PhD thesis

Transmission of *Porcine reproductive and respiratory syndrome virus* (PRRSV): assessment of the reproduction rate (R) in different conditions.

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Declara

Que la memòria titulada: "Transmission of *Porcine reproductive and respiratory syndrome virus* (PRRSV): *assessment of the basic reproduction rate in different conditions*", presentada per l'Emanuela Pileri per a l'obtenció del grau de Doctor en Veterinària, s'ha realitzat sota la meva direcció en el programa de doctorat de Medicina i Sanitat Animals, del Departament de Sanitat i d'Anatomia Animals, opció Sanitat Animal.

I per a que consti als efectes oportuns, signo la present declaració en Bellaterra, 16 de Setembre de 2015:

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SUMMARY

The available scientific literature on the quantification of *Porcine reproductive and respiratory syndrome virus* (PRRSV) transmission is very limited. This information is crucial to foresee the potential of vaccination as a mean of control of the infection at a population level. In view of the scarcity of data about the reproduction rate (R) of PRRSV, the aim of the present thesis was to determine this value under field and experimental conditions using weaners/growers as a model. For achieving that purpose the thesis was organized in three studies.

Study 1 was directed to preliminary assess R of PRRSV in two endemically infected farrow-to-finish farms designated as F1 and F2. In both cases, a whole batch of weaners was followed serologically from weaning to slaughtering age. Based on serological data, the average time needed to reach 50% of infected pigs was 7 and 3.5 weeks in F1 and F2, respectively. R value for PRRSV transmission was estimated to be 3.53 ($CI_{95\%}$: 2.89-4.18) and 7.11 ($CI_{95\%}$: 3.55-10.68) for F1 and F2, respectively.

The second study was directed to assess the efficacy of the vaccination in an experimental study of transmission by contact in groups. Ninety-eight three-week-old piglets were selected for the study. Animals were divided in two groups: V and NV. Pigs in group V were vaccinated with a commercial live attenuated and adjuvanted PRRSV genotype 1 vaccine, while piglets in group NV were kept as unvaccinated controls. Five weeks later, 14 NV piglets (from now on, designated as seeders (S)) were inoculated intranasally (IN) with PRRSV genotype 1 isolate 3267. Meanwhile, the remaining V and NV pigs were distributed separately in groups of five pigs in pens of different rooms, resulting 8 V groups and 6 replicas of NV animals. Two days after the inoculation of S one inoculated pig was introduced in each V or NV pen. Animals were left in contact for 21 days being sampled (blood, nasal and fecal swabs) at days 0, 3, 7, 10, 14, 17 and 21 after contact with S. After that time, all NV developed viremia while only 52.5% of V pigs were detected as viremic at any given moment. The calculation of the average 50% survival period showed significant differences between groups, being necessary only 7 days of contact with a S to infect 50% of the NV pigs

while 21 days of contact were needed for infecting 50% of V pigs ($p<0.05$). The R value was significantly different between groups as well: 2.78 ($CI_{95\%}$: 2.13-3.43) and 0.53 ($CI_{95\%}$: 0.19-0.76) for NV and for V pigs, respectively ($p<0.05$). Moreover, vaccination significantly reduced the biological parameters related to transmission, such as the duration of viremia and shedding of virus by the nasal and fecal routes. Globally, the results of study 2 suggested that the use of mass vaccination in piglets have the potential to stop, or at least for significantly slowing, transmission of PRRSV in piglets.

Since the design of the study number 2 was based in a model of transmission in groups, the next step was to assess the transmission in one-to-one experiments (study 3). In this way, it was possible estimate the vaccine efficacy in a scenario where the probability of transmission between animals was the highest possible. In this case, forty-four three-week-old piglets were initially selected. Twenty of them were vaccinated as above and 24 were kept as unvaccinated controls. Four weeks later, 18 of the unvaccinated were separated and inoculated IN with the same genotype 1 isolate as in study 2 (these inoculated pigs were designated as S again). Two days later, S pigs were mixed in a 1:1 basis with V and NV pigs, namely 1S:1V (n=12) and 1S:1NV (n=6) and were then followed for 21 days. Once a V pig was detected as viremic as a result of the contact with a S pig, the infected V (V_{inf}) was transferred (in less than 24 h) to a new pen where it was left in contact with a new V (reserved from the initial stock, n=8, from now V_c) for at least 14 days. All NV and V pigs became viremic after contacting S pigs; however, the average duration of viremia was significantly reduced from 12.5±2.7 to 5.5±4.3 days in average comparing V pigs to NV ($p<0.05$). Also, V animals needed on average 13.6±3 days in order to become infected from the S, namely one week later compared to NV (5.4±2.7 days, $p< 0.05$). Transmission from V_{inf} to V_c pigs occurred in 7/8 animals (87.5%) but the mean length of viremia as well as the area under the curve (AUC) for viral load in sera of V_c pigs were also significantly reduced (AUC: 0.98, 0.87 and 0.79 for NV, V_{inf} and V_c, respectively, $p<0.05$). The delayed transmission observed in the V_{inf} and the significant reduction of viremia in V contacts indicated that even in the worst-case scenario, vaccination was

able to reduce the global infection pressure. In addition, the results suggested that under more natural conditions, the reduction of transmission from V_{inf} to other V pigs would have been even more substantially reduced.

In conclusion, the data shown in the present thesis indicated that for genotype 1 subtype 1 PRRSV, mass vaccination of pigs can be a tool effective to stop or to significantly reduce the transmission of the virus. Therefore, if combined with herd management and biosecurity measures, vaccination can contribute to the success of control and eventually eradication plans at a farm or regional levels.

RESUMEN

La bibliografía científica disponible acerca de la cuantificación de la transmisión del *Virus del síndrome reproductivo y respiratorio porcino* (VSRRP) es escasa a pesar de que esta información resulta crucial para poder estimar el potencial de la vacunación como medio de control de la infección a escala poblacional. En vista de la escasez de datos sobre la tasa de reproducción (R) del VSRRP, el objetivo de la presente tesis fue determinar este parámetro tanto en condiciones de campo como experimentalmente, usando como modelo cerdos destetados y de engorde. Para esta finalidad, la tesis se organizó en 3 estudios.

El primer estudio tuvo como objetivo estimar de forma preliminar el valor de R en dos granjas de ciclo cerrado (F1 y F2) infectadas de forma endémica por el VSRRP. En ambos casos, se siguió serológicamente un lote entero de lechones desde el destete hasta su envío a matadero. En base a los resultados de la serología, el tiempo medio necesario de supervivencia hasta alcanzar una incidencia acumulada del 50% fue de 7 y 3,5 semanas para F1 y F2, respectivamente. El valor de R para la transmisión del VSRRP se estimó en 3,53 ($CI_{95\%}$: 2,89-4.18) y 7,11 ($CI_{95\%}$: 3,55-10,68) para F1 y F2, respectivamente.

El segundo estudio pretendía determinar la eficacia de la vacunación en un modelo experimental de transmisión por contacto en grupos. Para este estudio se seleccionaron 98 lechones de tres semanas de edad que dividieron en dos grupos: V y NV. Los V se vacunaron con una vacuna comercial atenuada y adyuvantada del genotipo 1, mientras que los NV no recibieron ningún tratamiento manteniéndose como controles. Cinco semanas más tarde, 14 lechones NV (que se designaron con el nombre de “semillas”, S) se inocularon por vía intranasal (IN) con la cepa 3267 del VSRRP (genotipo 1). Los V y NV estantes se distribuyeron en grupos de 5 animales por corral en diferentes salas, formando así 8 y 6 réplicas de V y NV, respectivamente. Dos días después de la inoculación, en cada corral de V o NV se introdujo un animal S y se siguió al grupo durante 21 días llevándose a cabo un muestreo consistente en la toma de sangre e hisopos nasales y fecales a los 0, 3, 7, 10, 14, 17 y 21 días tras el contacto con el S. Al finalizar el estudio, todos los NV habían desarrollado viremia

mientras que sólo el 52.5% de los V se había infectado. El cálculo del tiempo medio de supervivencia mostró diferencias significativas entre los dos grupos, siendo necesarios 7 días de contacto con el S para infectar al 50% de los NV, mientras que para los V se necesitaron 21 días ($p<0,05$). También el valor de R fue significativamente diferente para los dos tratamientos: 2,78 ($CI_{95\%}$: 2,13-3,43) y 0,53 ($CI_{95\%}$: 0,19-0,76) para los NV y V, respectivamente ($p<0,05$). Además, la vacunación redujo significativamente los parámetros biológicos relacionados con la transmisión tales como la duración de la viremia y del periodo de excreción del virus por las vías nasal y fecal. En conjunto, los resultados del estudio 2 sugieren que el uso de la vacunación masiva en lechones tiene el potencial de parar, o por lo menos de ralentizar significativamente, la transmisión del VSRRP.

Considerando que el diseño del estudio 2 estaba basado en un modelo de transmisión en grupos, el paso siguiente fue estimar la transmisión del virus con experimentos de tipo uno a uno (estudio 3). De este modo era posible evaluar la eficacia vacunal en un contexto donde la posibilidad de transmisión del virus entre animales era la más alta posible. En este caso, se seleccionaron 44 lechones de tres semanas de vida, de los cuales 20 se vacunaron como en el estudio anterior mientras que 24 se dejaron como controles no vacunados. Cuatro semanas más tarde, se separaron 18 de los NV y se inocularon IN con el mismo aislado de genotipo 1 usado en el estudio 2 (de nuevo designados como semillas, S). Dos días después, cada S se reagrupó con un V o un NV formando así 12 réplicas de 1S:1V y seis réplicas de 1S:1NV. Los animales se siguieron por 21 días con muestreos periódicos cada 2-3 días. Cuando se detectaba que un V desarrollaba viremia (RT-PCR) como consecuencia del contacto con el S, el V infectado (V_{inf}) se trasladaba en menos de 24 horas a un nuevo corral en el que se le dejaba en contacto con un nuevo V no infectado, que se había reservado desde el inicio del estudio con este fin (n=8; designados como V_c). Todos los NV y V se infectaron tras el contacto con el S. No obstante, la duración media de la viremia se redujo significativamente desde 12,5±2,7 días en los NV, a 5,5±4,3 días en los V ($p<0,005$). Además, los V tardaron en infectarse una media de 13.6±3 días; es decir, necesitaron una semana más de contacto con los S respecto a lo que necesitaron los NV para infectarse (5,4±2,7 días, $p< 0,05$).

La transmisión desde los V_{inf} a los V_c se produjo en 7/8 animales (87.5%). Sin embargo, la duración media de la viremia así como el área bajo la curva (AUC) de la carga vírica en suero se redujeron significativamente en los V_c (AUC: 0.98, 0.87, 0.79 para NV, V_{inf} y V_c, respectivamente, $p<0.05$). La ralentización de la transmisión observada en los V_{inf} y la reducción significativa de la viremia en todos los V, indican que incluso en el peor escenario, la vacunación fue capaz de reducir la presión de infección global. Además, los resultados sugieren que bajo circunstancias más naturales, la reducción de la transmisión desde los V_{inf} a los otros V habría sido aún más sustancial.

En conclusión, los resultados de la presente tesis indican que para el VSRRP de genotipo 1 y subtipo 1, la vacunación masiva de los animales puede ser una herramienta efectiva para parar o disminuir significativamente la transmisión. Por tanto, si se combina con el manejo de los animales y las medidas de bioseguridad, la vacunación puede contribuir al éxito de los programas de control y eventualmente erradicación de la infección en la granja o a escala regional.

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PPRS) has become one of the most important diseases of intensive pig production worldwide. The economic impact of PRRS in breeding and farrowing units is caused mostly by a reduction in the number of weaned pigs and by an impairment of the farrowing rates. In growing-finishing pigs, mortality may increase and secondary infections become more prevalent leading to slower growth rates, non-uniform performance, persistent respiratory disease and increased antimicrobial usage.

In 2005, Neumann *et al.* (2005) calculated the economic impact of PRRS in the US swine industry using the productivity data of 10 different farms (including farrow-to-finish, farrow-to-weaning, grower-finisher and breeding herds) and the PRRSV prevalence in USA assessed from the National Animal Health Monitoring System (NAHMS). Results indicated that most of the PRRS-related costs during an outbreak were attributable to the impact of the disease in growing pigs, while only 12% of the economic impact corresponded to reproductive losses. In global, the total annual cost related to PRRS outbreaks in USA in 2005 was estimated in $ 560 million, which comprised $ 67 million for the breeding-farrowing phase, $ 201 million for the nursery phase and $ 292 million for the grower-finisher phase of production. More recently, Holtkamp *et al.* (2013) estimated anew the total annual cost of PRRS in the USA including also indirect costs associated to PRRS outbreaks (medications, vaccination, biosecurity etc.). In this case, data were collected from 80 farms (with different production systems), from 2005 to 2010. Considering the productivity losses exclusively, the total annual cost of PRRS outbreaks in USA was estimated to be $ 663 million/year, representing a 10% increase compared to Neumann *et al.* (2005). Moreover, the relative proportion of losses due to reproductive disorders increased from 12% in 2005 to 45% in 2010. These differences on the estimated costs in a 5-years period could be due to changes on the incidence and severity of PRRS outbreaks as well as changes in pork production strategies and in the methods used for controlling the disease. Nevertheless, reliable data to support or to refute these speculations are not available. In Europe, the economic impact of PRRS outbreaks was

also estimated by Nieuwenhuis *et al.* (2012) taking into account the productivity data of nine Dutch sow herds (from 250 to 1,200 sows per farm) and the associated control costs. Average losses were estimated in 126 €/sow, slightly more than the $ 121/sow described by Neumann *et al.* (2005).

Regarding the endemic disease, losses are difficult to evaluate since the infection is often subclinical and most of the negative impact, especially in weaning and finishing pigs, is due to secondary infections, to a reduction in the growing rate and to control measures after the outbreaks. Therefore, Nieuwenhuis *et al.* (2012) indicated that annual financial losses during the endemic phase of PRRS in the studied Dutch herds ranged between 2.5 € to 109.5 €/sow depending on the control strategy.

With the objective of reducing the negative impact on production and the economic effects of the disease, substantial efforts have been made to control and eventually eradicate PRRS. Nevertheless, the high genetic diversity of PRRS virus (PRRSV), the limited cross-protection between strains and the possibility of reintroduction of the virus to the farm, implicate that PRRS control is yet a challenge. Unfortunately, PRRS continues being one of the most devastating pig diseases in all pig-producing countries.

1.1 History of PRRS

In 1987 outbreaks of a previously unrecognized disease characterized by severe reproductive losses, respiratory disease, reduction in the growth rate and increased mortalities were reported in the United States (Hill, 1990; Keffaber, 1989; Loula, 1991). Initially the disease was named "Mystery Swine Disease" (MSD) since the etiological agent was unknown and this term became common usage in the United States (Hill, 1990). Shortly thereafter a similar disease to MSD was observed in Europe (Wensvoort *et al.,* 1991) and in 1990 more than 3,000 outbreaks occurred in Germany (Lindhaus, 1991; OIE, 1992). In the following four years, the disease spread to almost all countries of Europe. In Asia, the first outbreaks occurred in Japan in 1988 (Hirose *et al.,* 1995).

At first, several agents were proposed as the cause of MSD: encephalomyocarditis virus, classical swine fever virus, porcine enterovirus, porcine parvovirus, etc. The etiology of the disease was discovered in 1991 in the Netherlands when a new virus was isolated in porcine alveolar macrophage cultures (Wensvoort *et al.*, 1991) and the Koch's postulates were fulfilled (Terpstra et *al.*, 1991; Wensvoort *et al.*, 1991). This first viral isolate was designated as Lelystad virus (LV) and became the prototype of this new virus species. In the same year, the causative agent of MSD was also isolated in the United States (Benfield et *al.*, 1992; Collins *et al.*, 1992) and the North American prototype was designated as VR-2332 virus. The term "porcine reproductive and respiratory syndrome virus" was introduced to design the new agent during the first international congress on the disease in 1992.

Early genetic analysis showed that LV and VR-2332 belonged to the same species, but the nucleotide homology between them was only around the 55-70% (Meulenberg *et al.*,1997; Nelson *et al.*,1993).Therefore, European and American isolates were classified as distinct genotypes (Meng *et al.*,1995) now designated as type 1 and type 2, respectively (King *et al.*, 2012). Nowadays, both genotypes of PRRSV share worldwide distributions, with type 1 being predominant in Europe and type 2 being predominant in North America and Asia.

Isolates belonging to the same genotype have a great genetic diversity, but the largest heterogeneity is observed among genotype 1 strains, which are actually classified in 4 different subtypes: a pan-European subtype 1 mainly distributed in Western and Central Europe, but also in North America and Asia, and the Eastern European and Russian subtypes 2, 3 and 4 (Stadejek *et al.*, 2006; Stadejek *et al.*, 2008). Phylogenetic analysis places the most recent common ancestor for type 1 and type 2 PRRSV at least 100 years back in time from the first appearance of the disease in USA, Europe and Asia, suggesting that they evolved separately prior to their emergence as clinical entities (Stadejek, 2011). The great genetic diversity in lineages from Eastern Europe and the apparent existence of a common ancestor of the genotype 1 PRRSV strains between 1946 and 1967, suggest that PRRSV may have emerged in the former Soviet Union. After the Second World War, especially after the breakdown of the iron

curtain, the ancestor of the subtype 1 of PRRSV could have found its way to Central and Western Europe. Moreover, extensive crossbreeding programs of Western European with local Eastern European, Asian or Caucasian breeds along with the construction of big pig farms, created an environment allowing PRRSV to emerge and spread in Europe. Also Asian type 1 PRRSV strains are closely related to Western European ones. It is thought that they were dispersed in Asia with imported breeding pigs or semen before the laboratory tools to diagnose PRRS became widely available. Regarding type 2 PRRSV, its diversity resembles that of the subtype 1 PRRSV in Western Europe but, at the same time, Asian and North American type 2 PRRSV strains are closely related (Stadejek, 2011). Thus, it is difficult to determine where the genotype 2 originated. Nauwynck *et al.* (2012) suggested that the virus entered in North America by the importing of minipigs.

1.2 Clinical signs of PRRS

Clinical presentation of PRRS varies greatly between herds, being reproductive disorders and respiratory distress the typical manifestations in sows and weaning-finishing pigs, respectively. Variations in the course of the infection in the herd can be explained by different virulences of PRRSV isolates, the age of the pigs at the time of infection, the reproduction stage of sows, the coexistence of other pathogens and the level of immunity in the herd, among others things.

Typically, a PRRS outbreak begins in one or more stages of production and quickly spreads to all production stages. This initial phase lasts 2 or more weeks and is characterized by anorexia, fever and lethargy in animals of different ages. In breeding herds is relatively common to see that sows stop eating for 1-2 days. This anorexia is seen moving along the sows' herd. Then, the reproductive outbreak starts and may last for 1-5 months depending on the epidemiological circumstances (Done et *al.*, 1996; Nodelijk, 2002; Stevenson *et al.*, 1993).

Reproductive disorders include abortion, premature birth, late term birth, increased incidence of fresh or autolytic stillborns, foetal death with or without mummification, and weak-born piglets that die shortly after birth (Rossow *et al.*, 1998). Clinical

manifestations in sows depend on their reproductive status, so the risk of transplacental infection and abortion, stillborns o weak-born piglets increase as the gestation progresses (Kranker *et al.*, 1998; Mengeling *et al.*, 1994; Prieto *et al.*, 1996a, 1996b; Prieto *et al.*, 1997a, 1997b). This increased susceptibility at the later stages of gestation can be explained by an increase of foetal macrophages permissiveness to the virus (Karniychuk and Nauwynck, 2009; Mateusen *et al.*, 2007) and also by a more efficient materno-fetal capillary exchange as gestation progresses (Prieto *et al.*, 1997a).

Some cases of severe acute disease in sows have been described as a consequence of infection with high virulent PRRSV variants. For instance, in 1996-1997 an atypical PRRS form designed as "swine abortion and mortality syndrome" (SAMS) appeared in United States. This syndrome was characterized by 5-10% of sow and gilt mortality and 10-50% of mid- or late-term abortions (Halbur and Bush, 1997). Similar outbreaks still occurs periodically every 4-6 years. A high virulent variant of PRRSV, the MN-184 strain, was isolated from atypical PRRS cases occurred in Minnesota in 2001 (Han *et al.*, 2006). In Europe, atypical PRRS outbreaks were described in Italy in 2002-2003 (Martelli *et al.*, 2003) and also in the Eastern part of Belarus in January 2007 (Karniychuk *et al.*, 2010). In Belarus, a nucleus herd of 5,000 sows experienced reproductive disorders, a high mortality rate before weaning and up to 70% of mortality in growing pigs, being the last one the most characteristic finding of this outbreak. The virus isolated from the samples collected during the outbreak was called Lena strain and resulted to be a highly pathogenic genotype 1, subtype 3 isolate (Karniychuk *et al.*, 2010). Lastly, a hypervirulent strain of PRRSV is also circulating in South-east Asia from 2006 (Zhou and Yang, 2010). Depending on the farm, mortality rates up to 20% and 70% were observed in finishing and weaning pigs, respectively. Abortion rates ≥40% and sow mortality around 10% were also commonly observed in these farms.

During the phase of late-term reproductive failure, high mortality, respiratory distress and secondary infections are observed in neonatal piglets as a consequence of the transplacental and early postnatal infections (Hopper *et al.*, 1992; Loula, 1991;

Mengeling *et al.*, 1998). Respiratory signs such tachypnea and/or dyspnea without coughing in addition to a poor growth performance and increased bacterial infections, are seen in weanling and grower pigs. In adult animals and boars the infection can be asymptomatic. Nevertheless, infected boars can shed virus in semen and show increased sperm abnormalities (Prieto *et al.*, 1996c).

Once reproductive performance and preweaning mortality return near pre-outbreak levels, endemic infection of most herds continues. In endemic farms infections are often subclinical, although regular or occasional outbreaks of typical acute PRRS can be observed in the different productive stages, depending on the availability and number of susceptible animals (Dee and Joo, 1994b).

1.3 Etiological agent

1.3.1 Taxonomy and genomic organization

PRRSV is a small, enveloped, positive-sense, single-stranded RNA virus (Benfield *et al.*, 1992), classified within the genus *Arterivirus*, family *Arteriviridae*, together with *Equine arteritis virus* (EAV), *Lactate dehydrogenase-elevating virus* (LDV), and *Simian haemorrhagic fever virus* (SHFV) (Cavanagh *et al.*,1997; King *et al.*, 2012). Members of the genus *Arterivirus* share about 55% of their nucleotide sequences and all of them share also the cell specificity for macrophages, the ability to produce persistent and asymptomatic infections and a high genetic diversity (Conzelmann *et al.*, 1993; Snijder and Meulenberg, 1998).

Genomic organization of PRRSV is similar to that of other arteriviruses. The RNA genome is approximately 15.1 kilobases in length, with at least 11 open reading frames (ORFs) flanked by 5' and 3' untranslated regions (UTRs). Downstream the 5'-UTR, the ORFs 1a and 1b comprise 80% of the genome (Meulenberg *et al.*, 1997). They are translated in two large poly-proteins (pp1a and pp1ab) which are then processed into at least 14 non-structural proteins (nsp) including 4 proteases (nsp1α, nsp1β, nsp2 and nsp4), the RNA-dependent RNA polymerase (nsp9), the helicase (nsp10) and a endoribonuclease (nsp11) (den Boon *et al.*,1995; Snijder and Meulenberg, 1998). Recently, two additional nsp namely nsp2TF and nsp2N have been discovered (Fang

et al., 2012; Li *et al.*, 2014). The viral proteins are originated by means of a ribosomial frameshift mechanism from a conserved alternative ORF that overlaps the nsp2-encoding region of ORF1a. This alternative mechanism of transcription is activated by the nsp1β of the virus. Fang *et al.* (2012) showed that when nsp2TF expression was prevented the virus exhibited a lower growth rate and a clearly reduced plaque size, although nsp2TF is not essential for replication.

Eight ORFs downstream of ORF1ab, at the 3'end of the viral genome, encode the viral structural proteins which expression is accomplished by formation of sub-genomic mRNAs (sgRNA) (Snijder and Meulenberg, 1998). The most abundant structural protein of PRRSV is the nucleocapsid protein (N), encoded by ORF7 (Dea *et al.*, 2000; Meulenberg *et al.*, 1995). It interacts with the viral RNA in the assembly of the infectious particle and it is the main responsible for the induction of antibodies during the infection (Loemba *et al.*, 1996). Due to its abundant expression and antigeniticy it is largely used as a target for immunodiagnostic assays.

The two major envelope proteins are the non-glycosylated matrix (M) protein and the glycoprotein 5 (GP5), encoded by ORFs 6 and 5, respectively. GP5 and M form heterodimers that are essential for virion formation and for the viral infectivity (Das et al., 2010; Delputte *et al.*, 2002; Wissink *et al.*, 2005). Recently, an additional small protein encoded by the ORF5, called ORF5a, has been described and appears to be involved in the replication process, as shown by the decrease of viral titers when deleted (Firth *et al.*, 2011; Johnson *et al.*, 2011).

Finally, glycoproteins 2, 3 and 4 (GP2, GP3 and GP4, encoded by ORF2a, ORF3 and ORF4, respectively) form a trimeric envelop protein complex. The presence of all three proteins, alone or through the interaction with GP5 is essential for viral infectivity (Welch *et al.*, 2004; Wissink *et al.*, 2005). Protein E that interacts with the mentioned GP2-GP3-GP4 trimer, is a small non-glycosylated protein product of ORF2b that is also important for the viral replication and for the cellular tropism of the virus (Tian *et al.*, 2012).

1.3.2 Stability of the virus in the environment and disinfection

Being an enveloped RNA virus, PRRSV results fairly labile outside the host and it is quickly inactivated by heat and drying; however, PRRSV can remain infectious for an extended period of time under specific conditions of temperature, moisture and pH. Bloemraad *et al.* (1994) demonstrated that LV was stable for several months in cell culture medium at pH 7.5 and temperatures of -70°C and -20°C. Moreover, the estimated half-life of the virus at 4°C was around 6 days, although low titers of infectious VR-2332 isolate can still be detected for at least 30 days (Benfield *et al.*, 1992).

Regarding disinfection, complete inactivation of the virus is accomplished in 1 minute using iodine (0.0075%) or quaternary ammonium compound (0.0063%) (Shirai *et al.*, 2000). Complete inactivation of PRRSV is also achieved with chlorine, although a higher disinfectant concentration (0.03%) and a longer exposure time (10 minutes) were needed (Shirai *et al.*, 2000). Similarly, 10 min. of ultraviolet light exposure completely inactivated the virus on commonly farm surface and materials (Dee *et al.*, 2011).

