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ABSTRACT

Highly pathogenic (HP) isolates of the PRRS virus startd emerging in North America and Asia in the late 1990s. More recently, they have emerged in Europe. These isolates are characterized by high viral loads, severe general clinical signs and high mortality, in sows or in weaners and growers. Their genome shows a discontinuous aminoacidic deletion in the non-structural protein 2. This present study was aimed at characterizing the clinical, pathological and immunological features of a highly pathogenetic, Italian PRRSV-1 subtype 1 isolate (PRRSV1_PR40/2014), following experimental infection in conventional 4 week-old pigs. The PRRSV1_PR40/2014 infected group showed severe clinical signs (high fever and dispnoea). Pathological lesions, including severe lymphocytopenia in lymph nodes and the thymus gland, higher serum PRRSV genome copies and lower virus neutralizing antibody titre were observed when compared to homologous virus. The genetic analysis of the strain, and the phenotipic features observed in the field and reproduced in the experimental study, confirmed the high pathogenicity of the Italian PRRSV-1 subtype 1 PR40 isolate.

KEYWORDS

PRRSV-1, highly pathogenic, clinical signs, viremia, immunity

INTRODUCTION

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most important diseases causing significant economic losses to the pig industry worldwide. Two genotypes of the virus have been identified: the European or genotype 1 (PRRSV-1) and the American or genotype 2 (PRRS-2). Genetic analysis based on ORF7 size of a number of European PRRSV-1 isolates shows the existence of four subtypes: Pan-European subtype 1, East European subtypes 2 and 3, and a fourth subtype in Western Europe (Stadejek et al., 2013). A large genetic variability has been shown to exist between and within both genotypes. The intrinsic genomic

instability of the virus and the selective pressure in the field determine continuous genetic changes (Murtaugh et al., 2010), associated also to different patterns of virulence and pathogenicity. In recent years, these genomic changes led to the emergence of “highly pathogenic” (HP) variants (Lunney et al, 2010). These isolates can cause severe disease and death in all ages, including adults. The infection features high viral load in blood and tissues, high fever and severe general and respiratory clinical signs, increased mortality in nursery pigs and growers, and “abortion storm” and mortality in sows (SAMS) (Mengeling et al., 1998). Numerous outbreaks due to isolates with the features of a highly virulent PRRSV have been reported since the late nineties in USA (MN184 prototype strain) (Brockmeier et al., 2012). In 2006 the infection gained more attention, when a devastating "high fever disease" appeared in South-east Asia (An et al., 2011) affecting two million pigs and with death of 400,000 animals (Leedom Larson, 2016). The disease was diagnosed as an “atypical” form of PRRSV (Tian et al., 2007) caused by the prototype JXA1 strain belonging to PRRSV-2. It spread throughout China and Vietnam with huge economic losses for the swine industry. A highly virulent PRRSV (NADC30 strains) also appeared in the USA (Brockmeier et al., 2012) and a NADC30-like strain in China (Li et al., 2016a; Zhang et al., 2016a; Zhao et al., 2015; Zhou et al., 2015). They all carry the same genetic marker MN184.

In Europe, a PRRSV-1 subtype 3 designed “Lena” was involved in outbreaks of severe reproductive and respiratory disorders associated with high mortality in Belarus in 2006 (Karniychuk et al., 2010) and a strain of subtype 2 (prototype Bor) has been shown to be more virulent than subtype 1 (Karniychuk et al., 2010). More recently in Western Europe (Belgium), a HP PRRSV-1 subtype 1 (PRRSV-1.1), prototype 13V091 strain, was reported to possess a highly pathogenic pattern (Frydas et al., 2015). Baased on these reports, it is largely accepted that there is a geographical demarcation between Eastern European areas with highly virulent strains type 1 subtype 3 (Lena-like strains) and Western European areas with low virulent strains type 1 subtypes 1 (Lelystad-like strains). Currently, a major issue of concern is growing evidence of recombination among high virulent/HP-PRRSV isolates in the field (Li et al., 2016b; Zhao et al., 2015), leading to potential complications and limitations to PRRS diagnosis and control (Han et al., 2017).

