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

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26 **Abstract**

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

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45

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

47 1. Introduction

48 At first, the equine alphaherpesviruses equine herpesvirus type 1 (EHV-1) and equine
49 herpesvirus type 4 (EHV-4) were considered as 2 subtypes of the same virus, designated
50 EHV-1 subtype 1 and EHV-1 subtype 2 respectively (Sabine *et al.*, 1981; Studdert *et al.*,
51 1981). Additional sequence information confirmed that EHV-1 and EHV-4 are actually two
52 closely related, but distinct viruses, with an amino acid (aa) sequence identity ranging
53 between 54.9% and 96.4% (Telford *et al.*, 1998). To date, EHV-1 and EHV-4 are both
54 classified as members in the order *Herpesvirales*, subfamily *Alphaherpesvirinae*, genus
55 *Varicellovirus* (Davison *et al.*, 2009).

56 EHV-1 and EHV-4 are major causative agents of respiratory disease in the horse (Allen and
57 Bryans, 1986; Crabb and Studdert, 1995; Reed and Toribio, 2004). Initial infection starts with
58 replication of the virus in epithelial cells lining the upper respiratory tract (URT). EHV-1 does
59 not only have a tropism for epithelial cells, but also targets mononuclear leukocytes, misusing
60 these cells for viral transportation through the basement membrane (BM) barrier (Kydd *et al.*,
61 1994; Gryspeerdt *et al.*, 2010). Indeed, we showed in a previous study that EHV-1-induced
62 plaques cannot breach the BM, but that EHV-1 penetrates the deeper tissues of the respiratory
63 tract in a more discrete manner, using migrating individual mononuclear cells as a Trojan
64 horse (Vandekerckhove *et al.*, 2010). Carried by these mononuclear leukocytes, EHV-1 can
65 easily spread through the body via a cell-associated viremia, reaching its target organs, the
66 pregnant uterus and the central nervous system. At these secondary replication sites, EHV-1
67 replicates in endothelial cells, causing vasculitis, thrombosis and disseminated ischemic
68 necrosis, resulting in abortion and nervous system disorders (Smith *et al.*, 1996; Wilson,
69 1997; Smith and Borchers; 2001; Reed and Toribio, 2004; Brosnahan and Osterrieder, 2009).
70 In contrast, EHV-4 infection remains mostly restricted to the URT and a cell-associated
71 viremia, following primary replication in the URT is very rare (Patel *et al.*, 1982; van

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

82

83

84 **2. Materials and Methods**

85 *2.1. Donor horses*

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

93

94 2.2. *Cultivation of the nasal mucosal explants*

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

113

114 2.3. *Virus*

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

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

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

126

127 2.4. *Inoculation of the nasal mucosal explants*

128 All explants were inoculated after 24 h of culture. Inoculation took place by immersion of the
129 explant in 1 ml of inoculum, containing $10^{6.5}$ TCID₅₀ of EHV-1 or EHV-4 for 1 h at 37 °C and
130 5 % CO₂. After incubation, explants were washed twice with warm medium and transferred
131 back to their gauze. At several time points post inoculation (pi), explants were collected,
132 embedded in methylcellulose medium (Methocel[®] MC, Sigma-Aldrich) and frozen at -70 °C.
133 Nasal explants of three horses were collected. Of each horse, several explants were made and
134 inoculated with different EHV-1 isolates (03P37, parental RacL11) and EHV-4 isolates (VLS
135 829, EQ₁ 012, V01-3-13), and collected at different time points pi (0, 24, 48 and 72 hpi).

136

137 2.5. *Plaque analysis and quantification of individual infected cells*

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

142 labeled goat anti-mouse antibodies (Molecular Probes (Invitrogen)). In a second step, viral
143 proteins were stained by incubation with biotinylated equine polyclonal anti-EHV-1 IgG (van
144 der Meulen *et al.*, 2003), followed by streptavidin-FITC[®] (Molecular Probes (Invitrogen)).
145 This polyclonal antibody against EHV-1 shows cross-reactivity with EHV-4 and was
146 therefore also used for staining of EHV-4. Antibodies were incubated for 1 h at 37 °C and 5
147 % CO₂. Finally, cryosections were washed three times in PBS and mounted with glycerin-
148 DABCO (Janssen Chimica, Beerse, Belgium). To reproducibly analyse the replication
149 characteristics of several EHV-1 isolates, we used a system set up by Glorieux *et al.* (2009)
150 and optimized by Vandekerckhove *et al.* (2010). Briefly, penetration of the virus through the
151 BM was inspected and virus plaque latitudes were measured in one hundred consecutive
152 cryosections using the ImageJ 1.28 software that is freely available from the National Institute
153 of Mental Health webpage (<http://rsb.info.nih.gov/ij/docs/intro.html>). Plaque latitude was
154 measured by means of the line tool in ImageJ. Number of plaques and number of individual
155 infected cells below the BM were counted by confocal microscopy (Leica TCS SP2 laser
156 scanning spectral confocal system, Leica Microsystems GmbH, Wetzlar, Germany) and the
157 Leica confocal software.

