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Efficacy of a modified-live virus vaccine in pigs experimentally infected with a highly pathogenic porcine reproductive and respiratory syndrome virus type 1 (HP-PRRSV-1)

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ABSTRACT

PRRS is one of the main viral diseases in pig production, causing huge economic losses to the swine industry worldwide. The virus shows an intrinsic genomic instability and is able to change continuously, with the emergence of new strains, with different pathogenicity patterns. Commercially available vaccines only partially prevent or counteract the disease and the correlated losses. Moreover, the emergence of highly virulent and pathogenetic isolates represents a particular concern for PRRS control and diagnosis. The purpose of this study was to evaluate the efficacy of a modified-live virus (MLV) PRRSV-1 commercial vaccine in reducing the severity of the disease and minimizing losses upon challenge with a highly pathogenic PRRSV-1.1 Italian isolate (PRRSV-1_PR40/2014). Four different groups were compared: C (unvaccinated-uninfected), VAC-C (vaccinated-uninfected), PR40 (unvaccinated-infected) and VAC-PR40 (vaccinated-infected). The tested vaccine provided partial, but statistically significant clinical, virological and pathological protection after challenge under experimental conditions. In particular, vaccinated animals showed reduced viremia in terms of duration and magnitude, reduced respiratory signs and pathological lesions. Vaccination was able to trigger adaptive immunity able to respond efficiently also against the HP PR40 isolate. Vaccinated animals showed higher average daily weight gain, even during the viremic period, compared to non-vaccinated challenged pigs.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus belonging to the family *Arteriviridae*. Since its appearance in the late 1980s, PRRS is considered the costliest disease in swine production worldwide (Chand et al., 2012). Economic losses due to PRRS are mainly associated with reproductive failure in late gestating sows, and systemic and respiratory disease in growers/fatteners affecting growth performance and increasing morbidity and mortality (Holtkamp et al., 2013). PRRS control is still a challenging, unsolved issue. The development of programs capable of long-term virus elimination from swine-dense regions have failed, mainly because of the extreme genetic diversity of the isolates, the efficient horizontal and vertical transmission and sophisticated immune evasion strategies of the

virus (Niederwerder et al., 2015). Available vaccines, among which modified-live virus (MLV) vaccines are the most routinely used, can only partially prevent the disease, reducing clinical signs and viremia, with acceptable costs for pig farmers when compared to other strategies (Charentantanakul, 2012).

Beginning in the late '90s, outbreaks of "atypical" PRRS, featuring high morbidity and mortality, were observed in the USA (Brockmeier et al., 2012). Similar outbreaks in South-east Asia in 2006 were called "high fever disease" (An et al., 2011) and the isolates involved were identified as highly pathogenic PRRS viruses (HP-PRRSV) (Shi et al., 2010). Several cases were also reported in Europe: in 2006 a PRRSV-1.3 (Lena) and in 2009 a PRRSV-1.2 (BOR59) were found in Belarus (Karniychuk et al., 2010; Stadejek et al., 2017). More recently, two HP-PRRSV-1.1 strains (13V091 and PRRSV-1_PR40/2014) were isolated in Belgium (Frydas et al., 2015) and in Italy (Canelli et al., 2017),

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tively. Partial heterologous protection has been reported by using attenuated vaccines against PRRSV genotypes 1 and 2, upon challenge with different HP-PRRSV isolates, providing reduction of economic losses (Roca et al., 2012; Wei et al., 2013; Trus et al., 2014; Do et al., 2015; Bonckaert et al., 2016).

The present study aims at evaluating the protective efficacy of a commercial MLV PRRSV-1 vaccine upon experimental challenge with a HP-PRRSV Italian isolate (PRRSV-1_PR40/2014), responsible for severe clinical outcomes in the field.

2. Materials and methods

2.1. Vaccine and challenge virus

Vaccination was performed with a commercial MLV vaccine, based on the European PRRSV-1, namely the DV strain (Porcilis[®] PRRS, MSD-Animal Health; vaccine batch A208AD01). The vaccine was administered intramuscularly (IM) at a dose of 2 ml (containing at least 10^4 TCID₅₀ of PRRSV strain DV). The Italian HP-PRRSV-1_PR40/2014 strain (Canelli et al., 2017), reported below as PR40, was used as challenge virus.

As a preliminary analysis, the full genome sequence of the vaccine (KF991509.2) and challenge (MF346695.1) strains were compared in order to define the homology percentage. Particularly, the evolutionary divergence of the main glycoprotein (GP) coding regions was analysed. Analyses were conducted using the Kimura2-parameter model for nucleotide sequences (Kimura, 1980) and Poisson correction for amino acid sequences (Zuckerkindl and Pauling, 1965). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The two strains share 89.7% and 85.2% of nucleotide and amino acidic identity, respectively, for the total genome: therefore, they can be considered as heterologous strains. The amino acid divergence of glycosylated membrane-associated minor structural proteins (GP2, GP3 and GP4) and major structural proteins, GP5, M (GP6) and N (GP7) proteins are reported in Table 1.

