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Annick C. Gryspeerdt, A.P. Vandekerckhove, B. Garré, F. Barbé, G.R. van de Walle, et al.. Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. *Veterinary Microbiology*, 2010, 142 (3-4), pp.242. 10.1016/j.vetmic.2009.10.015 . hal-00587285

HAL Id: hal-00587285

<https://hal.science/hal-00587285>

Submitted on 20 Apr 2011

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Accepted Manuscript

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PII: S0378-1135(09)00535-5
DOI: doi:10.1016/j.vetmic.2009.10.015
Reference: VETMIC 4643

To appear in: *VETMIC*

Received date: 10-7-2009
Revised date: 13-10-2009
Accepted date: 16-10-2009

Please cite this article as: Gryspeerdt, A.C., Vandekerckhove, A.P., Garré, B., Barbé, F., Van de Walle, G.R., Nauwynck, H.J., Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.10.015

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1 **Differences in replication kinetics and cell tropism between neurovirulent**
2 **and non-neurovirulent EHV1 strains during the acute phase of infection in**
3 **horses**

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10
11 Number of text pages: 30

12 Number of tables: 1

13 Number of figures: 8

14 Shortened running title: *In vivo* differences of EHV1 strains replication

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23

24 **Abstract**

25 Equine herpesvirus 1 (EHV1) replicates in the respiratory tract of horses, after which infected
26 leukocytes transport virus throughout the body, resulting in abortion or nervous system
27 disorders. Two EHV1 strains circulate in the field: neurovirulent and non-neurovirulent. To
28 investigate differences in replication in the upper respiratory tract (URT), an experimental
29 inoculation study in ponies was performed with both strains. Two groups of six ponies, were
30 inoculated intranasally with $10^{6.5}$ TCID₅₀ of either strain. Clinical signs, nasal shedding and
31 viremia were evaluated. At early time points post inoculation (pi), one pony of each group
32 was euthanized. Tissues were collected for titration and immunostainings. Number and size of
33 EHV1-induced plaques were calculated, and individual EHV1-infected cells were quantified
34 and characterized. Inoculation with either strain resulted in nasal shedding and replication in
35 several tissues of the URT. Both strains replicated in a plaquewise manner in epithelium of
36 the nasal mucosa, but replication in epithelium of the nasopharynx was largely limited to non-
37 neurovirulent EHV1. Plaques were never able to cross the basement membrane, but individual
38 infected cells were noticed in the connective tissue of all examined tissues for both strains.
39 The total number of these cells however, was 3-7 times lower with non-neurovirulent EHV1
40 compared to neurovirulent EHV1. CD172a⁺ cells and CD5⁺ lymphocytes were important
41 target cells for both strains. Interestingly, in lymph nodes, B-lymphocytes were also important
42 target cells for EHV1, irrespective of the strain. Viremia was detected very early pi and
43 infected cells were mainly CD172a⁺ for both strains. In summary, these results are valuable
44 for understanding EHV1 pathogenesis at the port of entry, the URT.

45

46

47 Keywords: EHV1, pathogenesis, strain differences, carrier cells

48

49 **Introduction**

50 Infectious respiratory tract disease has been recognized as the cause of major health problems
51 in horses. The most important respiratory pathogen is equine herpesvirus 1 (EHV1) and
52 infections with this virus cause serious economic losses in the horse industry worldwide
53 (Allen and Bryans, 1986; Ostlund, 1993; van Maanen, 2002). The upper respiratory tract is
54 the first line of defence against respiratory pathogens and is also the primary replication site
55 of EHV1, where it causes upper respiratory disorders (Kydd et al., 1994a, b). In addition,
56 EHV1 can spread via infected blood leukocytes to internal organs, for example the pregnant
57 uterus, causing symptoms such as abortion and neonatal foal death (Allen and Bryans, 1986).
58 EHV1 can also reach the central nervous system, where replication in endothelial cells results
59 in severe nervous system disorders with frequent fatal outcome. Neurological disease in
60 horses caused by infection with certain isolates of EHV1 is a severe condition which is poorly
61 understood (McCartan et al., 1995; Stierstorfer et al., 2002; van der Meulen et al., 2003a). The
62 pathogenesis for developing this devastating condition is largely unknown and full protection
63 against these secondary and severe symptoms can not be obtained by vaccination with the
64 currently available vaccines (Heldens et al., 2001; Goodman et al., 2006; van der Meulen et
65 al., 2007). Therefore, an improvement of the existing vaccines and/or the development of
66 alternative strategies for the prevention and treatment of EHV1-induced infections are
67 necessary. Importantly, this implies a firm notice about the pathogenesis of EHV1 during the
68 acute phase of infection.

69 It has been suggested that distinct isolates of EHV1, differing in pathogenic capacity,
70 circulate in the field. Nugent et al. (2006) indicated that a single nucleotide polymorphism
71 (SNP) in the DNA polymerase is strongly associated with neurological versus non-
72 neurological disease outbreaks. Studies in naturally infected horses with nervous system
73 disorders have revealed a more robust cell-associated viremia in horses infected with

74 neurovirulent isolates of EHV1, in contrast to horses infected with non-neurovirulent isolates
75 (Allen and Breathnach, 2006). This was further confirmed by reverse genetic experiments of
76 EHV1 strains with a sole mutation of the DNA polymerase SNP, where experimental
77 inoculation with such strains altered neurologic disease in horses (Goodman et al., 2007; Van
78 de Walle et al., 2009). Moreover, *in vitro* experiments with these strains revealed differences
79 in leukocyte tropism which could explain the difference in clinical outcome (Goodman et al.,
80 2007). Still, the exact identity of carrier cells susceptible to EHV1 infection and hereby
81 responsible for dissemination of infectious virus to sites of secondary replication, is a matter
82 of debate. Blood samples from experimentally infected ponies were collected and examined
83 for the presence of neurovirulent EHV1 (Scott et al., 1983). Hereby, T-lymphocytes were
84 found to be the most important cell fraction to harbor virus *in vivo*. However, no full
85 characterization of these cells was performed. Different studies with *in vitro* EHV1-infected
86 leukocytes have also been performed to determine the identity of these cells. Hereby, mainly
87 monocytes were susceptible to infection with non-neurovirulent EHV1 (van der Meulen et al.,
88 2000). After stimulation with mitogens, T-lymphocytes became more susceptible, followed
89 by B-lymphocytes (van der Meulen et al., 2001). Despite these studies, information on
90 possible differences between neurovirulent and non-neurovirulent EHV1 strains at the port of
91 entry, and identity of carrier cells during viremia remains limited.

92 To investigate this, six Shetland ponies were experimentally inoculated with a
93 neurovirulent EHV1 strain and six with a non-neurovirulent EHV1 strain. These animals were
94 subsequently euthanized to collect different tissues of the upper respiratory tract. Tissues that
95 were positive for EHV1 on titration were stained with markers for different cell types to
96 determine and quantify the carrier cells of EHV1 in the upper respiratory tract. In addition,
97 peripheral blood mononuclear cells (PBMC) were also collected to quantify and identify the
98 infected cell type during cell-associated viremia.

99

100 **Materials and methods**

101

102 *Horses*

103 Twelve male Shetland ponies, between the age of 6 months and 2 years, were used in
104 this study. They were housed inside isolated stables. They were fed daily with a commercial,
105 complete feed. Drinking water and hay were supplied *ad libitum*.

106 Prior to the experiment, ponies were monitored for 4 weeks. Rectal temperatures were
107 measured daily and complement-dependent seroneutralization (SN)-tests and
108 immunoperoxidase monolayer assays (IPMA) were performed weekly to determine EHV-
109 specific antibody titers.

110

111 *Virus and inoculation*

112 Ponies were divided into two groups of 6. For the first group the Belgian EHV1 strain
113 03P37, isolated from the blood of a paralytic horse in 2003, was used for inoculation (Garre et
114 al., 2009; van der Meulen et al., 2003a). The second group was inoculated with the Belgian
115 strain 97P70, which was isolated from an aborted fetus (van der Meulen et al., 2006). The
116 strains were typed in the DNA polymerase as D₇₅₂ and N₇₅₂ respectively in cooperation with
117 the Animal Health Trust in the United Kingdom (Nugent et al., 2006). Based on their origin
118 and genotyping, these strains will be referred as neurovirulent and non-neurovirulent in this
119 paper. Virus stocks used for inoculation were at the 6th passage; 2 passages in rabbit kidney
120 cells (RK13) and 4 subsequent passages in equine embryonic lung cells (EEL). Both viruses
121 had virtually identical single-step growth properties in RK13 cells for intra- and extracellular
122 virus yields at all tested time points post infection (data not shown).

123 Pones were inoculated oronasally with 20 ml of a virus suspension containing $10^{6.5}$
124 tissue culture infectious dose₅₀ (TCID₅₀). Ten ml of the virus suspension was administered
125 intranasally (5 ml per nostril) using a thin probe and 10 ml was inoculated orally with a
126 syringe. The virus titer was confirmed by titration of the inoculum.

