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**Equine alphaherpesviruses (EHV-1 and EHV-4) differ in their efficiency to
infect mononuclear cells during early steps of infection in nasal mucosal
explants**

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Abstract

Equine herpesvirus type 1 (EHV-1) replicates extensively in the epithelium of the upper respiratory tract, after which it can spread throughout the body via a cell-associated viremia in mononuclear leukocytes reaching the pregnant uterus and central nervous system. In a previous study, we were able to mimic the *in vivo* situation in an *in vitro* respiratory mucosal explant system. A plaquewise spread of EHV-1 was observed in the epithelial cells, whereas in the connective tissue below the basement membrane (BM), EHV-1-infected mononuclear leukocytes were noticed. Equine herpesvirus type 4 (EHV-4), a close relative of EHV-1, can also cause mild respiratory disease, but a cell-associated viremia in leukocytes is scarce and secondary symptoms are rarely observed. Based on this striking difference in pathogenicity, we aimed to evaluate how EHV-4 behaves in equine mucosal explants. Upon inoculation of equine mucosal explants with the EHV-4 strains VLS 829, EQ₁ 012 and V01-3-13, replication of EHV-4 in epithelial cells was evidenced by the presence of viral plaques in the epithelium. Interestingly, EHV-4-infected mononuclear leukocytes in the connective tissue below the BM were extremely rare and were only present for one of the three strains. The inefficient capacity of EHV-4 to infect mononuclear cells explains in part the rarity of EHV-4-induced viremia, and subsequently, the rarity of EHV-4-induced abortion or EHM.

Key words: EHV-4; hampered spread; basement membrane barrier; mononuclear cells

1. Introduction

At first, the equine alphaherpesviruses equine herpesvirus type 1 (EHV-1) and equine herpesvirus type 4 (EHV-4) were considered as 2 subtypes of the same virus, designated EHV-1 subtype 1 and EHV-1 subtype 2 respectively (Sabine *et al.*, 1981; Studdert *et al.*, 1981). Additional sequence information confirmed that EHV-1 and EHV-4 are actually two closely related, but distinct viruses, with an amino acid (aa) sequence identity ranging between 54.9% and 96.4% (Telford *et al.*, 1998). To date, EHV-1 and EHV-4 are both classified as members in the order *Herpesvirales*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Davison *et al.*, 2009).

EHV-1 and EHV-4 are major causative agents of respiratory disease in the horse (Allen and Bryans, 1986; Crabb and Studdert, 1995; Reed and Toribio, 2004). Initial infection starts with replication of the virus in epithelial cells lining the upper respiratory tract (URT). EHV-1 does not only have a tropism for epithelial cells, but also targets mononuclear leukocytes, misusing these cells for viral transportation through the basement membrane (BM) barrier (Kydd *et al.*, 1994; Gryspeerdt *et al.*, 2010). Indeed, we showed in a previous study that EHV-1-induced plaques cannot breach the BM, but that EHV-1 penetrates the deeper tissues of the respiratory tract in a more discrete manner, using migrating individual mononuclear cells as a Trojan horse (Vandekerckhove *et al.*, 2010). Carried by these mononuclear leukocytes, EHV-1 can easily spread through the body via a cell-associated viremia, reaching its target organs, the pregnant uterus and the central nervous system. At these secondary replication sites, EHV-1 replicates in endothelial cells, causing vasculitis, thrombosis and disseminated ischemic necrosis, resulting in abortion and nervous system disorders (Smith *et al.*, 1996; Wilson, 1997; Smith and Borchers, 2001; Reed and Toribio, 2004; Brosnahan and Osterrieder, 2009). In contrast, EHV-4 infection remains mostly restricted to the URT and a cell-associated viremia, following primary replication in the URT is very rare (Patel *et al.*, 1982; van

Maanen, 2002; Patel and Heldens, 2005). What determines this difference in pathogenic potential between EHV-1 and EHV-4 is unknown up till now. Strikingly, pathogenicity seems to be correlated with host cell range. EHV-1 has a broad host range and can replicate in equine, human, mouse, monkey, hamster, rabbit, pig, bovine, canine, feline and even avian cells (Studdert and Blackney, 1979; Trapp *et al.*, 2005), whilst EHV-4 replication seems to be mainly restricted to equine cells. Furthermore, EHV-1 can infect mice and cause respiratory disease while this is not the case for EHV-4 (Awan *et al.*, 1990; Azmi and Field, 1993). In this study, we aimed to evaluate how EHV-4 interacts with the respiratory mucosa upon inoculation of equine respiratory mucosal explants and we wanted to compare these replication kinetics with those of its close relative, EHV-1.

