Age- and strain-dependent differences in the outcome of experimental infections of domestic pigs with wild boar pseudorabies virus isolates

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Although pseudorabies virus (PRV) has been eradicated in domestic swine in many countries, its presence in wild boars remains a threat for a reintroduction into the currently unprotected swine population. To assess the possible impact of such a reintroduction in a naive herd, an in vivo infection study using two genetically characterized wild boar PRV isolates (BEL24043 and BEL20075) representative for wild boar strains circulating in south-western and central Europe and the virulent NIA3 reference strain was performed in 2- and 15-week-old domestic pigs. Our study revealed an attenuated nature of both wild boar strains in 15-week-old pigs. In contrast, it showed the capacity of strain BEL24043 to induce severe clinical symptoms and mortality in young piglets, thereby confirming that the known age dependency of disease outcome after PRV infection also holds for wild boar isolates. Despite the absence of clinical disease in 15-week-old sows, both wild boar PRV strains were able to induce seroconversion, but to a different extent. Importantly, differences in infection and transmission capacity of both strains were observed in 15-week-old sows. Strain BEL24043 induced a more prolonged and disseminated infection than strain BEL20075 and was able to spread efficiently to contact animals, indicative of its capacity to induce a sustained infection. In conclusion, it was shown that a reintroduction of a wild boar isolate into the domestic swine population could have serious economic consequences due to the induction of clinical symptoms in piglets and by jeopardizing the PRV-negative status.

INTRODUCTION

Pseudorabies virus (PRV; also known as Aujeszky’s disease virus or Suid herpesvirus 1), is the causative agent of Aujeszky’s disease. The virus belongs to the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus. Infection in its natural host, members of the family Suidae, is characterized by respiratory, reproductive and neurological symptoms. The severity of the symptoms depends on the virulence of the strain and the age of the pig. Morbidity and mortality associated with PRV infection are higher in younger pigs, and are typically associated with symptoms of the central nervous system, whereas older swine mostly exhibit symptoms of respiratory and reproductive disease (Pomeranz et al., 2005). As a result of the high economic impact of the virus, large-scale vaccination programmes were set up which led to the eradication of the virus in domestic swine in several European countries, including Belgium, which has been officially PRV-free since 2011 (Decision 2011/648/EU).

Seroprevalence studies have, however, shown that the virus is still circulating within the wild boar population (Müller et al., 2011) which could pose a risk for a reintroduction of the virus into the currently unprotected domestic swine populations. Despite the fact that epidemiological studies suggest that such reintroductions do not occur frequently as PRV infection in domestic pigs and wild boar represent distinct infection cycles (Capua et al., 1997a, b; Müller et al., 1998; Pannwitz et al., 2012), some recent reintroductions in France and the USA have been attributed to PRV circulating in wild boars (ProMED archive number 20100923.3442) (Hahn et al., 2010).
Only limited information on the virulence of wild boar PRV isolates is available from two experimental infection studies and some field observations (Hahn et al., 1997; Müller et al., 2001). These studies suggest an attenuated nature for PRV isolates of wild boar origin in adult pigs, but this has not yet been confirmed for young piglets. The existence of an important genetic diversity among European wild boar strains has been shown and therefore care should be taken when extrapolating these results to all wild boar isolates. Phylogenetic studies using a part of the glycoprotein C (gC) fragment have shown that PRV isolates from wild boar cluster in two clades, A and B. Clade A is a heterogeneous group consisting of wild boar isolates from eastern Europe, but also all known domestic swine isolates are located within this clade. Clade B consists of strains originating from south-western Europe and is further subdivided into two genetic variants differing in only one amino acid in the gC fragment (Müller et al., 2010; Verpoest et al., 2014b).

As there is a growing concern for a reintroduction of the virus into the currently unprotected swine population, we performed an in vivo infection study in domestic pigs with isolates of wild boar origin to evaluate the possible impact of such a reintroduction in a naive herd. In a first experiment, 15-week-old domestic pigs were either inoculated with one of two different wild boar PRV isolates or the PRV-NIA3 reference strain to assess the virulence and the transmission capacity of these wild boar isolates in domestic swine. The wild boar isolates were genetically characterized in detail previously. They represent the two genetic variants of clade B and are therefore likely representative for wild boar strains circulating within south-western and central Europe. In a second experiment, 2-week-old piglets were inoculated with one wild boar PRV isolate or the NIA3 strain to evaluate if morbidity and mortality were also age dependent, as has been observed for domestic swine isolates.