127

128 *Serological examination*

129 For the seroneutralization (SN)-test, two-fold dilution series of the sera were prepared
130 in MEM. Fifty micro liter of these serial dilutions were incubated for 23 hours (h) at 37°C
131 with a fixed number of infectious virus (300 TCID₅₀ of EHV1 strain Arabica in 50 µl).
132 Hereafter, 25 µl of guinea pig complement was added. After 1 h of incubation, the mixture of
133 serum, virus and complement was added to RK13 cells. Inoculated cultures were further
134 incubated at 37°C in an atmosphere containing 5% CO₂. After 7 days of incubation, the
135 cultures were analysed for the presence of cytopathic effect. The neutralization titer was
136 calculated as the reciprocal of the highest dilution of the serum that was able to completely
137 block EHV1 infection in RK13 cells.

138 For the immunoperoxidase monolayer assay (IPMA), RK13 cells were inoculated with
139 10^3 TCID₅₀ of the EHV1 strain 97P70. After 28 h, cells were washed, dried at 37°C for 1 h
140 and stored at -20°C until use. Plates were thawed and subsequently fixed with 4%
141 paraformaldehyde and a solution containing 1% hydrogen peroxide in methanol. Following
142 extensive washing, serial 2-fold dilutions of the sera were added and cells were incubated for
143 1 h at 37°C. Cells were incubated with peroxidase-labeled goat anti-horse antibodies (Jackson
144 ImmunoResearch Laboratories Inc., PA, USA) and after 1 h, a substrate solution of 3-amino-
145 9-ethylcarbazole in 0.05 M acetate buffer with 0.05% hydrogen was added to each well.
146 Following 20 minutes of incubation at 37°C, substrate solution was replaced with acetate
147 buffer to block the enzymatic staining reaction. The IPMA titer was calculated as the

148 reciprocal value of the highest serum dilution that induced visual staining of infected RK13
149 cells as determined by light microscopy.

150

151 *Clinical examination*

152 Horses were monitored daily for clinical signs by physical examination and
153 measurement of rectal temperatures. Fever was defined as a rectal temperature $\geq 38.5^{\circ}\text{C}$.
154 During clinical examination, nasal discharge (serous, mucous or purulent), lymph node
155 swelling, tachypnea and coughing were evaluated. In addition, neurological exams were
156 performed.

157

158 *Virological examination*

159 Nasopharyngeal mucus samples were taken daily until euthanasia. Immediately after
160 collection, swabs were immersed in transport medium containing phosphate-buffered saline
161 (PBS) supplemented with 10% fetal calf serum (FCS), 1000 U/ml penicillin, 1 mg/ml
162 streptomycin and 0.5 mg/ml kanamycin. EHV1 in nasopharyngeal secretions was titrated on
163 monolayers of RK13 cells, exactly as described previously (van der Meulen et al., 2003b).

164 On 1, 2, 3, 4, 5 and 7 days post inoculation, one pony of each group was euthanized
165 with an overdose of Natriumpentobarbital[®] (Kela, Hoogstraten, Belgium). After euthanasia,
166 different tissue samples were collected from the upper respiratory tract. Tissues from
167 slaughterhouse horses were collected and served as uninfected control samples. Per tissue,
168 two equal squares of 1 cm^2 were collected from both left and right side of the head. From
169 each side, one square was frozen immediately with (immunofluorescence) or without (virus
170 titration) methylcellulose medium (Methocel[®]MC, Sigma-Aldrich, St. Louis, MO, USA) at -
171 70°C . To determine viral replication, 20% suspensions of collected tissues were made and

172 titrated on RK13 cells. Titers of infected tissues were determined after 7 days by means of
173 Reed and Muench's formula (Reed and Muench, 1938).

174 The experimental design was approved by the local ethical committee of the Faculty
175 of Veterinary Medicine.

176

177 *Quantification and characterization of individual infected cells in tissues of the upper*
178 *respiratory tract*

179 Immunofluorescent double stainings were used to quantify and characterize individual
180 infected cells in tissues that were EHV1-positive after titration on RK13 cells. First,
181 cryosections (16 µm) were incubated with either monoclonal antibody (mAb) HT23A,
182 DH59B or 1.9/3.2 (VMRD Inc, Pullman, WA, USA) against CD5 (T-lymphocytes), CD172a
183 (cells from the monocyte lineage, CML) or IgM (B-lymphocytes) respectively, and
184 subsequently incubated with Texas Red[®]-labeled goat anti-mouse Abs (Molecular Probes,
185 Eugene, OR, USA). Epithelial cells were identified based on the pancytokeratin marker
186 MNF116 (Abcam, Cambridge, UK) and/or morphological features. Second, viral EHV1
187 proteins were stained by incubation with biotinylated polyclonal horse anti-EHV1 IgG (van
188 der Meulen et al., 2000), followed by streptavidin-FITC[®] (Molecular Probes, Eugene, OR,
189 USA). As controls, (i) stainings were performed on uninfected tissue and (ii) isotype control
190 antibodies were included. All cryosections were analyzed using confocal microscopy (Leica
191 TCS SP2 Laser scanning spectral confocal system, Leica Microsystems GmbH, Wetzlar,
192 Germany). Number and latitude of EHV1-induced plaques were calculated using the software
193 program ImageJ. Latitude was used to evaluate plaque size and therefore the degree of cell-to-
194 cell spread. In order to quantify infected cells in nasal septum, two main regions were taken
195 into account, on the one hand regions with and on the other hand regions without plaques in
196 the epithelium. These regions were then subdivided into three equal regions underneath the

197 epithelium: A, B and C for regions with epithelial plaques and D, E and F for regions without
198 epithelial plaques (Fig 2A). For nasopharynx, tubal/nasopharyngeal tonsils and lymph nodes,
199 subdivisions into different zones of functional importance were made (Fig 3A and 4A
200 respectively). All regions were evaluated for the presence of plaques and/or individual
201 infected cells. In addition, the total amount of infected cells (positive for staining with anti-
202 EHV1 Abs), equine immune cells (positive for staining with the mAbs against immune cell
203 markers) and infected equine immune cells (double positive) were determined.

204

205 *Quantification and characterization of viremia*

206 Heparinized blood samples were taken daily until euthanasia and peripheral blood
207 mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Paque,
208 according to manufacturer's instructions (Pharmacia Biotech AB, Uppsala, Sweden). To
209 determine the magnitude of viremia, co-cultivation of PBMC on RK13 cells was performed as
210 previously described (van der Meulen et al., 2003b). Remaining PBMC were resuspended in
211 RPMI supplemented with 30% FCS and 20% Dimethyl Sulfoxide (DMSO) and frozen in
212 liquid nitrogen for later examination to identify the nature of infected cells. Hereby, a double
213 immunofluorescence staining was performed on acetone-fixed cell smears of PBMC. EHV1
214 expression was detected using a biotinylated polyclonal horse anti-EHV1 IgG (van der
215 Meulen et al., 2003b), followed by streptavidin-FITC[®]. The identity of infected cells was
216 determined using the mAbs HT23A, DH59B or 1.9/3.2, as described above, followed by the
217 incubation with Texas Red[®]-labeled goat anti-mouse Abs. Samples were analyzed using
218 confocal laser scanning microscopy.

219

220 **Results**

221

222 *EHV-status of the ponies before inoculation*

223 The inclusion criterium for ponies used in this study was a complete absence of EHV-
224 specific antibodies by either SN-test (<2) or IPMA (<10). In addition, during the 4-week
225 observation period prior to the experiment, (i) no raise in temperature was noted, (ii) no EHV-
226 specific antibodies were detected, and (iii) no virus was isolated from nasopharyngeal swabs.
227 The use of naïve horses is an important tool to minimize variation in clinical and virological
228 outcome due to differences in immunological status (Cornick et al., 1990; Heldens et al.,
229 2001; Matsumura et al., 1996).

230

231 *Experimental inoculation with either strain induces fever and upper respiratory tract disease*
232 *in ponies*

233 All ponies developed fever upon EHV1 inoculation with exception of the two ponies
234 that were euthanized 24 hours post inoculation (hpi). Fever started at 48 hpi for the
235 neurovirulent strain (5/5 animals) and at 36 (3/5 animals) or 48 hpi (2/5 animals) for the non-
236 neurovirulent strain. High temperatures lasted for the complete observation period, with peak
237 temperatures of 40.7°C for the neurovirulent strain and 40.1°C for the non-neurovirulent
238 strain. Serous nasal discharge was noted after inoculation with both strains, starting at 1 day
239 post inoculation (dpi). A transition to purulent nasal discharge was seen in both groups from 3
240 dpi. Breathing problems, consisting of tachypnea or coughing, were observed in two ponies of
241 each group. Swelling of mandibular lymph nodes was observed in the majority of the ponies
242 and occurred from 1 dpi in 2/6 animals inoculated with a neurovirulent strain and in 4/6
243 animals inoculated with a non-neurovirulent strain. Painful retropharyngeal lymph nodes were
244 noted in 5/6 ponies inoculated with a non-neurovirulent strain, in contrast to only 2 ponies
245 inoculated with a neurovirulent strain. None of the ponies showed neurological signs,
246 irrespective of the strain used for inoculation. The latter is not too unexpected, as the

247 incubation period of EHV1-induced neurological disorders is normally 6 to 10 days (Edington
248 et al., 1986; Jackson et al., 1977; Mumford and Edington, 1980). Moreover, nervous system
249 disorders have been described to occur more frequently in older horses and certain breeds
250 (Garre et al., 2009; Goehring et al., 2006; Greenwood and Simson, 1980; Jackson et al., 1977;
251 McCartan et al., 1995).