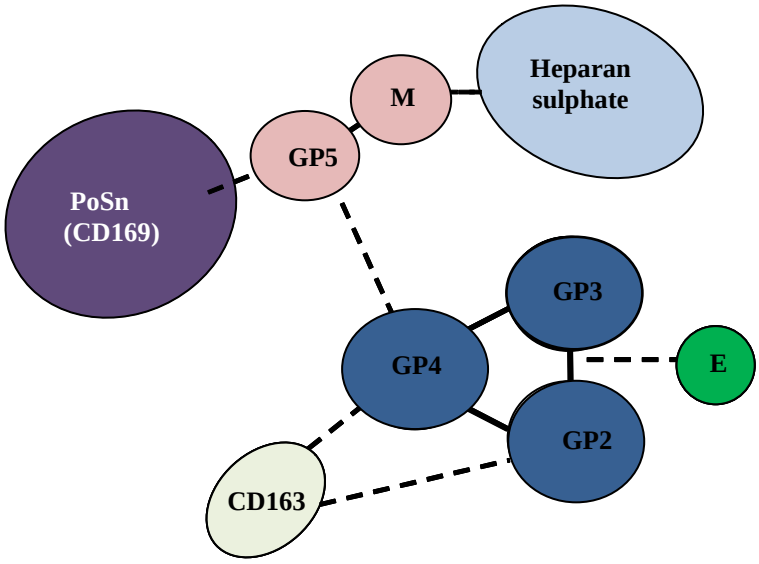
1.4 Pathogenesis of PRRS

1.4.1 Permissive cells and viral entry

PRRS virus has a very strict cellular tropism being pulmonary alveolar macrophages (PAMs) and macrophages of lymphoid tissues and placenta the predominant differentiated cells supporting virus replication in vivo (Duan *et al.*, 1997a, 1997b; Wensvoort *et al.*, 1991). Conversely, monocyte-derived cells such as peripheral blood monocytes, peritoneal macrophages and bone marrow progenitor cells are resistant to viral infection (Duan *et al.*, 1997a, 1997b). Recently it has been described that PRRSV is also able to infect and replicated in bone marrow (Bm) and monocyte-derived (Mo) dendritic cells (DC) (BmDC, MoDC) while pulmonary DCs (Charerntantanakul *et al.*, 2006; Flores-Mendoza *et al.*, 2008; Loving *et al.*, 2007; Silva-Campa *et al.*, 2010; Wang *et al.*, 2007) and plasmacytoid DCs are not permissive to the virus (Calzada-Nova *et al.*, 2011).

In vitro, PRRSV was first isolated on primary cultures of PAMs (Wensvoort *et al.*, 1991) and so far, these cells remain the only non-genetically modified porcine cells that can be used for viral propagation. On the other hand, the continuous cell lines MARC-145 and CL2621 (subclones of MA-104, an African green monkey kidney cell line) permit the complete replication cycle of several PRRSV isolates (Benfield *et al.*, 1992; Collins *et al.*, 1992; Kim *et al.*, 1993) and are routinely used for in vitro propagation of the virus and for the large scale production of PRRSV vaccine strains. Although it was thought that European and American strains replicated mostly in MAPs and continuous cell lines, respectively, it has been shown that several genotype 1 strains can replicate also in MARC-145 and that most of genotype 2 isolates growth in both MAPs and continuous cell lines. Moreover, some genotype 2 strain grow better in MAPs than in continuous cell lines (Fuentes de Abin *et al.*, 2009).

Figure 1. Interactions between PRRSV and host cell receptors during the establishment of the infection.



The tropism of PRRSV is determined by the interaction of viral surface proteins with specific membrane receptors in the permissive cells. At least 6 molecules have been described as potential cellular receptors for PRRSV, which include heparan sulphate, vimentin, CD151, CD163 (cysteine-rich scavenger receptor), porcine sialohadesin (PoSn, also named Siglec-1 or CD169) and DC-SIGN (dendritic cell-specific

intracellular adhesion molecule-3-grabbing non integrin, also known as CD209) (Zhang and Yoo, 2015). Among these, PoSn and CD163 are expressed exclusively in macrophages and have been considered as the most important putative receptors for PRRSV.

Early studies showed that the infection of susceptible cells starts with a low affinity interaction between the GP5/M heterodimer of the virus and the heparan sulphate of PAMs, followed by high affinity interaction between sialic acid residues of GP5 and the PoSn (Delputte *et al.*, 2002; Delputte and Nauwynck, 2004; Delputte *et al.*, 2004; Van Breedam *et al.*, 2010a; Van Gorp *et al.*, 2010) (Figure 1). Thus, the multimeric complex formed by GP2a, GP3 and GP4, specifically interact with the CD163 mediating the viral internalization into macrophages (Das *et al.*, 2010) through clathrin-mediated endocytosis (Nauwynck *et al.*, 1999). Once inside the endosoma, a pH drop along with the activity of the E catepsin, lead to the uncoating and release of the viral RNA into the cytoplasm and the replication process starts (Calvert *et al.*, 2007; Lee *et al.*, 2010; Misinzo *et al.*, 2008; Van Breedam *et al.*, 2010b; Van Gorp *et al.*, 2008; Van Gorp *et al.*, 2009).

Although this could be considered as the most common mechanism of PRRSV entry into permissive cells, several studies indicated that alternative processes of infection involving different cellular receptors, and consequently a great range of permissive cells, may also exist (Frydas *et al.*, 2013; Frydas *et al.*, 2015; Huang *et al.*, 2009; Shanmukhappa *et al.*, 2007). For example, PRRSV is able to infect Marc-145 cells very efficiently, but this cell line does not express PoSn. Therefore, vimentin expressed in Marc-145 has been described to interact with other cytoskeletal filaments to mediate the transport of the virus in the cytosol (Kim *et al.*, 2006). Prather *et al.* (2013) also showed that PoSn gene-knockout piglets challenged with a genotype 2 strain, developed viremia and antibody responses similar to those of control pigs, clearly demonstrating that PoSn is not required for the infection with PRRSV. Moreover, two recently studies by Frydas *et al.* (2013, 2015) showed that Lena strain and an highly pathogenic genotype 1, subtype 1 isolate are able to replicated more efficiently in nasal mucosa than LV or LV-like strains and that they have the capacity

to infect a broader range of cell types, including $CD163^{+}PoSn^{-}$ and to a lesser extent $CD163^{-}PoSn^{-}$ cells. Those cells seem to have specific functions in antigen capturing and presentation but their characterization is still lacking. These studies further confirm that: 1) the presence of PoSn is not required for the PRRSV infection; 2) alternative receptors can be used for the viral entry and, 3) PRRSV tropism may be dependent of the viral strain.

The evidence of infection in $CD163^{-}PoSn^{-}$ cells of the nasal mucosa suggests that even CD163 is dispensable for the virus entry. In this sense, CD151 was proposed as alternative receptor for PRRSV in cells lacking CD163. Shanmukhappa *et al.* (2007) demonstrated that BHK-21 cells, that are $CD163^{-}Sn^{-}$ and not susceptible for PRRSV infection, became susceptible after over-expression of CD151 by gene transfection. In addition, the PRRSV infection in MARC-145 is completely blocked by anti-CD151 antibody, indicating that this receptor is one of the key molecules facilitating or allowing PRRSV infection. As a matter of fact, CD151 was found to interact specifically with the PRRSV 3'-UTR and was proposed to be involved in the fusion between the viral envelope and the endosome as well as in the re-localization of the ribonucleoprotein complexes to promote viral replication (Shanmukhappa *et al.,* 2007). Lastly, porcine DC-SIGN is expressed on myeloid-derived DCs and macrophages present in skin, lymphoid tissues and placenta (Subramaniam *et al.*, 2014). This molecule can be involved in the infection of cells as shown in transfected BHK-21 cells (Huang *et al.*, 2009). In summary, the utilization of different receptors by PRRSV is still controversial and debatable. Nevertheless, CD163 remains the core receptor of PRRSV and the main determinant on susceptibility of cells (Zhang and Yoo, 2015).

1.4.2 Replication cycle of PRRSV

Once the virus enters in susceptible cells and starts the replication process, viral antigen can be detected in the cytoplasm from 6 h post-infection and completely assembled virions can be detected in infected cells at 9 h post-infection (Pol *et al.,* 1997). Viral release takes place by lysis of infected cells and seems to be also involved in the induction of apoptosis in neighbour uninfected cells (Kim *et al.,* 2002; Lee and

Kleiboeker, 2007). Cellular necrosis and apoptosis, as well as the secretion of pro-inflammatory cytokines from PRRSV infected macrophages (i.e. IL-10 and TNF-α), have important implication on the pathogenesis of the disease (Labarque *et al.*, 2003).

Pigs can be infected by several routes of exposure, including intranasal, intramuscular, oral, sexual and transplacental. Following exposure, replication occurs primarily in local permissive macrophages of lymphoid tissues and then the virus rapidly spreads throughout the body by the lympho-hematic way. Viremia starts as early as 12 h post-infection (Rossow *et al.*, 1995) and viral load peaks in serum around 7-10 days post-infection (dpi). The duration of viremia varies depending on the PRRSV strain and on the age of the animal (Cho et *al.*, 2006a; Klinge *et al.*, 2009; Van Der Linden *et al.*, 2003a). Younger pigs generally replicate virus to higher titers and for longer time than older pigs (Klinge *et al.*, 2009; Van Der Linden *et al.*, 2003a). Several studies indicated that the period of viremia may range from few weeks (generally less than four) in older animals to up to three months in young pigs (Allende *et al.*, 2000; Díaz *et al.*, 2012; Horter *et al.*, 2002; Van Der Linden *et al.*, 2003a; Wills *et al.*, 2003). The lung and the lymphoid organs, such as tonsil, Peyer's patches, thymus and spleen (Duan *et al.*, 1997b; Lamontagne *et al.*, 2003; Lawson *et al.*, 1997; Sur *et al.*, 1996) are the tissues with the higher viral loads. The virus in lung is usually detected from 1 day post-exposure until 28 dpi (Halbur *et al.*, 1996; Sur *et al.*, 1996) although it has been described until 72 days post-exposure in young pigs (Bierk *et al.*, 2001b).

The viremic phase of the infection is followed by a period of confinement of the virus in secondary lymphoid tissues and low viral replication. PRRSV antigen could be detected by RT-PCR in serum and tonsils until 251 dpi (Wills *et al.*, 2003) and infectious virus could be isolated from oropharyngeal scrapings until 157 dpi (Wills *et al.*, 1997c). Moreover, viral genome can be present in serum and tonsils until 132 days after birth in piglets surviving congenital infection (Benfield *et al.*, 2000b). Transmission of PRRSV from congenitally infected piglets to sentinel animals was shown until 112 days after birth (Rowland *et al.*, 2003).

In a study by Horter *et al.* (2002), 51/59 pigs were confirmed to carry the virus in oropharyngeal scrapings or tonsil between 63 and 105 dpi. Moreover, 10/11 pigs

euthanized at 105 dpi, harboured infectious virus as demonstrated by viral isolation and/or swine bioassay. Infectious PRRSV was also detected by swine bioassay in 2/5 pigs at 150 dpi (Allende *et al.,* 2000). Nevertheless, the proportion of animals harbouring the virus dropped substantially from 84 dpi. This fact indicated that most of pigs clear the virus between 3 and 4 months after infection and that the presence of PRRSV in tissues for long time after the end of viremia cannot be considered as a true state of viral persistence (Allende *et al.,* 2000; Wills *et al.,* 2003).

Obviously, the mere detection of PRRSV genome in tissues of chronically infected pigs cannot be considered as an evidence of shedding of the virus or as an evidence of contagiousness. The only evidence of contagiousness of an infected pig is its ability to infect a susceptible one after a period of contact. Therefore, Bierk *et al.* (2001a) demonstrated that non-viremic sows were able to transmit the infection by direct contact to PRRSV-naïve sows at 49, 56 and 84 dpi. Likewise, non viremic grower pigs (6-7 months of age) transmitted the virus to naïve sentinels until 62 dpi (Wills *et al.,* 2002). Conversely, sows with a detectable amount of viral genome in sera and nasal secretions until 77 and 48 dpi, respectively, were infectious by contact to naïve sows only until 42 dpi (Charpin *et al.,* 2012).

Regarding the ability of chronically infected pigs to transmit the virus to susceptible animals, it is worth to note that circumstances causing stress such as farrowing, regrouping etc., might induce a reactivation of viral replication and shedding. For example, Albina *et al.* (1994) demonstrated reactivation of PRRSV shedding after corticosteroid treatment at 15 weeks after the initial seroconversion of the animal.

Mechanisms of viral persistence in the host have not been clearly identified yet. Rowland *et al.* (1999) suggested that persistence could be associated with the selection of viral subpopulations/quasispecies. It was thought that the immune system can play a role in this selection. For instance, changes in the sequence of GP5 (Allende *et al.,* 2000; Rowland *et al.,* 1999), GP4 and GP3 (Allende *et al.,* 2000) were observed in viruses isolated from chronically infected pigs. However, the effect of positive natural selection for immune evasiveness in maintaining quasispecies variation did not was demonstrated (Chang *et al.,* 2002; Goldberg *et al.,* 2003).

A possible explanation for the maintenance of PRRSV in lymphoid tissues was offered by Díaz *et al.* (2005). The authors suggested that early in the infection, the virus can induce the release of immunomodulatory cytokines, i.e. interleukin 10 (IL-10), which can inhibit the cell-mediate immune response (CMI) against the virus. As the infection progresses, the number of permissive macrophages available in the lung decreases and the CMI achieves the confinement of the virus in the lymphoid tissues. Here, the viral replication continues but gradually declines as the number of permissive macrophages decreases. Thus, the immune response is finally able to clear the infection, and this moment would correspond with the increase of interferon-γ-secreting cells (IFN-γ-SC) in blood and the development of neutralizing antibodies (NA).

1.4.3 Viral shedding

The development of viremia and the bodily distribution of susceptible macrophages lead to the shedding of PRRSV by multiple routes. In fact, the presence of the virus in nasal secretions, saliva, urine, feces, mammary gland secretions and semen is well documented in several studies (Christianson *et al.*, 1993; Christopher-Hennings *et al.*, 1995a; 1995b; Christopher-Hennings *et al.*, 2001; Kang *et al.*, 2010; Kittawornrat *et al.*, 2010; Nielsen *et al.*, 1997; Prickett *et al.*, 2008; Rossow *et al.*, 1994; 1995; Swenson *et al.*, 1994; Wagstrom *et al.*, 2001;Wills *et al.*, 1997b;Yoon *et al.*, 1993).

Regarding viral shedding by the nasal and fecal routes, in both cases it appears to be irregular or sporadic. For instance, Rossow *et al.* (1994) isolated PRRSV VR-2332 only in two faecal swabs from two different pigs at 28 dpi, whereas the same authors were not able to detect the virus in faeces of gnotobiotic pigs experimentally infected with the same PRRSV isolate (Rossow *et al.*, 1995). Similarly, the presence of virus in nasal secretions was described in 2/105 nasal swabs collected from experimentally inoculated pigs during a 28-day observational period (Rossow *et al.*, 1994) but no virus was isolated in nasal secretions from infected gnotobiotic pigs (Rossow *et al.*, 1995). Christianson *et al.* (1993) inoculated sows around 50 days of gestation with the VR-2332 isolate. The study showed that faecal swabs were positive for viral isolation on 2, 4-6, 8 and 9 dpi, whilst shedding by the nasal route was observed from 3 to 9 dpi. Conversely, Yoon *et al.* (1993) described intermittent nasal and faecal shedding until

38 dpi in experimentally inoculated piglets. Charpin *et al.* (2012) Indicated that the viral load in nasal secretions of inoculated piglets increased very rapidly, reaching a maximum at 2 dpi, and then decreased steadily until 48 dpi. No RT-PCR positive nasal swabs were detected after 49 dpi (Charpin *et al.*, 2012).

Regarding shedding in oral fluids, Wills *et al.* (1997b) isolated the virus at least once in 5/6 inoculated pigs. PRRSV was also recovered intermittently from the saliva of one pig up to 42 dpi (Wills *et al.,* 1997b). Prickett *et al.* (2008) assessed viral shedding in oral swabs as well as in pen-based oral fluids of pigs inoculated with a genotype 2 PRRSV strain for a 63-days period. Oral fluids were positive by real time RT-PCR from 3 dpi to 4-5 weeks post-inoculation, with sporadic positive samples thereafter. Moreover, viral load in serum and oral fluid samples followed a similar pattern, although oral fluids usually present a lower concentration of virus (Prickett *et al.*, 2008). Conversely, Kittawornrat *et al.* (2010) found that serum contains equal or higher concentration of virus than oral fluids for the first 14 dpi while the amount of virus was higher in oral fluids from 21 dpi onwards. In all cases, shedding in oral fluids is detected early in the course of infection (76% and 100% of qRT-PCR positive samples at 2 and 4 dpi, respectively) independently of the virus isolate used as inoculum (Kittawornrat *et al.,* 2010). These results, together with the fact that collection of oral fluids is easy and not invasive, suggest that oral fluids can be very useful for monitoring purposes. It is worth to note, that the presence of virus in oral fluids and the relative consistency of that shedding over time, have also important implications in the PRRSV transmission between animals. As explained elsewhere in the present review, the infectious dose required for the establishment of infection by the parenteral route is low. Therefore, common pig behavior, i.e., tail-biting and ear-biting, can results in exchange of saliva and blood increasing the probability of PRRSV transmission between animals.

As previously commented, PPRSV can be also detected in urine (Rossow *et al.*, 1994; Wills *et al.,* 1997b) and mammary gland secretions (Kang *et al.,* 2010; Wagstrom *et al.*, 2001). In experimentally infected sows, PRRSV was detected by RT-PCR in the first day of (Kang *et al*., 2010). Wagstrom *et al.* (2001) showed that naïve sows

inoculated late in gestation shed PRRSV in colostrum and milk but only for a limited number of days and in low concentrations, as determined by virus isolation and titration. In addition, vaccination of sows appeared to prevent shedding during subsequent lactations and the virus was not detected in any of the milk samples collected from 181 sows of 8 endemically infected herds. These results suggest that colostrum and milk can be a source of virus for the offspring but their contribution in PRRSV transmission is probably limited in endemic farms (Wagstrom *et al.,* 2001).

Regarding PRRSV shedding in semen of infected boars, viral genome was detected by PCR as early as 3 dpi and up to 92 dpi in 1/4 boars inoculated with the VR-2332 isolate (Christopher-Hennings *et al.,* 1995a). Infectious virus in semen was intermittently detected by viral isolation and/or swine bioassay from 3 until 43 dpi in boars infected experimentally, in spite of the duration of viremia that was less than 14 days (Christopher-Hennings *et al.,* 1995a; 1995b; Christopher-Hennings *et al.,* 2001; Nielsen *et al.,* 1997; Swenson *et al.,* 1994). Moreover, PRRSV was isolated from the bulbourethral gland of 1 boar at 101 dpi, suggesting that the male reproductive tract could be a possible long-term source of persistent virus and that viremia is not an adequate indicator of virus shedding in semen (Christopher-Hennings *et al.,* 1995a).

PRRSV can also be detected in aerosol samples obtained from experimentally infected pigs. Cho *et al.* (2006b) inoculated intranasally two groups of pigs with the MN-30100 and the MN-184 isolates, respectively. Then animals were anesthetized; then aerosol samples (1,000 breaths/sample) were collected on alternating days from 1 to 21 dpi and were analyzed by qRT-PCT. Results showed that a small number of pigs inoculated with PRRSV MN-30100 shed intermittently throughout the sampling period, whereas more consistent shedding was observed in a larger number of pigs inoculated with PRRSV MN-184. Although the difference in the mean concentrations of PRRSV RNA in aerosols from pigs infected with PRRSV MN-30100 or PRRSV MN-184 was not significant, the logistic regression analysis showed that inoculation with PRRSV MN-184 resulted in a significantly higher likelihood of aerosol shedding than inoculation with PRRSV MN-30100. These results supported the notion that PRRSV transmission by aerosol is dependent on the PRRSV strain involved.

1.5 Transmission of PRRSV

The minimum infectious dose of PRRSV varies depending on the route of exposure. Hermann *et al.* (2005) evaluated the infectious dose 50 (ID_{50}) for the oral and intranasal routes. Exposure of pigs to isolate VR-2332 resulted in an ID_{50} of $10^{5.3}$ and $10^{4.0}$ $TCID_{50}$ for the oral and intranasal route, respectively. The same authors found that inoculating pigs by the intramuscular route, the ID_{50} was $10^{2.2}$ $TCID_{50}$ whilst Yoon *et al.* (1999) reported that ≤10 PRRSV particles of the isolate ISU-P were enough to infect pigs parenterally. Differences in the infectivity among PRRSV isolates were also observed for other transmission routes. Cutler *et al.* (2011) calculated that the ID_{50} for the aerosol exposure to isolate MN-184 was less than 2 $TCID_{50}$. In contrast, Hermann *et al.* (2009) reported an ID_{50} of $10^{3.1}$ $TCID_{50}$ for the aerosol exposure using isolate VR-2332. Regarding the sexual transmission, Benfield *et al.*, (2000a) estimated the ID_{50} for exposure via artificial insemination to be $10^{3.3}$ $TCID_{50}$.

According to the available data (table 1) the percutaneous exposure is the route with the lowest minimum infectious dose. In the field, potential parenteral exposure could be frequent and would include standard practices such as ear notching, tail docking, teeth clipping and injection of drugs and vaccines. For example, Otake *et al.* (2002d) demonstrate that indirect transmission of PRRSV is achieved by using contaminated needles. Likewise, a study by Baker *et al.* (2012) showed that hematogenous transmission of MN-184 isolate can occur via repeated use of the same needle between pigs and that the use of needle-free injection device (NFID) can reduce, but did not fully prevent, this type of transmission. Moreover, normal pig behavior commonly results in parental exposure, i.e., bites, cuts, scrapes and/or abrasions that occur during aggressive interaction among pigs. In this sense, Bierk *et al.* (2001a) demonstrated that aggressive behavior between carrier sows and susceptible contacts may play a role in PRRSV transmission. Other behaviors that results in exchange of blood and saliva, i.e., tail-biting and ear-biting, may surely contribute to transmission of PRRSV infection.

Table 1. Maximum viral load in tissues and secretions and minimum infectious dose required for the establishment of the infection by different routes of exposure. Modified from Mateu and Díaz (2007).

Tissue or secretion	Viral load	Route of infection	Minimum infectious dose
Saliva	$10^{4,3}$ $DICT_{50}$/ml	Oral	$10^{5,3}$ $DICT_{50}$
Nasal secretions	$10^{4,3}$ $DICT_{50}$/ml	Intranasal	$10^{4,0}$ $DICT_{50}$
Blood	$10^{4,0}$ $DICT_{50}$/ml	Sexual	$10^{3,3}$ $DICT_{50}$
Semen	$10^{6,0}$ $DICT_{50}$/ml	Parenteral	$10^{2,2}$ $DICT_{50}$

Viral shedding from infected pigs in saliva, nasal secretion, urine and feces, results in environmental contamination and creates the potential for PRRSV transmission by fomites. Otake *et al.* (2002c) demonstrated that infectious virus could be isolated from boots, coveralls and also from the hands of personnel that contacted with experimentally infected pigs when standard sanitation protocols (change of boots and coveralls, washing of hands etc.) were not used. Conversely, Pirtle and Beran (1996) detected PRRSV by virus isolation in alfalfa, wood shavings, straw, plastic, boot rubber and stainless steel but only the same day of the experimental contamination of these materials. In the same study, the virus was isolated from city and well waters until day 9 post-contamination suggesting that contamination of drinking water and lagoons could serve as a source of infection. Indirect transmission between pigs could be potentially achieved by contact with mechanical vectors (flies, mosquitoes, etc.) although their role is in any case minor.

1.5.1 Transmission within herds

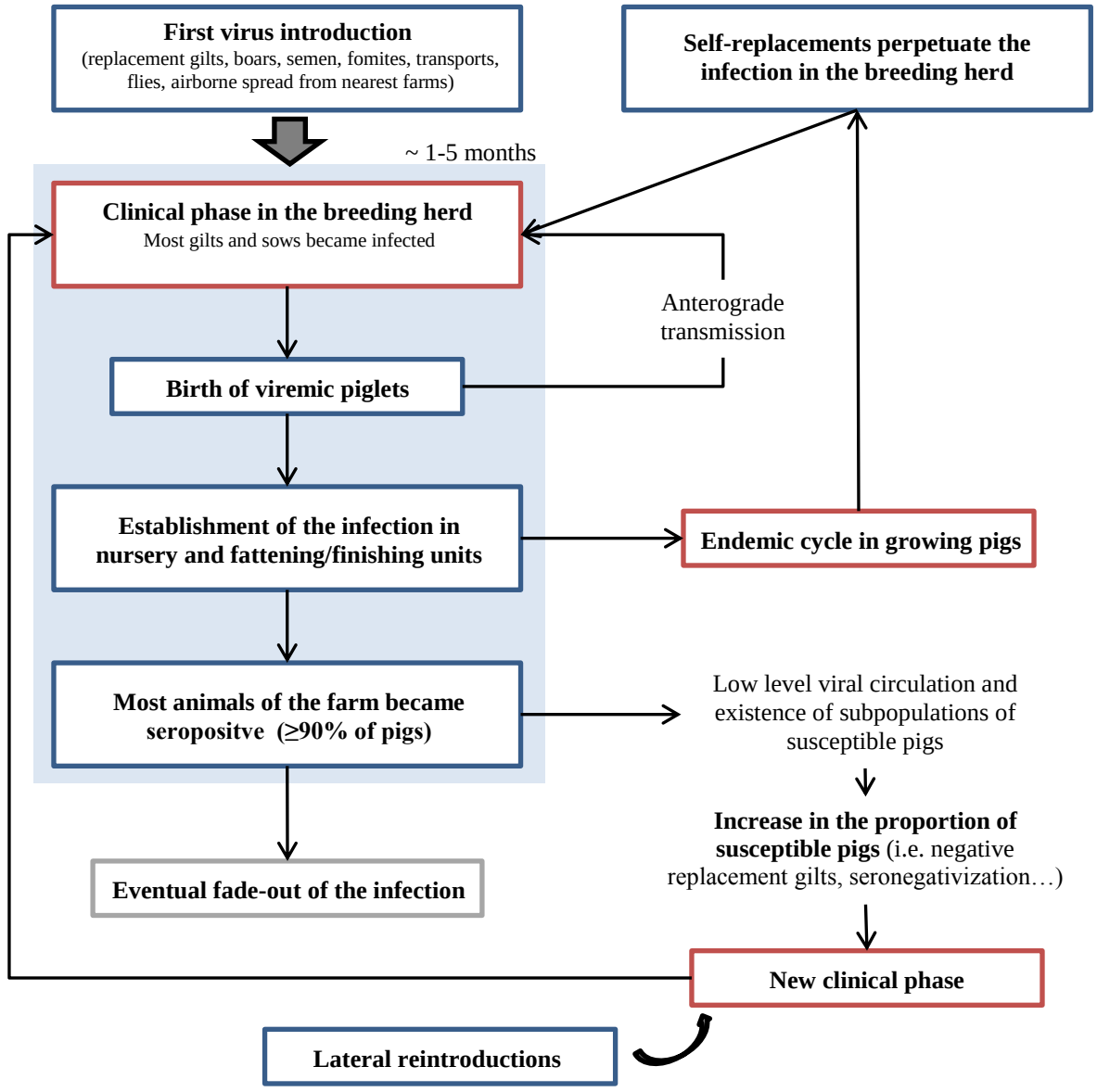
The spread of PRRSV within a farm is mainly due to the comingling of infected and susceptible animals. When PRRSV enters in an immunologically naïve herd, all ages of pigs are affected and a clinical outbreak occurs. Viral circulation begins in one or more stages of production, commonly in the breeding herd, and the virus spreads to all production stages in about 2-3 weeks (Figure 2). Sows can transmit the virus to their offspring by the transplacental route and/or by direct contact during lactation. Piglets infected congenitally or very early in life can harbor the virus for several months and

can contribute to the spread of the infection in the following productive stages. As the infection progresses, the proportion of immune pig increases and that of susceptible animals decreases. This leads to the decline phase of the epidemic in 1-5 months, depending on the herd size and the time needed to achieve a protective immunity on the majority of pigs (Done *et al.,* 1996; Nodelijk, 2002; Stevenson *et al.,* 1993). At this point, the infection can fade out or, in most of cases, becomes endemic.