2. Materials and Methods

2.1. Donor horses

Material from slaughter horses was used to obtain nasal explants. Horses negative for nasal/ocular discharge and lung pathology were selected. All horses were between 5 and 7 years old, as determined by inspection of dental incisive architecture (Muylle *et al.*, 1996). A complement-dependent seroneutralization (SN)-test was performed on the serum of all horses and EHV-specific antibody titres ranged between 24 and 96. Nasal explants of at least three individual horses were used to evaluate replication kinetics of several EHV-1 and EHV-4 isolates.

2.2. *Cultivation of the nasal mucosal explants*

The cultivation of nasal mucosal explants was performed exactly as previously described (Vandekerckhove *et al.*, 2009). In brief, immediately after slaughter, the head was removed from the carcass and longitudinally sawn into 2 equal sections. Tissue from the deep intranasal part of the septum was collected. The tissues were transported on ice in phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamicin (Invitrogen, Paisley, UK), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium), 1 mg/mL kanamycin (Sigma-Aldrich, St. Louis, MO, USA) and 5 µg/mL fungizone (Bristol-Myers Squibb, New York, USA), to the laboratory. Mucosal explants were stripped from the surface of the different tissues by use of surgical blades (Swann-Morton). The stripped mucosa of each tissue was divided into equal explants of 25 mm² and placed epithelium upwards on fine-meshed gauze for culture at an air-liquid interface. Only a thin film of serum-free medium (50 % Roswell Park Memorial Institute medium (RPMI, Invitrogen)/50 % Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 0.3 mg/mL glutamine (BDH Biochemical, Poole, UK), 1 µg/mL gentamicin (Invitrogen), 0.1 mg/mL streptomycin (Certa) and 100 U/mL penicillin (Continental Pharma)) covered the explants, thereby mimicking the air/liquid interface found in the respiratory tract of the living animal. Explants were maintained at 37 °C in an atmosphere containing 5 % CO₂.

2.3. *Virus*

Three different EHV-4 strains were used in this study. EHV-4 isolate VLS 829 was provided by the Office International d'Epizooties Reference Laboratory (University of Kentucky, Lexington, KY) and was identified as EHV-4 by restriction fragment length polymorphism

(RFLP) (Van de Walle *et al.*, 2007). Strain EQ₁ 012 was obtained from Intervet (Heldens *et al.*, 2001) and strain V01-3-13 was isolated from a horse with respiratory disease and obtained from the Institut für Virologie in Berlin (kind gift of Dr. K. Borchers).

The EHV-1 isolate 03P37 was isolated from peripheral blood mononuclear cells (PBMC) of a paralytic horse in 2003 and typed as neurovirulent by the Animal Health Trust in the United Kingdom (Nugent *et al.*, 2006). EHV-1 strain RacL11 was isolated in the late 1950s from an aborted foal and exhibits high virulence in the natural host and laboratory animals (Reczko & Mayr, 1963; Mayr *et al.*, 1968).

2.4. *Inoculation of the nasal mucosal explants*

All explants were inoculated after 24 h of culture. Inoculation took place by immersion of the explant in 1 ml of inoculum, containing $10^{6.5}$ TCID₅₀ of EHV-1 or EHV-4 for 1 h at 37 °C and 5 % CO₂. After incubation, explants were washed twice with warm medium and transferred back to their gauze. At several time points post inoculation (pi), explants were collected, embedded in methylcellulose medium (Methocel® MC, Sigma-Aldrich) and frozen at -70 °C. Nasal explants of three horses were collected. Of each horse, several explants were made and inoculated with different EHV-1 isolates (03P37, parental RacL11) and EHV-4 isolates (VLS 829, EQ₁ 012, V01-3-13), and collected at different time points pi (0, 24, 48 and 72 hpi).