252

253 *Experimental inoculation with either strain results in nasal shedding and viral replication in*
254 *the upper respiratory tract, but differences were observed in the preferential epithelial*
255 *replication site between strains*

256 When evaluating nasal shedding, by titrations of nasal swab samples, five out of six
257 ponies inoculated with the neurovirulent strain shed virus from 1 dpi, with a peak excretion at
258 2 dpi (Fig 1). Nasal shedding then slowly decreased until 7 dpi. For the non-neurovirulent
259 strain, 6 out of 6 ponies shed virus at 1 dpi, reaching a peak at 2 dpi with remaining high titers
260 until 7 dpi (Fig 1).

261 Next, different tissue samples were collected from the upper respiratory tract of one
262 pony per group per day for viral titrations. Virus could not be isolated from any tissue at 1 dpi
263 for the neurovirulent strain, but was found in the nasopharynx of ponies inoculated with the
264 non-neurovirulent strain (Fig 2). From 2 dpi, virus was mainly recovered from different parts
265 of the nasal septum and the nasopharynx for both strains (Fig 2). Septum and nasopharynx
266 remained positive for EHV1 until 7 dpi, with the exception of day 5 for the neurovirulent
267 strain where no virus was detected in the nasopharynx (Fig 2) and day 4 for the non-
268 neurovirulent strain where the distal nasal septum was negative (Fig 2). No difference in virus
269 titers could be noticed in the nasal septum, but titers found in the nasopharynx seemed to be
270 higher after inoculation with the non-neurovirulent strain of EHV1 compared to the
271 neurovirulent strain (Fig 2). However, because of the limited number of animals used, we

272 should be careful to draw any conclusions based on these results. Virus was also found in
273 lymphoid tissues such as the tubal and nasopharyngeal tonsils and mandibular lymph nodes
274 (Fig 2). In general, titers found in tubal and nasopharyngeal tonsils (Fig 2) were higher for the
275 non-neurovirulent strain. Titers detected in mandibular lymph nodes started rising from 3 dpi
276 (neurovirulent strain) or 4 dpi (non-neurovirulent strain), and at 7dpi, mandibular lymph
277 nodes were positive for the non-neurovirulent strain only (Fig 2). EHV1 could also be isolated
278 for both strains at different time points pi from the ethmoid, the cranial part of the trachea and
279 3 different tonsils (data not shown).

280 Finally, replication of EHV1 was evaluated in the epithelium of different tissues of the
281 upper respiratory tract by immunofluorescence. Hereby, EHV1-induced plaques were
282 analyzed and plaque latitudes measured. In the epithelium of the entire nasal septum, EHV1-
283 induced plaques were seen at different time points pi for both strains (Fig 3A). These plaques
284 were defined as groups of infected epithelial cells. Since results for proximal, intermedial and
285 distal nasal septum were virtually identical, only results for the intermedial nasal septum are
286 shown. Plaques were not present in the epithelium at 0 and 1 dpi (Fig 3A). However,
287 individual infected cells were observed at 1 dpi, which were identified as CD172a⁺ cells, as
288 determined by the marker DH59B, or epithelial cells, as determined by the pancytokeratin
289 marker (data not shown). For the neurovirulent strain, EHV1-induced plaques were observed
290 in the epithelium of the nasal mucosa starting from 2 till 7 dpi (Fig 3A). At 2, 3 and 4 dpi,
291 these plaques consisted of epithelial cells and rarely one or two CD172a⁺ cells. From 5 dpi,
292 EHV1-positive cells became separated from the basement membrane (BM) and these cells
293 were exclusively positive for the DH59B marker which identifies cells of the monocytic
294 lineage (CML) (Fig 3A). This observation was not seen in ponies inoculated with a non-
295 neurovirulent strain. There, plaques were present in the epithelium of the nasal mucosa and
296 nasopharynx from 2 till 7 dpi (Fig 3A & B). These plaques consisted of epithelial cells and

297 rarely some CD172a⁺ cells, but no separation of EHV1-positive cells was observed (Fig 3A).
298 No differences were observed in both number (data not shown) and size (Fig 3A) of EHV1-
299 induced plaques in the intermedial nasal mucosa at different time points pi for both strains. In
300 the epithelium of the nasopharynx, plaques could be found for the non-neurovirulent strain
301 from 2 till 7 dpi, with the exception of 4 dpi (Fig 3B). In contrast, no plaques were found for
302 the neurovirulent strain in the nasopharyngeal epithelium, with the exception of one small
303 plaque at 3 dpi (Fig 3B). Interestingly, plaques were not able to cross the BM at any time
304 point pi, but single EHV1-infected cells could be noticed in the connective tissue from 1 dpi.

305 Taken together, these results show that EHV1 infection results in nasal shedding and
306 replication in several tissues of the upper respiratory tract, irrespective of its neuropathogenic
307 potential. However, whereas both types of EHV1 replicate equally in the epithelium of the
308 nasal mucosa, replication in the nasopharynx was limited to non-neurovirulent EHV1.

309
310 *Neurovirulent EHV1 uses mainly CD172a⁺ cells as carrier cells in tissues of the upper*
311 *respiratory tract, whereas non-neurovirulent EHV1 shows a tropism for both CD172a⁺ cells*
312 *and CD5⁺ cells*

313 As mentioned before, EHV1-induced plaques never crossed the BM, irrespective of
314 the strain used. However, individual infected cells were observed in the underlying connective
315 tissue and the total number was 3-7 times lower with non-neurovirulent EHV1 compared to
316 neurovirulent EHV1. To identify the nature of these cells, cryosections of tissues were made
317 and double immunofluorescence stainings were performed.

318 **Nasal septum.** In the connective tissue of the nasal septum, no differences were
319 observed in the total amount of infected cells present beneath epithelium with plaques or
320 without plaques, with the exception of 5 and 7 dpi for the neurovirulent strain, where a

321 marked increase in infected cells was observed in region A (Fig 4). Such increase was not
322 seen in the regions located underneath epithelium without plaques (Fig 4).

323 **Nasopharynx.** EHV1-infected cells were present in the connective tissue of the
324 nasopharynx from 1 till 7 dpi, irrespective of the strain used, however, the localization of
325 these cells appeared to be strain-dependent (Fig 5). Whereas for the neurovirulent strain,
326 single infected cells were mainly found in epithelium (region A) and connective tissue (region
327 B), single infected cells were located in connective tissue (region B) and lymphocyte
328 aggregates (region C) for the non-neurovirulent strain (Fig 5).

329 **Tubal and nasopharyngeal tonsils.** For both EHV1 strains, single infected cells were
330 observed starting from 2 dpi (Fig 6). They were mostly present in the connective tissue
331 underneath the epithelium (region B) and lymphocyte aggregates (region C, Fig 6), but single
332 infected cells could also be observed in epithelium/fundus (region A), deeper connective
333 tissue (region D), blood vessels (region E) and glands (region F) (Fig 6).

334 **Mandibular lymph nodes.** For the neurovirulent strain, most of the infected cells
335 were found in the marginal sinus of the mandibular lymph nodes (region C, Fig 7). Their
336 number increased until 4 dpi and rapidly decreased afterwards, with rarely any infected cells
337 left at 7 dpi. Furthermore, infected cells were also observed in the capsule and trabecula
338 (region A), the medulla (region F) and in fewer amounts in vessels of the capsule and the
339 cortical nodules (regions B and D, Fig 7). In these regions a peak number of infected cells
340 was observed at 4 and 5 dpi (Fig 7). For the non-neurovirulent strain, single cells were only
341 found from 4 till 7 dpi and were mostly found in the marginal sinus (region C) and medulla
342 (region F) (Fig 7).

343 Immunofluorescence stainings, using mAbs against immune cell surface markers
344 showed that in all tissues of the upper respiratory tract, infected cells were largely CD172a⁺
345 CML, followed by a slightly lower amount of infected CD5⁺ lymphocytes when the

346 neurovirulent EHV1 strain was used for inoculation (Table I). These results were very similar
347 to what was observed with the non-neurovirulent strain, although a more substantial amount
348 of infected CD5⁺ lymphocytes was observed in these different tissues of the upper respiratory
349 tract (Table I). IgM⁺ lymphocytes were always present, but rarely infected with the exception
350 of the mandibular (Table I) and retropharyngeal (data not shown) lymph nodes, were these
351 cells were an important target cell type for both strains.

352

353 *Ponies became viremic upon experimental inoculation with either strain, and CD172a⁺ cells*
354 *were the most important carrier cells in the blood*

355 Transmission of EHV1 from PBMC to susceptible RK13 cells, as determined by co-
356 cultivation, was observed in 5 out of 6 ponies upon experimental inoculation with
357 neurovirulent EHV1 (Fig 8). No differences in the amount of infected PBMC was noted,
358 except for one pony, which showed a higher level of viremia at 4 and 5 dpi compared to the
359 other infected ponies (Fig 8). For the non-neurovirulent strain, viremia was observed in all six
360 infected ponies, with no differences in magnitude for the first 4 dpi (Fig A). However, at day
361 5 and 7 pi, the non-neurovirulent strain seemed to retain a higher viremia level than the
362 neurovirulent strain, but due to the low amount of animals left at those days, no conclusions
363 could be drawn (Fig 8).

