Pseudorabies virus isolates from domestic pigs and wild boars show no apparent \textit{in vitro} differences in replication kinetics and sensitivity to interferon-induced antiviral status

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Pseudorabies virus is the causative agent of Aujeszky’s disease. Domestic pigs and wild boars are its natural hosts, and strains circulating within both populations differ in their capacity to induce clinical disease. Cell biological and molecular explanations for the observed differences in virulence are, however, lacking. Different virulence determinants that can be assessed \textit{in vitro} were determined for five domestic swine strains, four wild boar strains and the NIA3 reference strain. Replication kinetics and plaque formation capacity in continuous swine testicular cells and different primary porcine cell lines were highly similar for isolates from both populations. Treatment of these cell lines with IFN$_\alpha$, IFN$_\gamma$ or a combination of both provoked similar plaque-reducing effects for all strains. In conclusion, our results indicate that isolates from domestic swine and wild boar differ neither in intrinsic replication and dissemination capacity nor in sensitivity to antiviral effects of IFNs.

Aujeszky’s disease is an economically important disease in domestic swine caused by suid herpesvirus 1, also called Aujeszky’s disease virus or pseudorabies virus (PRV). The virus belongs to the family \textit{Herpesviridae}, subfamily \textit{Alpha-herpesvirinae}, genus \textit{Varicellovirus}. Members of the family Suidae are the only natural hosts of the virus and infection is characterized by respiratory, reproductive and neurological symptoms. The severity of the symptoms depends on the age of the swine and the virulence of the strain (Pomeranz \textit{et al.}, 2005). Aujeszky’s disease occurred only sporadically before the 1960s, and pigs were considered to be only a reservoir for the virus. Since the early 1980s, an increase in clinical outbreaks has been reported, coinciding with the emergence of more virulent strains and the intensification of swine production (Pomeranz \textit{et al.}, 2005; Nauwynck \textit{et al.}, 2007). In view of the great economic impact of the virus, large-scale vaccination programmes were set up, which led to the eradication of the virus in the domestic swine population in several European countries, including Belgium (Decision 2011/648/EU). PRV remains, however, present within the wild boar population (Czaplicki \textit{et al.}, 2006; Müller \textit{et al.}, 2011) and poses a risk for reintroduction of the virus in the currently unprotected domestic swine population. PRV strains circulating in wild boars are considered to be attenuated based on field observations and experimental infection studies (Hahn \textit{et al.}, 1997; Müller \textit{et al.}, 2001). The risk of transmission to domestic swine is considered low based on the limited number of observed reintroductions in countries free of Aujeszky’s disease (Pannwitz \textit{et al.}, 2012). The presence of genetic differences in the glycoprotein C (gC) segment of different wild boar PRV isolates and the high genetic resemblance between some isolates from wild boar and domestic swine (Müller \textit{et al.}, 2010; Verpoest \textit{et al.}, 2014) suggest, however, that one should be careful when generalizing the proposed attenuated nature of wild boar PRV strains.

Cell biological and molecular explanations for the observed differences between wild boar and domestic swine isolates in their capacity to induce clinical disease are lacking. Therefore, we wanted to evaluate if differences in \textit{in vitro} virulence between strains from domestic swine and wild boar exist that could help to explain the observed differences in \textit{in vivo} virulence. If such differences would be found, this could lead to the use of \textit{in vitro} models to assess the virulence of newly isolated strains from wild boar and predict the potential consequences associated with a reintroduction of such strains in the domestic swine population.
To evaluate if differences in in vivo virulence are also reflected in in vitro assays used to assess PRV replication and spreading capacity and sensitivity to IFN-induced antiviral mechanisms, four and five well characterized isolates of wild boar and domestic swine origins, respectively, and the PRV reference strain NIA3 were selected. Strains of wild boar origin were collected from a wolf and hunting dogs that were diagnosed with Aujeszky’s disease after being fed with offal of asymptomatic wild boar shot during hunting. Phylogenetic analysis of a part of the gC gene, performed in accordance with literature standards (Goldberg et al., 2001; Hahn et al., 2010; Müller et al., 2010), showed that these isolates cluster together with other wild boar isolates from south-western Europe in clade B and can therefore be considered as representative strains (Verpoest et al., 2014). The virulence of two of these wild boar strains, BEL20075 and BEL24043, was tested in vivo in 2- and 15-week-old domestic pigs, and indicated the attenuated nature of these strains in adult pigs (Verpoest et al., 2016). Domestic swine strains were directly obtained from domestic swine that were euthanized after experiencing important clinical symptoms typical for Aujeszky’s disease. The strains clustered within the heterogeneous clade A, which contained all the domestic swine isolates so far, and also wild boar isolates from eastern Europe.