The main genetic characteristic of highly pathogenic/virulent isolates of both emerging and re-emerging PRRSV-1 and PRRSV-2 is the presence of discontinuous deletions in the NSP2-coding region. This is considered a genetic marker of highly virulent and highly pathogenic isolates. This region is one of the most variable regions of the viral genome, and it is essential for viral replication and is considered an important gene for monitoring the genetic and epidemiological evolution of PRRSV (Bautista et al., 1996; Fang et al., 2004; Fang et al., 2007; Gao et al., 2004; Han et al., 2006; Ropp et al., 2004; Yoshii et al., 2008). Moreover, the relationship between NSP2 variation and biological properties such as PRRSV virulence, pathogenicity, tissue tropism and cell invasion ability has also been the subject of recent study (Chen et al., 2009; Wang and Zhang, 2014; Wang et al., 2013). The NSP2 gene is able to evolve rapidly, possibly due to the selective pressure of the host immune system on the virus (Chen et al., 2010). In general, the instability of the virus is associated

with immune-evasion and, consequently, with the dysregulated or delayed induction of a protective immune response against the virus (Butler et al., 2014). Different PRRSV isolates were observed to affect differently type I IFN-dependent pathway, as well as the pro-inflammatory (TNF-α, IL-1, IL-8) and IFN-γ/IL-10 ratio (Diaz et al., 2012; Darwich et al., 2011; Gimeno et al., 2011, Ferrari et al., 2013; Martelli et al., 2009). Furthermore, there is evidence that HP-PRRSV replicates predominantly in the thymus (Butler et al., 2014), resulting in thymus atrophy with consequent disfunction of host immune regulation.

In 2014, an Italian PRRSV-1 (PRRSV1_PR40/2014) was isolated in our laboratory from an atypical PRRS outbreak in weaners, characterized by an unusuallly long-lasting, severe respiratory and systemic disease (anorexia, persistent high fever) associated with high mortality (up to 50%).
Through challenge in susceptible pigs under experimental conditions, this study aims at (a) confirming the pathogenicity and the clinical features of the Italian isolate (PRRSV-1_PR40/2014); (b) comparing the infection dynamics of this virus with a "conventional" isolate (PRRSV-1_PR11/2014) and a negative/control; (c) verifying whether the differences in virulence/pathogenicity are associated with different virological, serological, pathological and immunological patterns.

MATERIAL AND METHODS

Viruses

Origin

PRRSV-1_PR40/2014, hereafter named PR40, was isolated from a weaner belonging to a commercial farrow to finish 1500-sows herd, experiencing an uncommon PRRS outbreak characterized by high fever, severe respiratory and systemic clinical signs and high post-weaning mortality (up to 50%). Bacterial pathogens were also found in the weaners suffering from respiratory disorders and the outbreak ran out in six months.
The "conventional" virus (PRRSV-1_PR11/2014), hereafter named PR11, was isolated from the lungs of a grower suffering from respiratory disease associated with PRRSV infection. Morbidity and mortality of the outbreak were compatible with a more common field episode of the syndrome.
In both cases, PRRSV was demonstrated firstly by RT-PCR (Martelli et al., 2013) and then isolated on Porcine Alveolar Macrophages (PAMs). The isolates were confirmed by a PRRSV-specific staining on cells and again by RT-PCR (Martelli et al., 2013), and all tested negative for other relevant viruses (PCV2, SIV, ADV).

Sequencing and phylogenetic analysis

The two isolates were submitted to full genome sequence analysis. Total RNA was extracted and converted into first-strand cDNA. Double-stranded cDNA was synthesized and amplified simultaneously with random primers via whole genome amplification method. Shotgun DNA library was constructed from the amplified ds cDNA and subjected to massive parallel sequencing on Illumina MiSeq System at 2x300bp module

following the manufacturer's instruction. The obtained reads were initially trimmed with quality filter and mapped to the reference genome. The mapped reads were assembled into consensus contig for a complete viral genome. Recombination events were determined with SimPlot version 3.5.1.

All PRRSV-1 strains with full genome sequences deposited in GenBank were used to construct the data set. Genetic analyses were conducted in MEGA7. The phylogenetic tree was constructed using the Neighbor-Joining method. The genetic distances were computed using the Kimura 2-parameter model, with 1000 bootstrap replicates.

PRRSV-1_PR40/2014 was obtained with a size of 14,678bp (GenBank accession number MF346695). Genetic analysis showed that PR40 is a PRRSV-1 subtype 1 (PRRSV1.1_PR40/2014). The nucleotide sequence of the ORF5 showed a similarity of 85.9% to Lelystad Virus (LV). For both ORF5 and ORF7 phylogenies, PR40 was grouped within a cluster consisting of Italian sequences, defined in the subtype 1. Two-discontinuous deletions of 42nt (14 aa) and 366nt (121 aa) was identified in the NSP2 region, in particular at aa positions 693-814 and 1061- 1074 of the PRRSV genome (positions are relative to Lelystad virus genome), aa 310-431 and 678-691 of the NSP2. This second deletion is located in the region of nsp2 able to tolerate several 100- to 200-aa deletions, without affect the virus viability and replication (Han et al., 2007). A 6nt (2aa) deletion was observed also in the ORF4 (aa 244-245 of the PRRSV genome). The discontinuous deletion in the nsp2 gene was not due to any recombinant event. From the phylogenetic analysis of the total genome, PR40 resulted in a different lineage and, in particular, in a different branch of a cluster composed by South Korean, Danish and Austrian isolates (Figure 1).