364 In addition, around 2×10^7 mononuclear cells for each strain were analyzed by
365 confocal microscopy to detect infected cells and further characterize their nature. These cells
366 were a mixture of PBMC from three ponies collected at 2, 3 and 4 dpi. For the neurovirulent
367 strain, fourteen EHV1-infected cells were found and eleven were characterized as CD172a⁺
368 cells (78.5%), whereas three were CD5⁺ (21.5%). No infected B-lymphocytes were found. For
369 the non-neurovirulent strain, nine EHV1-infected cells were identified, consisting of seven

370 CD172a⁺ cells (77.8%), one CD5⁺ T-lymphocyte (11.0%) and one cell remained unidentified.

371 Also here, no infected B-lymphocytes were found.

372 These data indicate that CD172a⁺ cells appear to be the main target cell during EHV1-

373 induced viremia, irrespective of the neuropathogenicity of EHV1.

374

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375 **Discussion**

376 Despite several studies conducted on the pathogenesis of equine herpesvirus type 1
377 (EHV1), it still remains unclear how this virus invades the tissues of the upper respiratory
378 tract and invades the deeper tissues to initiate a cell-associated viremia. Furthermore, two
379 strains of EHV1 circulate in the field, namely non-neurovirulent and neurovirulent strains, the
380 latter causing neurological disease, a severe condition which is poorly understood. In the
381 present study, we used the two strains of EHV1 to gain more insight into the pathogenesis of
382 these strains during the acute phase of infection in the upper respiratory tract *in vivo*. To this
383 end, Shetland ponies were experimentally inoculated with either a non-neurovirulent or a
384 neurovirulent EHV1 strain and upon euthanasia, several tissues from the upper respiratory
385 tract were collected and analyzed.

386 It was found that EHV1 has different approaches to successfully establish an infection
387 in the upper respiratory tract. Firstly, we observed that EHV1, irrespective of the strain, can
388 replicate in the epithelium of the nasal septum and/or nasopharynx by means of virus-induced
389 plaques. This replication appears to occur in a restricted way, as no increase in number or size
390 of plaques was observed over time. In addition, a repulsion of EHV1-induced plaques was
391 observed from 5 dpi onwards for the neurovirulent strain, and a decrease in plaque latitude
392 was seen for the non-neurovirulent strain starting at day 3 pi. This reduction in replication
393 might be attributed to the production of interferon (IFN), as (i) the presence of interferon in
394 nasal secretions after experimental inoculation of horses with EHV1 has previously been
395 described and (ii) peak concentrations of IFN- α were noted at day 4 and day 7 pi (Bridges and
396 Edington, 1986; Edington et al., 1989). In the present study, IFN- α was also found in nasal
397 swabs of experimentally infected ponies (data not shown), which further points towards the
398 importance of IFN to limit EHV1 replication in the epithelium of the upper respiratory tract.
399 Interestingly, even though the non-neurovirulent strain was able to replicate in a plaquewise

400 manner in the epithelium of the nasopharynx, no plaques could be observed at any time point
401 pi in the nasopharynx after inoculation with the neurovirulent strain. As the epithelial
402 structure of both tissues is identical, with the exception of the presence of lymphoid
403 aggregates in the nasopharynx, the reason for this observation remains elusive. Another
404 interesting observation for both EHV1 strains was that we were unable to observe EHV1-
405 infected plaques under the basement membrane (BM) at any time point pi, indicating that
406 EHV1 does not reach the underlying tissue by breaking down the BM. This is in striking
407 contrast to previous findings with another and closely related alphaherpesvirus, pseudorabies
408 virus (PRV), which has been shown to cross the BM in a plaque wise manner between 12 and
409 24 hpi in a porcine respiratory explant model (Glorieux et al., 2009). This indicates that
410 different alphaherpesviruses may use different mechanisms to invade the underlying
411 connective tissue. Indeed, whereas PRV was shown to break down the BM and replicates
412 further in the underlying tissue in a plaque wise manner, our study with EHV1 demonstrated
413 only single infected cells below the BM. This is in agreement with previous studies on EHV1,
414 where the presence of single infected cells in tissues of the upper respiratory tract has been
415 reported (Edington et al., 1986; Kydd et al., 1994). We could further demonstrate that these
416 infected cells in the underlying tissue were immune cells (as discussed in more detail below),
417 which makes it tempting to speculate that immune cells in the epithelium become infected
418 with EHV1 and are responsible for transporting the virus to deeper connective tissue. Indeed,
419 we did found EHV1-infected cells in the epithelium of nasal septum and/or nasopharynx,
420 which were further characterized to belong to the monocytic lineage (CML) by using the cell
421 surface maker CD172a. Not only differences concerning the identity of the EHV1-infected
422 immune cells present in the underlying tissues were noticed between both strains, moreover,
423 differences concerning there localization were also observed. When analyzing the identity of
424 the single EHV1-infected cells in the connective tissue of nasal septum and nasopharynx,

425 several differences could be observed between the two EHV1 strains. Firstly, the total number
426 of infected cells in the nasal septum was 10 to 20 times higher for the neurovirulent strain
427 compared to the non-neurovirulent strain. Secondly, while individual infected cells in the
428 nasopharynx were mainly present in the connective tissue upon neurovirulent EHV1
429 inoculation, individual cells infected with the non-neurovirulent EHV1 seemed to migrate
430 deeper into the lymphoid follicles of the nasopharynx in a time wise manner. Thirdly,
431 CD172a⁺ CML were the most important carrier cells for both neurovirulent and non-
432 neurovirulent EHV1, although these cells seemed to be more important for the neurovirulent
433 strain. In addition, both strains were able to infect CD5⁺ T-lymphocytes, with a higher amount
434 of infected cells for the non-neurovirulent strain (Table I). When analyzing the data on the
435 identity of infected cells in lymphoid tissues of the upper respiratory tract, we again observed
436 that the most prominent target cells for EHV1 were CD172a⁺ CML, irrespective of the strain
437 used. This is in line with previous studies, where EHV1-infected macrophages and
438 lymphoblasts were detected in lymph nodes after inoculation with EHV1 isolates from both
439 an aborted fetus and a paralyzed mare (Patel et al., 1982). Moreover, another EHV1
440 experimental inoculation study showed that lymphocytes, monocytes, macrophages and
441 plasma cells in interstitium and lymph nodes all expressed EHV1 antigens, as determined by
442 immunohistochemical staining using anti-EHV1 polyclonal Abs (Kydd et al., 1994). In the
443 present study, the majority of EHV1-positive cells was found in the marginal sinus of lymph
444 nodes. At those sites, transport is possible to extra follicular interdigitating cells in the lymph
445 node where both mature and virgin lymphocytes have access to antigen, hereby initiating
446 humoral immune responses (Tew et al., 1990). Surprisingly, and in contrast to what was
447 observed in nasal septum and nasopharynx, IgM⁺ B-lymphocytes were also an important
448 target cell for EHV1. *In vitro* it has been previously shown that a 6- to 14-fold increase of
449 EHV1-infected peripheral blood mononuclear cells (PBMC) could be obtained after *in vitro*

450 stimulation with proliferating drugs such as pokeweed mitogen (PWM), concanavalin A
451 (ConA), phytohaemagglutinin (PHA), or ionomycin and phorbol dibutyrate (IONO/PDB)
452 (van der Meulen et al., 2001). Together with the fact that B-lymphocytes are known to
453 undergo extensive proliferation in lymphoid follicles (Fu and Chaplin, 1999), we would like
454 to reason that the elevated percentage of EHV1-infected B-lymphocytes observed in
455 lymphoid tissues, is due to the proliferation stage of these immune cells.

456 The onset of a cell-associated viremia could be detected as early as one day after
457 experimental inoculation with EHV1, indicating that EHV1-infected cells can enter the blood
458 stream very rapidly. Moreover, we identified these EHV1-positive cells as mainly CD172a⁺
459 CML. Despite the fact that only 14 infected cells were found, we strongly feel that this low
460 number is of relevance, as it is known that EHV1-induced viremia is generally very low with
461 numbers ranging from 1 to 10 positive cells/ 0.8×10^7 PBMC (van der Meulen et al., 2006).
462 Combining the results on the identity of EHV1-infected cells in the connective tissues of the
463 primary port of entry, the upper respiratory tract, with the results on the identity of the EHV1-
464 infected cells in the blood, we would like to hypothesize that the CD172a⁺ cells we observed
465 could be dendritic cells (DCs). The reasoning behind such hypothesis is as follows. The
466 marker DH59B that was used in the present study to identify CD172a⁺ cells is a pan
467 granulocyte/monocyte marker which has been used in several studies as a DC marker (Ahn et
468 al., 2002; Tumas et al., 1994). In addition, there is evidence indicating that local tissue DCs
469 are able to re-enter the bloodstream, which might facilitate the spread of pathogens from
470 tissue to tissue, carried by DCs serving as Trojan horses (Randolph et al., 2008). Finally, DCs
471 have been shown to play an important role during the pathogenesis of alphaherpesviruses in
472 general (Bosnjak et al., 2005; Novak and Peng, 2005; Pollara et al., 2005), and for EHV1 in
473 specific, both murine and equine blood-derived DCs are susceptible to EHV1 *in vitro* (Siedek
474 et al., 1999; Steinbach et al., 1998). Unfortunately, little is known about equine DCs in

