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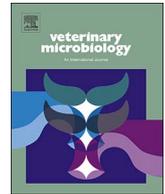
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Genetic and biological characterization of a Porcine Reproductive and Respiratory Syndrome Virus 2 (PRRSV-2) causing significant clinical disease in the field



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ABSTRACT

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the cause of severe reproductive and respiratory disease in swine worldwide. In Denmark, both PRRSV-1 and PRRSV-2 are circulating and approximately 35% of pig herds are seropositive for PRRSV.

In November 2010, a pig herd in the Northern part of Denmark experienced an infection with PRRSV-2 with clinical signs that were much more severe than normally reported from current Danish PRRSV-2 affected herds. Due to the clinical observations of reproductive failure in sows and high mortality in piglets, it was speculated that a new, more pathogenic or vaccine evading PRRSV strain had emerged in Denmark. The overall aim of the present study was to perform a genetic and biological characterization of the virus isolated from the diseased herd. Complete genome sequencing of isolates from this herd revealed that although the case strain had some unique genetic features including a deduced 3 amino acid deletion, it was in overall very similar to the other PRRS-2 viruses circulating in Denmark. In an experimental trial in growing pigs, no overt clinical signs or pathology were observed following intranasal inoculation with the new virus isolate. Virus shedding, acute phase protein responses and serological responses were comparable to those seen after experimental challenge with a Danish PRRSV-2 reference strain isolated in 1997. Vaccination with a commercial modified live PRRSV-2 vaccine had a clear reducing effect on virus shedding, magnitude, and duration of viremia and viral load in the lungs. Overall, the results indicate that the severe disease observed in the field was contributed by additional factors in combination with the PRRS virus infection.

1. Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) belongs to the order Nidovirales (Cavanagh, 1997) and has recently been reclassified to the Porartevirus genus in the Arteriviridae family (https://talk.ictvonline.org/ictv-reports/ictv_online_report). PRRSV is the cause of severe respiratory and reproductive disease in swine worldwide (Botner et al., 1994; Han et al., 2006; Karniychuk et al., 2010; Tian et al., 2007). PRRSV is an enveloped single-stranded positive-sense RNA virus with a genome size of 15.0–15.5 kb containing a 5'-end cap and a 3'-end polyadenine tail (Meulenbergh et al., 1998). The genome encodes at least 10 open reading frames (ORFs) (Firth et al.,

2011; Johnson et al., 2011; Meulenbergh et al., 1993; Wu et al., 2001). Due to the high genetic and serologic diversity between PRRSV isolates (Allende et al., 1999; Wensvoort et al., 1992) the virus was divided into two major genotypes; Type 1 and Type 2. Both genotypes are circulating globally (Shi et al., 2010). In Denmark, PRRSV-1 was detected for the first time in 1992, and PRRSV-2 was introduced in 1996 in connection with an extensive vaccination program (Botner et al., 1997; Madsen et al., 1998). Approximately 35% of the Danish herds are seropositive for antibodies against PRRSV (Kristensen et al., 2014; Lopes Antunes et al., 2015).

Occasionally, highly virulent PRRSV strains evolve, with significant impact on animal welfare (Balka et al., 2015; Han et al., 2006;

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Karniychuk et al., 2010; Sinn et al., 2016; Tian et al., 2007) and associated with huge economic losses (Holtkamp et al., 2013). In 2006, China experienced the emergence of a highly pathogenic strain of PRRSV, which was similar to other Chinese PRRSV-2 strains in ORF5, but exhibited unique differences in other ORFs (An et al., 2010; Tian et al., 2007). The PRRSV epidemic in China affected more than 2 million pigs with about 400,000 fatal cases which also included grown pigs (Tian et al., 2007) and subsequently spreading to other Southeast Asian countries (An et al., 2011; Feng et al., 2008). In general, PRRSV-2 in Europe are similar and share a high level of identity to the VR2332 vaccine strain, but more diverse and pathogenic strains have been reported in recent years (Balka et al., 2015; Jackova et al., 2013). Occurrence of highly pathogenic and diverse PRRSV strains has also been observed in Eastern Europe (Karniychuk et al., 2010; Morgan et al., 2013) and North America (Han et al., 2006) emphasizing the importance of monitoring the diversity of circulating PRRSV strains globally both with respect to the sensitivity and specificity of diagnostic tests as well as efficacy of available vaccines.

In November 2010, a severe case of PRRS with high mortality rate in piglets occurred in a farrow-to-finisher herd in the Northern part of Denmark. By real-time RT-PCR, PRRSV-2 was detected in lung tissue samples from 10-day-old piglets submitted to the Danish National Veterinary Institute for routine diagnostics. The samples were negative for swine influenza virus, porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae*. Vaccination with the Ingelvac® PRRS modified live vaccine (MLV) was initiated in the herd. Within the first 6 weeks after the initial observation of disease, pre-wean mortality increased up to 50% among the live-born piglets. After 6 weeks, pre-weaning mortality was back to normal however between week 6 and week 14 up to 75% of the fetuses were mummified. In February 2011 (12 weeks after the initial infection was recognized in piglets), pleura and lung tissues from dead fetuses and stillborn piglets were found positive for PRRSV-2 by real-time RT-PCR. The samples were negative for porcine parvovirus, PCV2 and *Leptospira* spp. Approximately 15 weeks following the initial infection, the number of live born piglets had returned to normal (33 weaned piglets per sow per year). Total losses of piglets until weaning for the 15-week period were around 30%. The average loss in the nursery was 9.3% until 18 weeks after the initial infection. Nineteen weeks after the initial infection, post weaning mortality was back to normal (2.2%).

Since the clinical impact in the infected herd appeared to be more severe than usually reported for Danish PRRSV-2 affected herds, it was speculated that a new, more pathogenic PRRSV strain had evolved in Denmark similar to the strains that had previously evolved in Minnesota (USA), China and Eastern Europe (Han et al., 2006; Karniychuk et al., 2010; Morgan et al., 2013; Tian et al., 2007). Thus, the overall aim of the present study was to make a genetic and biological characterization of the virus isolated from the case herd by full-genome sequence analysis of the isolate and by comparing the pathogenicity of the virus with an older Danish PRRSV-2 isolate by experimental infection of young pigs. The protective effect of a modified live virus (MLV) PRRSV-2 vaccine against this recent Danish PRRSV-2 isolate was also assessed.

