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Pathogenicity of three genetically diverse strains of PRRSV Type 1 in specific pathogen free pigs

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ABSTRACT

Studies from Eastern European countries proved that porcine reproductive and respiratory syndrome virus Type 1 (PRRSV-1) harbours high genetic diversity and that genetically divergent subtypes 2–4 circulate in this area.

In the present study, we compared the pathogenicity of two different PRRSV-1 subtype 2 strains and a strain representing PRRSV-1 subtype 1. Four groups of 8-week-old specific pathogen free pigs were either infected with subtype 2 strain ILI6, subtype 2 strain or BOR59, subtype 1 strain 18794, or mock inoculated.

The most pronounced clinical signs were observed in pigs infected with BOR59. Pigs from both subtype 2 strain infected groups exhibited significantly elevated mean body temperatures on DPI 2 compared to the other two groups, the difference remaining significant up to DPI 13 for the BOR59 group, only. The pigs in the latter group also displayed significantly highest levels of early viremia together with the most rapid APP response.

Overall, the results indicated that BOR59 strain can be considered a highly pathogenic strain, similarly to subtype 3 strains Lena and SU1-bel, while the virulence of the other subtype 2 strain ILI6 was intermediate between BOR59 and subtype 1 strain.

1. Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) belongs to the *Arteriviridae* family within the order *Nidovirales* (Faaberg et al., 2012). PRRSV is the cause of severe respiratory and reproductive disease in swine worldwide. The virus emerged as a swine pathogen in North America and Europe nearly simultaneously in the period 1980–1990 (Zimmerman et al., 2012). Quickly, nucleotide sequence comparisons of the prototypical isolates Lelystad virus and VR-2332 revealed that the European (EU) and North American (NA) isolates were only distantly related. Later, the EU and NA genotypes were officially designated as Type 1 (PRRSV-1) and Type 2 (PRRSV-2), respectively (Faaberg et al., 2012). Following the expansion of our understanding of arterivirus evolution and discovery of many new genera in the family of *Arteriviridae*, it was recently proposed to separate PRRSV-1 and PRRSV-2 into two different species (Kuhn et al., 2016).

The results of bioinformatic analysis suggested that PRRSV existed at least 100 years back in time (Forsberg, 2005), and the most recent common ancestor of PRRSV-1 emerged in 1947–1968 (Ronald Forsberg, personal communication), or even earlier (Nguyen et al., 2014).

Initially, PRRSV-1 was thought to be genetically homogenous, but studies from Italy, Lithuania, Latvia, Belarus and the Russian Federation established that PRRSV-1 is even more diverse than PRRSV-2 (Forsberg et al., 2002; Le Gall et al., 1998; Stadejek et al., 2006, 2008, 2013, 2002; Suarez et al., 1996).

Previously, it has been suggested that the high genetic diversity in PRRSV-1 ORF5 and ORF7 sequences warranted definition of subtypes

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(Stadejek et al., 2006, 2008). Currently, subtypes 1 (Lelystad virus-like), 2, 3 and 4 are recognized and tentative evidence has been found for potential additional subtypes (Stadejek et al., 2013). In contrast, multiple genetic clades have been defined in PRRSV-2 but they comprise a degree of sequence diversity found in PRRSV-1 subtype 1 lineages alone (Shi et al., 2010).

The genetic subtyping or clustering the strains of PRRSV may be affected by genetic recombination between the strains of the same genotype, and the classification of viruses based on the analysis of small genetic fragments should not be overrated (Brar et al., 2014; Franzo et al., 2015; Lu et al., 2015; Martin-Valls et al., 2014; Shi et al., 2010; Zhao et al., 2015).

It has been shown that, in terms of virulence, antigenic characteristics and immunological responses following experimental infection, the Belarusian Lena subtype 3 strain differed from subtype 1 strains from Western Europe being generally more pathogenic for pigs (Karniychuk et al., 2010; Weesendorp et al., 2013a, 2014). Later, another subtype 3 Belarusian strain SU1-bel, which was characterized *in vitro* and in *in vivo* challenge experiments also proved to be significantly more virulent than Western European strains (Morgan et al., 2014, 2013; Salguero et al., 2015). At present, no data are available about the biological characteristics of East European PRRSV-1 strains from other genetic subtypes, e.g. of subtype 2 strains, which compose the second most prevalent cluster of PRRSV-1, present from Lithuania and Belarus in the west, to Khabarovsk Krai in the Russian Far East in the east (Stadejek et al., 2008).