475 general and mucosal DCs in specific in this species, and more research is needed to elucidate
476 the role of DCs in EHV1 infection and/or other viral infections in equines. Regardless, the
477 results presented here are in striking contrast to another *in vivo* study, where mainly blood T-
478 lymphocytes were found to harbor EHV1 (Scott et al., 1983). However, in the latter study,
479 leukocyte populations were solely separated by glass-bead columns and no direct
480 characterization was performed. A possible explanation for the discrepancy between cell
481 types infected during cell-associated viremia could be the strain used. In a previous study an
482 American strain, isolated from an aborted fetus, was used (Scott et al., 1983) whereas in the
483 present study, we used two Belgian EHV1 strains. Surprisingly, we observed CD172a⁺ cells
484 as the main carrier cell type for both the neurovirulent and the non-neurovirulent strain,
485 indicating that the EHV1 strain does not seem to correlate with a difference in cell tropism
486 during viremia. This is again in contrast to another study, where a difference in leukocyte
487 tropism between neuro- and non-neurovirulent EHV1 strains has been demonstrated
488 (Goodman et al., 2007). Hereby it was observed that the EHV1 strain Ab4, which has the
489 neuropathogenic genotype, mainly infected CD4⁺ lymphocytes, whereas mutating this strain
490 into the non-neuropathogenic genotype resulted in a preferential infection of monocytes and
491 B-cells. However, these experiments were performed with *in vitro* infected PBMC, which
492 most likely explains the discrepancy with our *in vivo* data.

493

494 **Conclusion**

495 Based on all the observations presented in this study, we would like to propose
496 following model on the pathogenesis of different EHV1 strains in the upper respiratory tract.
497 Upon infection, EHV1 replication in epithelial cells of the nasal septum, and nasopharynx for
498 the non-neurovirulent strain, results in a productive infection with plaque formation and nasal
499 virus shedding. This nasal shedding allows for transmission of virus to susceptible contact

500 animals and ensures that EHV1 can persist in the equine population. This productive infection
501 in epithelial cells, however, is self-limiting, most likely because of IFN- α production. In
502 addition, EHV1 seems to cross the BM through the help of CD172a⁺ carrier cells, probably
503 mucosal dendritic cells. We postulate that these carrier cells which become infected with
504 EHV1 can transport the virus across the basement membranes and through connective tissues
505 in the direction of vascular endothelial cells on the one hand and lymphatic epithelium on the
506 other hand. As a result, a cell-associated viremia is originated, together with a migration of
507 infected immune cells to local lymph nodes, hereby initiating the start of a specific immune
508 response. Although not observed in this study, the higher potential of the neurovirulent strain
509 to infect large amounts of carrier cells, could lead to a prolonged viremia, with a higher risk
510 of developing nervous system disorders.

511 Despite the fact that only a limited number of animals could be included in this experiment,
512 we still feel that this paper revealed important new insights about the pathogenesis of
513 neurovirulent and non-neurovirulent strains in the acute phase of infection in horses.

514

515 **Acknowledgements**

516 This research was supported by the Institute for the promotion of Innovation through
517 Science and Technology in Flanders (IWT-Vlaanderen). The authors like to thank C. Boone
518 and N. Monteiro for titrations, and C. Bracke for isolation of PBMC and performing SN-tests.

519

520 **Conflict of interest**

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

523

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633

634 **Figure legends**

635

636 **Figure 1. Nasal viral shedding.** Ponies were inoculated with a neurovirulent or a non-
637 neurovirulent strain of EHV1. At 0, 1, 2, 3, 4, 5 and 7 days post inoculation (dpi),
638 nasopharyngeal swabs were collected and titrated as described in material and methods.

639

640 **Figure 2. Viral replication in different tissues of the upper respiratory tract.** Ponies were
641 inoculated with either neurovirulent or non-neurovirulent EHV1 and euthanized at 0, 1, 2, 3,
642 4, 5 and 7 days post inoculation (dpi). Different tissues were collected and titrated as
643 described in materials and methods.

644

645 **Figure 3. Plaque formation in epithelium of intermedial nasal septum and nasopharynx.**

646 Ponies were inoculated with either neurovirulent or non-neurovirulent EHV1 and euthanized

647 at 0, 1, 2, 3, 4, 5 and 7 days post inoculation (dpi). Tissues of the intermedial part of the nasal
648 septum (A) and nasopharynx (B) were collected and latitude of plaques were determined. For
649 each tissue, ten consecutive sections were made and the total number of individual plaques
650 was determined. This number is presented above the relevant histogram. The largest
651 measurement of latitude of each individual plaque was used to determine plaque latitude. The
652 sum was used to calculate the mean plaque latitude \pm SEM. Representative
653 immunofluorescence pictures of EHV1-induced plaques in the intermedial nasal septum are
654 shown for neurovirulent or non-neurovirulent EHV1 for each dpi. BM: basement membrane,
655 objective 20x, NI: non-infected epithelial cells.

656

657 **Figure 4. Quantification and identification of single EHV1-infected cells in different**
658 **zones of the intermedial nasal septum for neurovirulent and non-neurovirulent strains.**

659 Every tenth section of a serially sectioned block of tissue was collected. Ten sections were
660 analysed for each cell marker separately, giving a total number of 30 sections. The total
661 number of infected cells present in the intermedial nasal septum per zone and per day, is
662 shown in the upper right table. The results for the identification of single infected cells are
663 shown in the lower graphs and are represented as the mean of ten sections \pm SEM. ROI:
664 region of interest.

665

666 **Figure 5. Quantification and identification of single EHV1-infected cells in different**
667 **zones of the nasopharynx for neurovirulent and non-neurovirulent strains.** Every tenth

668 section of a serially sectioned block of tissue was collected. Ten sections were analysed for
669 each cell marker separately, giving a total number of 30 sections. The total number of infected
670 cells present in the nasopharynx per zone and per day, is shown in the upper right table. The

671 results for the identification of single infected cells are shown in the lower graphs and are
672 represented as the mean of ten sections \pm SEM. ROI: region of interest.

673

674 **Figure 6. Quantification and identification of single EHV1-infected cells in different**
675 **zones of the tubal and nasopharyngeal tonsils for neurovirulent and non-neurovirulent**
676 **strains.** Every tenth section of a serially sectioned block of tissue was collected. Ten sections
677 were analysed for each cell marker separately, giving a total number of 30 sections. The total
678 number of infected cells present in the tubal and nasopharyngeal tonsils per zone and per day,
679 is shown in the upper right table. The results for the identification of single infected cells are
680 shown in the lower graphs and are represented as the mean of ten sections \pm SEM. ROI:
681 region of interest.

682

683 **Figure 7. Quantification and identification of single EHV1-infected cells in different**
684 **zones of the mandibular lymph node for neurovirulent and non-neurovirulent strains.**
685 Every tenth section of a serially sectioned block of tissue was collected. Ten sections were
686 analysed for each cell marker separately, giving a total number of 30 sections. The total
687 number of infected cells present in the mandibular lymph node per zone and per day, is shown
688 in the upper right table. The results for the identification of single infected cells are shown in
689 the lower graphs and are represented as the mean of ten sections \pm SEM. ROI: region of
690 interest.

691

692 **Figure 8. Quantification of viremia for neurovirulent and non-neurovirulent strains.**
693 Ponies were inoculated with a neurovirulent or a non-neurovirulent strain of EHV1. At 0, 1, 2,
694 3, 4, 5 and 7 days post inoculation (dpi), blood samples were collected and cocultivation of
695 PBMC was performed as mentioned in materials and methods.

Table I. Percentage of marker positive EHV1-infected individual cells in different tissues of the upper respiratory tract

Region of interest	Percentage of marker positive EHV1-infected individual cells					
	Neurovirulent strain			Non-neurovirulent strain		
	CD172a ⁺	CD5 ⁺	IgM ⁺	CD172a ⁺	CD5 ⁺	IgM ⁺
Nasal septum	53.4 ± 22.8	16.2 ± 11.7	0.3 ± 0.8	43.9 ± 12.8	23.6 ± 8.6	0
Nasopharynx	69.5 ± 20.7	0	8.3 ± 20.4	37.9 ± 35.5	13.6 ± 5.7	1.1 ± 2.7
Tubal and nasopharyngeal tonsils	66.4 ± 20.7	11.2 ± 12.8	4.8 ± 5.6	28.7 ± 3.6	21.2 ± 5.9	0.5 ± 1.0
Mandibular lymph nodes	71.3 ± 17.9	7.3 ± 9.5	8.3 ± 8.8	42.6 ± 21	26.7 ± 5.7	7.7 ± 10.8