For the other isolate (PRRSV1_PR11/2014) only partial sequences were obtained. Anyway, the strain was confirmed as a PRRSV-1.1 with a similarity of 90,4% in ORF5 to LV.

Study design

Animals and experimental infection

Seventeen 6 week-old conventional pigs from seventeen different litters of a PRRSV-free herd were included in the study. The study was conducted in a biosafety level 2 (BSL-2) facility. No relevant pathogens (PRRSV, SIV, PCV2) were detected in the animals. The animals were randomly allocated in three separate rooms six days prior to inoculation (acclimation period) and assigned to three different groups. PR40 group (PR40): 7 pigs inoculated intra-nasally (IN) with PRRSV1_ PR40/2014 at T=0 (day of inoculation); PR11 group (PR11): 7 piglets inoculated IN with PRRSV1_PR11/2014 at T=0; Control (C): 3 animals inoculated IN with medium only (MOCK/negative control) serving as a reference group for the comparison of the clinical, virological and pathological findings. In inoculated pigs, a dose of 10^5 TCD_{50}/pig in 2 ml, 1 ml in each nostril was administered. Blood samples were collected at the day of inclusion (-6 days post inoculation-dpi) and on days 0, 3, 7, 10, 14, 17, 21, 28 and 35 post inoculation. Sera were separated in two different vials, one stored at –70°C for virus detection and the other at -20°C for PRRSV-specific antibody detection. The

individual body weight was recorded at 0, 21 and 35 dpi. Animals suffering from severe clinical signs with a fatal prognosis were humanely euthanized according to standard protocols. All the survivors were humanely euthanized at 35 dpi, the end of the experiment. At death, necropsies were performed for gross pathology and for organ sampling for histopathology. Tissue samples were stored at -70°C and/or fixed in formalin for further examination.

Clinical monitoring

Daily monitoring was performed from 0 to 35 dpi for rectal temperature (≥40°C=fever), general condition score (appetite: 0-normal, 1-decreased, 2- absent; level of consciousness: 0- normal, 1-compromised/depressed, 2-agonic) and respiratory disease. Respiratory signs were also scored from 0 to 6 as follows: 0 = normal; 1 = mild dyspnea and/or tachypnea when stressed; 2 = mild dyspnea and/or tachypnea at rest; 3 = moderate dyspnea and/or tachypnea when stressed; 4 = moderate dyspnea and/or tachypnea at rest; 5 = severe dyspnea and/or tachypnea when stressed; 6 = severe dyspnea and/or tachypnea at rest (Karniychuk et al., 2010). Stress was induced by holding the pig for 45 sec. At the end of the experiment the sum of the different scores was calculated to obtain the clinical score (0-10). The veterinarian responsible for the daily clinical monitoring was blinded to the treatment groups.

Pathology

Pigs were necropsied in case of death or euthanasia. The presence and the severity of gross lung lesions were evaluated and recorded. Samples from lungs, bronchial lymph-nodes and thymus were collected from each pig for virological, histological and immuno-histochemical analyses. Samples from heart, spleen, liver, lungs, and intestine were collected for bacteriology. Lung tissue areas with gross macroscopic alterations (colour, consistency) were specifically assessed and the proportion of affected lung was estimated.

For histology, tissues (lungs, bronchial lymph-nodes and thymus) were formalin-fixed, dehydrated through graded alcohols and embedded in paraffin wax; sections were stained with a routine haematoxylin and eosin (HE) protocol. The presence of pneumonia (i.e. presence of focal or diffuse alterations with interstitial pneumonia or atelectasia, the extent of infiltration of alveolar septae and perivascular/peribronchiolar area with mononuclear cells) in each slide was estimated. To describe the severity of lesions, a histological score (0=no findings, 1 = mild focal manifestation, 2 = moderate, multifocal manifestation, 3 = moderate diffuse manifestation, 4 = severe diffuse manifestation and 5 = very severe extended manifestation) was used as previously described (Weesendorp et al., 2014). Immunohistochemistry was performed on sections of thymus and bronchial lymph-nodes to differentiate T and B lymphocytes, using primary antibodies anti-CD3 (Rat anti-human CD3 clone CD3-12, AbD Serotec) and anti-CD79α (Mouse anti-human CD79α clone HM57, AbDSerotec), respectively. Target antigen signals were amplified by avidin-biotin complex (ABC) detection method (Vectastain Elite ABC kit of Vector Laboratories) followed by incubation with DAB chromogen substrate (DAKO Cytomation Liquid DAB + Substrate Chromogen System). Slides were counterstained with MAYER's haematoxylin solution. CD3 and CD79α positive cells were enumerated from three different areas

in five microscopic fields per slide at a 20x objective magnification, slightly modified from the protocol described by Weesendorp et al., 2014. The score for lymphocytopenia in lymphoid organs was as follow: 0= normal, 1 = mild lymphocytopenia, 2 = moderate lymphocytopenia, 3 = diffuse lymphocytopenia and 4 = very severe extended lymphocytes depletion.

