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# Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus

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## Abstract

The use of a live attenuated porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in piglets has been associated with reproductive disorders in non-vaccinated sows. Vaccine-derived virus (VDV) has been isolated from fetuses, stillborn pigs, and dead piglets, indicating that the live vaccine spread from vaccinated piglets to non-vaccinated sows, and that the virus might be implicated in the severe reproductive problems observed. In the present study, one such VDV isolate was used to experimentally infect pregnant sows in the last trimester. The chosen isolate, which had more than 99.6% identity to the attenuated vaccine virus, originated from the lungs of a stillborn pig from a swine herd with a sudden high level of stillborn pigs and increased piglet mortality in the nursing period. Intranasal inoculation of sows with the virus isolate resulted in congenital infection, foetal death, and preweaning pig mortality. As such, the present study showed that vaccine-derived PRRSV can cause disease in swine consistent with PRRS. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Pig-viruses; Porcine reproductive and respiratory syndrome virus (PRRSV); Attenuated vaccine; Field isolate; Experimental infection

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## 1. Introduction

The first case of porcine reproductive and respiratory syndrome (PRRS) in Denmark was diagnosed in 1992 (Bøtner et al., 1994). Subsequently, the infection spread slowly among Danish swine herds, and in 1996 the herd prevalence was about 30% (Mortensen and Strandbygaard, 1996). From mid-October 1996, there was a sudden increase in the number

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of samples (foetuses, weakborn and stillborn piglets) submitted to the Danish Veterinary Institute for Virus Research (DVIVR) for PRRS virus (PRRSV) examination. The samples originated from herds with acute PRRS-like clinical signs, characterised by increased numbers of late term abortions and stillborn piglets, and increased mortality in the nursing period (Bøtner et al., 1997). In the vast majority of these herds, the appearance of the clinical problems coincided with recent vaccination using the attenuated live “Ingelvac<sup>®</sup> PRRS MLV” vaccine (Boehringer Ingelheim Animal Health, MO, USA) which is based on the pathogenic American PRRSV field isolate, VR-2332 (Chladek et al., 1995).

PRRSV, identified as vaccine-derived by the use of monoclonal antibodies, RT-PCR, and nucleotide sequencing was isolated from dead or weakborn piglets from more than 100 herds within a period of 8 months (Bøtner et al., 1997). This was the first time that American type PRRSV had been isolated from clinical cases in Denmark, and this was epidemiologically closely linked to the use of the live vaccine (Bøtner, 1998a; Mortensen, 2000). Furthermore, during the period from November 1996 to the end of December 1997, a total of 350 previously PRRSV-seronegative herds became infected with the vaccine virus, judged by serological examination (Sørensen et al., 1998), even though they had never used the vaccine. Many of these herds also experienced acute PRRS-like symptoms (Bøtner et al., 1997), and American type PRRSV was isolated from foetuses/stillborn piglets from more than 50 of these herds (Bøtner, 1998a,b).

Altogether, these observations showed that the live vaccine virus had an untoward tendency to spread, not only within the vaccinated herds but also to neighbouring non-vaccinating herds. During that spread among pigs, the vaccine virus reverted genetically (Madsen et al., 1998; Storgaard et al., 1999; Nielsen et al., 2001), which apparently lead to the observed clinical problems (Bøtner, 1998a; Mortensen, 2000).

In the present study, we have evaluated the pathogenicity of one of the field isolates of PRRS vaccine-derived virus (PRRS-VDV) by experimental infection of late term pregnant sows.

## 2. Materials and methods

### 2.1. Animals

Ten healthy, pregnant sows were procured from a PRRSV-seronegative swine herd. The sows had been routinely vaccinated against porcine parvovirus (PPV) prior to breeding. Approximately 2 months before estimated farrowing, the sows were transferred to the animal isolation units at DVIVR.

### 2.2. Challenge virus

The PRRS-VDV (DVIVR reference: PRRSV 19407B) used as challenge virus had been isolated in January 1997 from the lungs of a stillborn pig. This pig originated from a swine herd with a sudden high occurrence of stillborn pigs and increased piglet mortality in the nursing period, consistent with an acute outbreak of PRRS. By serological examination, the herd had been tested free for specific antibodies to PRRSV both 4 and 1 month prior to the onset of clinical signs, and PRRS vaccination had not been carried out in the herd.

Initially, the virus isolate was characterised as American type by an immuno peroxidase monolayer assay (IPMA) using monoclonal antibodies (mAb) reacting with European (WBE4, Drew et al., 1995) or American (VO17, Nelson et al., 1993) PRRSV types as described by Bøtner et al. (1999). In addition, the complete genome sequence of this isolate was determined, and found to be more than 99.6% identical to the live attenuated vaccine virus, and proved to be a direct derivative of this vaccine (Nielsen et al., 2001). The GenBank Accession No. of the isolate is AF 303356. A third MARC-145 cell culture (Kim et al., 1993) passage of this isolate with a titre of  $10^7$  TCID<sub>50</sub>/ml was used as inoculum.

