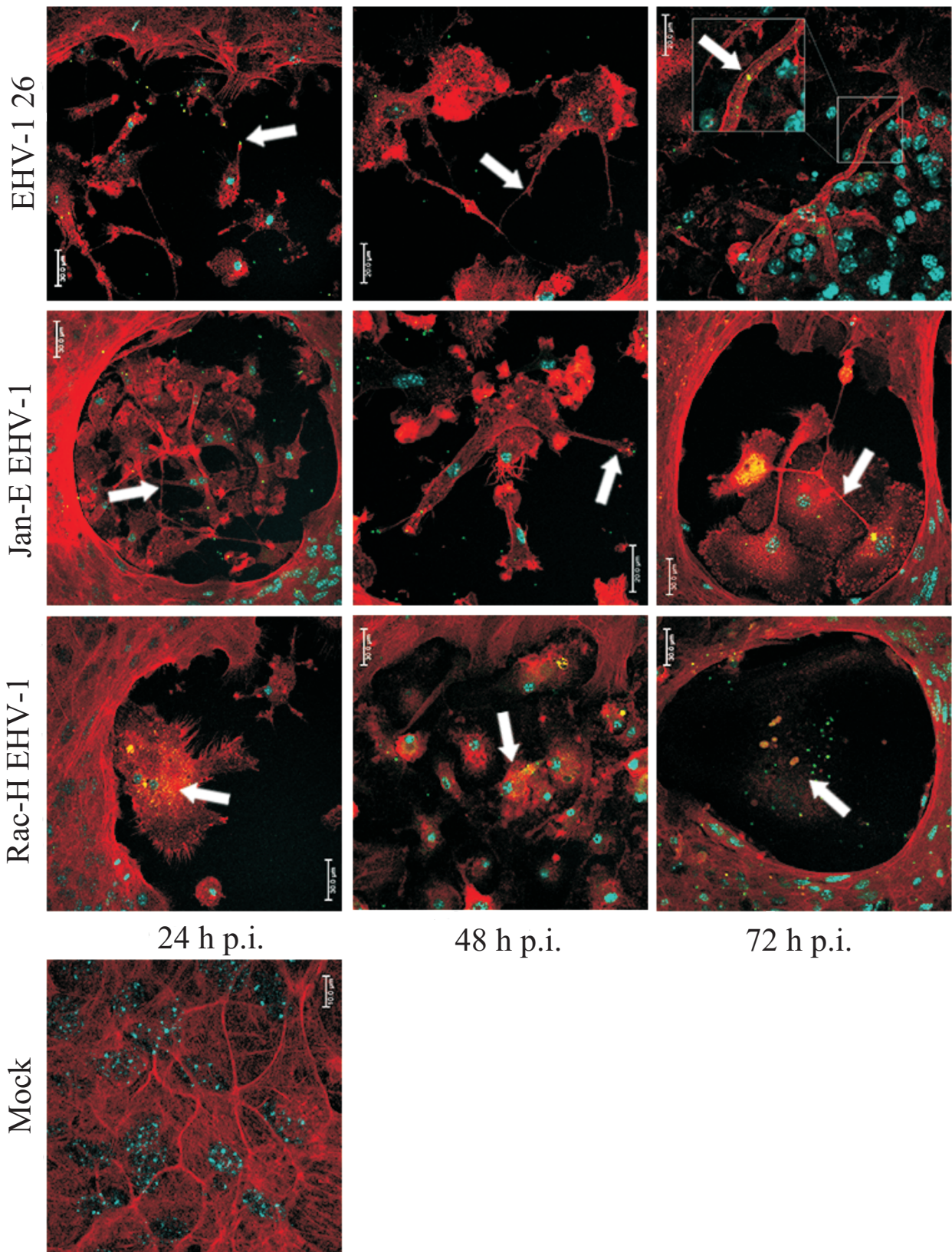


Fig. 2. The effect of EHV-1 infection on the cell morphology and actin filaments in primary murine neurons. Confocal images of mock-treated, Rac-H EHV-1-infected, Jan-E EHV-1-infected and EHV-1 26-infected neuronal cells (24, 48 and 72 h p.i.). Actin filaments are shown in red, EHV-1 antigens in green and DNA in blue.

Table 1. Results of real-time PCR quantification of non-neuropathogenic and neuropathogenic EHV-1 strains DNA.