The pathologists performing pathological investigations were blinded to the treatment group of the animals.

Viremia and virus re-isolation

RNA was extracted from serum by using Trizol LS (Invitrogen), following the manufacturer's instructions. Serum virus RNA copy number was evaluated by using a quantitative real time RT-PCR (qtRT-PCR), as previously described (Martelli et al., 2013).

Viruses used for the infection were re-isolated from sera of infected pigs with the highest copy numbers in qtRT-PCR, by one-passage cultivation on PAMs and adapted to MARC-145 cells. The MARC-145 adapted viruses were confirmed as homologous with the original strains of the infection by sequencing of the ORF5 and ORF7, titrated and used as serum neutralization assay (SN) antigens.

Serology

The measurement of the total antibody response was performed on sera by using the PRRSV-Abs ELISA (HerdChek PRRSV X3, IDEXX Laboratories Inc., Westbrook, Maine, USA) kit, according to the manufacturer's instructions. Samples were considered positive with a sample-to-positive (S/P) ratio ≥0,4.

A virus-neutralization (VN) test against homologous PRRSV isolate (PR40 and PR11) was done as previously described (Kim et al., 2011). The virus neutralizing antibody titre was determined as the reciprocal of the highest dilution that inhibited cytopathic effect in 100% of the well. Serum samples were considered to be positive if the titre was grater than 2.0 (log_2) (Zuckermann et al., 2007).

Data and Statistical analyses

In order to account the not-independency of the repeated observations made on the same subjects, both Mixed Effects Linear models (LMEm, where time was considered a categorical variable, as in a split-plot experiment) and Mixed Effects Generalized Additive models (GAMm, where time was treated as a continuous variable) were applied. The effect of the time (day of the observation), of the treatment (i.e. the estimated difference in viral load or antibody levels between the groups) and their interaction (i.e. the different shape of the response) were considered, as in a split-plot experiment.

The control group was excluded from the regression models, since it was assumed that a difference in response (i.e. cDNA copy number or S/P ratios) between inoculated and un-inoculated subjects existed by design, while the attention focused mainly on the difference between the treatment groups.

The p-values reported are derived from the LME models, where an autoregressive correlation structure was imposed to the residuals. Correction for within-time heteroscedasticity was applied in the analysis of body

weight growth, since the residual variance increased with the weight itself. To samples tested negative by qtRT-PCR, ELISA and VN were assigned a value corresponding to the minimum detectable cDNA copy numbers, S/P value and titer, respectively. Log-transformation of the cDNA copy number and VN titer variable was applied in order to meet the distributional assumptions needed by the regression models. Analyses and graphics were produced with R (version 3.4.0) (R Core Team, 2017), using the packages "nlme" (Pinheiro and Bates, 2000) and "ggplot2" (Wickham, 2009).

RESULTS

Clinical monitoring

Mortality rate was similar in the two infected groups; three pigs per group required euthanasia during the study (PR40 group: at 10, 14, 28 dpi; PR11 group: at 14 (2 pigs) and 17 dpi) due to severely compromised clinical conditions. All the other pigs survived until the end of study.

In PR40 pigs, rectal temperature reached values ≥41°C in all pigs, and, on average, it was above 40 °C from day 5 to day 24 pi (Figure 2a); in PR11 group, pigs showed fever from 7 to 15 dpi. The difference of rectal temperature between the infected groups was significant ($p<0.05$). None of the controls was feverish during the whole experiment.

After 3 and 5 days pi, pigs in PR40 and PR11 groups respectively, started to show mild (clinical score <5) and moderate respiratory signs and lethargy (clinical score >5) (Figure 2b). The average clinical score in animals belonging to PR40 group increased over 7 from day 7 pi. Clinical signs were characterized by severe respiratory disorders associated with dyspnoea and thumping, anorexia and lethargy. This pattern was maintained until the end of the study. During the second week pi, two of the PR40 euthanized animals presented with cyanosis of the ears, nose and belly. The average clinical score between the two groups resulted significantly different ($p< 0.05$). The negative-controls did not show respiratory signs at any time of the experiment, and the rectal temperature was constantly within the normal range.