The maintenance of the infection within a farm is basically due to the persistent infection in carrier animals and the continual availability of susceptible pigs. The latter can be added to the population by replacement, by birth of piglets from seronegative sows, by loss of passive immunity in young pigs, or by loss of active immunity in previously infected pigs (Nodelijk *et al.,* 2003). As a result, PRRSV can circulate in the farm for several years. For instance, a longitudinal study conducted in a Dutch breeding herd by Nodelijk *et al.* (2000) shown that seroprevalence in sows during an acute outbreak was 86–95% and that sows that initially escaped the infection, did seroconvert at a later stage, indicating the existence of subpopulations that permit a low level of viral circulation. In sows, the cycle of infection can be maintained by transmission between them but also by anterograde transmission of the virus from nurseries or finishing units (Bierk *et al.,* 2001b).

It is generally acknowledged that most infections are subclinical in chronically infected herds. For instance, Bilodeau *et al.* (1994) detected viral circulation in a farrow-to-finish farm using sentinel pigs several months after the cessation of the outbreak. The subclinical infection was also detected in a neighboring barn that ever experienced PRRSV and was situated 50 meters from the main farm. Likewise, Stevenson *et al.* (1993) monitored 6-8 week-old piglets of two farrow-to-finish farms that experienced a reproductive outbreak 2.5 years before. They found that most of the necropsied pigs were positive in lungs and spleen by viral isolation, confirming that PRRSV was circulating in nursery pigs despite both farms were clinically “healthy” for several years since the original outbreaks.

Figure 2. Common patterns of circulation of PRRSV in a farm.



1.5.1.1 Quantification of PRRSV transmission

The knowledge of the dynamics of PRRSV circulation within a herd and the quantification of the virus transmission in a pig population are key points for the development of prevention and control strategies of the infection as well as for estimating the impact of such interventions. Unfortunately, to the date only few studies are available regarding this topic. Usually, the reproduction rate (R) is used for

quantification purposes. R is defined as the average number of cases infected by one infectious case (Diekmann *et al.,* 1990) and equals to the duration of the infectious period multiplied by the transmission parameter β (effective contacts between infectious and susceptible pigs per unit of time). The higher the R value, the greater and faster is the spread in the population. Moreover, when R<1, the infection tends to fade-out with time.

Nodelijk *et al.* (2000) quantified the transmission within a herd using the serological data coming from a longitudinal study of a closed breeding farm (115 sows) that experienced a major outbreak 6 years before. The results indicated that during the first wave of the epidemic seroconverted 80% and 49% of sows and rearing pigs, respectively. Four years after the epidemic and until the end of the study none of the pigs seroconverted and all sera were negative for PRRSV, indicating the total fade-out of the virus. In the same study, the reproduction rate for sows was estimated to be 3.0 ($CI_{95\%}$: 1.5-6.0) for sows devoid of previous immunity, assuming that the infectious periods of pigs lasted 56 days and that no lifelong immunity existed after infection. Charpin *et al.* (2012) estimated an R of 2.6 ($CI_{95\%}$: 1.8-3.3) for naive sows, using an experimental model of transmission by contact between inoculated and susceptible pigs introduced at different times post-infection. In that same work, the average duration of contagiousness was estimated to be of 14.8 days, with a peak of infectivity at 9 dpi and a negligible probability of transmission after 42 dpi, although inoculated sows were positive by means RT-PCR in sera until 77 dpi. Another transmission experiment to quantify the PRRSV transmission in piglets was performed by Rose *et al.* (2015). In this case, two susceptible pigs were kept in contact for 49 days with two experimentally inoculated piglets (six replicates in total) and monitored by RT-PCR in sera. They found that the average period of contagiousness, calculated on the basis of the duration of viremia, was of 22.6 days and that R for piglets was 5.4 ($CI_{95\%}$: 2.9-9.0).

Globally, it can be concluded that R for genotype 1 PRRSV might range between 2.6 to 5.4 in naïve pigs. Compared to other common swine pathogens, such as classical

swine fever virus (R= 15; Klinkenberg *et al.*, 2002) PRRSV seems not to be that transmissible.

As previously commented, the estimation of the reproduction rate of PRRSV is also a useful tool to predict for how long the infection could circulate in a herd, especially in the case of endemically infected farms. In fact, the spontaneous extinction of a virus in a population is mainly determined by the transmissibility of the virus, the duration of the infectious period and the existence of susceptible pigs in the population. Nodelijk *et al.* (2000) estimated by using a Montecarlo simulation that the average time for PRRSV to fade-out was about 6 years in a closed herd of 115 sows whereas it was as long as 80 years in a closed herd of 230 sows. These estimations are in accordance with those of Evans *et al.* (2010), indicating that the persistence of the infection is more likely as the herd size increases and when the gilt pool is not properly isolated sows. In addition, PRRSV fade-out seems to be less likely to occur when the infection is established in the farrowing house and piglets because of the retrograde transmission from infected nurseries or finishers to the breeding herd (Evans *et al.*, 2010).

1.5.2 Transmission between farms.

The virus may reach a farm in several ways but purchase of subclinically infected pigs is considered the most common route for virus introduction (Carlsson *et al.*, 2009; Mortensen *et al.*, 2002; Thakur *et al.*, 2015). A simulation study of PRRS spread in Ontario conducted by Thakur *et al.* (2015) indicated that animal movements from one farm to another can result in outbreaks in previously negative farms. The estimated number of newly infected farms could range, on average, from 31% to 37% of the total herds in the considered area. If the simulated outbreak started in a farrowing farm, then the infection spread to a larger number of farms of different characteristics: nurseries, finishing herds, etc. Conversely, the estimated spread of PRRS from finishing herds resulted in a low number of newly infected neighboring herds. Moreover, finishing farms seem to be those with the highest risk of receiving the infection from other production stages. Thakur *et al.* (2015) also found that the estimated number of newly infected farms could reach the 42-49% of the total herds when trucks for the shipment

of animals were shared between farms. This highlights the contribution of transport vehicles in PRRSV epidemiology.

Several works indicated that trucks, trailers and other vehicles used for transporting pigs, animal products, feed, offal and contaminated equipment are a potential risk for the spread of PRRS. For example, Dee *et al.*, (2004a) demonstrated that pigs may become infected after been housed for two hours in trailers artificially contaminated with $\geq 10^3$ $TCID_{50}$/ml of the MN-30100 PRRSV isolate. In the same study, transmission of PRRSV was also observed in 3/4 trials where two PRRSV-naïve sentinel pigs were placed for two hours in a trailer previously contaminated by experimentally inoculated pigs. Other two works simulated a coordinated sequence of events that mimicked common farm worker behavior in order to assess the mechanical transmission of PRRSV by fomites (boots and containers), vehicle sanitation, transports and the movement of personnel (Dee *et al.*, 2002; Dee *et al.*, 2003). Results showed that infectious virus can be isolated from the ventral surface of transport vehicles, the truck wash floor, the floor mat of the trailers, drivers' boots and also from the surface of various types of containers commonly used in swine farms. When the study was conducted during the cold season (<0°C), infectious virus was recovered from at least one sampling point in 5/10 replicates of the trial and viral RNA was detected by RT-PCR at all sampling points in 7/10 replicates (Dee *et al.*, 2002). Conversely, a significant decrease in the number of sampling points positive by virus isolation and/or by RT-PCR was observed during warm weather (>15°C), suggesting that mechanical transmission of PRRSV is less frequent during the spring and summer seasons (Dee *et al.*, 2003). Therefore, a proper sanitation of vehicles and trucks are mandatory for preventing the spread of the disease.

Treatment of vehicles by washing at high temperature (80°C) followed by phenol disinfection and overnight drying, was effective for a complete sanitation of trailers (Dee *et al.*, 2004a). Alternatively, the use of thermo-assisted drying and decontamination (TADD) system or the glutaraldehyde fumigation had an equivalent efficacy to overnight drying for the complete trailer decontamination (Dee *et al.*, 2004b; Dee *et al.*, 2005).

The use of contaminated semen is also an important way for the introduction of PRRSV in a farm. For instance, Bøtner *et al. (*1997) demonstrated that the clinical outbreaks occurring in Danish PRRS-free breeding herds in July of 1996 were caused by a genotype 2 isolate previously unrecognized in that country. The virus was found to be 99% similar to the live vaccine used in boars since December 1995. Shedding in semen of a reverting strain was identified as the source of the infection for naïve sows. Semen imported from Germany was also identified as the origin of the introduction of PRRSV in five Swiss herds in November 2012 (Nathues *et al.,* 2014). Fortunately, the outbreaks were quickly detected and could be controlled successfully.

Proximity of infected herds has been considered a hazard resulting in increased risk of introduction of the virus by aerosol transmission (Lager *et al.,* 2002; Mortensen *et al.,* 2002; Velasova *et al.,* 2012). Nevertheless, the airborne transmission of PRRSV and its implication on the area spread of the disease appears controversial, because it seems to be dependent on the strain and on the environmental factors. Torremorell *et al.* (1997) demonstrated the airborne transmission between pigs inoculated experimentally with the strain VR-2332 and naïve pigs located at 1 meter of distance, while transmission was not observed when the MN-1b isolate was used instead. Furthermore, airborne transmission occurred at distances of 1 and 2.5 meters using the MN-30100 and the VR-2402 isolates, respectively (Trincado *et al.,* 2004b; Wills *et al.,* 1997a). When the aerosol transmission of MN-30100 and VR-2402 isolates was evaluated in field conditions, neither positive air samples (by RT-PCR) nor infection in susceptible pigs were observed at different distances between the building where infected pigs were housed and the trailer of susceptible pigs (Otake *et al.,* 2002a; Trincado *et al.,* 2004b). However, using a source population of 300 grower-finisher pigs experimentally inoculated with the MN-184 isolate, infectious virus was detected from the exhausted air of the facility up to 4.7 km from the infected herd (Dee *et al.,* 2009). Evidence of long-distance airborne dispersion of PRRSV up to 9.1 km was also demonstrated from a herd experimentally infected with the MN-184 strain but not for the MN-1182 and MN-1262 PRRSV isolates (Otake *et al.,* 2010). However, the probability of infection of sentinel pigs by the contaminated aerosols was not assessed. In the same study, the viral load in air samples decreased from 10^4 $TCID_{50}$, observed

in the proximity of the infected barn, to 10^1 $TCID_{50}$ at 9.1 km from the source population facility. This indicates that the virus dispersion and/or degradation during airborne transport are important factors influencing potential transmission. Also directional winds of low velocity, low temperatures, high relative humidity and low sunlight levels are factors favorable to the airborne spread (Dee *et al.,* 2010). Collectively, the abovementioned data indicated that airborne transmission of PRRSV is possible, but with limitations. The characteristics of the PRRSV strain and the adequate atmospheric conditions are the main elements conditioning the actual airborne transmission of PRRSV.

Other factors that can be involved in pathogen transmission between farms are the presence of biological or mechanical vectors for the virus. Mice, rats and birds, are not susceptible to PRRSV. At one point, Zimmerman *et al.* (1997) reported that PRRSV replicated in mallard ducks (*Anas platyrhynchos*) but no one have been able to confirm this (Trincado *et al.,* 2004a) so, it is considered that avian species are not involved in PRRSV epidemiology.

Besides domestic pigs, only feral swine are susceptible to PRRSV infection. Infection have been confirmed by RT-PCR in wild boars of Italy (Bonilauri *et al.,* 2006), Germany (Reiner *et al.,* 2009) and Slovakia (Vilcek *et al.,* 2015) while serological evidences have been reported in Croatia (Roic *et al.,* 2012), France (Albina *et al.,* 2000) Germany (Oslage *et al.,* 1994), and also United States (Baroch *et al.,* 2015). The detection in wild boars of PRRSV viruses similar to commercial life vaccines indicates that the virus has been probably transmitted from domestic pigs to wild boars (Reiner *et al.,* 2009; Vilcek *et al.,* 2015). Thus, the role of feral swine in PRRSV area spread could be considered of limited relevance.

Regarding the role of arthropods, Schurrer *et al.* (2005) demonstrated that houseflies can harbor the virus for up to 48 hours, although they did not support PRRSV replication. Moreover, contaminated flies were shown to be able to transmit the infection to susceptible pigs (Otake *et al.,* 2004; Otake *et al.,* 2004). Similar results were obtained for mosquitoes by Otake *et al.* (2002b), which demonstrated the PRRSV transmission from experimentally inoculated to susceptible pigs in 2/4 trials. All of

these studies suggest that mosquitoes and houseflies could contribute to the virus transmission between pigs, and that they probably can play a role also in the PRRSV spread among neighbourhood farms (Moon, 2002; Schurrer *et al.,* 2006). However, in spite of the potential significance of these data, they require a careful interpretation since the three studies reported here used a highly artificial exposure models and thus cannot mimic field conditions. Moreover, movements of houseflies between farms are limited by several factors, including the existence of ventilation systems and filters and the environmental conditions, such as temperature, relative humidity and wind direction and speed (Otake *et al.,* 2004). Therefore, the implication of arthropods in the PRRSV transmission between farms is considered to be minor or even questionable.

The risk of introduction of PRRS in countries free of the disease through importation of contaminated meat and pork products has been also evaluated. In fact, pigs may become infected after ingestion of meat samples negative by virus isolation but positive by means of RT-PCR (Magar and Larochelle, 2004; Van Der Linden *et al.*, 2003b). Nevertheless, after conventional post-slaughter handling and freezing or after traditional manufacturing of pork products, the amount of infectious PRRSV in these products is very low or even negligible (Guarino *et al.*, 2013; Van Der Linden *et al.*, 2003b). Therefore, the likelihood of importing the disease in PRRSV-free countries with imports of meat or pork products is limited but has to be taken into account (Hall and Neumann, 2013).

1.6 Factors affecting the transmission

In the following sections, the most relevant aspects concerning the factors influencing the PRRSV transmission between pigs are summarized.

1.6.1 Age of the pigs at the time of the infection

Klinge *et al.* (2009) showed that piglets of 3 weeks of age had significantly longer viremias than finishers or adult pigs, regardless of the PRRSV isolate used as inoculum. Likewise, 2-month-old pigs had significant higher viral amounts in lymph nodes, lung and tracheobronchial swabs than 6-month-old animals, independently of

the virulence of the challenge strain (Cho *et al.*, 2006a, 2006b). In addition, Thanawongnuwech *et al.* (1998) showed that pulmonary macrophages from 4-week-old pigs yielded a higher virus titer than pulmonary macrophages from 4-month-old pigs. Therefore, it can be assumed that contagiousness of younger animals is greater than those of finishers or adult pigs and that the contribution of piglets in the virus transmission is higher than this of a sow or a finishing pig.

1.6.2 Virulence of PRRSV isolates

Regarding the replication characteristics of different PRRSV strains, Cho *et al.*, (2006a) showed that pigs inoculated with the highly virulent strain MN-184 had significantly higher viral loads in sera and tonsils compared to pigs infected with the lower virulent MN-30100 isolate. Therefore, viral shedding in aerosols was longer and more consistent in pigs infected with the MN-184 isolate than in pigs infected with a low virulence strain (Cho *et al.*, 2006b). Likewise, boars infected with the MN-184 isolate showed higher viral titers in serum and shed significantly higher amounts of virus in oral fluids at 7 and 14 dpi than pigs inoculated with strains of lesser virulence (Kittawornrat *et al.*, 2010). Increased replication efficiency and higher viral titers in serum were also observed for the highly pathogenic strains HuN4 (Liu *et al.*, 2010) and Lena (Weesendorp *et al.*, 2013), although in these cases the viral shedding was not assessed. Moreover, Frydas *et al.* (2013, 2015) discovered that some highly pathogenic PRRSV strains had the capacity to infect a broader range of cells (including $CD163^{+}Sn^{-}$ and $CD163^{-}Sn^{-}$ cells) and are able to colonize and replicate more efficiently in nasal mucosa compared to conventional strains of lower virulence. Collectively, these data suggest that pigs infected with highly virulent strains could shed higher amounts of virus than pigs infected with low virulent strains. However, experiments aimed to compare the transmissibility of PRRSV strains of different virulence are still lacking.

1.6.3 Immune response against PRRSV

The immune response against the virus is surely the most important factor influencing the course of the infection and consequently the susceptibility and infectiousness of

pigs. The adaptive immune response against PRRSV is characterized by the following aspects: 1) a strong and rapid antibody response that is devoid of neutralizing antibodies (NA) for at least the first 4-5 weeks after infection and 2) the generation of a cell-mediated immune response that is initially slow and oscillating. This adaptive response suggests that the virus has the ability to subvert the immune system and, as seen later in this review, it interferes with key elements of the host innate immunity.

1.6.3.1 Innate immune response

As previously described, target cells of PRRSV are differentiated macrophages, BmDC and MoDC. All of these cells play a key role in the immune response against the virus since they are able to sense pathogens and to induce the release of cytokines (i.e. type 1 Interferons and tumor necrosis factor α (TNF-α)) that created an antiviral state in the host. Moreover, these cells are "professional" antigen presenting cells (APC), representing the bridge between the innate and the adaptive immune response. Replication of PRRSV in macrophages and DC results in the death of the infected cells and alters their functionality. For example, several studies demonstrated that PRRSV can modulate the expression of MHC I, MHC II and CD80/86 molecules in infected cells (Chang *et al.,* 2008; Gimeno *et al.*,2011; Wang *et al.,* 2007), a fact that might result in a deficient antigen presentation to T cells. Moreover, PRRSV seems to affect the expression of TLRs (Chaung *et al.,* 2010; Kuzemtseva *et al.,* 2014; Liu *et al.,* 2009; Miller *et al.,* 2009) as well as their signaling pathways and the normal cytokine release from APC.

Type I interferons (IFN) are important antiviral cytokines that play an important role in conditioning the milieu where the recognition of the antigen takes place. Previous studies have shown that PRRSV is sensitive to interferons, and recombinant porcine IFN-β can inhibit PRRSV replication and protect pigs from the infection (Albina *et al.,* 1998; Miller *et al.*, 2004). Nevertheless, infected pigs produce very low, if any, levels of IFN-α in the respiratory tract even when the virus is actively replicating in the lung (Albina *et al.,* 1998). Gimeno *et al.* (2011) also observed the lack of IFN-α release from different types of APC (PAMs, PBMC and BmDC) when infected with a panel of PRRSV isolates.

Plasmacytoid dendritic cells (pDC), do not support PRRSV replication but can release copious amounts of IFN-α after stimulation with different type 1 and type 2 PRRSV isolates (Baumann *et al.,* 2013). However, the IFN-α response elicited by TGEV or TLR9 agonists (strong inducers of type I IFN) was completely abolished in pDC exposed to a genotype 2 strain (Calzada-Nova *et al.,* 2010; Calzada-Nova *et al.,* 2011). Thus, although pDC contribute in the immune response against PRRSV, their activity seems to be dependent to a certain extent on the viral strain.

Besides type I IFN, two other important cytokines for the immunology and pathogenicity of PRRSV are TNF-α and IL-10. TNF-α is a pro-inflammatory cytokine mainly produced by macrophages, that induces an antiviral state in uninfected neighboring cells, helps the recruitment of lymphocytes to the foci of the infection and induces selective cytolysis of virus-infected cells (Huang *et al.,* 2014). Previous studies indicated that recombinant porcine TNF-α clearly reduced PRRSV replication in cell cultures (López-Fuertes *et al.,* 2000). However, PRRSV seems to have evolved anti-TNF-α strategies and contradictory results about this cytokine are present in the literature. For example, Hou *et al.,* (2012) showed that the highly pathogenic PRRSV strains HV and JX were weaker inducers of TNF-α compared to the conventional strain CH-1a. Moreover, HV and JX strains also desensitized macrophages to TLR4- and TLR3-induced TNF-α production, which might partially contribute to the pathogenesis of these isolates. Conversely, the highly pathogenic strains Lena and HuN4 are both strong inducers of TNF-α, a fact that may explain the high fever observed in pigs early after infection (Liu *et al.,* 2010; Weesendorp *et al.,* 2013). Down-regulation of the TNF-α production was also observed for other conventional strains and has been associated with nsp1a, nsp1b and nsp2 (Chen *et al.,* 2010; Darwich *et al.,* 2011; Gimeno *et al.,* 2011; Subramaniam *et al.,* 2010).

With regards to IL-10, this is a pleotropic cytokine mainly produced by macrophages and DC in response to TLR stimulation or to endogen stimuli such as apoptosis. Also B cells and various subsets of T cells, including regulatory T cells (Treg), are capable of releasing IL-10 during an infection. Interleukin 10 may acts in autocrine manner to suppress proinflammatory activity of APC, limiting the production of proinflammatory

cytokines (i.e. IL-1, IL-6, IL-12 and TNF-α) and inhibiting the MCH II and CD80/86 expression. IL-10 can also act directly on CD4 T cells, inhibiting their proliferation and reducing the release of IL-2, INF-γ, IL-4 and IL-5 by Th1 and Th2 cells. In this sense, IL-10 is a homeostatic cytokine but if the source and timing of IL-10 secretion are inappropriate (e.g. produced too early or too late during the infection) the result could be an exacerbation of the infection and substantial tissue damage (Couper *et al.*, 2008). Thus, IL-10 production can be of potential benefit to both the host, by limiting injury, and to the pathogen by allowing persistent infection and thereby favoring transmission. Available literature shows contradictory results about the role of IL-10 in PRRSV infection. For instance, some studies indicated that PRRSV infection induced an increase of the IL-10 production in vitro and in vivo, especially during the phase of viremia (Díaz *et al.*, 2005; Gimeno *et al.*, 2011; Peng *et al.*, 2009; Silva-Campa *et al.*, 2009; Suradhat and Thanawongnuwech, 2003). Therefore, it could be supposed that the IL-10 released early in the infection could be one of the elements favoring an extended duration of the viremia as well as the maintenance of the virus in tissues. Nevertheless, other in vitro and in vivo studies did not report any increase of that cytokine during a PRRSV infection (Silva-Campa *et al.*, 2010; Subramaniam *et al.*, 2011). In contrast Gimeno *et al.* (2011) showed that the induction of IL-10 release from APC is dependent on the PRRSV strain used.

The implication of Treg in the course of PRRSV infection has been suggested. In a broad sense, Treg include different types of T cells involved in the suppression of the activation of the immune system and in the immune homeostasis. Regulatory T cells can be generated centrally (in the thymus; natural Treg) without any specific stimuli, or in the periphery, as a response to a specific pathogen (induced Treg, iTreg). iTreg produce IL-10 or TGF-β and upon stimulation with a recall antigen downregulate or inhibit the proliferation and the effector capabilities of other T cells. Thus, iTreg could also contribute to the lack of clearance of the infection.

Development of Treg has been related to DC activity. For example, Silva-Campa *et al.* (2009) showed that PRRSV-specific Treg can be generated in vitro after stimulation of lymphocytes with DC infected with genotype 2 PRRSV. Interestingly, the induction of

Treg appeared to be related with TGF-β release from infected DC, but not with IL-10 release. In a subsequent work from the same group, stimulation of DC with different type 1 PRRSV strains did not produce significant amounts of TGF- β, while each strain induced different results for IL-10 (Silva-Campa *et al.,* 2010). In this case, no iTreg were generated. Nevertheless, Wongyanin *et al.* (2010) showed that Treg generated both in vitro and in vivo using genotype 2 PRRSV were at least partially induced by an increase in IL-10 release from stimulated DC. Therefore, different mechanisms of induction of virus-specific Treg could exist and they could be influenced by the viral strain. However, up to now no clear evidence exists about the correlation of the development of Treg with extended periods of viremia or more severe lesions.