2.5. *Plaque analysis and quantification of individual infected cells*

At 0, 24, 48 and 72 hpi, one hundred consecutive cryosections of 20 µm were made of the frozen explants and the cryosections were fixed in methanol for 20 min (-20 °C, 100 %). Subsequently, the basement membrane (BM) of the tissues was stained with monoclonal mouse anti-collagen VII antibodies (Sigma-Aldrich), followed by secondary Texas Red®-

labeled goat anti-mouse antibodies (Molecular Probes (Invitrogen)). In a second step, viral proteins were stained by incubation with biotinylated equine polyclonal anti-EHV-1 IgG (van der Meulen *et al*, 2003), followed by streptavidin-FITC® (Molecular Probes (Invitrogen)). This polyclonal antibody against EHV-1 shows cross-reactivity with EHV-4 and was therefore also used for staining of EHV-4. Antibodies were incubated for 1 h at 37 °C and 5 % CO₂. Finally, cryosections were washed three times in PBS and mounted with glycerin-DABCO (Janssen Chimica, Beerse, Belgium). To reproducibly analyse the replication characteristics of several EHV-1 isolates, we used a system set up by Glorieux *et al*. (2009) and optimized by Vandekerckhove *et al*. (2010). Briefly, penetration of the virus through the BM was inspected and virus plaque latitudes were measured in one hundred consecutive cryosections using the ImageJ 1.28 software that is freely available from the National Institute of Mental Health webpage (<http://rsb.info.nih.gov/ij/docs/intro.html>). Plaque latitude was measured by means of the line tool in ImageJ. Number of plaques and number of individual infected cells below the BM were counted by confocal microscopy (Leica TCS SP2 laser scanning spectral confocal system, Leica Microsystems GmbH, Wetzlar, Germany) and the Leica confocal software.

2.6. Statistical analysis

The data were processed by the SPSS software (SPSS) for analysis of variance (ANOVA). The data are presented as means + standard deviations. Results with *P* values of ≤ 0.05 were considered significant.

3. Results

3.1. *EHV-4 strains replicate plaquewise in the epithelial cells of respiratory mucosal explants but are severely impaired in infecting mononuclear cells*

Replication kinetics of several EHV-4 strains were evaluated in our respiratory nasal mucosal explant system. To this end, nasal explants of three individual horses were inoculated with $10^{6.5}$ TCID₅₀/ml of the EHV-4 isolates VLS 829, EQ₁ 012, V01-3-13 and of each animal and for every strain used, one explant was examined at each collected time point (0, 24, 48 and 72 hpi).

No significant differences were seen in the results obtained with different EHV-4 strains, and hence, results will not be discussed separately for each strain. In general, viral epithelial plaques were visible starting from 24 hpi (Figure 1A) and their latitudes increased over time (Figure 1B).

These results are similar to what is normally observed upon inoculation of nasal mucosal explants with EHV-1. Indeed, when comparing the replication kinetics of the EHV-4 strains with the replication kinetics of the EHV-1 strains 03P37 and RacL11 (Figure 2), following observations were made. Viral plaques in the epithelium were seen from 24 hpi onwards. In addition, plaque latitude of these epithelial plaques increased over time, with significant differences between the EHV-1 plaque latitudes and the EHV-4 plaque latitudes at all observed time points (Figure 2B). The number of plaques and plaque latitudes increased significantly between 24 and 48 hpi, but no longer between 48 and 72 hpi (Figure 2A).

When evaluating infection below the BM, no individual infected cells were noticed at any observed time point pi for the EHV-4 strains VLS 829 and V01-3-13, and for strain EQ₁ 012, only three EHV-4-infected cells were found below the BM in the explant of 1 horse at 72 hpi (Table I). As these individual infected cells below the BM were only noticed very rarely, this implies that there is hardly any passing of virus through the BM via individual infected

immune cells, which is in striking contrast to what is normally observed for EHV-1 strains. Indeed, upon inoculation with strains 03P37 and RacL11, the presence of single infected cells in the underlying connective tissue was observed from 24 hpi onwards and the number of these single EHV-1-infected cells increased over time with an average of 21 ± 4.4 cells at 24 hpi, 135 ± 58.3 cells at 48 hpi, and 546 ± 94.3 cells at 72 hpi for strain 03P37, and an average of 6 ± 1.2 cells at 24 hpi, 78.7 ± 20.3 cells at 48 hpi, and 156.3 ± 16.3 at 72 hpi for strain RacL11 (Table I). EHV-1 strain 03P37 infected significantly more cells below the BM than EHV-1 strain RacL11.