### *2.3. Experimental design*

Each of six pregnant sows (principal sows) was challenged with PRRS-VDV. One millilitre of challenge virus ( $10^7$  TCID<sub>50</sub>) was diluted to a total volume of 4 ml in Eagle's minimum essential medium (EMEM) and given intranasally to each sow, 2 ml per nostril. Sows 721, 781, and 836 were inoculated on day 86 of gestation, and sows 641, 870, and 882 on day 93. At the same stages of gestation, four sows (control sows) were sham-inoculated with EMEM: sows 840 and 888 on day 86 of gestation, and sows 761 and 883 on day 93 of gestation. General health, appetite, and rectal body temperature of the sows were monitored daily.

For virological and serological examination, blood samples were collected from sows on day 0, 4, 7, and 14 post inoculation (PI), at farrowing, and on day 33 post farrowing (PF). Live piglets were blood-sampled before ingestion of colostrum, and on days 10 and 33 after birth. From stillborn pigs and from piglets dying during the observation period, lung and spleen tissue, and thoracic fluid were collected for examination. The sows and surviving piglets were euthanised on day 33 PF, and lung and spleen tissues were collected from all pigs. Piglets were allowed to suckle until termination of the experiment. This observation period was chosen to parallel the average preweaning period for Danish pigs.

### *2.4. Pathological examination*

All piglets and foetuses were subjected to necropsy. Crown-rump (c-r) length of dead foetuses was determined for an estimation of foetal age (Marrable and Ashdown, 1967). Tissue samples from the lungs were collected for histopathological examination. At least, one sample was taken from each of the three left lobes and one from the right apical lobe. On macroscopical indication, other organs were subjected to histopathological examination. The samples were fixed in 4% formaldehyde, trimmed, embedded in paraffin wax (Paraplast<sup>®</sup>, Bie & Berntsen, Copenhagen, Denmark) and cut at 5  $\mu$ m. All slides were routinely stained with hematoxylin and eosin (H&E). Histopathological examination was not attempted on severely autolysed or mummified foetuses.

### *2.5. Virus isolation*

Porcine pulmonary alveolar macrophages (PPAM) are found to be the most sensitive system for isolation of European PRRSV field strains whereas the vaccine strain, when taken directly from the vial, grows in the MARC-145 cell line and not in the PPAM (Bøtner et al., 1999). After in vivo passages in swine herds, however, the vaccine virus is able to

replicate in PPAM (Bøtner et al., 1999). To ensure detection of any type of PRRSV that might be present, clinical samples (serum, thoracic fluids, and lung/spleen tissue homogenates) were passaged in parallel in PPAM and in the MARC-145 cell line. After two passages, the cell cultures were stained using the mAb's reacting with European or American type PRRSV, as described above. Furthermore, the tissue homogenates were examined for the presence of other porcine viruses known to be present among Danish pigs, according to the standard procedures used at DVIVR. Briefly, the samples were inoculated onto monolayers of primary pig kidney cells and grown for two passages. The second cell culture passage was stained with specific antibodies against porcine adenovirus, swine influenza virus, porcine enterovirus, PPV, porcine respiratory coronavirus, haemagglutinating encephalomyelitis virus, and porcine circovirus type 1 (PCV1) and 2 (PCV2).

### *2.6. RT-PCR*

RNA was isolated as previously described (Oleksiewicz et al., 1998) from the challenge virus, and also directly from the lung tissue from which the challenge virus was originally isolated. Furthermore, RNA was isolated from the lungs of two dead piglets in the current experiment. All four RNA samples were subjected to RT-PCR using an American specific ORF5 PCR, an European specific ORF5 PCR, and a common ORF7 PCR, as previously described (Oleksiewicz et al., 1998). PCR amplicons were sequenced, compared to each other, and to the ORF5 and ORF7 sequences of the vaccine virus, essentially as described by Storgaard et al. (1999).

### *2.7. Serological examination*

Sera and thoracic fluids were examined for antibodies to PRRSV in an IPMA, carried out as a double test using a Danish PRRSV strain (IPMA/DK) and the vaccine virus (IPMA/VAC) in parallel (Sørensen et al., 1998). In addition, precolostral serum samples from liveborn pigs, thoracic fluids from stillborn pigs, and sera from the sows were tested for antibodies to PCV1 and PCV2 by an IPMA comparable to the IPMA for PRRS described by Bøtner et al. (1994), but using glucosamine-treated PK 15 cell cultures infected with either PCV1 or PCV2. In addition, sow sera were tested for antibodies to swine influenza virus, subtypes H1N1 and H3N2, by the haemagglutination inhibition test (Palmer et al., 1975), and for antibodies to PPV (Madsen et al., 1997).