2. Materials and methods

2.1. Case samples

All samples were collected from the case herd between November 2010 and March 2011. In November 2010, lung samples from nine 10-day-old piglets were obtained. In February 2011, lungs from 7 mummified fetuses, and 13 lungs and 11 pleura samples from stillborn piglets were collected and finally, in March 2011, lungs and livers from 3 stillborn piglets and lungs and/or hearts and livers from 11 mummified fetuses were collected. From the samples submitted in November 2010, one virus, designated DK-2010-10-13-1 (KF183946), was isolated

from a 2nd passage on in-house culture stock of Marc-145 cells a derivative of African green monkey kidney cells using general cell culture procedures (Kim et al., 1993).

2.2. Screening of case samples by real-time RT-PCR

RNA was extracted from lung tissue, serum and nasal swabs as previously described (Stadejek et al., 2017). Total RNA from cell culture supernatants for full genome sequencing was purified using QIAamp Viral RNA Mini Kit (QIAGEN). PRRSV positive samples and negative controls were included in each 24-sample batch of RNA purifications. RNA extracted from lung tissue and pleura from case animals and fetuses were screened for PRRSV using a modification of a previously published primer-probe energy transfer (PriProET) assay (Balka et al., 2009). The fluorophore on the probe was changed to ATTO663 and the reaction was run in RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen).

2.3. Cycle sequencing and next generation sequencing

PCR amplification of ORF5 and ORF7 was performed as previously described (Kvisgaard et al., 2013a). PCR amplification of partial-NSP2 was carried out using the primers described by Zhou et al. (Zhou et al., 2009) and the same PCR cycle conditions as for the ORF5 and ORF7 amplifications. PCR products were sequenced by cycle sequencing using the Sanger method with the PCR primers as sequencing primers (Sanger et al., 1977). The sequencing was conducted by LGC Genomics GmbH (Berlin, Germany). Full-genome cDNA synthesis with Superscript III First-Strand Synthesis System (Invitrogen) and long range PCR amplifications were performed as described by Kvisgaard et al. (2013b). The complete genomes of DK-2010-10-13-1 (KF183946) and DK-2011-88005-A8-PI (KF183947) were obtained using next generation sequencing methods described elsewhere (Kvisgaard et al., 2013a, 2013b).

2.4. Sequence data analysis

Data analysis of sequences obtained from cycle sequencing was carried out using the commercial software CLC Main Workbench v. 6.6.2 (CLC BIO, Aarhus, Denmark). Contigs of ORF5 and ORF7 were produced from assembling the raw data obtained for cycle sequencing against the reference sequence VR2332 (PRU87392). The complete genomes of DK-2010-10-13-1 (KF183946) and DK-2011-88005-A8-PI (KF183947) were obtained by the mapping of reads using the Burrows-Wheeler aligner (BWA) (Kvisgaard et al., 2013b). Amino acid (aa) sequences were predicted from the nucleotide (nt) sequences using CLC Main Workbench v. 6.6.2. Nt and aa sequences were aligned using MUSCLE (MULTiple Sequence Comparison by Log-Expectation).

2.5. Inoculum and virus propagation in cell culture

The two virus isolates used in the experimental infection study were propagated in Marc-145 cells (in house cell culture stock) using standard procedures (Kim et al., 1993). For both virus isolates used in the experimental infection study, a fresh second passage of cell culture supernatant was used as inoculation material. The virus titers of the isolates were determined using an immunoperoxidase monolayer assay (IPMA) as described previously (Botner et al., 1994; Markussen and Have, 1992). SDOW17 PRRSV monoclonal antibody (RTI, LLC) was used as primary antibody and horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (DAKO) as the secondary antibody. The DK-1997-19407B (KC862576) isolate, an older Danish PRRS-2 virus (Nielsen et al., 2002), had a titer of 5.4 log₁₀ TCID₅₀/ml. The DK-2010-10-13-1 isolate had a titer of 5.9 log₁₀ TCID₅₀/ml. One ml of cell culture supernatant was diluted in cell culture medium to a total volume of 4 ml and administered intra-nasally to each pig with 2 ml per

nostril.

2.6. Experimental set-up

Twenty-eight 4-week-old pigs (Landrace X Yorkshire X Duroc cross-breeds) of both sexes were purchased from a commercial pig herd seropositive for *Actinobacillus pleuropneumoniae* (AP) serotypes 6 and 12, but declared free from a range of important swine pathogens including *Mycoplasma hyopneumoniae*, PRRSV and AP serotypes 1, 2, 3, 4, 5, 7, 8, 9 and 10 according to the Danish Specific Pathogen Free (SPF) system. The pigs were blindly divided into one control group with four pigs (group 1) and three experimental groups (groups 2–4) each with eight pigs. The randomization did not follow any specific rule. The different groups were housed in separate sections of the BSL3 animal isolation facilities at the National Veterinary Institute, Lindholm. After an acclimatization period of 5 days, the pigs in group 4 (pig nos. 21–28) were vaccinated intramuscularly (i.m) with 2 ml of the Ingelvac® PRRS MLV vaccine (Boehringer Ingelheim Animal Health; Ingelheim, Germany). Four weeks later, at day post infection (DPI) 0, the pigs in group 1 (pig nos. 1–4) were sham-inoculated intranasally (i.n.) with MEM. The pigs in group 2 (pig nos. 5–12) and group 3 (pig nos. 13–20) were inoculated i.n. with the DK-1997–19407 B and the case DK-2010-10-13-1 isolate, respectively. The vaccinated pigs in group 4 (pig nos. 21–28) were challenged with the DK-2010-10-13-1 case isolate. The pigs in groups 1 and 4 were euthanized 29 DPI and the other two groups were euthanized 30 DPI. Euthanasia was performed by intravenous injection of pentobarbiturate (50 mg/kg) followed by exsanguination by cutting *arteria axillaris*. All procedures of animal handling and experimentation were approved by the Danish Animal Experiments Inspectorate (2012-15-2934-00681).

2.7. Clinical examination

Individual pigs were subjected to daily clinical examination and the rectal temperatures were measured. To avoid influence of handling on the body temperature recordings, these were usually carried out while pigs were eating. In order to obtain a semi-quantitative measure for comparison of clinical disease between the four groups, all pigs were scored using the clinical scoring (CS) system developed for swine fever infections (Mittelholzer et al., 2000) adapted for PRRSV experiments by focusing on PRRS typical clinical signs in growing pigs. All pigs were evaluated clinically twice daily and all deviations from normal recorded.