**RESULTS**

**PRV infection of 15-week-old sows**

**Clinical symptoms.** Inoculation of 15-week-old pigs with the NIA3 reference strain quickly led to severe respiratory and neurological disease. Starting from 2 days post-inoculation (p.i.), a decrease in the general condition of the sows was observed, associated with a rise of rectal body temperature during acute infection (≥41 °C in all pigs), and reduced appetite and mobility. At 4 days p.i., the first neurological symptoms were observed. One pig exhibited excessive salivation, and another was trembling and showed paresis of the hind limbs. Over the next days, respiratory symptoms developed in all pigs and the neurological symptoms worsened. Two pigs had to be euthanized at 5 days p.i. and two pigs at 7 days p.i. as they developed the typical neurological symptoms associated with Aujeszky’s disease, such as scratching, lack of coordination, circling, seizures and paralysis. The remaining two animals were euthanized at 14 days p.i. as a result of persistent respiratory symptoms.

In contrast, no clinical symptoms were observed when pigs of the same age were inoculated with a 10-fold higher dose of the wild boar isolates BEL24043 or BEL20075. The general condition of the animals, food consumption, mobility and rectal body temperature remained apparently unaffected throughout the experiment. The contact animals added to the BEL24043 and BEL20075 infection groups also remained unaffected during the entire experiment.

**Viraemia.** Serum samples of all 15-week-old sows inoculated with the different PRV strains and of all the contact pigs were tested for the presence of viral DNA by quantitative (q) PCR at several time points after inoculation (Fig. 1a). All NIA3 and most BEL24043 (5/6) infected pigs became viraemic for at least 1 day between 3 and 7 days p.i. Importantly, two of the contact pigs present in the pen with the BEL24043-infected sows also developed viraemia, indicating that virus transmission had occurred. In contrast, the number of pigs that became viraemic in the BEL20075 group was clearly lower as only one serum sample of the inoculated and none of the contact animals became positive. This was, however, not significantly different when compared with the BEL24043 group (Fisher’s exact test: P = 0.080 for inoculated animals and P = 0.455 for contact animals). Virus concentrations found in the positive serum samples were low but comparable for all strains tested with high cycle threshold (Ct) values between 35 and 40 (data not shown). However, the BEL24043-inoculated pigs had a significantly (P = 0.044) longer viraemia (1.2 days) compared with the BEL20075 infection group (0.2 days). However, this was not the case when the mean viraemia was compared for the contact animals of both groups (P = 0.145).

**Nasal and vaginal virus shedding.** qPCR results indicated that 15-week-old pigs already started to shed the NIA3 strain at the nasal and vaginal mucosa at 1 and 2 days p.i., respectively, and virus was shed until the day of euthanasia (Fig. 1b, c).

Infection with the wild boar isolate BEL24043 resulted in a prolonged nasal and vaginal virus excretion starting from 2 and 3 days p.i., respectively, and lasted until 17 days p.i. in some animals. Importantly, nasal and genital shedding by all contact animals in this pen evidenced efficient virus transmission. Contact animals were already found positive in nasal and genital swabs 2 days after introduction in the pen. Interestingly, the first positive vaginal swabs for both the inoculated and the contact sows were found at 3 days p.i. These positive vaginal swabs in contact animals were likely the result of nose-to-vagina contact with pigs that nasally shed the virus. This is supported by the fact that the initial Ct values found in vaginal swabs of the contact
animals were very high (data not shown) and only decreased later on.

A completely different shedding pattern was observed in pigs inoculated with the wild boar isolate BEL20075. Only two nasal swabs from two different pigs were found to be positive for viral DNA by qPCR. Sequencing of the gC fragment present in those samples confirmed that the positive PCR signal resulted from infection with the BEL20075 strain and excluded the possibility that they resulted from contamination or transmission of the NIA3 or BEL24043 strain. Although, the number of inoculated animals that shed the virus nasally in the BEL20075 group was lower compared with the BEL24043 group, this difference was again not statistically significant ($P=0.061$) in Fisher’s exact tests. The period of nasal shedding was, however, significantly longer for both the inoculated and contact animals of the BEL24043 group (mean nasal shedding period of 10.7 and 11.8 days, respectively) compared with the BEL20075 group (mean nasal shedding period of 0.3 and 0 days, respectively) ($P<0.001$ for both the infected and contact animals).

