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Different replication characteristics of historical pseudorabies virus strains in porcine respiratory nasal mucosa explants

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Abstract

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

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Virus strains

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

Except for Becker, of which the passage history was unknown, all viruses were used at second passage in swine testicle (ST) cells. Virus was diluted in serum-free medium to final concentrations of 3.10^6 $TCID_{50}$/600 µl.

2.2. Inoculation procedure

Porcine respiratory nasal explants were obtained as described previously (Glorieux *et al*., 2007). Just before inoculation, explants were taken from their gauze, placed in a 24-well plate with the epithelial surface upwards and washed twice with warm serum-free medium. Afterwards, they were inoculated with 600 µl inoculum containing 3.10^6 $TCID_{50}$ of PRV. After incubation for 1h at 37°C and 5% CO_2, explants were washed three times with warm serum-free medium and transferred again to the gauze. At different time points post inoculation (pi), explants were collected, embedded in methocel® (Fluka) and frozen at -70°C.

2.3. Immunofluorescence staining

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

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

2.4. Confocal microscopy

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

2.5. Plaque analysis

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

2.6. Statistical analysis

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

3. Results & discussion

3.1. Evolution of 89V87 virus spread

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

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

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

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

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

Before the 1960s, Aujeszky's disease occurred sporadically. Pigs were only considered as reservoir during this time period. During the 1960-70s, the density of 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 uncharacterizd 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 (Pl) 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 ± 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)

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

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

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

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

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

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

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

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352
353
354

Captions:

Table 1: Quantification of the number of single infected cells (I) and plaques of infected cells (Pl) at 12 and 36hpi per $2mm^2$.

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

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

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

Table 1

Origin	PRV strain	Number of ... at 12hpi			Number of ... at 36hpi			Pl 36hpi / Total 12hpi
		I	Pl	Total	I	Pl	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 1