2.8. Blood and nasal swab sampling

Blood samples were collected from all pigs on DPIs –28, 0, 3, 7, 10, 14, 21, 28, and 29/30 (days of euthanasia) for clinical, virological, hematological and immunological examinations. Samples obtained on DPI –28 and 0 were collected prior to vaccination and inoculation, respectively. Non-stabilized whole-blood samples were collected in 5 or 10 ml vacutainers (Venoject; Terumo Europe) from the anterior vena cava and left on ice to coagulate for 15 min. Serum was separated by centrifugation at $2600 \times g$ for 10 min at 4 °C and stored at –80 °C for subsequent analysis. EDTA-stabilized blood samples were collected in 5 or 10 ml haematology vacutainers (Venoject; Terumo Europe) from the anterior vena cava. The freshly collected EDTA-stabilized samples were used for total white blood cell (WBC) counts and flow cytometry analysis. Nasal swabs were collected on the same days as blood samples except on –28 and 29/30 DPI. The swabs (FLOQSwabs™, COPAN) were placed in 1 ml PBS (pH 7.5) and stored at –80 °C until further analysis.

2.9. Tissue sampling

The experimentally inoculated pigs were necropsied for characterization of type, distribution, and severity of gross lesions and the

following tissue samples were collected: nasal mucous membrane, tonsil, trachea (middle part), tracheobronchial lymph node, liver and spleen. Two identical samples were taken from the lungs from the right apical- and diaphragmatic lobes, the intermediate lobe and from lesions if present. One of each lung sample was fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin wax and cut in 3–5 µm thick sections. For histopathological examination the sections were mounted on conventional glass slides, stained with hematoxylin and eosin, and evaluated blinded and systematically. The other tissue samples were stored at –80 °C for later analysis by real-time RT-PCR. Selected lungs samples were prepared and propagated in Marc-145 cell culture as described in Section 2.7.

2.10. Total WBC counts

Total WBC counts were carried out on freshly collected EDTA-stabilized blood samples using a semi-automated animal blood cell counter (Vet abc™, ABX, Montpellier, France). All samples were counted twice, and the mean value was calculated.

2.11. Flow cytometry analyses

Phenotyping of leukocytes in peripheral blood was carried out by flow cytometry, using single as well as triple labeling methods to define different sub-populations. Single-color analysis was applied to differentiate leukocyte populations and for B cell detection, as previously described (Nielsen et al., 2003). Phenotyping of peripheral blood T cell subpopulations was performed by triple-color flow cytometry using CD3/CD8/CD4 labelling, as previously described (Nielsen et al., 2003). Selection of gates was as described elsewhere (Summerfield et al., 2001). This permitted the identification of the T cell subpopulations as CD3⁺CD4⁺CD8[–] naive Th cells, CD3⁺CD4⁺CD8⁺ as memory/activated Th cells, CD3⁺CD4[–]CD8⁺ as Tc cells, CD3⁺CD4[–]CD8^{low/–} as γδ T cells. NK cells were defined as CD3[–]CD4[–]CD8⁺ cells.

2.12. Quantification of viral load in experimentally infected pigs

For quantitative real-time RT-PCR, the previously published “Kleiboeker mod-1” primers and probe targeting PRRSV-2 ORF7 3'UTR (Wernike et al., 2012) were used. All samples were tested in duplicates.

Quantifications of viral load in the experimental samples were performed against standard curves matching each of the inoculation virus isolates and imported into each real-time RT-PCR run with adjustment according to a PRRSV RNA calibrator sample included in triplicate.

Standard curves for quantification of DK-2010-10-13-1 virus were obtained using a five-fold dilution series of 5.9 log₁₀ TCID₅₀/ml of DK-2010-10-13-1 inoculum virus isolate spiked 1:100 into PRRSV negative swine serum and nasal swab material, respectively. RNA was extracted from each spiked dilution and tested in triplicate by real-time RT-PCR in at least two independent PCR experiments. The serum and nasal swab standard curves had a PCR efficiency of 97% ($R^2 = 0.99$, slope = –3.398) and 91% ($R^2 = 0.99$; slope = –3.547) respectively, and their range of quantification covered 7 and 6 log₅ dilution steps corresponding to concentrations 0.5 – 8.0 × 10³ and 0.52–8.1 × 10³ TCID₅₀/ml equivalents, respectively. Similarly, standard curves for quantification of DK-1997–19407 B virus in serum and nasal swabs were produced by spiking of 5.4 log₁₀ TCID₅₀/ml DK-1997–19407B inoculum virus isolate in PRRSV negative serum and nasal swab material. The PCR efficiencies were 98% ($R^2 = 0.98$; slope = –3.374) and 93% ($R^2 = 0.99$; slope = –3.909), respectively, and both covered 6 log₅ dilution steps corresponding to concentrations 0.5–1.4 × 10³ and 0.14–2.3 × 10³ TCID₅₀/ml equivalents, respectively.

Similarly, standard curves for quantification of DK-2010-10-13-1 and DK-1997–19407 B in lung tissue were obtained by spiking of the corresponding virus isolates diluted 1:200 in Buffer RLT with 1% β-

mercaptoethanol and used for preparation of 5-fold dilution series in PRRSV negative lung tissue homogenate. The DK-2010-10-13-1 lung tissue standard curve had an efficiency of 90% ($R_2 = 0.99$; slope = -3.589) and covered 8 log₅ dilution steps corresponding to a concentration of $0.07\text{--}4.0 \times 10^3$ TCID₅₀/ml equivalents. The DK-1997-19407B lung tissue standard curve had an efficiency of 92% ($R_2 = 0.99$; slope = -3.534) and covered 8 log₅ dilution steps corresponding to a concentration of $0.02\text{--}1.58 \times 10^3$ TCID₅₀/ml equivalents.

2.13. Serology

Antibodies in sera from experimentally infected pigs were analyzed using a PRRSV immunoperoxidase monolayer assay (IPMA) (Sorensen et al., 1997) and a blocking enzyme-linked immunosorbent assay (ELISA) (Sorensen et al., 1998). For IPMA, the serum was initially diluted 1:50 and then tested using a fivefold dilution series (1:50-1:6250). The results were expressed as the highest dilution generating a positive signal (titer). For ELISA, sera were diluted 1:2 and the results expressed as blocking percentage (OD%). A sample was considered positive if the OD was below 44%.

2.14. Acute phase proteins

Plasma levels of haptoglobin and C-reactive protein (CRP) were determined by a sandwich ELISA as previously described (Heegaard et al., 2009; Stadejek et al., 2017).

2.15. Statistics

Unpaired *t*-tests with 95% confidence interval and two-sided ANOVA tests were performed for the comparisons of responses between groups using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

3.1. Case samples were positive for PRRSV-2 and shared high level of genetic identity

All nine lungs from 10 day-old piglets collected during the initial outbreak in November 2010 were found positive for PRRSV-2 by real-time RT-PCR and also the lungs from two fetuses and three stillborn piglets and pleura from one stillborn piglet collected in February 2011 tested positive for PRRSV-2. Similarly, lungs from one stillborn piglet and nine mummified fetuses that were collected in the farm in March 2011 were positive for PRRSV-2, however, the Ct value was rather high (Ct > 39).