In the first part of this study, the basic in vitro replication and spreading capacity of PRV isolates originating from both populations were compared by one-step growth curves and plaque size assays. In vitro growth kinetics for the different PRV isolates were determined as described before (Klupp et al., 2000). In brief, monolayers of continuous swine testicular (ST) cells or primary porcine cells established from kidney, lung, skin and testicular tissue from 7-14-day-old piglets were inoculated at an m.o.i. of 10. After 1 h incubation at 4 °C the inoculum was removed and the temperature was shifted to 37 °C to allow viral entry. One hour post-attachment (p.a.), extracellular virus was inactivated by citric acid buffer treatment for 2 min. At 1, 4, 8, 12, 24 and 48 h p.a., cells and supernatant were collected and titrated on ST cells. The detection limit of this assay was 10^2 TCID_{50} ml^{-1}. To analyse the capacity of the different PRV strains to spread to neighbouring cells, ST cells were inoculated with 1000 TCID_{50} of the different PRV isolates per well (resulting in approximately 50 plaques per well). The inoculum was washed away after 1 h and replaced by medium containing 1 % methylcellulose. At 36 h post-infection (p.i.), cells were fixed with methanol and plaques were visualized using a PRV-specific fluorescein-conjugated antiserum. For each isolate, the mean plaque diameter of 15 plaques was determined using ImageJ. Both experiments were performed in triplicate and statistical analysis of data was carried out using SPSS software. Viral titres and plaque sizes were compared between all individual strains by one-way ANOVA and Bonferroni post-hoc tests. Student t-tests were used to analyse whether an overall difference in virus replication and plaque size formation existed between domestic swine and wild boar strains. For this purpose, the mean values of the three independent replicates of each strain were used. P values < 0.05 were considered to be significant.

The performance of both the one-step growth curve assay and the plaque reduction assay were first validated during preliminary experiments with PRV-NIA3 WT strain, isogenic PRV NIA3-US3 null strain and the rescue strain. PRV NIA3-US3 null is known to be associated with limited but significantly reduced growth kinetics and plaque formation (Klopfeisch et al., 2006; Van Minnebrugge et al., 2003). A significant greater than tenfold reduction in viral titre at 24 and 48 h p.a. (Fig. 1a) and a significant 20 % reduction of the plaque diameter (Table 1) were observed for the NIA-US3 null strain compared with the rescue strain and the NIA3 strain, indicating that these in vitro assays are suitable to detect differences in growth kinetics and plaque formation if present.

When the replication kinetics of the different PRV strains under study were subsequently determined, similar one-step growth curves were observed for the different PRV isolates in all cell lines tested (Fig. 1). Progeny virus was first detected 8 h p.a. and this was followed by a rapid increase of the viral titres, reaching a plateau level at 24 h p.a. On all cell types tested, the one-way ANOVA analysis showed that differences between the mean viral titres of individual strains were present at one or several time points. Two-by-two comparisons, however, only occasionally detected significant differences between individual strains. The domestic swine isolates BEL50, BEL71 and to a lesser extent BEL69 showed a faster production of infectious virus than other strains at some early time points, but this was not consistent for all cell types and time points tested. On ST cells, only the domestic swine isolate BEL50 and the wild boar isolate BEL20075 were significantly different from each other at 48 h p.a., while the final titres of the other strains did not differ more than 0.36(log TCID_{50} ml^{-1}). For the primary porcine cell lines, no significant differences were found between titres of the PRV isolates at 48 h p.a. and they did not differ more than 0.75(log TCID_{50} ml^{-1}) from each other.