### *2.8. Bacteriology*

Tissue samples of lung, liver, spleen, and small intestine were examined for the presence of potentially pathogenic bacteria and mycoplasmas according to standard operation procedures at the Danish Veterinary Laboratory (DVL).

### *2.9. Statistical analysis*

The data on reproductive performance were analysed using Minitab Ver. 12 (Minitab, State College, PA). Statistical significance was determined by Kruskal–Wallis non-parametric test.

### 3. Results

The temperature of the sows remained within normal physiological range throughout the experiment, except for control sow 761 which reached 40 °C on day 2 PF (see below). None of the sows required obstetrical assistance. Gestation lengths did not differ significantly between principal sows (range 113–117 days) and control sows (range 114–115 days). Control sow 761 which had delivered nine live and one stillborn pig developed a severe metritis/mastitis on day 2 PF. Therefore, this sow and her litter were excluded from the experiment and euthanised. The remaining three control sows delivered a total of 39 live piglets (Table 1). Five of these were small and had birth weights lower than 900 g. However, the average birth weight for the control piglets was still 1297 g (sd 286 g). Neither stillborn pigs, mummified foetuses nor weakborn piglets were seen in the three control litters. However, six control piglets died between day 2 and 13 after birth (Table 1). The finding of traumatic lesions in the head and/or body together with milk-filled stomachs, indicated these piglets to be crushed to death by the dams. In fact, no other explanations of death could be found for these piglets. The remaining control piglets survived with no other clinical signs than occasional slight diarrhoea.

Table 1  
Reproductive performance of sows challenged with a PRRS vaccine-derived virus on 86 or 93 days of gestation

Experimental group	Sow ID	Number of pigs			
		Total	Born alive	Born dead <sup>a</sup>	Postnatal deaths
Principals	641 <sup>b</sup>	17	11	6	1
	870 <sup>b</sup>	12	12	0	2
	882 <sup>b</sup>	18	12 <sup>c</sup>	6	4
	721 <sup>d</sup>	13	11	2	0
	781 <sup>d</sup>	11	9 <sup>e</sup>	2	1
	836 <sup>d</sup>	15	11 <sup>b</sup>	4	5
Average		14.3	11.0 (77%) <sup>f</sup>	3.3 (23%) <sup>f,*</sup>	2.2 (20%) <sup>g,*</sup>
Controls	883 <sup>b</sup>	15	15	0	1 <sup>h</sup>
	840 <sup>d</sup>	13	13	0	2 <sup>h</sup>
	888 <sup>d</sup>	11	11	0	3 <sup>h</sup>
Average		13.0	13.0 (100%)	0.0 (0%)	2.0 (15%)

<sup>a</sup> Including stillborn pigs, and dead and mummified foetuses.

<sup>b</sup> Challenge on day 93 of gestation.

<sup>c</sup> Including four weakborn pigs.

<sup>d</sup> Challenge on day 86 of gestation.

<sup>e</sup> Including one weakborn pig.

<sup>f</sup> Percent of total number of pigs.

<sup>g</sup> Deaths in percent of pigs born alive.

<sup>h</sup> Crushed by sows, see text for further details.

\* The level of deadborn pigs differed significantly ( $p < 0.05$ ) between principals and controls. The level of postnatal deaths only differed significantly between principals and controls, if piglets crushed to death by the sow were excluded. These were only found in the control group.

Clinically, the principal sows did not appear to be affected by the PRRSV infection, except for sow 882 which had decreased milk production. Therefore, the litter of this sow was supplied with milk replacer from day 2 after birth. In total, the six principal sows delivered 66 liveborn pigs and 20 deadborn pigs, an average of 3.3 or 25% deadborn pigs per principal sow (Table 1). The level of delivered deadborn pigs was significantly higher ( $p < 0.05$ ) for the principal sows than for the three control sows which did not deliver any deadborn pigs (Table 1). There was no significant difference between principal and control sows in the level of total pigs born. The deadborn principal pigs comprised two stillborn pigs, and 18 dead foetuses with varying degrees of autolysis and mummification. Ten liveborn principal pigs had birth weights lower than 900 g, and the average birth weight of liveborn principal pigs was 1225 g (sd 283 g). Eight piglets appeared weak at birth, and one of these with a birth weight just below 400 g was euthanised immediately after blood sampling. In general, neonatal pigs in the control group appeared considerably more vigorous and thrifty compared to those in the principal group. During the observation period, another 13 principal piglets died (Table 1). None of these deaths could be attributed to crushing by the sows. Furthermore, the health status of the surviving principals varied considerably. Varying degrees of unthriftiness were seen between as well as within the principal litters. Neither coughing nor other clear signs of respiratory disease were observed in any of the principal piglets, and as time progressed, the general health improved for the majority of the principal piglets. At the end of the observation period, however, only the surviving piglets from sows 641 and 721 appeared to have fully recovered.

