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1 **Different replication characteristics of historical**
2 **pseudorabies virus strains in porcine respiratory nasal**
3 **mucosa explants**

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5
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33

34 **Abstract**

35 Different alphaherpesviruses, including pseudorabies virus (PRV), are able to cross
36 the basement membrane barrier in nasal respiratory epithelium. As a first step in
37 investigating this invasion process, a detailed quantitative analysis system was set up
38 to determine the kinetics of horizontal and vertical virus spread in nasal explants,
39 using the virulent PRV strain 89V87. Plaque latitudes, total depths, depths measured
40 from the basement membrane and volumes were determined at 0, 12, 24 and 36h post
41 inoculation (pi). PRV 89V87 was found to spread in a plaquewise manner and to cross
42 the basement membrane between 12 and 24hpi.

43 During the 1960s-1970s, an increase in PRV virulence has been reported. To analyze
44 potential differences in efficiency of infection and spread for different historical PRV
45 strains, single infected cells and plaques of infected cells were quantified at 12 and
46 36hpi in nasal mucosa explants for seven European PRV strains, isolated in the 1960s
47 (Becker, NIA1), the 1970s (NS374, NIA3, 75V19) and later (89V87, 00V72). All
48 viruses were used at second passage in cell culture, except for the Becker strain,
49 which had an unknown passage history. Older strains, Becker, NIA1 and/or NS374,
50 showed lower numbers of primary infectious centers, lower capacity to form plaques
51 and/or lower capacity to cross the basement membrane. The observed differences in
52 virus-mucosa interactions may aid in understanding the virulence increase of PRV.
53 The quantitative assay established here will be of use in unravelling the mechanism of
54 alphaherpesvirus-mediated invasion through the basement membrane.

55

56 **Keywords:** PRV, porcine respiratory nasal explants, quantitative assay, historical
57 strains

58

59

60 1. Introduction

61

62 Pseudorabies virus (PRV, Aujeszky's disease virus) is an alphaherpesvirus that causes
63 respiratory problems, nervous symptoms and abortion. In general, the pathogenesis of
64 most alphaherpesviruses follows a specific pattern. First, they replicate in the
65 epithelial cells of the upper respiratory tract. In contrast with most respiratory viruses,
66 replication of alphaherpesviruses is often not restricted to epithelial cells. From the
67 primary site of infection, they may quickly penetrate through the basement membrane
68 to the underlying lamina propria. By crossing the basement membrane, the virus may
69 reach blood vessels and nerves in the lamina propria whereafter it may spread to
70 internal organs. The latter can lead to abortion and nervous symptoms (Appel *et al.*,
71 1969; Sabo *et al.*, 1969; Wittmann *et al.*, 1980; Wyler *et al.*, 1989; Gibson *et al.*,
72 1992; Maeda *et al.*, 1998). Thus, a crucial step in the invasion mechanism of PRV, is
73 the crossing of the basement membrane barrier. *In vivo* pig experiments with PRV
74 illustrated invasion of the stroma and infection of axons of olfactory nerves within
75 24hpi (Pol *et al.*, 1989). The mechanism of this quick invasion process through the
76 basement membrane barrier is still unknown.

77

78 On the European continent, in Northern Ireland, as well as in the United States, a
79 similar increase in severity of PRV-induced Aujeszky's disease has been observed
80 during the 1960-70s, consisting of increased fatal encephalitis in young piglets, and
81 more prominent disease, viremia, and abortions in older animals (Lamont and Shanks,
82 1939; Gordon and Luke, 1955; McFerran and Dow, 1962; McFerran and Dow, 1965;
83 Olander *et al.*, 1966; reviewed by Baskerville *et al.*, 1973; Lee and Wilson, 1979;
84 Nauwynck *et al.*, 2007).

85

86 Recently, explant cultures of porcine respiratory nasal mucosa, cultured on fine-
87 meshed gauze at an air-liquid interface, have been set up *in vitro* (Glorieux *et al.*,
88 2007). In the present study, a quantitative and reproducible analysis system was
89 established to study the kinetics of horizontal and vertical spread of PRV (including
90 crossing of the basement membrane) in porcine respiratory nasal mucosa. The *in vitro*
91 model was subsequently used to examine whether different historical PRV strains
92 (Becker, NIA1, NS374, NIA3, 75V19, 89V87 and 00V72), isolated over a timeperiod
93 extending from the 1960s to 2000, behaved differently in the respiratory nasal
94 mucosa. The results may aid in explaining the increased virulence of PRV over the
95 past decades.