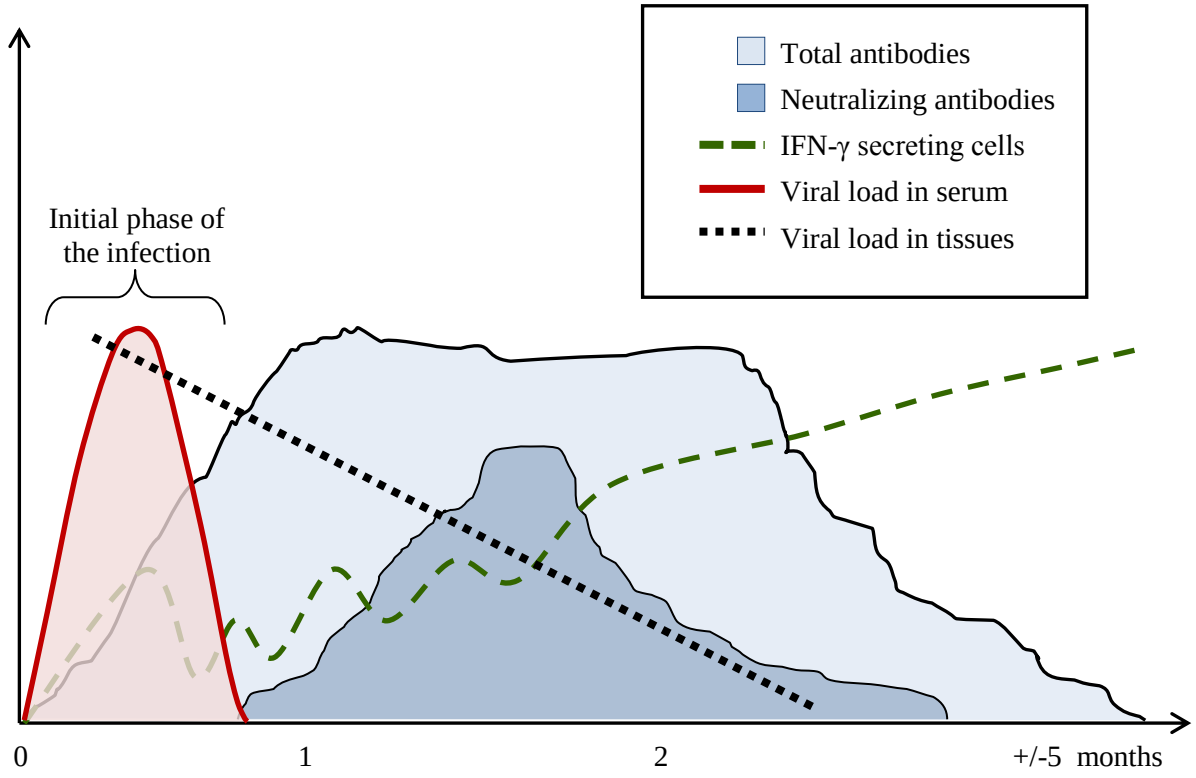
All the data presented above indicate that PRRSV have different strategies to subvert the immune system of the host and that many of them are dependent on the strain.

1.6.3.2 Adaptive immune response

Figure 3 shows the chronological events observed during the infection with PRRSV. The early infection period is characterized by a phase of viremia, which may last several weeks. Then, viremia ceases and the virus is only detected in lymphoid tissues. The virus is cleared from the host weeks to months after the cease of viremia.

Most pigs seroconvert between 7 and 14 dpi (Figure 3) (Labarque *et al.,* 2000; Meier et al., 2003; Nelson et al., 1994; Yoon et al., 1995). In the primary antibody response, IgM are predominant and can be detected until 42 dpi (Loemba *et al.,* 1996; Park *et al.,* 1995) whereas IgG appear around the second week of infection and peak between 21 and 42 dpi (Nelson *et al.,* 1994). After peaking, IgG levels remain in a plateau for several months and then start to decrease. These early antibodies are not protective and may also contribute to the phenomenon of antibody-dependent enhancement (ADE) of viral replication (Cancel-Tirado *et al.,* 2004; Yoon *et al.,* 1996). Early antibodies are mainly directed against the N protein (Yoon *et al.,* 1995) and to a lesser extent to the M and GP5 proteins (Loemba *et al.,* 1996; Nelson *et al.,* 1994).

Figure 3. Schematic representation of the immune response against PRRSV. Modified from Osorio et al., (2004).



Specific antibodies against GP3 were also detected in some animals (Gonin *et al.*, 1998). Regarding the non-structural proteins of the virus, different studies showed that the nsp1, nsp2 and nsp7 are also involved in the development of antibodies (Brown *et al.*, 2009; de Lima *et al.*, 2006; Oleksiewicz *et al.*, 2001). For example, antibodies against nsp2 follow a similar pattern to that of antibodies against N protein, although in the long term, anti-nsp2 antibodies persist longer than anti-N antibodies (Mulupuri *et al.*, 2008).

Neutralizing antibodies usually develop later on the infection. Different PRRSV isolates have different immunogenicity and some strains do not induce NA at all. Beside this there is also a notorious variation based on the idiosyncrasy of each individual. In general, NA appear around the 4th week post-infection (Figure 3) (Kim *et al.*, 2007; Loemba *et al.*, 1996) and their titers are usually low (below 1:32–1:64) in pigs inoculated with wild-type viruses (Díaz *et al.*, 2005; Loemba *et al.*, 1996; Meier *et al.*, 2003). Some authors reported that exists a certain correlation between the appearance of these antibodies and the cease of the viremia in infected animals

(Murtaugh *et al.,* 2002; Plagemann, 2004). Likewise, Labarque *et al.* (2000) showed that the clearance of PRRSV from the lungs coincides with the appearance of NA. Nevertheless, after experimental infection, viremia may be resolved without detectable levels of NA (Díaz *et al.,* 2006; Díaz *et al.,* 2012; Plagemann, 2006) and in some cases, viremia coexists for weeks with NA (Díaz *et al.,* 2012; Plagemann, 2006). A different picture arises when protection before infection is considered. For example, the passive transfer of serum with high NA titers (≥1:16) to pregnant sows protects them against reproductive failure and blocks transplacental infection (Osorio *et al.,* 2002). Likewise, passive transfer of NA at a titer of 1:8 or higher protected piglets against the development of viremia, whilst sterilizing immunity was attained at NA titers of 1:32 (Lopez *et al.,* 2007). These results suggest that certain levels of NA can be effective in protecting pigs from the infection, although the cross-reactivity of such antibodies against field strains is very difficult to be predicted.

Several hypotheses have been postulated to explain the delayed development of NA, the low titers of NA observed in immunized pigs and their implication in the clearance of infection. As seen previously in this review, a poor cytokine milieu and variations in the expression of molecules related to antigen presentation could predispose to a delayed mobilization of cells from the adaptive immune system, resulting in a slow NA response to PRRSV (Chang *et al.*, 2008; Flores-Mendoza *et al.,* 2008).

The hypothesis of the existence of a decoy epitope was proposed by Ostrowski *et al.* (2002). These authors identified two epitopes (A and B) located in the ectodomain of PRRSV GP5. The non-neutralizing epitope A was immunodominant, attracting thus the attention of the immune system. As a result, the development of NA against the B epitope was delayed for at least three weeks.

Other neutralizing epitopes has been described in GP2 (Weiland *et al.,* 1999; Yang *et al.,* 2000), GP3, GP4 and M protein (Vanhee *et al.,* 2011; Weiland *et al.,* 1999; Yang *et al.,* 2000). Vanhee *et al.* (2011) indicated that the linear epitopes predicted in GP4 of type 1 PRRSV were recognized by sera of all animals and serial passage of PRRVS in presence to NA directed to GP4 may produce the emergence of neutralization-resistant variants (Costers *et al.,* 2010). Furthermore, antigenic regions in GP3 were

recognized by a majority of the animals, while linear epitopes in GP2, GP5 and M were recognized by only few animals.

Another mechanism suggested to explain the NA response against PRRSV is the phenomenon of glycan shielding. Ansari *et al.* (2006) showed that mutant viruses lacking glycosylation at N34, N51 or both positions of GP5 were more susceptible to neutralization by sera obtained from pigs infected with the wild-type virus. Also, NA titers in sera of pigs inoculated with the mutants were higher (homologous and heterologous neutralization assay) than those of pigs inoculated with the wild-type virus. Similarly, Vu *et al.* (2011) found that the isolate PRRSV-01 obtained from a clinical case and naturally lacking glycosylation on the N151 of GP3 and N51 of GP5, was able to induce an atypically rapid and robust homologous NA response, with NA detected from 14 dpi and NA titers ranging from 1:512 to 1:2048 at 42 dpi. Moreover, sera from pigs infected with this virus possessed great neutralizing activities against mutant viruses where glycosylation in GP3 and GP5 were reintroduced. Infection with hyperglycosilated mutants conversely induced a poor NA response, in both homologous and heterologous neutralizing assay. However, Martínez-Lobo *et al.* (2011) indicated that differences in cross-neutralization in sera raised against different genotype 1 strains could not be related strictly to the sequence and number of glycosylations of the known GP3, GP4 or GP5 neutralizing epitopes and suggested that the conformational characteristics of the epitopes could also have a role on the cross-reactivity.

The CMI against PRRSV also shown unusual characteristics if compared with this observed in common viral infections (Meier *et al.*, 2003). Early studies showed that CMI can be detected from four weeks post-exposure (Figure 3) (Bautista and Molitor, 1997; Díaz *et al.*, 2005; Díaz *et al.*, 2006; López Fuertes *et al.*, 1999). Once detected, virus-specific IFN-γ secreting cells (IFN-γ-SC) fluctuate for the next ten weeks, reaching then the highest level around 25 weeks post-infection, followed by a steady state for several months (Díaz *et al.*, 2005; Díaz *et al.*, 2006; Meier *et al.*, 2003).

IFN-γ against PRRSV is mainly produced by double positive CD4/CD8 T cells, and to a lesser extent by $CD4^{-}CD8\alpha\beta^{+}$ cells (Meier *et al.*, 2003). Although mechanisms

governing the uncommon CMI against PRRSV are not well defined yet, the virus-induced impairment of the innate immune response may be considered as the main factor involved. In this sense, Royaee *et al.* (2004) showed that the pigs secreting highest levels of IFN-α after infection were also the animals showing the highest frequencies of virus-specific IFN-γ-SC.

Several studies indicated that the clearance of the infection was correlated with the levels of IFN-γ-SC (Díaz *et al.*, 2005; Díaz *et al.*, 2012; Mateu and Diaz, 2008). However, as seen for the NA, resolution of viremia can be achieved even without apparent development of specific IFN-γ-SC and the ability to induce such type of immune response seems to be dependent on the viral strain (Díaz *et al.*, 2012). Previous immunization of pigs with PRRSV isolates inducing high levels of IFN-γ-SC was shown to prevent the development of viremia after homologous or heterologous challenge (Díaz *et al.*, 2006; Díaz *et al.*, 2012). At the same time, Dotti *et al.* (2011) demonstrated that pigs with poor IFN-γ response after vaccination can be protected from a PRRSV challenge if high levels of NA are present. Therefore, both CMI and NA can be considered as beneficial in protecting pigs from the infection and in the clearance of the virus from the host, although their role as correlates of protection against PRRSV is debatable.

1.6.3.3 Vaccination against PRRSV

Vaccination of sows and piglets is one of the strategies commonly used for controlling PRRS together with management and biosecurity measures. At present, several commercial attenuated (modified live vaccines, MLV) or inactivated (IV) vaccines based on both type 1 and type 2 PRRSV strains are available (Table 2). Protection afforded by these vaccines has to be evaluated at both individual and population levels. In the first case, the main objective of vaccination is to protect pigs from the infection and reduce clinical signs, whereas at population level, the aim of vaccination strategies for controlling PRRS is also to reduce the economic losses associated with the disease and to stop the virus transmission.

With regards to inactivated vaccines, their efficacy in protecting pigs from the infection and also in reducing clinical manifestations of the disease is generally scarce, since they induce very low NA and CMI in a single application (Bassaganya-Riera *et al.,* 2004; Díaz *et al.,* 2006; Kim *et al.,* 2011; Misinzo *et al.,* 2006; Piras *et al.,* 2005; Zuckermann *et al.,* 2007). However, when applied to previously immunized pigs, IV are able to induce a high and fast anamnestic NA response (Misinzo *et al.,* 2006; Nilubol *et al.,* 2004). Therefore, the use of inactivated vaccines in the field is generally limited to recall purposes, especially in pregnant sows before mating (Geldhof *et al.,* 2013).

Vaccination strategies with MLV are currently predominating. Modified live vaccines are able to replicate in the host and their behavior, in terms of development of viremia and induced host's immune response, resembles that of mildly virulent PRRSV strains. Both virological and clinical protection afforded by MLV vaccination after challenge with a genetically homologous PRRSV are generally good (Díaz *et al.,* 2006; Lager *et al.,* 1997; Lager *et al.,* 1999; Li *et al.,* 2014; Zuckermann *et al.,* 2007) whereas in the case of exposure to a heterologous PRRSV strains, the relative efficacy of these vaccines range widely, from 50% (Osorio *et al.,* 1998) to 85% (Scortti *et al.,* 2006).

For example Lager *et al.* (1999) demonstrated that pregnant gilts infected with the American isolate NADC-8 and challenged with LV late in gestation had only partial protection against transplacental infection (virus crossed the placenta in 1/7 gilts), whereas gilts inoculated with the homologous virus were fully protected. Similarly, Scortti *et al.* (2006) vaccinated two groups of gilts with two different commercial MLV vaccines (both of genotype 1) and challenged them at 90 days of gestation with a heterologous European field strain. After challenge, viremia was detected in 57% and 40% of the vaccinated gilts and the challenge virus was also detected in some of their piglets, being the protection conferred by both MLV vaccines only partial. However, vaccination provided a statistically significant level of protection with regards to the incidence of congenital infection, reproductive performance, and piglet health and viability.

Table 2. Commercial vaccines against PRRSV currently available in Europe and America. The genotype of the vaccine strain is indicated between brackets. Modified from *http://www.cfsph.iastate.edu/Vaccines/disease_list.php?disease=porcine-reproductive-respiratory-syndrome&lang=es*

Vaccine	Manufacturer	Description	Approved Use
Ingelvac® PRRS MLV	Boehringer Ingelheim Vetmedica	Attenuated (2)	Pigs of all ages
Ingelvac® PRRS-ATP	Boehringer Ingelheim Vetmedica	Attenuated (2)	Weaner and growing pigs
ReproCyc® PRRS-PLE	Boehringer Ingelheim Vetmedica	Attenuated (2)	Gilts/sows
Ingelvac 3FLEX®	Boehringer Ingelheim Vetmedica	Attenuated (2)	Weaner and growing pigs
Fostera® PRRS	Zoetis	Attenuated (2)	Pigs of all ages
Porcilis PRRS®	MSD Animal Health	Attenuated (1)	Pigs of all ages
Unistrain PRRS®	Laboratories Hipra	Attenuated (1)	Weaner and growing pigs
Pyrsvac-183®	Laboratories Syva	Attenuated (1)	Pigs of all ages
Progressis®/ Ingelvac® PRRS KV	Merial	Inactivated (1)	Gilts, Sows
Suipravac PRRS	Laboratories Hipra	Inactivated (1)	Gilts, Sows
Suvaxyn PRRS	Zoetis	Inactivated (1)	Gilts, Sows
Suivac PRRS-INe/ Suivac PRRS-IN	Dyntec spd. s.r.o.	Inactivated (1 and 2)	Pigs of all ages

After vaccination with a genotype 1 MLV vaccine, pigs were mostly negative for virus in serum or BAL fluid after challenge with LV (Labarque *et al.*, 2004). In contrast, vaccinated pigs developed viremia over 15 days and were positive for virus in BAL fluid when challenged with an Italian variant strain that had 84% similarity in ORF5 to the vaccine strain. Partial clinical and/or virological protection of vaccinated piglets against heterologous challenge was also demonstrated by Martelli *et al.* (2007), Roca *et al.* (2012), Li *et al.* (2014) and others.

In spite of the data presented above, the prediction of the potential efficacy of MLV against heterologous PRRSV isolates cannot be done based in genetic similarities (Mateu and Diaz, 2008; Prieto *et al.,* 2008). For example, in a work of Díaz *et al.* (2006) pigs were vaccinated with two different European-type vaccines (V1 and V3) and then were challenged with a strain similar to one of the vaccines and slightly different to the other (92–96% similarity). The heterologous V3 vaccine produced apparently sterilizing immunity, while piglets vaccinated with the homologous V1 vaccine become infected. Analyzing the immune response generated by each vaccine, results showed that V3 vaccine induced higher levels of IFN-γ-SC, whereas V1 induced IL-10 release by PMBCs. Therefore, it could be concluded that for inducing protection, the ability of each strain to induce a strong CMI was more important than the genetic similarity. Díaz *et al.* (2012) also demonstrated that animals primo-inoculated with strain 3267 did not developed viremia when challenged with the heterologous strain 3262. Conversely, animals primo-inoculated with the 3262 strain developed viremia after challenge at 84 dpi regardless of the strain used. These results can be attributable to the fact that the primo-inoculation with strain 3267 induced a high NA response and that such NA were also capable of neutralizing strain 3262, whereas the primo-inoculation with the 3262 strain did not induce any NA response.

Under field conditions, different degrees of protection from PRRSV infection are seen in animals vaccinated with a MLV. As a matter of fact, given the genetic diversity of PRRSV (Murtaugh *et al.,* 2010), all challenge situations in the field can be considered as heterologous.

Stadejek *et al.* (2005) evaluated for example the efficacy of MLV vaccination in a farrow-to-finish herd where a Polish wild type PRRSV strain was circulating from several years prior to the start of the vaccination program of PRRS control. Twelve piglets of such farm were vaccinated with a genotype 1 MLV at 14 days of age and then were followed until 132 days of life. At 68 and 92 days post-vaccination, only two pigs had become infected with the field strain, despite the fact that the MLV and the wild type of the farm were only 82.6% similar (ORF5). Similarly, Martelli *et al.* (2009) assessed the efficacy of vaccination of piglet against natural exposure to a

PRRSV field strain belonging to the Italian cluster of genotype 1 PRRSV (84% of identity with the ORF5 of the MLV). In the post-exposure period, wild type virus was only detected in 59% of the sera of vaccinated pigs and also clinical signs were significantly reduced in vaccinated animals compared to the unvaccinated ones.

As previously commented, at a population level, the efficacy of current vaccines cannot be only evaluated in virological terms since the goal of vaccination strategies is not merely to protect pigs from the infection, but also to reduce the economic losses associated with the disease and to stop the viral circulation in a farm. The cease of virus transmission in a herd can be achieved by vaccination if the MLV has the ability to reduce the susceptibility of pigs against the infection and, at the same time, is capable to reduce the contagiousness of the individuals that eventually became infected. In this sense, the potential efficacy of MLV vaccines in the field can be estimated indirectly, assessing the biological parameters related to transmission (i.e. duration of viremia and shedding of the virus, number of chronically infected pigs etc.) and directly, determining the reproduction rate of PRRSV.

Cano *et al.* (2007b) demonstrated that repeated immunizations with MLV vaccine in pigs previously infected with a homologous isolate significantly reduced the number of persistently infected pigs at 127 dpi and also reduced the viral shedding after 97 dpi, although this strategy was not capable of eliminating completely the circulation of the wild type virus. In a similar study, Linhares *et al.* (2012) showed that vaccination of pigs after challenge significantly reduced viral shedding in oral fluids and the presence of virus in the air, although the magnitude and duration of viremia in vaccinated pigs was similar to the unvaccinated ones. A reduction on PRRSV shedding, but not in the proportion of chronically infected pigs was also observed after heterologous vaccination by Cano *et al.* (2007a).

Although the abovementioned works demonstrated the efficacy of mass vaccination in reducing the biological parameters related to the virus transmission, few studies have dealt with the assessment of R in vaccinated and naïve pigs. Nodelijk *et al.* (2001) were the first that evaluated the effect of vaccination on PRRSV transmission. However, they used a genotype 2 MLV and a genotype 1 challenge virus (LV), being

thus a worst-case scenario. The authors performed three different trials of transmission by contact. In experiment A, 5 vaccinated (V) pigs and 5 unvaccinated ones (NV) were challenged with LV and then comingled with other 5 V and 5 NV, respectively, in order to expose them to the challenge virus. In experiment B, one V pig was inoculated with LV and placed in contact with other 9 V pigs; the same protocol was used for NV pigs. Finally, in trial C transmission of PRRSV among 10 pairs of vaccinated pigs was compared with 10 pairs of unvaccinated pigs by means of multiple one-to-one experiments. Virological results showed that most vaccinated pigs (> 60%) became infected in experiments A and B, and all of them became viremic in experiment C. Thus, R value in vaccinated pigs as estimated from the pooled data of trials A and B, was 1.5 ($CI_{95\%}$: 0.7-44.8) whilst it could not be determined (infinite) for NV pigs. The study failed therefore in demonstrating a significant reduction of PRRSV transmission after vaccination. However, considering that the vaccine and challenge strains were of different genotypes and that pigs were intranasally inoculated, R value was probably overestimated. This phenomenon is partially confirmed by the evidence that V inoculated pigs had a significantly longer duration of viremia and higher viral loads compared to V pigs infected by contact.

Another study about the efficacy of vaccination in reducing PRRSV transmission was performed by Mondaca-Fernández *et al.* (2007). In that case animals were vaccinated with a genotype 2 MLV and challenged also with a genotype 2 PRRSV, the MN-30100 isolate. The authors failed in their objective since the challenge strain resulted to be little contagious, as indicated by the lack of infection in the exposed NV pigs. Thus, R values were 0.59 ($CI_{95\%}$: 0.13- 3.21) and 0.26 ($CI_{95\%}$: 0.01-2.26) for the vaccinated and non-vaccinated groups of pigs, respectively. The difference was not significant but considering the low transmissibility of the challenge strain it can be argued that PRRSV transmission could not be properly assessed.

Recently, Rose *et al.* (2015) showed a significant reduction on PRRSV transmission in vaccinated pigs. In that study, piglets of 3 weeks of age were inoculated with genotype 1 MLV and 12 of them were then challenged with a genotype 1 strain (92.7% of sequence homology with the MLV) at 31 dpv. Then, inoculated pigs were put in

contact with 12 vaccinated piglets during 49 days (6 replicates of 2:2 contact trials in total). The experiment was replicated simultaneously with unvaccinated piglets. Among the contact pigs, the challenge strain was detected in serum of only one V whereas all contact NV were infected. Consequently, R was significantly reduced from 5.42 ($CI_{95\%}$: 2.94-9.04) in NV pigs to 0.30 ($CI_{95\%}$: 0.05-0.96) in V animals, indicating that piglet vaccination would be a useful tool to control virus propagation within the population. However, results of this study have to be carefully interpreted since they are difficult to be extrapolated to other PRRSV strains. In addition, the outcome of the strategies of PRRS control into a farm can be influenced by other factors such as the pattern of viral circulation into the herd, the management of pigs and the biosecurity measure, among others.

HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Porcine reproductive and respiratory syndrome virus (PRRSV) is considered the most costly among common pig pathogens, being more than 50% of the cost of PRRS attributable to the impact of the disease on weaners and growers.

Immunization of gilts and sows, and eventually of piglets, can be crucial for controlling PRRS. The efficacy of current PRRSV vaccines in terms of protecting against the infection ranges from complete to very partial. This efficacy mainly depends, but probably not only, on the antigenic similarity between the vaccine strain and the challenge isolate. In practical terms, this means that when in the field a vaccinated animal is confronted to a circulating PRRSV isolate, the outcome of such challenge can be a complete protection against the infection (sterilizing immunity), a subclinical infection with very mild clinical signs (partial protection) or the development of clinical disease.

However, repeated mass vaccination could be effective for control and eventual eradication provided that it is able to reduce the reproduction rate (R) of PRRSV below one. Although it is a common believe that the virus spreads quickly among weaners and fatteners, the number of studies dealing with the calculation of R for PRRSV transmission in these populations is very scarce. Therefore, the starting hypothesis of the present thesis is that the efficacy of current genotype 1 vaccines will be enough to result in a reduction of R to values <1 when applied “en masse” to piglets.

2.2 Objectives

1. To preliminary estimate the reproduction rate (R) of genotype 1 PRRSV in weaners and fatteners pigs under field conditions in endemically infected farms.

2. To assess the transmission of genotype 1 subtype 1 PRRSV in vaccinated and unvaccinated pigs by means of two different experimental models of transmission by contact, representing two different scenarios for virus transmission.

3. To evaluate the effect of vaccination on the biological parameters that can be related with transmission of the virus, such as the duration of viremia and the nasal/fecal shedding periods as well as the amount of virus in sera and lymphoid tissues.

FIRST STUDY

Estimation of the reproduction rate (R) of *Porcine reproductive and respiratory syndrome virus* in pigs of two endemic farrow-to-finish farms.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, enveloped, positive-sense single-stranded RNA virus belonging to the family *Arteriviridae*, Genus *Arterivirus* (Cavanagh *et al.*, 1997). Under natural conditions, the virus is transmitted either vertically, namely, from sows to fetuses late in gestation (Lager, 1994; Rossow *et al.*, 1994; Terpstra *et al.*, 1991) or horizontally, by oral or nasal fluids, in the form of aerosols or trough injured or abraded skin (Hermann *et al.*, 2005; Wills *et al.*, 1997). From a population point of view, the spread of PRRSV within the farm is basically related to the pig flow (unidirectional or not), the management system (all in/all out versus continuous flow) and to the internal biosecurity, all of them factors that favor or hinder contact between different groups of pigs within the farm.

The assessment of the basic preproduction rate (R), namely the number of new cases produced by a single infected individual, is a way to quantify the transmission of a pathogen. When $R>1$ the infection spreads in the population in an epidemic form while when $R<1$ the infection tends to fade out with time. R value depends on the infectiousness of the pathogen for a given species, on the susceptibility to infection of the individuals in the population and on the likelihood of an effective contact that, in turn, depends on the density of susceptible and infectious animals among other things.

One of the most common ways to reduce R in a population is by decreasing the proportion of susceptible pigs or by making the pigs more resistant to the infection. This can be achieved by vaccination. If a vaccine is able to reduce R below 1, the infection might still enter in the population but will not spread.

Up to the date, only a few works have been published regarding the quantification of PRRSV transmission, mostly under experimental conditions (Charpin *et al.*, 2012; Mondaca-Fernández *et al.*, 2007; Nodelijk *et al.*, 2001; Rose *et al.*, 2015) or based on estimates after some field data (Nodelijk *et al.*, 2000). Regarding weaners and fatteners, Charpin *et al.* (2012) and Rose *et al.* (2015) calculated R for experimental contact models with genotype 1 PRRSV obtaining values that ranged from 2.6 to 5.4, respectively. These values do not fit the general assumption that, under natural

conditions the virus spreads very quickly. Thus, the aim of the present study was to estimate the basic reproduction rate of genotype 1 PRRSV transmission in weaners and fatteners under field conditions, by means of a longitudinal follow-up of two endemically infected farms.

MATERIALS AND METHODS

Selected herds

Two farrow-to-finish farms, previously confirmed to be endemic for PRRSV, as shown by the presence of seropositive pigs at 10 weeks of age, were selected for the present study. Farm 1 (F1) had 300 sows and farm 2 (F2) had 90 sows in the breeding stock. None of the farms vaccinated against PRRSV. Both farms were located in a high pig density area of Catalonia (NE of Spain).

