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EPIDEMIOLOGY AND CONTROL OF PRRSV INFECTION IN THE BREEDING HERD

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SUMMARY

1 INTRODUCTION

Since its appearance, Porcine Reproductive and Respiratory Syndrome (PRRS) has devastated the worldwide swine industry with tremendous economic losses (Meng, 2000), estimated in a study by Neumann et al. in 2005 to be in excess of $560 million each year in the United States. At present, in spite of all our progress, PRRS is still a frustrating disease and our understanding of the disease is still far from complete. The clinical features of PRRS are well known, a global picture of the epidemiology has been drawn, management procedures have been devised to control the disease and vaccines are available. Nevertheless, infection by PRRSV is still widespread, the virus is frequently reintroduced to farms after eradication and immune response induced is still poorly understood (Mateu and Diaz, 2008).

Moreover, the high heterogeneity among PRRSV isolates is likely to be the main obstacle to effective control of PRRSV infection using current commercial vaccines, since the immunity induced by one strain may be only partial against a different strain, even within the same genotype (Meng, 2000; Mateu and Diaz, 2008; Kimman et al., 2009). Therefore, adoption of strict biosecurity measures and acclimatization of replacement gilts by exposure to the specific PRRSV circulating on farm are commonly considered the most sound strategies to control PRRS in breeding herds (Batista et al., 2004; Fano et al., 2005; Pesente et al., 2006; Vashisht et al., 2008). Furthermore, control of PRRSV cannot rule out a regular and accurate survey of the herd stability, depending on correct sample-testing selection, diagnostics assessment and the sensitivity of the test used. A completed herd history, reliable production records, assessment of clinical signs and a combination of the proper diagnostic tests, adequate interpretation of results and the practitioner's experience are the best resources a veterinarian can use to successfully control PRRS and its economic impact on swine production (Batista, 2005). Finally, sequencing of PRRSV isolates followed by phylogenetic analysis is a powerful tool to monitor the spread of PRRSV intra and inter farms and allows a fuller understanding of the success or failure of the control programme (Pesente et al., 2006).

The purposes of my research were to better understand the epidemiology and the diffusion of PRRSV infection in two positive breeding herds and to evaluate the effectiveness and the critical points of the gilts acclimatization programmes, using adequate diagnostic tools and phylogenetic analysis of PRRSV isolates. I also took part in the development of a quantitative real time polymerase chain

reaction for PRRSV diagnosis in serum and tissues and evaluated the effectiveness of vaccination against Porcine Circovirus type-2 (PCV2) in a farm coinfected with both PRRSV and PCV2.

2 ETIOLOGY

PRRS was first recognized in some swine farms of the United States in 1987 (Hill, 1990), with a clinical picture including severe reproductive losses, postweaning pneumonia, reduction of growth performance and increased mortality (Zimmerman et al., 2006). In 1991 the Lelystad Virus (Wensvoort et al., 1992), better known as PRRS virus (PRRSV), was identified as the causal agent (Meulenberg et al., 1997) and shortly thereafter the virus was isolated also in the U.S. (Collins, 1991). Because of the divergent evolution of the virus in the two continents, PRRSV was classified into two distinct genotypes (55-70% nucleotide identity), European (EU) type (Type 1) and North-American (US) type (Type 2) (Nelsen et al., 1999). Current infection rates remain to be accurately determined in most areas of the world. Estimates in some countries have been as high as 80% while other countries claim to be free of the infection (Zimmerman et al., 2006).

2.1 TAXONOMY AND VIRAL CHARACTERISTICS

PRRSV is a small RNA virus in the order *Nidovirales*, family *Arteriviridae*, that includes also lactate-dehydrogenase-elevating virus of mice, equine arteritis virus and simian hemorrhagic fever virus (Zimmerman et al., 2006). These viruses can induce a prolonged viremia in the presence of antibodies, replicate in macrophages and produce persistent infection (cfr 3.2) (Cho and Dee, 2006).

Being an enveloped virus, PRRSV survivability outside of the host is affected by (Cho and Dee, 2006):

- temperature: PRRSV can survive for more than 4 months at temperatures ranging from -70 to -20°C, while viability decreases with increasing temperature;
- pH: PRRSV remains stable at pH ranging from 6.5 to 7.5, while infectivity is reduced at pH <6.0 or >7.65;
- exposure to detergents: lipid solvents are particularly efficient at disrupting the viral envelope and inactivating replication.

2.2 GENOMIC ORGANIZATION

PRRSV is a single positive-stranded RNA virus (Scortti et al., 2006). The genome is approximately 15 kb in length and contains eight open reading frames (ORFs) (Meulenberg et al., 1993). ORFs 1a and 1b comprise 80% of the genome and encode the viral RNA polymerase; ORFs 2, 3 and 4 encode virion-associated proteins designed as GP2, GP3 and GP4; ORFs 5, 6 and 7 encode envelope (E), membrane (M) and nucleocapsid (N) proteins, respectively. Therefore, the order of PRRS genes is: 5' - viral polymerase (ORFs 1a/1b) - GP2 (ORF2) - GP3 (ORF3) - GP4 (ORF4) - E (ORF5) - M (ORF6) - N (ORF7) - 3' (Meng, 2000).

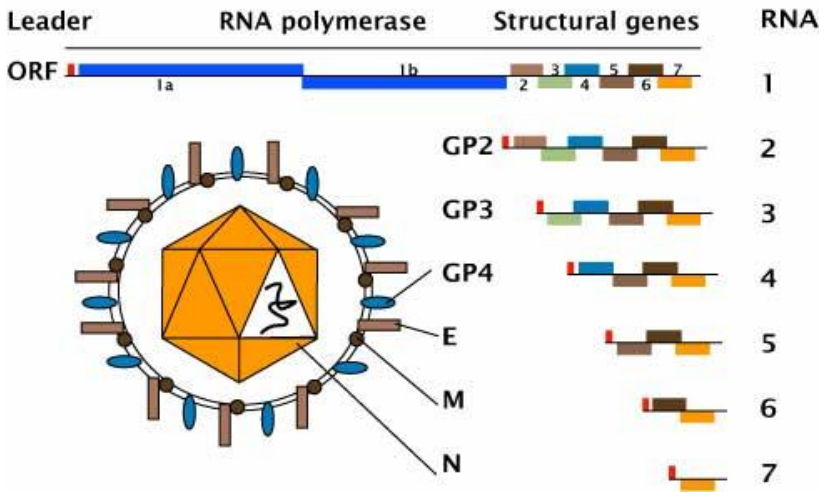

Figure 1 *Genomic organization of PRRSV*
(http://www.porcilis-prrs.com/microbiology-prrsv-structure.asp)

In particular, the primary envelope protein, encoded by ORF5, appears to be the most variable protein when EU type and US type isolates are compared, most likely because it is potentially exposed to selective pressures (Prieto et al., 2009). In addition, ORF5 exhibits the highest degree of genetic diversity also among viruses of the same genotype (Lee et al., 2009). Therefore, ORF5 is considered to be an important target for analyzing the genetic variation and evolution, as well as molecular epidemiology of PRRSV (L. Zhou et al., 2009; Y.-J. Zhou et al., 2009), and previously phylogenetic analyses have mainly focused on complete or partial ORF5 sequences (Kang et al., 2004).

On the contrary, ORF7 region is highly conserved between all PRRSV isolates, EU as well as US types (Oleksiewicz et al., 1998), so that ORF7 represents an important target for viral RNA detection (Yoon et al., 2008) (cfr. par. 7.2). Moreover it encodes for the highly antigenic nucleocapsid protein: the N protein is not important from an immunogenic or immunoprotective standpoint, but its abundant expression and antigenicity make it an ideal assay for diagnostic assays (Zimmerman et al., 2006).

2.3 GENOMIC DIVERSITY

A great biological, antigenic, genetic and pathogenic heterogeneity exists among PRRSV strains (Meng, 2000), also among isolates of the same genotype (Batista et al., 2004). The genetic variability was initially demonstrated within the US genotype, but recent reports have shown that genetic diversity of EU PRRSV is at least as high as that of US types (Prieto et al., 2009). Moreover, Forsberg et al. (2002) defined a marked geographical pattern after analyzing PRRSV isolates from different European countries, namely a cluster of Lelystad-like isolates, a cluster of purely Danish isolates and a cluster of extremely diverse Italian-like isolates. However, a study carried out in Spain by Prieto et al. (2009) has demonstrated that PRRSV variability cannot be attributed to geographical origin entirely, since it is possible to find isolates that are not closely related to other isolates from the same geographical area. These results support the theory proposed by Pesch et al. (2005) that there is no strict correlation between the degree of genetic relatedness and geographical origin for European PRRSV strains. Prieto et al. (2009) also suggested an increase in divergence between early and recent isolates of approximately 0.5% per year, but the analysis of the phylogenetic tree indicated that the heterogeneity observed does not appear to be due to temporal evolution exclusively. Early and recent isolates are grouped into different clusters independently of the time of isolation, indicating co-circulation of different variants at the same time. The same results were obtained also by Yoon et al. (2008), clearly indicating that neither the date nor the place of collection influenced the observed sequence variations.

Thus, the variability of PRRSV observed in the field might be the consequence of four different phenomena:

- co-circulation of different variants of PRRSV that might have evolved independently;
- introduction of new variants in a swine population;
- discovery of previously undetected variants that might have been replicating in the population;
- reversion to virulence of a vaccine virus able to be transmitted between swine herds.

Moreover, RNA recombination can provide a powerful and effective mechanism for evolution of an RNA virus. The ability to exchange genetic information may allow RNA viruses to adapt a changing environment and to escape a selection pressure, such as neutralizing antibodies, thus providing the recombinant virus with an evolutionary advantage. Sequence analyses of field isolates of PRRSV suggest that RNA recombination of PRRSV may occur in nature and provide evidence for intragenic recombination in ORFs 2-5 and in ORF7 among PRRSV isolates. The frequency of recombination, crossover sites and the clinical implications of PRRSV RNA recombination need to be studied (Meng, 2000).

Altogether, this genomic diversity among PRRSV strains seems to influence both the clinical

presentation of the disease and the respiratory and/or reproductive virulence. It has also a great significance for vaccine strain selection (Meng, 2000) and for the performance of diagnostic assays (Zimmerman et al., 2006). Finally, since genetically diverse PRRSV variants may coexist in the same farm, it must be taken into account in the control of the infection.

3 PATHOGENESIS

Although PRRSV was initially described as an acute infection causing respiratory distress in young animals and late-term abortions in sows, later the virus was demonstrated to establish persistent infections (Suarez, 2000). Today, two stages can be considered:

- an *acute stage* including the first 2 weeks of infection during which maximal virus titers are recovered from all susceptible organs throughout the body;
- a *persistent stage* characterized by lower levels of virus replication only in some organs.

3.1 ACUTE STAGE OF INFECTION

PRRSV infection most frequently occurs by the respiratory route. After exposure, virus replicates primarily in local susceptible macrophages of the respiratory tract and then it rapidly spreads to lymphoid organs and to other tissues (Van Reeth, 1997; Zimmerman et al., 2006), as heart and brain, either in the blood alone or in association with leukocytes or monocytes (Prieto and Castro, 2000). Cells known to support replication include pulmonary alveolar macrophages (PAM) and intravascular macrophages (PIM) in the lung and macrophages in lymphoid tissues (Zimmerman et al., 2006). Thus, lungs appear to be the portal of entry as well as the target organ (Van Reeth, 1997).

Virulent strains of PRRSV cause viremia as early as 12 hours in some pigs and in all pigs by 24 hours postinoculation. Viral titers increase rapidly and peak in serum, lymph nodes and lung by day 7-14, with the highest viral titers consistently reported in lung (Zimmerman et al., 2006).

After peaking, virus titers in serum decrease rapidly. Most pigs are no longer viremic by 28 days postinfection (DPI), although viral RNA has been detected in serum by reverse trascription polymerase chain reaction (RT-PCR) up to 251 DPI (Wills et al., 2003).

Pathogenesis in gestating sows

In gilts and sows the incubation period varies greatly, but the first clinical signs usually appear between 2-7 DPI. The virus primarily replicates in alveolar macrophages and then spreads to the rest of the body, reaching the reproductive tract, crossing the placenta in the free form or associated with maternal cells and leading to the development of clinical signs associated with reproduction. However, the importance of infection in early and mid-gestation is relative low and the venereal transmission of

PRRSV may have an insignificant effect or no effect on conception and fertilization rates, as virus is not detectable in fetuses before implantation (Prieto and Castro, 2000; Cheon and Chae, 2001).

On the contrary, the likelihood of transplacental infection and its effects increase as gestation progresses. Infection in late-gestation causes lesions of the placenta and umbilical cord and leads to fetal hypoxia that explain the great proportion of late-term abortions and stillborn and weak piglets occurring during natural outbreaks of the disease.

Finally, another remarkable finding is the detection of persistent infections related to in utero infections, with viremic pigs at birth by infecting sows in late gestation. These animals can easily develop a marked respiratory distress and are more susceptible to secondary diseases (Prieto and Castro, 2000).

Pathogenesis in boars

Boars are important in the transmission of PRRSV through semen and this has been demonstrated in both experimental and field studies: persistence of virus shedding in semen has been detected for as long as 92 days post inoculation (Cristopher-Hennings, 2000). The testicular infection by PRRSV centers on two types of cells: (i) epithelial germ cells of the seminiferous tubes and (ii) macrophages, which are located in the interstitium of the testis (Sur et al., 2000).

Since vasectomized boars can shed PRRSV in semen (Cristopher-Hennings, 2000) and the presence of virus in semen ceases when viremia subsides (Sur et al., 2000), the suggested mechanism for PRRSV entry into boar semen may primarily be through systemic distribution to monocytes and tissue macrophages into semen and not exclusively through replication in the testis and epididymis.

The testicular infection leads to the formation of multi-nucleated giant cells and abundant germ cell depletion and death by apoptosis. Simultaneously, in the ejaculate there is a significant increase in the number of immature sperm cells infected with PRRSV and responsible for the venereal transmission of PRRSV (Sur et al., 2000).

3.2 PERSISTENT STAGE OF INFECTION

As described above, PRRSV causes a prolonged acute infection, in which the viremic period may last for 4 to 5 weeks, followed by a persistent infection in lymphoid tissues lasting several months. Persistent infection is defined as “the continued presence of a pathogen in a host beyond the acute symptomatic phase of infection”. The persistence of PRRSV involves a continuous low level of viral replication but is not a true steady-state persistent infection (Batista et al., 2004; Zimmerman et al., 2006).

PRRSV persistence has been detected in lymphoid tissues up to 157 DPI in weaned pigs (Wills et al., 1997), while piglets born alive to females infected in late gestation tend to be viremic for extended periods of time, even 11 weeks after birth (Suarez, 2000). In contrast, a study by Batista et al., 2004 has demonstrated that PRRSV RNA is detectable up to 135 DPI in lymphoid tissues of gilts, although a reduction in the number of persistently infected animals was observed between 100 and 135 DPI, while sera samples were positive to RT-PCR up to 30 DPI.

A study carried out by Bierk et al. (2001) demonstrated that PRRSV can persist in non-pregnant sows for up to 86 DPI and persistently infected sows can transmit PRRSV to naïve contact animals over extended periods. However, it has been demonstrated that shedding of the virus from experimentally infected animals was not detected from 90 to 180 DPI (Batista et al., 2002a), even if stress or immunodepression can induce re-excretion at more than 15 weeks after initial seroconversion (Van Reeth, 1997).

3.3 DUAL INFECTIONS WITH OTHER PATHOGENS

There is clinical evidence that PRRSV is associated with outbreaks of other pathogens and consequently experimental dual infections have been performed.

Experimental studies found that PRRSV-infected pigs showed an increased incidence of septicemia, meningitis and mortality when challenged with *Streptococcus suis* (*S. suis*) (Galina et al., 1994; Thanawongnuwech et al., 2000) and increased pulmonary infections with *Bordetella bronchiseptica* (*B. bronchiseptica*) (Brockmeier at al., 2000). In contrast, experimental infection of PRRSV with *Pasteurella multocida* (*P. multocida*) failed to identify an increase in clinical disease (Carvalho et al., 1997). Primary infection with both PRRSV and *B. bronchiseptica*, however, predisposed pigs to secondary pulmonary infection with *P. multocida* (Brockmeier et al., 2000).

Furthermore, in contrast to the expected exacerbation of mycoplasmal pneumonia by PRRSV and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), infection with *M. hyopneumoniae* potentiates and prolongs PRRSV-induced pneumonia clinically, macroscopically and microscopically (Thacker et al., 2000). Moreover, a study examining the effects of PRRSV infection on subsequent infection with *Haemophilus parasuis* (*H. parasuis*) (Solano et al. 1997) and *Actinobacillus pleuropneumoniae* (App) (Pol et al., 1997) indicated that pigs infected with both PRRSV and *H. parasuis* or App actually had equal or fewer clinical signs and lesions than pigs infected with *H. parasuis* or App alone.