All genital swabs from the BEL20075 infection group remained negative, and no evidence for virus transmission to contact pigs was found for this virus strain based on qPCR analysis of both nasal and genital swabs.

Antibody response: PRV-specific antibodies. The development of PRV-specific antibodies was followed up over time by ELISA and virus neutralization tests (VNT) (Fig. 2). In 15-week-old pigs, PRV-specific antibodies were detected by ELISA in all remaining NIA3-infected pigs at 7 days p.i., whilst VNT results only became

Fig. 1. qPCR analysis of (a) serum, (b) nasal and (c) genital swabs after inoculation of 15-week-old sows with different PRV strains. Six 15-week-old sows were intranasally inoculated with different PRV isolates: the virulent NIA3 reference strain, the wild boar isolate BEL24043 and the wild boar isolate BEL20075. At 1 day p.i., six susceptible contact sows were added to the pens with animals inoculated with the wild boar strains. The number of surviving pigs over time is indicated by the grey line, and the number of pigs that tested positive by qPCR in serum, nasal and genital swabs over time is indicated for every infection group (black bars) as well as their contact animals (grey bars).
positive at 10 days p.i. In the BEL24043-infected group, antibodies were first detected at 10 days p.i. both by ELISA and VNT. An antibody response was also detected in the contact pigs of the BEL24043-infected group starting from 14 and 17 days p.i. by the use of ELISA and VNT, respectively. In contrast, no antibodies were detected by VNT in the serum samples of animals inoculated with the wild boar isolate BEL20075 nor in their contact animals. However, ELISA results revealed the presence of PRV-specific antibodies in three BEL20075-infected pigs and in one of their contact animals from 10 and 21 days p.i., respectively. This resulted in a significant higher number of VNT-positive animals in the BEL24043-infected and contact animals compared with the BEL20075 group (P = 0.002 and 0.002, respectively). When the presence of antibodies was compared by ELISA, however, this difference was only significant for the contact animals (P = 0.015), but not for the infected animals (P = 0.182).

**Virus detection in organs collected at euthanasia by qPCR.** Important differences were found in the distribution of the strains over the different organs at the time of euthanasia. It should, however, be kept in mind that NIA3-infected animals were euthanized during acute infection, whilst pigs infected with the wild boar strains were euthanized at 28 days p.i. For NIA3-inoculated pigs, tonsil and brain samples of all pigs, and lung, kidney, liver and spleen of some pigs, tested positive. For the BEL24043-inoculated pigs, tonsils of all animals, and spleen and brainstem of one and two animals, respectively, were still positive at 28 days p.i. Transmission of the BEL24043 strain was evidenced by the detection of viral DNA in tonsils of five out of six contact animals. In contrast, none of the organs collected from BEL20075-inoculated pigs or their contact pigs tested positive.

**Transmission ratios for wild boar PRV isolates.** For both wild boar strains, the transmission rate β was calculated based on the results obtained with the different diagnostic tests. The transmission rate β enables the calculation of the reproductive ratio R0, that indicates the mean number of secondary infections due to one infected individual, depending on the infectious period. As time intervals between sampling points were irregular in this study, no exact calculation of the infectious period could be done. Therefore, we calculated the reproductive ratios for hypothetical infectious periods of 1, 5, 10 and 15 days (Table 1).

However, based on the mean period of nasal shedding detected by qPCR, we can estimate the infectious period. For the wild boar isolate BEL24043, our study indicated that nasal shedding lasted for a mean of 10.7 and 11.8 days for the inoculated and contact animals,
Six 15-week-old sows were intranasally inoculated with the wild boar PRV isolates BEL24043 and BEL20075. At 1 day p.i., six susceptible contact sows were added to the pens. The transmission rate $R_0$ (95 % CI) was estimated using a generalized linear mixed model for the different diagnostic tests to detect virus transmission to contact animals. As time intervals between sampling periods were >1 day, the reproductive rate $R_t$ (95 % CI) was simulated for different infectious periods of 1, 5, 10 and 15 days. For the wild boar strain BEL20075 it was impossible to calculate transmission parameters for VNT, and virus detection in serum, nasal and genital swabs, as no transmission of the virus could be observed based on these diagnostic tests.