In conclusion, these experiments indicate that (i) EHV-4 spreads plaquewise in epithelial cells of nasal mucosal explants, similar to EHV-1 albeit to a lesser extent, and (ii) EHV-4, in contrast to EHV-1, has no marked tropism for mononuclear leukocytes as single infected cells were rarely observed below the basement membrane at all time points pi.

4. Discussion

The subfamily of the *Alphaherpesvirinae* is, within the family of the *Herpesviridae*, an extensive subfamily containing numerous mammal, bird and reptile viruses (Davison *et al.*, 2009). Pseudorabies virus (PRV or Suid herpesvirus 1, SHV-1), bovine herpesvirus 1 (BoHV-1), equine herpesvirus 1 (EHV-1) and -4 (EHV-4) are clinically and economically the most relevant veterinary mammalian viruses belonging to the alphaherpesvirus subfamily. Although these viruses share many similarities, differences between the viruses become apparent when considering the clinical picture and pathogenesis at the level of the upper respiratory tract (URT). While EHV-1 and EHV-4 cause mild respiratory problems, PRV and BoHV-1 have the possibility to cause severe respiratory disease accompanied by pustular, necrotic lesions that progress to large haemorrhagic and ulcerated areas in the respiratory

214 mucosa (Allen and Bryans, 1986, Crabb and Studdert, 1995; Reed and Toribio, 2004;
 215 Nauwynck *et al.*, 2007; Nandi *et al.*, 2009). Besides EHV-1, PRV and BoHV-1 can easily
 216 spread through the body via a cell-associated viremia, whereas EHV-4-induced viremia is an
 217 exceptional event (Nyaga and McKercher, 1980; Patel *et al.*, 1982; Wang *et al.*, 1988;
 218 Nauwynck and Pensaert, 1995; van Maanen, 2002; Patel and Heldens, 2005). These
 219 differences can partially be explained by the diverse replication and invasion strategies of
 220 these viruses in the respiratory mucosa. By means of respiratory mucosal explants, it was
 221 found that PRV and BoHV-1 spread horizontally as well as vertically in a plaque-wise manner
 222 in the epithelium, and that virus-induced plaques penetrated the BM barrier (Glorieux *et al.*,
 223 2007; Steukers *et al.*, 2010). In contrast, the spread of EHV-1-induced plaques was solely
 224 lateral, as plaques never crossed the BM at any time point *in vitro*, implying that the BM functions
 225 as an absolute barrier (Gryspeerd *et al.*, 2010; Vandekerckhove *et al.*, 2010). However, a
 226 marked tropism of EHV-1 for mononuclear leukocytes was observed and EHV-1-infected
 227 mononuclear leukocytes were present both in the epithelium and below the BM in the vicinity
 228 of virus-induced plaques *in vitro* but also *in vivo* (Gryspeerd *et al.*, 2010; Vandekerckhove *et al.*,
 229 2010). Hence, these data suggest that closely related alphaherpesviruses use distinct ways
 230 of crossing the BM barrier and invading the respiratory mucosa. In the present study,
 231 replication of several EHV-4 strains was assessed for the first time in nasal mucosal explants
 232 of the horse. Thereby, it was found that EHV-4-induced virus plaques were present in the
 233 epithelium, starting from 24 hours post inoculation (hpi), although the lateral spread of EHV-
 234 4 was less extensive when compared to EHV-1. This is in accordance with the *in vivo*
 235 situation as respiratory signs following an infection with EHV-4 are less severe when
 236 compared to those following an EHV-1 infection (Heldens *et al.*, 2001; Patel *et al.*, 2003;
 237 Patel and Heldens, 2005). Remarkably, individual EHV-4-infected cells below the BM were
 238 only noticed very rarely and were only present for one of the three strains, implying that

