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Research paper

Comparative evaluation of immune responses of swine in PRRS-stable and unstable herds

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ABSTRACT

Porcine Reproductive and Respiratory Syndrome (PRRS) is an elusive model of host/virus relationship in which disease is determined by virus pathogenicity, pig breed susceptibility and phenotype, microbial infectious pressure and environmental conditions. Successful disease control in PRRS-endemic Countries corresponds to "stability", i.e. a condition with no clinical signs of PRRS in the breeding-herd population and no viremia in weaning-age pigs. The aim of this work was to compare the profile and time-course of humoral and cell-mediated immunity in stable and unstable herds, respectively. In particular, we investigated PRRS virus (PRRSV) in serum and group oral fluid samples by Real-time RT-PCR, PRRSV-specific IgA and IgG in oral fluids, serum IgG antibody and the cell-mediated response (PRRSV-specific release of interferon-gamma) in whole blood samples. These parameters were measured in order to identify possible discrepancies in the development and kinetics of the immune response against PRRSV. PRRS-free gilts got regularly infected after entering PRRS-stable and unstable farms. In an open cycle, unstable pig farm PRRSV infection could be demonstrated in all groups of pigs, including suckling piglets. Four main results should be highlighted: A) the precocity of the Ab response in group oral fluids was generally similar to that recorded in sera; B) circulation of PRRSV was consistently detected in all age groups in the unstable herds, as opposed to the stable ones; C) an early, balanced, IgA and IgG response in oral fluids was only observed in the stable herds; D) an early IFN-gamma response after PRRSV infection was often observed in stable herds, as opposed to the unstable ones. These were characterized by IFN-gamma responses in piglets, likely due to transfer of maternal immunity. Most important, the mucosal IgA response was associated with cessation of virus excretion in oral fluid samples of PRRS-unstable herds. The above findings indicate that a peculiar profile of immune response to PRRSV can be found in PRRS-stable herds. Therefore, the outlined immune parameters can represent a useful readout system to evaluate successful adaptation to PRRSV based on acclimatization of breeding animals and management of pig flow.

1. Introduction

Porcine Reproductive and Respiratory Disease (PRRS) is still a major cause of direct and indirect losses in swine farms worldwide. The causative agent (PRRS Virus, PRRSV) is an enveloped, positive-strand RNA virus of the *Arteriviridae* family. Whereas PRRSV infection is present in the large majority of pig farms, the prevalence of both PRRS and PRRS-associated diseases is highly variable. Two swine Arterivirus types have been identified to date as etiological agents: the European (EU) type I, with the first strain isolated in 1991 and named "Lelystad"; the North American (NA) type II, isolated in 1992 with the acronym ATCC VR-2332 (Nelsen et al., 1999). Several disease signs can be detected on farm depending on pig age and production phase: pyrexia, respiratory problems, slow growth, decreased feed efficiency, lethargy, anorexia, fever, cough and dyspnea in all ages (general infectious syndrome); pneumonia, conjunctivitis, secondary bacterial infection in weaners and fatteners; "Blue ear", reproductive problems, mortality in sows; infertility in boars (Zimmerman et al., 2006).

Experimental infection of sows with PRRSV in late gestation consistently reproduces disease, whereas in other cases PRRS is an elusive model of host/virus relationship, in which full-blown disease is the possible final outcome of a complex interaction between host, virus and

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environmental conditions. Despite extensive studies, widespread surveillance and disease control activities on farm, PRRS is still a major threat to swine farms, often causing high direct and indirect losses and these amount each year to 664 million dollars in USA, only (Holtkamp, 2013).

Different reasons account for the present conditions. Basically, disease control measures may have faulty foundations, since critical issues of PRRS virus pathogenicity and virus/host interaction in vaccinated and naïve animals are still ill-defined. In this scenario, the control of PRRS in infected farms is generally pursued by established procedures founded on a combination of management and biosafety measures. These may be integrated by the use of live attenuated or inactivated vaccines, as well as by acclimatization programs aimed at a controlled exposure of gilts to the circulating PRRSV strains before the reproductive period.

On the whole, disease control programs generally aim at "stabilization" of the herd as a priority, meant as a condition in which clinical signs of PRRS are absent in the breeding-herd population, and PRRSV is no more transmitted from sows to their offspring (Holtkamp et al., 2011). In this respect, correlates of clinical protection are actually lacking. In general, there is often evidence of a poor response of the innate immune system to PRRSV infection, as well as of an abnormal delay in the onset of neutralizing antibody and interferon (IFN)-γ responses (Niekamp et al., 2007), which paves the way to prolonged virus persistence in the host. Yet, cessation of viremia often takes place before the onset of the above features of adaptive immunity (Mateu and Diaz, 2008).

Owing to the above, our working hypothesis implied that a successful acclimatization program in a PRRS-stable herd could be correlated with peculiar profiles of the immune response to PRRSV. Accordingly, we decided to investigate the time-course of the immune response in pigs exposed to PRRSV in PRRS-stable and unstable herds. These were strictly selected on the basis of the following criteria for stability: i) no disease in the breeding herd sectors; ii) no virus transmission to the progeny. Accordingly, stable farms were either open cycle operations with no disease in the breeding herd sectors and no virus transmission to the progeny, or site 2 farms of multi-site operations with PRRS-free breeding stock. In this respect, it was our understanding that the term "stability" should be restricted in our study to the pre-weaning period. Our aim was to define the main features of the pigs' immune response related to the early control of PRRS virus infection on farm.

2. Materials and methods

2.1. Animals

Four PRRS-stable farms were selected on the basis of clinical and laboratory data on sows and piglets. All of them showed compliance with the above criteria of stability.

Farm S1. The first part of our study was conducted in an open cycle pig farm (PIC® – Camborough® genetics for the sow) in Veneto Region (Italy), whose commercial target is to sell growers weighing 30–35 kg, born of 350–400 sows. The herd has been positive for PRRSV infection for many years; replacement animals are vaccinated first with a live attenuated vaccine at around 140 days of age and then with an inactivated one 3 weeks later. There is evidence of PRRSV circulation, and the herd can be defined as clinically stable. About forty, 4-week old replacement gilts are introduced every two months from a PRRSV-free farm. On the day of arrival, gilts are housed in internal huts for at least 6–7 weeks without any deliberate exposure to PRRSV-infected animals.