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>Transmission rate $\beta$ (95 % CI)</th>
<th>Simulated reproductive ratio $R_0$ (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>5 days</td>
</tr>
<tr>
<td>BEL24043</td>
<td>0.94 (0.41–2.19)</td>
<td>0.94 (0.00–2.85)</td>
</tr>
<tr>
<td>VNT</td>
<td>38.55 (0.00–\infty)</td>
<td>38.55 (4.76–72.34)</td>
</tr>
<tr>
<td>ELISA gB</td>
<td>0.31 (0.08–1.23)</td>
<td>0.31 (0.00–2.30)</td>
</tr>
<tr>
<td>Viraemia (qPCR)</td>
<td>3.39 (1.28–8.96)</td>
<td>3.39 (0.00–2.30)</td>
</tr>
<tr>
<td>Nasal swabs (qPCR)</td>
<td>54.59 (0.00–\infty)</td>
<td>54.59 (0.00–2.30)</td>
</tr>
<tr>
<td>Vaginal swabs (qPCR)</td>
<td>5.38 (1.99–14.56)</td>
<td>5.38 (0.34–10.42)</td>
</tr>
<tr>
<td>Overall tests</td>
<td>0.10 (0.01–0.70)</td>
<td>0.10 (0.00–2.39)</td>
</tr>
<tr>
<td>BEL20075</td>
<td>0.08 (0.01–0.54)</td>
<td>0.08 (0.00–2.36)</td>
</tr>
</tbody>
</table>

respectively. Therefore, based on an infectious period of a minimum 10 days, a reproductive ratio $R_0 > 1$ was found for all diagnostic tests used, indicating that introduction of the BEL24043 strain in a domestic swine population would result in a sustained infection (Table 1). However, results were only significantly different from unity for the reproductive ratio $R_0$ based on ELISA and nasal swabs. The statistical analysis shows that the reproductive ratio associated with qPCR results found in genital swabs was not reliable as the first positive swabs in the inoculated and the contact sows were both found at 3 days p.i., as described above. Seeing that the differences in reproductive ratios were dependent on the diagnostic test used, it was decided to also determine an overall reproductive ratio, taking into account all tests. Thereby, an animal was considered positive once it had scored positive in one of the tests. The transmission ratio simulated for an infectious period of 10 days and its 95 % confidence intervals were far above unity, confirming that a reintroduction of this strain would result in a sustained infection.

As no transmission of the wild boar strain BEL20075 to contact animals was observed based on the VNT and qPCR analysis of serum, nasal and genital swabs, no transmission parameters could be calculated based on these tests. Transmission was only detected by ELISA where one of the contact animals became positive. Based on nasal shedding as detected by qPCR, it could be assumed that virus shedding occurs for a maximum of 1 day for both the inoculated and the contact animals. This corresponds to a reproductive ratio $R_0$ of 0.10. This is $< 1$, indicating that a reintroduction of this strain will probably not lead to a sustained infection in the population.

**PRV infection of 2-week-old piglets.** Inoculation of 2-week-old piglets with the NIA3 reference strain led to general depression of all piglets, associated with severe diarrhoea and vomiting. The animals exhibited complete exhaustion and were unable to stand up, so that neurological symptoms could only be observed to a limited extent. All piglets were euthanized at 3 or 4 days p.i. Interestingly, infection with the wild boar isolate BEL24043 also induced respiratory and neurological disease in 2-week-old piglets. The symptoms were, however, less severe than after NIA3 infection and only one piglet had to be euthanized at 6 days p.i. based on ethical grounds.