There is also evidence of PRRSV interaction with other respiratory viruses, such as Porcine Respiratory Coronavirus (PRCV) and Swine Influenza Virus (SIV). Van Reeth et al. in 1996 have focused on dual infections with PRRSV followed by PRCV or H1N1-SIV. The results showed that

under experimental conditions dual PRRSV-PRCV and PRRSV-H1N1 infections caused more severe disease and growth retardation than each of the single virus infections.

Finally, Porcine Circovirus type 2 (PCV2)-associated diseases (PCVAD) recently emerged as a major problem worldwide. The most common manifestations of PCVAD are severe systemic and respiratory disease. In both of these manifestations, PRRSV is commonly present along with PCV2 (Opriessnig et al., 2008). In pigs experimentally coinfected with PRRSV and PCV2, PRRSV has been shown to enhance disease and upregulation of PCV2 replication was observed, probably due to the fact that monocyte/macrophage cell types are common targets of both the viruses (Allan et al., 2000).

3.4 PORCINE RESPIRATORY DISEASE COMPLEX

The term porcine respiratory disease complex (PRDC) is used to describe pneumonia of multiple etiology causing clinical disease and failure to gain weight later in the finishing process. The etiology of PRDC varies between and within production systems and over time within the same system. When infections with primary respiratory agents become complicated with opportunistic bacteria, serious and chronic respiratory disease results and the most economic loss is incurred (Brockmeier et al., 2000).

Primary agents in pigs include (i) viral agents as PRRSV, SIV, pseudorabies virus (PRV), PCV2 and PRCV and (ii) bacterial agents as *M. hyopneumoniae*, *B. bronchiseptica* and App. The most common opportunistic agent is *P. multocida*, but other opportunistic agents include *H. parasuis*, *S. suis*, *Actinobacillus suis* and *Arcanobacterium pyogenes*.

Noninfectious causes (management and environmental factors) are significant contributors to respiratory disease, either by increasing transmission and spread of the pathogens or by creating unfavorable conditions which result in increased stress or damage to the respiratory tract. Overcrowding and/or improper ventilation can lead to overheating or chilling and increased ammonia and dust levels. Also management practices, such as continuous pig flow and mixing of pigs from multiple sources and age groups, contribute to the spread of disease (Brockmeier et al., 2000).

4 CLINICAL SIGNS

Clinical disease in a herd is primarily the consequence of acute viremia in individuals and transplacental transmission resulting in reproductive failure. Clinical epidemics occur when PRRSV enters an immunologically naïve herd or production site and all ages are affected. Endemic PRRS occurs in herds that have homologous immunity to the infecting strain of PRRSV and in which clinical disease is observed only in susceptible subpopulation (Zimmerman et al., 2006).

4.1 EPIDEMIC INFECTION

The first phase of PRRS epidemics lasts 2 or more weeks and is characterized by anorexia and lethargy in animals of all ages, as a result of acute viremia. Clinically affected animals may also be lymphopenic, pyretic with rectal temperatures from 39-41°C, hyperpneic and dyspneic, or have transient cutaneous hyperemia or cyanosis on extremities.

The second phase may begin before the first one is completed and continues for 1-4 months, characterized by reproductive failure in sows and high preweaning mortality in piglets.

Sows: in the phase of acute illness an increase in the number of abortions (usually 2-3%), returns to estrus and nonpregnant sows is observed. Observed inconsistently are also agalactia, incoordination and exacerbation of endemic diseases. Mortality in sows is typically 1-4% during acute illness and is sometimes associated with pulmonary edema and cystitis/nephritis, even if cases of severe acute PRRS have been described with 10-50% abortions and up to 10% mortality. Reproductive failure includes low conception and farrowing rates, decreasing number of liveborn piglets, high incidence of weak-born, stillborn and mummified piglets (Zimmerman et al., 2006).

Boars: in addition to anorexia, lethargy and respiratory signs, boars may lack libido and have variable reduction in semen quality. Changes in sperm occur 2-10 weeks after infection and include reduced motility and acrosomial defects (Prieto and Castro, 2005; Zimmerman et al. 2006).

Suckling pigs: high preweaning mortality is observed in pigs born prematurely and at term associated with listlessness, diarrhea, emaciation, splay-legged posture, hyperpnea and dyspnea. Less common are tremors and paddling, anemia and bacterial polyarthritis and meningitis (Zimmerman et al., 2006).

Weanling and grower-finisher pigs: acute PRRSV infection is characterized by anorexia, lethargy,

cutaneous hyperemia, hyperpnea and dyspnea without coughing, rough hair coats and reduced average daily gain. Higher incidence of endemic diseases and elevated mortality are also reported (Zimmerman et al., 2006).

4.2 ENDEMIC INFECTION

Once introduced into a farm, PRRSV usually becomes endemic, leading to regular or occasional outbreaks of typical acute PRRS in susceptible subpopulations of animals, such as nursery or grower pigs and replacement gilts and boars (Stevenson et al., 1993; Zimmerman et al., 2006).

In nursery pigs, elevations in mortality mostly occur in winter months, suggesting that environmental factors, such as lowered ambient temperature, increased range in fluctuation of ambient temperature, lowered ventilation rates and elevated relative humidity, probably contribute to recirculation of virus and decrease in immune responses (Stevenson et al., 1993).

The reproductive consequences depend on the number of animals infected and the stage of their reproductive cycle when infected. If few gilts are infected, there may be scattered abortions, irregular returns to estrus, non-pregnant gilts and late-term reproductive failure. Alternatively, gilts may escape exposure to PRRSV until there is a significant subpopulation of susceptible gilts in various stages of gestation. In this case, endemic PRRS in the breeding herd manifests as periodic mini-outbreaks of PRRS (Zimmerman et al., 2006).

5 LESIONS

The most characteristic lesions of PRRSV are seen in young pigs affected with the respiratory facet of the syndrome. Gross and microscopic lesions are consistently observed from 4 to 28 or more DPI in lung and lymph nodes, where most viral replication takes place. The severity and the distribution of lesions are related to the virulence of the strain of PRRSV to which pigs are exposed. Gross lesions are suggestive of PRRSV, but are not diagnostic since a variety of other viral and bacterial diseases can cause similar lesions. Typical microscopic lesions of PRRSV sometimes allow a strong presumptive diagnosis. However, a definitive diagnosis always requires demonstration of PRRSV (Mengeling and Lager, 2000; Zimmerman et al., 2006).

5.1 MACROSCOPIC LESIONS

Lung: interstitial pneumonia occurs from 3 to 28 DPI, most severe 10-14 DPI. Mild lesions are in cranial lungs or diffuse and affected parenchyma is resilient, slightly firm, non-collapsing, mottled gray to tan and moist. Severe lesions are diffusely distributed and parenchyma is mottled or diffusely red-tan, non-collapsing, firm, rubbery and very moist (Zimmerman et al., 2006). Without complications, resolution begins shortly thereafter and by 4 weeks after initial exposure the extent of lung involvement has usually decreased markedly (Mengeling and Lager, 2000).

Lymph nodes: macroscopic lesions are presented mainly as tan to grey, enlarged lymph nodes, which are often several times the size of normal nodes for the same size pig (Mengeling and Lager, 2000). Early PI, enlarged nodes are edematous, tan and moderately firm. Later, nodes are firm and white or light tan in a nodular or diffuse pattern (Zimmerman et al., 2006). The may remain enlarged for 6 or more weeks (Mengeling and Lager, 2000).

Fetuses: PRRSV-infected litters contain variable numbers of normal pigs, small weak pigs and dead pigs that are either fresh stillborn, autolytic stillborn or mummified fetuses. Gross fetal lesions include perirenal edema, edema of the splenic ligament, mesenteric edema, ascities, hydrothorax and hydroperitoneum. Segmental hemorrhagic enlargement of the umbilical cord of weak liveborn and stillborn pigs is sometimes present (Zimmerman et al., 2006).

5.2 MICROSCOPIC LESIONS

Lung: alveolar septa are expanded by macrophages, lymphocytes and plasma cells. Alveoli may contain necrotic macrophages, cell debris and serous fluid, while lymphocytes and plasma cells form cuffs around airways and blood vessels.

Lymph nodes: microscopic lesions are predominantly in germinal centers that appear necrotic and depleted. The cortices may contain small cystic spaces that are variably lined by endothelium and contain proteinaceous fluid, lymphocytes and multinucleate prokaryocytes.

Heart: mild to moderate multifocal lymphohistiocytic vasculitis and perivascular myocarditis may develop in the heart 9 or more DPI.

Brain: mild lymphohistiocytic leukoencephalitis or encephalitis involving cerebellum, cerebrum and brainstem may develop 7 or more DPI. Segmental cuffing of blood vessels by lymphocytes and macrophages and multifocal gliosis may also occur.

Kidney: mild periglomerular and peritubular lymphohistiocytic aggregates were described. Affected vessels have swollen endothelium, pooled subendothelial proteinaceous fluid, intramural and perivascular aggregates of lymphocytes and macrophages.

Nasal mucosa: epithelium may have clumped or absent cilia and epithelial cell swelling, loss or squamous metaplasia. By 7 DPI, lymphocytes and macrophages are in the epithelium and propiria mucosa.

Uterus: myometrium and endometrium are edematous with lymphohistiocytic perivascular cuffs. There may be also segmental lymphohistiocytic vasculitis in small vessels and microseparations between endometrial epithelium and placental trophoblasts.

Testicles: atrophy of seminiferous tubules is observed 7-25 DPI, associated with apoptosis and depletion of germ cells (Zimmerman et al., 2006).

6 IMMUNOLOGY

Molitor et al. in 1997 defined the immune system as a "double-edged sword" for PRRSV infection. On the one edge, PRRSV has a predilection for immune cells (cfr 3.1) and the disease manifestations can be linked directly to changes in the immune system: the extensive replication in cells of immune lineage indeed leads to immunosuppression, precipitates secondary infection and mediates disease. On the other edge, the virus stimulates immunity post-infection protecting from re-infection. Thus, the immune system appears to be intimately involved in both the disease process and protection from disease.

6.1 IMMUNOSUPPRESSION

Clinical and experimental studies suggest that PRRSV modulates host responses based on two observations: (i) secondary infections are common following PRRSV infection and (ii) experimental infection with PRRSV precipitates clinical disease in piglets challenged with other pathogens (par. 3.3) (Molitor et al., 1997).

Viruses act as immune modulators in four principal ways:

1. by interfering with antigen presentation;
2. by the induction of apoptosis of cells involved in an immunological response;
3. by acting as cytokines or cytokine inhibitors, affecting their production or action;
4. by inhibiting the complement.

In particular, effects of PRRSV infection are directly on the infected macrophages. These effects are due to the viral replication in the cells, culminating in cell death usually within 24 hours p.i., and include the loss of the ability of the macrophage populations to synthesize superoxide and, consequently, the loss of their phagocytic function. (Drew, 2000).

6.2 HUMORAL IMMUNITY

Circulating antibodies against PRRSV are detectable in some pigs by 5-7 DPI and all animals seroconverted by 14 DPI. IgM responses reach a peak at 14-21 DPI and rapidly decrease, being undetectable by 35-42 DPI. IgG titres are detected 7-10 DPI, peak at 21-28 DPI, remain constant for a

period of months and then decline to low levels by 300 DPI (Drew, 2000; Zimmerman et al., 2006; Mateu and Diaz, 2008). Studies of the immune response to individual viral proteins showed that antibodies directed against the protein N are most abundant and thus are detected by most diagnostic tests. Antibody responses are mounted to a lesser extent also to proteins M and E (Drew, 2000). The rapid IgM and IgG response does not correspond to neutralizing antibodies (NAs) and is not correlated with protection.

NAs appear in serum 3 weeks after infection, but they are consistently detected only by 28 DPI. However, there is variation in the NAs responses of individual pigs, including the kinetics of their appearance and the level of titers. NAs are mainly directed against protein E, which contains the major neutralization epitope. Also proteins GP4 and M contain neutralizing epitopes, but they seem to be of minor biological significance (Mateu and Diaz, 2008).

Although the humoral immunity is presumed to play an important role in resistance to reinfection and in prevention or reduction of viral spread, the concurrent detection of NAs and infectious PRRSV in blood and the delay in NAs appearance relative to the changes in viral load in lung and serum suggest that NAs play a secondary role in adaptive immune response to PRRSV (Zimmerman et al., 2006).

A different picture arises when protection before infection is considered (Batista et al., 2004; Mateu and Diaz, 2008). Since transfer of NAs to pregnant sows protects them against reproductive failure, blocks transplacental infection and protect piglets against the development of clinical symptoms (Osorio et al., 2002), a vaccine capable of inducing NAs should prevent clinical disease and be a key tool in eradication of PRRSV (Mateu and Diaz, 2008).

6.3 CELL-MEDIATED IMMUNITY

The complexity of the regulation of the immune response of pigs to PRRSV is clearly indicated by the concomitant gradual decline of humoral immunity while the cell-mediated immunity increases (Meier et al., 2000). Antigen-specific lymphocyte proliferation response is first detected at 4 weeks PI, peaks at 7 weeks PI and declines 11 weeks PI. The response and decrease in lag time of the same animals to a secondary exposure to virus results in a T-cell proliferation response which increases in magnitude (Molitor et al., 1997). T-cell responses have been detected against all viral proteins and are initially weak and transient, while increases steadily for 1-2 years PI (Zimmerman et al., 2006).

Cytokine responses are mainly interferon (IFN)-γ and, to a lesser extent, interleukin (IL)-2. The cell-mediated immune response is highly variable and transient, ranging from insignificant to high (Zimmerman et al., 2006). Differences may be due to the viral strain and/or to the sensitivity of the diagnostic assay. Batista et al. in 2004 suggested that IFN-γ was involved in the clearance of virus,

since it was detected as early as 14 DPI, peaking between 50 and 70 DPI, and PRRSV-specific IFN-γ-producing cells were detected early after infection and persisted at low levels until 135 DPI. However, this finding contrast with previous results reported by Meier et al. in 2000, indicating that the presence of virus-specific IFN-γ-producing cells is not detectable until 8-10 weeks PI and then increases gradually up to 9-10 months PI and persists at high levels until 2 years PI.

The unusual characteristics of the adaptive immune response to PRRSV suggest that the virus strongly modulates the immune response by altering the cytokine patterns of macrophages, inhibiting key cytokines, such as IFN-α, as well as by down-regulating the expression of molecules involved in antigen presentation (Mateu and Diaz, 2008).

6.4 CROSS-PROTECTION

Given the genetic diversity of PRRSV strains within one genotype, cross-protection against heterologous field isolates is a key issue in disease prevention strategies that include vaccination. The question then is whether this genetic diversity might influence the efficacy of a homologous or heterologous vaccine. Several studies (Meng, 2000; Mengeling et al., 2003; Prieto et al., 2008; Kimman et al., 2009; Martelli et al., 2009) have investigated this issue, with contrasting findings.

Early studies demonstrated a high level of protection with strains homologous to an immunizing virus and that attenuated live vaccines were effective in reducing disease severity, duration of viremia, virus shedding and the frequency of heterologous PRRSV infection (Zimmerman et al., 2006).

Nevertheless, in following studies it appeared clear that current vaccines, based on a single PRRSV strain, are not or only partially effective in protecting against infections with genetically diverse field strains of PRRSV (Kimman et al., 2009). However, protection against PRRSV infection by a strain different to the one used as a vaccine is somewhat more complex than a matter of genetic similarity (Mateu and Diaz, 2008), also considering that the degree of genetic homology between vaccine strain and challenge isolate is not a good predictor of vaccine efficacy (Prieto et al., 2008). Furthermore, Martelli et al. in 2009 concluded that vaccine efficacy is associated with an efficient cell-mediated immune response. More precisely, the efficacy of a vaccine is not only related with its immunological properties but also to the characteristics of the challenging strain to trigger an immune response. In conclusion, it is a current opinion that the ability of each strain to induce a strong cell-mediated immune response is more important than the genetic similarity between the vaccine and the field strains for inducing clinical protection.

6.5 MATERNAL IMMUNITY

No specific study has evaluated the effect of maternal immunity on piglet susceptibility to PRRSV infection, but indirect inferences suggest that immune sows provide maternal protection to piglets. Anti-PRRSV antibodies are present in colostrum at the same concentration as in blood and PRRSV infections increase in pigs as maternal antibodies decline (Zimmerman et al., 2006).

Pigs born from PRRSV-infected dams maintain maternal antibody titers until 4-8 weeks of age using the indirect ELISA (Molitor et al., 1997). However, a study carried out by Morrison et al. in 1992 showed that pigs from immune dams were protected from experimental challenge, but pigs that received anti-PRRSV sera failed to be protected from challenge: these results documented that immunity can be transferred via colostrum, but antibodies by themselves fail to totally protect, confirming a primary role of cell-mediated immunity in protection from disease.

7 DIAGNOSIS

Diagnosis of PRRSV is based on (Zimmerman et al., 2006):

1. subjective information: history, clinical signs, gross and microscopic lesions;
2. objective information: herd production records, detection of virus, serology.