Farm S2. This is a mixed open/close cycle farm in Lombardy Region, in which about 80% of the pigs are sold at a 30-kg b.w., whilst the remaining part (20%) is held until they reach usual market weight (160 kg b.w.) in a fattening unit. The farm has an intermediate level of biosecurity, i.e. the access is not strictly regulated. It consists of 2 pens for breeders (about 750), and 3 others for weaning, growing-finishing and fattening stages. At a distance of about 20 m from the growing-finishing-fattening pens, 4-month old PRRS-free gilts are introduced and housed in a quarantine box for about 60 days. The box is neither closed, nor separated. An All-In-All-Out (AIAO) production system is applied with no access regulation.

Farm S3. This is an open cycle farm in Lombardy Region, in which all the pigs are sold at a 30-kg b.w (about 3 months of age). The farm has a moderate level of biosecurity and access is partially regulated. The farm consists of a pen hosting the delivery and the cage gestation units, whereas a second pen is divided into two sectors, for the box gestation and weaning phases, respectively. The 5-month old replacement gilts are introduced every two months in the box gestation unit, within 6 boxes, and an AIAO production system is not applied. The gilts remain in the 6 boxes until they reach 120-kg b.w. when they reach the sexual maturity.

Farm S4. This is a site 1 of a multi-site pig production system, in which 25-day old piglets are moved to site 2. The farm has a high level of biosecurity, consisting of a pen intended for the delivery rooms and 3 others for cage and box gestation phases. There are about 2900 breeding sows. The quarantine structure is located at about 1.5 km from the breeding core, and managed as another farm (controlled entry and dedicated staff). The 28-day old replacement gilts arrive every two months and remain in the quarantine unit for three months. The quarantine unit consists of separate rooms, each hosting one group of replacement gilts.

Two PRRS-unstable herds were also monitored.

Farm U1. An open cycle, PRRS-unstable pig farm (Goland genetics) was selected for our study in Veneto Region (Italy) because of the persistent PRRS cases and PRRSV circulation, evidenced by clinical inspections and virological examinations. The herd includes about six hundred sows. All the pigs are sold at a 30-kg b.w (about 3 months of age). The external biosecurity level is high with respect to fencing and access regulation. Internal biosecurity is of intermediate level, the main critical point being the lack of a real, one-way pig forward flow. Replacent gilts (4-week old, 7–8-kg b.w.) are purchased from a PRRS-free farm and directly introduced into the herd within rooms of same age pigs. No specific acclimatization program is followed.

Farm U2. A second unstable herd was selected in Lombardy Region (Italy). This is classified as a farrow-to finish operation with some 1000 Danish sows. The farm is characterized by poor biosecurity because of two reasons: i) it neighbors another pig fattening farm with a wide distribution and circulation of PRRSV, whose vehicles are allowed to pass through its premises; ii) there is no quarantine: gilts weighing 70 kg are directly introduced every two months into the gestation unit and housed in boxes adjoining adult breeding animals.

2.2. Samplings

Samplings aimed to depict the time-course of the immune response to PRRSV of replacement gilts. This scheme was replaced by a cross-sectional study in farm U1 because of the ongoing PRRS cases in different age groups.

Farm S1. Eight, 30-day old gilts of three consecutive replacement groups were ear-tagged, grouped together and then followed longitudinally at the following times: T1 = day 1; T2 = day 49 (animals exiting the huts); T3 = day 63; T4 = day 77; T5 = day 91; T6 = day 105 after arrival (six samplings for each group).

Farms S2-S4. 15-subject groups of gilts per farm were monitored at 15 days-intervals over 2–3 months from their day of arrival (T1) onwards. Farm S2: one group of 4-month old PRRS-free gilts and six samplings. T1 at arrival at 4 months of age. Farm S3: two groups of 5-month old PRRS-free gilts and four samplings. T1 at arrival at 5 months of age. Farm S4: one group of 25-day old PRRS-free gilts and six samplings. T1 at 28 days of age.

Farm U1. A cross-sectional sampling was carried out at the same

time on diseased pig groups (about three hundred pigs each) aged 28, 42, 56 and 70 days, respectively. Eight animals per group were included in the sampling.

Farm U2. One group of 15 PRRS-free gilts weighing 70 kg was monitored 6 times at 15-day intervals starting from the arrival day. T1 at arrival at 6 months of age.

2.3. Processing of samples

Venous blood was drawn in vacuum tubes without anticoagulant to obtain serum. A second aliquot of blood was drawn in heparinized vacuum tubes for cell-mediated immunity assays. Group oral fluids (OF) were collected by a cotton thread cord with a frayed end hanging at about 5 cm from the snout of the animals. The exposure time was one hour for 4-week old pigs down to 20 min for the 3-month old ones in order to ensure adequate imbibition of the rope without compromising its integrity. The cord was then placed into a stomacher bag, and transported to the laboratory under refrigerated conditions. It was manually squeezed and the collected liquid was centrifuged at 1800 rpm for 10 min at 5 °C, the supernatant was recovered into test tubes and frozen at −20 °C until analysis.

2.4. Laboratory tests

PRRSV viremia and its presence in oral fluids were investigated in the two herds of Veneto Region (S1 and U1) by Real-time RT-PCR for PRRSV ORF 7 gene, as previously described (Drigo et al., 2014), using validated primers and probes for both EU and NA PRRSV (Lurchachaiwong et al., 2008). RNA was extracted from 200 microliters of blood serum or oral fluids by an automatic procedure, using the QIACube apparatus (Qiagen). The limit of detection (LoD) of EU PRRSV target is 125 copies/μL.