96

97 **2. Materials and methods**

98 **2.1. Virus strains**

99 Five PRV strains from the European continent, Becker, NS374, 75V19, 89V87 and
100 00V72, and two Northern Irish strains, NIA1 and NIA3, were used. The precise origin
101 of the Becker strain is unknown. Becker made reports of Aujeszky's disease between
102 1958 and 1966. The Becker strain is often referred to as from 1967 (Becker, 1967).
103 The strains NS374, a low virulent Belgian virus strain, and 75V19, a virulent Belgian
104 virus strain, were isolated in respectively 1971 and 1975 from the brains of pigs with
105 nervous symptoms (Nauwynck *et al.*, 2007). 89V87, a highly virulent virus strain,
106 was isolated in 1989 in Belgium from an aborted foetus (Nauwynck and Pensaert,
107 1992). 00V72, a highly virulent Belgian virus strain, was isolated in 2000 from an
108 aborted foetus. The low virulent Northern Irish strain NIA1 was isolated in 1962 by
109 Mc Ferran and Dow. In 1971, a virulent Northern Irish strain, NIA3, was isolated
110 from 14-18-weeks old pigs (Mc Ferran *et al.*, 1979).

111 Except for Becker, of which the passage history was unknown, all viruses were used
112 at second passage in swine testicle (ST) cells. Virus was diluted in serum-free
113 medium to final concentrations of $3 \cdot 10^6$ TCID₅₀/600 μ l.

114

115 **2.2. Inoculation procedure**

116 Porcine respiratory nasal explants were obtained as described previously (Glorieux *et*
117 *al.*, 2007). Just before inoculation, explants were taken from their gauze, placed in a
118 24-well plate with the epithelial surface upwards and washed twice with warm serum-
119 free medium. Afterwards, they were inoculated with 600 μ l inoculum containing $3 \cdot 10^6$
120 TCID₅₀ of PRV. After incubation for 1h at 37°C and 5% CO₂, explants were washed
121 three times with warm serum-free medium and transferred again to the gauze. At
122 different time points post inoculation (pi), explants were collected, embedded in
123 methocel[®] (Fluka) and frozen at -70°C.

124

125 **2.3. Immunofluorescence staining**

126 Cryosections were made and fixed in methanol (-20°C, 100%) for 25 minutes. The
127 cryosections were first incubated with antibodies to visualise collagen IV. Collagen
128 IV is a component of the basement membrane and of the extracellular matrix of the
129 lamina propria. Staining collagen IV enables to distinguish the epithelium from the
130 underlying basement membrane. Therefore, cryosections were incubated (1h, 37°C)
131 with goat anti-collagen IV antibodies (Southern Biotech) (1:50 in PBS). Then,
132 cryosections were washed three times in PBS and incubated (1h, 37°C) with
133 biotinylated rabbit anti-goat IgG antibodies (Sigma) (1:100 in PBS). After this
134 incubation step, cryosections were washed three times with PBS and incubated (1h,
135 37°C) with streptavidin-Texas Red (Molecular Probes) (1:50 in PBS). PRV-infected

136 cells were detected by using FITC-labeled porcine polyclonal anti-PRV antibodies
137 (Nauwynck and Pensaert, 1995) (1:100 in PBS) (1h, 37°C). Finally, cryosections were
138 washed three times in PBS and mounted with glycerin-DABCO.

139

140 **2.4. Confocal microscopy**

141 Image series of the immunofluorescence stained cryosections were acquired using a
142 Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Heidelberg,
143 Germany). A Gre/Ne 543nm laser was used to excite Texas Red-fluorochromes. An
144 Argon 488nm laser was used to excite FITC-fluorochromes.

145

146 **2.5. Plaque analysis**

147 Using the software imaging system ImageJ, the plaque latitude and plaque penetration
148 depth (total penetration depth and depth from the basement membrane) were
149 measured. By combining serial sections, which cover an entire plaque, and counting
150 the voxels positive for PRV-FITC using ImageJ, the volumes of the plaques were
151 obtained.

152

153 **2.6. Statistical analysis**

154 The data were statistically evaluated by analysis of variance (ANOVA) using SPSS
155 software (SPSS Inc., Chicago, Illinois, USA). Differences in results that had P values
156 of ≤ 0.05 were considered significant. The data shown represent means + SD of
157 independent experiments.

