

Protection and immune response in pigs intradermally vaccinated against porcine reproductive and respiratory syndrome (PRRS) and subsequently exposed to a heterologous European (Italian cluster) field strain

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Abstract

The purpose of this study was to assess the immune response in pigs intradermally vaccinated with a commercially available attenuated porcine reproductive and respiratory virus (PRRSV) vaccine (Porcilis[®] PRRS) and subsequently exposed to a heterologous (Italian cluster) field strain of virulent PRRSV. A total of 18, 4-week-old pigs seronegative for PRRSV were allocated to 1 of 3 groups (groups A, B, and C). At 5 weeks of age, pigs of groups A ($n=6$ pigs) and B ($n=6$ pigs) were vaccinated intramuscularly and intradermally, respectively, with Porcilis[®] PRRS. The more conventional intramuscular route of vaccination was included for comparative purposes with the intradermal route of vaccination (performed with the I.D.A.L.[®] vaccinator). Pigs of group C ($n=6$ pigs) were kept as nonvaccinated controls. At post-vaccination (PV) days 7, 14, 21, 28, and 35, blood samples were collected for detection of vaccine virus (PCR) and antibodies (ELISA), and for changes in PBMC (flow cytometry). At PV day 35, pigs of all groups were each exposed (challenged) intranasally to a heterologous field strain (78% ORF5 sequence homology between vaccine and field virus) belonging to the Italian cluster of the European genotype of PRRSV. At post-challenge (PC) days 0, 3, 7, 10, 13, and 17, blood samples were collected for detection and quantitation of virus and antibodies, and for changes in PBMC as described above for blood samples collected PV. Throughout the experiment all pigs were observed daily for clinical signs. At PC days 7 and 17, two pigs and four pigs, respectively, of each group were euthanized and examined for macroscopic lesions. Following vaccination some pigs of groups A and B had a detectable viremia that in two pigs (one pig of group A and one pig of group B) lasted until PV day 28. However, all pigs (groups A, B, and C) remained clinically normal. All vaccinated pigs developed a serological response (ELISA) to PRRSV. Presumptive evidence for vaccine-induced protective immunity against the heterologous challenge strain was provided by finding that viremia following challenge was generally less (incidence) and significantly less (titers) in vaccinated pigs than in nonvaccinated pigs. No differences were apparent between pigs vaccinated intramuscularly and those vaccinated intradermally. The absence of virulent-virus-induced clinical signs and macroscopic lesions in nonvaccinated as well as in vaccinated pigs precluded a more definitive evaluation of the magnitude of protective immunity provided by vaccination or by the route of vaccination. Some likely treatment-associated changes in lymphocyte subpopulations were observed among the three treatment groups. These changes and their potential relationship to protective immunity are discussed.
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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the *Arteriviridae*, is widespread among the swine population, causing reproductive failure in sows and respiratory disorders in pigs of all ages, and has an economic impact on the pig industry worldwide.

The studies on PRRSV pathogenesis have shown a complex interaction of the virus with its host's inflammatory and immune response. In contrast with a typical acute viral infection resolving in 1–2 weeks by an effective clearance, PRRSV gives rise to a long-term infection, characterized by prolonged viremia and by a persistent virus detection in lymphoid tissue for several months in nonviremic animals.

The immune response to PRRSV is partially ineffective, although the mechanism for this is virtually unknown; a relatively low or altered activation of inflammatory and innate response has been speculated as having an important role in accounting for the persistence of PRRSV infection and its low and retarded activation of acquired immune response [1–4].

Several studies have been performed to investigate the humoral and cellular immune response after PRRSV infection in pigs; however, experimental and field evidence has confirmed that the current knowledge of innate and adaptive immune response remains incomplete and many questions are as yet unanswered.

In spite of the observation that the exposure to the virus induces a protective immunity against re-exposure to the homologous virus, it has also been demonstrated that infected pigs develop a persistent infection in lymphoid tissue and they can become re-infected and clinically affected with a heterologous virus. This supports the view that the immunobiological aspects of PRRSV infection are complex and that the infection can induce both subversion and activation of the immune response [3].