In Farm 1 (Figure 1-A), piglets were weaned at 4 weeks of age being housed in three separated outdoor modules, with capacity for about 35 piglets, with no temperature or ventilation control systems. Then, from 10 to 15 weeks of age, pigs were allocated in four pens of a different building. Each pen had a capacity for up to 30 pigs and after that they were transferred to the finishing facilities, where they remained in 8 pens with capacity for up to 15 finishing pigs each until sent to the slaughterhouse at 24 weeks of age. Fattening and finishing facilities had natural ventilation and open separations between pens. Regarding management practices, an "all in/all out" (AIAO) system was implemented in the nurseries but no afterwards. Distribution of pigs in the different pens throughout the different phases of production was done at random, therefore mixing of animals occurred each time that animals were moved.

In farm 2 (Figure 1-B), piglets were weaned and moved to the nursery at 4 weeks of age, where they remained until they were 11 weeks old. The nursery was distributed in 7 pens plus an infirmary pen. Each pen had capacity for up to 15 piglets. Then, pigs were moved to pens for fatteners until sent to the slaughterhouse at 21 weeks of age. These pens could allocate up to 12 finishing pigs. Whereas nurseries were equipped with forced ventilation system, fattening units had natural ventilation. Both facilities had open separation between pens. Farm 2 was managed in an "all in/all out" system.

Figure 1-A. Schematic representation of Farm 1 facilities. Closed and open separations between pens are represented by solid and dashed lines, respectively.

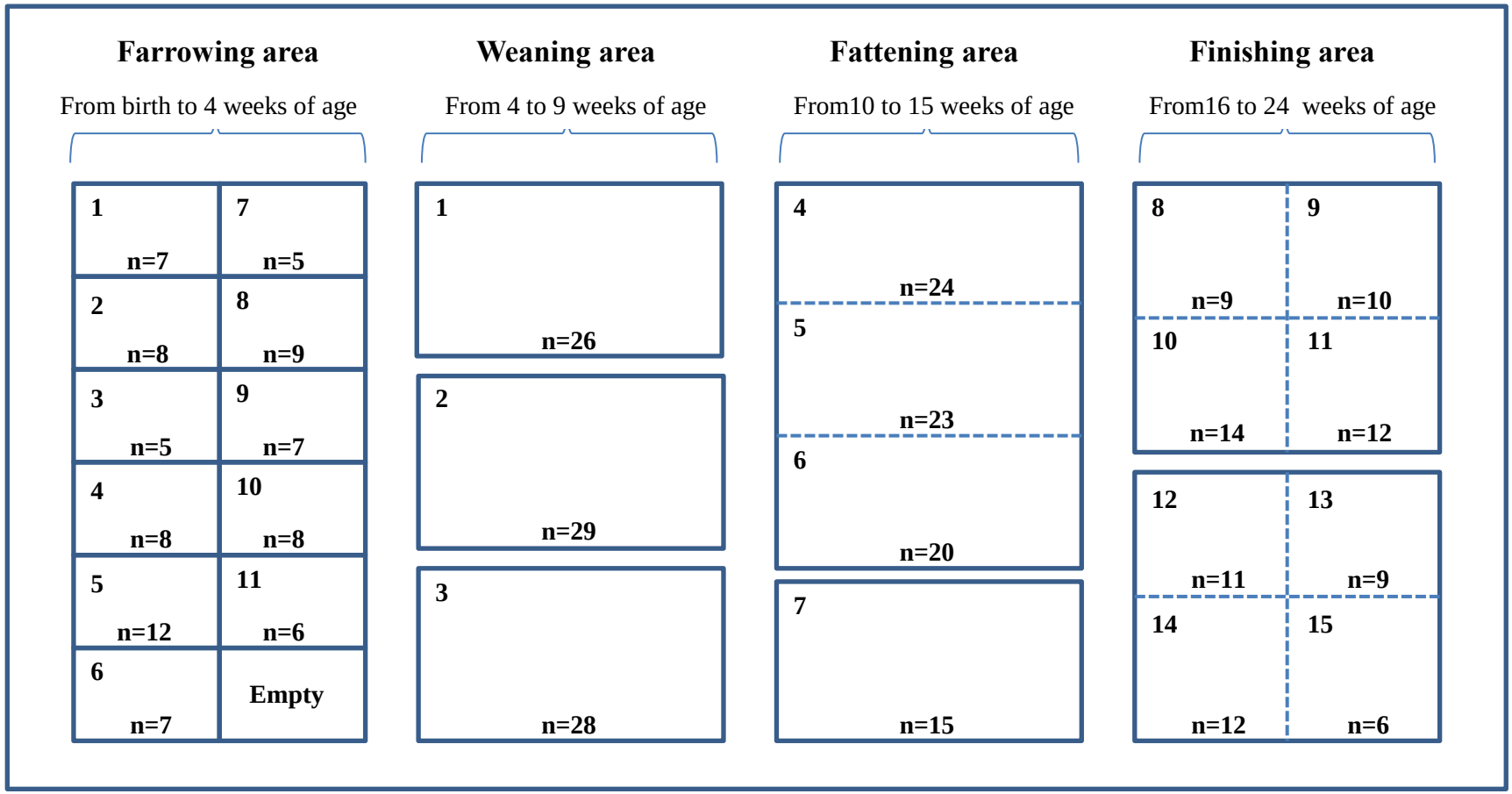
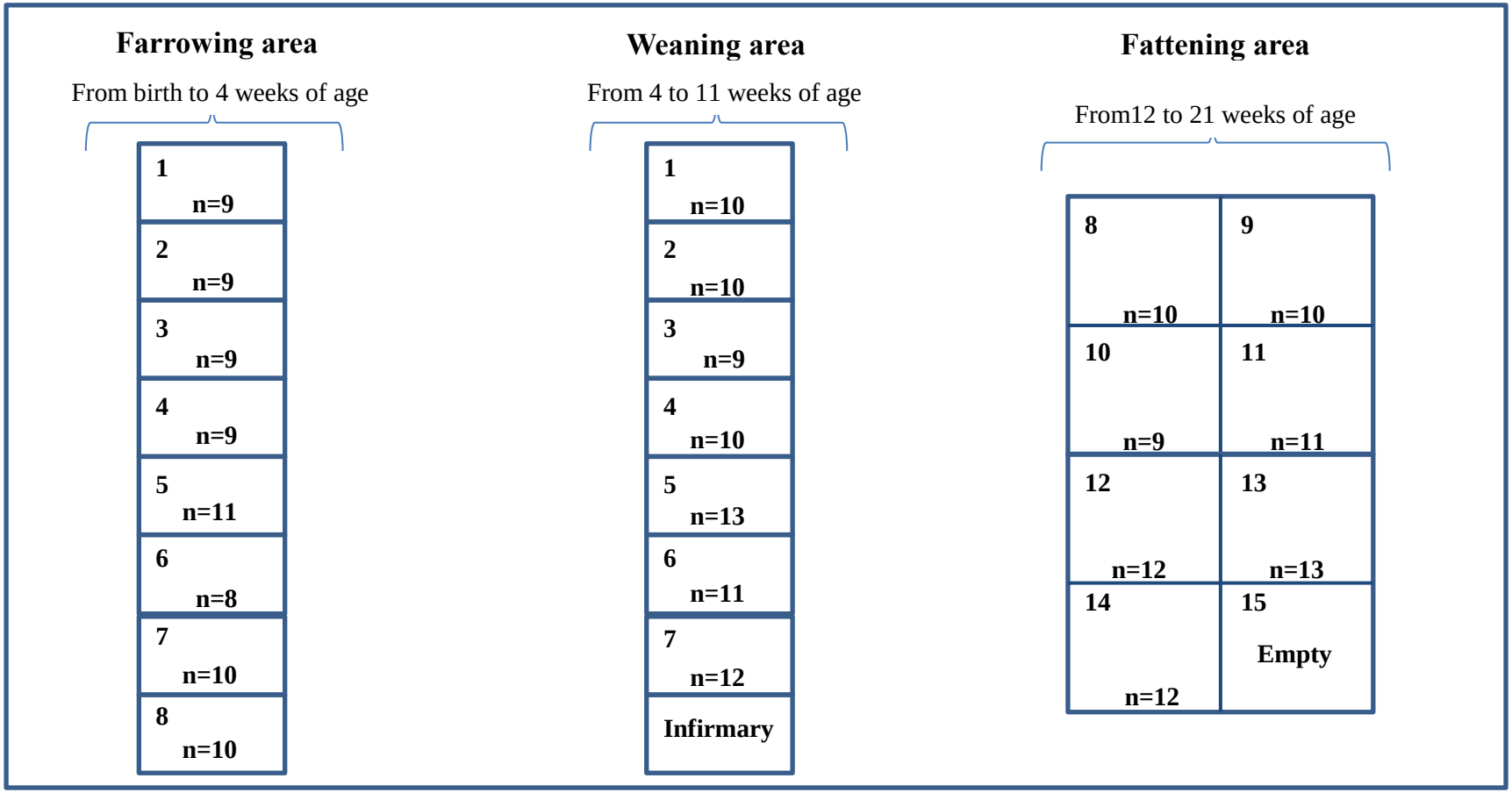


Figure 1-B. Schematic representation of Farm 2 facilities. Solid lines indicate closed separations between pens.



The follow-up of F1 started in July and ended in December. A whole batch of 3-week-old piglets (n=84, coming from 11 litters) was ear-tagged and followed until sent to the slaughterhouse. Blood sampling was carried out weekly between 3 and 13 weeks of age, and afterwards animals were bled at 15, 17 and 24 weeks of age. Every time the farm was visited the distribution of pigs per pen was recorded.

The period of study of F2 started in January and ended in June. A whole batch of weaned pigs (n=75, coming from 8 litters) was ear-tagged and followed from 3 weeks of age until sent to the slaughterhouse at 21 weeks of age. Blood samples were collected weekly and the distribution of pigs per pen was recorded every time the farm was visited.

Assessment of the infection

Serum was obtained from blood samples by centrifugation at 1,500 x g for 10 minutes. Then, sera were analyzed for the presence of PRRSV-specific antibodies by means of a commercial ELISA (Idexx PRRS X3 Ab Test®, Iddex Laboratories). Results were expressed as sample to positive control (S/P) optical density ratios. According to the manufacturer, samples with S/P $\geq$0.4 were considered to be positive. Seroconversion was defined as a change from negative to positive ELISA status. Animals were also tested by RT-PCR (Mateu *et al.,* 2003) when entering the nurseries in order to determine the flow of viremic animals from the maternity area.

Data analysis

Based on the serological the results, the seroprevalence and the cumulative incidence (CI) of the infection were calculated based on the seroconversion observed at each sampling time. Seroprevalence was defined as the number of seropositive animals by ELISA divided by the whole population size. Conversely, CI was calculated as the number of new seroconversions observed at a given sampling point, divided by the number of seronegative individuals presented at the previous sampling day (susceptible pigs). In the case of F1, considering that the interval between each consecutive testing of animals varied depending on the age of pigs, the CI values obtained at each sampling point were also adjusted for a one-week period.

Regarding transmission parameters, both the transmission coefficient β and the reproduction ratio (R) were calculated separately for every pen. The average R for the whole F1 and F2 were also estimated.

In order to quantify the transmission of the virus, for each time interval Pi (i.e., the interval between two consecutive sampling) pigs were classified as "infectious" (I) once seroconversion was detected, "recovered" (R), after the end of their infectious period and, "susceptible" (S), when animals were negative by ELISA. When seroconversion occurred in a pen and a new case (C) was detected, S pigs decreased by one (S-1) whereas the number of I pig increased by one (I+1). Therefore, the whole pen population was made by I+R+S. The transition from S to I occurred according to the transmission coefficient β that represents the adequate contacts between a given infectious animal and a susceptible one in the interval Pi. Animals recovered from the infection were considered as lifelong immune against PRRSV reinfection caused by the virus isolate circulating in the farm. β coefficient was estimated within each pen using a previously described method (Perez *et al.,* 2002; Van Roermund *et al.*, 1999): $\ln(C) = \ln(\beta * S * I / N)$ where β was the exponent of $\ln(Ca) - \ln(S*I/N)$ being Ca the number of new infections at the end of each Pi and N the total number of animals in each interval. Finally, the reproduction ratio (R) was calculated using the following formula: $R=\beta*D$; where D= duration of the infectious period and β= average number of individuals that are newly infected from one infectious individual per unit of time (Vynnycky and White, 2010).

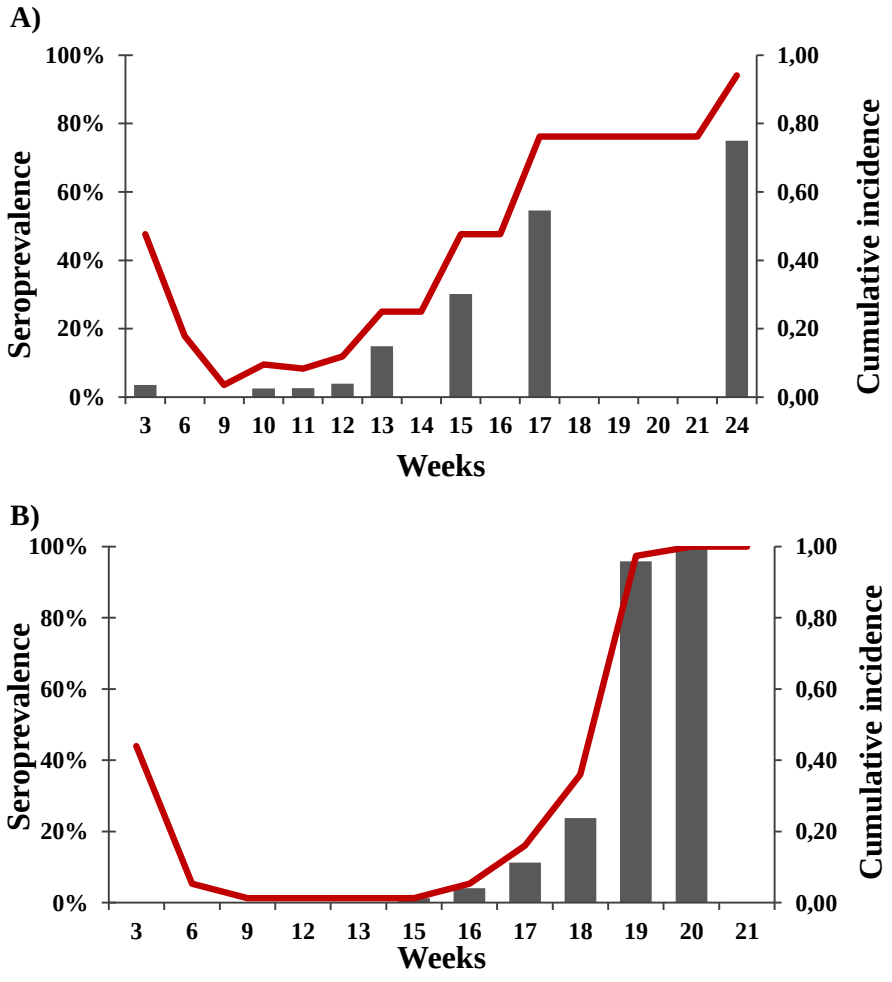
Based on previous published data, the duration of the infectious period (IP) of each pig was established to be 4 weeks. Charpin *et al.* (2012) showed that the evolution of pigs' infectiousness was mainly correlated with the time-course of viral genome load in blood. Thus, if the average duration of viremia was described to be around 5 and 3 weeks for young and old pigs, respectively, (Klinge *et al.*, 2009; Van Der Linden *et al.*, 2003), the infectious period can be reasonably assumed as lasting 4 weeks, regardless of the age of pigs.

RESULTS

Dynamics of the viral circulation

Figure 2 shows the seroprevalence and CI throughout the observational period for F1 and F2, respectively. In F1, 37/84 pigs (44.1%, $CI_{95\%}$: 33.4%-55.3%) were seropositive at 3 weeks of age, due probably to the presence of maternal-derived antibodies (MDA). Five animals were identified as viremic, one in nursery pen 1, and two in the other two nursery pens. Three of these pigs were seropositive until the end of the study suggesting that they were infected at birth or shortly afterwards. The viremic animals were then distributed at least one per pen in the subsequent phase. At six weeks of age 12/84 (14.3%, $CI_{95\%}$: 7.9%-24.0%) were still positive by ELISA among them those already seropositive at 3 weeks of age.

Figure 2. Seroprevalence (lines) and cumulative incidence of cases (solid bars) in farm 1 (A) and farm 2 (B).



Seroconversions due to PRRSV infection started at 10 weeks of age, after the decay of MDA. Seroprevalence at 10 weeks of age was 9.5% ($CI_{95\%}$: 4.5%-18.4%) whereas the CI was of 0.02 new cases/ week. At 17 weeks of age, most of pigs had seroconverted (seroprevalence: 76.2%, $CI_{95\%}$: 65.4%-84.5%)) and the IC was 0.27 new cases/ week. At end of the study (24 weeks of age) the seroprevalence was 94.0% ($CI_{95\%}$: 86.0%-97.8) with an IC of 0.11 new cases per week. On average, 0.10±0.9 new cases/ week were detected in F1 during the entire observational period. Considering the onset of seroconversions, most of infections in this farm took place during the weaning and fattening period. Fourteen weeks were needed for the virus to spread to the majority of pigs, and some animals (5/84; 5.9%) remained seronegative even at the end of finishing period. Based on the CI data, the time needed to 50% of pigs became infected was around 7 weeks.

Regarding F2, no viremic pigs were detected at the beginning of the study. In this case, MDA were also present in 33/75 pigs (44.0%; $CI_{95\%}$: 32.7%-55.9%) and 4/75 pigs (5.4%; $CI_{95\%}$: 1.7%-13.8%) at 3 and 6 weeks of age, respectively. One pig positive by ELISA at 3 weeks of age become negative for PRRSV antibodies at 15 weeks of age and then it seroconverted again at 19 weeks of age. For the other pigs, seroconversions were detected from 15 weeks of age, when the seroprevalence was 2.7% ($CI_{95\%}$: 0.5%-10.2%) and the CI was of 0.01 new cases/ week. Most animals had already seroconverted by 19 weeks of age (seroprevalence: 97.3%, $CI_{95\%}$: 89.8%-99.5%) with a CI of 0.96 cases per week. At 20 weeks of age, one week before the end of the finishing period, all pigs were seropositive. On average, 0.21±0.38 new cases/ week were detected in F2 during the whole study. Considering the onset of seroconversions, PRRSV infections were mainly taking place during the fattening/finishing period. Moreover, the virus lasted around 5 weeks in order to spread to the totality of the followed animals of the farm. Based on the CI data, the time needed to reach 50% of infected pigs was around 3.5 weeks. Figure 3 depicts the temporal and spatial distribution of cases in F1 and F2.

Figure 3-A. Temporal and spatial distribution of cases in Farm 1 from the first detection of seroconversions. Filled circles represent new seroconversions whilst empty circles depict individuals that have seroconverted in the previous weeks. The total number of pigs present in each pen (n) was also indicated.

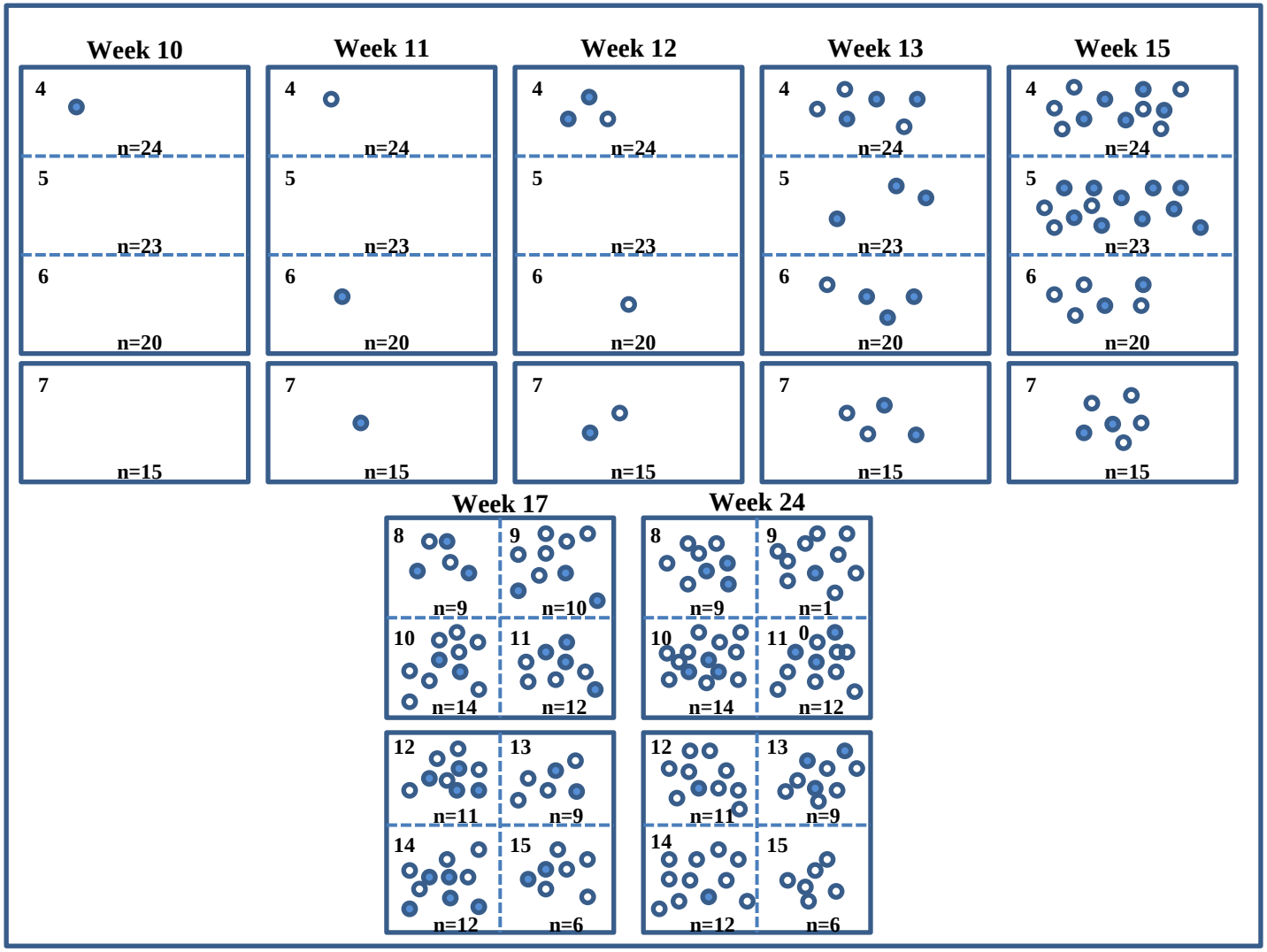
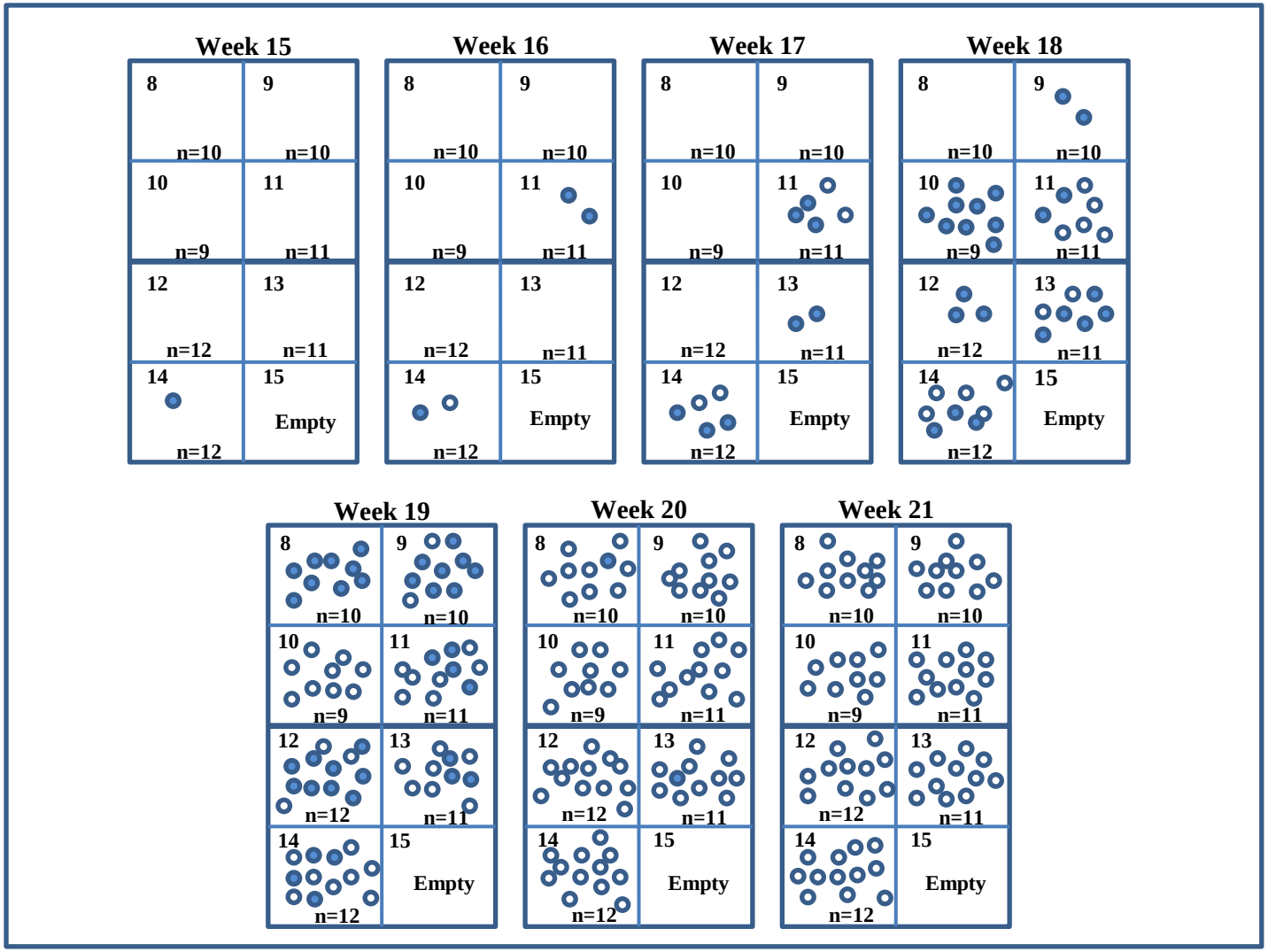


Figure 3-B. Temporal and spatial distribution of cases in Farm 2 from the first detection of seroconversions. Filled circles represent new seroconversions whilst empty circles depict individuals that have seroconverted in the previous weeks. The total number of pigs present in each pen (n) was also indicated.