Virus strain [h p.i.]	ED cells [CCID <sub>50</sub> ]	Primary murine neurons [CCID <sub>50</sub> ]
Rac-H EHV-1 [24 h p.i.]	1.49 ± 0.47 x 10 <sup>7</sup>	1.19 ± 0.18 x 10 <sup>5</sup>
Rac-H EHV-1 [48 h p.i.]	2.95 ± 0.14 x 10 <sup>7</sup>	1.26 ± 0.13 x 10 <sup>5</sup>
Rac-H EHV-1 [72 h p.i.]	–	0.18 ± 0.04 x 10 <sup>5</sup>
Jan-E EHV-1 [24 h p.i.]	0.46 ± 0.08 x 10 <sup>7</sup>	2.15 ± 0.45 x 10 <sup>5</sup>
Jan-E EHV-1 [48 h p.i.]	1.82 ± 0.13 x 10 <sup>7</sup>	1.95 ± 0.24 x 10 <sup>5</sup>
Jan-E EHV-1 [72 h p.i.]	–	0.07 ± 0.01 x 10 <sup>5</sup>
EHV-1 26 [24 h p.i.]	0.86 ± 0.02 x 10 <sup>7</sup>	2.96 ± 0.22 x 10 <sup>5</sup>
EHV-1 26 [48 h p.i.]	1.31 ± 0.03 x 10 <sup>7</sup>	3.08 ± 0.78 x 10 <sup>5</sup>
EHV-1 26 [72 h p.i.]	–	0.60 ± 0.12 x 10 <sup>5</sup>

Table 2. The statistical analysis between EHV-1 strains. The statistical analysis between the strains was performed at various time points after infection primary murine neurons and ED cells, in order to compare the replication kinetics of non-neuropathogenic and neuropathogenic EHV-1 strains.

Compared samples	Statistical significance [p value]	
	ED cells	primary murine neurons
Jan-E EHV-1 [24 h p.i.] vs [48 h p.i.]	p = 0.0002***	p = 0.1200
Jan-E EHV-1 [48 h p.i.] vs [72 h p.i.]	–	p = 0.0030**
Rac-H EHV-1 [24 h p.i.] vs [48 h p.i.]	p = 0.0276*	p = 0.0543
Rac-H EHV-1 [48 h p.i.] vs [72 h p.i.]	–	p = 0.0013**
EHV-1 26 [24 h p.i.] vs [48 h p.i.]	p = 0.0033**	p = 0.4257
EHV-1 26 [48 h p.i.] vs [72 h p.i.]	–	p = 0.0113*
Rac-H EHV-1 vs Jan-E EHV-1 [24 h p.i.]	p = 0.0423*	p = 0.0127*
Rac-H EHV-1 vs Jan-E EHV-1 26 [24 h p.i.]	p = 0.0811	p = 0.0083**
Jan-E EHV-1 vs EHV-1 26 [24 h p.i.]	p = 0.0029**	p = 0.0856
Rac-H EHV-1 vs Jan-E EHV-1 [48 h p.i.]	p < 0.00001***	p = 0.0038**
Rac-H EHV-1 vs Jan-E EHV-1 26 [48 h p.i.]	p = 0.0020**	p = 0.0199*
Jan-E EHV-1 vs EHV-1 26 [48 h p.i.]	p = 0.0163*	p = 0.0344*
Rac-H EHV-1 vs Jan-E EHV-1 [72 h p.i.]	–	p = 0.0392*
Rac-H EHV-1 vs Jan-E EHV-1 26 [72 h p.i.]	–	p = 0.0069**
Jan-E EHV-1 vs EHV-1 26 [72 h p.i.]	–	p = 0.0116*

Real-time PCR technique was applied to detect viral DNA in ED and murine neuronal cells. A statistically significant increase in the amount of viral DNA was demonstrated for all used EHV-1 strains when compared to the non-infected neurons. The ED cells infected with EHV-1 showed high CCID<sub>50</sub> (10<sup>7</sup>) values from 24 h p.i. to 48 h p.i. It was not possible to perform the measurement at 72 h p.i., because the cells were entirely lysed (Table 1). In primary murine neurons we also detected a statistically significant increase in viral DNA, from 24 h p.i. until 72 h p.i. (CCID<sub>50</sub> ~ 10<sup>5</sup>).

The statistical analysis between the strains was performed at various time points after infection of the ED cells and neurons, in order to compare the replication kinetics of non-neuropathogenic and neuropathogenic EHV-1 strains (Table 2). As regards the ED cells, statistically significant results of the comparison of replication 24 h p.i. were recorded for

strains Rac-H vs Jan-E (p<0.05), and Jan-E vs EHV-1 26 (p<0.01). At 48 h p.i., highly statistically significant differences were demonstrated by comparing the replication of strains Rac-H vs Jan-E (p<0.001), Rac-H vs EHV-1 26 (p<0.01) and Jan-E vs EHV-1 26 (p<0.05) (Table 2). The highest level of viral DNA was observed during the infection of ED cells with the non-neuropathogenic Rac-H EHV-1 strain (Table 1).

The statistical analysis between EHV-1 strains after infection of primary murine neurons showed statistically significant differences between the Rac-H strain and Jan-E and EHV-1 26 strains (p<0.05 and p<0.01). Concerning Jan-E vs EHV-1 26 strains, statistically significant differences were demonstrated 48 and 72 h p.i. (p<0.05) (Table 2). The highest level of viral DNA was detected during the infection of neurons with the neuropathogenic EHV-1 26 strain 24 and 48 h p.i. (Table 1).