All of this testifies to the variable features of the PRRS immune response and the consistent discrepancy between the experimental and field observations regarding the efficiency of the immune response, such as possible vaccine failure. Under experimental conditions, single or multiple exposure of pregnant gilts to porcine reproductive and respiratory syndrome virus (PRRSV) at various stages of gestation provided protection against subsequent exposure to the homologous strain [5], even if live virus vaccines sometimes fail to come up to expectations in the field, and the overall protective immunity in pig herds is generally inadequate [6]. The relative virulence and the degree of antigenic relatedness between vaccine and field strains are considered as being the two major variables to explain these differences in effectiveness. Genetic divergence and presumably at least some antigenic diversity among PRRSV strains raise questions about immunity, and particularly cross-protection.

A high degree of genetic variability has been demonstrated within both the North American [7,8,9] and European genotypes [10,11]. On the basis of such differences, isolates of the European genotype comprise at least four genetic clus-

ters; namely, Lelystad-like, Danish, Italian [10], and Belarus [12].

In earlier experiments, a significant reduction in virus titres was obtained in the lungs and blood of pigs vaccinated with an attenuated European-type vaccine and challenged with PRRSV isolates from the Lelystad cluster [13–16].

In addition, as the mean virus titres of the vaccinated pigs challenged with a heterologous isolate belonging to the Italian strain were significantly lower than those of the nonvaccinated control pigs, Labarque et al. [17] concluded that the genetic diversity within European-type PRRSV may affect the efficacy of the current European-type vaccines.

Different studies showed the importance of the route and system of vaccine administration for the induction of a protective immunity against pig viral diseases. In fact, different routes of vaccination against Aujeszky's disease (AD) in pigs were evaluated in term of clinical protection and induction of humoral and cellular immune response. Intradermal vaccination was proposed as a route of administration of AD vaccines inducing a stronger immune response and protection against challenge than intramuscular administration [18,19,41].

Since it is known that specialised antigen-presenting cells (APCs) are essential for effective induction of cell-mediated immunity [20,21] and that skin contains more specialised APCs [22] than muscle tissue, we speculated that intradermal administration of PRRS vaccine would induce a comparable immune responses to intramuscular vaccination and would give the same protection against challenge infection.

This is the first study carried out to determine how an attenuated PRRS vaccine administered via the intradermal route by using a needle-free device (I.D.A.L.[®]) can induce a protective immune response in pigs subsequently exposed to a genetically diverse PRRSV isolate.

In the present study pigs were vaccinated with an attenuated European-type vaccine and challenged with an Italian wild-type strain belonging to another genetic cluster, and the degree of reduction in virus quantities was assessed in the blood. Since in most cases a single PRRSV infection does not induce overt respiratory signs, especially under experimental circumstances and with European isolates [13–15,23], the clinical efficacy of attenuated PRRSV vaccines cannot be assessed. Identification and titration of the viruses, serological profiling, clinical signs, and lesions were the primary means by which vaccine safety and efficacy were evaluated.

Moreover, the cell-mediated immune response after vaccination and experimental exposure to a challenge virus was characterized, since different lymphocyte subsets have critical roles in the innate and acquired immune response against viruses, and their changes are important for evaluating phenotypic and functional efficiency of the immune response [24,25,40], time-related changes in peripheral lymphocyte subpopulations were assayed by flow cytometry in order to monitor the immune modulation in intradermally and intramuscularly vaccinated pigs subsequently challenged with the heterologous PRRSV isolate.

2. Materials and methods

2.1. Vaccine and challenge virus

The PRRSV vaccine used was the attenuated European virus strain DV (Porcilis® PRRS, Intervet BV, Boxmeer, The Netherlands). The vaccine was suspended in a tocopheryl acetate-containing aqueous adjuvant (Diluvac Forte®, Intervet BV, Boxmeer, The Netherlands).

A fifth passage on porcine alveolar macrophages (PAMs) of a wild-type Italian strain was used as the challenge virus. PRRSV was obtained from the pneumonic lungs of pigs which had died in an outbreak of respiratory disease during their growing phase. At the nucleotide level (ORF5), the percentage of identity for the Italian challenge virus strain with the vaccine strain was 78%.

2.2. Animals and experimental design

A total of 18 pigs were purchased from a PRRSV-seronegative farm at the age of 4 weeks. Pigs were divided into three groups (designated groups A, B, and C) and housed in isolation units with HEPA-filtered air.

At 5 weeks of age, the pigs of groups A ($n=6$ pigs) and B ($n=6$ pigs) were vaccinated intramuscularly (IM) and intradermally (ID) respectively with Porcilis® PRRS at a dose of $10^{4.5}$ TCID₅₀ per pig. The pigs of group C ($n=6$ pigs) were kept unvaccinated and served as control pigs. Intradermal vaccination was performed using the I.D.A.L.® vaccinator.