Four of the six piglets infected with NIA3 were found to be viraemic by qPCR for at least 1 day at 3 and 4 days p.i. (Table 2). After inoculation with the BEL24043 strain, only serum from two piglets tested positive at 7 and 10 days p.i., respectively. The NIA3-infected piglets had to be euthanized before the antibody response developed.
In the BEL24043-infected piglets, neutralizing antibodies were detected from 10 days p.i. by the VNT. Virus excretion occurred both at the nasal and genital mucosa after infection with both strains. Analysis of the organs collected at euthanasia showed that NIA3 had spread to the tonsils, lung and brain of all piglets, and also to the kidney, spleen and liver of most of them. As the general condition of BEL24043-infected piglets improved from 5 days p.i. onwards, we decided to euthanize the remaining piglets at 7 (piglets 2 and 3), 10 (piglet 4), 14 (piglet 5) and 21 (piglet 6) days p.i. to study the spread of the virus to the organs over time. qPCR showed that the BEL24043 strain had spread to the brain of all piglets, but that the spread to the other organs had occurred to a lesser extent than in NIA3-infected piglets. BEL24043 was found in three lungs, two tonsils and one kidney, but not in liver and spleen.

**DISCUSSION**

Based on previous experimental infection studies and field observations, an attenuated nature of wild boar strains has been assumed (Hahn et al., 1997; Müller et al., 2001). Our study partly confirms these results, as no clinical symptoms were observed after inoculation of 15-week-old domestic pigs with two different Belgian wild boar strains using a relatively high inoculation dose. As it is known that mortality and morbidity associated with PRV infection are age dependent, and more severe symptoms are expected in younger piglets, we

**Table 2. Inoculation of 2-week-old piglets with different PRV strains**

Six 2-week-old piglets were intranasally inoculated with the virulent NIA3 reference strain or the wild boar isolate BEL24043. The presence of viral DNA detected by qPCR in serum samples, nasal and genital swabs over time is shown, together with the results of the VNTs. Furthermore, the results of virus detection by qPCR in different organs collected at the moment of euthanasia are indicated.

<table>
<thead>
<tr>
<th>Strain (time/organ)</th>
<th>Piglet 1</th>
<th>Piglet 2</th>
<th>Piglet 3</th>
<th>Piglet 4</th>
<th>Piglet 5</th>
<th>Piglet 6</th>
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<tr>
<td><strong>NIA3</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 0</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 1</td>
<td>N</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 3</td>
<td>B/N/G*</td>
<td>N/G</td>
<td>N/G</td>
<td>N/G</td>
<td>B/N/G</td>
<td>N/G</td>
</tr>
<tr>
<td>Day 4</td>
<td>N*</td>
<td>N*</td>
<td>B/N/G*</td>
<td>B/N*</td>
<td>B/N*</td>
<td>–</td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Liver</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Spleen</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tonsils</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Brainstem</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cerebrum</td>
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<tr>
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<td>Day 3</td>
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<td>N</td>
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<tr>
<td>Day 5</td>
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<td>B/N/G</td>
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<tr>
<td>Day 10</td>
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<td>N/G</td>
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<td>B/G</td>
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<td>Day 14</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Day 17</td>
<td>–</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Lung</td>
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<tr>
<td>Kidney</td>
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<td>–</td>
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<tr>
<td>Liver</td>
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<td>Spleen</td>
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<td>Cerebellum</td>
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<td>Cerebrum</td>
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B, Positive serum sample; N, positive nasal swab; G, positive genital swab; NT, not tested.

*Euthanized on ethical grounds.
†Pig euthanized.
also infected 2-week-old piglets with one of the wild boar PRV strains. This infection induced a fast deterioration of their general condition and severe neurological symptoms. This interesting observation indicates that the known age dependency for symptoms upon infection with PRV strains circulating in domestic pigs also holds true for wild boar PRV isolates. This had been suggested previously by Hahn et al. (1997). Importantly, however, in contrast to an infection with the NIA3 strain, most piglets recovered after some days. In addition to the induction of respiratory and neurological symptoms in young piglets, it cannot be excluded that wild boar PRV isolates might also be able to induce reproductive disorders as described for strains from domestic pigs (Pomeranz et al., 2005), but this was not evaluated in this study.