158

159 3. Results & discussion

160 3.1. Evolution of 89V87 virus spread

161 A morphometric analysis of the evolution of 89V87 PRV spread in respiratory nasal
162 mucosa was performed (Glorieux *et al.*, 2007). Plaque latitudes, depths and depths
163 from the basement membrane were measured and plaque volumes were calculated.
164 PRV was found to spread in a plaquewise manner. The evolution of horizontal and
165 vertical spread of the wild type PRV strain 89V87 was investigated at 0, 12, 24 and
166 36hpi. Representative images of 89V87 plaque formation at 0, 12, 24 and 36hpi are
167 illustrated in Fig.1. Fig.2. shows mean values + SD of four independent experiment
168 for different parameters.

169

170 Plaque latitudes (Fig.2a) increased steadily over time (from 0.0 μm at 0hpi to 119.1
171 μm at 36hpi) and increased significantly between 0, 12 and 24hpi. Plaque depths
172 (Fig.2b) increased significantly between 0, 12 and 24hpi, and evolved from 0.0 μm at
173 0hpi to 113.0 μm at 36hpi. Plaque depths from the basement membrane (Fig.2c) also
174 increased significantly between 0, 12 and 24hpi and went from 0.0 μm at 0hpi to 40.7
175 μm at 36hpi. Hence, PRV was found to cross the basement membrane between 12 and
176 24hpi. This is consistent with *in vivo* observations made by Pol *et al.* (1989). To
177 calculate the plaque volume (Fig.2d), a stack of z-series was made for each
178 cryosection. Voxels positive for FITC-fluorescence were counted using the
179 voxelcounter of the software imaging system ImageJ. To cover an entire plaque,
180 different cryosections were combined. A significant increase in plaque volume was
181 again observed between 0, 12 and 24hpi, and plaque volumes evolved from 0.0 μm^3
182 to 283280.2 μm^3 between 0 and 36hpi.

183

184 We could demonstrate that the plaque latitudes, plaque depths, depths from the
185 basement membrane and plaque volumes increased significantly between 0, 12 and
186 24hpi. However, the increase in plaque dimensions was not significantly different
187 between 24 and 36hpi. Although speculative, it is possible that the infection is
188 accompanied by a local upregulation of cellular antiviral products which achieve their
189 inhibitory effect from a certain time point. This could result in a reduced lateral
190 spread. The distance covered laterally and in the depth evolved similarly with
191 increasing time pi. In this context, 12hpi was not taken into account, as a primary
192 infected epithelial cell with its elongated morphology has a larger depth compared to
193 its latitude. We can conclude that the data on the evolution of plaque formation
194 illustrate the highly invasive characteristics of PRV. PRV easily crosses the basement
195 membrane. Testing different specific genetically altered PRV strains in the current
196 model will enable the investigation of the exact mechanism underlying the efficient
197 basement membrane crossing.

198 In the present study, a quantitative, reproducible and complete 3-dimensional picture
199 of the horizontal and vertical spread of PRV in porcine respiratory nasal mucosa is
200 obtained by confocal microscopical analysis of serial tissue sections. This novel
201 approach is an ideal tool for studying cell biological and molecular aspects of the
202 invasion mechanisms of pathogens.

203

204 **3.2. Quantification of single infected cells and plaques of infected cells for** 205 **different PRV strains**

206 Before the 1960s, Aujeszky's disease occurred sporadically. Pigs were only
207 considered as reservoir during this time period. During the 1960-70s, the density of
208 pig populations increased and increasing incidences of severe clinical outbreaks were

209 reported. The increase in outbreak incidence is generally believed to be associated
210 with an as yet uncharacterized increase in virus virulence (reviewed by Nauwynck *et*
211 *al.*, 2007). Using porcine nasal mucosa explants, Pol *et al.* (1991) showed that NIA3
212 virus infected epithelial and stromal cells within 24hpi. Infection with non-virulent
213 Bartha virus was restricted to epithelial cells, even at 48hpi. Therefore, virulence
214 could be associated with larger spread in latitude and/or crossing of the basement
215 membrane barrier. These characteristics were taken into account to
216 examine whether different historical PRV strains, isolated over a timeperiod
217 extending from the 1960s to 2000, behaved differently in respiratory nasal mucosa.