A presumptive diagnosis of PRRS is suggested in any herd with reproductive problems in breeding animals and respiratory disease in pigs of any age. Production records in clinically active PRRS herds usually reveal increased abortions, early farrowings, stillbirths and preweaning mortality and a moderate decrease in growth rate and feed efficiency (Mengeling and Lager, 2000).

However, a diagnosis based only on clinical signs is difficult to achieve due to variation of signs among herds. Furthermore, no pathognomonic gross lesions have been demonstrated in infected pigs at necropsy and interstitial pneumonia is the only consistent lesion observed by histological examination (Bøtner, 1997). Thus, differential diagnoses must include many pathogens, such as parvovirus, PRV, hemoagglutinating encephalomyelitis virus, PCV2, enterovirus, SIV, classical swine fever virus, cytomegalovirus and leptospirosis (Zimmerman et al., 2006).

Therefore, adequate use and interpretation of the available diagnostic tests for PRRSV can provide very useful information (Batista, 2005). Several detection methods were developed over time and at present PRRS can be diagnosed by virus isolation (VI) or by detecting the antibodies, antigens or nucleic acid in infected tissues and body fluids (Martínez et al., 2008).

7.1 SEROLOGY

The demonstration of seroconversion or of increasing titers of PRRSV specific antibodies is the most definitive method to diagnose PRRSV infection in a population. However, serology is not a valid approach for diagnosis of PRRSV in previously infected or vaccinated herds, because serologic assays do not differentiate among antibodies resulting from the initial infection, reinfection or vaccination. Therefore, single serum samples are of limited use and do not prove a causal role for PRRSV in a clinical diagnosis. Thus, in herd monitoring serology should be used in combination with detection of viral nucleic acid. Moreover, detection of antibodies in nursing and nursery pigs may be due to the presence of the maternal immunity (cfr. par. 6.5).

Five serological tests to detect antibodies to PRRSV have been described: indirect fluorescent antibody (IFA), Enzyme-Linked Immunosorbent Assay (ELISA), blocking ELISA, serum-virus neutralization (VN) and immunoperoxidase monolayer assay (IPMA).

The commercial ELISA is the gold standard for detection of antibodies to PRRSV. The assay is sensitive, specific, standardized and rapid. The test putatively targets antibodies to the nucleocapsid antigens for both US and EU strains of PRRSV. Samples are classified as positive when the sample to positive (s/p) ratio greater than or equal to 0.4 (Zimmerman et al., 2006).

7.2 DETECTION OF PRRSV

PRRSV can be demonstrated by (i) the isolation of virus using cell coltures, (ii) the direct detection of viral antigen in tissue sections or (iii) the detection of virus-specific RNA (Bøtner, 1997).

Specimens for virus isolation and detection of viral RNA must be refrigerated at 4°C immediately after collection, because freezing may degrade viral RNA, and shipped to the diagnostic lab within 2 days. Since the virus is degraded by heat and has a narrow pH stability, sterility and submission of fresh tissues must be guaranteed in order to avoid pH alterations caused by bacterial contaminations (Zimmerman et al., 2006).

Virus Isolation

Isolation of PRRSV is done by using either porcine alveolar macrophages (PAMs) or sublines of the African monkey kidney cell lines, but in general the use of macrophages constitutes the most sensitive system for virus isolation (VI) (Bøtner, 1997). Furthermore, since not all PRRSV strains replicate in all cell types, at least two cell types should be used whenever possible.

Results can be obtained within 1 day after inoculation of cultures, but may take several weeks, depending on the amount of virus in the specimen.

Virus can be also detected in direct cultures of PAMs from pulmonary lavage collected either from live infected pigs or at necropsy.

VI is most successful using serum, lung, lymph nodes and tonsils collected between 4-28 DPI. In late abortions and early farrowings, same tissue samples from liveborn pigs are preferred, because mummies and stillborn fetuses rarely yield positive VI results due to tissue autolysis. For persistent infections tonsils, oropharyngeal scrapings and lymph nodes are better samples than serum and lung (Zimmerman et al., 2006).

The most notable exception is boar semen. Because of the toxicity of boar semen for cell cultures, PCR is usually the only test by which PRRSV can be identified in semen (Mengeling and Lager, 2000).

Detection of Viral Antigens

Viral antigens of PRRSV nucleocapsid are detected by immunohistochemistry (IHC) and immunofluorescence (IF). Lung, heart, kidney, lymph nodes, spleen, thymus and tonsils are used for IHC, while lung is the primary sample for IF.

The IHC is more sensitive than the IF and can be done using formalin fixed tissues. The IF is more rapid and economical, but requires fresh tissues. Viral antigens are best detected during peak viral replication (4-7 DPI) (Zimmerman et al., 2006).

However, actual application of IHC and IF is limited because of the level of technical skill required for their satisfactory performance and the experience required for their correct interpretation (Mengeling and Lager, 2000).

Detection of Viral Nucleic Acid

Recently, molecular-based tools have been used to diagnose PRRSV infections. These techniques are usually more sensitive than traditional methods: wide variability of the strains limits the usefulness of VI, with PRRSV being selective among cell types, serology has some disadvantages because antibody titres can fall rapidly after infection, sensitivity of IHC and IF is still a concern (Lurchachaiwong et al., 2008; Martínez et al., 2008).

End-point reverse transcription polymerase chain reaction (RT-PCR) is one of the most commonly used techniques for PRRSV detection in infected tissues, in serum and in "difficult" material for cell cultures, such as boar semen (Oleksiewicz et al., 1998; Batista, 2005; Martínez et al., 2008). Thus, RT-PCR offers several advantages over VI, IHC and IF (Zimmerman et al., 2006):

1. higher sensitivity and specificity;
2. detection of viral RNA in both acute and persistently infected pigs;
3. detection of viral RNA in autolyzed fetuses and in samples, such as semen and feces, that are toxic to cell cultures;
4. rapid turnaround time, with results available in 1-3 days;
5. PCR products can be utilized for sequencing, expanding the diagnostic utility of this assay.

In acute infections serum and tissues recommended for VI are also the preferred diagnostic samples for PCR. PRRSV nucleic acid has been detected up to 86 DPI in lymph nodes, 92 DPI in semen, 105 DPI

in oropharyngeal scrapings and 251 DPI in serum and tonsil homogenates (Zimmerman et al., 2006). However, conventional RT-PCR methods use multiple steps and time-consuming post-PCR analysis that has a high risk of cross-contamination and false-positive results (Martínez et al., 2008). Therefore, real-time RT-PCR technology has been developed for rapid, user-friendly detection of nucleic acid directly during the DNA amplification step and quantitative analysis (Lurchachaiwong et al., 2008).

Both probe- and SYBR Green-based real-time RT-PCR approaches have proven to be robust for different purposes. However, there are advantages and disadvantages to both methods.

SYBR Green is a dye that binds any double stranded DNA irrespective of the DNA sequence, thus avoiding potential failures to detect the amplicon due to sequence variation. However, SYBR Green-based assays are considered less specific than probe-based assays because non-specific amplification and the formation of primer dimmers can lead to erroneous results. These unspecific fluorescence signals would not even be detected by probe-based assays. Nevertheless, melting curve analysis (MCA) has been reported as a simple, straightforward way to check SYBR Green-based reactions for artefacts and to ensure reaction specifity (Martínez et al., 2008). ORF7 PCR exhibits equal sensitivity for EU and US genotypes, making a highly suited test for estimating type prevalence (Oleksiewicz et al., 1998). Differences in the amplicons of every genotype consistently resulted in differences in the melting temperature (T_m) values of around 1.5°C between the EU and US PRRSV PCR products. Moreover, SYBR Green assay is not able to discriminate between negative results obtained from truly negative samples from those due to amplification inhibition, RNA degradation or PCR failure. The development of an internal positive control (IPC) to be present in the same sample reaction tube is an actual improvement (Martínez et al., 2008).

Real-time RT-PCR technology using TaqMan primers and probes is more sensitive than SYBR Green assay with MCA (Lurchachaiwong et al., 2008) and achieve high specificity through the combination of gene-specific primers and probes (Martínez et al., 2008). However, TaqMan methods require a perfect match between the probes and the targeted region, otherwise the probe is displaced rather than hydrolyzed and the quencher molecule remains in the close proximity of the reporter. This can lead to signal loss, reduced sensitivity and possible false negative results. Considering that PRRSV has marked genetic variability and is one of the most rapidly evolving RNA virus, the design of a universal primer-probe detection system is difficult and the development of a sensitive and accurate real-time RT-PCR method effective in the presence of possible nucleotide substitutions is needed. The primer-probe energy transfer (PriProET) method uses a 5' exonuclease deficient enzyme for the polymerization that prevents the hydrolysis or displacement of the probe, offering more stable fluorescence signal. Thus, the PriProET method showed to tolerate at least five mismatches in certain regions of the probe-binding site without significant loss of sensitivity (Balka et al., 2009).

7.3 SEQUENCING

Precise identification of PRRSV isolates by determination of nucleotide sequence of viral genetic material can be a valuable tool for understanding the success or failure of PRRSV control, possible sources of introduction and area spread (Batista, 2005).

Sequencing is commonly done on ORF5 and ORF7. As said above (par. 2.2), ORF5 sequence is highly variable and there is an extensive databank of sequences available for comparison; thus, ORF5 is considered an important target for analyzing the genetic variation and evolution of PRRSV isolates (Indik et al., 2005; L. Zhou et al., 2009; Lee et al., 2010). On the contrary, ORF7 is more conserved, even if it may show a significant variability, with important consequences on PCR diagnostic tests (Pesente et al., 2006). Previous studies (Kang et al., 2004; Yoon et al., 2008) demonstrated that the phylogenetic tree derived from ORF7 most closely reflects the tree derived from the full-length genome, proving that ORF7 might be considered as the most useful marker for revealing the genetic relationships among different strains.

In general, sequencing may be used to (Zimmerman et al., 2006):

1. determine whether the reappearance of PRRS on a farm is due to the reemergence of a previously existing or a new virus strain;
2. determine whether PRRS outbreaks on farms are due to a single clone or multiple clones of virus;
3. track introduction of virus into a production system;
4. monitor spread of PRRSV strains within and between herds;
5. differentiate vaccine and field viruses and determine how closely related they are.

However, at present sequence information cannot be used to make inferences about biological properties of PRRSV. Therefore, genomic sequencing does not predict the likelihood of a vaccine successfully preventing the disease, the virulence of a strain, the clinical signs of the disease, growth characteristics of the virus, elicited immune response and the degree of protective immunity conferred (Batista, 2005; Prieto et al., 2008).

8 EPIDEMIOLOGY

Blaha (2000) defined the epidemiology of PRRSV infection as a "colorful" epidemiology. Any communicable disease has its specific epidemiological pattern, dividing diseases into two groups: on the one hand the endemic diseases, the occurrence is unlimited in time but limited in space, on the other hand the epidemic diseases, the occurrence of which is unlimited in space but limited in time. This epidemiological behavior is determined by (i) the biological properties of the causative agent, (ii) the characteristics of the pathogen-host interactions and (iii) socio-economic conditions, including in case of domestic swine herd size, animal movement, production systems etc. On the contrary, PRRS has characteristics that are clearly epidemic, others that are clearly endemic and most of them somewhere in between: the reproductive disease seems to have more characteristics of an epidemic infection, developing a pretty good protective immunity, whereas the respiratory disease seems to have more of the characteristics of an endemic infection, with a weak immune response and a greatly varying severity of clinical symptoms.

8.1 DIRECT ROUTES OF TRANSMISSION

The primary transmission route of PRRSV is via close contact between carrier and susceptible pigs, mainly through nose-to-nose contact or by contact with urine and feces (Albina, 1997). Infected animals may shed virus in saliva up to 42 DPI, nasal secretions up to 38 DPI, urine up to 28 DPI and feces (consequently in slurry) up to 35 DPI (Prieto and Castro, 2005). Moreover, pregnant susceptible sows infected in late gestation shed virus in mammary secretions (Zimmerman et al., 2006).

Shedding of virus in semen is of particular concern because of the widespread use of artificial insemination. PRRSV was detected in semen and bulbourethral gland of infected boars for up to 43 and 101 DPI, respectively. Viral RNA was detected in semen for up to 92 DPI (Cho and Dee, 2006; Zimmerman et al., 2006). However, duration of semen shedding varies widely among boars, influenced by (i) individual factors, (ii) PRRSV strain and (iii) diagnostic method used (Prieto and Castro, 2005).

Pigs are susceptible to PRRSV by several routes of exposure, including intranasal, intramuscular, oral, intrauterine and vaginal. The probability that a given dose of virus will result in infection differs by route of exposure: infectivity data indicate that pigs are extremely susceptible to infection via

parenteral exposure, less by oral and intranasal exposures. In field, possible parenteral exposures are represented by common husbandry practices, i.e. tail docking, tattooing and inoculations. Likewise, since PRRSV is shed in saliva for weeks PI, also aggressive behavior among pigs results in parenteral exposures, i.e. tail-biting, ear-biting, scrapes and cuts (Zimmerman et al., 2006).
PRRSV is also transmitted from viremic dams transplacentally to fetuses, resulting in fetal death and/or birth of infected piglets (cfr. par. 3.1).

8.2 INDIRECT ROUTES OF TRANSMISSION

Indirect transmission involves transmission by inanimate objects (equipment, instruments, clothing) or substances (water, food), living carriers (vectors) or aerosols (Zimmerman et al., 2006).
Boots and coveralls have been identified as potential sources of PRRSV to susceptible pigs. However, studies have demonstrated that certain intervention strategies, such as the use of disposable footwear, boot baths, wearing of gloves, showering and double-bagging products designed for entry into farms substantially reduced the mechanical spread of the virus.
Needles have also been recognized as an indirect means of PRRSV transmission, demonstrating the need for proper needle management (Cho and Dee, 2006).
Also **transport vehicles** have been investigated as a potential route of mechanical PRRSV transmission, even if PRRSV is highly unstable in solutions containing low concentrations of detergents and particularly susceptible to drying (Cho and Dee, 2006; Zimmerman et al., 2006).
Mosquitoes and flies have been shown to mechanically transmit PRRSV from infected to naïve pigs. The site of virus in insects is the intestinal tract, but insects are not biological vectors of PRRSV; therefore, the duration of retention of virus within the intestinal tract depends on virus load post-ingestion and environmental temperature. Control of on-farm insect populations may be carried out using a combination of screening of the air inlets, targeted insecticides and habitat management (Cho and Dee, 2006).
Although at present pigs are the only animals susceptible to PRRSV, presumably PRRSV entered domestic swine from an unidentified wildlife species (Zimmerman et al., 2006). A number of **mammals and birds** have been investigated, but none were capable of serving as mechanical or biological vectors (Cho and Dee, 2006). However, migratory waterfowl have been proposed as vectors of PRRSV spread between farms. Zimmerman et al. (1997) reported that pigs intranasally exposed to PRRSV isolated from feces of mallard ducks became viremic, seroconverted by ELISA and transmitted the virus to sentinel swine, but subsequent workers have not replicated these results.

Finally, feral swine are susceptible to PRRSV and in areas where they interact with domestic swine they could serve as a source of infection.

Airborne transmission was once considered the primary route of PRRSV transmission. Early data collected during the first outbreaks in England proposed that the virus could be spread through aerosols up to 3 km (Edwards et al., 1992). Actually, airborne transmission, along with arthropod-borne transmission, could explain the apparent long-distance transmission of PRRSV in the absence of other sources (Zimmerman et al., 2006). However, airborne transmission has been difficult to document and still remains highly controversial. Experimental studies have shown that aerosol transmission may occur over short distances: one trial demonstrated that experimentally infected pigs were able to transmit virus to sentinel pigs separated by 102 cm (Kristensen et al., 2004), while in another study, transmission from infected to susceptible pigs over a space of 1.0-2.5 meters has been successful in approximately 50% of the attempts (Otake et al., 2002). Overall, airborne spread is generally enhanced during winter when the temperature is low, humidity high and when wind speed and ultra-violet light exposure are low (Albina, 1997).

8.3 Persistent Infection in Breeding Herds

Persistence of PRRSV infection in a breeding herd may result from different mechanisms.

The chain of infection is maintained by a cycle of transmission from dams to pigs, either in utero or postpartum, or by commingling susceptible with infected animals in later stages of production. Indeed, after a PRRSV outbreak some pigs remain susceptible and can subsequently be infected at any time. New susceptible pigs can be added to a population by replacement, by birth of piglets from seronegative sows, by loss of passive or active immunity (Nodelijk et al., 2003). Therefore, where susceptible and infectious pigs are mixed, such as at weaning, a large proportion of the population may quickly become infected. However, marked differences in infection rates between groups, pens or rooms of animals may be observed in endemically infected herds (Zimmerman et al., 2006).

8.4 Risk Factors of Infection

In a regional PRRSV control programme in France, Le Potier et al. (1997) estimated that 56% of herds acquired the infection through the introduction of infected pigs, 20% through infected semen, 21% through fomites/slurry and 3% through unknown sources.