Apart from the potential direct production losses due to induced clinical disease, it is important to underline the observation that wild boar PRV strains are able to induce seroconversion of domestic pigs, as has also been shown in previous infection studies (Hahn et al., 1997; Müller et al., 2001). This could compromise the Aujeszky’s disease-free state of a country and lead to important economic consequences. Efficient monitoring seems crucial for the early detection of a potential reintroduction as the absence of clinical symptoms after infection with the wild boar PRV strains, as observed in this study, could allow the virus to spread unnoticed within the population. This is further emphasized by the fact that one of our tested isolates was capable of inducing a sustained infection upon introduction into a population of naive domestic pigs. It seems advisable that monitoring should especially focus on farms with free-ranging facilities, associated with an elevated risk of contact between wild boars and domestic pigs.

Our results indicate that qPCR analysis of nasal swabs represents the most efficient method for PRV detection during an acute outbreak, allowing more sensitive virus detection over a longer time period than PCR analysis in serum samples. This, in combination with the serological screening of serum samples by ELISA, seems to be the most efficient way to rapidly detect a reintroduction of a wild boar strain in the domestic population. It had already been shown that ELISA is more sensitive than the VNT to detect PRV-specific antibodies (Müller et al., 2001) and this was further confirmed in this study. However, individual sampling of animals remains labour intensive and the use of group sampling methods, such as saliva collection based on ropes, for the detection of both virus and antibodies could provide an easier and cheaper method for surveillance and screening against PRV, but still needs to be validated for this virus.

An important difference in infection and transmission capacity between both wild boar strains was observed. BEL24043 caused a more prolonged and disseminated infection in inoculated animals and spread more efficiently to contact pigs than the BEL20075 strain. This difference in virulence was somewhat surprising as both strains belong to clade B in the PRV phylogeny, and are supposed to be representative for strains circulating within south-western and central Europe (Verpoest et al., 2014b). The reason for these differences remains unknown and future sequencing studies will be required to reveal if genomic differences in regions outside the gC region exist that could provide an explanation for the reduced virulence of the BEL20075 strain. Furthermore, if cannot be excluded that the less efficient infection observed for the BEL20075 strain could be due to the choice of an intranasal infection route as it has been suggested that transmission of feral isolates can also occur through the venereal route (Romero et al., 1997, 2001), as opposed to domestic swine strains where transmission usually occurs by the oropharyngeal route after direct contact of infected and susceptible animals, and the ingestion or aspiration of infected aerosols, secretions and excretions (Pomeranz et al., 2005). Our results with the BEL24043 strain, however, clearly show that transmission of wild boar PRV isolates between domestic pigs can occur efficiently via the oropharyngeal and respiratory route. Interestingly, despite the apparent differences in in vivo virulence between both wild boar isolates, no differences were found in their in vitro growth and plaque-forming characteristics, and their sensitivity to type I and II IFNs (Verpoest et al., 2015).

The observed differences in virulence between both wild boar isolates belonging to clade B suggest that it might also be interesting to evaluate the virulence of strains belonging to clade A (Verpoest et al., 2014b) as those are genetically more diverse and cluster together with strains originating from domestic swine.

In conclusion, to the best of our knowledge this is the first study to show the attenuated nature of two genetically characterized isolates representative for strains circulating in south-western and central Europe in adult domestic pigs. Despite the absence of clinical symptoms upon inoculation, clear differences in virulence were observed between both strains. One strain was capable of inducing seroconversion and was efficiently transmitted to contact animals. Furthermore, the same isolate was capable of inducing severe clinical symptoms upon inoculation in 2-week-old piglets, thereby confirming that the known age dependency on the outcome of infection with PRV isolates from domestic pigs also holds true for wild boar strains. This had not been shown before for strains circulating in this population. Despite the fact that reports of PRV transmission from wild boars to the domestic population are rare, the current report thus indicates that a potential reintroduction could have serious economic consequences.

**METHODS**

**Animals and viruses.** The 15-week-old (n=36) and 2-week-old (n=18) Belgian Landrace sows were purchased from a commercial swine herd. The sows were in good condition and tested negative in serology for PRV and classical swine fever at the beginning of the experiment. The animals were housed in Biosafety Level 3 facilities on slatted floors (CODA-CERVA, Machelen, Belgium). Water was available ad libitum and pigs were fed once each day. The animals were randomly assigned to the different infection groups. Animal experiments were performed in accordance with the European Union and Belgian regulations on animal welfare in experimentation. The

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protocol was approved by the joined ethical committee of CODA-
CERVA and the Scientific Institute of Public Health Belgium (pro-
cedure agreement 121017-02).