The ORF5 and ORF7 sequences from case PRRSV-2 positive samples obtained in November 2010 were more than 99.2% identical between animals. The pairwise nt sequence identity for ORF5 and ORF7 of these viruses to Ingelvac PRRS MLV vaccine were 96.9-97.4% and 98.4-98.7%, respectively. Alignment of the deduced aa sequence of ORF5 revealed up to 10 aa differences in the glycoprotein 5 (GP5) of the case viruses compared to the vaccine strain (Supplementary Fig. 1). Since some of the aa positions in GP5 (e.g. G3, Y24, and N35) were identical to variants present in the HP-PRRSV strains from China (Supplementary Fig. 1), a partial fragment of 772 bp of the coding region of NSP2 covering the region containing the Chinese HP-PRRSV typical deletions were sequenced and compared to three Chinese HP-PRRSV strains. This analysis revealed that the case viruses did not harbor the typical 1 plus 29 aa deletions of the HP-PRRSV strains (Tian et al., 2007), but instead harbored a 3 aa deletion 28 aa downstream of the 1 plus 29 aa deletion (Supplementary Fig. 2). BLASTp searches of GenBank did not reveal any published sequences harboring this 3 aa deletion in nsp2 of PRRSV-2 but in addition to this deletion, several aa differences were present. The

pairwise partial nsp2 aa sequence similarities to the vaccine strain was 89.75–91.39%.

Comparison of ORF5, ORF7 and partial NSP2 sequences obtained from viruses from the initial infection in November 2010 to the sequences obtained from mummified fetuses and stillborn piglets sampled approximately 12 weeks later (February 2011) confirmed that it was the same virus strain that had persisted in the herd. The pairwise nt sequence identities were 99.3–99.8%, 99.7–100%, and 99.3–100%, for ORF5, ORF7, and partial NSP2 respectively. The aa alignments further confirmed the similarity of these viruses with 98.5–100%, 100%, and 98.8–100% pairwise aa identities in GP5, N, and partial nsp2 respectively.

Complete PRRSV genome sequences were obtained from viral RNA extracted from lung tissue and pleura from pigs submitted in November 2010 (DK-2010-10-13-1) and February 2011 (DK-2011-88005-A8-PI), respectively. The complete genomes were 15,402 nt long (excluding the poly(A)-tail) and the pairwise nt identity between the two genomes was 99.7%. The identity to the vaccine strain was 97.8–97.9%. Apart from the 3 aa deletion in nsp2 no further deletions were found. Thus, only limited genetic drifts occurred during the outbreak.

3.2. The genomic sequences of DK-2010-10-13-1 and DK-1997-19407B were very similar

The genetic identity between the two full genomes DK-2010-10-13-1 and DK-1997-19407B used as inoculum in the experimental infection study was 97.6%. In detail, they shared 96.7%, 98.7%, and 93.9% pairwise nt identity in ORF5, ORF7, and partial NSP2 respectively. In comparison, the full genome of DK-1997-19407B shared 99.4% identity to the vaccine strain with 98.6%, 100%, and 99% similarity in ORF5, ORF7, and partial NSP2 respectively. Thus, the strain was highly similar to the typical PRRSV-2 strains isolated in Europe during the last 15 years.

3.3. The challenge strains induced limited changes in clinical signs and body temperature and induced limited pathological changes in the lungs

Generally, all pigs remained healthy throughout the experimental period, and only few deviations from normal health were observed (data not shown). On DPI 18, Pig no. 7 (DK-1997-19407B) was slightly lethargic, had increased respiratory rate when eating and increased body temperature (40.2 °C). Short (13 days) episodes of semi-liquid feces were observed occasionally in all groups. Slightly increased body temperatures of short durations were observed on two occasions for group 4 (Fig. 1A) and could be related to restraint of pigs or to the episodes with semi-liquid feces. There were no statistical differences between the groups.

At necropsy, no lesions were seen in the control pigs and only minor lesions were observed in the inoculated pigs. Thus, in pigs inoculated with DK-1997-19407B, focal lobular consolidation in the cranial lung lobe ($n = 1$) and slightly enlarged tracheobronchial lymph nodes were seen ($n = 4$). Pig no. 7 was pale and had chronic adhesive pleuritis and pericarditis, and the spleens of three pigs were mottled with slightly depleted margins. In pigs inoculated with DK-2010-10-13-1, findings were mildly enlarged tracheobronchial lymph nodes ($n = 2$) and mottled spleens with slightly depleted margins ($n = 2$). Among the vaccinated pigs, small areas of condensation were seen in the cranial lung lobes ($n = 2$). In the dorsal lung lobes ecchymotic hemorrhages ($n = 1$) and areas of collapsed lung tissue (atelectasis) ($n = 1$) were present. Two pigs had slightly depleted margins of the spleen.

Histopathological examinations revealed only minor lesions. All pigs had mild to moderate hyperplasia of the bronchus-associated lymphoid tissue and areas of slightly thickened alveolar septa, which was regarded as a normal pulmonary response to environmental factors (Hansen et al., 2010). One of the control pigs (no. 3) had mild focal non-suppurative bronchopneumonia. Among the pigs inoculated with