Interestingly, when the growth kinetics of wild boar strains were compared with those of domestic swine strains for the different time points, the mean titre for the wild boar isolates was significantly lower than that of the domestic swine isolates on primary lung cells at 24 h p.a. (P = 0.010) and on primary testicular cells at 12 (P = 0.029), 24 (P = 0.011) and 48 (P = 0.034) h p.a., but again, these differences were not consistent for all cell types or time points tested.

Also, no significant differences were observed between the mean plaque diameters induced by the individual PRV isolates, indicating that all tested strains are equally capable of spreading to neighbouring cells in vitro (Table 1). Furthermore, no significant differences were observed when the
Fig. 1. One-step growth kinetics of PRV isolates from wild boar and domestic swine on continuous ST cells and different primary porcine cell lines. One-step growth kinetics were determined for the NIA3 strain, the US3 null mutant and the US3 null rescue strain on continuous ST cells (a) and for PRV strains of both domestic swine and wild boar origin on continuous ST cells (b) and primary porcine cells obtained from kidney (c), lung (d), skin (e) and testicular (f) tissue. Cells were inoculated at an m.o.i. of 10 and at different time points post-attachment, virus titres were determined on ST cells. Mean values of three independent assays (± SE) are shown. The detection limit of this assay was $10^{2.5}$ TCID$_{50}$ ml$^{-1}$ and is indicated by the horizontal line. Values of one-way ANOVAs with $P<0.05$ and results of Bonferroni post-hoc tests performed at the different time points post-attachment are shown. Time points marked with an asterisk indicate that significant differences between strains were found in ANOVA. Further significant differences found by two-by-two comparisons in Bonferroni post-hoc tests are indicated by a number, and the following strains were involved: (I) NIA3, NIA3 null rescue versus NIA3-US3 null; (II) BEL 20070, NIA3 versus BEL2, BEL60 and BEL71 versus BEL60; (III) BEL50 versus BEL60; (IV) BEL50 versus BEL20075; (V) BEL50 versus NIA3; (VI) BEL50 versus BEL20075, LUX20484, BEL60, BEL69; (VII) NIA3, BEL50, BEL71 versus BEL24043.
Table 1. Overview of the different PRV strains used, and plaque diameters obtained on ST cells

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Host species</th>
<th>Year</th>
<th>Country</th>
<th>GenBank accession no.</th>
<th>Plaque diameter (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEL20070</td>
<td>Hunting dog</td>
<td>2007</td>
<td>Belgium</td>
<td>KF779457</td>
<td>110 ± 7.8</td>
</tr>
<tr>
<td>BEL20075</td>
<td>Hunting dog</td>
<td>2007</td>
<td>Belgium</td>
<td>KF779458</td>
<td>90 ± 8.6</td>
</tr>
<tr>
<td>LUX20484</td>
<td>Hunting dog</td>
<td>2010</td>
<td>Luxembourg</td>
<td>KF779459</td>
<td>91 ± 1.7</td>
</tr>
<tr>
<td>BEL24043</td>
<td>Wolf</td>
<td>2011</td>
<td>Belgium</td>
<td>KF415193</td>
<td>94 ± 7.9</td>
</tr>
<tr>
<td>BEL50</td>
<td>Domestic swine</td>
<td>1973</td>
<td>Belgium</td>
<td>KF779461</td>
<td>97 ± 2.3</td>
</tr>
<tr>
<td>BEL2</td>
<td>Domestic swine</td>
<td>1988</td>
<td>Belgium</td>
<td>KF779460</td>
<td>95 ± 1.1</td>
</tr>
<tr>
<td>BEL60</td>
<td>Domestic swine</td>
<td>1988</td>
<td>Belgium</td>
<td>KF779463</td>
<td>100 ± 4.1</td>
</tr>
<tr>
<td>BEL69</td>
<td>Domestic swine</td>
<td>1989</td>
<td>Belgium</td>
<td>KF779467</td>
<td>98 ± 2.4</td>
</tr>
<tr>
<td>BEL71</td>
<td>Domestic swine</td>
<td>1989</td>
<td>Belgium</td>
<td>KF779468</td>
<td>107 ± 6.5</td>
</tr>
<tr>
<td>NIA3</td>
<td>Domestic swine</td>
<td>1971</td>
<td>Northern Ireland</td>
<td>KF779469</td>
<td>100 ± 3.8</td>
</tr>
<tr>
<td>US3 null NIA3 rescue†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>103 ± 2.2</td>
</tr>
<tr>
<td>US3 null NIA3†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>80 ± 1.5</td>
</tr>
</tbody>
</table>