Generally, the risk of a herd becoming PRRSV-positive increases with:

1. density of PRRSV-positive neighboring herds, within 500 meters (Le Potier et al., 1997);

2. increasing herd size (Weigel et al., 2000);
3. high number of animal introductions (Albina, 1997);
4. purchase of semen for artificial insemination (Weigel et al., 2000);
5. absence of quarantine for replacement boars and gilts (Albina, 1997; Weigel et al., 2000).

9 PREVENTION

The objective of a PRRSV prevention programme is either to stop the introduction of PRRSV into negative herds or the introduction of new strains into PRRSV-infected herds. Animals and semen are considered the primary sources of PRRSV, but the importance of other sources has become evident (Zimmerman et al., 2006).

Actually, under ideal conditions, biosecurity starts with the establishment of the production units in isolated areas, but biosecurity efforts should place particular emphasis on all procedures involving the movement of inputs and outputs from the farms (Zimmerman et al., 2006).

9.1 DIRECT ROUTES OF INFECTION

All replacement breeding stock entering a negative herd should originate from sources known to be PRRSV-negative by a regular schedule of herd testing (Nodelijk et al., 2003; Zimmerman et al., 2006). Vet-to-vet communication to review the current health of the herd prior to purchase any genetic material is recommended, followed by quarantine and testing of animals. Regardless of the herd serostatus, an isolation facility is a critical component of a PRRSV biosecurity programme. Isolation/quarantine facilities should be located greater than 120 meters from the breeding herd and ideally off-site. Incoming stock should be kept separate from resident stock for a minimum of 30 days and tested prior to introduction into the breeding herd by a combination of RT-PCR and ELISA (Batista, 2005; Zimmerman et al., 2006).

Semen for artificial insemination should come from PRRSV-negative boar studs that should be routinely monitored for PRRSV infection, testing both blood and semen (Nodelijk et al., 2003). Real-time RT-PCR is the best option to assure that semen is free of PRRSV. This assay also reduces processing time, therefore allowing delivery of semen the same day it was collected and processed (Batista, 2005).

9.2 INDIRECT ROUTES OF INFECTION

A manual of biosecurity protocols for the prevention of spread of PRRSV was developed by Pitkin et al. and is available in the American Association of Swine Veterinarians Foundation (AASV) website

(www.aasv.org/aasv/PRRSV_BiosecurityManual.pdf).

Facilities: swine facilities should be managed using all-in-all-out (AIAO) pig flow, thereby reducing the spread of PRRSV from older infected pigs to younger naïve animals. In conjunction with AIAO flow, it is important to properly sanitize facilities before introducing susceptible animals. Disinfection fist requires removal of all organic material. Thereafter, infectious agents are inactivated in a temperature- and contact time-dependent fashion specific to the agent and the disinfectant used (Cho and Dee, 2006). The application of disinfectants via a foamer allows for better visualization of where product has been applied and also prolongs the contact between the chemicals and the surfaces. Following cleaning, the facilities must be allowed adequate downtime or drying time after disinfection.

Needles: in order to reduce the risk of hematogenous spread of PRRSV, it is recommended to change needles between sows during third trimester injections or utilize needle-free technology.

Transport vehicles: stringent compliance with cleaning/disinfection and drying protocols is critical for sanitizing the trailers of transport vehicles. Potential risk points in the cab of the truck (pedals, floor mats, etc.) can be effectively sanitized using disinfectant spray. As with facilities, an adequate drying time after disinfection is the most important step in the sanitation protocol to completely inactivate the virus.

Personnel: personnel should practice one night of downtime before entering a farm. Moreover, shower-in-shower-out protocols have been proven to successfully decontaminate personnel contaminated with PRRSV prior to entry. Barn-specific coveralls should be available in all facilities and washed routinely. Likewise, disposable or facility-specific boots should be used; boots should never leave the farm and should be power-washed to remove feces from the soles. Use of footbaths can greatly help reduce the risk of PRRSV transfer between groups of pigs.

Fomites: all incoming supplies should be disinfected and allowed a minimum of 2 hours contact time prior to introduction. “Double-bagging” supplies is an acceptable method for reducing the risk of spread. A specific room should be used as a disinfection and drying room for fomites.

Insects: all inlets, windows and areas that could be accessed by insects should be covered with screens, in conjunction with the use of insect bait and insecticides; in order to maintain proper ventilation, screens must be cleaned regularly. In addition, cutting the grass and removal of standing water are also recommended for eliminating insect breeding areas.

Aerosols: in order to reduce the risk of airborne spread of PRRSV, the adaptation of filtration systems to swine facilities has come about. Installation of an air filtration system depends upon the individual producer’s budget, the swine density of the area, the level of acceptable risk and the type of production system. If an air filtration system is installed in a building that is ventilated using negative pressure, all areas of the barn that could serve as potential air leaks need to be sealed. In addition, double-door

entry/exit systems must be installed. The chamber between the external and the internal doors must contain an exhaust fan designed to clear all the air in the room, to prevent potentially contaminated air from entering the animal air space.

10 CONTROL

The objectives of PRRS control are to limit the effects of the virus circulation in the various stages of production, to reach and protect herd stability (Dee, 1998) and to promote the development of protective immunity to the specific PRRSV variants of the farm. In endemically infected herds PRRSV cycles because, in any given time, animals are in different stages of infection and immunity. The first step in breaking virus circulation in the breeding herd is to use replacement animals that have been exposed to PRRSV and developed immunity prior to their introduction into the herd (Zimmerman et al., 2006).

10.1 ACCLIMATIZATION

The key population in the breeding herd to PRRSV control is the replacement gilt pool (Batista et al., 2004; Zimmerman et al., 2006). The results of other studies (Pesente et al., 2006; Vashisht et al., 2008) indicate that PRRSV exposure in the herd can be controlled by managing the gilt population. Consistent acclimatization of incoming breeding stock to PRRSV results in the stabilization of clinical signs, improvements in production parameters and production of PRRSV negative piglets at weaning (Zimmerman et al., 2006).

Pesente et al. (2006) confirmed that use of acclimatization in conjunction with biosecurity measures might prevent PRRSV outbreak associated with endemic PRRSV strains. Acclimatization in substance consists of exposure of seronegative replacements to the homologous herd strain for a sufficient time to recover and develop specific immunity before being bred (Fano et al., 2005; Vashisht et al., 2008). These animals should be introduced into herd only after they are tested by RT-PCR and resulted no longer viremic, thus not constituting a source of infection to other sows (Zimmerman et al., 2006). Therefore, researchers recommend that gilts be purchased and acclimatized at an early age (Vashisht et al., 2008).

Several acclimatization programmes were described. Some systems exploit contact exposure between gilts and weaned pigs that are used as donor sources of virus. Contact exposure is a timesaving, laborsaving and economically efficient strategy (Vashisht et al., 2008). However, over time and as the breeding herd becomes immune, virus transmission within the breeding herd stops and the production of PRRSV negative animals at weaning increases. Thus, natural infection of the gilts may be difficult.

Therefore, it is important to make sure that donor pigs do really transmit PRRSV (Andreasen, 2000). Additional exposure methods in replacement gilts may include feedback with tissues from weak-born piglets and stillbirths in the face of outbreaks, the use of vaccine products and inoculation of negative replacement animals with serum collected from viremic pigs from the same farm (Zimmerman et al., 2006).

Serum inoculation is successful in assuring seroconversion in all the animals submitted to the programme (Batista et al., 2002b; Fano et al., 2005). However, it is labor-expensive and carries some inherent risks, because serum may bring with the possible transmission of the other pathogens. Thus, it requires thoughtful application, high quality control standards and a proper choice of time of injection because of the short-lasting immunity induced by PRRSV (Batista et al., 2002b).

10.2 VACCINATION

Currently, both attenuated live and inactivated PRRSV vaccines have been licensed for use. In particular the attenuated live vaccines have been widely used and have shown some efficacy in reducing disease occurrence and severity, as well as the duration of viremia and virus shedding (Scortti et al., 2006; Martelli et al., 2007; Kimman et al., 2009). Moreover, Alexopoulos et al. (2005) observed that vaccination of gilts and non-pregnant sows with an attenuated vaccine resulted in improvement of the health status and performance of gilts/sows and their litters. Nevertheless, there are still evidences of failure of current vaccine programmes, mainly due to problems of safety and efficacy (Kimman et al., 2009).

There have been several field reports concerning the reversion of attenuated vaccine strains to virulence (Kimman et al., 2009), as well as the spread of vaccine virus within herds, with its detection in non-vaccinate pigs (grosse Beilage et al., 2009). Producers using an attenuated live vaccine for the first time may experience a decrease in reproductive productivity, with increasing number of liveborn and weaned pigs. This will be particularly true if they vaccinate their entire herds at one point in time or if they vaccinate sows during gestation (Dewey et al., 1999).

It is further evident that protection conferred by current vaccines is not or only partially effective in protecting against re-infection and transplacental infections caused by heterologous strains (Scortti et al., 2006; Kimman et al., 2009). The absence of complete protection has frequently been attributed to antigenic differences between vaccine and challenge strains and the limited cross-reactivity between the strain used to immunize the pigs and the strain used for the challenge. The main neutralizing epitope of PRRSV has been identified in the N-terminal ectodomain of GP5: it is possible that the differences in amino acid sequence between vaccine and challenge strains in this region are sufficient

to change the conformation of the epitope and prevent cross-reactivity (Prieto et al., 2008). However, current opinion suggests that vaccine efficacy is associated with an efficient cell-mediated immunity: the efficacy of a given vaccine is not only related with its immunological properties but also to the characteristics of the challenging strain to trigger an immune response (Martelli et al., 2009). Therefore, the ability of each strain to induce a strong cell-mediated immune response is more important than the genetic similarity between the vaccine and the field strains for inducing clinical protection (Mateu and Diaz, 2008).

The complexity of the immune response to PRRSV and the ability of the virus to escape or modulate the host's immune system make it difficult to develop a vaccine that can be used to control and eradicate the disease. Such a vaccine should accomplish at least four requirements: universality, efficacy, safety and ability to differentiate vaccinated from infected animals (Mateu and Diaz, 2008).

1. The first line of investigation is the investigation and identification of the B and T cell epitopes involved in the development of protective immunity and of the common critical epitopes in both EU and US strains.
2. The second line of research is to determine which components of the virion are involved in the down-regulation or modulation of the host's immune system and the mechanism by which this occurs. In addition, studies on the relationship between genetic diversity and the immunopathological properties of different strains are needed.
3. Thirdly, any possibility of reversion to virulence should be eliminated and transmission of the vaccine virus among pigs should be minimal or non-existent. The easiest way to gain in safety is by using non-replicating vaccines, but it is unclear if they are able to induce NAs and adequate cell-mediated immune responses.
4. Fourthly, the development of a differential vaccine is highly desirable. However, since PRRSV is a virus with a relatively small genome, it is difficult to find targets for deletion. Therefore, an extensive study of essential and non-essential parts of the viral genome is need.

Altogether, it is still valid what Dee and Joo emphasized in 1997: it is important to view vaccination as a part of a control programme, not as the final solution. Practioners must be sure to collect adequate data and attempt to understand patterns of viral transmission, the risk of persistent infection and whether naïve subpopulations of animals exist within infected farms, prior to implementing its use.

10.3 MONITORING HERD STABILITY

Breeding herd stability assures that PRRSV is not actively circulating in gilts, sows and boars housed in the breeding herd (Dee, 1998). In general, the purpose of this screening is to assess the effectiveness

of implemented control measures (e.g. gilt acclimatization, mass vaccination, planned exposure or herd closure). It is critical that veterinarians understand the advantages and disadvantages of the currently available technology associated with diagnostic assays for PRRSV. Adequate use and interpretation of the available diagnostic tests for PRRSV can provide very useful information (Batista, 2005). Breeding herds should be closely monitored following an integrated approach using clinical, serological and molecular analysis. In particular, molecular analysis proved fundamental to assess the presence of a unique PRRSV variant on the farm, which can be considered one of the most important aspects and critical points for the success of the control programme (Pesente et al., 2006).

Once herd stability has been achieved, veterinarians are faced with two options: either continue control or proceed to eradicate PRRSV. The final decision will depend on the geographical location of the farm and the probability of lateral introductions (Batista, 2005).

11 ERADICATION

Over the last few years major advances have been made to define protocols that can successfully eliminate PRRSV from infected farms. Successful PRRSV elimination in the breeding herd relies on the introduction of negative non-exposed replacement animals at a time when virus is no longer circulating. A successful eradication plan also requires successful control strategies that prepare an immune virus-free population and the implementation of strict biosecurity measures in order to prevent the herds from becoming reinfected (Zimmerman et al., 2006). As the movement of infectious pigs between farms has been well established to be the main risk factor for PRRSV introduction, if farms are not closed herds, maintenance of their PRRSV-free status could be hampered by purchase of replacement stock or finishing pigs. Therefore, pigs should only be purchased from known PRRSV-free farms that participate in a PRRSV certification programme. Likewise, also semen should only be purchased from AI-centres known to be free of PRRSV (Nodelijk et al., 2003).

Creating a pool of infection-free herds by a certification programme requires protocols for entrance of herds into the programme and for surveillance of the certified herds. The entrance protocol should assure that newly certified herds pose no increased threat for other certified herds. The surveillance protocol should assure that if a certified herd becomes infected it will on average only infect less than one other herd (Nodelijk et al., 2003).

Several methods of eradication have been shown effective in eliminating PRRSV from positive herds. However, the scientific value of these reports is often limited by the low number of herds involved, inadequate sample size of pigs, relative short observation periods to evaluate long-term effects and lack of standardized conditions and controls. Moreover, it is questionable whether the results of American studies can be extrapolated to European conditions (Nodelijk et al., 2003).

11.1 DEPOPULATION & REPOPULATION

Total and partial depopulations have proved to be effective means for controlling the spread of PRRSV within and between herds (Blanquefort and Benoit, 2000).

Whole herd depopulation and repopulation has been used for the elimination of multiple swine pathogens including PRRSV. Key elements in maintaining this strategy include purchasing PRRSV-

negative animals and consistent diagnostic testing of each incoming group animals. However, despite its success, several disadvantages exist including inability to preserve genetic material and an increase in production down-time, thus resulting in high implementation costs (Cho and Dee, 2006). Therefore, this strategy may be the only feasible alternative for farrow-to-finish herds where ongoing replication of PRRSV in the growing population does not allow for the elimination of the virus through other measures (Zimmerman et al., 2006).

Partial depopulation is indicated for the elimination of the virus from growing pigs when shedding from the breeding population has completely stopped. This technique may be sufficient to eliminate the virus from small-sized farms, while in large units (>500 sows) it requires the application of additional strategies (Zimmerman et al., 2006).

11.2 TEST & REMOVAL

Test and removal methods have also resulted in the successful elimination of PRRSV from positive populations. The principle of test and removal is to collect sera from all breeding females and evaluate the samples using ELISA for the detection of PRRSV antibodies and RT-PCR for the detection of viral nucleic acid. Animals that are positive by either ELISA or PCR or both are immediately removed (Dee et al., 2000). Candidate herds for test and removal include herds with segregated production and more than 12 months since the last clinical episode of PRRS and herds where there is no indication of virus recirculation in the breeding herd and where the presence of persistently infected animals is considered a potential risk for failure of the programme (Zimmerman et al., 2006). Although highly successful in eliminating PRRSV from endemically infected populations, several disadvantages are present, such as the high cost of diagnostic procedures and the potential removal of previously exposed animals that no longer have the virus (Cho and Dee, 2006). In fact, the identification and culling of carrier pigs demands highly specific and sensitive diagnostic tests: test qualities are important as culling of false positive pigs involves extra costs while the presence of false negative pigs implies risk of transmission of virus. For a herd, reaching infection-free status by test and removal requires that infected animals are removed before they infect, on average, less than one other animal (R<1) (Nodelijk et al., 2003).

11.3 HERD CLOSURE

The basis of herd closure is the cessation of replacement gilt introduction for an extended period (4 to 8 months), depending on the status of the farm and pig flow. It results in the reduction in viral shedding and elimination of carrier animals. Although herd closure allows for the preservation of

genetics and retains minimal diagnostic costs, it can result in the production of an improper parity distribution and the development of a negative population of breeding animals over time. However, these effects can be minimized through the use of off-site breeding projects for replacement gilts (Cho and Dee, 2006; Zimmerman et al., 2006).

Experimental Part

12 STUDY A

Adoption of strict biosecurity measures and acclimatization of replacement gilts by exposure to the specific PRRSV strain circulating on farm are commonly considered sound strategies to control PRRS in breeding herds (Batista et al., 2004; Fano et al., 2005; Pesente et al., 2006; Vashisht et al., 2008). Aims of this policy are to avoid the introduction of new variants, to reach and protect herd stability eliminating virus circulation in breeding stock and to promote the development of protective immunity to the specific PRRSV variants of the farm. Thus, monitoring infection and immunity status of replacement animals, herd stability and PRRSV strains circulating on farm is critical to assess the effectiveness of the control strategies or to decide for their reinforcement.