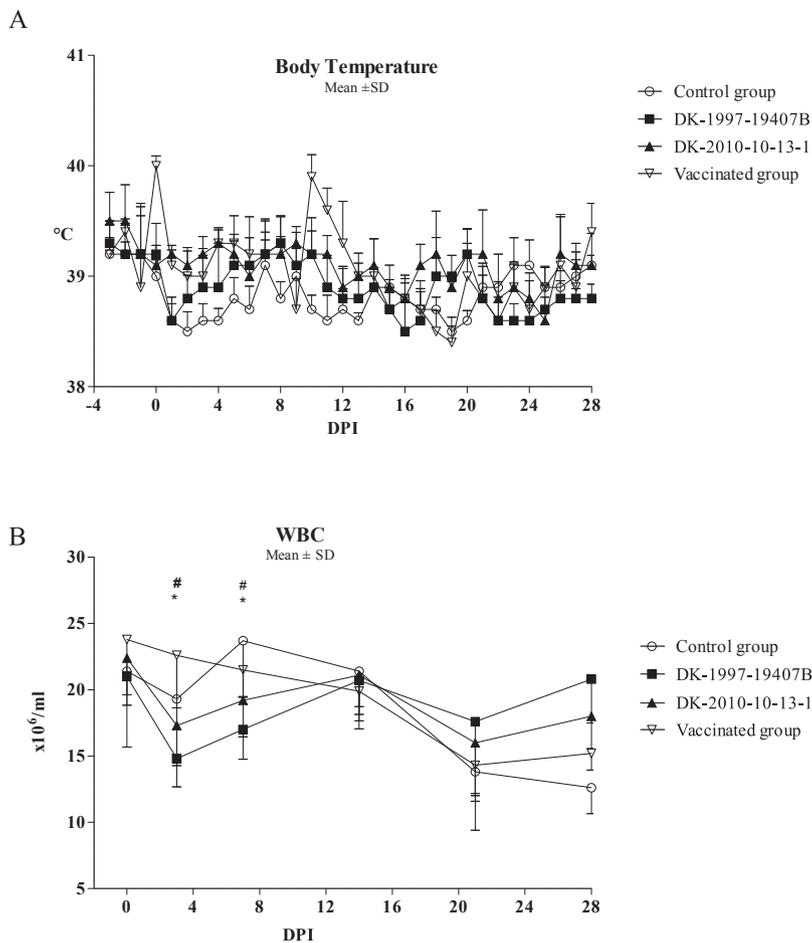


Fig. 1. Variations over time in body temperatures (A) and absolute numbers of total WBC (B) of uninfected control pigs (N = 4), DK-1997-19407B (N = 8) and DK-2010-10-13-1 (N = 8) and vaccinated (N = 8) pigs following challenged with two different strains of PRRSV. DPI: day post infection. There were no statistical differences in body temperature between the groups. * And # indicate statistical differences ($p < 0.05$) between the WBC counts of the DK-1997-19407B and DK-2010-10-13-1 group compared to the control and vaccinated pigs, respectively.

DK-1997-19407B, one (no. 9) had mild multifocal non-suppurative interstitial pneumonia and another (no. 7) had an abscess, and chronic suppurative bronchopneumonia and pleuritis. In pigs inoculated with DK-2010-10-13-1, mild multifocal non-suppurative interstitial pneumonia was seen (nos. 13, 14, 18). Findings among the vaccinated pigs were mild multifocal non-suppurative interstitial pneumonia (no. 21) and focal peracute suppurative bronchopneumonia (no. 24). Thus, overall the challenge strains induced weak clinical signs and limited pathology.

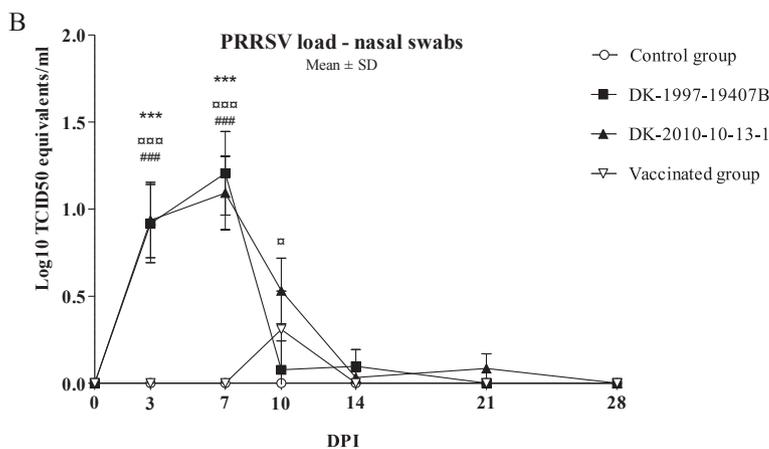
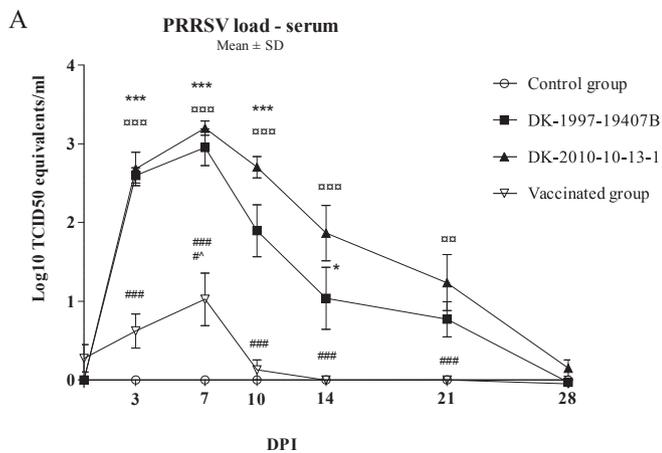
3.4. Challenge induced typical changes in WBC

The numbers of WBC counts developed almost in parallel for pigs inoculated with DK-1997-19407B and DK-2010-10-13-1 and for the control and vaccinated pigs, respectively (Fig. 1B). The decreased levels of WBC seen for pigs inoculated with DK-1997-19407B or DK-2010-10-13-1 at DPI 3 and 7, most pronounced for pigs inoculated with DK-1997-19407B, represented significant reductions of absolute lymphocyte counts compared to the control and vaccinated pigs ($p < 0.05$). This was characterized by significantly reduced levels of B cells in pigs inoculated with DK-1997-19407B or DK-2010-10-13-1, together with decreased numbers of CD8+ cells (T cells and NK cells) in pigs inoculated with DK-1997-19407B, only (data not shown). At DPI 14, WBC values for pigs inoculated with DK-1997-19407B or DK-2010-10-13-1 had returned to DPI 0 levels. The WBC counts of control and vaccinated pigs gradually decreased throughout the experimental period. Generally, the percentages of individual lymphocyte subpopulations did not change during the period (data not shown).

3.5. Both challenge strains induced viremia and nasal shedding of PRRSV, but vaccination had a clear effect on the level of virus

To compare the viral load of the pigs in the different challenge groups, the levels of PRRSV virus were quantified by real-time RT-PCR in all pigs inoculated with PRRSV (DK-1997-19407B, DK-2010-10-13-1, and vaccinated group). The viremia peaked around DPI 7 in all inoculated pigs (Fig. 2A), but the viral load was significantly lower in the vaccinated pigs compared to the DK-1997-19407B group at DPIs 3, 7, 10, and 14 and between the vaccinated group and the DK-2010-10-13-1 group at DPIs 3–21 (Fig. 2A). In pigs inoculated with DK-1997-19407B or DK-2010-10-13-1, the mean viral load reached approximately $3 \log_{10}$ TCID₅₀/ml equivalents and PRRSV remained detectable until the end of the experiment at DPI 28. The mean viral load was higher in the group of pigs inoculated with DK-2010-10-13-1 than with DK-1997-19407B at DPI 10 and 14 although the differences were not statistically significant. Low levels of PRRSV were detected at DPI 0 in serum from 4 out of 8 pigs in the vaccinated pigs and these results were reproduced from a second RNA extraction from the same serum samples. None of the other pigs were positive for PRRSV by real-time RT-PCR at DPI 0.