At 7 (T1), 14 (T2), 21 (T3), 28 (T4), and 35 (T5/D0) days post-vaccination (PV) blood was collected for vaccine virus detection by PCR, serological investigations and flow cytometry on PBMC.

Thirty-five days PV, all the groups were inoculated intranasally (2 ml in each nostril) with $10^{6.0}$ TCID₅₀ of the wild-type Italian PRRSV. After challenge, all pigs were monitored clinically by visual inspection and body (rectal) temperature was measured. At 0 (D0=challenge day=35 days PV), 3, 7, 10, 13 and 17 days post-challenge (dpc), blood was collected for PCR, determination of virus quantities and antibody titres in serum and flow cytometry in PBMC.

Sera were stored, after collection and separation, at -70°C .

At day 7 post-challenge, two pigs per group were euthanized for virological examinations and gross pathology. At the end of the experiment, all pigs were submitted to virological investigations and gross pathology.

2.3. Serological examinations

The presence and sample/positive (S/P) ratio of antibodies were determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Laboratories Inc., Westbrook, ME, USA) in accordance with the manufacturer's directions.

2.4. PCR, virus titrations, and sequencing of serum samples

All serum samples were submitted to PCR for virus detection. Serum samples collected after challenge were examined for virus by titration in PAMs, in accordance with standard procedures [13–15].

Briefly, 50 μl of 10-fold serial dilutions of the cell-free BAL fluids and of the serum samples were inoculated on 1-day cultivated PAMs, which were obtained from 4- to 6-week-old pigs from a PRRSV-negative farm. After 1 h incubation at 37°C , the inocula were replaced by medium. After 72 h at 37°C , PAMs were washed once with PBS and stained using an immunoperoxidase staining with monoclonal antibody against ORF7 [26] as described by Wensvoort et al. [27].

Viral isolates from sera and lungs were submitted to ORF5 sequencing as described by Oleksiewicz et al. [28].

2.5. Flow cytometry

The characterisation of lymphocyte subpopulations by flow cytometry was performed during the post-vaccination period and at 0 (D0), 3, 7, 10, 13, 17 days post-challenge (dpc). Briefly, 50 μl of heparinised blood were mixed with 5 μl of the specific antibodies in a plastic tube. After 15 min of incubation in the dark at room temperature, the cells were washed with phosphate buffer solution (PBS)/1% fetal calf serum (FCS) and centrifuged for 5 min at $400 \times g$. The contaminating red cells were lysed by treatment with NH_4Cl solution, pH 7.2, for 15 min at room temperature in the dark. The cell suspension was then washed twice with PBS/1% FCS, centrifuged for 5 min at $400 \times g$ and resuspended in 0.5 ml of PBS/1% FCS for flow cytometric analysis (Epics® XL-MCL, Coulter).

Cells were double-stained with anti-CD4 α -PE (clone 72-12-4) plus anti-CD8 α -FITC (clone 76-2-11) antibodies and anti-CD3 ϵ -PE (clone PPT3) plus anti-CD8 α -FITC antibodies; anti-CD14 (clone CAM36A) antibody was used as primary antibody coupled with goat anti-mouse IgG FITC-labelled secondary antibody for single staining.

Monoclonal antibodies were purchased from Valter Occhiena (Italy) and VMRD (USA); monocyte subset and the following lymphocyte subsets were phenotypically characterized [25,29,30]:

- T helper lymphocytes: CD3+CD4+CD8 α -;
- Cytotoxic T lymphocytes: CD3+CD4-CD8 α^{high} ;
- Double positive (DP) T lymphocytes (memory T helper): CD3+CD4+CD8 α^{low} ;
- Natural killer cells: CD3-CD8 α^{low} ;
- Monocytes: CD14+.

2.6. Gross examinations

Lung lesions (consolidation) and selected lymph nodes (sternal and deep cervical) were examined. Lungs and lymph nodes were examined for virus-induced changes.

2.7. Processing and analysis of BAL fluids

Lungs collected post-mortem were lavaged using a method described by Van Reeth et al. [31]. The right lung half was lavaged with 100 ml cold PBS via an 18-gauge blunt needle inserted through the trachea. The left main bronchus was cross-clamped to prevent lung lavage fluid from entering the left lung half.