The aim of the present study was to compare the pathogenicity of different PRRSV-1 strains by inoculation of groups of specific pathogen free pigs. Based on ORF5 sequence analysis, two strains from Belarus and Russian Federation and one strain from Denmark were classified as subtypes 2 and 1, respectively.

2. Material and methods

2.1. Virus strains

Three PRRSV-1 strains isolated in porcine alveolar macrophages (PAM) cultures derived from PRRS-negative pigs were used in this study. The strain 18794 was isolated from a Danish pig in 1993 (Botner et al., 1994). The strain ILI6 was isolated in 2009 from lung tissue of a Russian weaner pig. The strain BOR59 was isolated in 2009 from lung tissue of a Belarusian pig that died with respiratory disease symptoms. In order to prepare the various inocula, the strains were passaged and titrated in PAM cultures. The inoculum of 18794 was a 5th passage (titre of 5.4 log10 TCID50/ml). The inoculum of ILI6 was a 3rd passage (titre of 6.8 log10 TCID50/ml). The inoculum of BOR59 was a 2nd passage (titre of 4.4 log10 TCID50/ml).

2.2. Challenge experiment

The challenge experiment performed at the BSL3 animal facilities of DTU National Veterinary Institute was carried out in accordance with the Danish legislation on animal experiments (LBK nr 1306 – 23/11/2007) and EU regulations on the use of laboratory animals for research. The pigs used in the experiment were procured from the institute's high health pig production herd, which was tested free from infection with the following pathogens: encephalomyocarditis virus, hepatitis E virus, porcine circovirus type 1 and type 2 viruses, porcine cytomegalovirus, porcine epidemic diarrhoea virus, porcine parvovirus type 1, porcine respiratory coronavirus, PRRSV-1 and PRRSV-2, influenza A virus, transmissible gastroenteritis virus, *Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Brachyspira hyodysenteriae, Brachyspira pilosicoli* and *Brucella* spp., using *in-house* standard diagnostic methods.

Twenty-eight 4-week-old pigs were randomly divided into four groups that were housed separately. After 4 weeks of acclimatization, the pigs (now 8 weeks of age) were inoculated intranasally (i.n.) with 2 ml of virus inoculum in each nostril. Group 1 (pigs 1–7) was inoculated with the strain 18794, group 2 (pigs 8–14) was inoculated with ILI 6 and group 3 (pigs 15–21) was inoculated with BOR59. Group 4 (pigs 23–28) was mock-inoculated with 2 ml of Eagle's medium in each nostril and served as a PRRS negative control group.

Individual pigs were subjected to daily clinical examination and rectal body temperatures were recorded starting from −2 day post infection (DPI). In order to obtain a semi-quantitative measure for comparison of clinical disease between the 4 groups, a scoring system developed by Mittelholzer et al. (2000) and adapted to PRRS experiments was applied. All pigs were evaluated for overall well-being, respiration, eye disorders and appetite. Each parameter was scored as 0 (normal condition), 1 (mild disorder), 2 (moderate disorder) or 3 (severe disorder). The scores for individual pigs were added up to a cumulative clinical score (CS) per day.

Non-stabilized blood samples were collected from the anterior vena cava in 10 ml vacutainers (Venoject; Terumo Europe, Leuven, Belgium) on −2, 0, 3, 7, 10, 14, 21 DPI and at euthanasia. Serum was isolated (left to coagulate for 15 min and centrifuged at 3500 rpm for 10 min at 4 °C) and stored in aliquots at −80 °C for subsequent real time RT-PCR analysis, or at −20 °C for antibody and acute phase protein (APP) measurements. In parallel to the blood samplings, nasal swab samples were collected on the same days from right nostrils. The swabs were placed in 1 ml PBS (pH 7.5) and stored at −80 °C until further analysis with real time RT-PCR.

Euthanasia of the majority of the pigs was performed on 22 DPI (pigs 15, 16, 17, 18 and 19), 23 DPI (pigs 8, 9, 10, 11, 12, 13), or 24 DPI (pigs 1, 2, 3, 4, 5, 6) by intravenous injection of pentobarbiturate (50 mg/kg) followed by exsanguination by cutting arteria axillaris. In order to get additional information on the dissemination of virus in individual pigs during the course of infection, pigs representing the various groups (pigs 7, 14, 20, 21 and 28) were euthanized on 17 DPI. At necropsy, all pigs were subjected to macroscopic evaluation of respiratory tract lesions, and lung tissue samples were collected for real time RT-PCR and histopathological analysis. Lung sections for real time RT-PCR were stored in RNAlater according to instruction from the supplier (QIAGEN) and sections for histopathological evaluation were fixed in 10% buffered formalin.