To investigate the shedding of virus in the different groups, the level of virus was quantified in nasal swab samples. Virus shedding was detected in all pigs in the inoculated non-vaccinated groups from DPI 3 and peaked with approximately one \log_{10} TCID₅₀/ml equivalent at DPI 7 (Fig. 2B). In the group of pigs inoculated with DK-1997-19407B, virus was detected until DPI 7 in three pigs, until DPI 10 in four pigs, and until DPI 14 in one pig. The DK-2010-10-13-1 inoculated pigs were positive for virus in nasal swab samples until DPI 10 for six pigs, one pig was positive until DPI 14 and one pig had detectable levels of virus at



DPI 21. In the vaccinated pigs, only two out of the eight pigs showed detectable levels of virus in nasal swabs at DPI 10. Thus, the vaccinated pigs had decreased virus shedding compared to the two non-vaccinated groups. There was a significant differences in virus shedding between the vaccinated group and the DK-1997–19407B and at DPI 3 and 7 and between the vaccinated group and the DK-2010-10-13-1 group at PID 3, 7 and 10 (Fig. 2B).

Virus load in lung tissue samples were quantified by real-time RT-PCR and the TCID50/ml equivalents calculated. All lung samples from all control animals were negative by real-time RT-PCR (Fig. 3). In pigs inoculated with DK-1997–19407B or DK-2010-10-13-1, PRRSV was

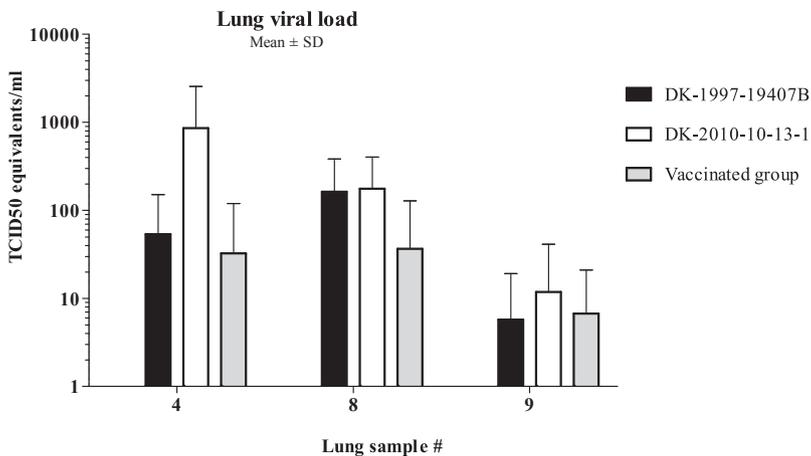


Fig. 2. Mean viral load (log₁₀ TCID₅₀ equivalents/ml) (± SD) after challenge in serum (A) and nasal swabs (B) quantified by real-time RT-PCR. DPI: Day post infection. N = 4 for the control group and N = 8 for the remaining three groups. *, **, *** Indicate significant difference between the control group and the DK-1997–19407B group at 0.001, 0.001 and 0.05 level, respectively. Similarly, □, □□, □□□ indicate significant differences between the control group and the DK-2010-10-13-1 group. #, ##, ### indicate significant differences between DK-2010-10-13-1 and DK-1997–19407B and the vaccinated group. #^ indicate a significant difference (p < 0.05) between the control group and the vaccinated group. No legends indicate that there were no significant differences.

detected in all tested lung samples (i.e. from right apical (4) and the intermediate (8) and diaphragmatic (9) lobes), except for lung samples number 8 and 9 from pig number 8 inoculated with DK-1997–19407B (Fig. 3). For the vaccinated animals, all pigs had at least one positive lung sample, but in total, five of the individual lung samples were negative. The highest virus load was detected in right apical- and the intermediate lobes and in general the amount of virus was highest in unvaccinated animals challenged with DK-2010-10-13-1 and lowest in the vaccinated pigs challenged with this virus (Fig. 3). No significant differences in virus load in the lungs were detected.

Four lung samples from pigs inoculated with DK-1997-19407B, four

Fig. 3. Mean viral load (log₁₀ TCID₅₀ equivalents) (± SD) at necropsy (DPI 29/30) in the right apical (4), intermediate (8) and the diaphragmatic lung lobes (9). Lung samples from control animals were PRRS virus negative (data not shown). No significant differences were seen between the groups.