158

159 2.6. *Statistical analysis*

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

163

164 **3. Results**

165 3.1. *EHV-4 strains replicate plaquewise in the epithelial cells of respiratory mucosal*
166 *explants but are severely impaired in infecting mononuclear cells*

167 Replication kinetics of several EHV-4 strains were evaluated in our respiratory nasal mucosal
168 explant system. To this end, nasal explants of three individual horses were inoculated with
169 $10^{6.5}$ TCID₅₀/ml of the EHV-4 isolates VLS 829, EQ₁ 012, V01-3-13 and of each animal and
170 for every strain used, one explant was examined at each collected time point (0, 24, 48 and 72
171 hpi).

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

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

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

189 immune cells, which is in striking contrast to what is normally observed for EHV-1 strains.
190 Indeed, upon inoculation with strains 03P37 and RacL11, the presence of single infected cells
191 in the underlying connective tissue was observed from 24 hpi onwards and the number of
192 these single EHV-1-infected cells increased over time with an average of 21 ± 4.4 cells at 24
193 hpi, 135 ± 58.3 cells at 48 hpi, and 546 ± 94.3 cells at 72 hpi for strain 03P37, and an average
194 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
195 RacL11 (Table I). EHV-1 strain 03P37 infected significantly more cells below the BM than
196 EHV-1 strain RacL11.

197

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

202

203 4. Discussion

204 The subfamily of the *Alphaherpesvirinae* is, within the family of the *Herpesviridae*, an
205 extensive subfamily containing numerous mammal, bird and reptile viruses (Davison *et al.*,
206 2009). Pseudorabies virus (PRV or Suid herpesvirus 1, SHV-1), bovine herpesvirus 1 (BoHV-
207 1), equine herpesvirus 1 (EHV-1) and -4 (EHV-4) are clinically and economically the most
208 relevant veterinary mammalian viruses belonging to the alphaherpesvirus subfamily.
209 Although these viruses share many similarities, differences between the viruses become
210 apparent when considering the clinical picture and pathogenesis at the level of the upper
211 respiratory tract (URT). While EHV-1 and EHV-4 cause mild respiratory problems, PRV and
212 BoHV-1 have the possibility to cause severe respiratory disease accompanied by pustular,
213 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 t_i , 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*
229 *al.*, 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

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

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

264 and, when compared to other previously tested EHV-1 strains, only a fraction of individual
265 infected cells below the BM was observed at 72 hpi (Vandekerckhove *et al.*, 2010). This is in
266 agreement with results obtained in *in vivo* studies comparing the pathogenic potential of
267 EHV-1 strains in the horse. Upon infection with Ab4, horses displayed a significantly longer
268 period of fever, a higher nasal virus excretion and a longer duration of viremia, when
269 compared to RacL11 (Goodman *et al.*, 2007; G. Van de Walle, personal communication).
270 This seems to indicate that RacL11 is an attenuated EHV-1 strain with less virulent potential
271 in the horse than other EHV-1 strains, such as Ab4. In mice however, inoculation with
272 RacL11 does result in severe symptoms such as a dramatic body weight loss within days of
273 infection, a strong inflammatory infiltration in the lungs and even death (von Einem *et al.*,
274 2007). The murine model is frequently used as an *in vivo* model for investigation of
275 virological and histological aspects of EHV-induced disease in the horse (Awan *et al.*, 1990;
276 Walker *et al.*, 1999). However, to which extent valid comparisons and extrapolations can be
277 made from mouse to horse remains highly questionable (Walker *et al.*, 1999). Concerning the
278 pathogenesis of equine herpesviruses, we conclude that our *in vitro* model appears to relate
279 more closely to the *in vivo* situation in the natural host than the murine model. Indeed, the
280 pathogenesis of an infection with EHV-1 strain RacL11 in the explant model closely
281 resembles the situation in the natural host, whereas the clinical picture in the mouse model is
282 much more dramatic (von Einem *et al.*, 2007). Also, EHV-4 replication in equine explants is
283 similar to the *in vivo* situation, with a solid replication in epithelial cells but an incapability to
284 infect mononuclear cells, whereas EHV-4 is incapable of infecting murine respiratory mucosa
285 (Awan *et al.*, 1990; Azmi and Field, 1993). This indicates that our *in vitro* respiratory
286 mucosal explant system is a valuable alternative model to provide novel information on
287 pathogenesis or differences in pathogenic potential of different EHV-4 strains, in addition to
288 the currently existing EHV-1/EHV-4 models.