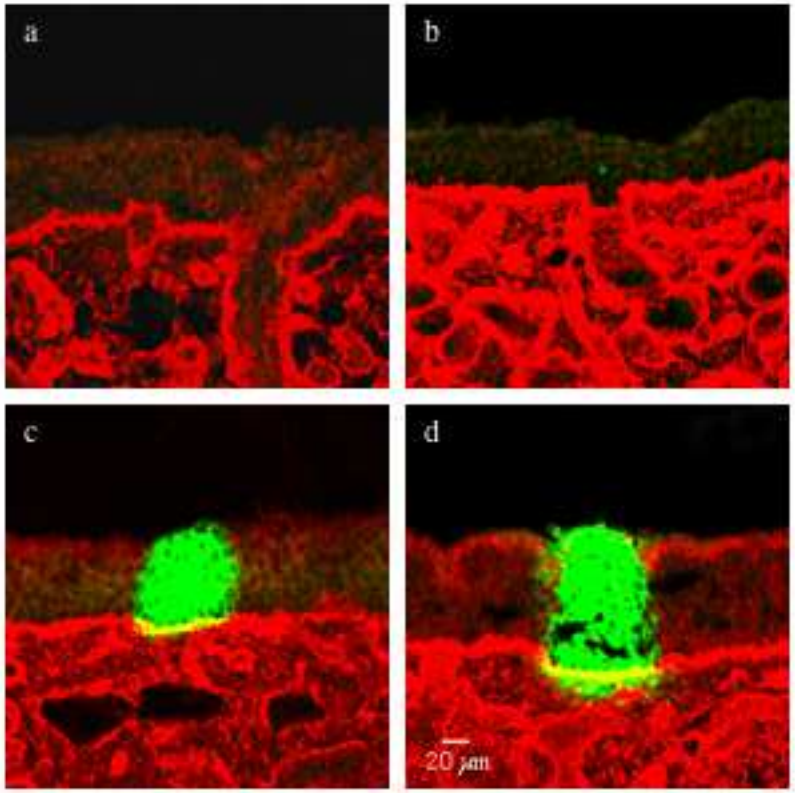


Figure 2

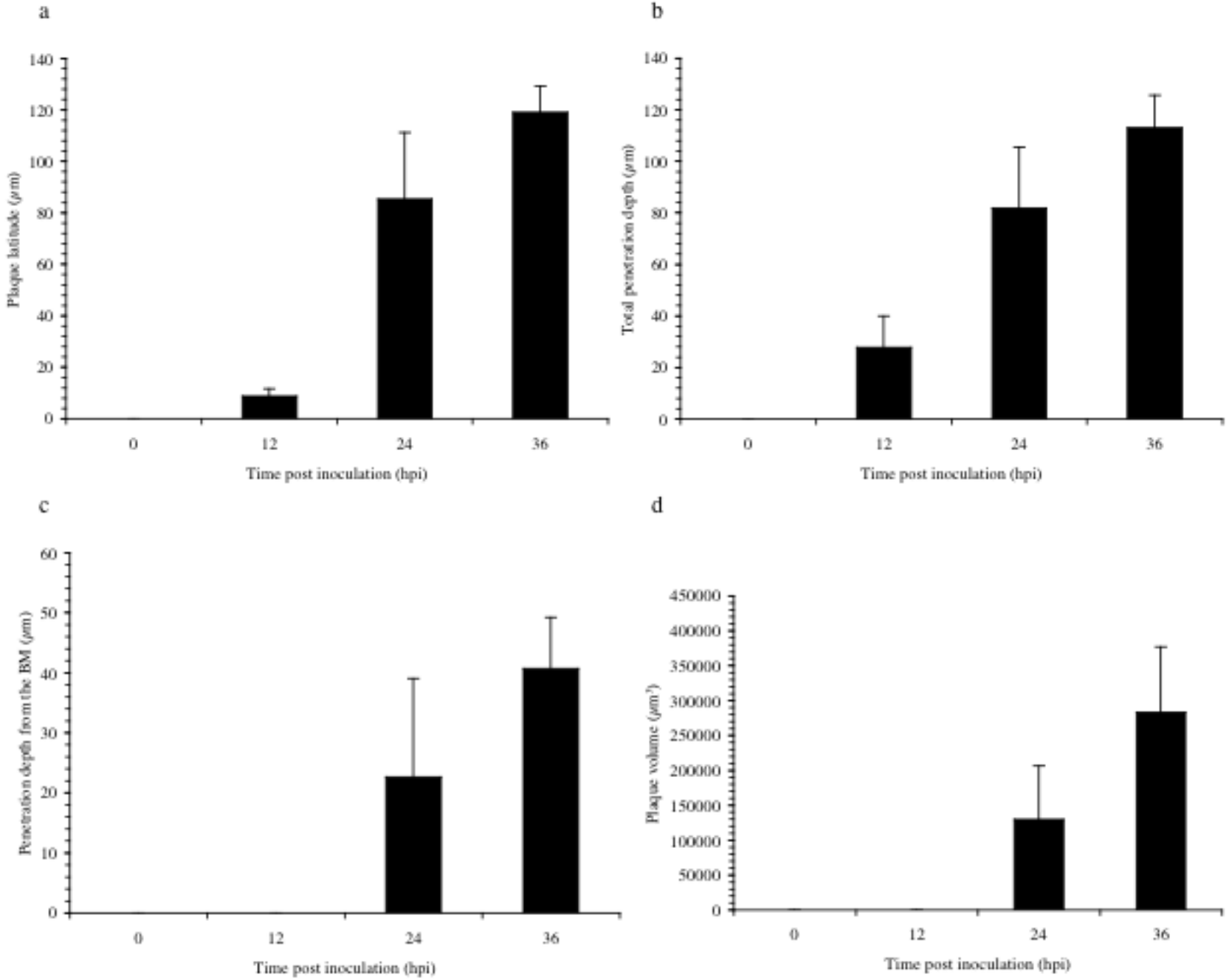
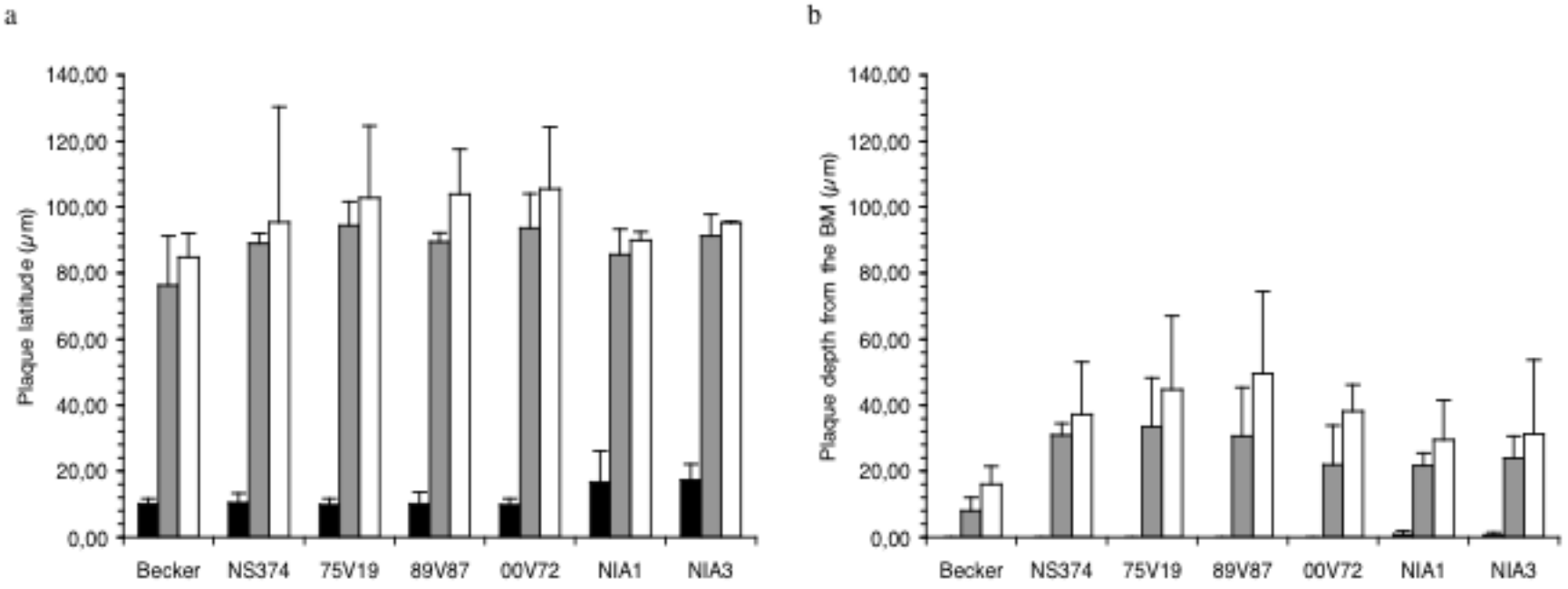

a
Plaque latitude (μm)
0
20
40
60
80
100
120
140
0
12
24
36
Time post inoculation (hpi)
b
Total penetration depth (μm)
0
20
40
60
80
100
120
140
0
12
24
36
Time post inoculation (hpi)
c
Penetration depth from the BM (μm)
0
10
20
30
40
50
60
0
12
24
36
Time post inoculation (hpi)
d
Plaque volume (μm³)
0
50000
100000
150000
200000
250000
300000
350000
400000
450000
0
12
24
36
Time post inoculation (hpi)

Figure 3


a
Plaque latitude (μm)
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
Becker
NS374
75V19
89V87
00V72
NIA1
NIA3
b
Plaque depth from the BM (μm)
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
Becker
NS374
75V19
89V87
00V72
NIA1
NIA3