2.2. Experimental infection

2.2.1. Animals and study design

The experimental study was approved by the Ethical Committee, according to the European and National rules on experimental infection studies and animal welfare. Sixteen 3-week-old conventional pigs from different litters belonging to a PRRSV-free herd were randomly allocated to four separated rooms of a biosafety level 2 (BSL-2) facility. After an acclimation period of six days, the following four groups were designated: 1) VAC-C: 2 pigs were IM-vaccinated against PRRSV at 4 weeks of age (35 days from inoculation) and left uninfected; 2) VAC-PR40: 6 pigs were IM-vaccinated against PRRSV at -35 days and intra-nasally (IN) infected with PR40 at 35 days post-vaccination (dpv)/ 0 days post-inoculation (dpi); 3) PR40: 5 pigs were IN infected with PR40 at 35 dpv / 0 dpi; 4) C: 3 animals were IN-inoculated with

Table 1

Estimates of evolutionary divergence between the PR40 strain (MF346695.1) and vaccine strain DV (KF991509.2) amino acid sequences (GP2-GP7).

PRRSV GLYCOPROTEINS	DIVERGENCE
GP2	0.147
GP3	0.235
GP4	0.155
GP5	0.139
GP6	0.078
GP7	0.128

medium only, and left as the non-vaccinated/non-infected negative control group.

The number of pigs belonging to each group was defined in accordance with and after revision of the above-mentioned Ethical Committee, in order to minimize the use of experimental animals. No relevant pathogens (PRRSV, swine influenza virus [SIV], and porcine circovirus type 2 [PCV2]) were detected in the animals at the beginning of the study. For both groups PR40 and VAC-PR40, a dose of 10^5 TCID₅₀/pig of PR40 in 2 ml, 1 ml/nosril was administered. Blood samples were collected from all animals at 0, 3, 7, 10, 14, 17, 21, 28, 35 dpi. Vaccinated animals were also bled at 0 (-35 dpi) and 21 (-14 dpi) dpv. Animals suffering from severe clinical signs with a fatal prognosis, and survived animals at 35 dpi were humanely euthanized, according to standard protocols.

2.2.2. Clinical, pathological and bacteriological analyses

Following vaccination (-35 to 0 dpi) and challenge (0–35 dpi), pigs were monitored daily for rectal temperature ($> 40^\circ\text{C}$ = fever) and clinical signs. Animals were scored for general conditions (appetite: 0 = normal, 1 = decreased, 2 = absent; level of consciousness: 0 = normal, 1 = compromised/depressed, 2 = agonic) and respiratory signs according to Karniychuk et al. (2010) from 0 to 35 dpi. Briefly, respiratory signs were 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. Stress was induced by holding the pig for 45 s. 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. Pigs were individually weighed at three time points (0, 21 and 35 dpi). Average daily weight gain (ADWG, g/day) for the intervals between infection and 21 and 35 dpi were calculated for each pig individually. In addition, mortality was recorded throughout the study period.

After spontaneous death or euthanasia, pigs were necropsied and the presence and severity of gross lung lesions were assessed and scored (lung lesion score (LLS) as defined by Halbur et al., 1995). In order to describe the severity of interstitial pneumonia lesions, a histological score (HS, 0–5) was used as previously described (Weesendorp et al., 2014). Samples from heart, spleen, liver, lungs, and intestine were collected for bacteriology and cultured on blood agar containing 10 µg/ml of nicotinamide adenine dinucleotide, serum agar and Hektoen agar. All agar plates were incubated at 37 °C for 48 h. Bacterial isolates were identified using Gram staining and standard biochemical procedures.

2.2.3. Haematological analyses

Haematological analyses were performed by using an automated analyser (CELL-DYN 3500 PLUS, Abbott Diagnostics, Lake Forest, IL, USA) on whole blood.

2.2.4. Viremia

Viral RNA copy number in serum was evaluated by using a quantitative RT-PCR (Martelli et al., 2013). RNA was extracted by using Trizol-LS (Invitrogen-ThermoFisher, Carlsbad, CA, USA), following the manufacturer's instructions.

2.2.5. Serology

All sera were tested for total PRRSV antibodies (Abs) by ELISA (HerdChek PRRSVX3, IDEXX Labs. Inc., Westbrook, ME, USA) according to the manufacturer's instructions. Samples with S/P ratio greater than or equal to 0.4 were considered positive. Virus neutralization

(VN) test was performed on MARC-145 cells using both the challenging strain PR40 and the vaccine DV strain as antigens, with a protocol previously described (Kim et al., 2011). The VN-antibody titer was determined as the reciprocal of the highest dilution that inhibited the cytopathic effect in 100% of the well. Serum samples were considered positive if the titer was greater than 2.0 (\log_2) (Zuckermann et al., 2007).