289

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

310

311 **5. Conclusion**

312 The following conclusions can be drawn from the observations presented in this study. EHV-
313 4 spreads plaquewise in epithelial cells of nasal mucosal explants, similar to EHV-1 albeit to a

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

324

325 **Conflict of interest statement**

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

328

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478 **Figure legends**

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

486

487 **Figure 2. Evolution of plaque formation in the epithelial cells of nasal explants for EHV-**
488 **1 and EHV-4.** Equine respiratory mucosal explants were inoculated ($10^{6.5}$ TCID₅₀/ml of
489 EHV) and at 0, 24, 48 and 72 hours post inoculation (hpi), explants were collected. **(A)**
490 Number of plaques was determined at 24, 48 and 72 hpi. **(B)** Plaque latitudes were always
491 measured for 10 plaques at every time point and for every horse. The values for EHV-1 are
492 the average of the 2 isolates 03P37 and RacL11, the values for EHV-4 are the average of the 3
493 isolates VLS 829, EQ₁ 012 and V01-3-13. All data shown represent means + SD of triplicate
494 independent experiments and *P* values for statistical significance are given for each strain.
495 Asterisks indicate statistically significant differences ($P \leq 0.05$). Representative confocal
496 photomicrographs are given, illustrating viral plaques in the epithelium (green) at 72 hpi
497 (objective 20x) for EHV-1 isolate 03P37 **(C)** and EHV-4 isolate VLS 829 **(D)**. Single EHV-1-
498 infected cells are clearly visible in the connective tissue below the basement membrane (red)
499 for EHV-1 isolate 03P37 (c). For EHV-4 isolate VLS 829 (d), single EHV-4-infected cells in
500 the connective tissue are absent.