Body weight and ADWG

The ADWG (g/pig/day) was calculated from the individual weight. Considering the overall study period (0-35dpi) the PR40-ADWG remained the lowest one ($p<0.05$) (Figure 2c). In particular, PR40 pigs showed the poorest ($p<0,05$) weight gain in the first 21 days pi (155g/day), compared to PR11 (256g/day) and control pigs (422g/day). During the period 21-35 dpi, the three groups showed similar ADWG, likely due to the fact that most of the infected pigs with the worse conditions had died by 21 dpi and the surviving infected pigs recovered in the last period.

Pathology

Gross lesions

Pigs in the negative control group had no remarkable lung lesions. Interstitial pneumonia, on th other hand, was consistently evident in both infected groups, independently from the isolate (Figure 3a). In some cases, lesions referable to secondary bacterial infections were also observed. Lungs from the PR40 group showed multifocal to coalescing areas of atelectasia, congestion and alveolar and interlobular edema, with a mottled and multifocal tanned appearance. Lesions were more diffuse and gross lung lesion mean score was higher than 3 in the PR40 group, compared to the PR11 group. In both infected groups, hypoplasia of the thymus was detected. This condition was more marked in the PR40 group, where three pigs out of seven showed an almost complete atrophy of the cervical part of the thymus.

Microscopic lesions

Comparative microscopic investigation of lung sections stained with HE showed different degrees of severity of interstitial pneumonia in both groups, characterized by alveolar septa thickening due to the infiltration of variable numbers of lymphocytes and macrophages (Figure 3a). Interestingly, higher severity was seen in all PR40 pigs. In case of secondary infections, bronchi were filled with bacteria. Bronchial lymph-nodes and thymus gland from PR40 pigs showed, respectively, atrophy of germinal centres and lymphocytopenia of lobular medulla, caused by severe lymphocyte depletion (average l score 3,2 vs 2,8 of the PR11 one). This was confirmed by IHC (Figure 3b). Thymus from PR40 animals showed markedly reduced T cells subpopulation. In lymph nodes, B cells were very scarce and located in the germinal centres of mildly activated follicles, while T cells were located all around the follicles and with very low numbers in the paracortical area. These patterns were also present in the PR11 group, but they were less severe. Negative controls did not show any microscopic alterations.

Bacteriology

Streptococcus suis was isolated from animals euthanized in the first two weeks in both infected groups. In one pig *E. coli* was diffusely detected from all sampled organs. No other bacteria were isolated.

Viremia and virus re-isolation

All animals were PRRSV negative before inoculation (0 dpi). Three dpi, serum virus RNA copy number averages in both infected groups started to increase, reaching their peaks at 7 dpi in the PR40 group, and at 10 dpi in the PR11 group. Viremia was higher for the whole duration of the study in PR40 pigs, (Figure 4). The observed differences in shape (and level) of the cDNA copy number between the two groups during the time is not attributable to chance alone ($p<0.1$). PRRSV was not detected in sera from animals in the control group at any time during the experiment.

The two viruses were re-isolated from the sera of infected pigs during the viremic period (7 dpi PR40; 10 dpi PR11) with one passage on PAMs and then adapted to MARC-145 cells. The ORF5 and ORF7 genes were sequenced and identity with the inoculated viruses was confirmed.

Serology

Sera from all pigs at arrival and at inoculation were all negative (S/P < 0.4) for ELISA antibodies to PRRSV. In the PR40 group seventy percent of the animals were positive for PRRSV antibodies at 7 dpi, while animals in the PR11 group were not. At 10 dpi all animals from the two infected groups were positive. The observed differences in course and level of S/P ratios between the two groups is not attributable to chance alone ($p=0.00018$) (Figure 5a). Pigs in the control group remained negative throughout the study.

Virus-neutralizing antibodies (Figure 5b) were detectable two weeks post-infection in PR40 pigs but at a lesser extent compared to PR11 pigs for the whole duration of the study. The VN response in PR11 pigs peaked 21 days pi.

DISCUSSION

PRRSV infection is one of the most important causes of economic losses in swine industry worldwide. PRRSV is characterized by extensive genetic differences not only between the two recognized genotypes, but also within them, leading to the sub-classification into different subtypes. The differences among strains and isolates are responsible for varying pathogenicity patterns *in vivo* (Martinez-Lobo et al., 2011). Recently, some virulent variants of the virus (called highly pathogenic strains) caused severe PRRS outbreaks in many countries in Southeast Asia and in Europe, with unusually high mortality rates of pigs of all ages.