2.2.6. PRRSV-specific IFN- γ ELISpot

PBMC were isolated by Histopaque-1077[®] density gradient as reported in Ferrari et al. (2013). An IFN- γ ELISpot assay was used for the quantification of PRRSV-specific IFN- γ secreting cell (IFN- γ SC) frequencies in PBMC as previously described (Ferrari et al., 2013; Martelli et al., 2013). Specifically, PBMC were plated and ex vivo stimulated with DV or PR40 at 1 multiplicity of infection (MOI). The plates were analyzed by using an AID[®] ELISpot Reader and Software v.6.0 (Autoimmun Diagnostika, Straßberg, Germany) and the PRRSV-specific IFN- γ SC frequencies were expressed as number of IFN- γ SC/10⁶ PBMC.

2.3. Statistical analysis

Mixed Effects Linear models (LMEm; time was considered a categorical variable) and Mixed Effects Generalized Additive models (GAMm; time was treated as a continuous variable) were applied for accounting the not-independency of the repeated observations done on the same subjects. The effect of the day of observation, of treatment and their interaction were considered. The p-values reported are derived from the LMEm, where an autoregressive correlation structure was imposed to residuals. To analyze data and obtain graphics, the R software (R Core Team, 2017), with the packages nlme (Pinheiro et al., 2000) and ggplot2 (Wickham, 2009) was used. LLS and HS were analyzed by Chi-square and Fisher's exact test. Statistical comparison was performed between groups for each day, and within groups over time. For analysis between groups for each day, Kruskal-Wallis test was employed to detect an overall significant difference, followed by the Dunn's multiple comparisons test (for independent data) to identify the couple/s of groups responsible for the difference. The analysis within groups over time was conducted firstly with Friedman test and subsequently with Dunn's multiple comparisons test (for repeated data). In order to evaluate cross-reactivity of the DV vaccine against PR40, data on the IFN- γ secreting cell responses of the vaccinated groups with both stimulation were compared with Kruskal-Wallis test. A p-value < 0.05 was considered as statistically significant.

3. Results

3.1. Experimental infection

3.1.1. Clinical, pathological and bacteriological analyses

3.1.1.1. Reduction of clinical disease was observed in the vaccinated-infected group compared to unvaccinated infected pigs. Rectal temperature and a clinical score based on general conditions and respiratory signs were measured in order to monitor the clinical signs induced by PRRSV in groups experimentally infected with PR40. Groups C and VAC-C did not show either fever or clinical signs for the whole duration of the study. Specifically, from 9 to 16 dpi the VAC-PR40 group showed mild fever (mean 40.2 ± 0.5 °C, always below 40.5 °C), with a statistically significant ($p < 0.05$) difference compared to pigs belonging to the PR40 group, which showed high fever (up to 41.0 °C), continuously from 5 to 25 dpi (Fig. 1a; mean group values \pm standard deviation (sd) are detailed in Fig. 1S). The VAC-PR40 group also showed mild clinical signs starting from 15 dpi (with the exception of one pig showing respiratory signs at 10 dpi), until 21 dpi, with a clinical score below 5 in all animals. Conversely, PR40 pigs showed severe clinical signs exceeding 7 as clinical score, from 7 to 35 dpi. All pigs in the

VAC-PR40 group survived until the end of the trial. On the contrary, in the PR40 group, 3 out of 5 pigs died during the first 28 dpi.

3.1.1.2. PRRSV vaccination had a remarkable effect on the average daily weight gain of infected pigs. The average daily weight gain was monitored throughout the experiment. In the first period after infection (0–21 dpi), inclusive of the viremic period, ADWG was 909 ± 24 g (mean \pm sd) for the VAC-C group, 718 ± 85 g for the VAC-PR40 group and only 317 ± 10 g for the PR40 group. In the post-viremic period (21–35 dpi), the values of ADWG were quite similar, with better results for VAC-C pigs. In the overall study period (0–35 dpi), the PR40 group showed the lowest daily weight gain with 723 ± 8 g, compared to the VAC-PR40 group (879 ± 44 g). Statistical analysis detected an overall significant difference ($p < 0.05$), even though the Dunn's test did not identify any couple of groups responsible for this difference.