### *3.1. Pathology*

In the principal group, the two stillborn pigs had c-r lengths of 24 and 27 cm, respectively, but no other gross lesions could be seen. The remaining deadborn foetuses, which revealed a wide range from mild to severe autolysis to different stages of mummification process, had c-r lengths within the range of 17.5–27.5 cm, indicating times of death during the last third of gestation. However, one mummified principal foetus from sow 836 had a c-r length of 13 cm, indicating that this foetus had died before the virus challenge of the sow.

At necropsy days 33 after birth, more than 50% of the liveborn principal piglets showed macroscopic visible lesions of the lungs, often with all lobes affected. The changes were characterised by multifocal tan-mottled areas, but also cases of severe pleuritis and necrotising pneumonia were observed. Abscesses of varying size and numbers were seen in the lungs from five of the principal piglets. Furthermore, about 20% of the principal piglets showed other pathological changes like pericarditis, peritonitis, enteritis, and subcutaneous abscesses. The only gross lesions in the controls were seen in two piglets showing a moderate reddish discolouration of the lungs.

Histopathological evaluation of 74 principal pigs and 39 control pigs revealed that 73% of the former showed changes of the lungs characterised by interstitial pneumonia predominantly of lobular distribution. The intensity of the lesions varied but was dominated by mononuclear cell infiltration (lymphocytes, plasma cells, and macrophages)

of alveolar septae and interstitia together with scattered luminae with cellular debris of epithelial cells, mononuclear cells and granulocytes. Broncho-interstitial pneumonia associated with fibrinous pleuritis or necrotising pneumonia was seen in 5% of the principals. Heart tissue of three principal piglets with gross lesions revealed two animals with necrotising myocarditis and one animal with chronic pericarditis. Twenty-three percent of the examined principal piglets did not show any pathological lesions. The only histopathological changes seen in the control piglets were mild interstitial pneumonia in two piglets verifying the macroscopic discoloration of the lungs observed in these piglets.

### 3.2. Virus isolation and serology

*Sows.* PRRSV was detected in serum from all the principals from day 4 PI (Table 2). On day 14 PI, all principal sows had seroconverted to PRRSV with antibody responses confirming infection with American type PRRSV, i.e. high titers in the IPMA/VAC in parallel with no or low levels of antibodies detected in the IPMA/DK. Neither PRRSV nor PRRSV-specific antibodies could be detected in the control sows throughout the experiment. Furthermore, no other viruses were isolated from the sows, and antibody titers to swine influenzavirus, PPV, or PCV1 and PCV2 did not increase during the observation period.

*Piglets.* PRRSV was isolated from 52% of the examined liveborn principal piglets at birth (precolostral serum samples) and from 19% of the examined deadborn principals (Table 3), confirming that the virus had crossed the placental barrier and subsequently had infected foetuses. Furthermore, almost half of the live-born piglets were PRRSV-antibody positive with responses confirming infection with American type virus. Four of the dead

Table 2  
Examination for PRRSV and homologous antibody in sows challenged with a PRRS vaccine-derived virus on day 86 or 93 of gestation

Experimental group	Sow ID	Day 0 PI <sup>a</sup>	Day 4 PI	Day 7 PI	Day 14 PI	At farrowing	Day 33 after farrowing
Principals	641 <sup>b</sup>	-/- <sup>c</sup>	+/-	+/-	-/1250 (<50)	+/250 (<50)	-/250 (<50)
	870 <sup>b</sup>	-/-	+/-	+/-	+/250 (50)	+/250 (50)	-/1250 (50)
	882 <sup>b</sup>	-/-	+/-	+/-	-/250 (<50)	-/250 (<50)	-/250 (<50)
	721 <sup>d</sup>	-/-	+/-	+/-	-/250 (<50)	-/250 (50)	-/1250 (50)
	781 <sup>d</sup>	-/-	+/-	+/-	+/1250 (<50)	-/250 (<50)	-/250 (<50)
	836 <sup>d</sup>	-/-	+/-	+/-	+/1250 (<50)	-/250 (50)	-/250 (50)
Controls	883 <sup>b</sup>	All samples were negative for PRRSV and homologous antibodies.					
	840 <sup>d</sup>						
	888 <sup>d</sup>						

<sup>a</sup> PI: post inoculation.

<sup>b</sup> Challenge on day 93 of gestation.

<sup>c</sup> PRRSV/PRRSV-antibody (when positive, the IPMA VAC titer is shown with the IPMA DK titer in parenthesis). The sera were tested in the dilutions 1:50, 1:250, 1:1250, 1:6250.

<sup>d</sup> Challenge on day 86 of gestation.