In this study, a PRRSV strain (PRRSV1_PR40/2014) isolated on PAMs from an “atypical PRRS outbreak” in Italy, characterized by severe clinical signs in growers, including high fever, respiratory distress and high mortality rates, was tested for its genetic and phenotypic features. The clinical and pathological findings are similar to what is seen in the field conditions.

The total genome of the PR40 was obtained and phylogenetically analyzed. The results indicated that it clusters in a different lineage within the 1 genotype, subtype 1 and that it carries two aminoacidic deletions in NSP2 coding region. NSP2 is a multi-domain and multi-functional protein, and the NSP-2 gene is the most variable region within the PRRSV viral genome (Fang et al., 2004). The Chinese HP-PRRSV strains and Lena have a common feature, namely a discontinuous deletion in the NSP2 coding region (Li et al. 2007; Zhou et al., 2008). The relationship between this deletion and the strain’s virulence is difficult to determine (Wang et al., 2013) and the results from different studies are contradictory (Shen et al., 2000; Fang et al., 2004; Han et al., 2006; Kim et al., 2009; Wang et al., 2013). However, it should be considered that the epitope regions of NSP2 may also play a role in the modulation of host immunity: NSP2 is a viral antagonist of host defence and it appears to be a highly immunogenic protein (Chen et al., 2010). PRRSV NSP2 is also involved in the production of inflammatory cytokines, so that the effect of NSP2 variation on inflammatory responses has also been observed (Burgara-Estrella et al., 2013; Chen et al., 2010; Faaberg et al., 2010). Liu

et al. (2015) reported that deletion of the NSP2 hypervariable regions (D323-433 or D628-747) significantly reduces the production of IL-1β and IL-2. Analyses to determine if the deletions in the NSP2 of the PR40 isolate are involved in the induction of proinflammatory cytokines are ongoing. Results from the present study of the PR40 isolate would strengthen the hypothesis that deletions in this region is an important genetic characteristic for, and associated with, different pathogenicity *in vivo*, and is strongly correlated with different host immuno-modulation of the isolate.

In the present study, a comparative experimental infection was performed to reproduce the clinical signs induced by PR40 and to better characterize the phenotypic/pathological features of this isolate. Viral pathogenicity depends on multiple prominent factors, including viral propagation efficiency in the host, tissue tropism and immune evasion capability, together with the associated secondary bacterial infections (Han et al., 2017). Strongly virulent/highly pathogenic PRRSV strains exhibit longer and higher levels of viremia and viral loads in the tissues, they induced faster and more intense humoral immune responses and more severe clinical signs in infected animals (Frydas and Nauwynck, 2016; Johnson et al., 2004; Karniychuk et al., 2010). Here, virulence-related parameters, such as clinical signs, fever, and mortality rate, were compared between PR40 and PR11 isolates. Moreover, ADWG, viremia, serological profiles, gross and microscopic lesions, were also obtained and analysed. Results from these analyses indicate that PR40 possesses the characteristics of a highly pathogenic PRRSV isolate when compared to PR11, a conventional PRRSV isolate. Pigs infected with PR40 showed significantly more severe clinical signs, and longer duration of fever. PR40 and PR11 pigs started to show fever at 2 dpi and 3 dpi, respectively, and the rectal temperature mean in the PR40 group remained over 40°C from 5 to 24 dpi. No differences in mortality were observed comparing the two experimental groups. Secondary infections with other bacterial pathogens could have played an important role in the exacerbation of the symptoms in experimental conditions, precluding the possibility of recording differences between the two groups.

Macroscopic lesions showed moderate to severe interstitial pneumonia in both groups. Histopathology confirmed that extension and the severity of the interstitial pneumonia, thymus atrophy as well lymphocytopenia of the bronchial lymph-nodes are highly suggestive features of the high pathogenicity of PR40 PRRSV. Subject infected with high virulent strains showed severe decrease of B and, in particular, of T subpopulations in lymphoid organs. In fact, it was already described that highly virulent PRRSVs induce severe thymus atrophy, lead to Tcell depletion, affecting the development of naïve T cells, and the nature and quality of newly developed T cell existing in the thymus and predisposing piglets to a weak cellular immunity (Han et al. 2017). These effects are confirmed in the conditions of this study. As a consequence, PR40, like other HP-PRRSVs seems to induce a delayed, low-level neutralizing antibody response.

The differences in terms of duration of viremia and viral load between the two experimental groups (significantly higher in the PR40 group) are in accordance with the outcomes of HP-PRRSV infections described in both field and experimental cases by other authors (Guo et al., 2013; Zhou et al., 2008;

Karniychuk et al., 2010; Frydas et al., 2015; Sun et al., 2016; Stadejek et al., 2017). Despite a very similar serological profile in ELISA, virus-neutralizing antibody levels rose one week later and with lower titres in PR40 inoculated pigs, as compared to PR11. This lower and late virus-neutralizing antibody response to the homologous virus could be a specific characteristic of the PR40 isolate in terms of dys-regulation of host immunity.