3.1.1.3. Gross and histological lesions: the vaccinated-infected group showed reduced pathological lesions. Gross and tissue lesions were investigated in lungs, bronchial lymph nodes and thymus. No significant macroscopic or microscopic lung lesions were observed in C and VAC-C pigs. Main gross lesions in pigs of the PR40 group were thymus hypoplasia or atrophy and interstitial pneumonia, and bronchial lymph node hyperplasia. In PR40 pigs, LLS was higher in animals died within 28 dpi (individual LLS in order by day of death: 56.0; 44.5; 25.0). In the PR40 group, *Streptococcus suis* was isolated in two animals died within 28 dpi. No other relevant pathogens were detected in tissues collected from other pigs at necropsy. In VAC-PR40 pigs, LLSs (mean LLS value \pm sd: 9.4 ± 2.6) were significantly lower ($p < 0.05$) than in PR40 pigs. Microscopically, interstitial pneumonia with lymphocyte and monocyte septal infiltration was observed in the PR40 group (mean HS $3.75 \pm$ sd 0.55). Bronchial lymph nodes and thymus from PR40 pigs showed atrophy of germinal centres and lymphocytopenia of the lobular medulla, respectively. The microscopical lesions in lymph nodes and, particularly, in thymus (marked stroma inflammatory infiltration, disappearance of the cortico-medullary boundary, inflammation) were more evident in the animals died until 14 dpi. Pigs in the VAC-PR40 group showed similar lymphocyte depletion, but at significantly lower ($p < 0.05$) extent (mean HS $1.60 \pm$ sd 0.37).

3.1.2. PRRSV vaccination positively modulated lymphocyte and red blood cell counts as well as biochemical parameters compared to unvaccinated-infected pigs.

Haematological analyses were performed in peripheral blood to evaluate potential changes of major cell subpopulations and biochemical markers which can be modulated after PRRSV infection. White blood cell counts did not show significant differences between groups; however, VAC-PR40 and PR40 groups showed an early and transient reduction at 7 dpi (Fig. 2 S). Lymphocyte counts (Fig. 1b) and percentages (Fig. 2S) maintained slightly higher levels in the VAC-C group during the first 2 weeks pi compared to the other groups. In the PR40 group, lymphocyte counts and percentages decreased markedly from 7 dpi to 14 dpi ($p < 0.05$) and then gradually increased. Overall, the lymphocyte percentages in the PR40 group resulted lower compared to the other groups for the whole study period ($p < 0.05$). Lymphocytes in the VAC-PR40 raised as observed in the PR40 group from 2 weeks pi onwards. Neutrophil counts did not vary significantly within all groups except for VAC-PR40 pigs, for which a significant decrease was assessed between 0 and 28 dpi. Neutrophil counts (Fig. 1c) and percentages (Fig. 2S) showed a strong increase and lasting higher levels in the PR40 group ($p < 0.05$) during the whole study period compared to the other groups while VAC-PR40 pigs showed no trigger of this inflammatory subpopulation. Red blood cells, haemoglobin and haematocrit showed significantly lower levels in the PR40 group from 14 to 35 dpi compared to all the other groups while platelets showed significantly lower levels in the PR40 group from 14 to 21 dpi (Fig. 3S).