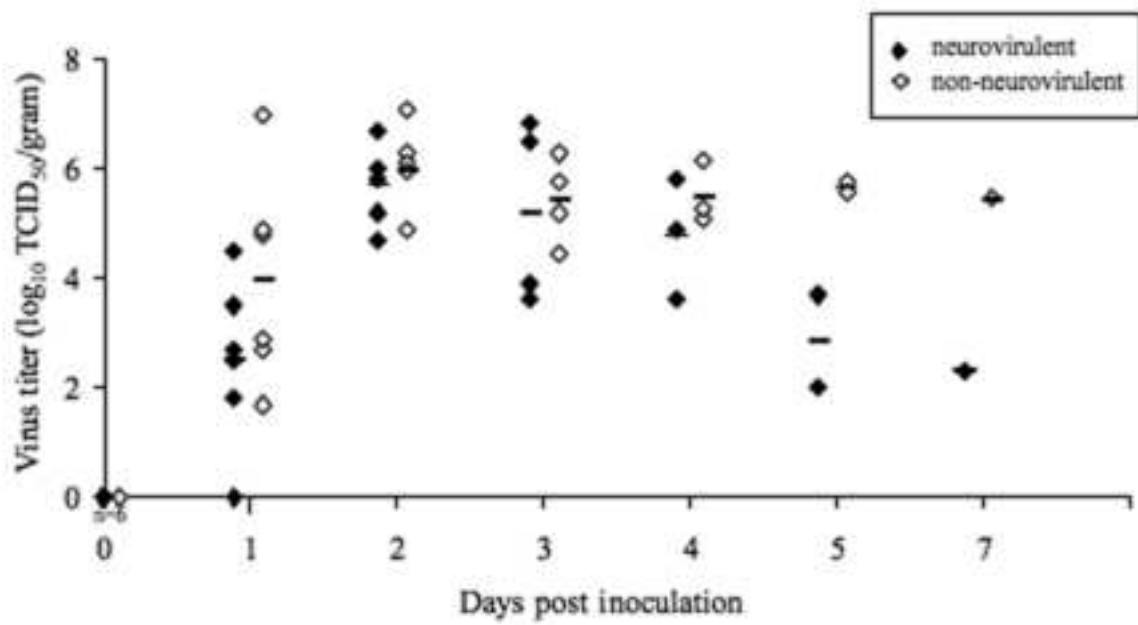
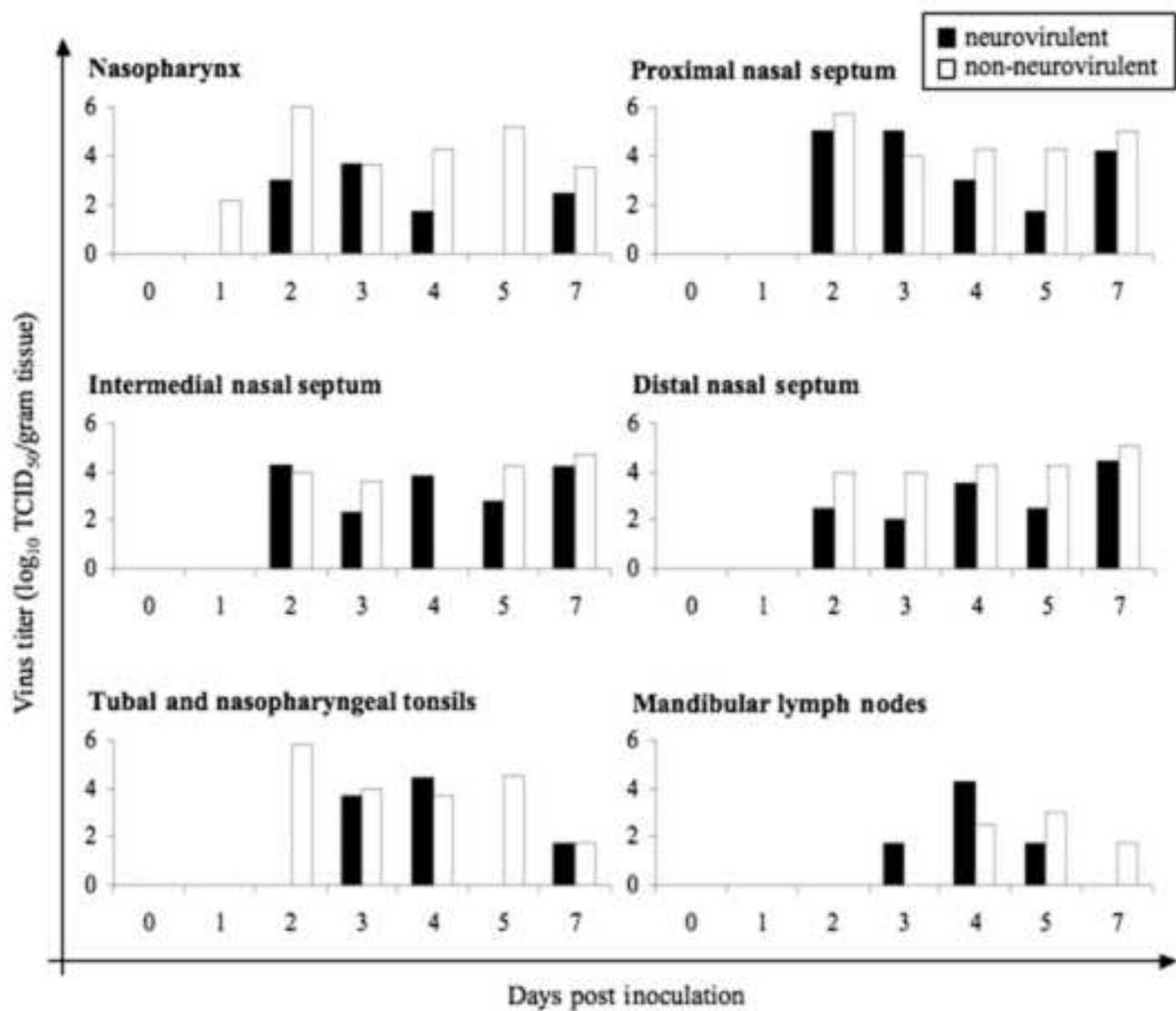
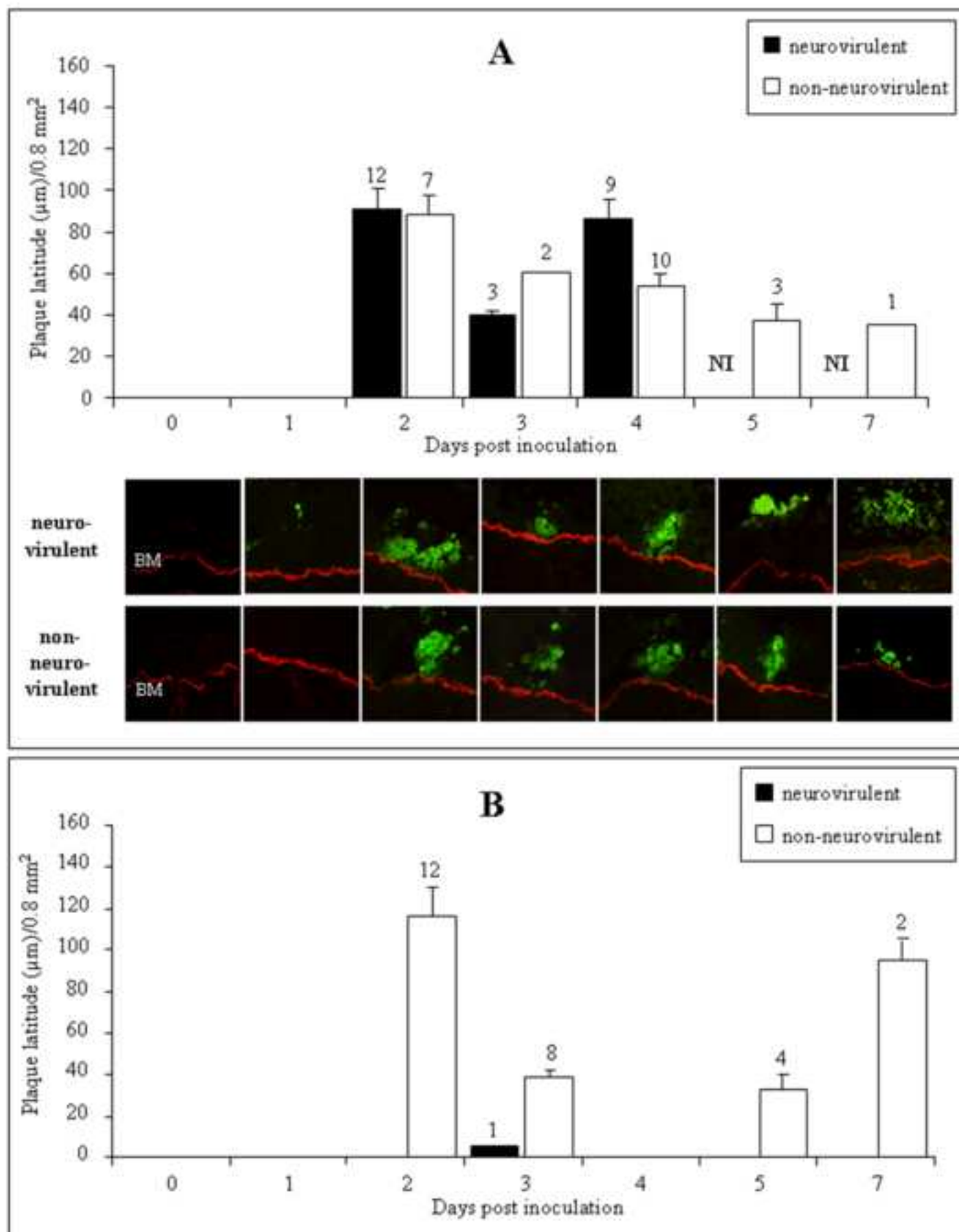
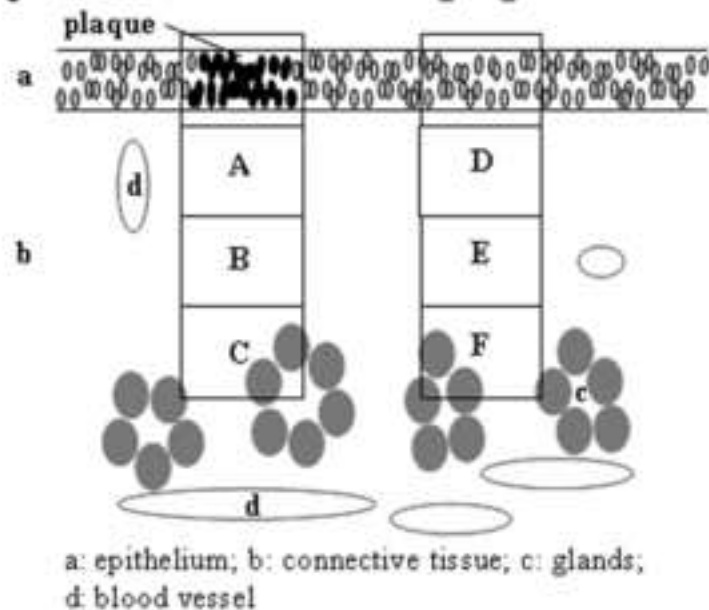