2.3. PRRSV quantification with real time RT-PCR

Total RNA was extracted from 100 µl serum and nasal swabs in PBS with QIAsymphony RNA Kit automated on QIAsymphony SP extraction robot with the protocol CT 400 V5, according to instructions provided by the supplier (QIAGEN, Denmark). Lung tissue samples stored in RNAlater (QIAGEN) were initially prepared as a 5% homogenates in RLT buffer (QIAGEN) containing 1% β-mercaptoethanol (Sigma-Aldrich) by homogenization on TissueLyser II (QIAGEN, Denmark) at 30 Hz for 3 min and clarified by centrifugation for 3 min at 12,000 rpm. Total RNA was extracted from 600 µl lung tissue homogenate using RNeasy Mini Kit (QIAGEN) with the large sample protocol V2 automated on the QIAcube (QIAGEN) according to instructions from the supplier. The RNA was stored at −80 °C until use.

In order to quantify PRRSV load in sera, nasal swabs PCR primers and a probe were designed based on ORF2 sequences of 18794, ILI6 and BOR59. The sequences of the primers and the probe were as follows: FW: 5′-TTYGGGTTCACHGTCGCAG-3′; Rev: 5′-GACCTTCGATARTTCGGGAG-3′; Probe: 5′-FAM-CAGAGCGCGAACGGAGAAKCGCG-BHQ1-3′ and were synthesized by Eurofins Genomics (Germany). The PCR reactions contained 300 nM of each primer and 200 nM probe, 1x QIAGEN OneStep RT-PCR Buffer, 0.4 mM dNTP each nucleotide, 1 µl QIAGEN OneStep RT-PCR Enzyme mix and 2 µl RNA in a total volume of 25 µl. Amplifications were performed on Rotor-Gene Q (QIAGEN®) with the following temperature profile: 30 min at 50° C for reverse transcription followed by 15 min at 95° C, and 45 cycles of 15 s at 94° C, 60 s at 60° C and 10 s of 72° C. Fluorescent signals were collected during the extension step at

each cycle in the Green channel and analyzed with Rotor-Gene Q software version 2.0.2 (QIAGEN) setting NTC threshold at 10% and the normalized fluorescence threshold limit at 0.01 for cycle threshold (Ct) value determination, starting normalization from cycle 2. Samples were tested in duplicates and were considered positive when both replicates had Ct below 37. The efficiency of PCR amplification of all three PRRSV strains was between 90 and 100%.

Quantification of viral load in experimental samples was performed against a standard curve constructed from a 5-fold dilution series of 5.4 log10 TCID50/ml of the 18794 strain. The standard curve had a PCR efficiency of 101% (R^2 = 1.01; slope −3.309) covering 9 dilution steps corresponding to 1 − 2.5E + 05 TCID50/ml equivalents (equal to 1.3–5.4 log TCID50 equivalents).

2.4. PRRSV antibody detection

Antibodies in sera from all pigs were analysed using a PRRSV genotype discriminating immunoperoxidase monolayer assay (IPMA) (Sorensen et al., 1997) and a genotype discriminating blocking 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 (titre). In ELISA, the serum samples were tested diluted 1:2 and the results were expressed as blocking percentage (OD%). A sample was considered positive if the OD% was below 44.

2.5. Acute phase protein (APP) quantification

The concentration of haptoglobin (Hp) in serum was determined by a sandwich ELISA using an in-house mouse anti-porcine Hp monoclonal antibody as catching antibody and a commercial rabbit anti-human Hp detection antibody (DAKO) as previously described (Sorensen et al., 2006). The serum concentration of C-reactive protein (CRP) was analysed by a sandwich ELISA using dendrimer-coupled cytidine diphosphocholine (a CRP-binding ligand) in the coating layer as described by Heegaard et al. (2009) employing polyclonal rabbit anti-human antibodies with cross-reactivity towards porcine CRP (DAKO) followed by peroxidase-conjugated goat anti rabbit antibody for detection (DAKO).

2.6. Histopathological analysis

Lung sections fixed in 10% buffered formalin were dehydrated through a graded ethanol and xylene baths and embedded in paraffin wax. Sections of 3–4 μm were stained with haematoxylin and eosin (HE) and microscopic lesions were scored as 0, no lesion; 1, mild; 2, moderate or 3, severe (Table 1). The evaluation included interstitial pneumonia infiltration of eosinophils, and hyperplasia of lymphoid follicles. Interstitial changes were evaluated at magnification 10x (objective lens) and 10x (eyepiece). Infiltration of eosinophils was assessed by the average number of cells observed in 30 different high-power fields (HPF), using a magnification of 40x (objective lens) and 10x (eyepiece) on the area of about 0.015 mm^2 (area of one field of view). Hyperplasia of lymphoid follicles in lung parenchyma and BALT was observed at magnification of 4x (objective lens) and 10x (eyepiece) in one field of view. Only visible and activated lymphoid follicles were counted. The details of scoring are presented in Table 1. The microscopic evaluation was performed in a blinded fashion using a standard light microscope Olympus BX41 and CellSens software (Olympus).