In the present study we performed serological, virological and productive surveys in an endemically infected farm in order to:

1. evaluate the effectiveness of an acclimatization strategy in terms of prevalence and titers of antibodies to PRRSV;
2. assess the viral population of the farm and its evolution over time through ORF5 and ORF7 phylogenetic analysis;
3. compare herd performances before and after the incoming of a new variant.

12.1 MATERIALS AND METHODS

Study farm

The study has been performed from April 2007 to February 2010 in an all-in-all-out, farrow-to-wean farm of approximately 1,550 productive sows, located in an endemically PRRSV-affected area (Veneto, Italy). The average number of piglets weaned per sow per year during the period of study was 25.6.

The first PRRS outbreak occurred in the herd in 1995, followed by sporadic reproductive failures. Rigorous biosecurity measures, such as perimeter fence, shower-in system, control of rodents, disinfection of trucks, isolated freezer for fetuses and dead animals, were subsequently implemented and a gilt acclimatization programme was developed. During the study two PRRS outbreaks occurred in the farm, in October-November 2008 and January-February 2010.

Acclimatization programme

The replacement rate of the farm was about 40%: 52 3-week-old PRRSV-naïve gilts were monthly purchased from a certified PRRSV-free breeder multiplier farm and submitted to the following acclimatization programme.

1. On arrival gilts were housed in a clean and disinfected nursery room in 5 pens of 10-11 animals each, separated each other by pens containing 3-week-old weanling pigs, promoting direct contacts among animals.
2. One month later, gilts and weanlings were moved to a grower unit. All of the 52 gilts were placed in the same room, in 3 pens of 17-18 gilts each and separated each other by 2 pens containing 20 growing pigs.
3. At about 3 months of age growing pigs were sold, while gilts were moved to the finisher unit. There were 4 rooms in the finisher unit and 4 pens in each room (Figure 2). Thirteen gilts were placed in each pen and 12 nursery pigs in 4 smaller pens between the pens of the gilts, as potential source of PRRSV (Figure 3). These animals were selected on the basis of the results of a virological survey previously carried out in the farm, demonstrating that pigs of 6-8 weeks of age have the highest probability of being viremic. Pigs were turned over every 2 weeks and removed one month prior to the introduction of gilts into the stimulation/mating unit.
4. Aim of the programme was to bring to the first service 8-month-old gilts after they became immune but no longer viremic. As from 230 days of age and about 150 kg of weight, the gilts were moved to the stimulation unit in batches of 9-11 animals every week. After the first estrus was detected, they were bred by artificial insemination and housed in gestation pens until one week prior to farrowing, when they were moved to farrowing crates. The semen used for artificial insemination was obtained from boars stabulated in the farm and periodically tested for PRRSV by RT-PCR.

Figure 2 *Finisher unit for replacement gilts*

Figure 3 *Nursery pigs used as virus sources*

Sample collection

Anterior vena cava blood samples were collected by venipuncture with blood collection vacuum tubes, stored in ice during transportation to the laboratory and refrigerated at 4°C if the laboratory was not available on the day of sampling. In the laboratory serum was harvested from blood samples by centrifugation for 10 minutes at 2500 g/min. Sera were aliquoted into 1.5 mL Eppendorf tubes and up to 5 samples were pooled to be tested by RT-PCR as a single sample. Both aliquotes and pools of serum were stored at -80°C until analyzed.

- From April 2007 to March 2009, sera were collected from 1185 gilts belonging to 24 consecutive monthly batches at the moment of their move from the finisher unit into the stimulation one.
- From October 2007 and October 2009, 7 cross-sectional surveys were carried out on a total of 802 pigs between 2 and 13 weeks of age.
- On the occasion of the two PRRS outbreaks occurred in the farm during the study, thoracic fluid was collected from some aborted fetuses.

Serological tests

The presence and the s/p ratio of antibodies to PRRSV in gilts were determined using the HerdCheck PRRS Virus Antibody Test Kit 2XR (IDEXX Laboratoires), in accordance with the manufacturer's directions. Gilts were considered seropositive if the s/p ratio was ≥0.4.

Biomolecular analyses

Extraction

Viral RNA was extracted from 200 μL of serum or thoracic fluid using the High Pure Viral RNA Kit (Roche Diagnostic), following the manufacturer's instructions. Pools of 5 samples each were analyzed in duplicate by real-time RT-PCR to detect viral RNA.

Real-time RT-PCR

Four μL were used as template for real-time RT-PCR, combined with the reaction mixture to a final volume of 15 μL. The reaction was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen), following the manufacturer's instructions. After a holding step at 50°C for 5 min for reverse transcription and a preincubation step at 95°C for 5 min in order to activate the HotStartTaq DNA polymerase, amplification was performed during 40 cycles including denaturation (95°C for 10 sec), annealing and extension (60°C for 30 sec). The SYBR Green fluorescent signal was determined for each cycle at the end of the extension step.

Table 1
Description of the primers used for RT-PCR

RT-PCR type	Primer sequence	Primer specificity	Size of RT-PCR products	Reference
Real-Time ORF7 RT-PCR	*Forward primer* 5' GGG GAA TGG CCA GYC AGT CA 3' *Reverse primer* 5' GCC AGR GGA AAA TGK GGC TTC TC 3'	EU-type PRRSV	125 bp	Lurchachaiwong et al., 2008
ORF7 RT-PCR	*Forward primer* 5' GCC CCT GCC CAC CAC G 3' *Reverse primer* 5' TCG CCC TAA TTG AAT AGG TGA 3'	All known PRRSV-types	637 bp	Oleksiewicz et al., 1998
ORF5 RT-PCR	*Forward primer* 5' CTT TGT GCT TTA CCC GGT 3' *Reverse primer* 5' GGG CGT ATA TCA TTA TAG GTG 3'	EU-type PRRSV	448 bp	Pesente et al., 2006

After amplification, melting curve analysis was performed by heating the amplified products from 64°C to 90°C. Samples sharing the same T_m point were interpreted as belonging to the same strain. Primer sequences are reported in Table 1. EU standard strain was included as positive control in each PCR experiment. Pools were considered positive when the average cycle threshold (Ct) value was below 38, the curve in the amplification plot showed exponential increase and a PRRSV-specific melting curve (between 83.0°C and 85°C) was obtained (Martínez et al., 2008).

In case of positive pools, RNA was extracted from individual samples and submitted to real-time RT-PCR at the same conditions applied to pools.

Conventional RT-PCR

Samples testing positive to real-time RT-PCR were further analyzed using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). An adequate quantity of RNA, based on Ct value of real-time RT-PCR, was reverse transcribed and amplified in a 50 µL reaction mixture, following manufacturer's instructions. Depending on the PCR primer pair used, ORF5 or ORF7 was amplified (Table 1).

For the ORF7 amplification, according to the previous study described by Oleksiewicz et al. (1998), highly conserved primer-binding sites were chosen and the primer sequences yielding a 637 bp amplicon were used. The reverse transcription was performed at 50°C for 30 min, whereas the amplification reaction consisted of an initial step at 95°C for 2 min, followed by 45 cycles of 15 sec at 95°C, 20 sec at 55°C and 40 sec at 68°C, and a final extension step at 68°C for 5 min.

After the reverse transcription, the ORF5 amplification programme instead consisted of an initial denaturation of 2 min at 94°C, followed by 40 cycles at 94°C for 15 sec, 55°C for 30 sec and 68°C for 45 sec, and terminated with an elongation step at 68°C for 7 min.

The products were analyzed by gel electrophoresis in 3% agarose gel, stained with SYBR Safe (Invitrogen) and photographed.

Purification and Sequencing

DNA templates of RT-PCR positive samples were purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics), according to the manufacturer's instructions.

Both strands of the purified product were sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI PRISM 3100 Genetic Analyxer (Applied Biosystems), following the manufacturer's instructions. The consensus sequences were obtained using the software ChromasPro 1.42(Technelysium).

Phylogenetic Analysis

The multiple alignment of full length ORF7 (387 bp) and of partial ORF5 (330 bp) was performed using CLUSTAL W (Larkin et al., 2007). The phylogenetic trees were generated by the distance-based neighbor-joining method using MEGA 4 software (Kumar et al., 2008).

Statistical analysis

Data obtained from the farm database were submitted to statistical analysis using SPSS 18.0 software (SPSS Inc.). Differences in number of liveborn piglets between seropositive and seronegative sows were evaluated by Student's *t* test and Chi square test was used to compare the frequency of stillborn and mummified piglets, considering a number ≥3 as a unique category. The alpha level of statistical significance was set to 0.05.

12.2 RESULTS

Replacement gilts immunity and infection

Serological status of acclimatized gilts was evaluated considering prevalence of seropositive animals and mean s/p value (Figure 4). After a first period of low effectiveness of the acclimatization programme, especially in July and November 2007, when prevalence did not reach 60%, both values increased in the subsequent months, reaching or exceeding 80% of seroprevalence.

Viremia was detected, especially in wintertime, in 9 out of the 24 groups of gilts, and in 3 of them 100% of pools resulted positive (Figure 4).

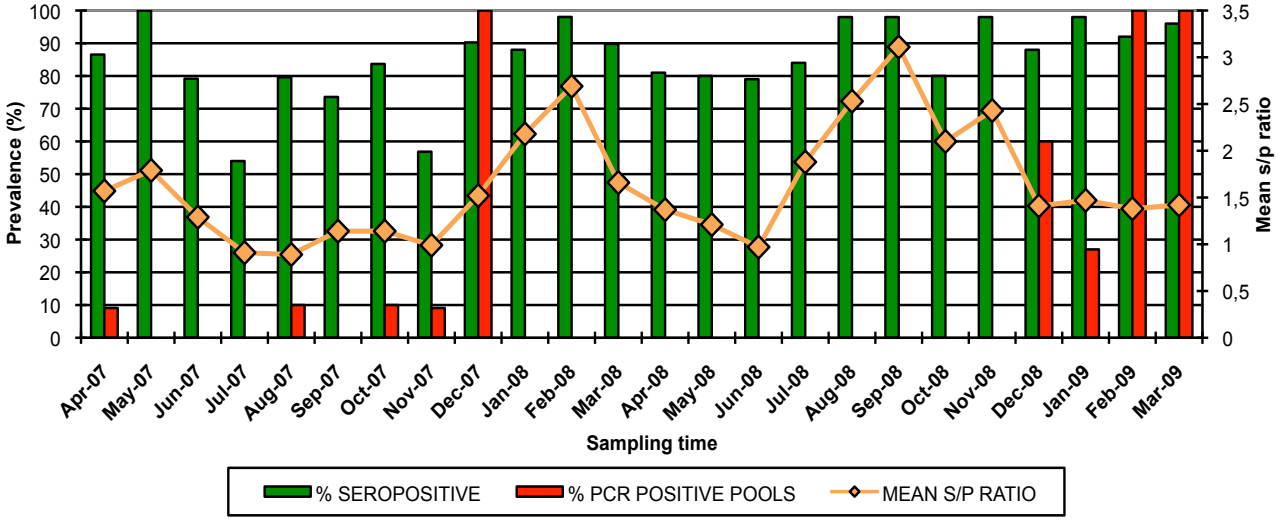

Figure 4 *Serological and virological data of the 24 groups of gilts at the end of acclimatization*

Cross-sectional surveys

A great variability was observed in prevalence of positive pools obtained from the cross-sectional surveys (Table 2), ranging from 2/16 positive pools in July 2008 to 23/23 in February and May 2009.

Table 2
Results of the cross-sectional virological surveys on pools of 5 pigs from farrowing rooms, nursery and growing unit

Date of sampling	Number of pools	Number of positive pools	% positive pools
October, 2007	40	38	95%
May, 2008	23	17	74%
July, 2008	16	2	13%
October, 2008	20	16	80%
February, 2009	23	23	100%
May, 2009	22	22	100%
October, 2009	17	11	65%
TOTAL	161	129	80%

Herd reproductive performances

No statistically significant differences were observed in number of liveborn piglets per litter at first farrowing between seropositive (10.34±2.90) and seronegative (10.10±2.65) gilts. Serological status did not affect even frequencies of stillborn and mummified piglets at first farrowing (Table 3).

Table 3
Table of frequencies of stillborn and/or mummified piglets per litter of seronegative and seropositive gilts

	Number/litter	Serological status	
		Negative	Positive
Stillborn piglets	0	133 (85.9%)	834 (88.8%)
	1	14 (9.0%)	82 (8.7%)
	2	5 (3.2%)	14 (1.5%)
	≥3	3 (1.9%)	10 (1.0%)
	TOTAL	155 (100%)	940 (100%)
Mummified piglets	0	142 (91.7%)	861 (91.7%)
	1	9 (5.8%)	41 (4.4%)
	2	1 (0.6%)	19 (2.0%)
	≥3	3 (1.9%)	19 (2.0%)
	TOTAL	155 (100%)	940 (100%)

Table 4
Monthly herd reproductive performances

Month	Farrowing rate (%)	Abortions	Fertility rate (%)	Liveborn piglets/litter	Weaned piglets/litter
April, 2007	84.7	1	91.1	11.44	11.10
May, 2007	80.2	2	84.6	11.83	11.50
June, 2007	87.2	4	83.6	11.93	11.16
July, 2007	89.1	0	81.3	11.81	10.94
August, 2007	89.4	1	86.0	11.13	10.33
September, 2007	83.1	1	93.9	11.48	10.86
October, 2007	84.4	0	91.6	11.25	10.45
November, 2007	79.7	0	90.4	10.77	10.15
December, 2007	88.3	0	91.0	10.59	9.97
January, 2008	91.0	2	93.5	11.26	10.57
February, 2008	92.5	0	91.3	11.22	10.89
March, 2008	90.1	3	93.7	11.47	10.84
April, 2008	90.5	1	87.6	11.62	10.86
May, 2008	92.8	1	89.5	11.69	11.20
June, 2008	92.1	1	84.7	11.63	11.08
July, 2008	94.6	1	80.7	11.73	11.28
August, 2008	84.8	1	85.9	11.46	10.74
September, 2008	86.6	1	86.2	11.20	10.72
October, 2008	82.0	10	92.1	10.97	10.61
November, 2008	76.0	14	88.1	10.15	9.69
December, 2008	83.5	3	89.5	10.32	9.72
January, 2009	87.8	0	87.3	10.76	10.28
February, 2009	90.4	3	86.2	10.89	10.58
March, 2009	86.9	1	89.3	10.99	10.71

During the PRRS outbreak of October-November 2008, farrowing rate decreased dramatically, whereas fertility rate did not show evident variations, contrary to what was usually observed in the hot seasons. In the months following the outbreak, all the parameters considered in the analysis except the fertility rate increased, however without reaching the levels of the previous months (Table 4) (Figures 5 and 6).