Calculation of R

The estimated reproduction ratio (R) as well the transmission parameter β of each pen of F1 and F2 are shown in the table 1.

Table 1. Transmission parameter estimates for each pen and for the overall farm.

Farm	*Period (weeks)*	*Pen*	*β*	*Overall β*	*R*	*Overall R*
1	10-15	4	0.77	0.91 [0.73; 1.08]	3.08	3.36 [2.93; 4.32]
		5	1.11		4.44	
		6	1.00		4.00	
		7	0.75		2.99	
	16-24	8	1.02	0.87 [0.64; 1.10]	4.07	3.48 [2.55; 4.42]
		9	0.81		3.22	
		10	1.06		4.25	
		11	0.63		2.50	
		12	0.47		1.89	
		13	0.95		3.80	
		14	0.54		2.14	
		15	1.50		6.00	
	10-24	Whole farm		0.88 [0.72; 1.04]		3.53 [2.89; 4.18]
Farm	*Period (weeks)*	*Pen*	*β*	*Overall β*	*R*	*Overall R*
2	5-21	1	1.11	1.78 [0.89; 2.67]	4.44	7.11 [3.55; 10.68]
		2	1.25		5.00	
		3	ND		ND	
		4	1.18		4.70	
		5	*4.00	**1.33 [1.10; 1.57]	*16.0	**5.34 [4.41; 6.26]
		6	1.78		7.11	
		7	1.35		5.42	

* Outlier β and R values.

** Average β and R of farm 2 excluding the outlier values for the calculation.

ND, Not determined.

In the case of F1, β and R could be assessed in two different period of the study: 1) between 10 and 15 weeks of age, when pigs were distributed in 4 pens and, 2) between 16 and 24 of age, when animals were housed in 7 different pens. In the first sampling interval (10-15 weeks of age), β parameter was estimated to be on average 0.91 ($CI_{95\%}$: 0.73-1.08) and R was on average 3.36 ($CI_{95\%}$: 2.93-4.32). In the second one (16-24

weeks of age), β and R were on average 0.87 ($CI_{95\%}$: 0.64-1.10) and 3.48 ($CI_{95\%}$: 2.55-4.42), respectively. Thus, for F1 as a whole, the average β per week was 0.88 ($CI_{95\%}$: 0.72-1.04) and consequently, R was 3.53 ($CI_{95\%}$: 2.89-4.18).

For F2, the transmission parameters could be assessed from 12 to 21 weeks of age. During this period, pigs were distributed in 7 different pens. In one of them, R and β could not be determined because all of the animals became seropositive at the same time and we were not able to determine which one/s started the infection chain. Conversely, in another pen three positive pigs started the infection chain at 18 weeks of age and in the following week, all remaining pigs in that pen (n=9) became infected at the same time, leading to both β and R significantly higher (β=4 and R= 16) than those estimated for the other pens in F2 and also in F1. Therefore, we considered that this pen was an outlier. Considering the outlier pen, in F2 the average β coefficient was estimated to be 1.78 ($CI_{95\%}$: 0.89-2.67) whereas the average R was 7.11 ($CI_{95\%}$: 3.55-10.68). When data of the outlier was excluded from this calculation, β and R were 1.33 ($CI_{95\%}$: 1.10-1.57) and 5.34 ($CI_{95\%}$: 4.41-6.26), respectively.

DISCUSSION

Among the factors that hinder the effective control of PRRS, the limited understanding of PRRSV spread and the relatively poor efficacy of current vaccines are significant elements. Surprisingly, there are very few available studies dealing with the quantification of PRRSV transmission. The experimental studies available in the literature (Charpin *et al.,* 2012; Rose *et al.,* 2015) suggest that for genotype 1 virus, transmission in growing and fattening pigs could be lower than what is intuitively assumed.

The aim of the present study was to assess the dynamics of circulation of PRRSV in two farrow-to-finish farms endemics for the infection and the calculation of R for weaners and fatteners.

The chosen model, namely an observational study in farrow-to-finish farms, has both advantages and disadvantages. The latter are evident: lack of control of the extraneous factors that can affect the result and the difficulties for a precise definition of the

"who-infected-who" cycles. For example, in one pen of F2 R could not be calculated because all pigs of that pen seroconverted at the same time. Shortening bleeding intervals would have permitted a better definition of the infection chain of each individual but would have created serious welfare issues. In addition, a more scattered distribution of the transmission events would have resulted in a lower β coefficient per week and, accordingly, a lower R estimate. Therefore, in any case our results will overestimate R.

In our opinion, the advantages clearly surpass the disadvantages. Firstly, the type of farms selected are an almost worst model: farms with an endemically established infection, with no active measures of control, mixing of animals at different times (increasing thus the likelihood of effective contact) and almost no internal biosecurity rules. Under this scenario, it can be expected that transmission of the virus would be maximized. Secondly, the selection of natural conditions mimics better the real events of transmission that are almost impossible to mimic in experimental facilities. In our case, F1 represented probably an unstable farm as seen by the initial PCR results and by the evolution of the antibody profiles while F2 was probably a stable farm (no viremic newborns) with an endemic cycle of circulation among weaners and fatteners.

With regards to the calculation of R it is important to note here that we considered that pigs were immune to re-infection, assuming that only one strain circulated in the farm. In our opinion, it is reasonable to assume that in practical terms immunity to homologous re-infection is full or almost full and, that most farms will suffer only infection by one PRRSV isolate at a time.

We also assumed an infectious period for the average pig of 4 weeks based on the duration of viremia in field cases (Klinge *et al.*, 2009; Van Der Linden *et al.*, 2003). Besides this, for genotype 1 Charpin *et al.* (2012) demonstrated that sentinel pigs can be infected by contact with experimentally inoculated animals until 28 days post-infection and although transmission is possible after the cease of the viremia (Bierk *et al.*, 2001; Wills *et al.*, 2002) it is reasonable to think that the vast majority of new cases will be originated by pigs during the viremic phase, when shedding of the virus is higher.

A detailed examination of the results shows that in F1 there was probably some protective effect of MDA since no seroconversion occurred until 10 weeks of age. Considering that pigs develop antibodies to PRRSV between 7 and 14 days after the onset of the infection (Nelson *et al.*, 1994; Yoon *et al.*, 1995), earliest transmission in nurseries probably occurred at the end of that phase (7-9 weeks of age). Interestingly, for F1 R was more or less constant for the two periods from 10 to 24 weeks of age, indicating that the conditions for transmission did not change substantially all along the observation period in spite of changing pens and mixing pigs. For F2 the source of infection is unclear but can be assumed that the virus entered in the studied population after 10-12 weeks of age. In this case, differences between pens were noticed but it is difficult to estimate the causes beyond chance.

Our estimation of R in the two farms ranged from 3.5 to 5.3 values that agree with those of previous experimental studies in piglets (Charpin *et al.*, 2012; Rose *et al.*, 2015). Taking these values in consideration, it can be calculated the hypothetical efficacy needed for a PRRSV vaccine to stop transmission in nursery/fattening phases. If R is the value representing transmission from an average infectious individual to an average naïve pig, vaccine efficacy can be estimated from the formula Rq= R*(1-q*Ve) where Rq is the R value for vaccinated pigs, R is the value for unvaccinated ones, q is the coverage of vaccination and Ve is the vaccine efficacy (Diekmann *et al.*, 2012). If all pigs were to be vaccinated (100% coverage), then Rq= R *(1-Ve). Assuming that the infection will not cause an outbreak when Rq<1, then 1= R *(1-Ve). In our case, the worst scenario R= 5.3 would result in a Ve≥81.13% and the best scenario R=3.5, will result in Ve≥71.42% which are just fairly good vaccination efficacies. It is important to note here that within the present context, the concept of efficacy does not refer to sterilizing immunity but to the ability of vaccines to reduce the transmission of the infection. This can be accomplished merely by increasing the minimum infectious dose required to infect a vaccinated pig and by decreasing the amount of virus shed by vaccinated pigs if infected.

In conclusion, the present study offers a reliable estimation of the range of R values for genotype 1 PRRSV transmission under field conditions. Moreover, these results

suggest that for mildly virulent genotype 1 subtype 1 PRRSV isolates sterilizing immunity is probably not needed to stop circulation of the virus, opening a new way to re-examine vaccine efficacy.

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SECOND STUDY

Vaccination with a genotype 1 modified live vaccine against *porcine reproductive and respiratory syndrome virus* significantly reduces viremia, viral shedding and transmission of the virus in a quasi-natural experimental model.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is probably the most costly among the common diseases of pigs. Recent estimates from Europe and North America indicate that the reproductive efficiency of infected herds is decreased about 1.4 weaned pigs/sow or 1.7 sold feeder pigs/ sow (Holtkamp *et al.,* 2013; Nieuwenhuis *et al.,* 2012). To these figures it has to be added the cost caused by increased mortality, excess medication, loss of productive days, etc. As a matter of fact, about 50% of the cost of the disease can be attributed to the impact on weaners and grower pigs (Holtkamp *et al.*, 2013).

Control of PRRSV relies in four different aspects: early diagnosis and monitoring, biosecurity, herd management and immunization. At present, several commercial vaccines (including live attenuated and inactivated) are marketed but their efficacy is considered to be only partial, in the sense that vaccinated animals can be infected if confronted to a heterologous strain. Given the genetic diversity of PRRSV (Murtaugh *et al.,* 2010), in practical terms all challenge situations in the field can be considered as heterologous.

Most often PRRSV vaccines are applied to the breeding herd because vaccination is efficient in preventing reproductive problems although does not avoid completely the development of viremia in sows (Scortti *et al.,* 2006a; Scortti *et al.,* 2006b). In contrast, vaccination of piglets is more controversial. Firstly, because respiratory disease caused by PRRSV, particularly by genotype 1 isolates, is not always overt (Martínez-Lobo *et al.,* 2011) and depends on the interaction with other pathogens (Van Gucht *et al.,* 2004). Therefore, the beneficial effect of vaccination is more difficult to evaluate. Secondly, because when a high proportion of viremic piglets arrive to the weaning units, the time needed to induce an effective immunity is probably longer than the time needed for the infection to spread to the majority of animals.

In recent years, the notion of the need of regional or area-wide strategies for controlling PRRS is gaining importance (Corzo *et al.,* 2010).This is particularly true for areas of high pig density where the risk of re-introduction of the virus from

external sources (e.g. by proximity) is important. In such circumstances, any intervention leading to the decrease of the likelihood of transmission of the virus within or between farms is positive for the purpose of controlling the infection. Thus, vaccination could significantly contribute to the control of the infection if: a) decreased the probability of being infected and, b) reduced the efficiency of vaccinated animals to transmit the infection in the event of getting infected. If vaccines were able to fulfill these requirements, vaccination should result in a decrease in the proportion of infected pigs among vaccinated animals because of a reduction of the reproduction ratio (R) (namely the expected number of secondary cases produced by a single infected individual). Actually, eradication of other important swine infections such as Aujeszky's disease virus has been achieved in many countries by the use of vaccines that were not 100% protective in virological terms but that reduced R significantly below 1 (Bouma *et al.*, 2005).

In the case of PRRSV, very few studies (Charpin *et al.*, 2012; Mondaca-Fernández *et al.*, 2007; Nodelijk *et al.*, 2000; Nodelijk *et al.*, 2001; Velthius *et al.*, 2002) dealt with the evaluation of virus transmission either to vaccinated or to unvaccinated pigs and, in some cases, the results were obtained using viruses of different genotype for vaccination and challenge (Nodelijk *et al.*, 2001). Moreover, most models used direct inoculation of vaccinated pigs which is probably very far from the natural way of contagion. Interestingly, when a model of contact between infected and vaccinated or naïve pigs was used, R0 was below 1 even among unvaccinated pigs, probably because of the low virulence of the isolate (Mondaca-Fernández *et al.*, 2007).

The present study was designed to assess the transmission of genotype 1 PRRSV in vaccinated piglets using a contact model resembling natural conditions for transmission with a well-characterized wild type strain. Also, the course of the infection in vaccinated and unvaccinated pigs was evaluated in order to determine how vaccination could contribute to the decrease of viral shedding.

MATERIALS AND METHODS

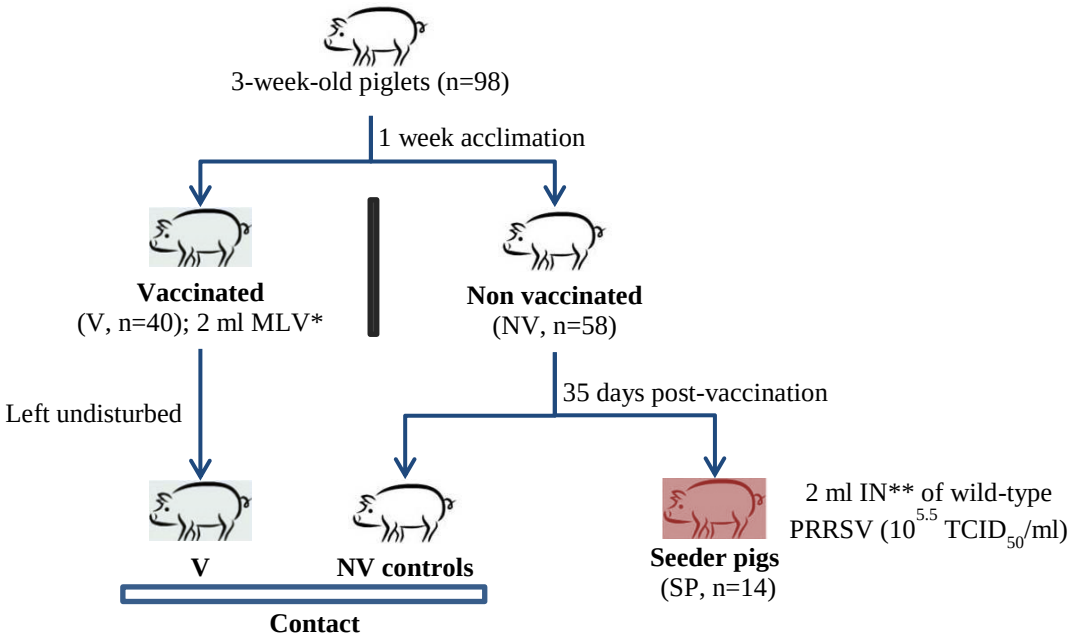
Animals and experimental design

Figure 1 summarizes the design of the experiment. Ninety-eight three-week-old piglets (Landrace x Pietrain) were obtained from a PRRSV and Aujeszky's disease virus negative farm. Animals were vaccinated at weaning (3 weeks of age) against *porcine circovirus type 2 (PCV2)* and *Lawsonia intracellularis.* The experiment was approved by the Ethics Commission for Human and Animal Experimentation of the Universitat Autònoma de Barcelona and by the the Departament de Medi Ambient i Habitatge (nº 5796) of the Autonomous Catalan Government.

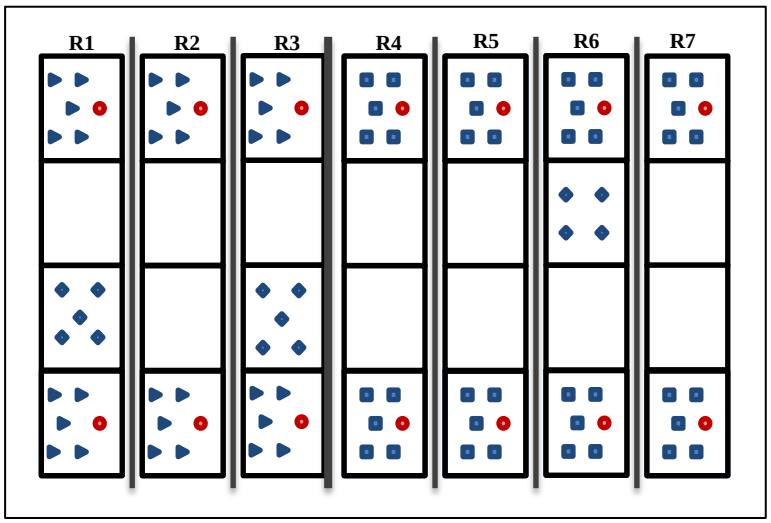
The experimental facilities were conventional weaning units with physical separation between rooms (solid walls, no air filtering). No other animals than those included in the study were housed for the duration of the experiment. After arrival to the experimental farm, piglets were left to acclimatize for one week. Animals were ear-tagged and randomly divided (random numbers) in two groups, designated as V (n=40) and NV (n=58) that were housed in separated rooms with no physical contact between them. V pigs were administered intramuscularly a 2 ml dose of a commercial modified live PRRSV vaccine (MLV) (PORCILIS PRRS® MSD Animal Health) according to manufacturer instructions. Group NV was left unvaccinated and remained as naïve controls. On the 35^{th} day post-vaccination (dpv), 14 NV pigs were separated, housed in an isolated room and inoculated intranasally with 2 ml (1 ml/nostril) of a suspension containing $10^{5.5}$ $TCID_{50}$/ ml of a genotype 1 PRRSV strain designated as 3267 (Darwich *et al.*, 2011; Díaz *et al.*, 2012; Gimeno *et al.*, 2011). The inoculated animals were designated as "seeder" pigs (SP). In parallel, the remaining V and NV animals were distributed in groups of 5 pigs each allocated in seven rooms, four of them allocating V pigs and three allocating NV animals. In each room two groups of animals were housed but direct physical contact between pens within the same treatment was avoided by using continuous pen separations and leaving an empty space between groups. Nevertheless, the air space was shared between pigs. Two days later, at 37 dpv, one SP was introduced into each pen to expose uninfected pigs to PRRSV (1 SP: 5 V and 1 SP: 5 NV pigs). Additionally, 2 sentinel groups of 5 NV pigs

Figure 1. Design of the experiment (A) and distribution of pigs during the exposure phase, from 37 to 58 dpv (B).

A) Piglets were received at 3 weeks of age and divided in two groups, V and NV. V pigs (n=40) were vaccinated with a commercial PRRSV vaccine while NV (n=58) were kept as controls. Thirty-five days later, NV pigs were further divided in two groups, SP (n=14) that were intranasally inoculated with a wild type genotype 1 PRRSV and the rest of the animals remained as NV.



B) NV and V pigs were then distributed in the rooms and pens. Each room (R1 to R7) contained 4 pens with continuous separation between them. Two days after the inoculation, SP animals were entered in the different pens for the starting of the contact exposure phase. The final distribution is represented as follows: circles represent SP; triangles, squares and rhombus represent unvaccinated, vaccinated and sentinel pigs, respectively.



Vaccine, challenge virus and cell culture

The MLV vaccine used **(**PORCILIS PRRS® MSD Animal Health) is based on the DV strain of PRRSV, belonging to the Lelystad-like cluster. Vaccine virus was resuspended in the adjuvant (tocopheryl acetate-containing aqueous adjuvant; Diluvac Forte®) as recommended by the manufacturer. Each vaccine dose contained at least 10^4 $TCID_{50}$/ml.

The inoculation of SP pigs was done with a genotype 1 subtype 1 PRRSV 3267 strain, [Genbank accession number JF276435] that has been described in previous studies (Darwich *et al.*, 2011; Díaz *et al.*, 2012; Gimeno *et al.*, 2011). The strain was isolated in 2006 from the serum of a boar of a Portuguese farm and causes a mild respiratory process in piglets and a viremia lasting three or more weeks. Viral stock was produced from sixth passage in porcine alveolar macrophages (PAM). The viral stock and PAM used for producing it were shown to be free of PCV2, *Mycoplasma hyopneumoniae*, and Torque-Teno Sus virus by PCR (Mattsson *et al.*, 1995; Quintana *et al.*, 2001; Segalés *et al.*, 2009). Overall nucleotide similarity (ORFs 1-7) between challenge and vaccine virus was 93.4% (Darwich *et al.*, 2011). Regarding the ORF5 sequence, the homology between the considered strains was 94% (Darwich *et al.*, 2011).

Evaluation of the immune response

Sera were analyzed for specific PRRSV antibodies by means of a commercial ELISA (Idexx PRRS X3 Ab Test®, Iddex Laboratories). Results were expressed as sample to positive control (S/P) optical density ratios. According to the manufacturer, samples with S/P $\geq$0.4 were considered to be positive.

Additionally, virus neutralization test (VNT) was performed with sera collected at 35 dpv and with sera collected at the end of the experiment (58 dpv). The VNT was performed following a previously described method (Díaz *et al.*, 2012) using either MARC-145 cells or PAM for vaccine and challenge virus, respectively (the vaccine virus replicates poorly in PAM). Briefly, sera were inactivated at 56ºC for 30 minutes, diluted (in duplicate) in a serial dilution from 1/2 to 1/256 and incubated overnight at 4ºC with an equal volume (50 µl) of either the vaccine or the challenge virus (2,000

$TCID_{50}$/ ml). VNT was done in duplicate and plates were incubated in parallel for 72 hours. At that time, cells were visually examined for the presence of cytopathic effect and then cultures were fixed in absolute ethanol at -20ºC. The presence of infected cells was revealed by the addition of an anti-PRRSV antibody (ICH5, Ingenasa, Madrid, Spain) and a fluorescein-labeled anti-mouse IgG antibody. Neutralization was considered to occur when less than 10 fluorescent foci were observed at dilutions ≥1:4. Results were expressed as $\log_2$ of the reciprocal of the titer.

The ELISPOT for the enumeration of PRRSV-specific IFN-γ-secreting cells (IFN- γ-SC) was performed according to Diaz *et al.* (2005) at 35 dpv and at 47 dpv (namely, 10 days post-exposure to SP pigs). In both cases the vaccine and the challenge strain were used to stimulate PBMC at a multiplicity of infection of 0.1.

Virological analysis

For sera and swab suspensions, viral RNA was extracted in 96 well-plates using the BioSprint® 96 One-For-All Vet kit (Qiagen) and the BioSprint 96 workstation (Qiagen) according to manufacturer instructions. Briefly, 100 µl of each serum or swab suspension was used for the extraction and RNA was eluted with 75 µl of the corresponding buffer. Viral RNA in tissues was extracted using the Total RNA isolation Nucleospin® RNA II kit (Macherey-Nagel), following manufacturer directions. Viremia and presence of virus in nasal fluids, faeces or tissues was determined by means of a quantitative one step RT-PCR (qRT-PCR) targeting ORF 7 (Forward primer: 5'-AGTTGCTGGGTGCAATGATA-3; Reverse primer: 5'-AATGTGGCTTCTCAGGCTTT-3'; TaqMan probe 5'-6FAM-AAGTCCCCAGCGCCAGCAACC-TMR-3'). qRT-PCR was carried out in a 7500 Fast Real Time PCR System (Applied Biosystem) and consisted of one cycle at 50°C for 30 minutes followed for 15 minutes at 95°C, and 40 cycles at 94°C for 15 s and 60°C 1 minute.

Absolute quantification of PRRSV in samples was done by constructing a standard curve using in duplicate an in-house standard (from $2x10^9$ to $2x10^0$ genomic copies/ reaction) corresponding to a purified amplicon containing the whole ORF7 sequence

of strain 3267. Prior to the sample analysis, triplicates of a serial dilution of the standard (10^0-10^{-15} genomic copies) were tested by qRT-PCR in order to establish the efficiency and the analytical sensitivity of the test that resulted to be $\geq 10^3$ genomic copies/ ml. For accepting the results of any of the analysis, a coefficient of determination (R^2) of the standard curve $\geq$0.99 and a slope of the regression curve between -3.2 and -3.5 were required. Also, the standard had to produce a positive result with controls containing $2x10^0$ genomic copies/tube. Duplicates of negative controls of PCR (0 genomic copies) and positive and negative controls of RNA extraction were also included in each analysis. In any case, all samples were analysed in duplicate.

In order to ascertain whether the virus detected was the vaccine or the challenge virus, 23 samples were sequenced for the ORF5 (Mateu *et al.*, 2003). Of these, 15 were random sera selected among those representing the peak of viremia in V and NV groups (all 8 V replicas were represented and 4 samples came from NV groups). After vaccination 4 pigs were still positive by qRT-PCR in serum at 35dpv. For all those four samples, sequencing was attempted and samples from these pigs were re-sequenced using the last qRT-PCR positive sample after the challenge (days 14 or 21 post-exposure to SP).

Data analysis

Main variables examined in the present study and tests used for comparison between groups were: 1) proportion of viremic and shedder animals (χ^2 test); 2) viral load at the peak of viremia (Kruskal-Wallis test); and 3) average length of viremia and shedding (Kruskal-Wallis test). For the purpose of the present study, duration of viremia and shedding period were defined as the number of days between the first and the last occasion in which PRRSV was detected in sera or swabs respectively.

Regarding the dynamic of PRRSV transmission between animals, incidence was estimated from virological data. A survival analysis –time needed for reaching a 50% of infection in contact pigs- , was done by means of the Kaplan-Meier survival test.

In order to quantify the transmission of the virus in the contact animals, for each time interval P_i (i.e., the interval between two consecutive sampling) pigs were classified as "infectious" (I) if positive by qRT-PCR in sera, "recovered" (R) after ceasement of the viremia and "susceptible" (S) for naïve and vaccinated animals while negative by qRT-PCR. When transmission occurred in a pen and a new infectious case (C) is detected, S pigs decreased by one (S-1) whereas the number of I pig increased by one (I+1). Therefore, the whole pen population was made by I+R+S. The transition from S to I occurs according to the transmission coefficient β that represents the adequate contacts between a given infectious animal and a susceptible one in the interval P_i. Considering that the interval P_i between each consecutive testing of animals was 3-4 days (average =3.5 days) and, that the total observation period was 21 days, $21/P_i$ was used to represent the cases detected (C_a) if animals were tested exactly at this interval P_i for the whole 21-day period. β was estimated within each group using the method described previously (Perez *et al.,* 2002; Van Roermund *et al.,* 1999): $\ln(C) = \ln(\beta * S * I)/ N)$ where β was the exponent of $\ln(C_a) - \ln(S*I/N)$ being C_a the number of new infections at the end of each P_i and N the total number of animal in each interval. Finally, the reproduction ratio (R) of V and NV pigs was calculated using the following formula $R=\beta*D$ where D= duration of the infectious period and β= average number of individuals that are newly infected from one infectious individual per unit of time (Vynnycky and White, 2010).