Two Belgian PRV isolates from wild boar origin and the virulent PRV
NIA3 reference strain were used. The wild boar isolates BEL24043 and
BEL20075 were obtained from infected brain tissue of a wolf and a
hunting dog, respectively, that had been fed with offal of asympto-
matic wild boars shot during hunting. The wild boar strains have been
genetically characterized previously (Verpoest et al., 2014b). BEL24043 and BEL20075 belong to subgroup 1 and 2 of clade B
strains, respectively. A second passage of the wild boar strains
BEL24043 and BEL20075 and a third passage of the NIA3 strain were
used for inoculations.

Experimental design. As the outcome of a PRV infection largely
depends on the age of the pigs and the virulence of the strains,
experiments were conducted in sows of two age categories. In the first
experiment, six 15-week-old sows were either mock infected with PBS
or intranasally inoculated (1 ml per nostril) with the virulent NIA3
reference strain or with the wild boar isolates BEL24043 or BEL20075.
Due to the high costs of experimental infections in Biosafety Level 3
facilities only a limited number of groups and conditions could be
tested. Therefore, wild boar isolates were inoculated to a relatively
high dose of 10^5 TCID_{50} per animal to avoid the possibility that
potential negative results would be due to an inoculation dose that
was too low. For the NIA3 strain that was used as a positive control,
this high dose was expected to result in premature lethal infection
even in adult pigs (McFerran et al., 1979) and therefore a lower dose of
10^4 TCID_{50} per animal was used. At 24 h.p.i., six susceptible
contact pigs that had been kept apart were introduced into the pens
containing the pigs inoculated with the wild boar strains. No contact
animals were included in the control and NIA3-infected group. The
sows were monitored daily for clinical symptoms and rectal body
temperature. Blood and nasal and genital swabs were collected at
–4, 0, 1, 2, 3, 5, 7, 10, 14, 17, and 28 days p.i. Animals were euthanized and organs collected at the end of the experiment at
28 days p.i. or at intermediate time points when mandatory on ethical
grounds.

In the second experiment, six female 2-week-old piglets were intra-
 nasally inoculated (0.5 ml per nostril) with a final dose of 10^3 TCID_{50}
per animal of either the NIA3 reference strain or with the wild boar
isolate BEL24043. Six piglets were mock-inoculated with PBS and
held as a negative control group. Piglets were monitored daily for
clinical symptoms and rectal body temperature. Blood and nasal and
genital swabs were collected at 0, 1, 3, 5, 7, 10, 14, 17 and 21 days p.i.
Animals were euthanized and organs collected at the end of the experiment at 21 days p.i. or at intermediate time points when
mandatory on ethical grounds.

Blood was collected in serum tubes, and after centrifugation for
15 min at 2 000 x g at 37 °C before testing. Afterwards, a two times dilution series (1/2 to 1/256) was made in duplicate in MEM for each serum sample in
microtitre plates. Then, 50 μl PRV dilution containing 30–300
TCID_{50} as verified by back-titration of the virus dilution, was added to
the serum dilution in microtitre plates and incubated for 1 h at

37 °C. Thereafter, 100 μl PK15 cell suspension in MEM, sup-
plemented with 10 % FCS, penicillin (1000 U ml⁻¹), gentamicin
(50 μg ml⁻¹) and fungizone (250 ng ml⁻¹), was added to each well
and the microtitre plates were incubated for 4 days at 37 °C. Plates
were then examined under a light microscope for the presence of
plaques and the neutralizing titre of each serum sample was defined as
the highest serial dilution that was capable of completely neutralizing
the virus. For each VNT, a negative, weak positive and strong positive
reference serum was included, and VNTs were only validated if these
samples showed the expected values.