Another Real-time RT-PCR procedure for PRRSV was employed for serum and OF samples collected in the 4 herds of Lombardy Region (S2, S3, S4, U2). Briefly, viral RNA was extracted from 200 μL of sample using a commercial kit (NucleoMag® Vet kit, Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. An exogenous internal control RNA, was added to specimens prior to RNA extraction to verify the success of the procedure and the absence of inhibitors. The extraction was carried out on the Biosprint 96 instrument (Qiagen, Hilden, Germany), using the NucleoMag Vet 200 protocol. Nucleic acids were eluted into 100 μL of elution buffer and immediately subjected to RT-PCR or stored at −80 °C until used. RNA detection was done using the LSI VetMAX (TM) PRRSV EU/NA Real-Time PCR kit (Thermo Fisher), as specified by the manufacturer. The detection limit of EU PRRSV target is 9 copies of nucleic acids per reaction, corresponding to 0.642 genome copies/μL.

PRRSV antibody titers in serum and oral fluids were measured by ELISA using a commercial kit (Herdcheck PRRS X3 Antibody Test Kit, IDEXX). The kit was used according to the manufacturers' directions on serum samples.

The same kit was adapted to oral fluids by introducing HRP-conjugated, anti-swine IgG and IgA antisera, respectively, in the test procedure, and properly diluting the positive and negative controls of the kits to reach the usual threshold OD values. Also, as opposed to serum samples, 1:2-diluted oral fluids were reacted overnight at 4 °C with PRRSV Ag-coated strips. This procedure had been validated on oral fluids of pigs collected before and after experimental intranasal infection with a EU PRRSV strain (Amadori M., unpublished results). On the basis of our experimental data, a sample to positive (s/p) ratio of 0.4 was identified as threshold for IgG and IgA-positive samples. S/p values < 0.4 were scored negative. The IgG and IgA-specific s/p values of each sample were compared. The Ab response was considered unbalanced if the IgA-specific s/p value was ≤50% of the IgG-specific one.

The cell-mediated immune response to PRRSV was assessed by the PRRSV-specific IFN-gamma release assay on whole blood samples, as previously described (Dotti et al., 2011). Briefly, heparinized whole blood samples were stimulated overnight at 37 °C in 48-well tissue culture plates with PBS, cell-adapted EU PRRSV BS114 strain grown in MARC-145 cells (700,000 $TCID_{50}$/well) and a cryolysate of MARC-145 cells, respectively. Then, porcine IFN-gamma was measured by sandwich ELISA in the plasma fraction of each aliquot. Several T cell epitopes are located in glycoproteins 4, 5 and nucleocapsid (N) protein of PRRSV, and are largely conserved in the different isolates (Diaz et al., 2009); therefore, divergence between field and laboratory EU strains of PRRS virus would not be of major concern for the outcome of this assay.

2.5. Statistical analyses

The possible association between PRRSV viremia and IFN-γ response was investigated by Fisher's exact test (Graph Pad Prism 5, GraphPad Software Inc., La Jolla, CA). The threshold for significance was set at $P < 0.05$.

3. Results

3.1. PRRS-stable herds

In the PRRS-stable herd S1, the clinical status of gilts remained satisfactory during the course of the entire trial. Laboratory findings are shown in Table 1. The three groups of gilts under study got infected with PRRSV seven (group 1) to nine weeks (groups 2 and 3) after entering the herd. There were no major discrepancies between test results based on serum and mucosal antibodies in revealing PRRSV infection. Some gilts in group 1 tested positive in the IFN-γ release assay after the seroconversion and got negative at T6. Some later infected gilts (groups 2 and 3) were still positive instead at T6. All serum and OF samples tested negative for PRRSV RNA in Real-time RT-PCR, i.e. there was no evidence of PRRSV circulation in gilts despite seroconversion.

In the other PRRS-stable herds (S2 to S4), results were substantially in agreement with those of herd S1, albeit with distinct signs of virus circulation (results of Real-time RT-PCR, see Figs. 1–3). All the gilts under study remained healthy in the observation period, and got infected at different times after arrival as revealed by both PCR and antibody assays. There was a clear negative association ($P < 0.01$ at least) between PRRSV viremia and IFN-γ response, whereas neither serum antibody nor IFN-γ responses were associated with cessation of viremia. As in farm S1, there were obvious discrepancies in the development of PRRSV-specific IgA and IgG responses in oral fluids. Virus excretion in OF was generally associated with low IgA to IgG s/p ratios (prevailing IgG responses). Once again, mucosal and serum antibody assays did not differ in terms of diagnostic precocity in farms S3 and S4, whereas serum Ab provided an earlier indication (T2 vs. T3) in farm S2. No Ab test was carried out on T1 OF samples of group 2 gilts of farm S3 (Fig. 2); these also showed a subsequent time-course of PRRSV excretion and Ab response in OF in line with the aforementioned pattern.

3.2. PRRS-unstable herds

In the PRRS-unstable herd U1, there was an outbreak of "storm abortions", high mortality in suckling piglets (around 20% mortality rate), a respiratory syndrome in weaners, reduced fertility (80% farrowing rate) in the period of our study. Laboratory findings are shown in Table 2. In agreement with the clinical findings, pigs were shown to be PRRSV-infected from the suckling period onward, as shown by both Ab and PCR assays. All the pigs but the 70-day old ones were both viremic and excreting PRRSV in OF. But for one pig, cell-mediated immunity was not evidenced after the first sampling (at 28 days of age). The Ab response in OF samples was peculiar. After the infection in the suckling period, the Ab response was substantially skewed to IgG (serum transudate antibody) over 10 weeks, approximately. Only in the

Table 1
PRRS-stable herd S1: laboratory findings.