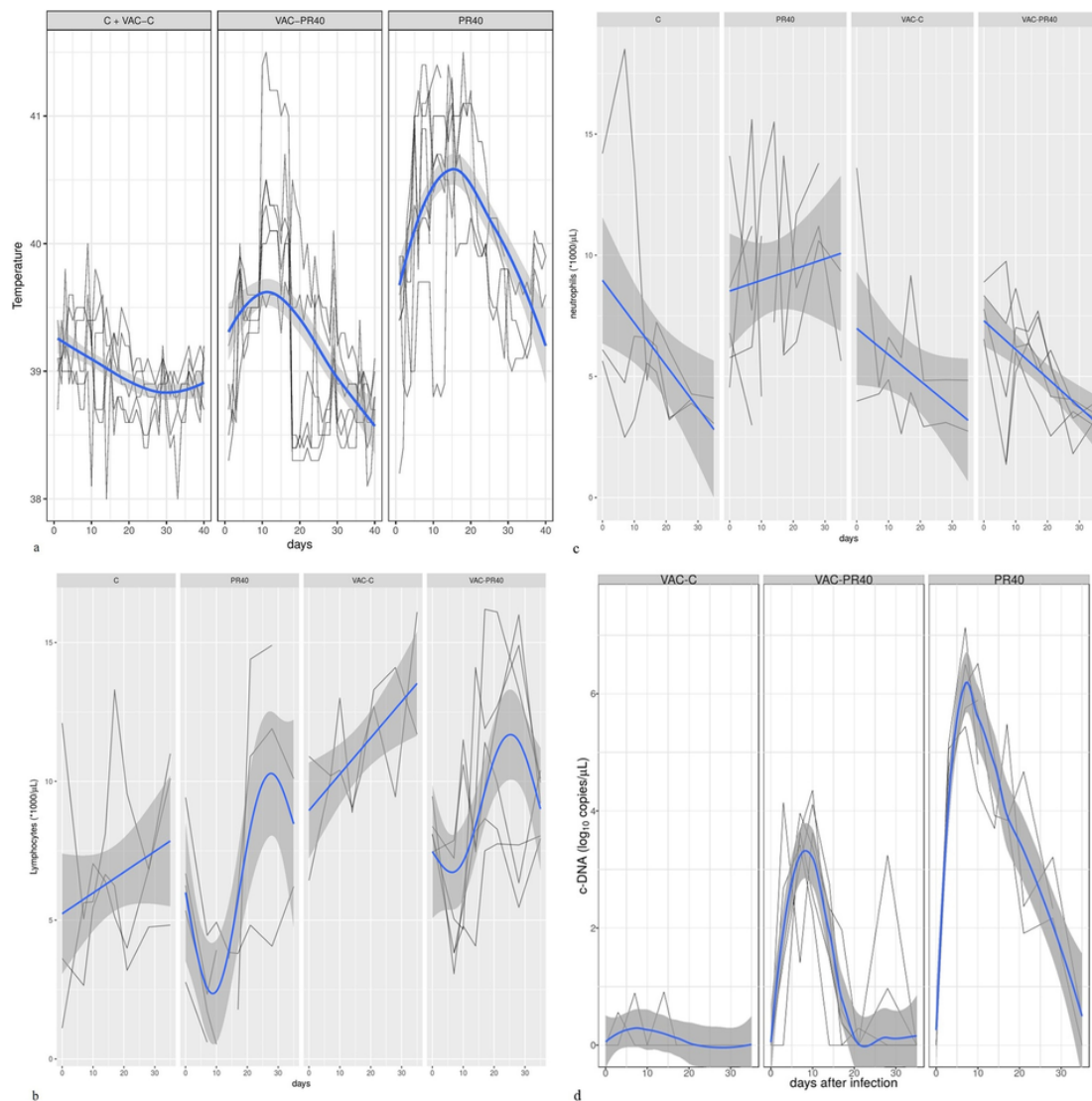


Fig. 1. (a) Rectal temperature of pigs after PRRSV experimental infection. Rectal temperature was recorded on a daily basis from 0 to 35 dpi (fever: if temperature $\geq 40^{\circ}\text{C}$). The central line represents the mean value in each group. (b) Levels of lymphocytes and (c) neutrophils in peripheral blood. The central line represents the mean value (values are expressed as cells/ μL) in each group. (d) PRRSV titres in serum. The central line represents the mean value (values are expressed as Log_{10} cDNA copies/ μL) in each group. c-DNA: complementary DNA. C: control unvaccinated-uninfected pigs; VAC-C: vaccinated uninfected pigs; VAC-PR40: vaccinated PR40-infected pigs; PR40: unvaccinated PR40-infected pigs.

3.1.3. Reduction of viremia duration and magnitude was detected in vaccinated-infected animals.

The presence and levels of PRRSV were evaluated in all pigs to testify and monitor the ongoing experimental infection by PR40 and monitor negativity in uninfected animals throughout the experimental period. Pigs from all groups were negative for PRRSV RNA at 0 dpv and 0 dpi. Serum RNA copy number means in the PR40 and VAC-PR40 groups started to increase at 3 dpi, with a peak at 7 dpi and 10 dpi, respectively. Viremia persisted until 28 dpi in the PR40 group. VAC-PR40 group showed lower titres of viremia compared to PR40 animals, which rapidly decreased after a peak at 10 dpi and became negative at 14 dpi (Fig. 1d). The observed differences in the pattern of cDNA copy number between the two groups (Tab. 1S) were statistically significant ($p < 0.05$). PRRSV was not detected in sera from animals of the C and VAC-C groups at any time point.

3.1.4. Serology: an earlier and boosted VN-Ab response was detected in the vaccinated group after infection.

The humoral response was evaluated both in terms of total ELISA antibodies and virus-neutralizing antibodies in serum. In all vaccinated animals, PRRSV-specific ELISA Abs were detected at -14 dpi (21 dpv), with a mean S/P value (mean \pm sd) of 0.94 ± 0.71 and 0.85 ± 0.45 for the VAC-PR40 group and VAC-C group, respectively, and throughout the post-infection period (Fig. 2a). The total antibody response started rising after 7 dpi in PR40 pigs, with the highest S/P values at 14 dpi. The ELISA Ab profile was lower compared to the VAC-PR40 group, where a booster effect was observed after 10 dpi. The difference in the S/P ratio between the two groups was significant ($p < 0.05$; Tab. 2S). VAC-PR40 pigs had a detectable but low (2^3 – 2^5) VN response to PR40 PRRSV isolate starting from 7 dpi until the end of the study (Fig. 2b). PR40 pigs showed VN-Abs to the homologous virus starting from 17 dpi, with a peak at 21 dpi, but the titer was very low ($< 2^3$).