Table 3

Examination for PRRSV and homologous antibody in pigs delivered by sows challenged with a PRRS vaccine-derived virus on day 86 or 93 of gestation

Experimental group	Sow ID	Day 0 <sup>a</sup>				Day 10 after birth <sup>b</sup>		Day 33 after birth			Day X <sup>c</sup> after birth	
		Virus isolation		Antibody		Virus isolation serum	Antibody serum	Virus isolation		Antibody serum	Virus isolation	
		Born alive	Born dead	Born alive	Born dead			Serum	Tissue samples		Serum <sup>d</sup>	Tissue samples
Principals	641 <sup>c</sup>	3/11 <sup>f</sup>	1/6	0/11	0/6	11/11	11/11	7/10	1/10	10/10	0/1	0/1
	870 <sup>c</sup>	12/12	0/0	8/12	0/0	12/12	12/12	9/10	1/10	10/10	2/2	2/2
	882 <sup>c</sup>	12/12	2/6	4/12	1/6	10/10	10/10	6/8	4/8	8/8	2/4	4/4
	721 <sup>g</sup>	1/10 <sup>h</sup>	0/1 <sup>i</sup>	1/10 <sup>h</sup>	0/1 <sup>i</sup>	11/11	11/11	11/11	7/11	11/11	–	–
	781 <sup>g</sup>	1/9	0/2	1/9	0/2	7/8	8/8	8/8	8/8	8/8	1/1	1/1
	836 <sup>g</sup>	5/11	0/1 <sup>j</sup>	2/11	0/1 <sup>j</sup>	10/10	10/10	4/6	4/6	6/6	5/5	5/5
Total		34/65	3/16	15/65	1/16	61/64	62/64	45/53	25/53	53/53	11/13	12/13
Controls	883 <sup>c</sup>	0/15	0/0	0/15	0/0	0/14	0/14	0/14	0/14	0/14	0/1	0/1
	840 <sup>g</sup>	0/13	0/0	0/13	0/0	0/11	0/11	0/11	0/11	0/11	0/2	0/2
	888 <sup>g</sup>	0/11	0/0	0/11	0/0	0/8	0/8	0/8	0/8	0/8	0/3	0/3
Total		0/39	0/0	0/39	0/0	0/33	0/33	0/33	0/33	0/33	0/6	0/6

<sup>a</sup> Precolostral serum from pigs born alive, and thoracic fluids from pigs born dead (including stillborn pigs, and dead and mummified foetuses).<sup>b</sup> Serum samples.<sup>c</sup> X represents other days of death before day 33.<sup>d</sup> Or thoracic fluids.<sup>e</sup> PRRSV-challenge on day 93 of gestation.<sup>f</sup> Number of positive pigs/number of pigs examined.<sup>g</sup> PRRSV-challenge on day 86 of gestation.<sup>h</sup> Not including one pig, from which we did not obtain a precolostral sample.<sup>i</sup> Not including one foetus, from which appropriate material for testing could not be obtained.<sup>j</sup> Not including three foetuses, from which appropriate material for testing could not be obtained.

foetuses were not examined due to severe autolysis or mummification. On day 10 after birth, all principal piglets, but one, were viremic, and PRRSV-antibody positive. Twelve of the 13 piglets which died before day 33 after birth were virus positive at necropsy. At necropsy on day 33, the majority of the piglets were still PRRSV-positive, either in serum and/or tissue, with the virus most frequently being isolated from serum samples. All PRRSV isolates recovered from principal sows and piglets were identified as American type by IPMA.

The two different cell types used for virus isolation were found to have almost equal sensitivity for the isolation of PRRS-VDV, the virus most often being isolated on both MARC cells and PPAM (data not shown). Neither virus nor specific antibodies could be detected in any of the control piglets. Finally, no other viruses than PRRS-VDV were detected in any of the piglets.

### 3.3. RT-PCR

The European specific ORF5 RT-PCR did not amplify any product from the inoculum, indicating that European type PRRSV was not present. To finally exclude the possibility that any trace fraction of the inoculum might have played a role in the outcome of the experimental infection, RT-PCR was likewise performed on two principal piglets. Again, only the American ORF5 and the common ORF7 RT-PCR became positive. These RT-PCR results showed that the inoculum was not contaminated with European type PRRSV. To investigate the genetic stability of the isolate, ORF5 and ORF7 amplicons obtained from the two principal piglets were DNA sequenced and compared with amplicons obtained before and after cell culture amplification. No genetic changes were found either during the cell culture amplification, the viral replication in the sow or the viral replication in the two piglets investigated.