2.7. Statistical analysis

Statistical analysis to compare mean viremia, nasal shedding and acute phase proteins concentration between groups was performed at each sampling point using a one-way ANOVA followed by post-hoc Tukey's test. If the assumptions of normality or equality of variances were not fulfilled (evaluated by Shapiro-Wilk and Levene's test respectively) non-parametric Kruskal-Wallis ANOVA test was applied. Clinical scores and microscopic lesions scores were compared based on Kruskal-Wallis ANOVA test. Calculations were performed with Statistica (Statsoft) software. Differences were considered statistically significant at $p < 0.05$.

Analysis of body temperature was performed using GraphPad In Stat version 3.00 (GraphPad Software, San Diego, CA). Student's *t*-test was used for comparison between means of the infected groups and the control group.

3. Results

3.1. Clinical signs

No clinical signs were observed in the uninfected control pigs. In groups 1 and 2, 3 pigs showed mild lethargy on a few days, only (DPI 6, 9 and 11, respectively) reflected by CS values of 1. All pigs in group 3 exhibited varying degrees of clinical signs characterized by mild lethargy, increased respiratory rate, conjunctival hyperaemia and reduced feed-intake. Starting from DPI 6, cumulative CS values of 1 to 5 were observed for an 8 day period with highest scores (3–5) observed in 4 pigs, all infected with BOR59. Between DPI 6 and 8, cumulative CS in group 3 was significantly higher compared to other groups ($p < 0.05$).

Body temperatures (BT) of all pigs remained within the range of 37.8 °C to 39.6 °C on DPI – 2-0, and BT did not exceed 39.6 °C in any of the controls post inoculation. Thus, BT higher than 39.6 °C were considered to represent fever.

In group 1, only 1 pig had fever of 40.7 °C on 5 DPI. Pigs from groups 2 and 3 exhibited significantly elevated mean BT on DPI 2 compared to pigs from group 1 and 4 ($p < 0.05$) (Fig. 1). Hereafter, the mean BT of group 2 was higher than the controls but not statistically significant at any time point. In group 2, fever with BT ranging between 39.7 °C and 40.5 °C was recorded in individual pigs for 1 to 4 days in the period from 2 to 10 DPI. Two pigs (11 and 12) in group 2 did not show elevated BT. In group 3, all pigs had fever of 39.7 °C to 41.6 °C between 2 DPI and 13 DPI. The duration of fever period was 4 to 6 days for 2 pigs (17 and 18) and 9 to 11 days for 4 pigs (15, 16, 20, 21) with the highest BT recorded for the latter pigs. The mean BT for group 3 pigs were significantly higher compared to the controls from DPI 3 to 13, the highest difference seen on DPI 6 ($p < 0.0001$)

Table 1
Scoring system of microscopic lesions.

Score	0	1	2	3
Interstitial pneumonia	Normal lung	Thickening alveolar septa and proliferation of pneumocytes type II	Honeycomb lung (fibrosis) in < 50% of field of view	Honeycomb lung (fibrosis) in > 50% of field of view
Eosinophil infiltration	0 − 1	2–3	4–5	> 5
Hyperplasia of lymphoid follicles	0 − 5	6–16	16–25	> 25