*Plaque diameter obtained after inoculation of the NIA3 strain, the US3 null mutant and the US3 null rescue strain or PRV strains originating from domestic swine and wild boar on continuous ST cells at low m.o.i. The percentage of plaque diameter compared with the NIA3 reference strain (mean ± se) is indicated.

†Strains described in Van Minnebruggen et al. (2003).

Overall plaque size of wild boar strains was compared with those of domestic swine strains.

The results described above indicate that the intrinsic replication and dissemination capacities of wild boar strains are not affected and that this probably does not account for the differences in in vivo virulence between strains from the two populations. Another possible virulence determinant that can be assessed in vitro is that of evasion mechanisms to counteract the IFN response. IFNs are known to limit peripheral replication of PRV in vivo as well as in vitro replication (Yao et al., 2007), and are also proposed to be involved in the induction or maintenance of PRV latency in trigeminal ganglia (De Regge et al., 2010; Van Opdenbosch et al., 2011). Several herpesviruses, including PRV, have evolved evasion mechanisms to counteract the IFN response (Brukman & Enquist, 2006a, b) and differences between PRV strains in these mechanisms may result in a different sensitivity to the antiviral effects of IFNs.

Therefore, in the second part of this study, the sensitivity of strains from both populations to antiviral effects induced by IFNs was evaluated by plaque reduction experiments. Since only limited differences were observed between the replication kinetics and the plaque diameter of the five domestic swine PRV strains, only three of them (BEL50, BEL60, BEL71) were retained for further experiments analysing the sensitivity of these strains to IFN-induced antiviral effects by plaque reduction experiments.

For each isolate, three wells with continuous ST cells or primary porcine cells were pretreated for 24 h with either IFNz (PBL InterferonSource), IFNγ (R&D systems, USA), or a combination of both IFNs, and three wells with non-treated cells were used as control. IFNz was used at a concentration of 50 ng ml⁻¹, corresponding to approximately 1500 U ml⁻¹, for continuous ST cells, primary kidney and lung cells, and at a concentration of 5 ng ml⁻¹, corresponding to approximately 150 U ml⁻¹, for primary skin and testicular cells since a cytotoxic effect was observed in these tissues at higher concentrations. After pretreatment, 1000 TCID₅₀ of the different strains was added per well (resulting in approximately 50 plaques per well) and after 1 h the inoculum was removed by washing twice with PBS. Fresh medium supplemented with the respective IFNs was added to the cells. Cells were fixed with methanol at 24 h.p.i. and immunofluorescence staining using a PRV-specific fluorescein-conjugated antiserum was performed to visualize plaques. The number of plaques in each well was counted and plaque reduction was determined as the percentage reduction of the number of plaques in the treated wells compared with the untreated wells. Mean percentages of plaque reduction and se of three independent experiments were calculated. Percentage plaque reduction was compared between all individual strains by ANOVA and Bonferroni post-hoc test. Student t-tests were used to analyse whether an overall difference in plaque reduction exists between domestic swine and wild boar strains. For this purpose, the mean values of the three independent replicates of each strain were used. P values <0.05 were considered to be significant.