### 3.4. Bacteriology

The detection of non-hemolytic *E. coli* in the intestines of 13 control pigs and 22 principal pigs could neither be related to intestinal disorders nor any other disease. Non-hemolytic streptococci were found in the liver, lung, and spleen from one principal piglet (from sow 882) which died 6 days after birth, and *Streptococcus dysgalactiae* was isolated from the lungs from one principal litter-mate with lobular interstitial pneumonia and enteritis which died on day 10 after birth. *Staphylococcus aureus* was detected in the lungs of two other piglets from sow 882, one of which had a lung absces and abscesses on the legs. *Staphylococcus hyicus* was isolated from the lungs of two principals (from sow 870) which became apathetic and reluctant to move, and accordingly were euthanised on day 20 and 23 after birth, respectively. Both had lung lesions, characterised as lobular interstitial pneumonia and focal necrotising pneumonia. The latter also had a number of abscesses on the legs. *Mycoplasma hyosynoviae* was identified in the lungs of one principal piglet (from sow 721) with lobular interstitial pneumonia. No bacteria were found in any of the stillborn pigs or dead foetuses. Finally, no other bacteria than the *E. coli* described above were found in any of the control pigs.

#### 4. Discussion

By sequence analysis, the current PRRS-VDV isolate has been proved to be a direct derivative of the live attenuated vaccine virus which had been used in Denmark for a period of approximately 6 months prior to the virus isolation (Nielsen et al., 2001).

Intranasal inoculation of PRRS-naive pregnant sows with the PRRS-VDV isolate in the last trimester resulted in a significantly higher level of deadborn pigs than observed in the control sows (Table 1). In addition, approximately 50% of the liveborn pigs from the principal sows were congenitally infected (Table 3). Perhaps as a result of the congenital infection, the preweaning mortality appeared to be higher in the principal group than in the control group (Table 1). However, this difference was not statistically significant, unless piglets crushed to death by the sows were excluded from the analysis. These were only found in the control group (Table 1).

The present findings are consistent with the observations from the field with appearance of PRRS-like symptoms in sow herds after vaccination of 3–18-week-old pigs with an attenuated live PRRS vaccine (Bøtner et al., 1997). As such, the present study provides strong evidence that PRRS-VDV isolated from the field can cause disease in swine consistent with PRRS. This assertion is based on fulfilment of the postulates of Koch (see, Levine, 1996): The virus isolated from the disease was cultured *in vitro* and characterised as PRRS-VDV. Inoculation of susceptible animals with the PRRS-VDV reproduced the disease, and the same PRRS-VDV strain was recovered from the experimentally induced disease.

The reproductive signs and losses, the gross lesions in dead foetuses, and the generally unhealthy appearance of many of the live-born principal pigs reported here were comparable with previous observations after experimental infection of sows with European as well as American PRRSV during late gestation (Terpstra et al., 1991; Christianson et al., 1992; Plana et al., 1992; Mengeling et al., 1994; Lager and Halbur, 1996; Kranker et al., 1998; Allende et al., 2000). Furthermore, the interstitial pneumonia demonstrated in approximately three-fourth of the principal piglets by histopathological examination was in accordance with the lesions typical for PRRS infection (Pol et al., 1991; Collins et al., 1992; Halbur et al., 1995; Kranker et al., 1998). In addition, we observed signs of severe bacterial pneumonia as well as generalised bacterial infection in a number of the congenitally infected piglets, but not in any of the control piglets. These findings are consistent with an immunosuppressive effect of *in utero* infection by PRRSV with resulting increased susceptibility to secondary bacterial infections, as also shown by Feng et al. (2001).

The low level of positive virus isolations in the stillborn piglets and dead foetuses found in the present study corresponds well to observations by others (Christianson et al., 1992; Mengeling et al., 1994) who did not detect PRRSV in autolysed foetuses from late term infected dams. The absence of infectious virus in these foetuses most probably reflects a destruction of virus caused by the autolysis, as also suggested by Christianson et al. (1992). That almost all piglets were found to be viremic at 10 days of age, was probably due to a horizontal transmission of virus from *in utero* infected pigs to non-infected litter-mates during the early period of life, as also described by Mengeling et al. (1998).

The severe PRRS problems observed in a huge proportion of Danish swine herds subsequent to the wide use of the live vaccine in 3–18 week-old piglets (Bøtner et al., 1997; Mortensen, 2000) have not been reported from any other country following the use of the

vaccine. This has raised the question as to whether the observed clinical symptoms were caused by the vaccine virus or by other pathogens. The present study, however, provides strong evidence that the problems were primarily the result of infection with the PRRS-VDV. The sporadic bacteriological findings did not indicate a primary role of bacteria. However, especially the pathogenic bacteria, e.g. staphylococci, found in some of the principal piglets may very well have played a secondary role in the development and severity of actual disease in individual animals. Furthermore, the fact that none of the sows seroconverted to any other viruses, and that attempts to isolate other viruses than PRRS-VDV remained negative, strongly indicated that no other viruses than PRRSV were involved. Finally, differentiating RT-PCR performed on both the original isolate, the virus inoculum itself, and on serum from two principal piglets excluded any role of European type PRRSV.