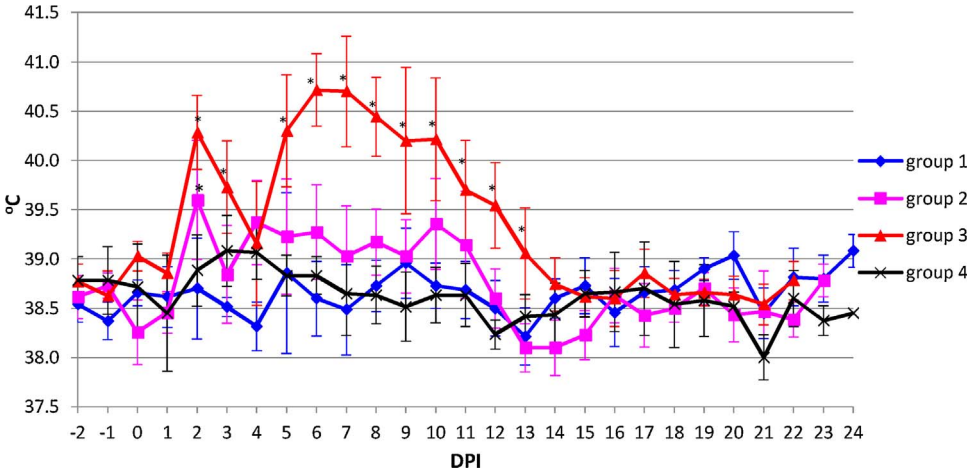

Fig. 1. Body temperature in pigs infected with PRRSV-1 strain 18794 (group 1), strain ILI6 (group2) and strain BOR59 (group 3), and uninfected control pigs (group 4). Data are expressed as mean ± SD. Statistically significant difference at DPI 2 between groups 2, 3 and groups 1, 4 as well as differences between group 3 and remaining groups between DPI 3–13 were marked with asterisk ($p < 0.05$).

3.2. Detection of PRRSV in serum, nasal secretions and lungs, and PRRSV – antibodies in serum

PRRSV was detected in serum in all infected pigs at 3 DPI and the viremia persisted until 14 DPI. All pigs from groups 1 and 3 and one pig from group 2 had low level of viremia at 21 DPI (Fig. 2). Viremia was significantly higher in group 3 infected with BOR59, at 3 DPI and 7 DPI, when in pigs 15 and 17 it reached about 3×10^5 TCID50 genomic copy equivalent/ml. On 10 DPI viremia in groups 2 and 3 was significantly higher than in group 1 ($p < 0.05$). No viremia was detected in group 4.

PRRSV could not be detected in nasal swab samples from any of the control pigs but in most pigs in the 3 infected groups at 3, 7 and 10 DPI (not shown). At 10 DPI only 2 out of 7 pigs from group 1 had virus positive nasal swab samples compared to 7 and 6 pigs in groups 2 and 3, respectively. In pig 12 from group 2 virus was detected also at 14 and 21 DPI. There were no significant differences between the groups in the virus detection levels in nasal swabs, the highest observed at 3 and 7 DPI.

PRRSV was detected in lungs from all inoculated pigs tested with varying Ct-values of 31.5 ± 5.1 (group 1), 30.9 ± 2.3 (group 2) and 29.8 ± 4.2 (group 3). However, the differences between the groups were not significant. PRRSV could not be detected in lung tissue samples from control animals.

All animals seroconverted at 7–10 DPI in both the ELISA and IPMA tests. No differences were seen in the serological profile between the three different strains used for challenge (data not shown).

3.3. Acute phase proteins response

An acute phase protein CRP response was observed as early as at 3 DPI, peaking at DPI 10, in all PRRSV infected groups (Fig. 3A). Globally, during the study the mean CRP concentration ranged from 3.5 µg/ml (± 1.0) to 23.0 µg/ml (± 10.9). BOR59 infected pigs showed earliest increase in CRP levels. In ILI 6 infected pigs, the increase of CRP level was slower and peaked at slightly higher level than in BOR59 infected pigs. Hp levels ranged from 0 to 5.6 mg/ml (± 2.7). An remarkably early response at 3 DPI only in BOR59 infected pigs ($p < 0.05$) (Fig. 3B).

3.4. Pathological examination

No gross lesions of the lungs were observed during necropsy at 17, 22, 23 or 24 DPI.

Microscopically, varying levels of lung lesions were observed in all pigs except one control animal (Table 2). The lesions included proliferation of pneumocytes type II, *mononuclear inflammatory cell infiltration* (mainly lymphocytes and macrophages) and thickening of alveolar septa. In addition, areas of fibroblast proliferation and fibrosis were observed in lung parenchyma ("honeycomb" pattern). The most affected were lungs of pigs 19 (necropsied at 22 DPI), 20 and 21 (necropsied at 17 DPI), infected with the strain BOR59, where fibrosis and cellular infiltration were particularly severe, and alveolar lung structure was completely lost. Similar severity was observed in pig 9 (necropsied at 23 DPI) infected with the ILI6 strain.