218

219 First, the numbers of single infected cells (I) and plaques of infected cells (PI) were
220 quantified at 12 and 36hpi for five PRV strains from the European continent (Becker,
221 NS374, 75V19, 89V87 and 00V72) and two Northern Irish strains (NIA1 and NIA3).
222 At 12hpi, single infected cells and plaques of infected cells were considered as
223 'primary infectious centers'. The ratio of plaques at 36hpi / primary infectious centers
224 at 12hpi was determined to evaluate the capacity to spread to plaques. Table 1
225 represents the mean values \pm SD of three independent experiments.

226 The numbers of primary infectious centers at 12hpi were similar for the oldest strains
227 Becker and NIA1, but significantly lower than those of the other more recent strains
228 from the European continent (NS374, 75V19, 89V87 and 00V72) and from Northern
229 Ireland (NIA3). Experiments were performed with a standardized method using the
230 same virus titer of each strain and therefore, the difference in number of infectious
231 centers cannot be attributed to differences in multiplicity of infection (moi). Although
232 speculative, one possible explanation may be found in PRV entry mediators, such as
233 herpesvirus entry mediator B (HveB, nectin 2), HveC (nectin 1) and HveD (CD155)

234 (Nixdorf *et al.*, 1999). Future research may show whether old PRV strains such as
235 Becker and NIA1 may use different receptors than the more recent strains or virus-
236 receptor binding characteristics may be different.

237 To analyse the capacity to spread, the ratio of the number of plaques at 36hpi /
238 number of primary infectious centers at 12hpi was determined. For strains from the
239 European continent, a ratio lower than 1.0 was obtained showing that only a part of
240 the primary infected cells expanded into plaques. For NIA strains, a higher ratio was
241 observed and plaques were already formed at 12hpi. More efficient cell-associated
242 spread (Yoon and Spear, 2002; Farnsworth and Johnson, 2006), fusion from without
243 (Klupp *et al.*, 2000), and/or other mechanisms perhaps may be involved. A lower
244 number of plaques at 36hpi was demonstrated for NIA1 compared to NIA3. For
245 Becker and NS374, a much lower ratio and a much lower number of plaques at 36hpi
246 were observed compared to the other European continent strains.

247

248 Second, the different virus strains were tested morphometrically for their penetration
249 characteristics through the nasal respiratory mucosa at 0, 12, 24 and 36hpi. Mean
250 values + SD of three independent experiments are illustrated in Fig. 3.

251 As NIA strains showed plaque formation within 12hpi, larger plaque latitudes were
252 obtained at 12hpi. Some plaques even penetrated the basement membrane within
253 12hpi. However, no differences were observed at 24 and 36hpi between the different
254 virus strains. For strains from the European continent, no significant differences could
255 be observed for the plaque latitude. For Becker, lower values were obtained for the
256 plaque depth from the basement membrane at 24 and 36hpi.

257 Taking these data together, some notable differences were observed between some of
258 the older and more recent PRV strains. Older strains Becker and NIA1 showed much

259 lower numbers of primary infectious centers at 12hpi, Becker and NS374 showed a
260 lower capacity to form plaques, and Becker had a reduced invasive capacity through
261 the basement membrane. However, it has to be pointed out that all viruses were used
262 at second passage in cell culture except for the Becker strain, of which the passage
263 history was unknown. Higher passages of Becker may have reduced its capacity to
264 spread in the explant model. Nevertheless, since differences were observed for
265 different older PRV strains (e.g. NIA1 and NS374), the current data appear to be in
266 line with previous reports, obtained in pigs, showing an increased severity in the
267 evolution of Aujeszky's disease over time, from the 1960-70s onwards.

268

269 Taking together, this study illustrates the utility of a quantitative and reproducible *in*
270 *vitro* respiratory nasal mucosa explant model to investigate the kinetic evolution of
271 alphaherpesvirus horizontal and vertical spread in a very important primary
272 replication site, the nasal mucosa. This model will be a very useful tool in unravelling
273 the invasion mechanism of alphaherpesviruses. Unravelling the exact mechanism of
274 PRV invasion through the basement membrane may open new strategies to interfere
275 with the invasion of alphaherpesviruses and their diseases.

276

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283

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- 352
- 353
- 354

355 **Captions:**

356

357 **Table 1:** Quantification of the number of single infected cells (I) and plaques of
358 infected cells (PI) at 12 and 36hpi per 2mm².

359

360 **Fig. 1.** Confocal photomicrographs illustrating the evolution of plaque formation of
361 the PRV strain 89V87 at 0 (a), 12 (b), 24 (c) and 36 (d) hpi. Collagen IV is
362 visualised by red fluorescence. Green fluorescence visualises PRV antigens.