Having excluded the role of other infections, it was of interest to consider why problems associated with the use of the live PRRSV vaccine were so pronounced in Denmark. We suggest that a number of unique circumstances existed regarding the use of the live PRRSV vaccine in Denmark, as outlined in the following: (i) No North American wild type of PRRSV was present in Denmark, so the spread of the vaccine virus was easy to monitor. (ii) The vaccine was used solely in piglets in herds seropositive towards European type of PRRSV, and with the majority of the herds having no or minor clinical PRRSV-associated problems at the time of vaccination. (iii) The simultaneous (within few months) use of the vaccine in more than 1100 herds made it much easier to detect vaccine reversion. (iv) Due to the coordinated vaccination-program, the vaccinating herds were followed very closely. (v) In Denmark, a serological test differentiating between European and American strains of PRRSV is routinely used. In summary, the situation in Denmark made it very simple to observe vaccine reversion. In contrast, due to the reasons given above, vaccine reversion might go unnoticed in other countries. In fact, studies from American laboratories are increasingly beginning to quote vaccine reversion as the cause of clinical problems (reviewed by Meng (2000)).

Recently, another study describing the experimental inoculation of pregnant sows with a putative vaccine virus revertant has been published (Allende et al., 2000). That particular isolate (16244B) had 212 nucleotide substitutions when compared to the vaccine virus (Allende et al., 2000) and produced reproductive disorders comparable to those caused by the VR-2332 isolate parental to the vaccine virus (Christianson et al., 1992). Compared to the present results, Allende et al. (2000) observed a more pronounced clinical effect after infection of pregnant sows at 90 days of gestation with the 16244B isolate. Thus, these authors found a reduced level of liveborn pigs (57% in contrast to 77% in our study) with a parallel increase of stillborn pigs (43% in contrast to 23% in our study). Furthermore, the rate of survival at 10 days after farrowing was much lower (29%) than in the present study (80%). The present PRRS-VDV isolate which was collected approximately 6 months after the introduction of the vaccine into Denmark had only 63 nucleotide differences when compared to the vaccine virus (Nielsen et al., 2001). This higher level of sequence identity to the attenuated vaccine virus may explain the apparently reduced mortality when compared to the 16244B isolate, and may indicate — as also speculated by others (Mengeling et al., 1999) — that the genetic reversion to virulence happens gradually with prolonged time of passage of the vaccine virus in the field.

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## References

- Allende, R., Kutish, G.F., Laegreid, W., Lu, Z., Lewis, T.L., Rock, D.L., Friesen, J., Galeota, J.A., Doster, A.R., Osorio, F.A., 2000. Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. *Arch. Virol.* 145, 1149–1161.
- Bøtner, A., 1998a. PRRS — the Danish experience. In: Proceedings of the XVIII Nordic Veterinary Congress, Helsinki, Finland, August 1998, pp. 122–125.
- Bøtner, A., 1998b. Field experiences with PRRS and with the use of a live vaccine in Denmark. In: Proceedings of the Swine Disease Conference for Swine Practitioners, Iowa State University, November 1998, pp. 59–65.
- Bøtner, A., Nielsen, J., Bille-Hansen, V., 1994. Isolation of porcine reproductive and respiratory syndrome (PRRS) virus in a Danish swine herd and experimental infection of pregnant gilts with the virus. *Vet. Microbiol.* 40, 351–360.
- Bøtner, A., Strandbygaard, B., Sørensen, K.J., Have, P., Madsen, K.G., Madsen, E., Smedegaard, Alexandersen, S., 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS-vaccine. *Vet. Rec.* 141, 497–499.
- Bøtner, A., Nielsen, J., Oleksiewicz, M.B., Storgaard, T., 1999. Heterologous challenge with porcine reproductive and respiratory syndrome (PRRS) vaccine virus: no evidence of reactivation of previous European-type PRRS virus infection. *Vet. Microbiol.* 68, 187–195.
- Chladek, D.W., Gorczyca, D.E., Harris, L.L., 1995. US Patent 5 476 778. US Department of Commerce, Patent and Trademark Office, Washington, DC, December 19.
- Christianson, W.T., Collins, J.E., Benfield, D.A., Harris, L., Gorczyca, D.E., Chladek, D.W., Morrison, R.B., Joo, H.S., 1992. Experimental reproduction of swine infertility syndrome in pregnant sows. *Am. J. Vet. Res.* 53, 485–488.
- Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., Gorczyca, D., Chladek, D., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. *J. Vet. Diagn. Invest.* 4, 117–126.
- Drew, T.W., Meulenberg, J.J.M., Sands, J.J., Paton, D.J., 1995. Protection, characterisation and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome virus (PRRSV). *J. Gen. Virol.* 76, 1361–1369.
- Feng, W., Laster, S.M., Tompkins, M., Brown, T., Xu, J.S., Altier, C., Gomez, W., Benfield, D., McCaw, M.B., 2001. In utero infection by porcine reproductive and respiratory syndrome virus is sufficient to increase susceptibility to challenge by *Streptococcus suis* Type II. *J. Virol.* 75, 4889–4895.
- Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet. Pathol.* 32, 648–660.
- Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. *Arch. Virol.* 133, 477–483.
- Kranker, S., Nielsen, J., Bille-Hansen, V., Bøtner, A., 1998. Experimental inoculation of swine at various stages of gestation with a Danish isolate of porcine reproductive and respiratory syndrome virus (PRRSV). *Vet. Microbiol.* 61, 21–31.
- Lager, K.M., Halbur, P.G., 1996. Gross and microscopic lesions in porcine fetuses infected with porcine reproductive and respiratory syndrome virus. *J. Vet. Diagn. Invest.* 8, 275–282.