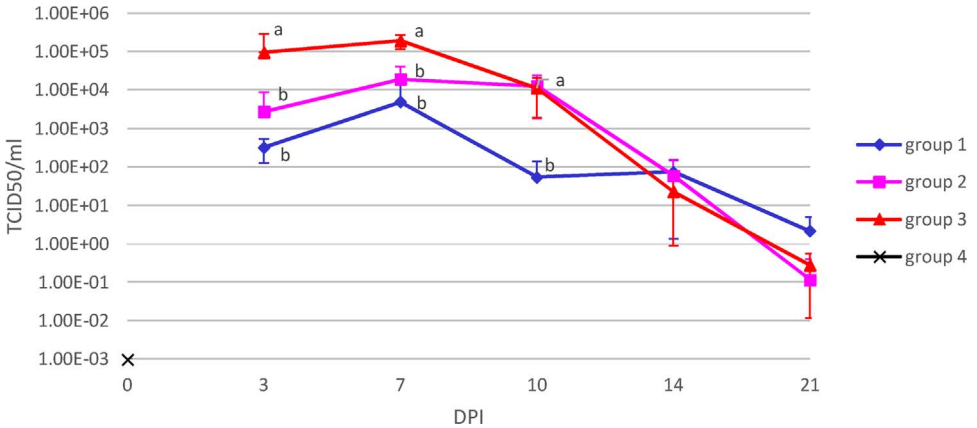

Fig. 2. Viremia in pigs infected with PRRSV-1 strain 18794 (group 1), strain ILI6 (group2) and strain BOR59 (group 3), and uninfected control pigs (group 4). Data are expressed as mean ± SD. Different superscripts denote significant statistical differences ($p < 0.05$).

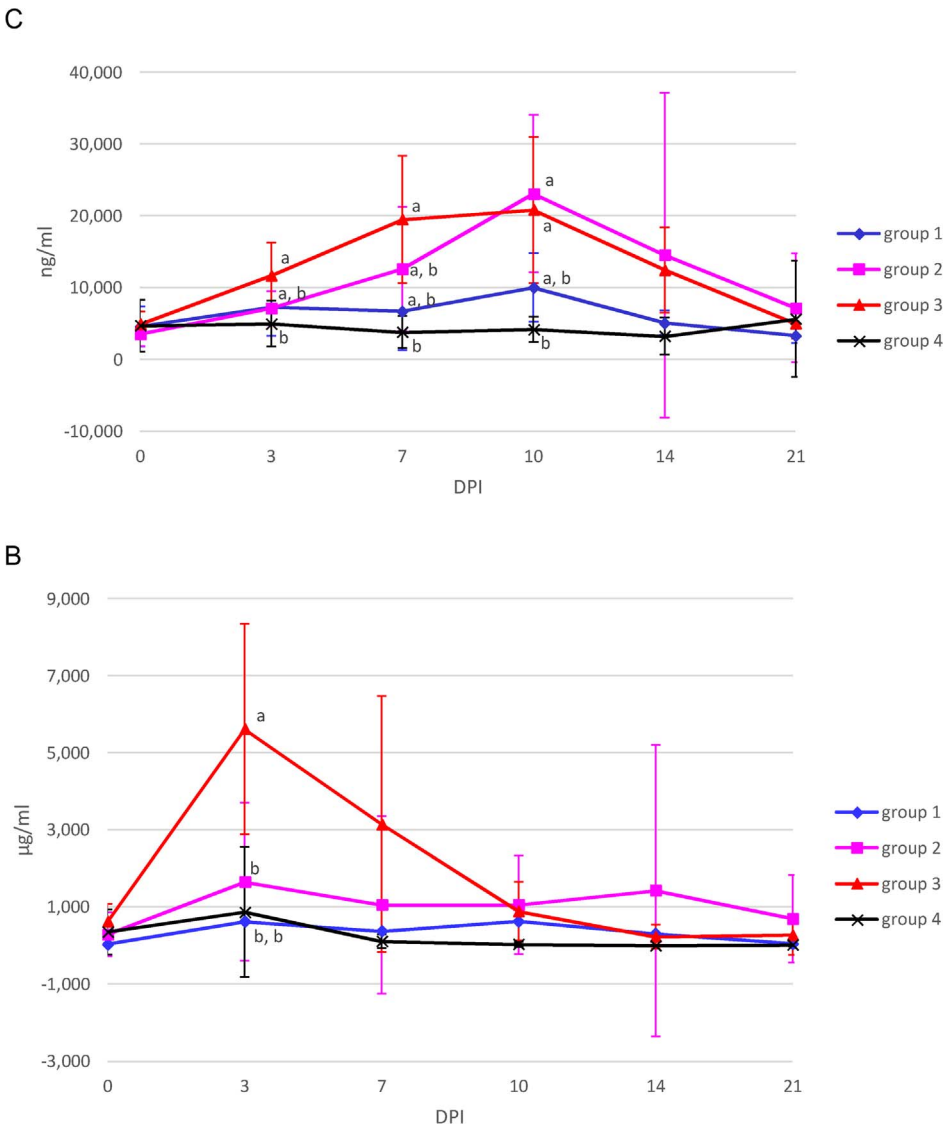

Fig. 3. Concentration of C-reactive protein (A) and haptoglobin (B) in serum from pigs infected with PRRSV-1 strain 18794 (group 1), strain ILI6 (group2) and strain BOR59 (group 3), and uninfected control pigs (group 4). Data are expressed as mean ± SD. Different superscripts denote significant differences ($p < 0.05$).