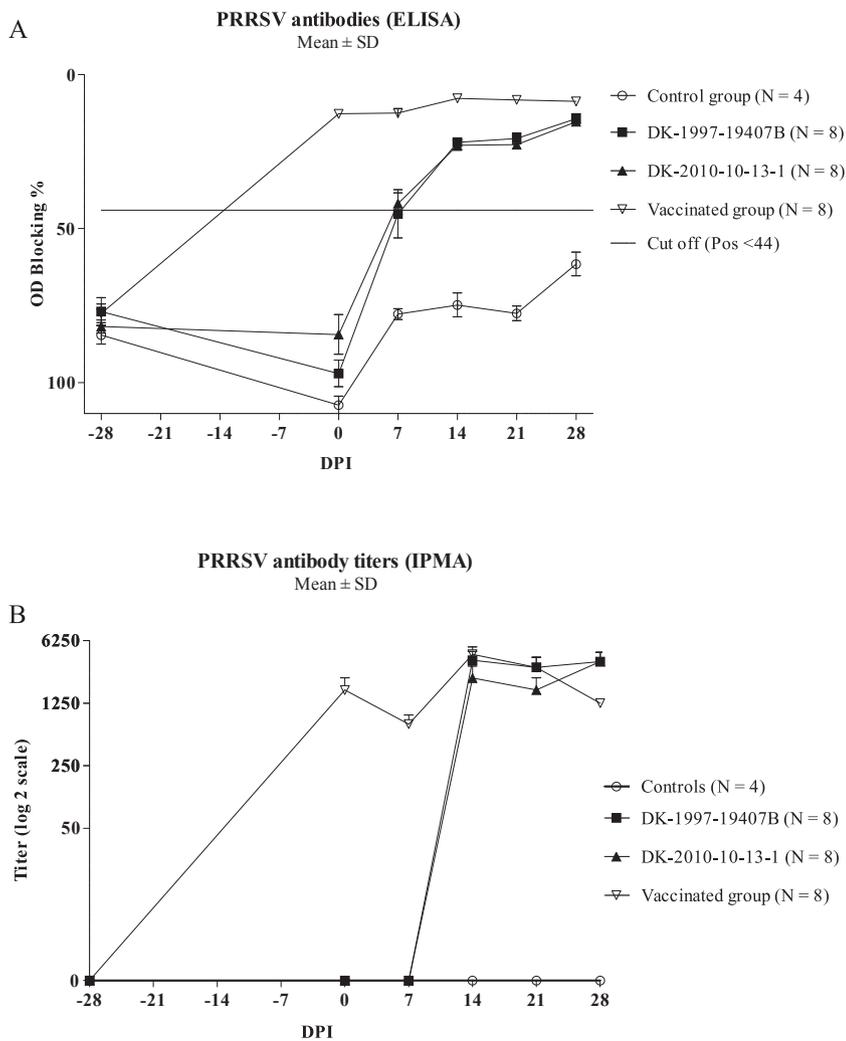


Fig. 4. Test of serum samples for PRRSV antibodies. (A) PRRSV antibody level (means \pm SD) in serum determined by blocking ELISA. A sample was positive when the blocking percentage was below 44% (cut off shown by the black horizontal line). (B) Antibody titers (means \pm SD) measured by immunoperoxidase monolayer assay (IPMA). The serum samples were tested in five five-fold dilutions (1:50–1:6250) and the results expressed as the highest dilution giving a positive signal. DPI: day post infection.

samples from pigs inoculated with DK-2010-10-13-1, and two samples from vaccinated pigs had \log_{10} TCID₅₀/ml equivalents above 2.40 and were tested for infectious PRRS virus by inoculation in Marc-145 cells to investigate if the PRRSV virus detected in the lungs by RT-PCR represented live virus. As controls, two PRRSV PCR negative lung samples from control animals were tested. Nine out of the 11 samples from infected pigs were positive by culturing whereas the remaining two samples and the samples from the control animals were negative. Thus, the lungs indeed contained infectious virus at necropsy.

3.6. Challenge of pigs with PRRSV led to seroconversion and induction of acute phase proteins

All animals were negative for PRRSV antibodies by ELISA at day –28 and only vaccinated animals had antibodies at DPI 0 (Fig. 4A). All pigs inoculated with DK-1997-19407B or DK-2010-10-13-1 seroconverted at DPI 7 followed by a steady increase in level of antibodies that remained high until the end of the study at DPI 28. A similar development in IPMA titers was observed (Fig. 4B) however, the pigs inoculated with DK-1997-19407B or DK-2010-10-13-1 seroconverted at DPI 14 in IPMA compared to DPI 7 in ELISA.

The acute phase responses were included as an objective indicator of the severity of infection. Results from measurements of the acute phase proteins C – Reactive Protein (CRP), and haptoglobin are shown in Fig. 5A and B, respectively. The control pigs and PRRSV inoculated pigs showed a clear CRP response following inoculations at DPI 0, peaking on DPI 7 and with a second peak at DPI 21 (only for pigs

inoculated with DK-1997-19407B). Similarly, haptoglobin peaked at around DPI 10 for pigs inoculated with DK-1997-19407B or DK-2010-10-13-1. The CRP and haptoglobin responses of the pigs in the vaccinated group were similar to the responses of the pigs in the sham inoculated control group and showed some random increases and decreases throughout the observation period.

There was a significant difference in haptoglobin concentration between control pigs and pigs inoculated with DK-1997-19407B when using an unpaired *t*-test with 95% confidence interval ($p = 0.04$) (Fig. 5B). No other significant differences were observed between the groups.

4. Discussion

The aim of the present study was to perform a genetic and biological characterization of PRRSV isolated from a herd with severe reproductive and respiratory clinical signs and to compare the virulence of the virus to an early Danish PRRSV-2 isolate under controlled experimental conditions. Although the case strain DK-2010-10-13-1 had some unique genetic features it was overall very similar to contemporary and earlier Danish PRRSV-2 viruses belonging to clade 5.1 (Kvisgaard et al., 2013a; Shi et al., 2010). Furthermore, the virus induced no overt clinical signs or pathology in pigs following intranasal inoculation.

Terms such as “high and low pathogenicity”, “high and low virulence” and “hot strains” are often used interchangeably to allocate PRRSV isolates into pathotypes, but efforts to link specific genetic

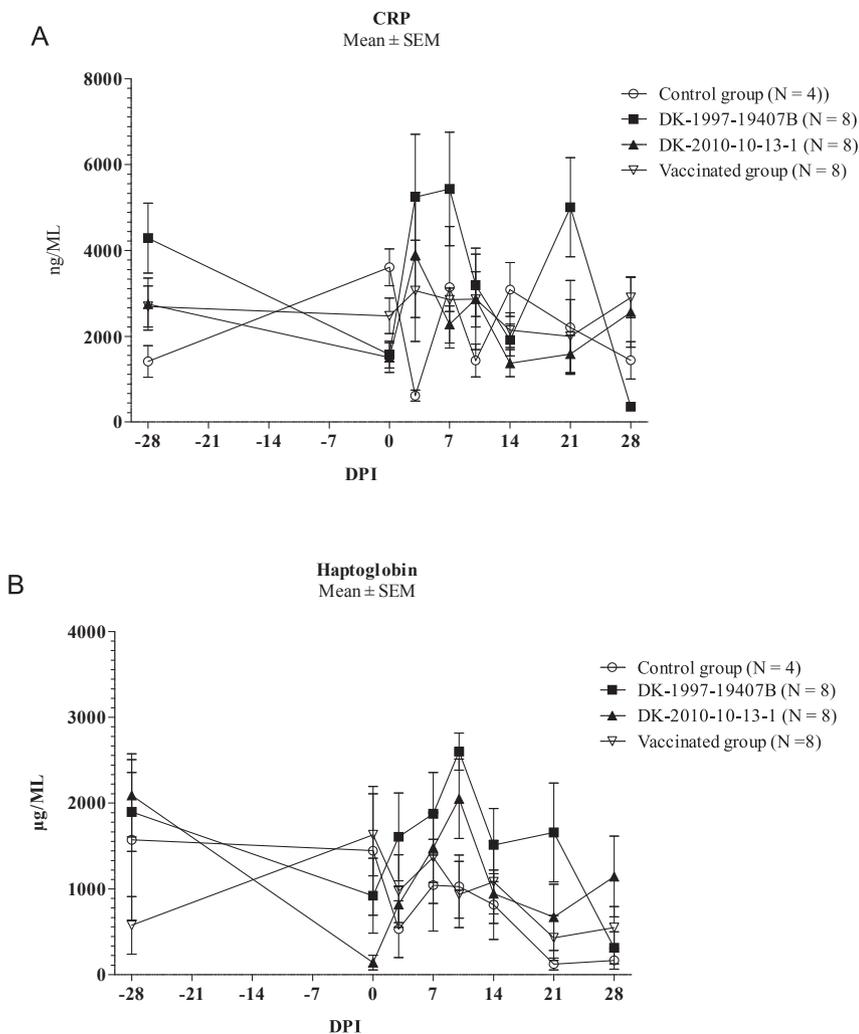


Fig. 5. Levels of acute phase proteins. Serum concentration of C-reactive protein (CRP) (A) and haptoglobin (B) in the four experimental groups expressed as group means (\pm SEM). DPI: day post infection.