IFN treatment of ST cells resulted in a reduction of plaque formation compared with non-treated cells for all PRV strains tested (Fig. 2a). Treatment of the cells with IFNz led to a plaque reduction between 67.95 and 85.76 % depending on the strain, but differences between these strains were not significant. At the concentrations used, IFNγ treatment was less capable of reducing plaque...
**Fig. 2.** Plaque reduction of PRV isolates from wild boar and domestic swine after IFN treatment of continuous ST cells and different primary porcine cell lines. The sensitivity of PRV strains of both domestic swine and wild boar origin to IFNs was compared by plaque reduction experiments on continuous ST cells (a) and primary porcine cells obtained from kidney (b), lung (c), skin (d) and testicular (e) tissue. Cells were pretreated for 24 h with either IFNα (100 U ml⁻¹), IFNγ (50 ng ml⁻¹ for ST and for kidney and lung primary porcine cells and 5 ng ml⁻¹ for skin and testicular porcine cells) or a combination of both IFNs. Afterwards, cells were inoculated with the different strains to obtain approximately 50 plaques per well. Mean percentage plaque reductions of three independent experiments (± se) are shown. Values of one-way ANOVA analyses for the different IFN treatments are shown for each cell line. If ANOVA indicated significant differences (P<0.05), Bonferroni post-hoc tests were performed, and significant differences in these two-by-two comparisons are indicated by an asterisk [NIA3 and BEL60 in (a)].
formation than IFNα, and led to a reduction of between 6.20 and 51.51%. Except for the difference in plaque reduction between the NIA3 reference strain and the domestic swine isolate BEL60, no significant differences were, however, observed in the post-hoc tests. No synergism between IFNα and IFNγ treatment was found compared with IFNα treatment alone. Furthermore, none of the treatments resulted in significant differences in overall mean plaque reduction between wild boar strains and domestic swine strains.

The plaque reduction experiments on primary porcine cells obtained from kidney, lung, skin and testicular tissue revealed comparable results (Fig. 2b–e). On all primary porcine cells tested, the high IFNγ concentrations were less potent than IFNα in inhibiting plaque formation, and combined treatment with both IFNs resulted in plaque reductions that were slightly, but not significantly, higher than those obtained for IFNα treatment alone. Although the one-way ANOVA analysis indicated significant differences between strains for the combined treatment on primary porcine kidney cells, and for both IFNα and IFNγ treatment on primary testicular cells, no significant differences in mean plaque reduction between strains were detected using the Bonferroni post-hoc tests. Furthermore, when the overall sensitivity of wild boar strains was compared with that of domestic swine strains, the mean plaque reduction after IFNγ treatment was significantly lower (P=0.006) for the wild boar isolates than for the domestic swine isolates on primary testicular cells. This was, however, not the case on the other cell types tested. Since these results did not reveal consistent differences between strains in their capacity to counteract the plaque-reducing effect of IFNs, differences in capacity to counteract the IFN response can probably not explain the attenuated nature of wild boar strains in vivo either.

Based on the overall results of this study, no differences in intrinsic viral replication, spread to neighbouring cells, and sensitivity to the IFN-induced antiviral response were found. Therefore, these basic in vitro assays seem unsuitable as a screening tool to evaluate the in vivo virulence of PRV strains and to help in the prediction of possible consequences of a reintroduction of a wild boar strain into the domestic swine population. Further studies examining other processes like PRV crossing of the basement membrane, virus spread to the blood vessels and trigeminal nerves, and efficiency of replication and spread in the nervous system may possibly reveal underlying determinants of the in vivo virulence of strains from both populations. Some in vitro models to study the interactions of herpesviruses with the nasal mucosa (Glorieux et al., 2009; Vandekerckhove et al., 2010) and replication and spread in trigeminal ganglion neurons (De Regge et al., 2006) have been developed and could be useful for such studies. These models are, however, expensive, labour intensive and time demanding and are not suitable to routinely screen the virulence of a high number of strains.

Acknowledgements

We thank Professor H. Nauwynck from Ghent University for the kind gift of the continuous ST cell line. This study was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment (RF11/6249) and the Federal Science Policy, BELSPO (BR/132/PI/melatin-PRV).

References