- Levine, A.J., 1996. The origins of virology. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), *Fields Virology*, 3rd Edition. Lippincot–Raven, Philadelphia, pp. 1–14.
- Madsen, E.S., Madsen, K.G., Nielsen, J., Jensen, Holm, M., Lei, J.C., Have, P., 1997. Detection of antibodies against porcine parvovirus nonstructural protein NS1 may distinguish between vaccinated and infected pigs. *Vet. Microbiol.* 54, 1–16.
- Madsen, K.G., Hansen, C.M., Madsen, E.S., Strandbygaard, B., Bøtner, A., Sørensen, K.J., 1998. Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. *Arch. Virol.* 143, 1683–1700.
- Marrable, A.W., Ashdown, R.R., 1967. Quantitative observations on pig embryos of known ages. *J. Agric. Sci. Camb.* 69, 443–447.
- Meng, X.J., 2000. Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. *Vet. Microbiol.* 74, 309–329.
- Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1994. Temporal characterization of transplacental infection of porcine fetuses with porcine reproductive and respiratory syndrome virus. *Am. J. Vet. Res.* 55, 1391–1398.
- Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1998. Clinical effects of porcine reproductive and respiratory syndrome virus on pigs during the early postnatal interval. *Am. J. Vet. Res.* 59, 52–55.
- Mengeling, W.L., Vorwald, A.C., Lager, K.M., Clouser, D.F., Wesley, R.D., 1999. Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. *AJVR* 60, 334–340.
- Mortensen, S., 2000. Herd testing and disease spread models in swine populations. Ph.D. Thesis. DSR Forlag, Copenhagen.
- Mortensen, S., Strandbygaard, B., 1996. PRRS spredes nu langsommere. *Veterinærinformation* 4, 35–36 (in Danish).
- Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield, D.A., 1993. Differentiation of US and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. *J. Clin. Microbiol.* 31, 3184–3189.
- Nielsen, H.S., Oleksiewicz, M.B., Forsberg, R., Stadejek, T., Bøtner, A., Storgaard, T., 2001. Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J. Gen. Virol.* 82, 1263–1272.
- Oleksiewicz, M.B., Bøtner, A., Madsen, K.G., Storgaard, T., 1998. Sensitive detection and typing of porcine reproductive and respiratory syndrome virus by RT-PCR amplification of whole viral genes. *Vet. Microbiol.* 64, 7–22.
- Palmer, D.F., Coleman, M.T., Dowdle, W.R., Schild, G.C., 1975. Advanced laboratory techniques for influenza diagnosis. US Department of Health, Education and Welfare, Immunology Series No. 6.
- Plana, J., Vayreda, M., Villarrasa, J., Bastons, M., Rosell, R., Martinez, M., San Gabriel, A., Pujols, J., Badiola, J.L., Ramos, J.A., Domingo, M., 1992. Porcine epidemic abortion and respiratory syndrome (mystery swine disease). Isolation in Spain of the causative agent and experimental reproduction of the disease. *Vet. Microbiol.* 33, 203–211.
- Pol, J.M.A., van Dijk, J.E., Wensvoort, G., Terpstra, C., 1991. Pathological, ultrastructural, and immunohistochemical changes caused by Lelystad virus in experimentally induced infections of mystery swine disease (synonym: porcine epidemic abortion and respiratory syndrome (PEARS)). *Vet. Q.* 13, 137–143.
- Sørensen, K.J., Strandbygaard, B., Bøtner, A., Madsen, E.S., Nielsen, J., Have, P., 1998. Blocking ELISA's for the distinction between antibodies against European and American strains of porcine reproductive and respiratory syndrome virus. *Vet. Microbiol.* 60, 169–177.
- Storgaard, T., Oleksiewicz, M.B., Bøtner, A., 1999. Examination of the selective pressures on a live PRRS vaccine virus. *Arch. Virol.* 144, 2389–2401.
- Terpstra, C., Wensvoort, G., Pol, J.M.A., 1991. Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet. Q.* 13, 131–136.