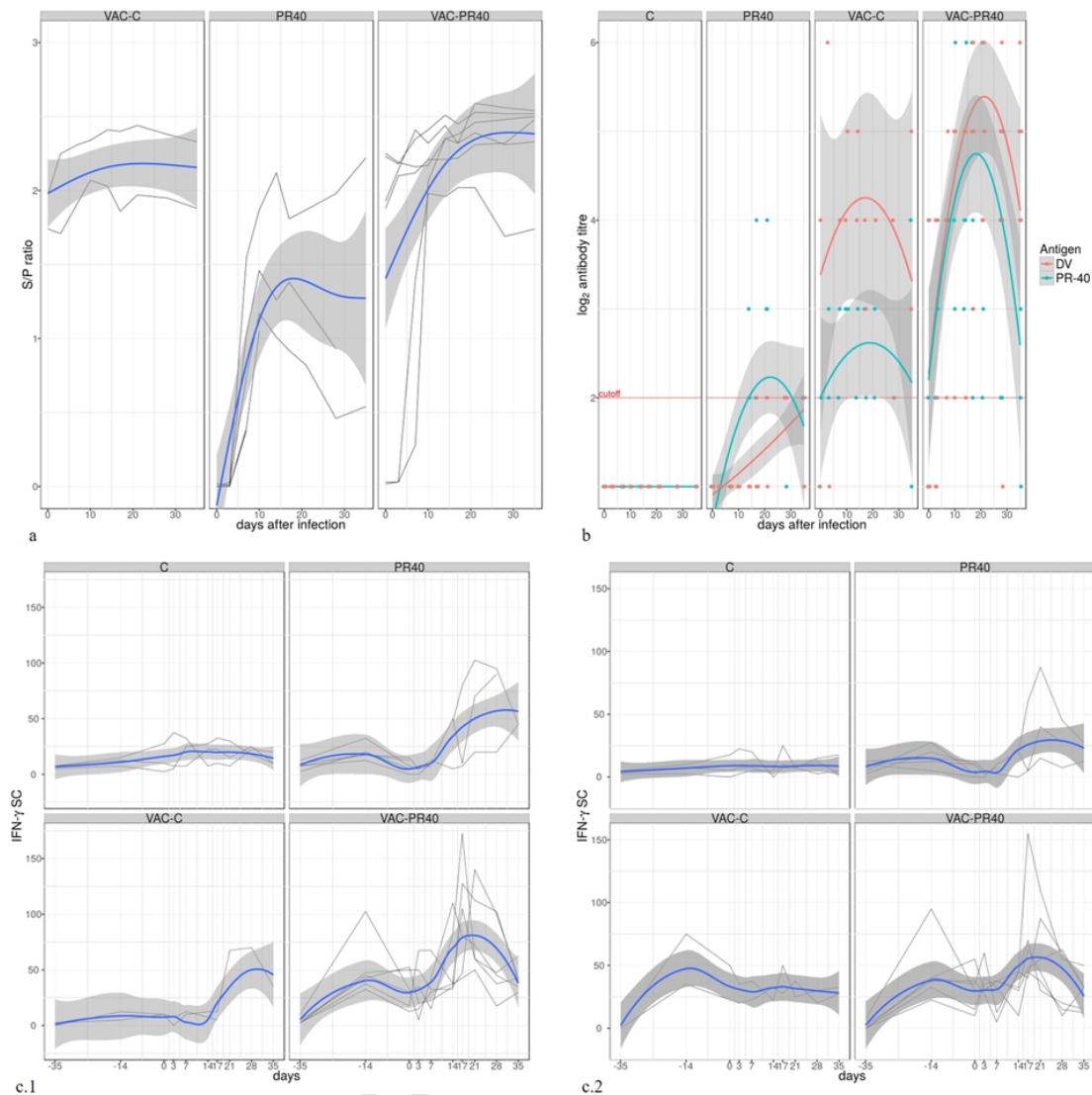


Fig. 2. PRRSV-specific antibodies in serum and PRRSV-specific IFN- γ secreting cells (SC) in PBMC. (a) ELISA S/P antibody ratios, (b) PR40-specific VN antibody titres (\log_2) (orange: VN-Ab titer to the DV strain; green: VN-Ab titer to the PR40 strain), (c) frequencies of IFN- γ SC expressed as number of IFN- γ SC/ 10^6 PBMC upon 20 h ex vivo re-stimulation with the PR40 strain (c.1), and with the DV vaccine strain (c.2). The central line represents the mean value in each group. C: control unvaccinated-uninfected pigs; VAC-C: vaccinated uninfected pigs; VAC-PR40: vaccinated PR40-infected pigs; PR40: unvaccinated PR40-infected pigs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.1.5. Vaccinated pigs mounted an IFN- γ secreting cell response able to cross-react to the PR40 strain.