## Discussion

Latest data indicate the existence of both neuropathogenic and non-neuropathogenic strains of EHV-1. The concept of EHV-1 neuropathogenicity indicates the ability to cause pathological changes in the nervous tissue which is associated with the occurrence of clinical symptoms (Cymerys et al. 2010). The neuropathogenic mechanism of the virus has been partially explained by other researchers. Epidemiological studies have shown that a single-nucleotide polymorphism in the EHV-1 DNA polymerase gene was associated with the outbreaks of highly lethal neurological disease in horses. It is known that neuropathogenic strains during replication in neuronal cells cause damage to the small blood vessels, which is accompanied by extravasation and blood clotting. However, the role of direct neuronal damage in the induction of neurological form of infection is not known (Sauerbrei et al. 2002, Goodman et al. 2007, Yamada et al. 2008, Smith et al. 2010).

In the present study, the infectivity and replication kinetics of neuropathogenic and non-neuropathogenic strains of EHV-1 were investigated using primary murine neurons and ED cells. Neurons are a special type of cells for herpesviruses, because the status of latent infection is established in these cells. Previously, we have demonstrated that cultured murine neurons are useful *in vitro* model to study neurotropism and neuroinvasiveness of EHV-1 (Cymerys et al. 2010, 2012). Although mouse is not a natural host for EHV1, it is a species susceptible to infection, in which the virus replicates in the nervous system (Awan et al. 1990, Walker et al. 1999). The ED culture is a homologous cell line, which was used to accurately determine the CPE character of non-neuropathogenic EHV-1 strains (Jan-E and Rac-H) (Turowska et al. 2010).

In the current study three EHV-1 strains were used: two non-neuropathogenic strains – the reference Rac-H and field Jan-E isolated from abortion case in Poland and one neuropathogenic strain 26, isolated in Hungary. Observation of the CPE caused by these viruses during infection of cultured murine neurons and ED cell cultures revealed that each strain affected both cell types in different manner causing different cytopathic effect (Fig. 1 and 2). Further analysis of the cytoskeleton rearrangement also indicated that each virus affects F-actin network in different way. The results are consistent with our previous studies (Turowska et al. 2010, Słońska et al. 2014). The Rac-H EHV-1 strain caused the destruction of the actin cytoskeleton in the infected neurons and ED cells. The Jan-E EHV-1 strain stimulated actin polymerization in the peripheral portion, which in case of

neuronal cultures could be observed as the formation of long, thin projections containing actin filaments. These actin-containing projections were also detected in the EHV-1 26-infected neurons. The above-mentioned structures seemed to contribute to the direct spread of virus particles to adjacent cells without being exposed to the extracellular space. It seems that the neuropathogenic EHV-1 strain uses the same strategy to infect adjacent cells as the Jan-E EHV-1 (Słońska et al. 2014). The literature data suggest that other herpesviruses also induce the formation of similar actin projections – HHV-1 in the Vero cells and BHK, VZV in the HFF cells or SuHV-1 in RK13 and PK15 cells (Favoreel et al. 2005, 2007).

The analysis using real-time PCR showed that both neuropathogenic and non-neuropathogenic strains of EHV-1 replicated in primary murine neuronal cells and ED cells without the need for adaptation. The highest level of viral DNA during the infection of ED cells was demonstrated for the non-neuropathogenic Rac-H EHV-1 strain. Probably the highest level of the Rac-H strain replication was due to the fact that it was passaged many times and thus it was well adapted to the ED cells. The analysis of the replication level of tested EHV-1 strains in primary murine neuronal culture showed that the highest level of viral DNA was observed for the neuropathogenic EHV-1 26 strain 24 and 48 h p.i.

According to these results, it can be concluded that the point mutation in the gene encoding the DNA polymerase of the EHV-1 strain 26 does interfere with the replication of neuropathogenic strains neither in neurons cultured *in vitro* nor the ED cell line. As mentioned before, in 2015, Stasiak et al. (2015) have confirmed for the first time the presence of neuropathogenic strains of EHV-1 in the Polish horse population. Restriction digestion of viral genomes isolated from aborted fetuses revealed that out of twenty samples infected with EHV-1, two were infected with the neuropathogenic strain (10%). Sequencing of neuropathogenic samples confirmed the presence of adenine at nucleotide position 2254. Interestingly, neurological disorders were confirmed in none of the analyzed cases. Considering our results, it is tempting to speculate that direct neuronal damage is not the result of the infection with neuronal form of EHV-1. As discussed above, neuropathogenic EHV-1 strains have also the ability to infect endothelial cells, which can lead to inflammation and thrombosis of small blood vessels in the brain or spinal cord. Neurological disorders, such as ataxia, muscle weakness and/or paralysis, bladder atony, recumbency, which may ultimately lead to death do not result from direct damage to neurons associated with EHV-1 replication (Yamada et al. 2008, Cymerys et al. 2010, 2012).

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