Greater differences existed between the experimental groups in regard to eosinophil infiltration, where again the lungs from the BOR59 infected pigs 18, 20 and 21, as well as the ILI6 pig 14 were most affected. Eosinophils accumulated around vessels and in the areas with severe fibrosis of the lung parenchyma. Occasionally, degranulated eosinophils were also visible. Also, a small amount of eosinophils was observed in healthy pigs. However, they were evenly distributed and degranulation was not observed.

Hyperplasia of lymphoid follicles was higher in BOR59 infected pigs than in 18794 and ILI6 infected pigs. The highest score was noted in lungs from BOR59 infected pigs 19, 20 and 21, where more than 30 activated lymphoid follicles were observed. In particular, numerous lymphoid follicles were observed in lungs with a high degree of fibrosis and severe cellular infiltration.

The most severe interstitial pneumonia, eosinophil infiltration and hyperplasia of lymphoid follicles were observed in the BOR59 infected pigs 21 and 22 that were necropsied at 17 DPI.

4. Discussion

In the present study, we compared clinical signs, virological parameters, seroconversion, APP response and histopathological lesions in pigs infected with either the Danish strain 18794 from subtype 1, the Russian strain ILI6 from subtype 2, and the Belarusian strain BOR59 from subtype 2, all belonging to PRRSV-1. The results indicated that BOR59 strain can be considered a highly virulent strain, similarly to subtype 3 strains Lena and SU1-bel (Karniychuk et al., 2010; Morgan et al., 2013; Weesendorp et al., 2013a, 2014).

The infection with the Danish strain 18794 did not cause significant clinical signs which is in agreement with the results of earlier studies on PRRSV-1 subtype 1 strains from the Netherlands, United Kingdom, Belgium or Korea (Frydas et al., 2015; Han et al., 2012, 2013; Karniychuk et al., 2010; Morgan et al., 2013; Nielsen and Botner, 1997; Weesendorp et al., 2013a). The mild clinical signs combined with the slightly elevated body temperatures including fever for a few days in pigs infected with the Russian ILI6 strain indicate that this is more virulent than 18794, but less virulent than BOR59, since the pigs infected with the latter strain exhibited more clinical signs, significantly higher body temperatures and more days with fever, together with earlier and higher level of viremia.

Clinical symptoms of PRRSV infection in the field depend on a variety of modifying factors such as differences in genetic susceptibility, environmental factors, immune status, management, virus strain differences or coinfections with other pathogens (Zimmerman et al., 2012).

Table 2
Results of histopathological evaluation of lung samples.

Inoculum	Pig	Necropsy on DPI	Interstitial pneumonia	Eosinophil infiltration	Hyperplasia of lymphoid follicles	Total lesion score
18794	1	24	1	1	0	2
	2	24	1	1	0	2
	3	24	2	1	0	3
	4	24	2	2	1	5
	5	24	2	2	3	7
	6	24	2	2	1	5
	7	17	2	1	1	4
ILI6	8	23	1	1	0	2
	9	23	3	2	1	6
	10	23	2	2	1	5
	11	23	2	1	1	4
	12	23	1	2	1	4
	13	23	2	2	0	4
	14	17	2	3	2	7
BOR59	15	22	1	1	0	2
	16	22	1	2	0	3
	17	22	1	2	1	4
	18	22	2	3	2	7
	19	22	3	2	3	8
	20	17	3	3	3	9
	21	17	3	3	3	9
Control	23	22	1	0	0	1
	24	Not analysed				
	25	23	0	0	0	0
	26	24	1	1	0	2
	27	24	1	2	1	4
	28	17	2	1	2	5

The generally mild clinical disease observed in the present study likely resulted from the use of pigs with a very high sanitary status thus avoiding exaggeration of secondary infections contributing to more overt clinical signs observed in conventional pigs.

Viremia is one of the measures of PRRSV virulence and immediately easier to compare between experiments than clinical signs. In the present study, using quantitative real time RT-PCR, viremia was detected in all challenge groups from 3 DPI to the end of sampling at 21 DPI. At this stage, all pigs infected with 18794 and BOR59 were viremic compared to only one pig infected with ILI6 strain. The significantly higher level of viremia detected in pigs infected with BOR59 at 3 and 7 DPI supports the higher virulence of this strain. However, the occurrence of viremia was analysed on predetermined days only, and as such the picture of viremia may not represent its complete dynamics.