The cellular response was investigated in terms of frequencies of IFN- γ secreting cells (SC) within PBMC upon ex vivo re-stimulation with the vaccine DV strain and the HP PR40 strain using an ELISPOT assay. The two vaccinated groups showed a significant IFN- γ response to the DV strain at -14 dpi (21 dpv) ($p < 0.05$) (Fig. 4S). The overall response in terms of IFN- γ SC specific to the DV and PR40 strains detected upon ex vivo re-stimulation with the PR40 strain (Fig. 2c.1) was comparable with the DV response in all groups (Fig. 2c.2). However, the recall with PR40 induced a higher mean peak response at 17 dpi in VAC-PR40 pigs. The VAC-PR40 group showed a significant increase after 14 dpi compared to VAC-C pigs and the other groups ($p < 0.05$) and returned to pre-infection values at 35 dpi. PR40 pigs re-stimulated with DV or PR40 had a significant peak response between 17 and 35 dpi compared to controls ($p < 0.05$), showing overall a more pronounced and long-lasting response upon re-stimulation with PR40 ($p < 0.05$). VAC-PR40 animals had an earlier and more intense response

compared to the PR40 group ($p < 0.05$). The response in VAC-C animals during the post-infection period was not subjected to any modulation (Fig. 4S).

4. Discussion

The continuous evolution of the PRRS virus has led to the emergence of new variants, so that in recent years, highly virulent/pathogenic isolates of the virus have been identified both for PRRSV-1 and PRRSV-2. Different vaccines have been recently tested in challenge trials with HP-PRRSV isolates, and partial protection has been reported (Roca et al., 2012; Wei et al., 2013; Trus et al., 2014; Do et al., 2015; Bonckaert et al., 2016). In the present study, the efficacy of a commercial, attenuated live European PRRSV-1 vaccine was tested against infection with a recently isolated HP-PRRSV-1.1 Italian isolate (PR40) under experimental conditions.

Upon challenge, PR40-infected animals showed all the typical respiratory and pathological features of infection due to a highly virulent isolate. VAC-PR40 pigs, compared to PR40 pigs, showed a statistically

significant reduction of clinical disease, in terms of severity and duration, less fever, no mortality and reduced pathological lesions. Interestingly, the effect of vaccination on weight gain in particular was noteworthy, despite the challenge: the animals in the VAC-PR40 group did not show a significant reduction of weight, as recorded in PR40 pigs. These results are very remarkable as ADWG and mortality are the most relevant performance variables affected by the PRRSV in growers (Holtkamp et al., 2013). The better general conditions and mild pathological features, due to the absence of secondary infections in the VAC-PR40 group, were also confirmed by the haematological investigations, in comparison with PR40 pigs at almost all time points.

High lymphocyte levels in VAC-PR40 pigs which resulted comparable with the levels in VAC-C pigs testify that infection did not induce lymphocyte depletion in PRRSV-primed animals upon infection. Concordantly, neutrophil levels and biochemical parameters testify an inflammatory status upon PR40 infection as previously reported and a depletion of red blood cells (Halbur et al., 2002; Amarilla et al., 2017) and platelets in unvaccinated-infected pigs only. The data can be interpreted partly in accordance with the conclusions drawn by Amarilla and coll. (2017) demonstrating hypoplasia of erythroid cells and hyperplasia of myeloid cells in the bone marrow of HP PRRSV-1 experimentally infected pigs. The transient thrombocytopenia can further support the hypothesis of a general hypoplasia of bone marrow precursors.

Viremia was detectable also in VAC-PR40 pigs, but the duration (3 weeks shorter) and the magnitude (3 log₁₀ lower at the peak) were reduced, compared to PR40 pigs. The VN-Ab profile after challenge in the two infected groups did not reach high titres (considering that the experimental study lasted only 35 dpi), but the comparison of the two groups in this period highlighted a significantly earlier and boosted VN-Ab response in the VAC-PR40 group.

Considering the cross-neutralizing activity developed from a viral PRRSV strain, the neutralizing epitopes of the virus have to be considered. These epitopes have been reported not only within the heterodimer GP5/M, but also in the envelope triplex GP2/GP3/GP4 (Vanhee et al., 2011). In this work, the similarity among the vaccine and challenge strains for the GP5 is 86.1% and 85.3%, 76.5 and 84.5% for GP2, GP3 and GP4, respectively. The divergence is quite high, but the amount of the neutralizing antibody response in vaccinated animals was rapid and quantitatively remarkable, indicating a good priming of the vaccine strain and suggesting the presence of cross-neutralizing epitopes. To date, the mechanism of antibody-mediated PRRSV neutralization is still unclear, due to conflicting data from various studies (Nan et al., 2017), and how to induce high titers of broadly neutralizing antibodies remains a tough task to tackle for all the PRRSV strains (Han et al., 2013).