The duration of the infectious period (IP) of each group was considered to be as the average length of viremia of all pigs in a group including the SP. For animals that remained viremic by the end of the experiment, the value of their viremic period was assumed to equal the mean duration of viremia for either all V or NV pigs as appropriate or, alternatively, the duration of the period with positive qRT-PCR results for that given pig, whatever the higher. R values for V and NV pigs were compared by means of the Student's T test. Level of significance for all statistical tests was set at 0.05. Statistical analyses were performed using StatsDirect v.2.7.9.

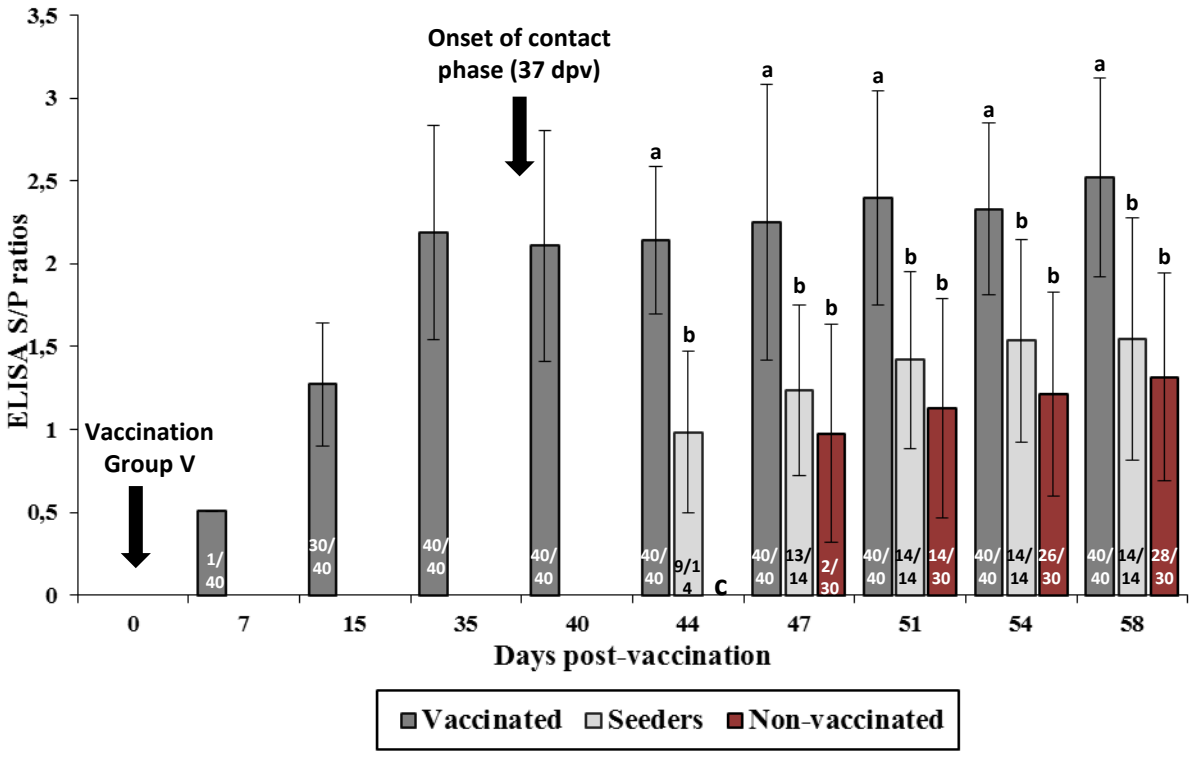
RESULTS

Immune response after vaccination

All vaccinated pigs seroconverted between 7 and 21 dpv (average S/P ratio at 35 dpv, before exposure to SP: 2.2±0.7). Determination of neutralizing antibodies (NA) at day 35 dpv (immediately before the contact with SP pigs) showed VNT titers between 2 log_2 and 4 log_2 when the vaccine virus was used but sera were negative against the challenge virus. All NV pigs were serologically negative (ELISA) by day 35 dpv.

The majority of NV had seroconverted by 51 dpv (14 post-exposure to SP pigs) and by day 58 dpv (21 post-exposure) 93.4% of NV pigs were seropositive by ELISA (average S/P ratio: 1.2±0.6) (Figure 2). Neither vaccinated nor unvaccinated pigs had developed NA against strain 3267 at the end of the study.

Figure 2. Serological response of pigs throughout the experiment (from 0 to 58 dpv). The graph represents average and standard deviations for ELISA S/P ratios for V, SP and NV pigs. Within the bar is shown the number of positive pigs over the total number of pigs in the group. Average ELISA S/P ratios were compared between treatment groups by using the Kruskal-Wallis test; different letters indicate statistically significant differences ($p<0.05$).



The IFN-γ ELISPOT showed that V animals developed cell-mediated immunity just before the contact phase (35 dpv) with an average frequency of 33±29 IFN-γ-SC per million PBMC for the vaccine virus and 68±47 against the challenge virus. At 47 dpv (10 days post-exposure) frequencies of IFN-γ-SC in V animals were 26±31 against vaccine virus and 41±33 against the challenge virus.

Transmission of PRRSV and virological data

At the end of the vaccination phase (35 dpv) all but four of the vaccinated pigs tested negative by qRT-PCR. All SP pigs remained viremic at least until day 19 post-inoculation and most of them (9/14; 64.3%, $CI_{95\%}$: 35.6-86.0%) remained so until the end of the study (23 days post-inoculation). The peak of viremia occurred between 7 and 10 days post-inoculation.

Table 1 summarizes the virological data for NV and V animals. All NV pigs (30/30) became viremic after contact with SP pigs while this proportion was only 53% (21/40) for V animals ($p<0.05$). Figure 3 shows the proportion of viremic pigs in each group distributed by days of exposure to the SP animal. Moreover, the average length of viremia (time between the first and the last day that virus could be detected in sera) was significantly shortened by vaccination, being 12.2±4 days versus 3.7±3.4 in NV and V pigs, respectively ($p<0.01$). Nevertheless, viral loads at the peak of viremia were similar between groups (5.5±1.1, 5.7±0.3 and 5.6±0.75 $\log_{10}$ of genomic copies/mL of serum, for NV, V and SP pigs, respectively).

The first positive qRT-PCR results in each group of NV animals (designated as 1st transmission wave and caused probably directly from the seeder pig) occurred between the 3rd and 7th day post-exposure. In contrast, the 1st transmission wave in V groups took place from 3 to 14 days post-exposure depending on the group. Thus, on average, 2±1.1 NV pigs/pen and 1.5±1.1 V pigs/ pen became infected in the first wave of infection. In subsequent waves of infection (new cases after the initial detection of transmission) differences between treatment groups were higher: 1.8±1 versus 0.9±0.8 and 1.6±0.5 versus 0.4±0.5 in the 2nd and 3rd waves for NV and V pigs, respectively. When the Kaplan-Meier survival analysis was performed, the mean 50% survival time

for V was 21 days ($CI_{95\%}$: 14.1-27.9) compared to 7 days ($CI_{95\%}$: 5.2-8.7) for NV animals ($p<0.01$) (Fig. 4). This reduction in the virus transmission in vaccinated groups was reflected in the R value that was calculated to be 2.78 ($CI_{95\%}$: 2.13-3.43) and 0.53 ($CI_{95\%}$: 0.19-0.76) for NV and V pigs, respectively ($p<0.05$) (Table 2). These data confirmed that, in the present scenario, vaccination resulted in a significant decrease in PRRSV transmission.

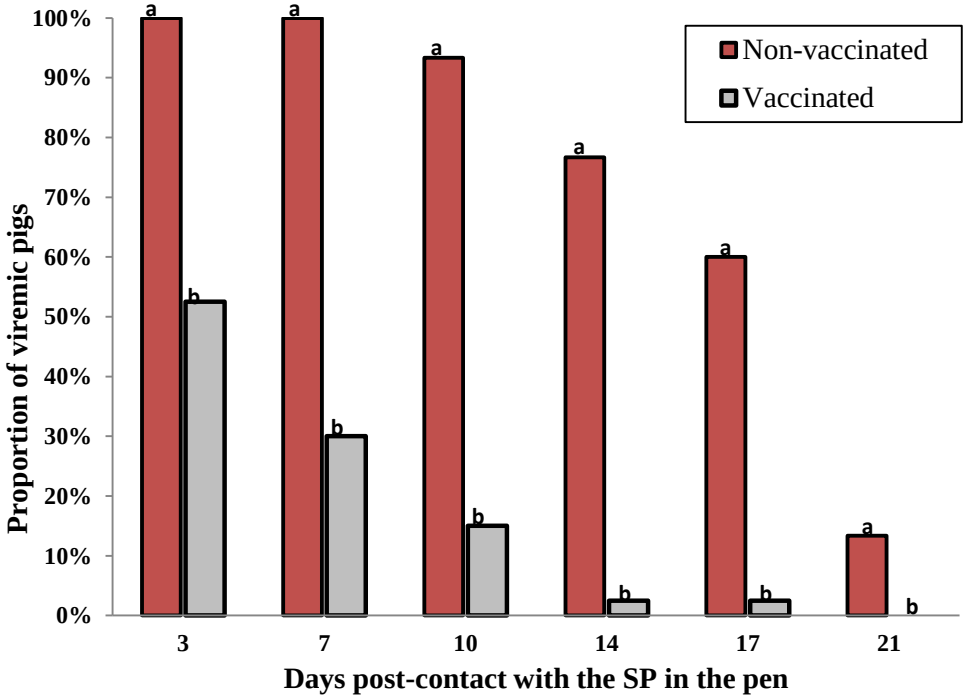
Table 1. Virological data for V and NV pigs during the exposure phase.

Group	Proportion of infected animals/total	Length viremia (days)[a]	Average date of first detection of viremia[a] (days)
NV	5/5	12.6±3.1 (7-14)	7.6±1.3 (7-10)
	5/5	15.8±1.6 (11-14)	7.4±2.9 (3-10)
	5/5	12.8±5.2 (4-18)	8.2±5.2 (3-17)
	5/5	12.8±5.9 (4-18)	6.0±3.0 (3-10)
	5/5	10.6±3.5 (7-14)	10.4±3.5 (7-14)
	5/5	11.4±4.7 (7-18)	9.6±4.7 (3-14)
Total or average	30/30	12.2±4.0	8.2±3.7
V	3/5	2±1.7 (1-3.9)	15±5.6 (10-21)
	4/5	6±5.4 (3-14)	13±4.2 (7-17)
	3/5	6±1.8 (3.9-7)	13.7±6.4 (1.-21)
	1/5	7±NA (NA)	14±NA (NA)
	2/5	1±0 (NA)	3±0 (NA)
	3/5	2.6±1.5 (1-3.9)	16±4 (14-21)
	1/5	7±NA (NA)	10±NA (1NA)
	4/5	3.8±2.5 (1-7)	12±2.3 (10-14)
Total or average	21/40[b]	3.7±3.4[b]	12.6±5.6

NA= does not apply.
[a] Average ± standard deviation (range).
[b] $p<0.05$

Regarding viral shedding, the main results are shown in the table 3. The number of infected contact animals that shed virus by the nasal route at any time was significantly higher in NV animals than V pigs (80.0% (25/30) versus 47.5% (19/40) respectively; $p<0.05$). Furthermore, the average duration of nasal shedding was 6.9±3.2 days in NV pigs compared to 2.1±1.8 days in V ones ($p<0.01$), showing that vaccination significantly reduced viral shedding by this route as well. It is worth to note that the period of nasal shedding differed among pigs within a given group (1-14 days in NV

Figure 3. Proportion of viremic pigs distributed by days of sampling after exposure to SP animals. The number of positive animals by qRT-PCR at each sampling day was compared between V and NV pigs by the χ^2-test. Different letters indicate statistically significant differences ($p<0.05$) between treatment groups.



and 1-11 in V). Fecal shedding was erratic in both treatment groups, and only 40% on infected NV pigs (12/30) had at least one positive fecal swab by qRT-PCR compared to 20% (8/40) of infected V animals ($p<0.01$). The average duration of fecal shedding was 5.2±2.5 days in NV pigs compared to 3.1±2.6 days in V animals ($p>0.05$).

The viral genome was detected by qRT-PCR in the lung of all analyzed NV pigs (4/4) at day 21 post-exposure. Lung samples of V pigs were also positive (5/5), although only one of these animals was still viremic at the end of the experiment.

Sequencing of the 15 randomly taken samples showed that the 4 NV and 10/11 V corresponded to the challenge isolate (>99.5% similarity). Interesting, the vaccine strain was detected at 10 days post-exposure to SP (47 dpv) in a V pig (>99.5% similarity) but that animal was positive for the challenge virus in the next sampling (51 dpv, >99.5% similarity). For V pigs yielding positive qRT-PCR results at 35 dpv, only two samples produced readable sequences (probably because low amount of viral

RNA) that resulted to be the vaccine virus (>99.5% similarity). In contrast, samples from the same group obtained later in the exposure phase corresponded to the challenge virus (>99.5% similarity).

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Figure 4. Graphic depiction of the Kaplan-Meier 50% survival analysis for V (continuous line) and NV (dotted line) pigs. This test indicated the period of time needed to reach the 50% of infected pigs in each treatment group. As indicate on the figure, the mean 50% survival time for V was 21 days compared to 7 days for NV animals. Different letters in the graph indicate statistically significant differences between groups ($p<0.01$), calculated by Kruskal-Wallis test.

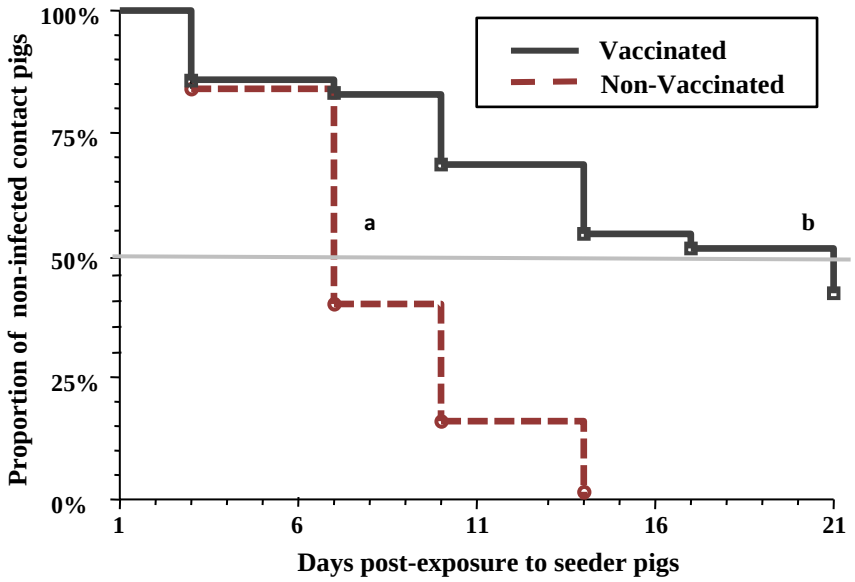

Table 2. Transmission parameter estimates for each replicate and for the overall treatment groups.

Group	IP[a] (days)	Average β	Overall β	R	Overall R
NV	14.0	0.30	0.21 [0.17; 0.25]	4.20	2.78 [2.13; 3.43]
	13.5	0.20		2.70	
	13.0	0.19		2.49	
	13.5	0.23		3.15	
	12.3	0.18		2.19	
	12.3	0.16		1.95	
V	4.5	0.13	0.10 [0.06; 0.13]	0.58	0.53 [0.19; 0.76]
	7.5	0.13		0.94	
	5.8	0.09		0.49	
	4.0	0.03		0.13	
	3.8	0.11		0.43	
	4.3	0.09		0.41	
	4.7	0.03		0.16	
	6.0	0.18		1.07	

IP, infectious period.

Table 3. Viral shedding by the nasal and fecal routes for V and NV pigs.

	Nasal swabs		Fecal swabs	
Group	**Proportion of shedder pigs/total**	**Duration of shedding[a] (days)**	**Proportion of shedder pigs/total**	**Duration of shedding[a] (days)**
NV	3/5	9.3±5 (4-14)	2/5	6.5±4.9 (3-10)
	5/5	6.8±4.5 (3-14)	3/5	3.7±3.1 (1-7)
	3/5	11.7±4 (7-14)	1/5	1±NA (NA)
	4/5	2.5±1.7 (1-4)	2/5	5.5±2.1 (4-7)
	5/5	6.2±3.3 (3-11)	2/5	7±5.7 (3-11)
	5/5	5.0±2.8 (1-7)	2/5	7.5±9.2 (1-14)
Total or average	25/30	6.9±3.2	12/30	5.2±2.5
V	4/5	1±0 (NA)	2/5	1±0 (NA)
	4/5	3.5±2.5 (1-7)	1/5	7±NA (NA)
	2/5	2.5±2.1 (1-4)	0/5	NA
	2/5	1±0 (NA)	1/5	1±NA (NA)
	1/5	1±NA (NA)	1/5	1±NA (NA)
	3/5	1±0 (NA)	1/5	3±NA (NA)
	1/5	1±NA (NA)	0/5	NA
	2/5	6±7.1 (1-11)	2/5	5.5±2.1 (4-7)
Total or average	19/40[b]	2.1±1.8[b]	8/40[b]	3.1±2.6

NA= does not apply.
[a] Average ± standard deviation (range).
[b] $p<0.05$

All sentinel pigs (10/10) housed in pens adjacent to NV+SP pens became infected by the end of the study, with an average of 9.5±3.6 days of duration of viremia and a Kaplan-Meier 50% survival time of 14 days. The average viral load in serum for those pigs at the peak of viremia (7 days post-infection) was 5.8±0.8 $\log_{10}$ of genomic copies/mL. Nasal and fecal shedding pattern in sentinel pigs was also similar to the NV pigs exposed to the SP: all sentinels were shedding virus by these routes (10/10) but with a high variation in the range of days that they did so (from 1 to 21 days). Conversely, in the case of sentinel pigs allocated contiguous to a V+SP pen, only 1/4 animals became infected; the only positive pig was infected by day 17 day after exposure.

DISCUSSION

The efficacy of current PRRSV vaccines in the field is not known with precision and very few data are available regarding their usefulness for reducing PRRSV transmission.

The present study was aimed to gain understanding on the potential of current vaccines when applied in mass vaccination in quasi natural conditions of transmission. The results show that vaccination with a commercial vaccine was effective in terms of reducing most of the biological parameters that can be related to transmission: proportion of infected animals, length of viremia and shedding. Moreover, the number of secondary cases (those appearing after the initial transmission) was also lower in vaccinated animals compared to unvaccinated ones; as a result R decreased to 0.53 in V from 2.78 in NV pigs. The reduction of the infectiousness of V pigs was also supported by the lack of virus transmission to naïve sentinels that shared the same air space with V.

The interpretation of the results of the present study has to be done considering different aspects including at least: the potential biases created by the experimental model, the vaccine and the challenge strain used and, the way in which transmission and infection were assessed.

The transmission model of this study was designed to mimic natural conditions. For this reason, infection was not caused by deliberate inoculation of pigs but by contact with inoculated animals (seeder pigs) that were introduced into the pens at the moment in which viremia was forecasted to start. The effectiveness of this design is demonstrated by two facts: a) at 7 days after exposure NV pigs had been effectively infected because of the contact with the SP pig and, b) pigs infected by contact had a virological course similar to SP pigs. In our opinion, this model fits better the way in which pigs are infected under field conditions and does not overwhelm the immune system with a high dose of virus (usually $>1x10^{5.5}$ $TCID_{50}$) inoculated in the nostrils or intramuscularly. Additionally, the value for R in non-vaccinated pigs is quite similar to the calculation of Charpin *et al.* (2012) with experimentally inoculated pigs ($R_0 = 2.6$) reinforcing the validity of our model.

A second point of discussion is the fact that the SP animals were left in contact with V or NV all throughout the exposure phase, creating a difficulty for the precise evaluation of the transmission. In fact, with such study design we are not able to surely differentiate if the infection is transmitted to the contact pigs from the SP or from another contact-infected animal. Nevertheless, our strategy was to consider that all first cases (namely, all cases in the first day at which viremic pigs were detected in the pen) were attributable to the transmission from the SP pig and that all other subsequent cases in the pen were caused by the infected V or NV. Within a pen, probably the SP contributed more to the transmission than the infected V pigs and therefore, we are probably overestimating transmission between vaccinated animals. This was a worst case scenario for V pigs and even then, the reduction of transmission to or from V pigs was evident. In other words, if the contribution of infected V was lower than our estimation, the efficacy of the vaccine would be higher. For a more precise calculation discriminating two different R that is, one representing transmission from a naïve infected pig to a V pig, and a second representing transmission from V infected pigs to other V pigs a different design would be need. That design should have included a larger number of pigs in order to obtain an accurate measurement of R between V pigs and that would have been extremely difficult to manage considering that the present experiment already included almost one hundred pigs.

Besides this, it can be argued that in field conditions pens of weaners will contain more than the 5 naïve animals that we used in our study (more often 10-20 animals). It cannot be discarded the idea that an increase in the ratio of susceptible animals could have probably increased the value of R. Conversely, the use of larger groups would have made extremely difficult to produce replicas of each pen as we did in our design. Similarly, the way in which the infectious period was calculated (average length of viremia of SP plus contact-infected pigs of each group) tended to overestimate transmission from infected-by-contact vaccinated pigs.

Regarding the vaccine and the challenge strain several points are worth to be discussed. The experimental model included one single vaccination in naïve pigs and then V and NV animals were left alone for 35 days before entering in contact with SP pigs. The reason for this long period was to let the pigs establish a solid immune response and to avoid interference with the detection of vaccine virus. Recently, it has been shown that after vaccination with the MLV used in our study, less than 40% of pigs remained viremic at three weeks post vaccination and most of lymphoid tissues were already negative at that time (Martínez-Lobo *et al.*, 2013). Thus, in our study it was expectable that most of pigs, if not all, were not viremic by vaccination at 35 dpv. In spite of our caution, in one pen at least two pigs were still positive by qRT-PCR because of the vaccination at 35dpv. Probably, a shorter period after vaccination could have influenced the results of the study. We also find that sera samples of a V pig showed sequences of MLV and 3267 strains (at 47 and 51 dpv, respectively), suggesting a possible coexistence of the two isolates or a recombination event between them. From our point of view, if some animals would yielded positive by qRT-PCR starting the exposure phase because the length of vaccine viremia, that would mean even a greater efficacy of the vaccine since the real number of infected pigs by the challenge virus would be lower. Thus, despite we are assuming again the worst case scenario for V pigs, the impact of vaccination on the viral transmission is still evident. Regarding the challenge strain 3267, when used in previous experiments (Díaz *et al.*, 2012) it was shown that most animals developed a viremia long enough to cover the entire exposure phase, being thus adequate for the purpose of the present study. The results of the infection with strain 3267 can be considered as typical of an average

genotype 1 subtype 1 isolate when inoculated in young pigs (Martínez-Lobo *et al.*, 2011). It is difficult to speculate what would have happened using a different heterologous strain but it is reasonable to think that virulence and antigenic similarity would play a role. For example, Nodelijk *et al.* (2001) estimated R using a genotype 2 MLV and a genotype 1 challenge strain (the prototype Lelystad virus) and were unable to clearly show vaccine efficacy (Nodelijk *et al.*, 2001).

Regarding the way in which transmission was assessed, the use of a sensitive qRT-PCR using samples taken every 3-4 days combined with sequencing is, in our opinion, a stringent approach. Certainly, the possibility of missing some V animals that were infected by contact with SP exists, particularly if they did not develop viremia at all or the length of the viremia was shorter than the interval of sampling. However, in both cases we considered that the potential contribution of those animals to transmission had to be very low, especially when shedding was not detected as well.

We have shown that V pigs had a probability of getting infected significantly lower than NV and that virus transmission within a pen of vaccinated pigs is lower than in unvaccinated groups. Moreover, since the length of viremia and shedding also decrease in V pigs, the viral load of vaccinated groups decreased as well. In practical terms, this probably means that vaccination has at least the potential for slowing the transmission of type 1 PRRSV and as a consequence, to limit the impact of infection. The decrease of the viral load in V pigs also probably resulted in a decrease of the likelihood of airborne transmission or transmission by fomites.

In our experience, the outcome of vaccination programs in European farms is diverse. While some farms report good results with the stabilization of the viral circulation, others report re-infections and failures to control the disease. To understand this it is necessary to consider that management of the animals (for example, movements within the farm), lateral introductions of the virus, incorrect vaccination practices and the immediate exposure of vaccinated pigs to the virus after vaccination are factors that will contribute to the final outcome of the vaccination program. All those factors were absent in our experiment. The present paper demonstrates that an experimental model of transmission by contact is adequate and useful to evaluate the efficacy of a PRRSV

vaccine. Also, in the present model, vaccination was efficacious enough to reduce transmission of the infection and all data suggested that vaccinated pigs were significantly less efficient to transmit the infection than naïve counterparts. These results indicate that there is a potential for the use of mass vaccination in control programs for piglets and that slight increases in vaccine efficacy might result in much better performance for control purposes.

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THIRD STUDY

Vaccination of piglets with a genotype 1 modified live PRRS virus vaccine delays transmission between pigs and decreases viral load in a one-to-one transmission experiment.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterized by abortion and other reproductive disorders in sows and respiratory disease in piglets and growing pigs (Done *et al.*, 1996; Rossow, 1998). Economic losses associated to PRRS make this disease one of the most costly for the swine industry worldwide. Thus, Holtkamp *et al.* (2013) estimated the annual cost of PRRS in USA to be about $663 million/year. More than 50% of such cost was attributed to the impact of the disease in weaners and grower pigs.

Control of PRRS virus (PRRSV) infection relies mainly in four pillars: diagnosis and monitoring, biosecurity, herd management and immunization. Vaccination against PRRSV is most often applied to sows in order to prevent reproductive problems (Mengeling *et al.*, 1999; Scortti *et al.*, 2006a; 2006b). The use of vaccines in piglets is more controversial since they have to be applied at least 3-4 weeks before the infection takes place and the result is usually poor if the virus is actively circulating in the breeding herd and weaners are infected early in live.

At present, several commercial vaccines are marketed, including modified live vaccines (MLV) and inactivated vaccines (IV). Initial immunization against PRRSV has to be done with MLV since IV are less immunogenic (Scortti *et al.*, 2007; Zuckermann *et al.*, 2007). In any case, heterologous protection is always partial (Geldhof *et al.*, 2012; Mengeling *et al.*, 1998; Scortti *et al.*, 2007).