motifs to different biological and immunological features have so far been unsuccessful. Thus, until now no clearly defined molecular marker of virulence has been established for PRRSV (Opriessnig et al., 2002; Wu et al., 2009). The 1 + 29 aa deletions seen in nsp2 of the so-called high pathogenic PRRSV strains in China were initially believed to be a virulence marker, however, experimental infections performed with chimeric infectious clones and the finding of a low pathogenic field virus also harboring the same deletions have subsequently shown that the 1 + 29 aa discontinuous deletion alone was not directly related to virulence (Li et al., 2010; Zhou et al., 2009). The nt sequences revealed that the DK-2010-10-13-1 isolate used as inoculum was 97.4% and 94.8% identical to the Ingelvac® PRRS MLV vaccine strain in ORF5 and partial NSP2, respectively. The pairwise nt identity between the sequences obtained from the viruses from the initial infection and those obtained approximately 12 weeks later were 99.3–99.8%, 99.7–100%, and 99.3–100% identical in ORF5, ORF7, and partial NSP2, respectively. Taken together, these results documented that the same virus was isolated 3 months apart and that this strain was less than 5% different from the vaccine strain. The strain belonged to the same cluster as PRRSV-2 normally found in Denmark (clade 5.1) (Kvisgaard et al., 2013a) which are all related to the Ingelvac® PRRS MLV vaccine strain. The level of identity between the two strains used for inoculation was overall 97.6%, approximately 96% in ORF5 and 94% in NSP2. The DK-2010-10-13-1 strain had a deletion of three aa in nsp2 not previously described for any PRRSV-2 strains (Kvisgaard et al., 2013a). This unique 3 aa deletion has subsequently been shown also to be present in PRRSV-2 samples collected from 6 out of 14 other Danish pig herds

(accession no. KF311033-57). Five of these samples were collected in 2010 and 2012 and one was collected in 2003. It is not known if these herds experienced severe clinical signs so the use of this 3 aa deletion as a predictor/marker of virulence is not supported by the available data.

Results from previous studies support the concept that the magnitude and duration of viremia is the best indicator of the virulence of a given PRRSV strain, however, a direct quantitative relationship between virulence and viral load has not been established (Haynes et al., 1997; Johnson et al., 2004; Weesendorp et al., 2013). In the present study, the level of virus in serum was higher from DPI 3 and onwards in pigs infected with the DK-2010-10-13-1 virus in comparison to the older Danish DK-1997–19407B virus and the virus load in lung tissue samples also tended to be higher. Furthermore, the viremia was shorter and less pronounced than described for other strains of PRRSV causing significant clinical disease and mortality in experimental trials (Johnson et al., 2004; Weesendorp et al., 2013). On the contrary, a more pronounced reduction of B- and CD8+ cells were seen in animals inoculated with the older Danish isolate DK-1997-19407B, indicating that this infection may compromise immune functions more severely than the DK-2010-10-13-1 virus. Thus, it is unlikely that the differences in duration and magnitude of viremia between the case strain and an older Danish PRRSV-2 virus can explain the severe clinical signs and mortalities seen in the herd from which this virus was isolated. With respect to pathology, no obvious differences were seen between the four groups and in general the changes were mild. This is in accordance with previous findings after experimental infection with PRRSV-2 (Halbur et al., 1995).

Neither overt clinical signs nor mortalities related to the PRRSV infection were seen in the present experimental study albeit the pigs were indeed infected as documented by the significant acute phase protein responses, the viremia, and the development of antibodies against PRRSV. There are many possible explanations for differences in clinical impact in the field and in experimental set up's. Indeed, others have previously failed to provoke severe clinical disease in growing pigs (Halbur et al., 1996) probably because the clinical impact are influenced by other factors such as management, and co-infections with swine influenza virus, PCV2, Mycoplasma and other bacterial infections (Harms et al., 2001; Schmitt et al., 2001). Indeed, the temporary decrease of absolute lymphocyte and B cell numbers in both unvaccinated groups of pigs indicated a state of immunosuppression following infection, which may prime the animals for secondary infections in the field.

The influence of other factors on the impact of PRRSV infection is also supported by observations from the field where a large variation in severity of outbreaks between herds are observed. The age of the pigs may also influence the outcome of the experimental study since the pigs in the present study was older than the pigs showing the most severe clinical signs in the herd. Furthermore, the applied model using non-pregnant pigs does not evaluate the impact on reproductive parameters. The impact of PRRSV on pigs may also depend on the breed and sex of the animals, however, it is doubtful that these parameters were responsible for the different outcome of the experimental trial and the field infection since pigs of both sexes were included in the experiment and the breed of the pigs used in the experimental study were Landrace X Yorkshire X Duroc cross-breeds which are similar to most Danish herds.

Vaccination of the pigs with a modified live PRRSV vaccine had a clear reducing effect on virus load in serum and lungs, viral secretion and acute phase responses despite the fact that the difference between the vaccine isolate and the case virus ranged from 3 to 6% in some genes.

In conclusion, the PRRS virus isolated from a herd experiencing severe clinical signs and significant mortalities, failed to induce overt clinical signs or pathology in a respiratory pig model. Full genome sequencing of the isolate revealed that the isolate was very similar to other Danish PRRSV-2 isolates. Combined, these data indicated that the severe disease seen in the field was caused by other factors than the PRRS virus. This confirmed that clinical impact in the herds is an unreliable indicator of virulence. Early identification of emerging PRRSV isolates with increased virulence is indeed very important for the further control of PRRSV, but it is too resource demanding to perform experimental infections each time such events are encountered in the field. Thus, research aimed at identifying molecular virulence makers of PRRSV are highly needed for fast clarification when suspicion of hot strains are reported from the field.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.vetmic.2017.10.001>.

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