ELISA. Serum samples were tested by an ELISA detecting antibodies
directed against the gB glycoprotein (PrioCHECK PRV gB Antibody
ELISA kit; Prionics) following the manufacturer’s instructions. For
each sample, the sample/positive (S/P) percentage was calculated. Test
results were considered negative if S/P < 50 % and positive if S/P > 50 %.

qPCR. Genomic DNA was extracted from the collected organs, serum
samples, and vaginal and nasal swabs using a QIAamp DNA kit
(Qiagen). The spin protocol ‘DNA Purification from Blood or Body
Fluids’ was used to isolate DNA from serum samples, and nasal and
vaginal swabs. For DNA extraction from all collected organs, except
the tonsils, ~0.5 cm^2 tissue was homogenized in 1 ml PBS by adding
10–15 silicon carbide beads of 1 mm (Biospec Products) and high-
speed shaking (4 min, 25 Hz) in a TissueLyser (Qiagen). After homo-
ogenization of tonsil samples, ~0.5 cm^2 tissue was homogenized in
1 ml PBS by adding two stainless steel beads of 5 mm (Qiagen) and
high-speed shaking (10 min, 30 Hz) in a TissueLyser. After hom-
ogenization of the collected tissues the spin protocol ‘DNA Purifi-
cation from Tissues’ was used to isolate DNA. The collected DNA was
then tested for the presence of PRV by a qPCR detecting a fragment of
the gB (gB-Forw: 5'-ACCCGCCGCTACTTATTAG-3'; gB-Rev:
5'-CTCTCAAGCAGGTCAGGT-3'; gB-probe: FAM-5'-ACGCAG-
GCAGGTTGAC-3'; TAMRA) gene. Amplification was done using a FastStart
TaqMan Probe Master kit (Roche) and reactions were run on a LightCycler 480 (Roche) under the following con-
ditions: initial denaturation at 95 °C for 10 min, followed by 45 cycles
of 15 s at 95 °C and 45 s at 60 °C, and a final step of 30 s at 40 °C
(Verpoest et al., 2014a).

Sequencing. A part of the gC gene was sequenced following the
protocol described previously (Verpoest et al., 2014b). Viral DNA was
amplified with DyNazyme Ext DNA polymerase (Thermo Fisher
Scientific) using previously published primers (Hahn et al., 2010;
Müller et al., 2010). Approximately 5 ng PCR product was used as
template for sequencing using the PCR primers described above using
a BigDye Terminator Sequencing kit (Applied Biosystems). Sequen-
cing reactions were purified with BigDye Xterminator reagent and run
on a 3130 Genetic Analyzer (Applied Biosystems). The sequences
obtained from positive serum and swab samples were compared with
the previously obtained sequences for the wild boar strains
BEL24043 and BEL20075 (GenBank accession numbers KF415193
and KP779458).

Statistical analysis. Fisher’s exact test was used to analyse whether
statistically significant differences existed between the number of
animals in the BEL24043 and BEL20075 group that were found to be
positive in the different diagnostic tests. Student’s t-test was used to
analyse whether differences in the period of viraemia and virus
shedding existed between animals infected with the different isolates.
This was done for both inoculated and contact animals. These tests
were carried out using SPSS software (IBM, Armonk, NY, USA).
P < 0.05 was considered to be significant.

Furthermore, a generalized linear mixed model was developed in SAS
version 9.2 (SAS, Cary, NC, USA) to estimate the transmission rate β

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for each of the diagnostic tests and strains used. The advantage of this longitudinal model in comparison with other methods used for the estimation of transmission parameters, such as the final size model or the transitional state model, is that this longitudinal model accounts for the time intervals between observations (Van der Goot et al., 2003; Velthuis et al., 2007). The parameter log β was estimated by modelling the number of new infections upon contact, using the offset function \( \ln(\Delta t/N) \) and complementary log-log function. A back transformation of log β was required to obtain β. As the time intervals between sampling were >1 day, we were unable to determine the exact infectious period, and therefore infectious periods of 1, 5, 10, and 15 days were simulated. This enabled the calculation of the transmission ratio \( R_0 \), indicating the mean number of secondary infections due to one infected individual, for the different infectious time periods (γ) given the strain and diagnostic test used based on the standard Susceptible–Infectious–Recovered model and using the following formula: \( R_0 = \beta \gamma \). The 95% confidence intervals (CIs) were obtained for the parameter log β. The goodness of fit of the model was assessed with Akaike’s Information Criterion. The 95% CIs of the parameter \( R_0 \) were calculated based on the following, taking into account the variance (Var) and the estimates (E) of each parameter: 

\[
\text{Var}(\beta^\gamma) = E(\beta)^2 \text{Var}(\gamma) + E(\gamma)^2 \text{Var}(\beta).
\]

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