Fig 1 Gryspeerdt *et al.*

Fig 2 Gryspeerdt *et al.*

Fig 3 Gryspeerdt *et al.*

Schematic overview of the structure of the nasal septum and different counting regions



Strain	Roi	Total number of single infected cells present at ... dpi/30 sections						
		0	1	2	3	4	5	7
Neurovirulent	A	-	-	34	17	34	137	82
	B	-	-	52	13	62	8	60
	C	-	-	24	8	40	4	18
	D	0	3	24	34	62	11	9
	E	0	0	24	10	58	11	5
	F	0	0	28	9	22	8	2
Non-neurovirulent	A	-	-	3	0	4	1	0
	B	-	-	4	3	15	0	0
	C	-	-	0	1	0	0	0
	D	0	0	6	0	19	8	0
	E	0	0	0	0	17	6	0
	F	0	0	0	0	25	3	0

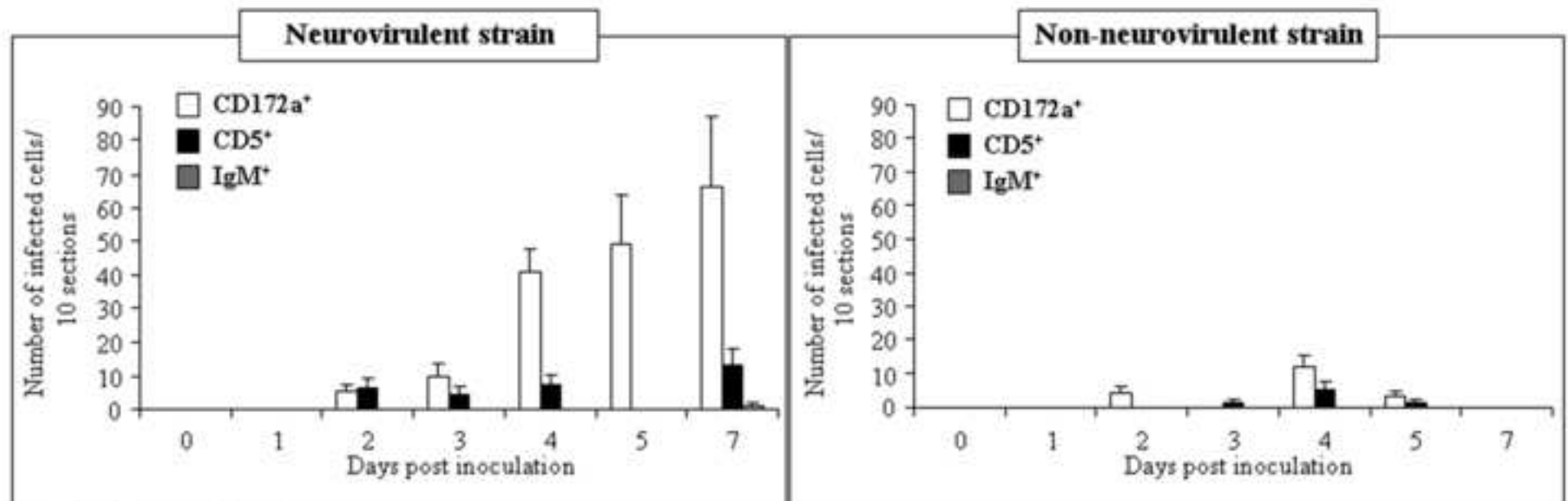
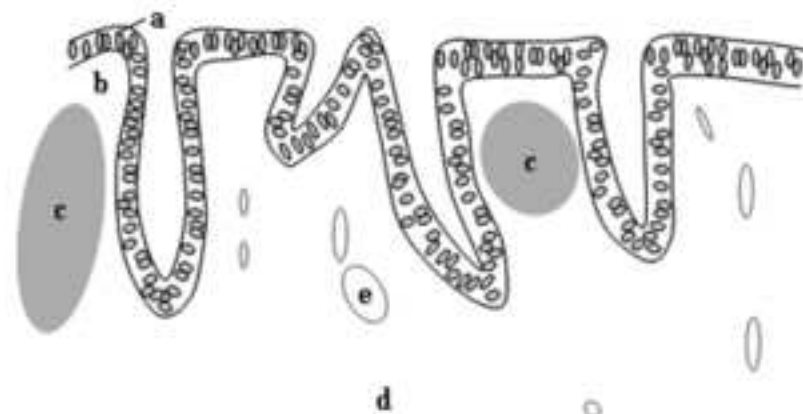


Fig 4 Gyspeerd *et al.*

Schematic overview of the structure of the nasopharynx and different counting regions



a: epithelium; b: connective tissue; c: lymphocyte aggregate; d: deeper connective tissue; e: blood vessels

Strain	Roi	Total number of single infected cells present at ... dpi/30 sections						
		0	1	2	3	4	5	7
Neurovirulent	a	0	3	3	7	2	0	1
	b	0	22	24	118	22	18	23
	c	0	0	0	0	0	0	0
	d	0	0	0	0	0	0	0
	e	0	0	0	0	0	0	0
Non-neurovirulent	a	0	0	0	0	0	0	0
	b	0	1	320	15	28	18	1
	c	0	0	0	0	99	16	1
	d	0	0	0	0	0	0	0
	e	0	0	0	0	0	1	0

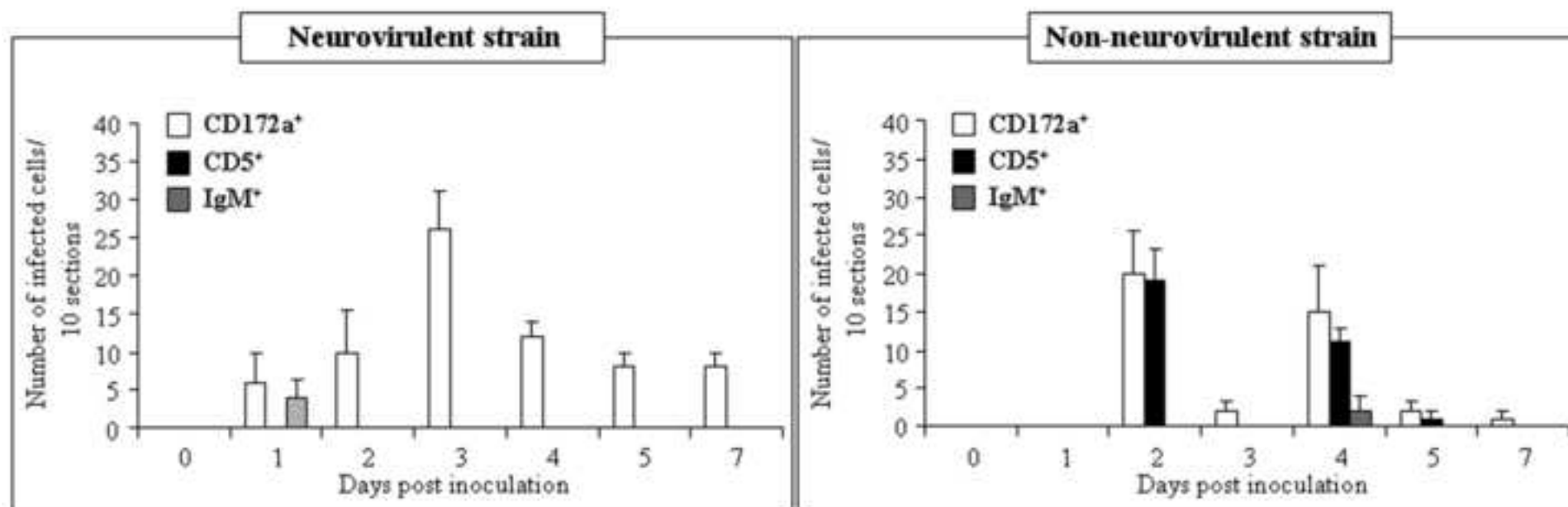
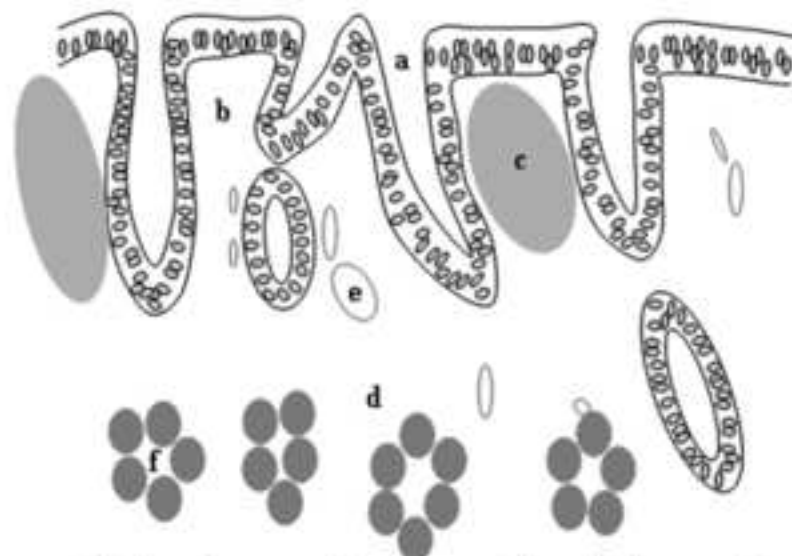