363

364 **Fig. 2.** Evolution of the plaque latitude (a), total penetration depth (b), penetration
365 depth from the basement membrane (c) and plaque volume (d) of the PRV
366 strain 89V87 at 0, 12, 24 and 36hpi. Data are represented as means + SD (error
367 bars).

368

369 **Fig. 3.** Kinetic evolution of the plaque latitude (a) and penetration depth from the
370 basement membrane (b) of the PRV strains Becker, NS374, 75V19, 89V87
371 and 00V72 from the European continent and of the Northern Irish strains,
372 NIA1 and NIA3, at 12 (black bars), 24 (grey bars) and 36hpi (white bars).
373 Data are represented as means + SD (error bars).

374

Table 1

Origin	PRV strain	Number of ... at 12hpi			Number of ... at 36hpi			PI 36hpi / Total 12hpi
		I	PI	Total	I	PI	Total	
European continent	Becker	8.1 ± 1.0*	0.0 ± 0.0	8.1 ± 1.0*	8.0 ± 2.4	2.7 ± 0.5	10.7 ± 1.9	0.3
	NS374	22.2 ± 2.8	0.0 ± 0.0	22.2 ± 2.8	14.6 ± 1.9	6.0 ± 2.7	20.7 ± 4.3	0.3
	75V19	23.5 ± 1.5	0.0 ± 0.0	23.5 ± 1.5	11.4 ± 2.3	12.4 ± 3.1	23.8 ± 4.9	0.5
	89V87	25.8 ± 2.8	0.0 ± 0.0	25.8 ± 2.8	11.2 ± 5.4	16.2 ± 6.3	27.5 ± 8.2	0.6
	00V72	18.5 ± 3.6	0.0 ± 0.0	18.5 ± 3.6	12.2 ± 1.7	11.4 ± 2.3	23.6 ± 3.0	0.6
Northern Ireland	NIA1	7.5 ± 1.6	1.7 ± 2.4	9.2 ± 4.0*	4.3 ± 3.1	15.9 ± 7.0	20.1 ± 3.9*	1.7
	NIA3	12.6 ± 4.3	3.5 ± 0.5	16.1 ± 3.8	9.0 ± 1.2	27.2 ± 8.6	36.2 ± 9.8	1.7

* = significantly different within each of the two groups of PRV strains ($p \leq 0.05$)

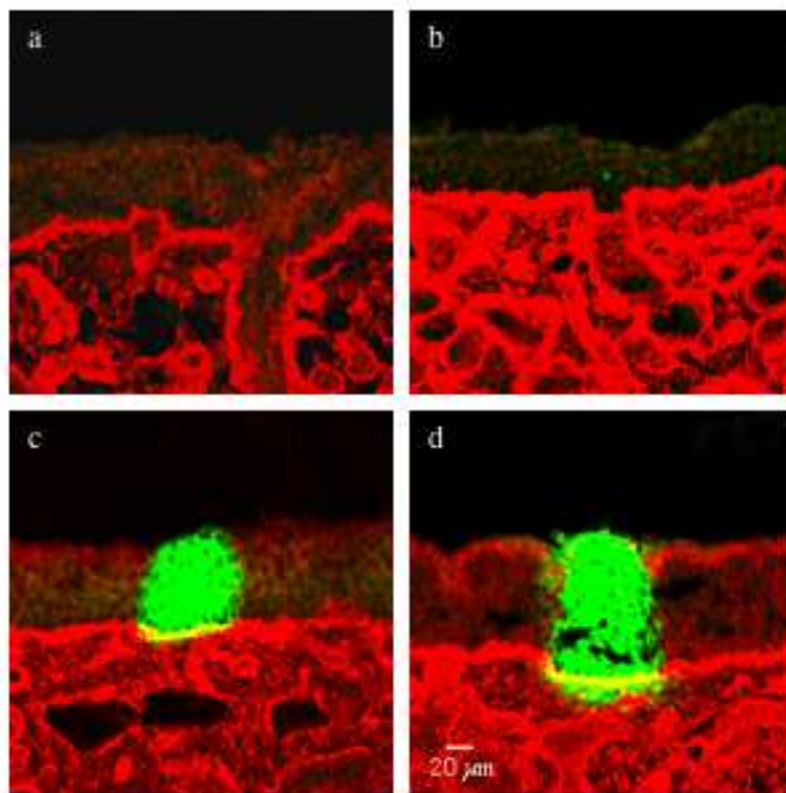


Figure 2