passing of the virus through the BM via individual infected mononuclear leukocytes is an exceptional event in comparison with EHV-1. Also this observation can be linked to the *in vivo* pathogenesis, since a cell-associated viremia following an EHV-4-induced respiratory infection is not at all consistent (Patel *et al.*, 1982). This was in agreement with a study on kinetics of EHV-4 viral DNA load in peripheral blood leukocytes (PBL) from foals during a field outbreak of respiratory disease, showing that EHV-4 DNA loads in PBLs were low, which indicates that EHV-4 viremia is rare (Pusterla *et al.*, 2005). Hence, the induction of abortion or nervous system disorders upon EHV-4 infection is also scarce (Patel *et al.*, 1982). Indeed, EHV-4 accounts for only 1 to 16% of herpesviral-induced abortions (Ostlund, 1993; Whitwell *et al.*, 1995a, b) and apart from a single case report in Europe (Meyer *et al.*, 1987), EHV-4 has not been found in association with herpesviral-induced neurologic disease (Ostlund, 1993). This limited capability of EHV-4, in contrast to EHV-1, to initiate a cell-associated viremia might be due to an inefficient infection of these circulating mononuclear cells. Indeed, it has been previously shown that EHV-4 is incapable of efficiently infecting *in vitro* cultured peripheral blood mononuclear cells (PBMC) and moreover, it was found by using mutational analysis that an RSD motif, present in EHV-1 gD but not in EHV-4 gD, is an important determinant for proper infection of mononuclear cells *in vitro* (Van de Walle *et al.*, 2008; Osterrieder and Van de Walle, 2010). As our study indicates that some strains are incapable of infecting nasal mucosal mononuclear cells, while some strains are capable of infecting very few mononuclear cells, this might imply that the latter strains are the ones able to cause abortion or nervous system disorders in the field.

Another interesting observation in our present study was that the EHV-1 strain RacL11 showed a mild infection pattern in mucosal explants when compared to infection patterns obtained upon inoculation with several other EHV-1 strains such as Ab4, NY03 and different Belgian field isolates (Vandekerckhove *et al.*, 2010). Epithelial plaques were smaller in size

and, when compared to other previously tested EHV-1 strains, only a fraction of individual infected cells below the BM was observed at 72 hpi (Vandekerckhove *et al.*, 2010). This is in agreement with results obtained in *in vivo* studies comparing the pathogenic potential of EHV-1 strains in the horse. Upon infection with Ab4, horses displayed a significantly longer period of fever, a higher nasal virus excretion and a longer duration of viremia, when compared to RacL11 (Goodman *et al.*, 2007; G. Van de Walle, personal communication). This seems to indicate that RacL11 is an attenuated EHV-1 strain with less virulent potential in the horse than other EHV-1 strains, such as Ab4. In mice however, inoculation with RacL11 does result in severe symptoms such as a dramatic body weight loss within days of infection, a strong inflammatory infiltration in the lungs and even death (von Einem *et al.*, 2007). The murine model is frequently used as an *in vivo* model for investigation of virological and histological aspects of EHV-induced disease in the horse (Awan *et al.*, 1990; Walker *et al.*, 1999). However, to which extent valid comparisons and extrapolations can be made from mouse to horse remains highly questionable (Walker *et al.*, 1999). Concerning the pathogenesis of equine herpesviruses, we conclude that our *in vitro* model appears to relate more closely to the *in vivo* situation in the natural host than the murine model. Indeed, the pathogenesis of an infection with EHV-1 strain RacL11 in the explant model closely resembles the situation in the natural host, whereas the clinical picture in the mouse model is much more dramatic (von Einem *et al.*, 2007). Also, EHV-4 replication in equine explants is similar to the *in vivo* situation, with a solid replication in epithelial cells but an incapability to infect mononuclear cells, whereas EHV-4 is incapable of infecting murine respiratory mucosa (Awan *et al.*, 1990; Azmi and Field, 1993). This indicates that our *in vitro* respiratory mucosal explant system is a valuable alternative model to provide novel information on pathogenesis or differences in pathogenic potential of different EHV-4 strains, in addition to the currently existing EHV-1/EHV-4 models.

Despite severe efforts from researchers worldwide, an adequate vaccine preventing the severe EHV-1-induced symptoms such as abortion and equine herpes myeloencephalopathy (EHM) remains to be developed (Patel & Heldens, 2005; Kydd *et al.*, 2006; Rosas *et al.*, 2006; Brosnahan and Osterrieder, 2009; Pusterla *et al.*, 2009). The effect of the existing vaccines, both inactivated and attenuated, seems to be limited to the alleviation of viral shedding and clinical signs. An adequate protection against viremia, and thus severe EHV-1-induced symptoms such as abortion and EHM, might be reached by impeding the virus to cross the basement membrane (BM) barrier via single mononuclear leukocytes. To this end, it is important to induce a local immunity. The best way to raise local immunity is to administer an attenuated EHV strain intranasally. For safety reasons, this EHV vaccine may no longer infect mononuclear cells. An attenuated EHV-4 strain cannot be used as a vaccine virus since a study in ponies has shown that the immunity induced by an EHV-4 infection is not cross-protective against an EHV-1 challenge (Edington and Bridges, 1990). The most promising route for the design of more efficacious vaccines remains recombinant technology. For instance, when the glycoprotein(s) of EHV-1 responsible for efficient infection of the nasal mucosal mononuclear cells is (are) identified, genes coding for this (these) glycoproteins in EHV-4 could be introduced in the genome of EHV-1. This could result in a recombinant virus that is able to induce a powerful local immune response upon local intranasal administration, but is safe due to its inefficiency to infect mononuclear cells, markedly reducing its capability to initiate a cell-associated viremia.