The BAL fluids were centrifuged ($400 \times g$, 10 min, 4°C) to separate the cells and the cell-free lavage fluid. Aliquots of the cell-free lavage fluid were stored at -70°C until virus titration on MARC-145 cells or PAMs.

2.8. Statistical analysis

Differences in virus titres were analyzed using analysis of variance (ANOVA). Samples which tested negative for virus were given a numeric value of $0.95 \log_{10} \text{TCID}_{50}$ per ml serum (detection limit $0.96 \log_{10}$). $p < 0.05$ was taken as the level of statistical significance. Time-related changes in blood lymphocytes were analyzed by Tukey's test and were performed using SPSS® 6.1 software.

3. Results

3.1. Clinical examination

None of the groups had any relevant clinical signs at any time during the vaccination period or during challenge exposure. No pigs died during the entire experimental period. Body temperatures fluctuated in both groups and no statistically significant differences were detected.

3.2. Response to vaccination

3.2.1. Recovery of vaccine virus from serum samples

The results of PCR from serum samples are shown in Table 1. Vaccine strain, identified by ORF5 sequencing, was detected in the post-vaccination period in vaccinated pigs (groups A and B). All nonvaccinated control pigs remained negative for virus.

Four out of the six pigs (group B) had vaccine virus in their sera at 7 days PV, and one was still PCR positive 28

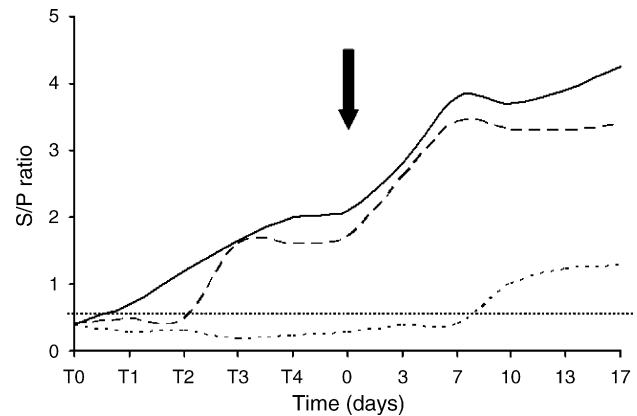


Fig. 1. Course of PRRSV ELISA antibodies in pig serum samples before (T0 to 0) and after challenge (0 to 17 days PC). The arrow indicates the challenge day. Straight line: group A (intramuscularly vaccinated pigs); sketched line: group B (intradermally vaccinated pigs); dotted line: group C (unvaccinated pigs); horizontal dotted line: detection limit.

days post-vaccination. One out of six pigs (group A) had vaccine virus at day 14 PV. Vaccine virus was detected in one pig per vaccinated group up to 28 days PV (i.e. 7 days before challenge). Thirty-five days PV no PCR positive serum samples were detected.

3.2.2. Serological response to vaccination

All the pigs were negative for PRRSV-specific antibodies at the start of the experiments (Fig. 1). After vaccination, all the pigs developed ELISA antibodies against PRRSV with a geometric mean S/P ratio of 2.0 in group A and 1.6 in group B at 35 days PV (i.e. challenge day).

Non-vaccinated pigs remained seronegative (S/P ratio < 0.4).

3.2.3. Changes in PBMC after vaccination

Changes in lymphocyte subpopulations between vaccinated and nonvaccinated animals were observed in the post-vaccination period. One week PV, in both groups of vaccinated animals (groups A and B) a decrease in $\text{CD4}^+\text{CD8}^-$ T cells and an increase in CD8^+ cells as compared to the control group were detected. The pattern of CD4^+ reduction persisted during the second week PV in both groups and also during the third week PV, albeit only in intradermally vaccinated animals. The time-related decrease in CD4^+ cells in both vaccinated groups was significantly different from that of the control group ($p < 0.05$). However, this appeared as a transitory change, since at the fourth and fifth week there was a gradual recovery of CD4^+ cell values in vaccinated animals.

The CD8^+ T lymphocyte percentage increased in the first and second week post-vaccination in both groups of vaccinated pigs. Thereafter, CD8^+ values in intradermally vaccinated animals resulted as being constantly and significantly higher up to the fifth week post-vaccination ($p < 0.01$).