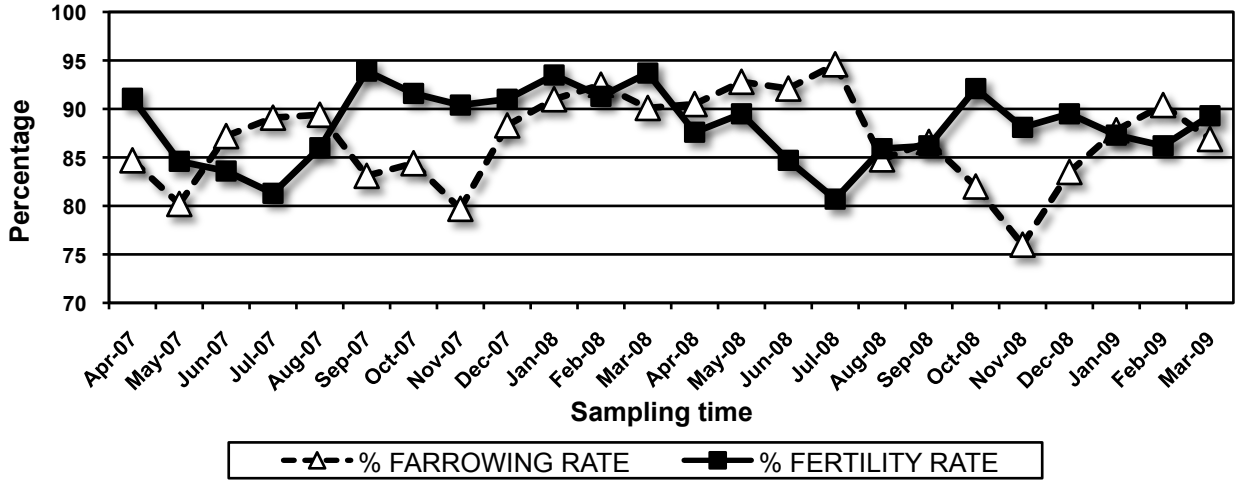

Figure 5 *Trend of monthly herd farrowing rate and fertility rate*

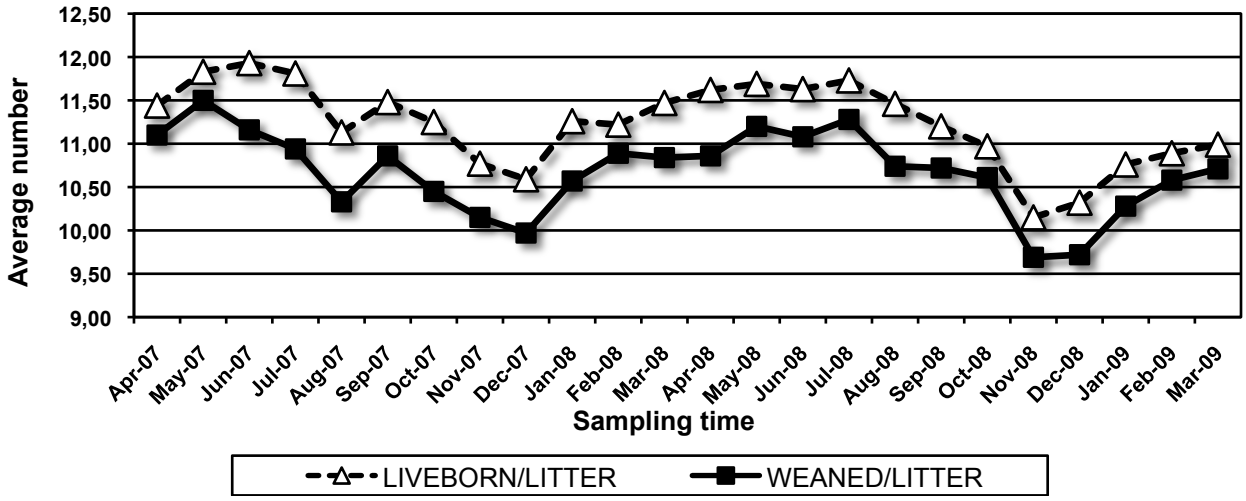

Figure 6 *Trend of monthly herd liveborn piglets/litter and weaned piglets/litter*

PRRSV phylogenesis

From samples resulted positive during the studies of this thesis, 81 sequences of full length ORF7 (Table 5) and 50 sequences of partial ORF5 were obtained. Phylogenetic tree based on ORF7 analysis of PRRSV isolates is represented in Figure 7, whereas the comparison between the phylogenetic trees based on ORF7 and ORF5 sequences, when sequencing of both the genomic regions was possible, is discussed in study B (Figure 12 pag. 69).

The phylogenetic analysis of ORF7 sequences included also the sequences of the 2 isolates used as positive controls for biomolecular diagnosis and 54 sequences obtained from GenBank database (www.ncbi.nlm.nih.gov/genbank/) (Table 6). In this study the phylogenetic analysis revealed the presence of a stable viral population in the farm, represented by a poorly divergent single homologous variant from October 2007 to May 2009 (p-distance of 1.4% of bases). However, as from May 2009 a new PRRSV variant was isolated and seemed to substitute the previous endemic variant, which was no longer isolated. Moreover, the sequence obtained from the thoracic fluid of the aborted fetuses during the outbreak of October-November 2008 (02_301008) largely differed from those of the endemic strain, with a nucleotidic p-distance of 9.4-10.8%. Finally, the 2 sequences relative to the outbreak of January-February 2010 (10_040210 and 11_040210) belong to the cluster of the endemic variant of study B (Figure 7).

Table 5
Origin of the ORF7 sequences investigated and analyzed in the studies of this thesis

Sample code (#_ddmmyy)	Region	Study	Sampled animals
01_070508	Veneto	A	Nursery/growing pigs
01_090708	Veneto	A	Nursery/growing pigs
01_110110	Veneto	C	Nursery/growing pigs
01_210109	Friuli V.G.	B	Aborted fetuses
01_250209	Veneto	A	Nursery/growing pigs
02_090708	Veneto	A	Nursery/growing pigs
02_210109	Friuli V.G.	B	Aborted fetuses
02_301008	Veneto	A	Aborted fetuses
03_090708	Veneto	A	Nursery/growing pigs
04_110110	Veneto	C	Nursery/growing pigs
4e_180609	Friuli V.G.	B	Replacement gilts
4u_180609	Friuli V.G.	B	Replacement gilts
06_180309	Veneto	A	Replacement gilts
07_070508	Veneto	A	Nursery/growing pigs
07_081008	Veneto	A	Nursery/growing pigs
07_170209	Veneto	A	Nursery/growing pigs
08_081008	Veneto	A	Nursery/growing pigs
08_170209	Veneto	A	Nursery/growing pigs
8e_180609	Friuli V.G.	B	Replacement gilts
09_170209	Veneto	A	Nursery/growing pigs
09_180309	Veneto	A	Replacement gilts
10_040210	Veneto	A	Aborted fetuses
10_070508	Veneto	A	Nursery/growing pigs
10_090708	Veneto	A	Nursery/growing pigs

10_170209	Veneto	A	Nursery/growing pigs
11_040210	Veneto	A	Aborted fetuses
13_070508	Veneto	A	Nursery/growing pigs
15_070508	Veneto	A	Nursery/growing pigs
16_070508	Veneto	A	Nursery/growing pigs
16_090409	Friuli V.G.	B	Replacement gilts
17_070508	Veneto	A	Nursery/growing pigs
18_180609	Friuli V.G.	B	Replacement gilts
19_070508	Veneto	A	Nursery/growing pigs
20_070508	Veneto	A	Nursery/growing pigs
20_090409	Friuli V.G.	B	Replacement gilts
21_240209	Friuli V.G.	B	Replacement gilts
22_070508	Veneto	A	Nursery/growing pigs
22_181209	Friuli V.G.	B	Nursery/growing pigs
24_160409	Friuli V.G.	B	Nursery/growing pigs
24_240209	Friuli V.G.	B	Replacement gilts
25_221107	Veneto	A	Replacement gilts
26_090708	Veneto	A	Nursery/growing pigs
26_180309	Veneto	A	Replacement gilts
27_180309	Veneto	A	Replacement gilts
28_090708	Veneto	A	Nursery/growing pigs
28_160409	Friuli V.G.	B	Nursery/growing pigs
28_180309	Veneto	A	Replacement gilts
29_090708	Veneto	A	Nursery/growing pigs
30_160409	Friuli V.G.	B	Nursery/growing pigs
30_180309	Veneto	A	Replacement gilts
31_181209	Friuli V.G.	B	Nursery/growing pigs
31_211009	Veneto	A	Nursery/growing pigs
34_181209	Friuli V.G.	B	Nursery/growing pigs
35_240209	Friuli V.G.	B	Replacement gilts
36_211009	Veneto	A	Nursery/growing pigs
36_240209	Friuli V.G.	B	Replacement gilts
37_240209	Friuli V.G.	B	Replacement gilts
39_240209	Friuli V.G.	B	Replacement gilts
40_211009	Veneto	A	Nursery/growing pigs
47_280509	Veneto	A	Nursery/growing pigs
48_280509	Veneto	A	Nursery/growing pigs
49_311007	Veneto	A	Replacement gilts
50_180309	Veneto	A	Replacement gilts
51_180309	Veneto	A	Replacement gilts
54_170209	Veneto	A	Nursery/growing pigs
56_081008	Veneto	A	Nursery/growing pigs
57_081008	Veneto	A	Nursery/growing pigs
59_081008	Veneto	A	Nursery/growing pigs
66_290708	Veneto	A	Nursery/growing pigs
66_301007	Veneto	A	Nursery/growing pigs
67_081008	Veneto	A	Nursery/growing pigs
67_290708	Veneto	A	Nursery/growing pigs
68_081008	Veneto	A	Nursery/growing pigs
68_290708	Veneto	A	Nursery/growing pigs
69_290708	Veneto	A	Nursery/growing pigs
70_290708	Veneto	A	Nursery/growing pigs
70_301007	Veneto	A	Nursery/growing pigs
76_081008	Veneto	A	Nursery/growing pigs
78_081008	Veneto	A	Nursery/growing pigs
79_081008	Veneto	A	Nursery/growing pigs
80_081008	Veneto	A	Nursery/growing pigs

Table 6
Origin of the ORF7 sequences obtained from GenBank

Sample code	Country of origin (region)	Sampling year	PRRSV type
A26843	Netherlands	1992	European
AB288356	Japan	1992	American
AF066183	USA	1998	American (RespPRRS vaccine)
AF176348	Canada	2002	American
AF512378	Austria	1996	European
AJ223078	Denmark	2001	American
Ame_Ctrl+	IZSVE	Unknown	American
AY035941	Denmark	2001	European
AY035948	Denmark	1995	European
AY150564	Denmark	2003	American
AY366525	USA	2004	European
AY395081	USA	2004	European
AY457635	China	2003	American
AY588319	Netherlands	2004	European
AY612613	South Korea	2005	American
AY749412	USA	2004	European
AY749418	Italy (Lombardia)	2003	European (ITA)
AY749419	Italy (Lombardia)	2003	European (ITA)
AY749420	Italy (Veneto)	2003	European (ITA)
AY749421	Italy (Lombardia)	2003	European (ITA)
AY749422	Italy (Lombardia)	2003	European (ITA)
AY749423	Italy (Veneto)	2003	European (ITA)
AY749424	Italy (Lombardia)	2003	European (ITA)
AY749425	Italy (Veneto)	2003	European (ITA)
AY749426	Italy (Lombardia)	2003	European (ITA)
AY749427	Italy (Lombardia)	2003	European (ITA)
AY749428	Italy (Veneto)	2003	European (ITA)
DQ009650	Spain	2005	European
DQ056373	Thailand	2005	American
DQ324710	Poland	2005	European
DQ473474	South Korea	2006	American
DQ489311	USA	2006	European
DQ779791	USA	2006	American
DQ864705	Thailand	2006	European
EF473138	China	2007	European
EF484033	USA	2007	American
EF641008	China	2007	American
EU071225	Poland	2007	European
EU076704	Hong Kong	2007	European
EU144079	China	2007	American
EU424166	South Korea	2008	European
EU864232	China	2005	American
Eur_Ctrl+	IZSVE	Unknown	European
FJ524377	USA	2009	American
FJ895329	China	2009	American
GQ451680	Spain	1995	European
GQ451684	Spain	2005	European
GQ451689	France	1993	European
GU067771	Spain	2009	European
L77914	UK	1997	European
M96262	Netherlands	1993	European (Lelystad Virus)
U87392	USA	1995	American
X92942	Spain	1995	European
Z92538	France	1997	European

STUDY A (Veneto)	STUDY B (Friuli V.G.)	STUDY C (Veneto)
• Endemic variant	• Endemic variant	• Variant used for experimental injection
• Abortions	• Abortions	
	• Gilts entering quarantine	
	• Gilts leaving quarantine	

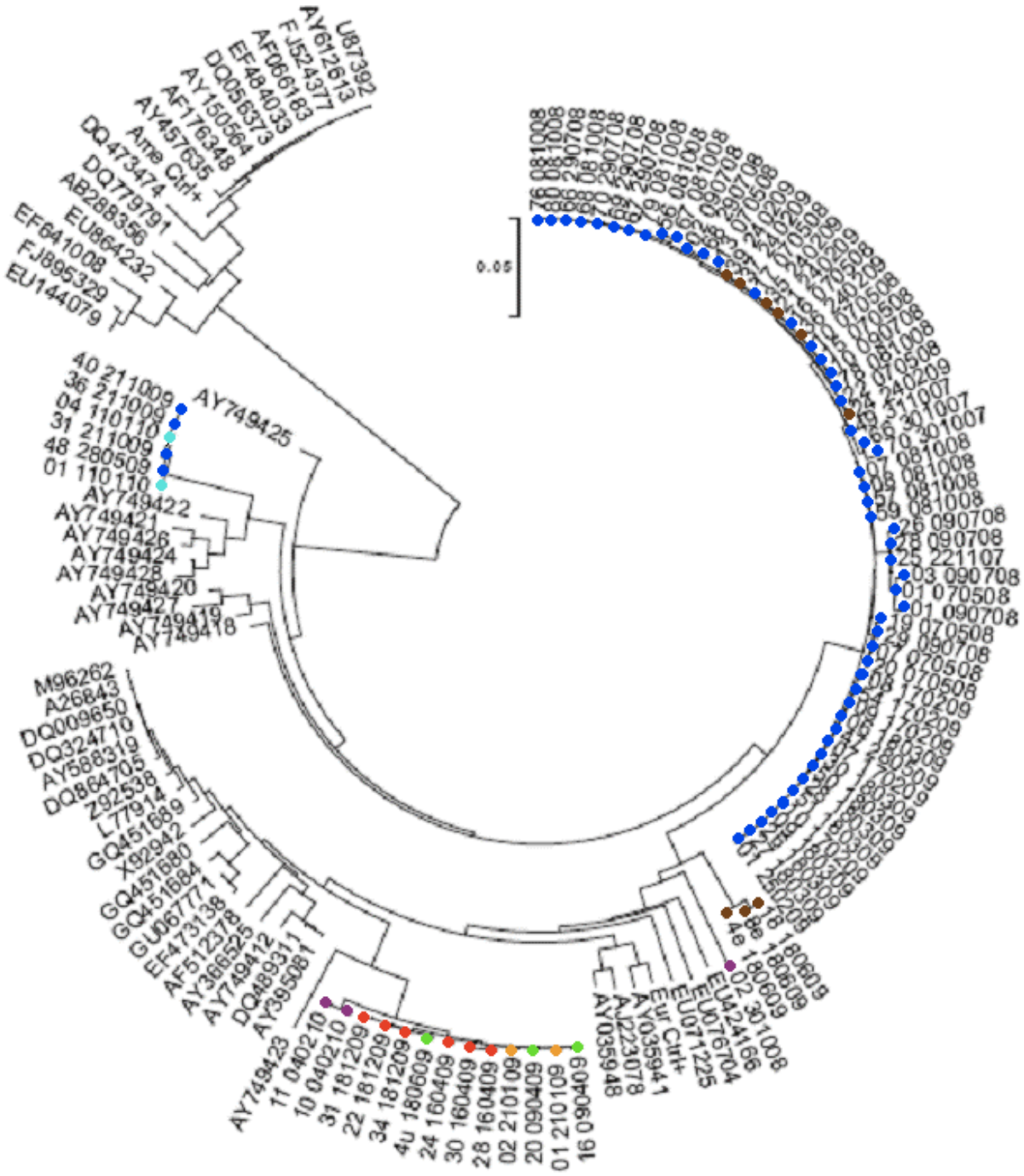

Figure 7 *Phylogenetic tree of ORF7 sequences*

12.3 DISCUSSION

Previous studies (Pesente et al., 2006; Vashisht et al., 2008) have suggested that acclimatization of gilts in conjunction with biosecurity measures is able to control PRRSV infection in endemically infected breeding farms and to maintain herd stability. In 1998 S. A. Dee defined the stability of an infected breeding herd as the absence of viral circulation in gilts, sows and boars. Considering the endemic infection experienced by most of the Italian herds, herd stability can be regarded as the absence of clinical signs in dams and piglets and the circulation of a unique or few homologous PRRSV variants. Therefore, the aims of an acclimatization programme are to develop specific immunity to the endemic PRRSV strains, to control virus circulation in the herd preventing its circulation in the reproductive units and to stabilize and make uniform the farm performances.
Although gilts acclimatization has become in the pig industry a very common strategy to cope with PRRSV infection, there are few analytical studies of its actual effectiveness (Batista et al., 2002b; Fano et al., 2005; Pesente et al., 2006; Vashisht et al., 2008).
In the present study a standardized acclimatization programme of gilts was evaluated in a farm for a 24-month period. The outcomes of the study suggest some considerations.

1. We observed a raising trend of the prevalence of seropositive gilts at the end of the acclimatization that has become stable in the course of the study. Likely reason of this improvement was a better management of pigs used as virus shedders in the growing unit. Owing to the results of the first groups of gilts, the turnover of potentially viremic pigs was speeded up, in order to increase the probability of an effective contact with naïve gilts. Contact exposure cannot assure a uniform transmission of the infection, but an 80% of seropositive gilts can be reasonably considered as effective. The lack of differences in performances (liveborn, stillborn and mummified piglets) between seropositive and seronegative gilts confirms that seronegative gilts included in an immunized and stable population may benefit from group immunity.

2. PRRSV does not survive long in the environment (Nodelijk et al., 2003; Zimmerman et al., 2006) and its infectivity is influenced by several environmental factors (temperature, ventilation, relative humidity). In this study this was confirmed by the higher occurrence of viremic gilts in the cold months. Beyond, studies have shown that PRRSV can persist for an extended period of time in individual pigs that remain potentially infectious (Bierk et al., 2001; Batista et al., 2002a; Wills et al., 2003; Batista et al., 2004). To avoid viral transmission from viremic gilts to pregnant sows losing their specific immunity, it is suggested to purchase and acclimatize young 3-week old gilts to ensure enough

time for viral clearance. For the same reason, both in fecundation-gestation unit and in farrowing rooms primiparous and pluriparous sows were kept apart.