GROUP 1	T1(day 1)	T2 (day 49)	T3 (day 63)	T4 (day 77)	T5 (day 91)	T6 (day 105)
Serum Ab: mean s/p	0	0.35	ND	0.92	0.76	0.86
Serum Ab positive/total	0/8	3/8	ND	8/8	7/8	8/8
OF s/p IgA	0 (neg)	0.61 (pos)	ND	0.49 (pos)	0.87 (pos)	0.75 (pos)
OF s/p IgG	0.10 (neg)	1.83 (pos)	ND	1.04 (pos)	1.16 (pos)	1.06 (pos)
IFN-γ test positive/total	ND	4/8	ND	ND	ND	0/8
GROUP 2	T1(day 1)	T2 (day 49)	T3 (day 63)	T4 (day 77)	T5 (day 91)	T6 (day 105)
Serum Ab: mean s/p	0.01	0.08	0.93	1.11	1.03	ND
Serum Ab positive/total	0/8	1/8	8/8	8/8	8/8	ND
OF s/p IgA	0.02 (neg)	0.21 (neg)	0.91 (pos)	0.53 (pos)	0.49 (pos)	ND
OF s/p IgG	0.12 (neg)	0.23 (neg)	0.84 (pos)	1.09 (pos)	0.86 (pos)	ND
IFN-γ test positive/total	ND	0/8	ND	ND	ND	4/8
GROUP 3	T1(day 1)	T2 (day 49)	T3 (day 63)	T4 (day 77)	T5 (day 91)	T6 (day 105)
Serum Ab: mean s/p	0	0.05	0.11	0.89	0.65	0.75
Serum Ab positive/total	0/8	0/8	1/7	7/7	7/7	6/7
OF s/p IgA	0 (neg)	0 (neg)	0.17 (neg)	0.07 (neg)	0.27 (neg)	0.8 (pos)
OF s/p IgG	0.04 (neg)	0.03 (neg)	0.28 (neg)	0.5 (pos)	1.0 (pos)	0.77 (pos)
IFN-γ test positive/total	ND	0/8	ND	ND	ND	2/7

Three groups of gilts were introduced in the quarantine sector. They were clinically inspected and samplings were carried out at the indicated times. Pos: test positive sample. Neg: test negative sample. ND: not done. Real time RT-PCR for PRRSV ORF 7 gene was always negative on both serum and OF samples (data not shown).

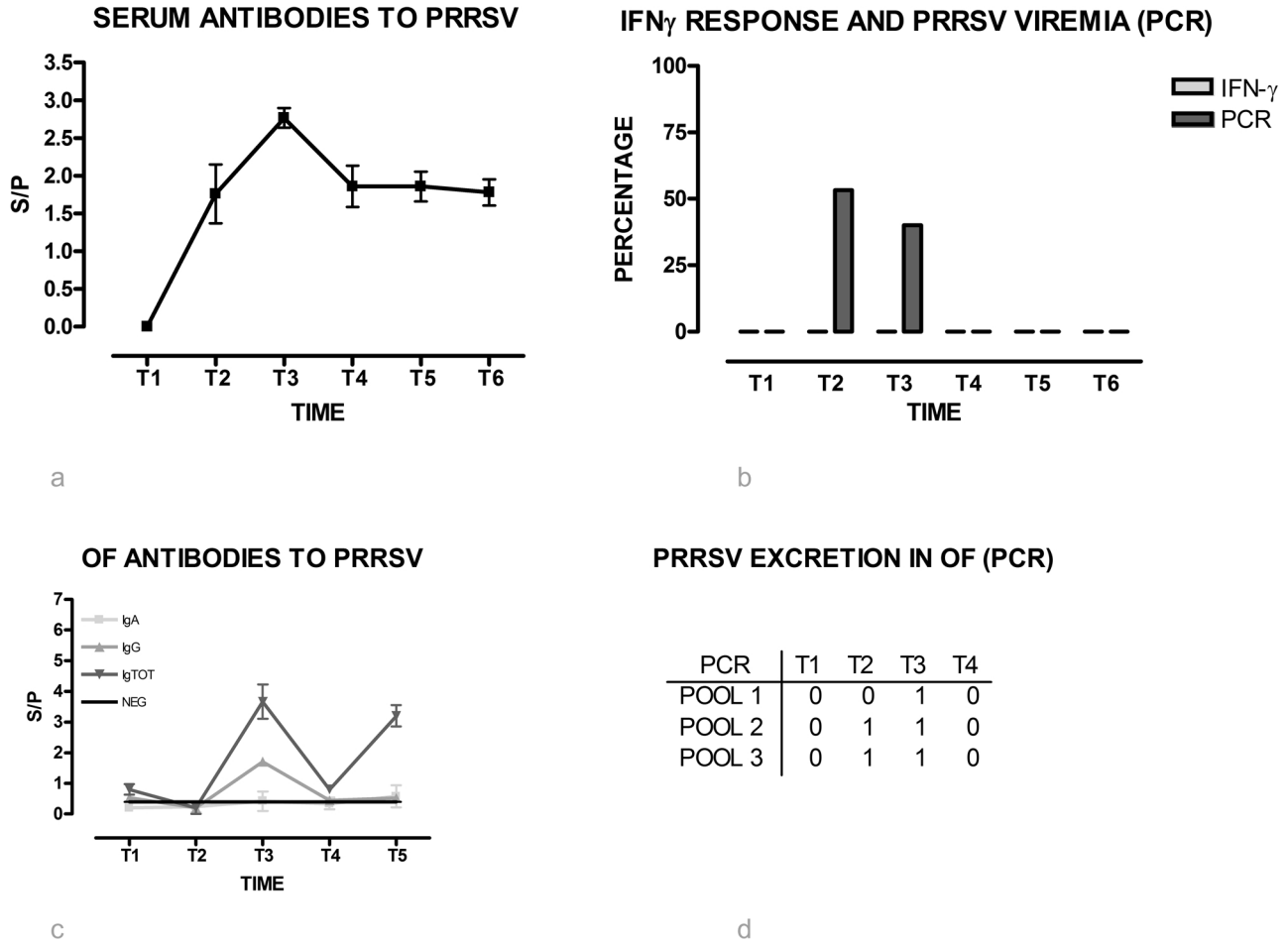
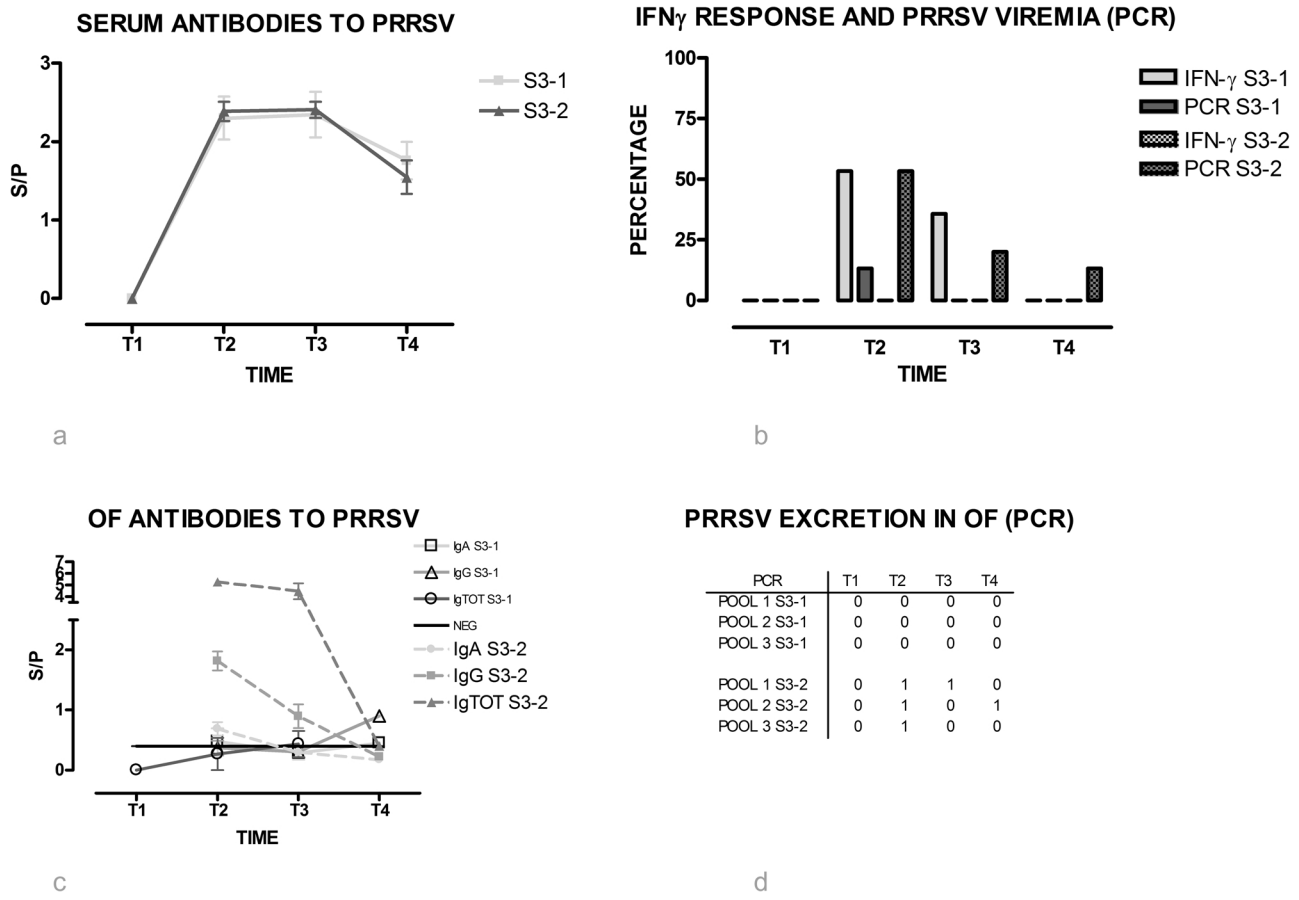