In addition to the VN-Ab response, lymphocyte counts in the blood were higher in VAC-C pigs, whereas strongly decreased at 7 dpi and gradually increased after 14 dpi in the VAC-PR40 and PR40 groups, suggesting the contribution of the cellular component in the control of infection and an early immune cell depletion in unvaccinated PR40-infected pigs. In fact, the cellular immune response evaluated by ELISPOT showed that the frequencies of PRRSV-specific IFN- γ SC in the VAC-PR40 group started to rise concomitantly with VN-Abs, maintaining a comparable profile during the whole study, while in PR40-infected pigs this increase occurred later, and showed a lesser extent.

These results show that the prompt decrease of the viremic titre in the VAC-PR40 group, compared to PR40 pigs, is associated with a more intense, simultaneous increase of both cellular and humoral adaptive immune components. As high PRRSV viremia is associated with the development of severe interstitial pneumonia (Han et al., 2013), the reduction of viremia duration and magnitude induced by vaccination is a critical point in controlling the consequences of infection. It is not clear how cellular and humoral immunity contribute to

inhibit viral replication in pigs (Iseki et al., 2017), but both play an essential role in PRRSV clearance (Wesley et al., 2006; Mulupuri et al., 2008), as also suggested by the results of this study.

In addition, the present work demonstrates that vaccination with a MLV can trigger an adaptive immune response able to be recalled also against a heterologous HP-PRRSV isolate. The ELISPOT PRRSV-specific IFN- γ SC response showed that vaccination induced cross-reactivity, in fact the *ex vivo* stimulation with the PR40 isolate could recall comparable, if not higher, PRRSV-specific reactive cells. The IFN- γ SC levels after vaccination (0 dpi) indicate that a significant PRRSV-specific T cell response was present in both vaccinated groups before infection differently from unvaccinated animals. After challenge, the anamnestic recalled response in vaccinated-infected pigs was induced after the viremia peak. The course of IFN- γ SC may be involved in the more efficient T cell response in VAC-PR40 pigs to counteract PRRSV infection early, before the peak response, observed at 17 dpi, whereas in unvaccinated pigs, viremia lasted also in the presence of the cellular and VN-antibody responses. Moreover, all vaccinated pigs showed an increased production of PRRSV-specific antibodies before challenge, and a strong stable response after challenge, with VN seroconversion at higher titres than in unvaccinated infected pigs, confirming the boosted reaction induced by the PR40 virus after vaccine priming with the heterologous DV vaccine strain. Noteworthy, the results show that T cells in vaccinated animals are recalled upon infection by a heterologous and highly virulent virus, not being dependent on high levels of IFN- γ SC frequencies in the blood before and early after infection. Overall, the data sustain the efficacy of vaccination. A certain amount of cross-protection among different PRRSV strains has also been previously reported (Mengeling et al., 2003; Opriessnig et al., 2005; Martelli et al., 2007, 2009; Ferrari et al., 2013; Weesendorp et al., 2013; Park et al., 2015), but it is always difficult to predict the degree of cross-immunity and its mechanisms.

Further studies on cellular immunity are ongoing to clarify its role, the interaction with the humoral compartment and the involvement of relevant cell subsets, immunoregulatory hormones such as GH/IGF-1 and adiponectin, and cytokines (Saleri et al., unpublished data). Specifically, in addition to T helper (Th) memory and cytotoxic T lymphocytes (CTL), some other lymphocyte subpopulations, such as natural killer T (NKT) cells and CD1-dependent CD8⁺/CD8⁻ γ/δ T lymphocytes, that share innate and specific properties, may have a role in this cross-recognition (Ferrari et al., 2018).

In conclusion, the tested MLV PRRSV-1 vaccine provided partial, but significant clinical, virological and pathological protection after challenge with a newly defined Italian highly pathogenic European PRRSV-1.1 strain. This study agrees with the results of other studies (Trus et al., 2014; Bonckaert et al., 2016; Iseki et al., 2017). However, it is worthy to note that, under field conditions, the onset of immunity could be adversely affected by the presence of confounding factors, including the presence of other pathogens and vaccination in the face of an ongoing active PRRSV infection (Philips et al., 2006), thus it is not possible to reliably predict the efficacy of the tested vaccine against other HP-PRRSV variants under field conditions.

Competing interests

The authors declare that they have no competing interests.

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Uncited references

Tamura et al. (2013) and Zuckerkandl and Pauling (1965), Kimura, 1980

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2018.10.001>.

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