The variability of prevalence of positive pools in the 5 cross-sectional surveys seems to reflect the classic picture of an endemic PRRSV infection well described by Stevenson et al. (1993) and Nodelijk et al. (2003). Persistence of PRRSV infection within a breeding herd needs a sufficient number of infectious and susceptible animals to be maintained. Susceptible pigs are represented by replacement gilts, by piglets from seronegative dams or losing their passive immunity and by previously infected pigs losing their active immunity. PRRSV spread in young pigs depends on the level of antibodies in their dams. Thus, the more effective the acclimatization programme, the higher the immune response of sows, the later the infection of piglets, the lower the virus circulation, the more difficult the transmission of infection to replacement gilts. A very accurate management is therefore required to maintain an endemic and stable pattern of infection. On the other hand, the possible eradication of the infection in a single farm should be still considered as highly risky, given its endemicity in the area.

3. Also farm biosecurity policy is responsible for the effectiveness of the acclimatization programme, restricting the circulation of PRRSV variants (Pesente et al., 2006). Previous studies demonstrated that both acclimatized (Pesente et al., 2006) and vaccinated (Scortti et al., 2006) sows are only partially protected against challenge with heterologous strains and that the incoming of a different PRRSV variant may severely affect health and performances of sows. Nonetheless, there are few evidences in literature about the real effectiveness of an acclimatization programme under field conditions towards challenge with new PRRSV strains (Vashisht et al., 2008).

In the present study the PRRS outbreak had severe consequences on the farrowing rate, due to the increase in the number of abortions. On the contrary, fertility rate did not suffer variations during the outbreak, suggesting that a careful pig-flow and good farming practices do not allow PRRSV to circulate in fecundation units and to involve first steps of gestation.

Furthermore, the trend of all the productive parameters returned constant in the months following the outbreak. This confirms that a good acclimatization programme associated with strict biosecurity measures allows to restore herd stability in few weeks after an outbreak.

Finally, sequencing and phylogenetic analysis confirmed to be useful tools to monitor the viral population and the spread of the infection within a farm (Pesente et al., 2006), allowing to detect the incoming of a PRRSV variant never detected before and to confirm that acclimatization supported the return to herd stability, re-establishing the circulation of the sole endemic variant in the months following the outbreak. However, the phylogenetic analysis seems to demonstrate the incoming of a new variant in May 2009 that was substituting the previous endemic strain, with following

consequences on the control of infection and on the sensitivity of the diagnostic assays (cfr. Study C). Moreover, the detection in the fetuses aborted during the outbreak of January-February 2010 of the endemic PRRSV isolate of the farm of study B stresses once again the importance of reaching and maintaining a high level of biosecurity, especially when two or more farms are closely linked by commercial and/or managerial relationships.

12.4 CONCLUSIONS

This study confirms that an extended acclimatization of gilts in PRRSV endemic areas represents an effective strategy to contain possibly severe losses associated with the infection. This practice, if associated with strict biosecurity measures, may allow to control PRRSV infection and to keep herd stability, even after a challenge with heterologous virus variants.

13 STUDY B

In order to control PRRSV infection in a breeding farm, besides implementation of effective biosecurity protocols and application of control programs, adequate use and interpretation of the currently available diagnostic tests for PRRSV can provide very useful information. In particular, both virus detection and strain identification are essential for screening swine imported from abroad and hence a rapid and accurate diagnostic method is crucial for detecting even low levels of virus contamination.

In this study we carried out a longitudinal survey in the replacement gilt pool after a severe PRRSV outbreak in the gestation unit close to isolation/quarantine boxes for replacement gilts. The aims of the study were to establish if these abortions were due to lateral introduction of PRRSV with the replacement gilts and, in that case, to adopt new strategies for PRRSV control improvement.

13.1 MATERIALS AND METHODS

Study farm

The study has been performed from January 2009 to December 2009 in an all-in-all-out, farrow-to-wean farm of approximately 2,580 productive sows, located in an endemically PRRSV-affected area (Friuli Venezia Giulia, Italy). The average number of piglets weaned per sow per year during the period of study was 22.9. The owners and the veterinarian of the farm, as well as the semen used for artificial insemination, are the same of the farm of study A. From October 2008 to January 2009 186 late-term abortions occurred in old sows held in the gestation unit.

Quarantine/acclimatization programme

The replacement rate of the farm B was about 49%: 200 3-week-old gilts were purchased every two months from a PRRSV positive commercial seedstock farm. However, gilts were guaranteed to be PRRSV-free and immunocompetent on the basis of the results of tests carried out on their dams. The sows of the seedstock source indeed were regularly tested via serology and detection of virus by conventional RT-PCR performed in pools of 10 sows each.

Nevertheless, the biosecurity policy adopted by farmers in farm B provided that, once arrived, replacement gilts were housed in 4 on-site internal isolation boxes, containing 2 pens each, and

submitted to quarantine. At 3 months of age, gilts were moved to a growing unit with the same age pigs of the farm and after another month to the finishing unit, where they were maintained alone. Like for the farm A, also in this farm the aim of the programme was to move to the stimulation unit and fecundate 8-month-old gilts after they became immune but no longer viremic.

Sample collection

Blood samples were collected from 3 consecutive groups of replacement gilts. Forty gilts of each group were individually identified upon arrival with numbered ear tags and sampled:

- the day after arrival;
- at the end of the quarantine period;
- entering the finishing unit, (samples on 20 gilts);
- entering the stimulation unit (samples on 20 gilts).

Two cross-sectional surveys were carried out in April and December 2009 from about 50 animals of 6-10 weeks of age. Thoracic fluid was collected from fetuses aborted in January 2009.

Serological tests

The presence and the s/p ratio of antibodies to PRRSV in gilts were determined using the HerdCheck PRRS Virus Antibody Test Kit 2XR (IDEXX Laboratoires), in accordance with the manufacturer's directions. Gilts were considered seropositive if the s/p ratio was ≥0.4.

Biomolecular analyses

Extraction of RNA, real-time RT-PCR and conventional RT-PCR were performed at the same conditions and using the same primers described in the study A (cfr. pag. 51). All the samples collected on the day after the arrival were tested by real-time PCR in pools of 5 samples each; samples collected at the end of the quarantine period from gilts 1-20 were tested as single samples, while samples from gilts 21-40 were pooled; all the samples collected from the 20 gilts at the entry to the finishing and the stimulation units were tested as single samples. Samples were considered positive when the average Ct value of the replicates was below 38, the curve in the amplification plot showed exponential increase and a PRRSV-specific melting curve (between 83.0°C and 85°C) was obtained (Martínez et al., 2008).

Purification and sequencing were performed as well as in study A: 20 ORF7 sequences and 12 ORF5 sequences were obtained from positive samples of gilts, pigs and aborted fetuses. Multiple alignment was performed using CLUSTAL W (Larkin et al., 2007) and the phylogenetic trees were generated by the distance-based neighbor-joining method using MEGA 4 software (Kumar et al., 2008).

13.2 RESULTS

Replacement gilts immunity

Upon arrival all the 3 groups of gilts showed only about 50% of seroprevalence, with low average s/p values (Figures 8 and 9). Moreover, the first group of gilts showed only 52% of seropositive animals also at the end of the quarantine period. However, in the finishing and stimulation units both data increased, up to 100% of seropositive animals (Figure 8), even if with different trends of antibodies titers between group 1 and 2 on one hand, in which the highest mean of s/p values was obtained after the quarantine period, and group 3 on the other hand, where the peak in antibodies titers was observed in the finishing unit (Figure 9).

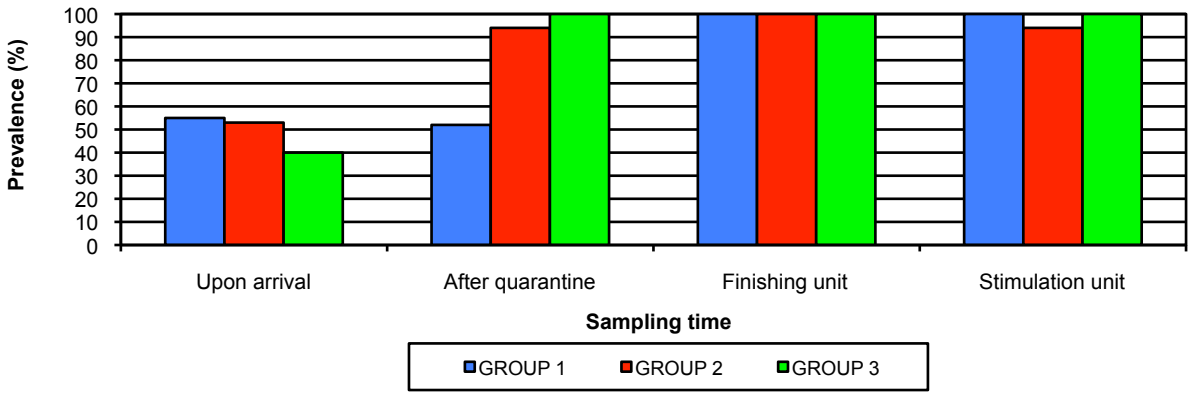

Figure 8 *Seroprevalence of the 3 groups of replacement gilts according to sampling time*

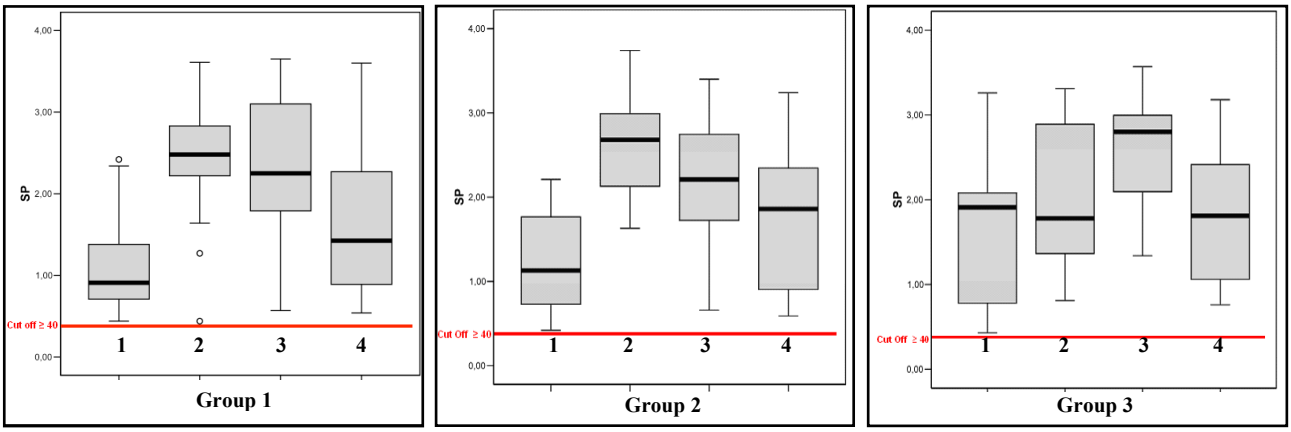

Figure 9 *Boxplots of s/p values of the 3 groups of replacement gilts upon arrival (1), after quarantine (2), in finishing unit (3) and in stimulation/fecundation unit (4)*

Replacement gilts infection

After confirming the presence of PRRSV in fetuses aborted during the outbreak preceding this study, real-time RT-PCR was used also to detect viral RNA in pools obtained from the replacement gilts upon their arrival into the farm. One hundred per cent of tested pools resulted positive, so that real-time PCR revealed that gilts were viremic before their entry into the quarantine, opposite to what the seedstock farmer had declared (Figure 10). Moreover, analysis of the Ct values has demonstrated high titers of PRRSV in serum of gilts one day after their arrival (Figure 11).

After the quarantine period gilts still resulted positive to PRRSV, from 65% of group 1 up to 100% of group 2. Although prevalence of positive animals tended to decrease over time and Ct values to increase, gilts of group 2 showed 59% of positive samples even upon their introduction into the stimulation/fecundation unit (Figure 10).

On the contrary, gilts of group 3 showed the preferable trend of both prevalence and average Ct values, with no positive animals and no detectable titers of virus in stimulation/fecundation unit.

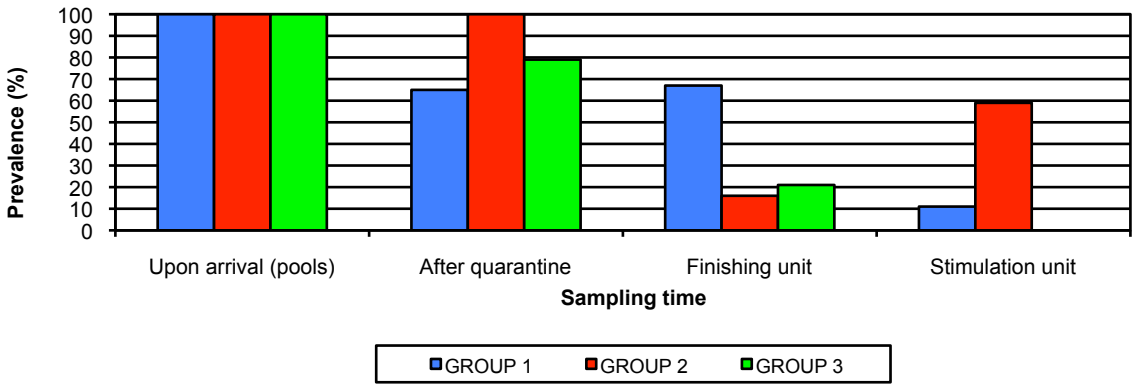

Figure 10 *Prevalence of PRRSV-positive gilts according to group of gilts and sampling time*

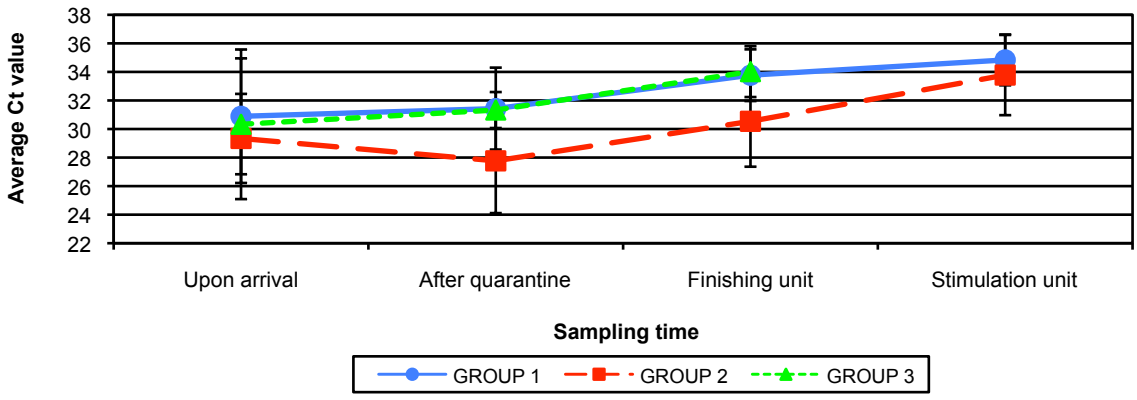

Figure 11 *Average Ct values of the 3 groups of replacement gilts according to sampling time*

Cross-sectional surveys

The results of the 2 cross-sectional surveys carried out in the nursery and growing pigs were 3/10 (30%) positive pools in April 2009 and 6/8 (75%) positive pools in December 2009.