Fig. 1. Laboratory results obtained in farm S2. Serum, whole blood and OF samples were collected from a single group of 15 gilts. The four panels depict the serum Ab response to PRRSV (upper left), the total, IgG and IgA Ab responses in OF (lower left), PRRSV viremia and IFN-γ response (upper right) and PRRSV excretion in OF (lower right), respectively. PRRSV excretion in OF is shown in terms of PCR positive (symbol 1) and negative (symbol 0) pools of 5 OF samples each. The prevalence of IFN-γ-positive pigs was assessed in PRRSV-viremic and non-viremic subjects, and shown to be significantly different by Fisher's exact test ($P < 0.001$). T1 at arrival at 4 months of age. Subsequent samplings (T2 – T6) at 15-day intervals.



PCR	T1	T2	T3	T4
POOL 1 S3-1	0	0	0	0
POOL 2 S3-1	0	0	0	0
POOL 3 S3-1	0	0	0	0
POOL 1 S3-2	0	1	1	0
POOL 2 S3-2	0	1	0	1
POOL 3 S3-2	0	1	0	0

Fig. 2. Laboratory results obtained in farm S3. Serum, whole blood and OF samples were collected from two groups of 15 gilts each (S3-1 and S3-2, respectively).The four panels depict the serum Ab response to PRRSV (upper left), the total, IgG and IgA Ab responses in OF (lower left), PRRSV viremia and IFN-γ response (upper right) and PRRSV excretion in OF (lower right), respectively. PRRSV excretion in OF is shown in terms of PCR positive (symbol 1) and negative (symbol 0) pools of 5 OF samples each in both groups of gilts. The prevalence of IFN-γ-positive pigs was assessed in PRRSV-viremic and non-viremic subjects, and shown to be significantly different by Fisher's exact test ($P < 0.001$). T1 at arrival at 5 months of age. Subsequent samplings (T2 – T4) at 15-day intervals. No Ab test was carried out on T1 OF samples of group 2 gilts.

70-day old pigs could we detect a balanced IgA to IgG response in OF samples, which was associated with absence of PRRSV excretion. Such data cannot be compared with the previous ones. Yet, they show the peculiarities of the immune response during clinically overt PRRS, a frequent outcome in unstable herds.