5. Conclusion

The following conclusions can be drawn from the observations presented in this study. EHV-4 spreads plaquewise in epithelial cells of nasal mucosal explants, similar to EHV-1 albeit to a

lesser extent. Remarkably, and in contrast to EHV-1, the EHV-4 strains in the present study have no marked tropism for mononuclear leukocytes as single EHV-4-infected cells are only rarely observed below the BM at all time points pi. Both these observations can be linked to the *in vivo* situation since respiratory signs following an infection with EHV-4 are less severe when compared to those following an EHV-1 infection, and since a cell-associated viremia following an EHV-4-induced respiratory infection is not at all consistent, and hence neither is the induction of abortion or nervous system disorders upon EHV-4 infection. The excellent correlation between the *in vitro* model and the *in vivo* situation in the horse indicates that our *in vitro* respiratory mucosal explant system is a valuable tool to provide novel information on pathogenesis or differences in pathogenic potential of different EHV-4 strains.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Figure legends

Figure 1. Evolution of plaque formation in the epithelial cells of nasal explants for several EHV-4 isolates (VLS 829, EQ₁ 012, V01-3-13). Equine respiratory mucosal explants were inoculated ($10^{6.5}$ TCID₅₀/ml of EHV-4) and at 0, 24, 48 and 72 hours post inoculation (hpi), explants were collected. One hundred consecutive sections were made and analysed. (A) Number of plaques was determined at 24, 48 and 72 hpi. (B) Plaque latitudes were always measured for 10 plaques at every time point and for every horse. All data represent means + SD of triplicate independent experiment.

Figure 2. Evolution of plaque formation in the epithelial cells of nasal explants for EHV-1 and EHV-4. Equine respiratory mucosal explants were inoculated ($10^{6.5}$ TCID₅₀/ml of EHV) and at 0, 24, 48 and 72 hours post inoculation (hpi), explants were collected. (A) Number of plaques was determined at 24, 48 and 72 hpi. (B) Plaque latitudes were always measured for 10 plaques at every time point and for every horse. The values for EHV-1 are the average of the 2 isolates 03P37 and RacL11, the values for EHV-4 are the average of the 3 isolates VLS 829, EQ₁ 012 and V01-3-13. All data shown represent means + SD of triplicate independent experiments and *P* values for statistical significance are given for each strain. Asterisks indicate statistically significant differences ($P \leq 0.05$). Representative confocal photomicrographs are given, illustrating viral plaques in the epithelium (green) at 72 hpi (objective 20x) for EHV-1 isolate 03P37 (C) and EHV-4 isolate VLS 829 (D). Single EHV-1-infected cells are clearly visible in the connective tissue below the basement membrane (red) for EHV-1 isolate 03P37 (c). For EHV-4 isolate VLS 829 (d), single EHV-4-infected cells in the connective tissue are absent.

502 Table 1. Quantification of EHV-infected cells below the BM

Virus designation		No of EHV-infected cells below the BM at ... hpi / 100 cryosections		
		24	48	72
EHV-4	VLS 829	0 ± 0.0	0 ± 0.0	0 ± 0.0
EHV-4	EQ1 012	0 ± 0.0	0 ± 0.0	1 ± 1.7
EHV-4	V01-3-13	0 ± 0.0	0 ± 0.0	0 ± 0.0
EHV-1	03P37	21 ± 4.4	135 ± 58.3	546 ± 94.3
EHV-1	RacL11	6 ± 1.2	78.7 ± 20.3	156.3 ± 16.3

503

Figure 1. Vandekerckhove *et al.*

Figure 2
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