Table 1
Results of PCR from pig sera during post-vaccination (PV) period

Time	A (IM)	B (ID)	C (ctrl)
T0 = vaccination	0/6	0/6	0/6
T1 = 7 dpv	0/6	4/6	0/6
T2 = 14 dpv	1/6	2/6	0/6
T3 = 21 dpv	2/6	0/6	0/6
T4 = 28 dpv	1/6	1/6	0/6
D0 = 35 dpv/challenge	0/6	0/6	0/6

dpv, days post-vaccination; IM, intramuscularly vaccinated pigs; ID, intradermally vaccinated pigs; ctrl, unvaccinated pigs.

Table 2
Results of PCR from pig sera during post-challenge (PC) period

Time	A (IM)	B (ID)	C (ctrl)
D1 = 3 dpc	5/6	5/6	6/6
D2 = 7 dpc	6/6	6/6	6/6
D3 = 10 dpc	4/4	4/4	4/4
D4 = 13 dpc	2/4	2/4	4/4
D5 = 17 dpc	0/4	1/4	1/4

dpc, days post-challenge; IM, intramuscularly vaccinated pigs; ID, intradermally vaccinated pigs; ctrl, unvaccinated pigs.

3.3. Response to challenge

3.3.1. Recovery of virus from serum samples and BAL fluids after challenge

In the post-challenge period, PRRSV was identified by PCR in 72.9% of serum samples from pigs of both vaccinated groups (A and B) and in 87.5% of sera from unvaccinated pigs (C) (Table 2).

The results of virus titrations of serum samples from the individual pigs of the different groups after challenge are shown in Fig. 2. Virus was isolated from serum samples of all nonvaccinated control pigs at day 3 PC, with peak virus titres at 7 days. All vaccinated pigs challenged with the Italian strain were virus positive at 7 days post-challenge. Mean virus titres of the vaccinated pigs challenged with the heterologous strain were significantly lower than those of the nonvaccinated control pigs ($p=0.0002$) at all the considered times (3, 7, 10, 13, and 17 days PC).

No statistically significant differences in virus titres were detected between the two vaccinated groups. At the time of euthanasia (7 days PC) of pigs of groups A (2 animals) and

B (2 animals), BAL fluids collected post-mortem were virus-negative in all pigs. Two out of two nonvaccinated control pigs (group C) were virus positive. PCR positive results were obtained from the sera of these animals. At the end of the experiment, three out of four samples of BAL fluid from pigs belonging to group A, none from group B and one out of four from group C (controls) were virus positive.

3.3.2. Serological response to challenge

The course of PRRSV-ELISA antibodies is shown in Fig. 1.

After challenge, all vaccinated pigs, via the intramuscular or the intradermal route, showed a significant and prompt booster increase in the S/P ratios.

In nonvaccinated pigs, antibodies were detectable at 10 days post-challenge.

3.3.3. Changes in PBMC after challenge

Within the first week after challenge, a CD4+ T lymphocyte transient reduction in all groups of vaccinated animals ($p<0.01$) was observed; this subset then rose significantly ($p<0.01$) at day 10 PC; during the whole post-challenge period, higher levels of CD4+ T cells were detected in vaccinated animals (Fig. 3). Controls did not show any significant changes in CD4+ T cells.

Seven days post-infection, a decrease ($p<0.01$) in peripheral double positive (CD4+CD8+) cells (Fig. 4) in all groups were observed. Ten days post-challenge, CD4+CD8+ cells recovered to pre-challenge values in all groups.

A rise in CD3-CD8 α^{low} lymphocyte percentage was observed at 3 days PC, with a peak at 7 days PC in all groups; this subset then normalised at 10 days PC (Fig. 5).