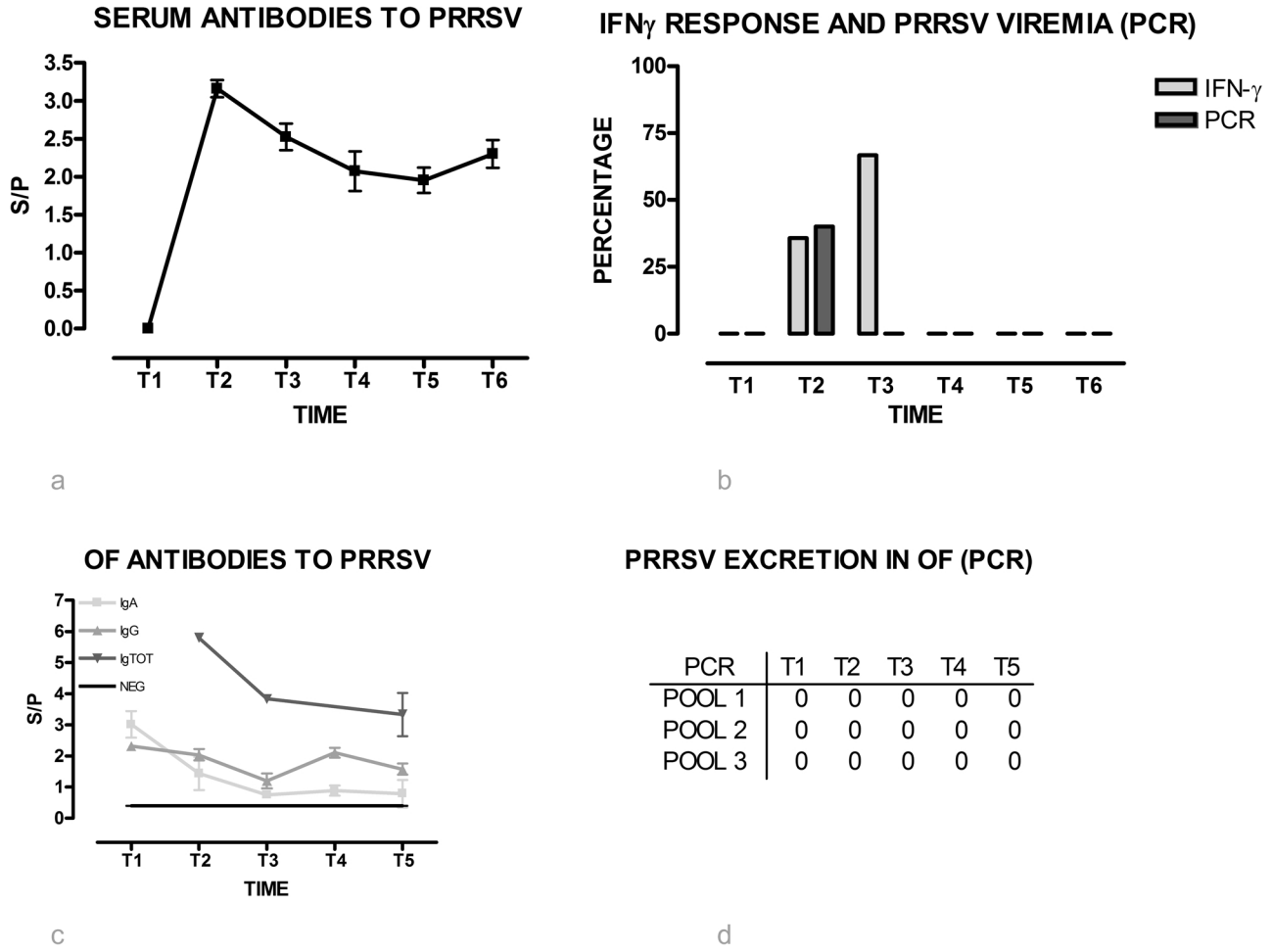
In PRRS-unstable herd U2, about 15% of weaners presented skin cyanosis in the thigh and ear regions, as well as blunting sensory and fever. The mortality stood at this stage to 10%. As for the breeding sector, a 15% abortion rate in the last third of pregnancy and an increase of unhealthy litters were observed during our study. Also, mortality in sows rose to 4%. Laboratory results are shown in Fig. 4. There was an early seroconversion of gilts at T2 and persistence of high serum Ab titers until T6. Viremia persisted at high titers between T2 and T5. There was no virus excretion in OF at T2, as opposed to T3 and T4, which once again coincided with rising IgG and decreasing IgA antibody in OF. The IFN-γ response was suppressed in the presence of viremia at T2, T3 and T4, and it was partially resumed in non-viremic pigs at T5 and T6 ($P < 0.001$).

4. Discussion

The field surveys carried out in PRRS-stable and unstable herds highlighted fundamental differences between the two kinds of farms in terms of clinical features, virological status and adaptive immune response to PRRSV infection. The authors are aware though the different dynamic seen between the herds might be partly accounted for by the age of the animal at exposure and by the infection pressure.

The IFN-γ response to PRRSV beyond the suckling period was either not observed or abnormally delayed after infection in the two PRRS-unstable herds under study. It should be stressed that our assay has very strict interpretation parameters, in that a cryo-lysate of the same cells used for propagation of PRRSV is used to rule out non-specific responses to stress antigens, which may be an important confounding factor in the assay (Amadori and Zanotti, 2016). The high prevalence of test-positive samples in the group of 28-day old pigs in herd U1 was probably due to maternal transfer of PRRSV-specific lymphocytes, in agreement with established models of maternal transfer of cell-mediated immunity in pigs (Bandrick et al., 2014). This may be traced back to activated lymphocytes and/or Transfer Factors (Viza et al., 2013) in colostrum and milk. Notably, there was a striking negative association between PRRSV viremia and IFN-γ response of pigs (Figs. 1–4), in agreement with the well-known immunosuppressive properties of PRRSV (Thanawongnuwech et al., 2000; Xiao et al., 2004). Please notice that our data do not imply that PRRSV viremia and suppression of T cell responses vary together. We compared qualitative (presence/absence), not quantitative data. This is the reason why results are expressed in terms of "negative association", and not of "negative correlation", for which a completely different statistical approach would be needed, like the non-parametric two-tails *rho* test of Spearman.