Fig 5 Gyspeerd *et al.*

Schematic overview of the structure of tubal and nasopharyngeal tonsils and different counting regions



a: epithelium; b: connective tissue; c: lymphoid aggregate; d: deeper connective tissue; e: blood vessel; f: glands

Strain	Roi	Total number of single infected cells present at ... dpi/30 sections						
		0	1	2	3	4	5	7
Neurovirulent	a	0	0	5	77	95	0	14
	b	0	0	6	273	538	1	51
	c	0	0	3	260	241	2	0
	d	0	0	0	86	150	2	7
	e	0	0	0	133	85	0	2
	f	0	0	0	38	117	1	13
Non-neurovirulent	a	0	0	7	23	7	18	5
	b	0	0	39	173	51	50	2
	c	0	0	10	34	31	487	8
	d	0	0	44	78	14	8	2
	e	0	0	0	5	3	0	2
	f	0	0	2	0	2	4	13

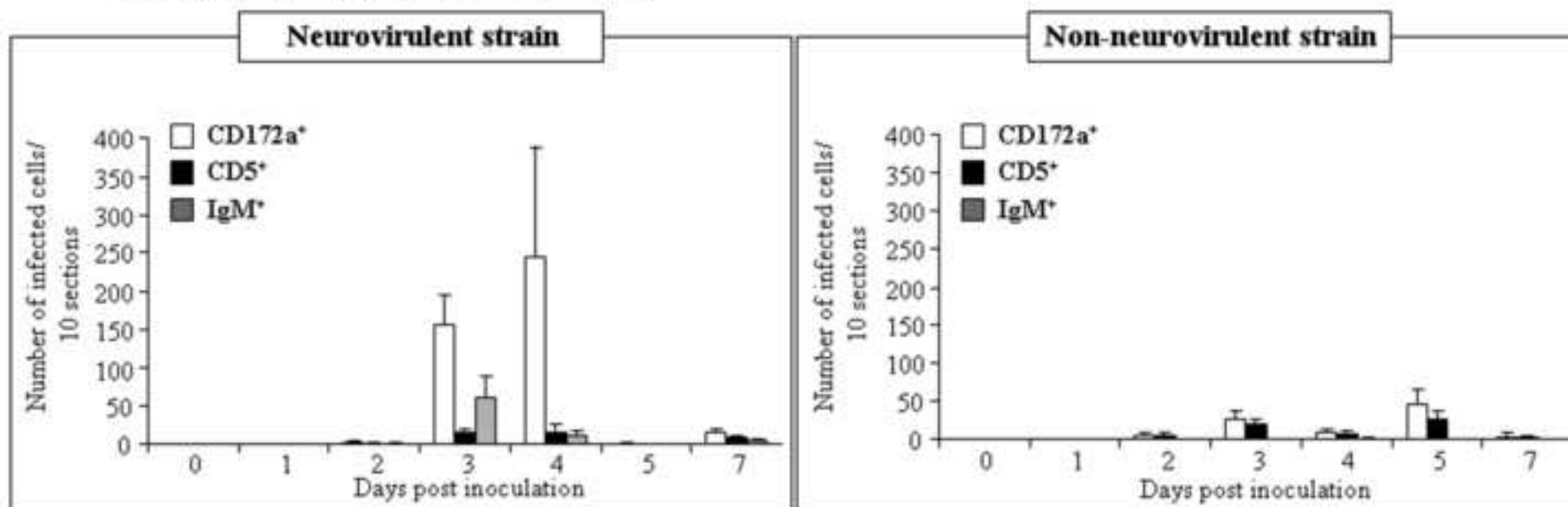
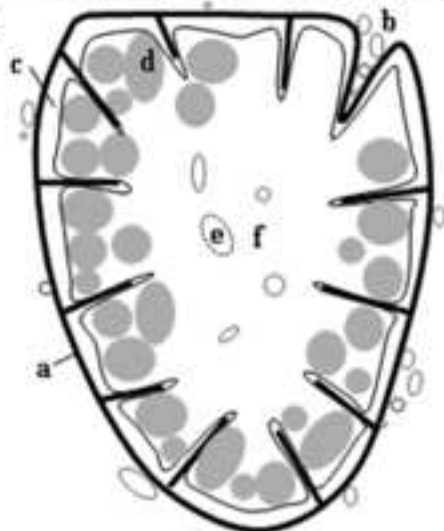


Fig 6 Gryspeerdt *et al.*

Schematic overview of the structure of the mandibular lymph node and different counting regions



a: capsule and trabecula; b: vessels of the capsule; c: marginal sinus; d: cortical nodule; e: vessels in medulla; f: medulla

Strain	Roi	Total number of single infected cells present at ... dpi/30 sections						
		0	1	2	3	4	5	7
Neurovirulent	a	0	0	0	40	10	162	1
	b	0	12	2	16	40	12	0
	c	0	17	6	106	846	170	34
	d	0	2	2	31	36	4	19
	e	0	0	0	3	1	0	0
	f	0	0	9	49	70	14	39
Non-neurovirulent	a	0	0	0	0	4	5	1
	b	0	0	0	0	0	0	0
	c	0	1	2	4	40	39	1
	d	0	0	0	0	5	17	8
	e	0	0	0	0	0	0	0
	f	0	0	0	0	41	82	1

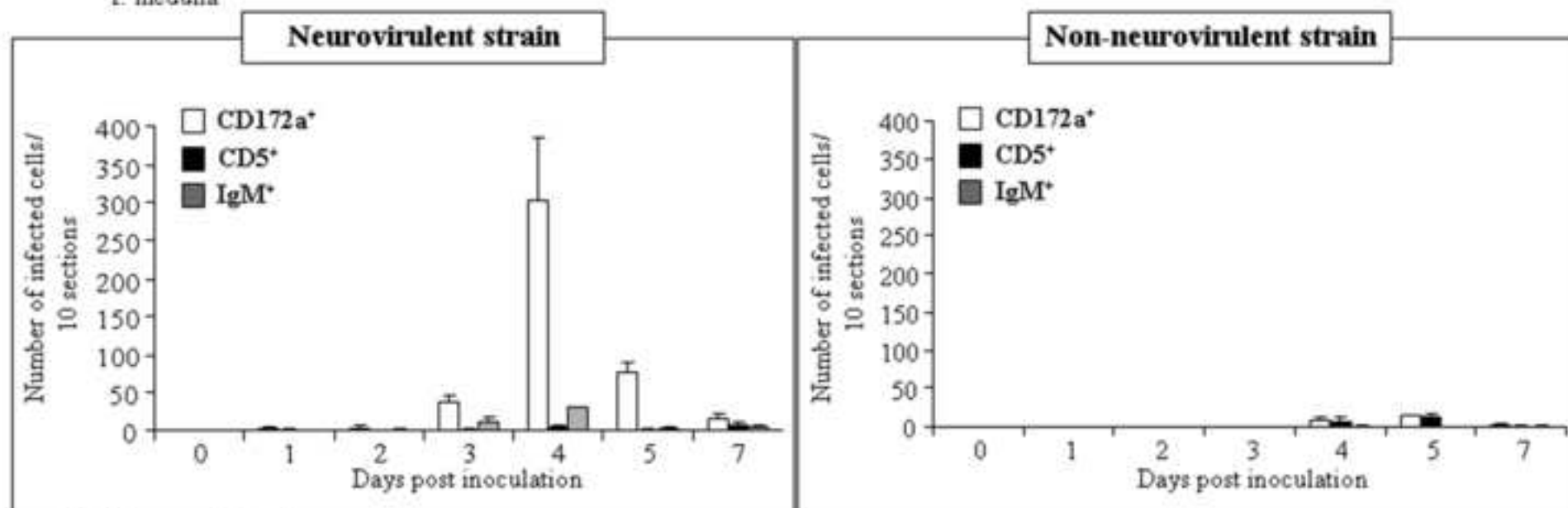


Fig 7 Gryspeerdt *et al.*