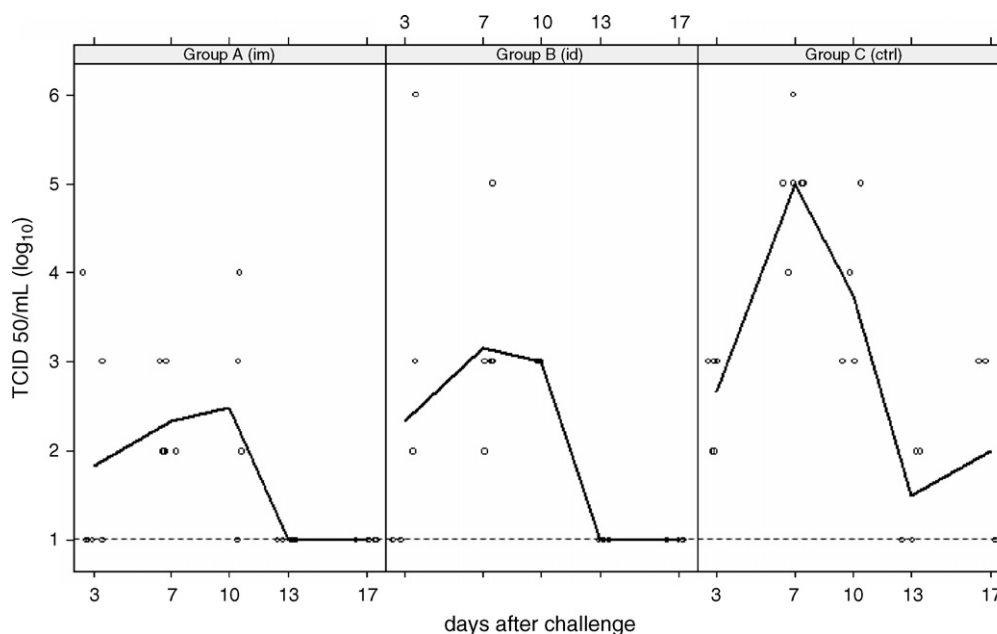


Fig. 2. Course of PRRSV titres (\log_{10}) in pig serum samples after challenge. Dots: individual virus titres; lines: group means; horizontal dotted line: detection limit; im: intramuscularly vaccinated pigs; id: intradermally vaccinated pigs; ctrl: unvaccinated pigs.

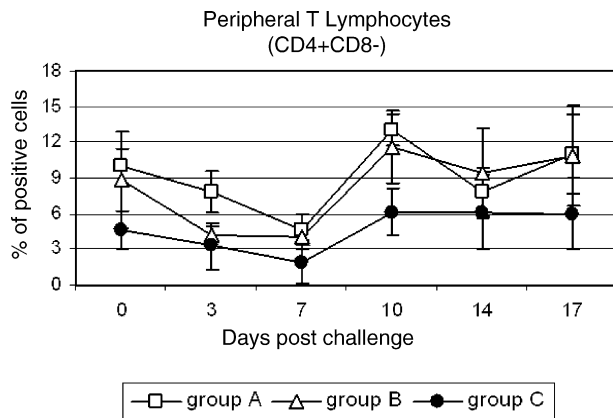


Fig. 3. Peripheral CD4+CD8- T lymphocytes in vaccinated and unvaccinated pigs during post-challenge period. Group A: intramuscularly vaccinated pigs; group B: intradermally vaccinated pigs; group C: unvaccinated pigs.

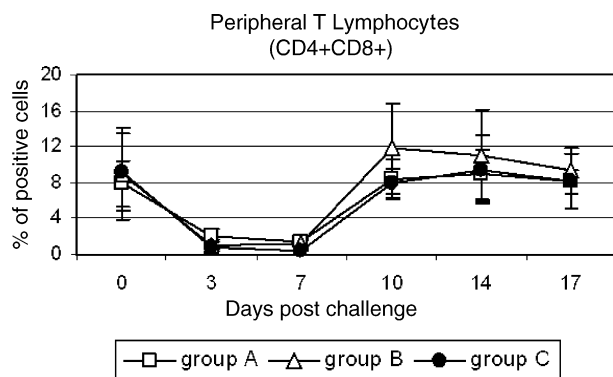


Fig. 4. Peripheral DP (CD4+CD8+) T lymphocytes in vaccinated and unvaccinated pigs during post-challenge period. Group A: intramuscularly vaccinated pigs; group B: intradermally vaccinated pigs; group C: unvaccinated pigs.

CD14+ cell trend (Fig. 6) in vaccinated animals was not significantly different from that of the controls. In all groups the time-related course of CD14+ cells showed a decrease at 7 days post-challenge.

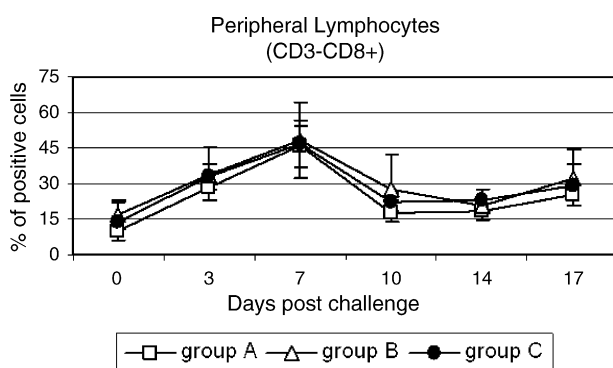


Fig. 5. Peripheral CD3-CD8^{low} lymphocytes in vaccinated and unvaccinated pigs during post-challenge period. Group A: intramuscularly vaccinated pigs; group B: intradermally vaccinated pigs; group C: unvaccinated pigs.