501

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

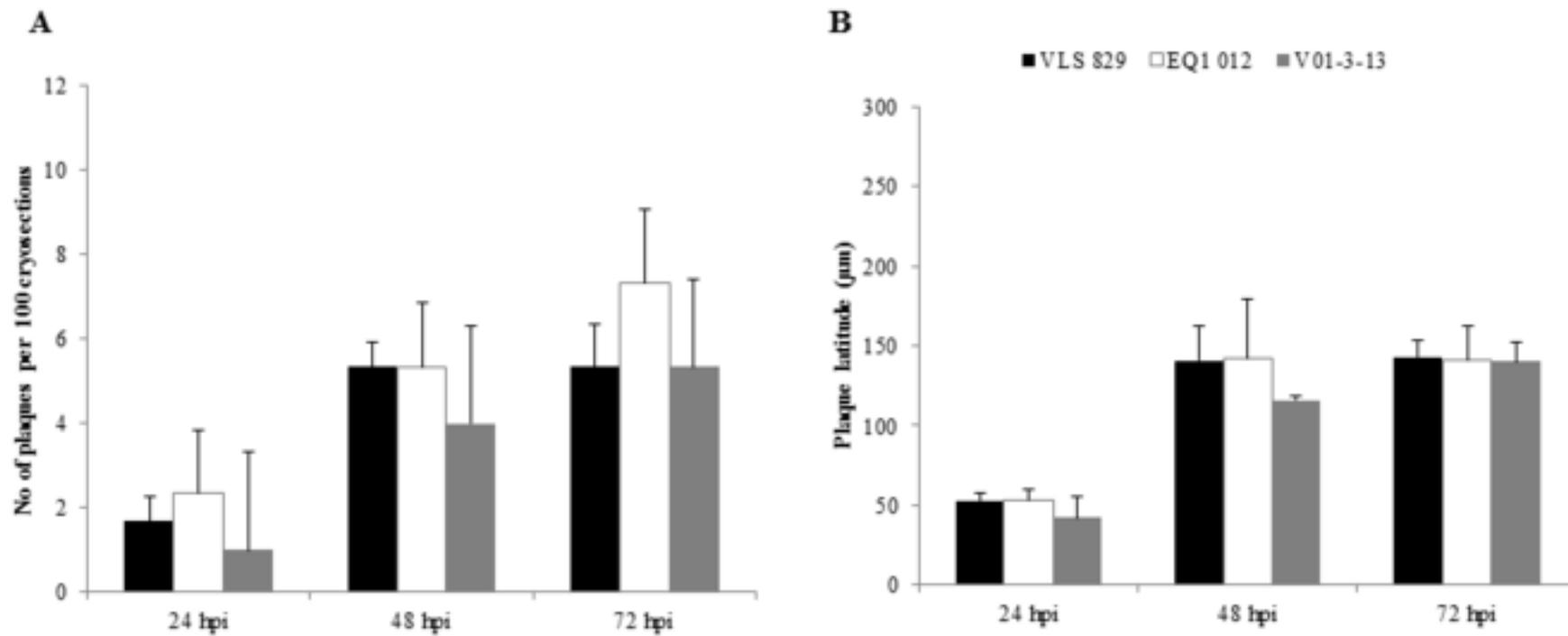


Figure 1. Vandekerckhove *et al.*

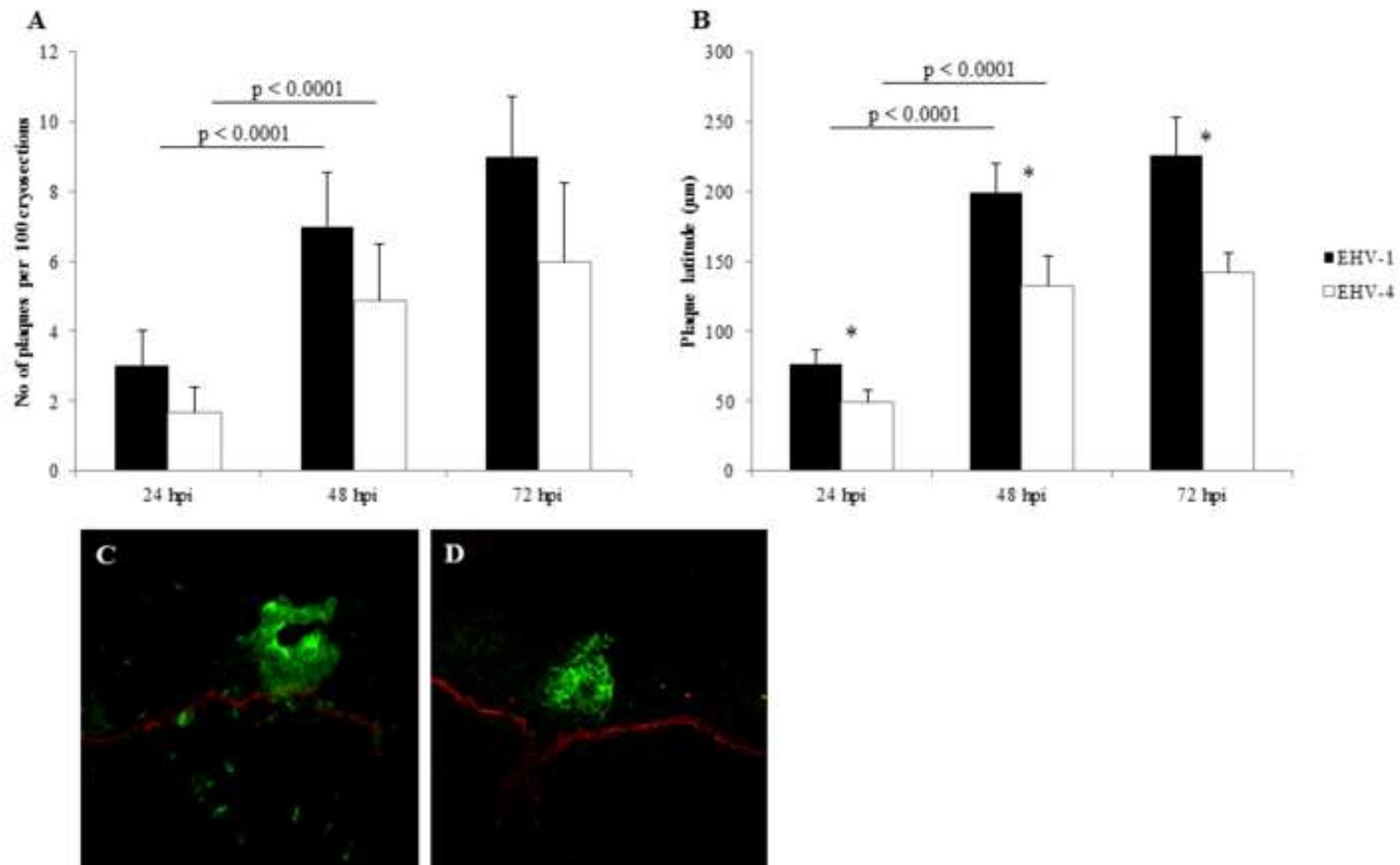


Figure 2. Vandekerckhove *et al.*