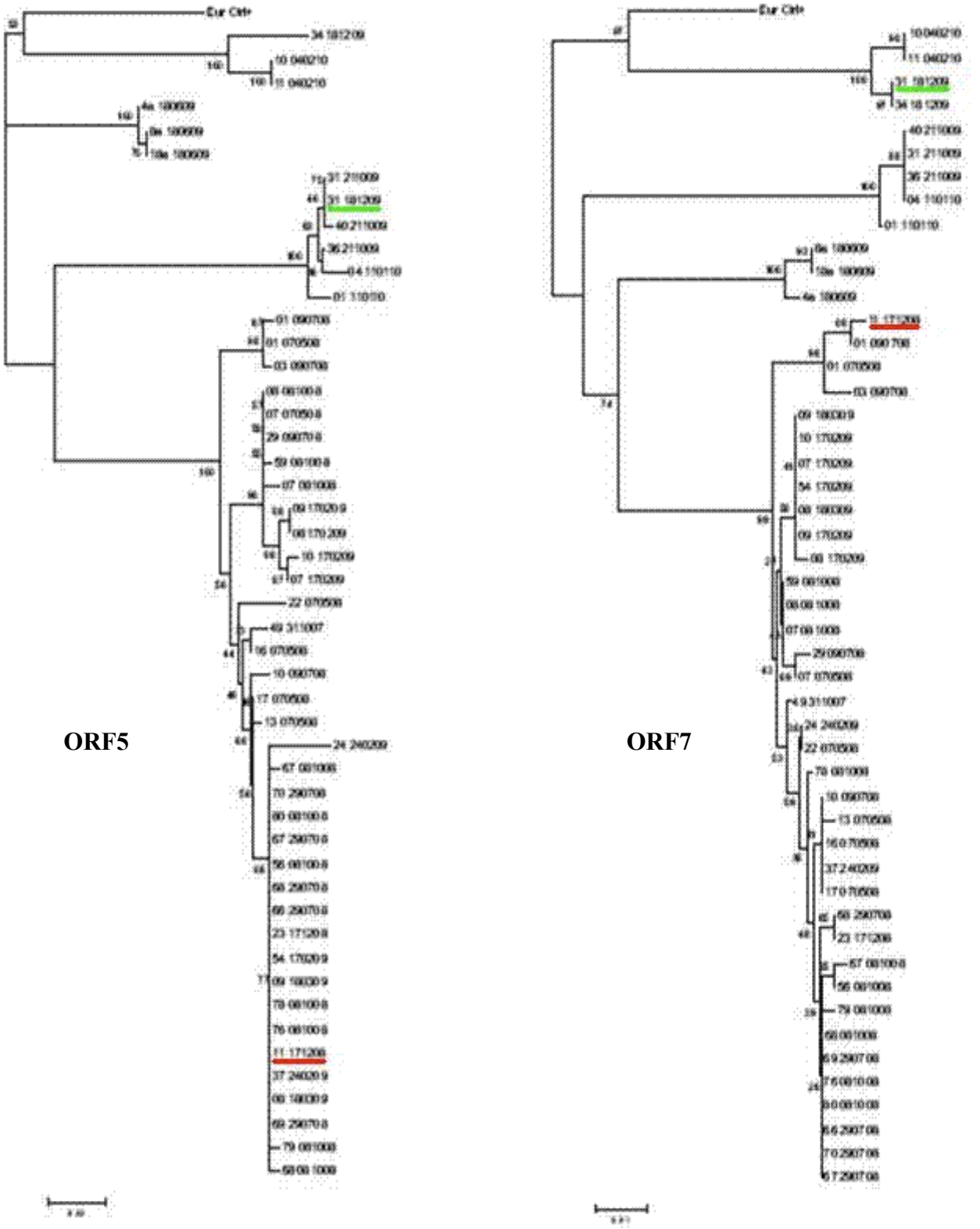

Figure 12 *Phylogenetic trees of ORF5 and ORF7 sequences*

Phylogenetic analysis

The phylogenetic analysis of ORF7 sequences demonstrated the presence of a unique endemic variant in the farm B during the whole duration of the study (Figure 7 pag. 61). Sequences obtained from the aborted fetuses revealed that abortions were caused by this endemic variant, although the replacement gilts tested upon their arrival showed to introduce at least 2 new variants of PRRSV, one of which belonged to the same cluster of the endemic isolate of the farm A. At the end of the quarantine period it was not detected the 2 viruses introduced by gilts, but only the endemic variant of the farm. Moreover, the outbreak of abortions occurred in farm A in February 2010 were caused by the endemic variant of farm B.

Finally, 2 isolates (11_171208 and 31_181209) belonged to different clusters when ORF5 and ORF7 sequences were compared by phylogenetic analysis (Figure 12).

13.3 DISCUSSION

After a severe PRRSV outbreak with scattered abortions occurred over few months, we decided to carry out this study to understand the source and the control of the infection. Abortions involved pluriparous sows in the gestation unit close to the isolation boxes for the quarantine of the replacement gilts. The most plausible hypothesis was that abortions were caused by the incoming of a new PRRSV variant with the replacement gilts, even if gilts were guaranteed to be PRRSV-free and immunocompetent on the basis of the results of tests carried out on their dams.

The analysis of the serological status of all the 3 groups of gilts upon their arrival into the farm has revealed that the maternal immunity in gilts was poor or absent. This is a symptom of a break in the herd stability of the seedstock source, which is confirmed by the detection of viremic animals at only 3 weeks of age. Therefore, introduction of viremic replacement animals imposes an isolated, enough long and well-managed quarantine period.

The phylogenetic analysis of the sequences obtained from gilts entering and leaving the quarantine has demonstrated that:

1. more PRRSV variants were introduced with replacement gilts;
2. two months of isolation are an enough time for viral clearance;
3. acclimatization of gilts started already during the quarantine period, even without the use of sources of virus, such as viremic pigs, fetuses or tissues.

Actually, the PRRSV variant detected in aborted fetuses was never detected in incoming gilts, while it belonged to the endemic variant of the farm. Nevertheless, it is likely that this endemic variant was introduced by previous groups of gilts that it was not possible to test.

Using a real-time RT-PCR allowed an increase in the sensitivity of the assay and a semi-quantitative evaluation of the viremia in the animals. In fact, the virological status of the replacement gilts was evaluated both inter- and intra-group during the whole isolation/acclimatization programme through the analysis of the Ct values. All the groups showed an increasing trend in the Ct values referable to the normal evolution of PRRSV infection. The group 2, that was the one with the lowest Ct values, showed also the major health and managerial problems.

Purchasing young replacement gilts (3 weeks of age) allows a long period of acclimatization. However, it results difficult to standardize an acclimatization programme when replacements are not homogenous for virological and serological status: only one of the groups tested in this study showed no viremia upon the introduction into the stimulation unit. Although PRRSV variants introduced with replacement gilts have never been detected in the cross-sectional surveys of the farm, it is unanswerable that incoming of more different strains is a substantial obstacle for the achieving of the herd stability.

Therefore, sensitive diagnostic assays and phylogenetic analysis of the sequences is essential tools to monitor the viral population of the farm and detect the incoming of new variants. Moreover, sequencing of both ORF5 and ORF7 permits to obtain more information about the circulating variants and their evolution over time, also considering the possible recombination among different strains and its consequences on the control strategies of the infection.

In conclusion, establishing an off-site holding facility for housing the replacement gilts until the proper time for introduction into the breeding herd is at hand would be the preferable solution for this farm. Thus, gilts should be tested to assess changes in serostatus and carry out virus detection on pooled samples: only animals with evidence of specific immunity and negative RT-PCR results should be brought into the herd. This would allow the farmer to maintain its genetic programme and a viable working relationship with the chosen seedstock source, even if the latter is PRRS-positive.

However, at present the solution chosen by the farmer is to purchase 4-month-old replacements and to hold them in an in-site isolated facility until their introduction into the stimulation unit.

13.4 CONCLUSIONS

Applying on field of assays of molecular biology and phylogenetic analysis is a valid tool for farmers and veterinarians in order to understand and face the challenges that PRRSV infection offers in the breeding herd. However, as demonstrated in this study, these tools remain inapplicable without a good management and strict biosecurity measures.

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APPENDIX 1

14 STUDY C

Experimental injection of gilts with PRRSV for validation of virological methods

Introduction

Genetic variability of PRRSV represents a continuous challenge for diagnosticians and it is critical to improve the currently available technology associated with diagnostic assays. Moreover, the validation and use of a quantitative real-time RT-PCR may have important implications in the evaluation of the acclimatization programmes of seronegative gilts.

The aims of this research was the validation of the available virological methods, based on a comparative evaluation of different PCR reactions on samples collected during an experimental infection trial. Furthermore, it was evaluated the possibility of submitting samples that can be drawn more easily than the anterior vena cava blood.

My operative unit was involved in planning and carrying out of the experimental injection and in collecting samples.

Materials and methods

The experimental injection was carried out in the farm of study A in January 2010. Twelve 3-week-old PRRSV-free replacement gilts were experimentally infected with the same PRRSV isolate circulating in the farm since May 2009 (Figure 7 pag. 61). After infection, gilts were hold in a box separated from other animals.

Prior to the arrival of the replacement animals, blood samples were collected from 10 nursery pigs and tested by real-time RT-PCR. Two out of them were selected as a source of field virus for inoculum preparation: blood samples were drawn from these piglets from the anterior vena cava by venipuncture. Serum was harvested by centrifugation for 10 minutes at 850 *g*, added with gentamicin to prevent growth of bacterial contaminations and stored for one day at +4°C. On the basis of a quantitative real-time RT-PCR, the nursery pig sera used to inoculate the study gilts were estimated to have PRRSV titers of $3x10^7$ copies/ml.

Upon arrival, the 12 gilts received both intramuscular and intranasal injections of 2 ml of PRRSV inoculum each. Samples were collected on days 0–1–3–7–10–14–21–28–35–42–49–76 post exposure. Samples included blood from anterior vena cava, nasal swabs, oral swabs and swabs soaked with

blood from the ear vein. Swabs were immersed in PBS and all the samples were stored at -20°C until the analysis. Samples were tested by classical and real-time RT-PCR with SYBR Green and TaqMan probes.

As a collateral remark, 2 out of the 12 experimentally infected gilts were euthanized on days 14 and 28 post exposure, in order to collect samples of blood/internal organs suitable to be used for the virological and serological proficiency tests yearly organized by the Istituto Zooprofilattico Sperimentale delle Venezie.

Results and discussion

The study is still in progress. The preliminary results can be summarized as follows.

- The first finding, that is very relevant for the pratical diagnostics, consists in the diagnostic failure of the real-time RT-PCR protocols with TaqMan probes that were initially selected for the present study. This is the consequence of 5 mismatches in the probe-binding site of the PRRSV isolate used for the experimental infection in comparison with the previous variant circulating in the farm, on which probes were designed. This notwithstanding already known finding has to be carefully taken into account, because it involved also the commercially available real-time RT-PCR kit.
- The second finding consists in the possibility of utilizing for diagnostic purposes also samples of peripherical blood (from ear vein) that can be collected more easily and at the same time in a less stressful way for the animal undergoing the sample collection.
- The third finding is given by the comparison of the analytical and diagnostic sensitivity trials of the different PCR protocols examined. For sure there is a gap affecting the analytical sensitivity of the two real-time protocols if compared with the classical RT-PCR, that can be partially explained by the different working volumes of the extracted RNA (10 times higher for the classical RT-PCR). However, at diagnostic level this difference is clearer, giving false negative results in the initial and in the late phases of viremia.
- The fourth finding consists in the need of putting under control the whole PCR reaction process by including an internal (universal) control, that in this study allowed to detect some false negative PCR results due to inhibiting substances.

Conclusions

This study permitted to evaluate the dynamics of PRRSV infection and the advantages and disadvantages of the available diagnostic assays. Moreover, new diagnostic approaches were studied, with major attention to the animal wellness.

APPENDIX 2

15 STUDY D

Effectiveness of sow vaccination with a commercially available killed PCV2 vaccine in a PCV2-positive PCVAD-free herd

Introduction

Evidence from field surveys suggests that co-infections of pigs with PRRSV and PCV2 in the field are common and that each virus promotes the replication of the other one.

The benefit of sows PCV2 vaccination in herds where clinical cases of PCVD are reported has been well documented (1). The effectiveness of such a vaccination is not so well known in PCV2-positive herds with neither clinical signs nor PCV2 anatomo-histopathologic lesions found in piglets.

The objective of this study was to assess the efficacy of sow vaccination in reducing mortality and improving growth of the offspring in a PCV2-positive but PMWS-negative herd.

Materials and methods

The study was carried out in a 170 sows farrow-to-finish farm using a 3-week farrowing batch management (23-25 sows per batch). PCV2 presence was confirmed from previous serological monitoring but no clinical signs evocative of PCVD could be noticed.

Six batches of sows were included: groups 1, 3 and 5 were vaccinated with 2 ml IM of a commercially available killed vaccine (CIRCOVAC®, Merial) 40-50 days and 20 days before farrowing; groups 2, 4 and 6 were not vaccinated and used as controls (1142 piglets overall). Mortality in piglets for the 6 groups was recorded. A sample of 50 animals (25 males and 25 females) each group was ear-tagged and weighted at weaning, around 68, 120 days of age and a week before slaughtering. Ten pigs randomly selected from the progeny of groups 1 and 2 were ear-tagged and serum sampled weekly. The sera were tested for PCV2 and antibodies ELISA.

Results and discussion

The PCV2 vaccination of sows in this PCV2-positive but PCVD-free herd induced a significant improvement in mortality rate of piglets (Figure 1). The seroprofiles obtained for the pigs belonging to the two experimental groups showed a better transfer of maternal immunity to the piglets of the

vaccinated dams (Figure 2). However, no seroconversion could be seen in any of the two groups up to 13 weeks of age. This may be due to a rather low virus pressure or to the fact that pigs were exposed to PCV2 later in their life. Increases in average daily gain (ADG) were observed, i.e. 0.110 kg in growing-finishing and 0.500 kg in finishing-slaughtering (Figure 3). Although such improvements are rather impressive, they did not result statistically significant, because of the very low number of weighed pigs (50 in each group).

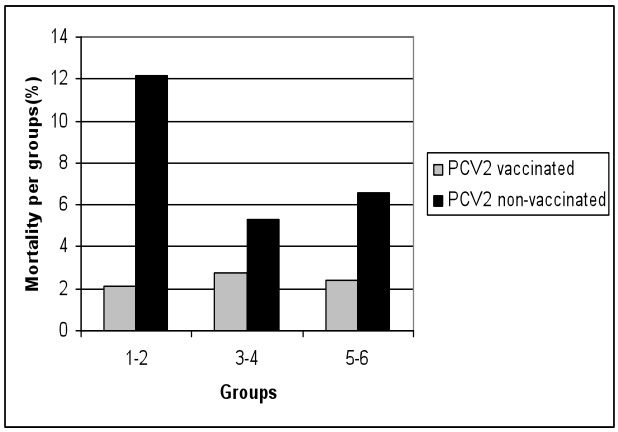

Figure 1 *Mortality rate per group*

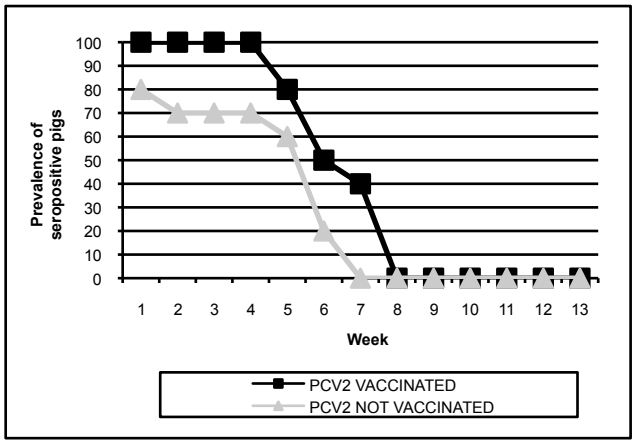

Figure 2 *Seroprevalence of piglets from vaccinated and not vaccinated dams*

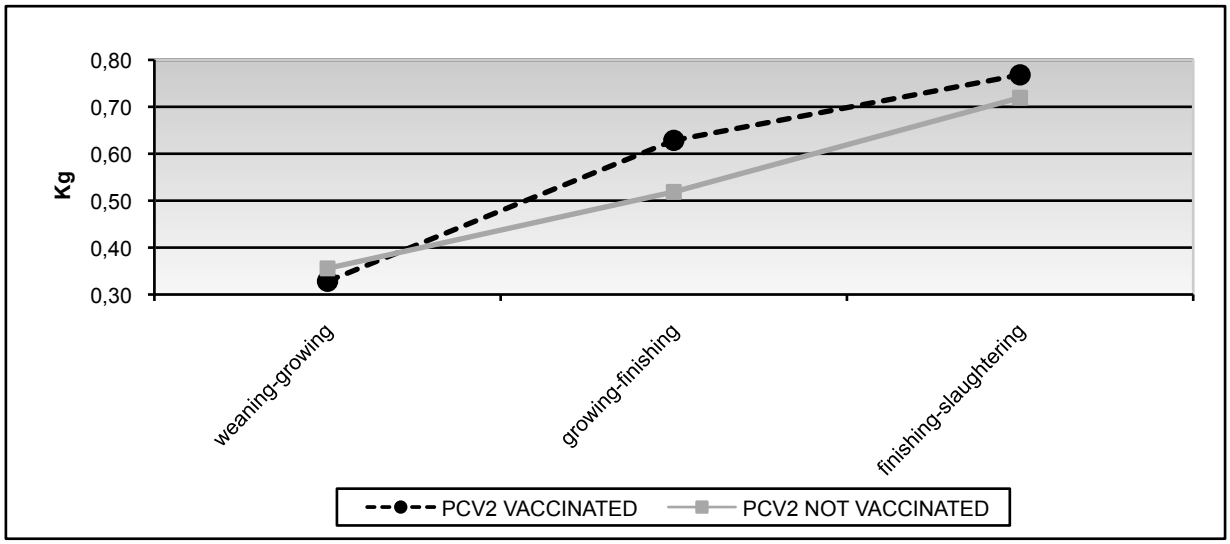

Figure 3 *Average daily gain of pigs from vaccinated and not vaccinated dams*

Conclusions

In summary, PCV2 vaccination in sows in a PCV2-positive PCVAD-free herd was effective at significantly reducing mortality rate of piglets, inducing a firm and lasting maternal immunity in the whole litter and increasing the ADG especially in growing and finishing hogs.

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