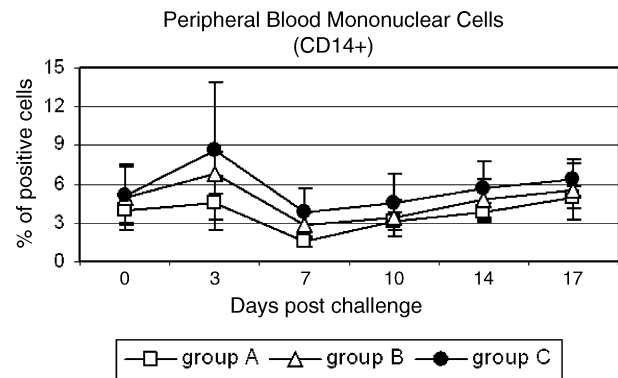


Fig. 6. Peripheral monocytes (CD14+) in vaccinated and unvaccinated pigs during post-challenge period. Group A: intramuscularly vaccinated pigs; group B: intradermally vaccinated pigs; group C: unvaccinated pigs.

4. Discussion

The present study was performed to evaluate the efficacy of vaccination using the commercially available attenuated PRRS vaccine belonging to the Lelystad cluster (i.e. Porcilis® PRRS) administered via the intradermal route using a needle-free vaccinator (I.D.A.L.®) in pigs experimentally exposed to a genetically divergent European PRRSV field isolate belonging to the Italian cluster.

The efficacy was compared to the effects of vaccination in intramuscularly vaccinated pigs and nonvaccinated controls. In addition, the immune response in vaccinated and control pigs was studied.

Criteria for determining efficacy were virus quantities in blood, the presence of virus in BAL of euthanized pigs, serological responses, and changes in peripheral T lymphocytes.

The results of PCR tests in blood collected after vaccination demonstrated that, like attenuated North American PRRSV strains [32,33], the European-type attenuated vaccine Porcilis® PRRS persists for several weeks after either intramuscular or intradermal administration. According to our results, the vaccine virus was not detected in either group by PCR from blood any later than 28 days post-vaccination.

It has been demonstrated that this vaccine virus can be isolated from blood [34] and in target organs, such as the lungs: according to the findings by Labarque et al. [17] the vaccine virus does not induce an attraction of inflammatory cells into the lungs, which may reflect its safety.

The first aim of this study was to evaluate how efficiently pigs which are vaccinated intradermally with an attenuated PRRSV vaccine based on a virus belonging to the Lelystad cluster are protected against an European wild-type strain from another cluster.

The fact that vaccination provided only partial virological protection following challenge might be explained by the genetic, and probably antigenic, differences between the strain used to prepare the vaccine (Lelystad-like cluster) and the strain used for challenge of immunity (Italian cluster). This would be consistent with a previous study with North

American PRRSV strains in which it was shown that the virus strain or strains identified in sera and BAL fluids of vaccinated pigs after exposure to a virulent virus were always those to which the pigs had not been exposed by vaccination [33].

The degree of virological protection is not affected by the route of administration of the vaccine, as pigs intradermally vaccinated did not show any differences in viral quantities in their blood.

In the present study, the differences in mean virus titres between nonvaccinated control pigs and pigs vaccinated either intramuscularly or intradermally and subsequently exposed to challenge with the Italian strain were constantly significant. This means that vaccine administration via either the intradermal or the intramuscular route reduces the viral titres of PRRSV in the blood and, as described by Labarque et al. [17] in the lungs, can confer some degree of virological cross-protection to heterologous isolates of PRRSV.

As measured by ELISA, vaccinated pigs (groups A and B) had a perceptible humoral immune response to vaccination by day 7. Seven days post-challenge, an increase in the S/P ratio was detected in both vaccinated groups. The extent of the increase was higher in vaccinated animals, related to the divergence of the challenge virus with the vaccine strain. The prompt increase in S/P ratio in vaccinated-challenged pigs is a consequence of the exposure to a heterologous isolate. This is consistent with the marked anamnestic increases in ELISA antibody responses described by McCaw et al. [35] after challenge with a heterologous strain of PRRSV.