The analysis of APP response performed in the present study provided an important insight into one of the less studied aspects of PRRSV infection. APPs are part of the systemic acute phase response and important components of the innate immune system. The concentration of APP in serum is altered in animals subjected to inflammation, infection or stress. In swine, Hp and CRP are the main APPs. Hp is a major antioxidant protective agent mediating removal of free hemoglobin (Alayash et al., 2013). CRP participates in the systemic response to inflammation. Its plasma concentration increases during inflammatory states, within hours after tissue injury or infection (Black et al., 2004).

The present work showed that the level of APP response can be variable towards different PRRSV-1 strains, likely reflecting the differences in their virulence. Similar differences in APPs profiles displayed by four PRRSV-1, subtype 1 strains were recently described by Saco et al. (2016) who identified strong correlation between APP (in particular Hp) concentration in serum and the severity of clinical signs. Clinical scores in pigs exposed to EU-17 PRRSV-1 strain inducing highest Hp response, and to JA142 PRRSV-2 strain inducing highest CRP response, where highest (Saco et al., 2016). In our study, BOR59 infected pigs showed the earliest increase in CRP levels but both BOR59 and ILI6 infected pigs had significantly higher levels of CRP at 10 DPI compared to pigs infected with the 18794 strain. Striking differences were observed in Hp levels where BOR95 pigs showed an unusual early response at 3 DPI while the response in other groups was absent or minimal (Fig. 3). It corresponds well with the previously reported greater and more common responsiveness of CRP than Hp towards PRRSV infection (Heegaard et al., 2011; Saco et al., 2016). This observation indicates that BOR59 infection is able to stimulate a rapid Hp response, possibly by activating neutrophils to release pre-stored Hp. Such a mechanism has been described for Hp (Theilgaard-Monch et al., 2006) but not for CRP. This finding is another indication that BOR59 is more virulent than ILI6 (and 18794).

It was proposed that the variability in clinical and APP response may be related to the different abilities of different PRRSV isolates to impact interferon and other cytokines production. In PRRSV infections, increased IL-6 levels have been described, while the data on IL-1β and TNF-α are conflicting (Borghetti et al., 2011; Darwich et al., 2010; Weesendorp et al., 2013b). Taking into account that the synthesis of APPs is mainly driven by these three pro-inflammatory cytokines, it is expected that more virulent isolates stimulating higher levels of cytokines will also induce stronger acute phase response and more severe clinical symptoms, as in the case of BOR59, and to a lesser extent ILI6.

Results of pathology evaluation were limited in this study as pigs were only necropsied at 17, 22, 23 or 24 DPI, rather late in the course of infection at stages when advanced recovery from clinical disease was observed. Nevertheless, despite wide range of lesions in each of the experimental groups, BOR59 infected pigs (especially those necropsied at 17 DPI) had generally most evident microscopic lung lesions. As with clinical signs, the lack of gross lesions together with the minor microscopic lesions could result from lack of co-infections in the used pigs of very high sanitary status.

The role of eosinophils in lung pathology remains unclear, but the long term consequences of eosinophil activation can result in increased pathological changes in the lungs. These cells produce eosinophilic cationic protein (ECP), which can damage the RNA virus, they are also able to present antigens to other cells and capable of phagocytosis and cytotoxicity (Giembycz and Lindsay, 1999; Gleich et al., 1986; Jacobsen et al., 2007). On the other hand, eosinophils may modify inflammation in the lungs through cytokines release and they can also promote fibrosis of the lungs by producing factors participating in transformation of fibroblasts into fibrocytes and myofibrocytes (Akuthota et al., 2010; Giembycz and Lindsay, 1999; Jacobsen et al., 2007). In general, the amount of eosinophils in lung tissues was small but highest in pigs infected with BOR59 strain.

In summary, our results suggest that the PRRSV-1 strain BOR59 is more virulent than ILI6 (and 18794), showing a more rapid and more quickly developing infection, as also supported by the detected early CRP and Hp responses. The results indicate that the strain BOR59 can be considered a high pathogenic PRRSV-1 virus, similarly to previously evaluated Belarusian Lena and SU1-bel (Karniychuk et al., 2010; Morgan et al., 2013). Pathogenicity of BOR59 for conventional pigs, as well as a deep genetic characterization of this strain, remains to be assessed. More efforts should be made in Europe and elsewhere to study complete genomes of PRRSV-1 and to study the genetic characteristics of unusually virulent strains, especially from Eastern European genetic subtypes.

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