PRRSV infection could be detected with similar efficiency and precocity by Ab tests on serum and OF samples. The slight discrepancy observed in farm S2 was probably due to low saliva contributions of the



PCR	T1	T2	T3	T4	T5
POOL 1	0	0	0	0	0
POOL 2	0	0	0	0	0
POOL 3	0	0	0	0	0

Fig. 3. Laboratory results obtained in farm S4. Serum, whole blood and OF samples were collected from a single group of 15 gilts. The four panels depict the serum Ab response to PRRSV (upper left), the total, IgG and IgA Ab responses in OF (lower left), PRRSV viremia and IFN-γ response (upper right) and PRRSV excretion in OF (lower right), respectively. PRRSV excretion in OF is shown in terms of PCR positive (symbol 1) and negative (symbol 0) pools of 5 OF samples each. The prevalence of IFN-γ-positive pigs was assessed in PRRSV-viremic and non-viremic subjects, and shown to be significantly different by Fisher's exact test ($P < 0.01$). T1 at arrival at 28 days of age. Subsequent samplings (T2 – T6) at 15-day intervals.

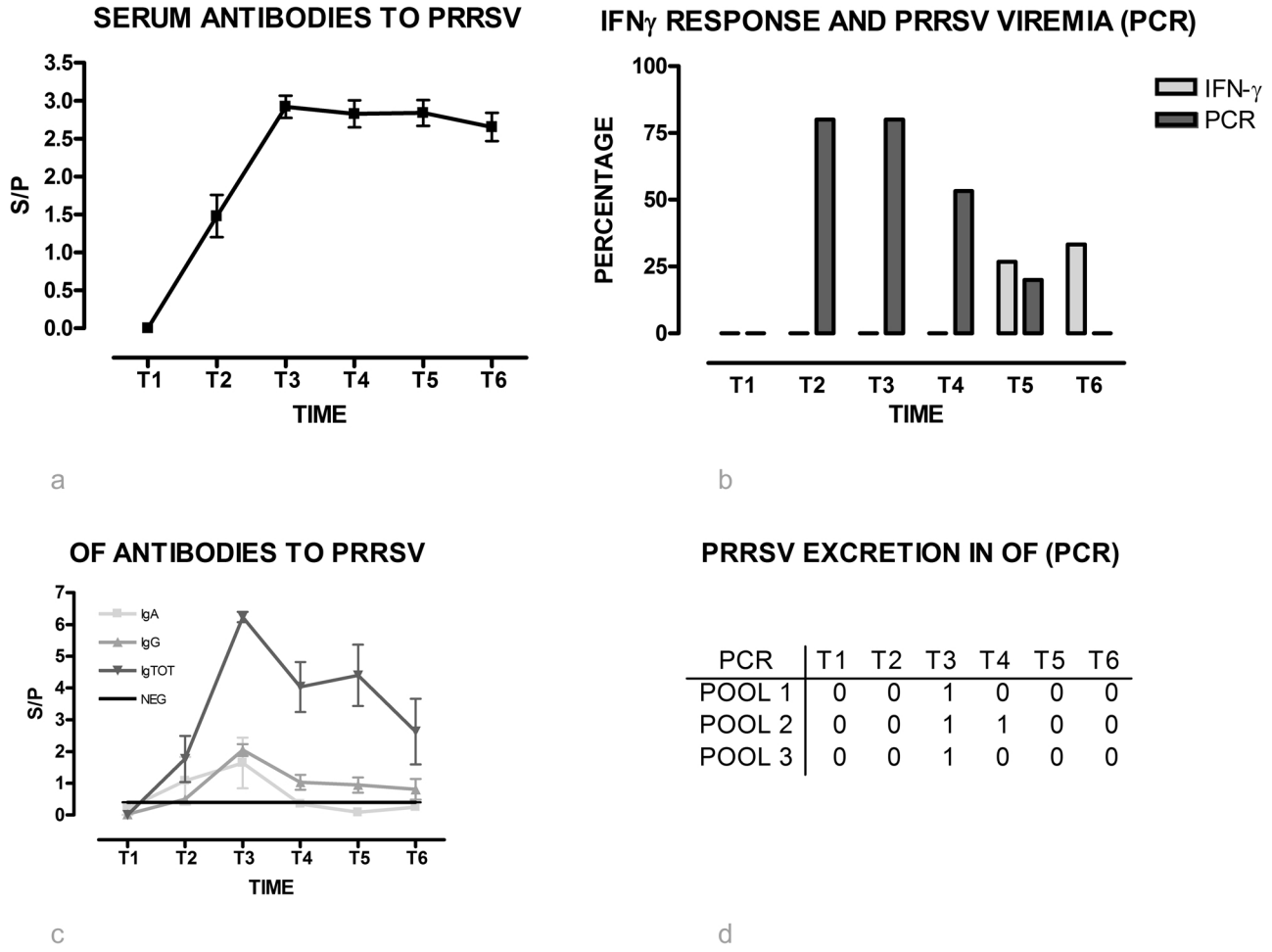
Table 2
PRRS-unstable farm U1: laboratory findings.