This study describes the main clinical, virological, pathological and serological features of an Italian highly pathogenic PRRSV field isolate. Further studies on the immunological properties are needed to better understand the complex interaction between host immune system and the PR40 PRRSV isolate, and to determine facorrs that may influence MLV vaccine efficacy.

Acknowledgements

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Figures and tables

Figure 1. Phylogenetic tree of complete genome nucleotide sequences of PRRSV type1. The tree was constructed using the Neighbor joining algorithm with Boostrap analyses (1000 replicates).

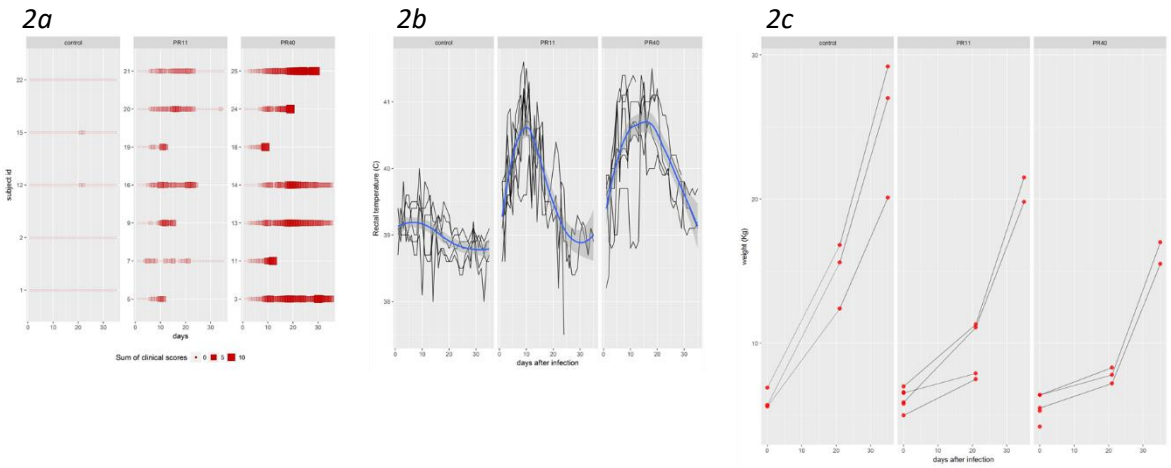

Figure 2. ***2a.*** *Body temperature* of pigs after infection to 35 days pi (green=C; violet= PR11; red=PR40). Temperature ≥40°C was considered as fever. Central line represents the mean value in each group. ***2b.*** *Clinical score* in each pigs per group (C, PR11, PR40). The grater the size and intensity of the marker, the grater the sum of clinical scores of the subject. ***2c.*** *Average daily weight gain* (g/day) calculated for each group during the first 21 dpi, from 21 to 35dpi and in the overall experiment period.