The time-related changes in lymphocytes and monocytes after vaccination and heterologous challenge allowed us to monitor the cell-mediated immune response and to provide some functional information on it.

The study demonstrated that vaccination mimics some changes in peripheral T-cell subpopulations observed during experimental PRRSV virus infection [40] and that the immune modulations after intradermal and intramuscular vaccinations are comparable.

The CD4+CD8[−] naïve T helper cells decrease in vaccinated groups (evident in intradermally vaccinated animals) as an expression of a transient chemotactic recruitment of naïve T helper cells into lymphoid tissues for the recognition and priming of a primary immune activation to the vaccine. The CD8⁺ increase (significantly higher in intradermally vaccinated pigs) also refers to an earlier immune activation induced by the vaccine stimulation.

In the post-challenge period, both T helper lymphocyte subsets (CD4⁺ and CD4+CD8 α +) showed a transient reduction within the first week PC in all groups. Shimizu et al. [40] described a transient decrease in CD4⁺ cells associated with an increase in CD8⁺ in naturally and experimentally infected pigs. These findings could express a recruitment of naïve or cross-reactive memory lymphocytes or both into the lymphoid tissues owing to chemokine production and chemokine receptor expression at inflammatory sites and lymph nodes after virus exposure.

Several studies have confirmed the functional role of CD4⁺ naïve and memory T cells during PRRSV-immune response, after field virus exposure or MLV vaccination [2,36]. In vaccinated animals there is a significant cell recruitment, and this could be explained by a more efficient chemotactic signal, probably deriving from the primary inflammatory signals induced by vaccination.

In contrast, to confirm an ineffective immunological T memory, the changes in memory T lymphocytes (CD4+CD8⁺) and cytotoxic T lymphocytes (CD3+CD8⁺; data not shown) did not show any proliferative effect in vaccinated and nonvaccinated animals after heterologous challenge. Apart from a great decrease in double positive (CD4+CD8⁺) cells within the first week PC (Fig. 4) as an expression of a strong cell trapping in lymph nodes, no significant differences between groups after 10 days PC, when blood CD4+CD8⁺ cell levels normalise, or any proliferative effects, were observed.

It is well known that NK cells play a pivotal role as a first line of defence in viral infection as effector cells, as IFN- γ producers, and in dendritic cell maturation, and that an efficient stimulation of these cells is very important for the correct priming of acquired immune cells [3].

CD3/CD8 double labelling elucidated the CD8⁺ cell rise as due to a natural cytotoxic (NK) cell (CD3[−]CD4[−]CD8^{low}) increase. During the first week after challenge, a rapid and great increase in NK was observed; the percentages of NK cells then decreased, maintaining the higher level up to 17 days PC. Our data on NK cells is consistent with the results by Samson et al. [25], which revealed a subsequent increase in NK percentage in BAL, with a peak between 14 and 21 days post-infection.

Moreover, the early increase of NK cells in blood might be related to an early production of IFN- γ in PRRSV infected pigs. In fact, Wesley et al. [37] detected serum concentration levels of IFN- γ peaking at about 10 days after PRRSV infection and returning to approximately baseline levels by day 22. This early production of IFN- γ probably results from activation of NK cells, and it may further activate additional NK contributing to the clearance of PRRSV from serum and to trigger a Th1 response.

The studies on PRRSV infection and replication in host alveolar macrophages continue to give conflicting results as to its effect on innate cell activation, antigen presentation and the triggering of acquired immunity [13–16,38,39].

Vaccination with a MLV-EU strain of PRRSV in pigs subsequently exposed to a heterologous virulent strain, even if not inducing a specific cross-response, could stimulate a stronger inflammatory innate response able to give rise to a reduction in infectivity of the challenge virus in serum and in the duration of acute infection, and possibly a more effective sterilizing immunity: in vaccinated animals a better activation of NK cells and a more efficient co-operation between NKs and macrophages could explain the quicker clearance of the heterologous virus from serum as compared to nonvaccinated-challenged pigs.

Furthermore, we firstly demonstrated that an attenuated PRRS vaccine administered via the intradermal route can efficiently induce a protective immune response in pigs subsequently exposed to a genetically diverse PRRSV isolate. We also have evaluated a needleless injector (I.D.A.L.[®]) for intradermal vaccination and this is important considering that needle-free applications have demonstrated some advantages such as safety for the executor, painlessness for the pig, absence of histological lesions and no iatrogenic transmission of pathogens.

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