	28-day old pigs	42-day old pigs	56-day old pigs	70-day old pigs
Serum Ab: mean s/p	1.19	0.77	1.72	1.45
Serum Ab: positive/ total	8/8	5/8	8/8	7/8
OF s/p IgA	2.38 (pos)	0.29 (neg)	0.78 (pos)	0.49 (pos)
OF s/p IgG	3.53 (pos)	1.98 (pos)	2.43	0.75 (pos)
IFN-γ test positive/ total	6/8	0/8	1/8	0/8
RT PCR serum samples	pos	pos	pos	pos
RT PCR OF samples	pos	pos	pos	NEG

A cross-sectional study was carried out at the same time in a PRRS-unstable herd in the presence of both respiratory and reproductive disease. Two pools of 4 serum samples each were analyzed by Real-time RT-PCR for PRRSV ORF 7 gene. Pos: test positive sample. Neg: test negative sample.

few seropositive pigs at T2. By matching the test results in the two assays (Table 1), 1–2 pigs out of 8 could be enough to detect the previous infection in the group. Most important, the isotype-specific Ab responses in OF samples were shown to differ between PRRS-stable and unstable herds. A balanced profile of Ab response (IgA to IgG ratio around 1) after infection was observed early in PRRS-stable herd S1, i.e. from the very beginning (group 2), after 40 days (group 1) and 30 days (group 3), approximately. The IgA response was similar in farm S4 and less pronounced in farms S2 and S3. Instead, after infection in the suckling period, such a balanced IgA to IgG ratio was only observed in 70-day old pigs in herd U1, which coincided with the clearance of PRRSV in OF samples and, presumably with lesser virus recirculation in the pen. Also, in farms S2 and S3 (group 2 gilts), virus excretion was always recorded in the presence of low IgA to IgG s/p ratios. Mucosal IgA express the secretory chain (SC) and can thus be internalized by PRRSV-infected cells, where IgA could perform an important mechanism of intracellular neutralization (Mazanec et al., 1992). On the contrary, high levels of PRRSV-specific IgG in OF samples were not predictive of control over virus excretion. This is reminiscent of long-lasting PRRSV viremia cases despite the presence of neutralizing antibody (Diaz et al., 2012), and of the poor, sometimes inverse correlation between serum Ab responses and clinical outcome after PRRSV challenge infection (Morgan et al., 2013; Diaz et al., 2013).

PRRSV excretion in OF samples did not always correlate with PRRSV viremia, which points at an important role of the lymphoid tissues of the oral cavity as niches of macrophage cells hosting PRRSV over a long time (O'Sullivan et al., 2011). This makes a case for a substantial re-appraisal of OF samples for monitoring the acclimatization of replacement gilts. This is generally evaluated on the basis of PRRSV viremia as indicator of an effective contact with the field virus. On the basis of our findings, this parameter should be integrated by



PCR	T1	T2	T3	T4	T5	T6
POOL 1	0	0	1	0	0	0
POOL 2	0	0	1	1	0	0
POOL 3	0	0	1	0	0	0

Fig. 4. Laboratory results obtained in farm U2. Serum, whole blood and OF samples were collected from a single group of 15 gilts. The four panels depict the serum Ab response to PRRSV (upper left), the total, IgG and IgA Ab responses in OF (lower left), PRRSV viremia and IFN-γ response (upper right) and PRRSV excretion in OF (lower right), respectively. PRRSV excretion in OF is shown in terms of PCR positive (symbol 1) and negative (symbol 0) pools of 5 OF samples each. The prevalence of IFN-γ-positive pigs was assessed in PRRSV-viremic and non-viremic subjects, and shown to be significantly different by Fisher's exact test ($P < 0.001$). T1 at arrival at 6 months of age. Subsequent samplings (T2–T6) at 15-day intervals.

proper checks on OF samples, demonstrating lack or cessation of virus excretion associated with a balanced IgA to IgG ratio. Also, lack or early cessation of viremia and an early IFN-γ response to PRRSV infection were further indicators of a favorable outcome of the host/pathogen relationship. In this respect, viremia should not be taken as a foundation of exposure to field PRRSV strains during the acclimatization period. Pigs may show in fact seroconversion without detectable viremia (see farm S1) in the framework of robust herd stability.

The above differences between PRRS-stable and unstable herds can be probably traced back to PRRSV infection in the suckling period and/or to the high global infectious pressure in unstable herds. PRRSV infection in the suckling period is conducive to high-titered viremia and reduced immune functions (Dwivedi et al., 2012). In this respect, stability definitely implies a better coping ability of animals since highly susceptible suckling piglets are not exposed to the PRRSV stressor. This is definitely the very foundation of herd stability, underlying successful disease control on farm. Under these conditions, correct acclimatization and/or vaccination procedures of gilts can be carried out and verified. Our study has indicated that stability for PRRS corresponds to a peculiar course of the immune responses. In particular, the timely occurrence of cell-mediated and mucosal IgA Ab responses were associated in our experience with absence (farm S1) or early cessation (farms S2-S4) of virus shedding in OF samples. Therefore, these immune parameters can represent a useful readout system to evaluate successful adaptation to PRRSV based on acclimatization of breeding animals and management of pig flow. A note of caution should be expressed though about possible age-related differences of the immune response of replacement gilts to PRRSV, having in mind the wide spectrum of operational schemes in pig farms. The herd-specific infectious pressure is likely to play a role, as well. This should be referred to high PRRSV circulation and long-lasting viremia of non-adult pigs, but also to the high microbial load in the environment following a high prevalence of opportunistic infections. This way, the association between PRRSV and other microbial and/or environmental stressors could underlie unfavorable outcomes of PRRSV infection in unstable herds. In this scenario, failure of acclimatization and disease control measures on the whole could be traced back to three kinds of reasons:

- poor biosecurity and/or farm management (Fablet et al., 2016);
- lean, PRRSV-susceptible animal phenotypes introduced in the farm (Petry et al., 2005);
- peculiar virus "immunotypes" of PRRSV (Gimeno et al., 2011), affecting the immune response of PRRSV-infected animals and causing a poor control of the inflammatory response to airborne LPS (Zhiping et al., 1996) and/or common environmental pathogens (Qiao et al., 2011).

The above-mentioned immune markers and the time-course thereof can be the basis of updated disease control programs on farms. In particular, they can define a global, coherent and predictive profile of

gilt acclimatization. Also, they can contribute to early detection of risks for animal health and welfare in pig farms, having in mind the primary pathogenic role of PRRSV in the Porcine Respiratory Disease Complex (PRDC) (Chae, 2016). Accordingly, a successful control of PRRS is likely to decrease the prevalence of secondary bacterial infections and antibiotic usage on farm, towards better food safety profiles in pork production.

Conflict of interest

The authors declare no conflict of interest. All funding bodies have been named in the acknowledgments.

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