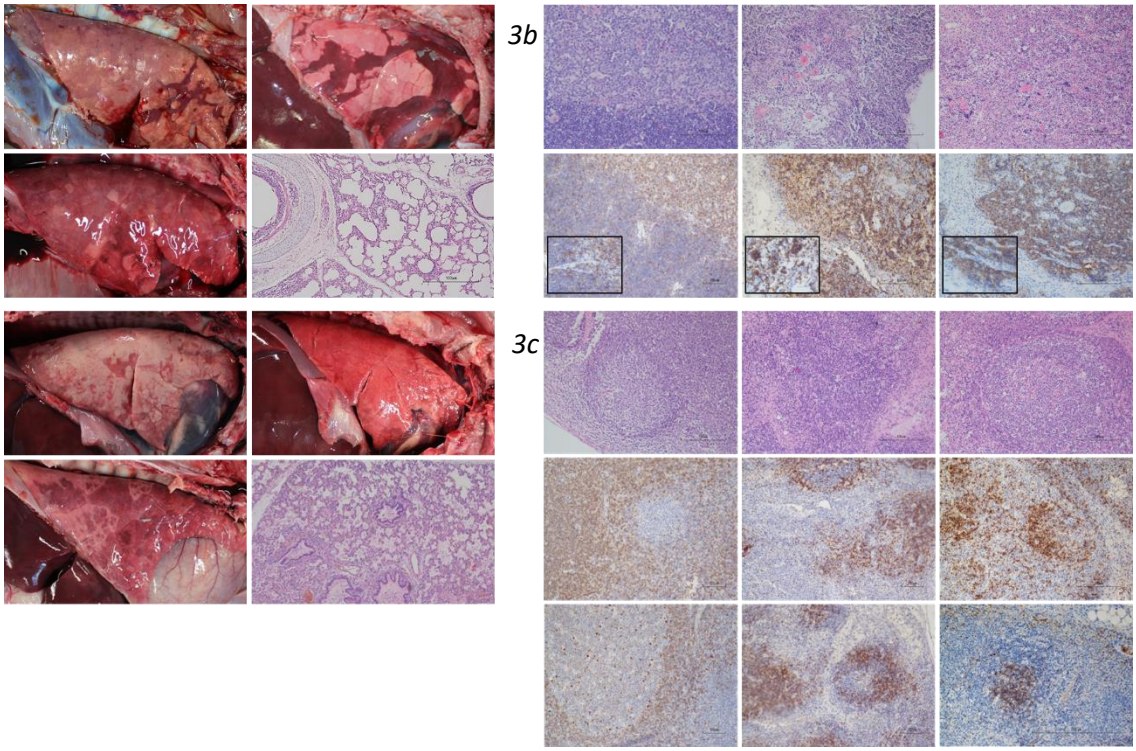

Figure 3. ***3a.*** *Representative gross pulmonary lesions* of three pigs/group and *histological* evidence of interstitial pneumonia in PR40 (above) and PR11 (below) groups. ***3b.*** Representative histology (above) and immunohistochemistry (CD3, 10X) stains (below) of thymus gland in the C, PR40 and PR11 groups (from left to right), respectively. ***3c.*** Representative histology (above) and immunohistochemistry (3c.1 CD3 10X; 3c2 CD79, 10X) stains (below) of bronchial lymph-nodes in the C, PR40 and PR11 groups (from left to right), respectively.

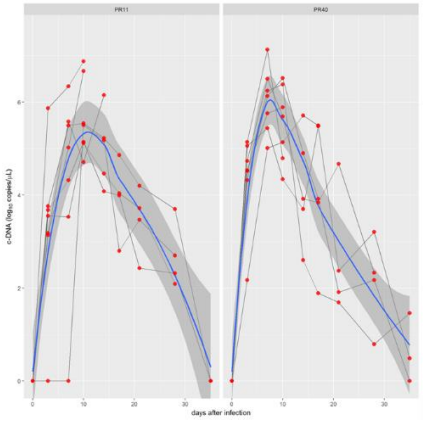

Figure 4. Mean (sd) c-DNA Log10 (copies/µL), by day and treatment.

5a *5b*

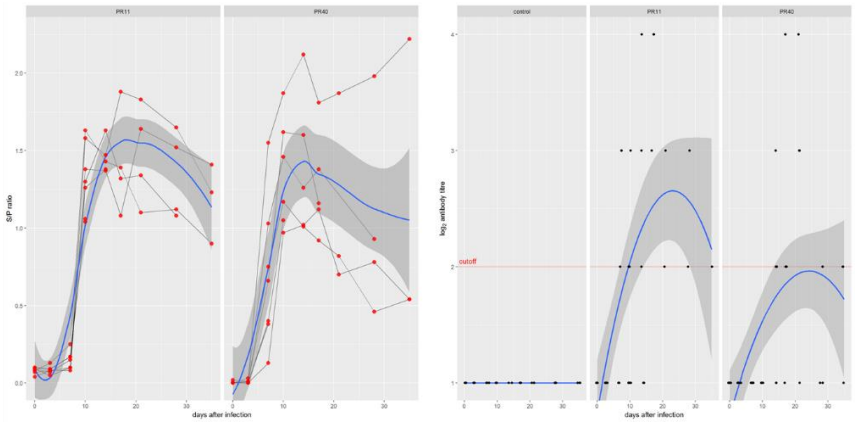

Figure 5 ***5a.*** PRRSV specific ELISA: S/P ratio by day and treatment. ***5b.*** VN antibodies titre (log2) specific to . the homolougous virus by day and treatment.

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DECLARATIONS

Ethics approval

The experimental study was approved by the Ethical Committee and by the Ministry of Health in Italy (171/2016-PR), according to European and National rules on experimental infection studies and animal welfare.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EC performed the experiment, analyzed the data and wrote the manuscript. ACat, LF, ED, GO, CB, VR and YL helped in carrying out the experiments and performed the laboratory investigations. PB

performed the PCR analyses. FCL performed the sequencing of viral strains. ACor, BPand VB performed the pathological investigations. GS followed the outbreak and the collection of the samples in the field. SG performed the statistics on data. PB, coordinated the laboratory investigations. PM designed the study, coordinated the work, and helped in writing the manuscript. All authors read and approved the final manuscript.