Recently, two independent studies have shown that in a genotype 1 subtype 1 model of infection by contact, the reproduction rate (R) in vaccinated pigs was significantly reduced to a range between 0.53 (Pileri *et al.*, 2015) and 0.30 (Rose *et al.*, 2015). In addition, vaccination was effective in terms of reducing most of the biological parameters that can be related to transmission, such as the proportion of infected animals and the length of viremia and nasal shedding.

The agreement between those two studies using different challenge isolates suggests that for genotype 1 PRRSV, vaccination has the potential to stop transmission. However, in those experiments the transmission model used was based in groups and thus the exact contribution of each participating pig is difficult to assess. This is particularly important for understanding the inter-individual variation. One-to-one experiments are a "worst-case scenario" but permit to examine the individual contribution of each pig to transmission and to determine the limits of vaccine efficacy (Velthuis *et al.*, 2002).

The aim of the present study was to assess the PRRSV transmission by contact to vaccinated and unvaccinated pigs in a one-to-one basis. Additionally, transmission from vaccinated pigs that were infected to other vaccinated animals was assessed as well.

MATERIALS AND METHODS

Animals and experimental design

Figure 1 summarizes the design of the experiment that was done under permission of the Ethics Commission for Human and Animal Experimentation of the Universitat Autònoma de Barcelona and by the the Departament de Medi Ambient i Habitatge (nº 5796) of the Autonomous Catalan Government.. Fifty three-week-old piglets (Landrace x Pietrain) were obtained from a PRRSV and Aujeszky's disease virus negative farm. Animals were vaccinated at weaning (3 weeks of age) against *porcine circovirus type 2 (PCV2)* and *Lawsonia intracellularis.*

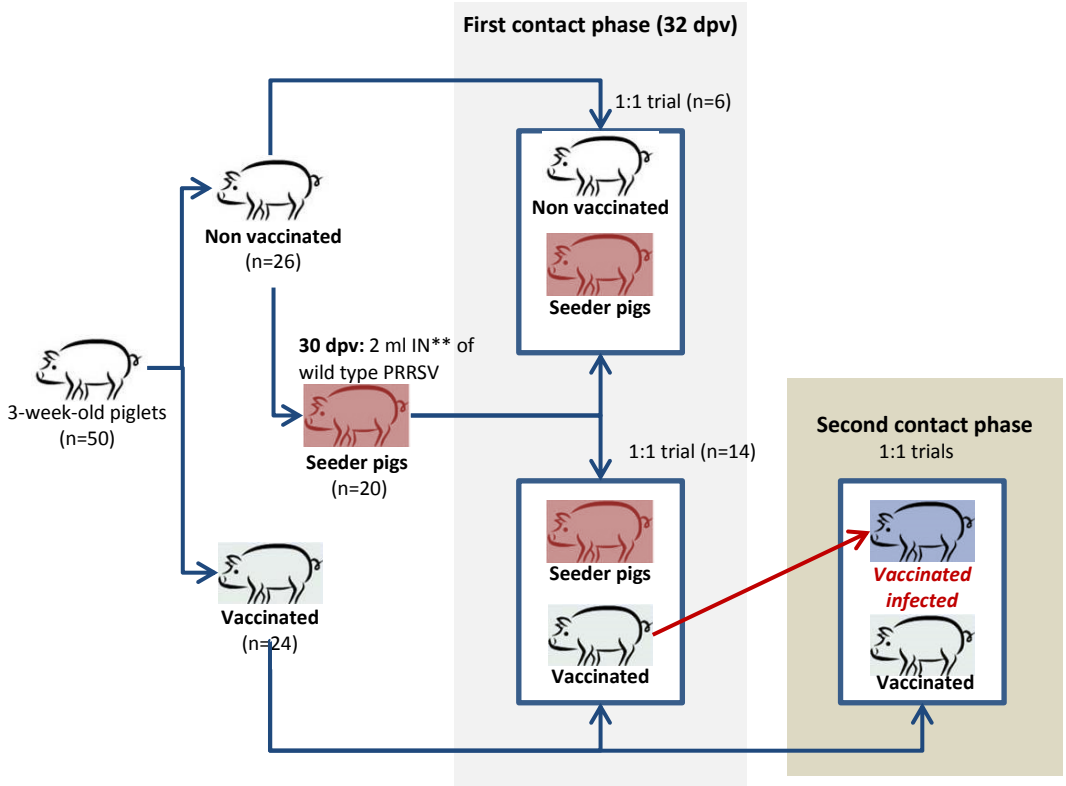
Experimental facilities were conventional weaning units (n=7) with physical separation between rooms (solid walls, no air filtering). Rooms 2 to 6 were structurally modified before the beginning of the study in order to have 12 individual pens each (0.8 m x 1.6 m) separated by solid walls. No other animals than those included in the study were housed in the facilities for the duration of the experiment.

After arrival to the experimental farm, piglets were left to acclimatize for 1 week. Then, they were ear-tagged and randomly divided (random numbers) in two groups,

designated as V (n=24) and NV (n=26) that were housed in rooms 1 and 7, respectively. V pigs were administered intramuscularly a 2 ml dose of a commercial modified live PRRSV vaccine (MLV) (PORCILIS PRRS®, MSD Animal Health) according to manufacturer instructions.

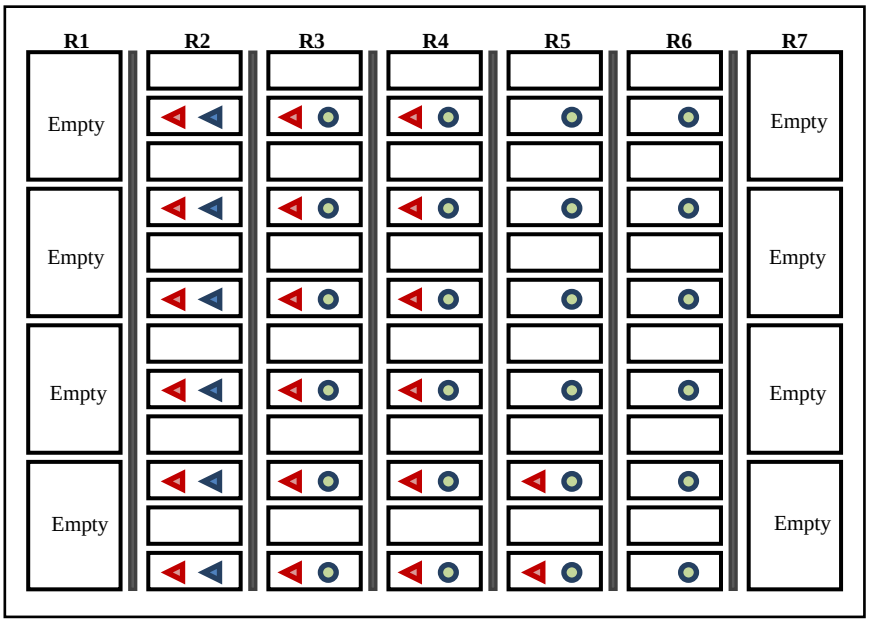
Group NV was left unvaccinated and remained as a naïve control. Twenty-eight days post-vaccination (dpv), all V and 6 NV were distributed in individual pens, from room 2 to 6 of the experimental facilities. Each 1:1 group was separated from the other by a solid partition and an empty space equal to the size of a pen; nevertheless, the air space was shared between animals.

Figure 1. Design of the experiment. Three-week-old piglets were divided in two groups, V and NV. V pigs (n=24) were vaccinated with a commercial MLV PRRSV vaccine while NV (n=26) were kept as controls. Thirty days later, 20 NV (namely) were separated in a different place and were inoculated intranasally with the wild-type genotype 1 PRRSV isolate 3267 (now on seeder pigs (S)). In parallel, the remaining NV and all V animals were distributed in individual pens. Two days later, namely at 32 days post-vaccination S pigs were mixed 1:1 with either an NV (six 1:1 replicas) or a V (12 1:1 replicas). This was the start of first contact phase. Follow-up of pigs was done for 21 days. During this 21-day period animals were bled periodically and each time a V pig was detected as viremic as a consequence of the virus transmission from a S, the infected V was immediately moved to another pen where it was mixed with a new V pig.



At 30 dpv, the remaining 20 NV pigs were housed in an isolated room and inoculated intranasally with 2 ml (1 ml/nostril) of a suspension containing 105.5 TCID50/ ml of the genotype 1 PRRSV strain 3267 (Genbank accession number JF276435) used in previous studies (Darwich *et al.*, 2011; Díaz *et al.*, 2012; Gimeno *et al.*, 2011; Pileri *et al.*, 2015). Viral stock was produced from a sixth passage in porcine alveolar macrophages (PAM) and was shown to be free of PCV2, Mycoplasma hyopneumoniae, and Torque-Teno Sus virus by PCR (Mattsson *et al.*, 1995; Quintana *et al.*, 2001; Segalés *et al.*, 2009). Overall nucleotide similarity (ORFs 1-7) between challenge and vaccine virus was 93.4% (Darwich *et al.*, 2011). The inoculated animals were designated then onwards as "seeder" pigs (S).

Figure 2. Distribution of animals at the beginning of the first contact phase (32 dpv). Rooms 2 to 7 contained 12 pens each with solid walls between them. An empty space was left between each occupied pen. Different treatments are represented as follows: Red triangles= seeder pigs, blue triangles = unvaccinated pigs, circles = vaccinated pigs.



Two days later (namely, 32 dpv) one S was introduced into each pen to expose either vaccinated or naïve pigs (first contact phase). Ten V pigs -designated from now on as V_c, were kept separately. As soon as a V pig was detected to be viremic as a result of the transmission of the virus from an S, the infected V (now on V_{inf}) was transferred (<24 h after detection) to a new pen where it was comingled with a V_c pig (second contact phase). For the first contact phase, pigs were maintained at maximum 21 days

and for the second contact phase the maximum exposure period was 14 days. Figure 2 depicts the distribution of pigs at the beginning of the first contact phase.

Collection and processing of samples

Special measures were taken in order to avoid transmission of PRRSV by fomites or personnel during the course of the experiment. Among others, personnel wore new coveralls and boots before entry in each room. Once inside a room, and before entering each pen, personnel wore additional disposable coveralls, boots, gloves, hairnets and face masks that were discarded after leaving the pen. Moreover, a separate restraining snare, needle and syringe was used for each animal and the sampling order was strictly kept for every sampling.

Blood samples were taken the day of vaccination to confirm the PRRSV-negative status of the animals. Then, pigs were bled at 14 and 30 dpv to assess the seroconversion. During the first exposure phase (from 32 to 53 dpv) blood samples were collected at days 0, 3, 5, 7, 10, 12, 14, 17, 19 and 21 after introduction of the S ping into the pen (namely, 32, 35, 37, 39, 42, 44, 46, 49, 51 and 53 dpv). For the newly V_{inf}:V_c groups created during the first exposure phase blood samples from these pigs were collected as above and also at 56, 58, 60, 63 and 65 dpv, depending on the starting date of the second exposure phase. Sera were obtained by centrifugation at 1,500 x g for 10 minutes and aliquots that were not analysed immediately were stored at -80ºC. Heparinized blood samples were taken at 28 and 49 dpv from 14 randomly selected pigs (6 NV and 8 V) in order to obtain peripheral blood mononuclear cells (PBMC).

Additionally, in order to evaluate the presence of macroscopic lesions and assess viral distribution, all pigs were euthanatized and necropsied at the end of the study. Samples of tonsils were collected and stored at -80°C until examined for the presence of virus.

Evaluation of the immune response

Sera were analyzed for specific PRRSV antibodies by means of a commercial ELISA (Idexx PRRS X3 Ab Test®, Iddex Laboratories). Results were expressed as sample to

positive control (S/P) optical density ratios. According to the manufacturer, samples with S/P ≥0.4 were considered to be positive.

The ELISPOT for the enumeration of PRRSV-specific IFN-γ-secreting cells (IFN- γ-SC) was performed at 28 dpv and 49 dpv (namely, 17 days post-exposure to S pigs) according to Díaz *et al.* (2005) using the challenge strain at a multiplicity of infection of 0.1.

Virological analysis

Quantitative one step RT-PCR (qRT-PCR) targeting viral ORF7 was carried out the same days of the collection of samples using the method described by Pileri *et al.*, (2015). Viral RNA was extracted from sera in 96 well-plates using the BioSprint® 96 One-For-All Vet kit (Qiagen) and the BioSprint 96 workstation (Qiagen) according to manufacturer instructions. Extraction of viral RNA from tonsils was done using the *Total RNA isolation Nucleospin® RNA II* kit (Macherey-Nagel), following manufacturer directions and starting with 1mg of tissue. The presence of virus in tissues was also determined by qRT-PCR as above.

For all V pigs, in order to discriminate between challenge and vaccine virus the first serum sample yielding a positive result by qRT-PCR and having $\geq 10^4$ genomic copies/ml was sequenced using previously described primers for the ORF5 (Mateu *et al.*, 2003).

Data analysis

Main variables examined in the present study and tests used for comparison between groups were: 1) proportion of viremic animals (χ^2 test); 2) viral load at the peak of viremia (Kruskal-Wallis test); and 3) average length of viremia (Kruskal-Wallis test). For the purpose of the present study, duration of viremia was defined as the number of days between the first and the last occasion in which PRRSV was detected in sera. In addition, the area under the curve (AUC) of viral load in sera over time of each group of pigs was calculated, as described by Schafer *et al.* (2001).

Level of significance for all statistical tests was set at 0.05. Statistical analyses were performed using StatsDirect v.3.0.9.7.

Additionally, in order to evaluate the presence of macroscopic lesions and assess viral distribution, all pigs were euthanatized and necropsied at the end of the study. Samples of tonsils were collected and stored at -80°C until examined for the presence of virus.

RESULTS

Immune response after vaccination

All V animals seroconverted before the onset of the contact phase (30 dpv) (average S/P: 3.1±0.9) while all NV pigs remained negative. At the end of the first exposure phase all NV had seroconverted as a result of contact with S pigs and showed an average S/P ratio of 2.2±0.3 that was similar to the S/P value of V pigs at that time 2.5±0.4.

Before the contact phase (28 dpv), 7/8 V pigs tested positive for the CMI using the challenge virus with an average frequency of 42±30 IFN-γ-SC/10^6 PBMC ($CI_{95\%}$: 19-64) whereas NV were negative. At 17 days post-contact (dpc), the average frequencies for V pigs increased (8/8 positive pigs, average frequency 68±44 IFN-γ-SC/10^6 PBMC; $CI_{95\%}$: 37-99). Regarding NV pigs in contact with S, the average frequency of IFN-γ-SC at 17 dpc was 27±15/10^6 PBMC ($CI_{95\%}$: 12-41; 4/6 responding pigs).

Virological data

All S pigs had become viremic by the second day post-inoculation (dpi) and remained so for at least the following 19 days. Most of them (12/18; 66.7%) were still viremic at the end of the contact phase (23 days post-inoculation). The peak of viremia occurred between 7 and 10 days post-inoculation.

At the end of the vaccination phase (30 dpv) 17 of 24 vaccinated pigs tested positive by qRT-PCR (all $\leq 10^4$ genomic copies/ml). Virus from four sera samples of these animals could be sequenced and resulted to be vaccine virus (>99.5% similarity). Two days later (32 dpv), at the start of the first contact phase, these pigs became negative

by qRT-PCR and from then onwards remained free of vaccine virus. However, two V pigs negative in sera for the presence of virus at 30 and 32 dpv were shown to harbor the vaccine virus (>99.5% similarity) when they were relocated with the corresponding V_c pigs at 3 and 7 dpc, respectively. Therefore, these two V pigs and the two V_c animals that they contacted, had to be excluded from the study. The infection with the challenge strain was confirmed by sequencing analysis in 12/12 remaining V_{inf} animals (>99.5% similarity). Regarding the V_c pigs remaining in the study (n=8), the sequencing analysis confirmed that 7 of them were infected by the challenge virus (>99.5% similarity).

Table 1 summarizes the virological data for NV, V_{inf} and V_c animals. All NV pigs became viremic after contacting S animals after an average contact period of 5.5±2 days. Most of the NV pigs (4/6) were still viremic at the end of the first contact phase. All V pigs (12/12) exposed to S animals also became infected during the exposure period although with a different pattern compared to NV. Thus, for V_{inf} pigs the first detection of viremia occurred at 13.6±3 dpc, namely one week later compared to NV ($p< 0.05$). Also, the mean duration of viremia was different for V_{inf} compared to NV: 15.5±2 days ($CI_{95\%}$: 13.4-17.6) versus 5.5±4 days ($CI_{95\%}$: 3.0-7.9) ($p< 0.05$). However, the V_{inf} group showed remarkable differences: eight animals had a short viremic period of 5 or less days (3.0±1.4) while the remaining four had a longer viremic period of more than one week (10.8±2.9). This was not seen for NV. Viral loads at the peak of viremia were similar between V_{inf} and NV groups (6.5±1.0 and 6.3±1.6 log_{10} of genomic copies/mL of serum, respectively) although the AUC for viral load in serum over time were 0.98 and 0.87, respectively ($p<0.05$).

In the second contact phase, transmission from V_{inf} to V_c pigs also occurred in 7/8 cases (87.5%; $CI_{95\%}$: 46.7%-99.3%). The average contact time needed to transmit the infection to V_c pigs was on average 5.3±1 days.

Figure 3 shows the number of viremic pigs in each group distributed by days of exposure to the S or the V_{inf} animals. The mean duration of viremia of the infected V_c was 4.9±3 days ($CI_{95\%}$: 2.3-7.3) similar to that of pigs infected in the first exposure phase although again, two different patterns were observed: two animals had viremias

Table 1. Virological data for NV, V_{inf} and Vc pigs during the first and second exposure phases.

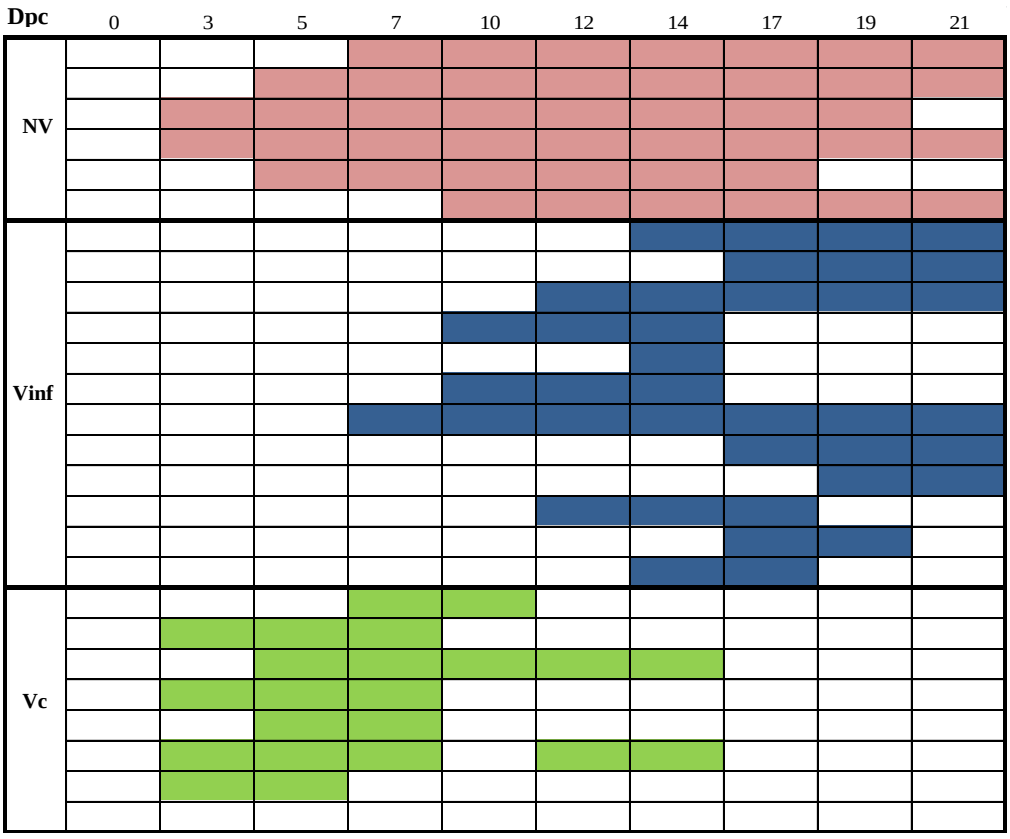
Group	Proportion of infected pigs/total	Length viremia (days)*	Average date of first detection of viremia (days)[a]	AUC
NV	6/6	12.5±2.7 (11-18)	5.4±2.7 (3-10)	0.98
V_{inf}	12/12	5.5±4.3 (1-14)[a]	13.6±3.6 (7-19)[a]	0.87
Vc	7/8	4.8±3.4 (2-10)[a]	5.3±1.7 (3-8)	0.79

* Average ± standard deviation (range)

[b] $p<0.05$

of 9-10 days and the rest averaged 3.0±1.4 days of viremia (range: 2-5 days). The viral loads at the peak of viremia were similar in all cases (average 5.4±1.4 $\log_{10}$ of genomic copies/mL of serum). The AUC of viral load for infected V_c was 0.79, less than for the other groups ($p<0.05$).

Figure 3. Individual virological data for NV, V_{inf} and Vc pigs after exposure to the seeder or an infected V. Filled squares represent positive results by qRT-PCR from sera. Dpc: days post-contact



The viral genome was detected by qRT-PCR in the tonsils of all infected pigs with no differences in viral load (7.4±2.5, 8.2±1.0, 8.1±1.2 genomic copies/g of tissue for NV, V_{inf} and infected V_c). Of interest, the vaccine virus (>99.5% similarity by sequencing) was detected in the tonsil of the V_c that was not infected in the second exposure phase.

DISCUSSION

Any intervention leading to a decrease in the likelihood of transmission of PRRSV within or between farms is favorable for the purpose of controlling the disease. Vaccination is one of the tools that can be used with this aim although the efficacy of current PRRSV vaccines for stopping transmission is not known with precision. Some studies have dealt with the evaluation of virus transmission to vaccinated and unvaccinated pigs (Mondaca-Fernández *et al.,* 2007; Nodelijk *et al.,* 2001; Pileri *et al.,* 2015; Rose *et al.,* 2015; Velthuis *et al.,* 2002) and for genotype 1, there are evidences that vaccination of piglets can significantly reduce the PRRSV transmission and the duration of viremia and shedding periods (Pileri *et al.,* 2015; Rose *et al.,* 2015). However, in Pileri *et al.* (2015) and Rose *et al.* (2015) the transmission model selected considered the use of pen-based groups and thus the individual contribution of each pig could not be assessed with precision.

The present study was conducted in a one-to-one basis in order to obtain individual estimates of virus transmission to and from vaccinated pigs as well as to assess individually the course of the infection in vaccinated pigs. This model represents a "worst-case scenario" scenario for assessing the effect of vaccination since the exposed animals have a continuous contact with an infected animal during the acute phase of the infection, when infectiousness can be considered at its maximum. As a consequence, the results have to be interpreted as the less expectable efficacy of the examined vaccine in a case of heterologous challenge.

The results of the present study show that vaccination with a commercial vaccine was effective in terms of reducing biological parameters that can be related to transmission, such the duration of viremia and viral load although in the conditions of the experiment all vaccinated animals became infected after 21 days of contact with S

pigs. In global, and compared with previous studies, the efficacy of the vaccine in the present model was lower. As indicated by Velthuis *et al.* (2007) in transmission experiments, the smaller the number of susceptible pigs, the higher the possibility that all susceptible pigs can be infected, even when the R<1.

Nevertheless, the time needed for an effective transmission was significantly delayed by vaccination, since V pigs become infected at least one week later compared to NV. This was not the case when Vc were exposed to V_{inf}. This is in apparent contradiction with the observations of the first contact phase. One first element for explaining this is that when the infected V_{inf} were introduced into the pens of Vc some fighting between pigs was observed in the first 24-48h until hierarchies were established. Bierk *et al.* (2001) indicated that aggressive behavior may play a role in PRRSV transmission in sows and a similar circumstance can occur in piglets. Also, the intramuscular route is assumed to be the one with the lowest minimal infectious dose (Hermann *et al.*, 2005) and thus the bite of a 30-40 Kg weight pig can well inoculate a sufficient amount of saliva to be effective in transmission. Besides this, V_{inf} were at maximum of its shorter viremia during those days and this, again, would be a worst-case scenario. Additionally, the stress resulting of this fighting may have also contributed to a decreased resistance to infection. However, with our data these are just hypothesis.

Interestingly, even in these conditions 1/8 Vc pigs withstand 14 days of contact with a V_{inf} without resulting infected, a fact that suggests that there was still full protection for some animals even in the worst conditions. It could be argued that the second exposure phase ended too soon and that if extended up to 21 days, infection could have reached 100%. Previous studies (Pileri *et al.*, 2015; Rose *et al.*, 2015) indicated that the duration of the infectious period in V_{inf} ranges between 5 and 12 days. Therefore, extending the second exposure phase should have not changed our outcomes.

In any case, vaccination resulted in a significant reduction of viremia in vaccinated pigs compared to NV. However, there was not a clear correlate between the strength of the cell-mediated immunity as measured in the ELISPOT and the duration of viremia. In our opinion, this result indicates the need for revising the value of the IFN-γ ELISPOT for measuring effective immunity in PRRSV or, at least, the need to refine

the test using different cell subsets or combined cytokine evaluations. The existence of "short viremic" and "long viremic" pigs suggests a role for the genetic background of pigs in the immunity developed by vaccination.

Regarding the vaccine and the challenge strain several points are worth to be discussed. According to Martínez-Lobo *et al.* (2013), most genotype 1 MLV replicate extensively in naïve piglets and this explains why 17 pigs were still positive by qRT-PCR because of vaccination at 30 dpv and two additional animals still presented MLV virus in sera at 35 and 39 dpv (the latter had to be eliminated from the study because they were moved to a Vc pen). The inability of our qRT-PCR to discriminate between vaccine and challenge viruses is a limitation and since we did not sequenced all qRT-PCR positive samples, it cannot be discarded that some of the positive results after contact with S could be vaccine virus. However, based on the short duration of viremia observed in V pigs in previous studies we preferred to favor sensitivity against specificity.

With regards on the challenge strain 3267, the resulting infection can be considered as typical of an average genotype 1 subtype 1 isolate when inoculated in young pigs (Martínez-Lobo *et al.*, 2011). It cannot be discarded that by using a more virulent isolate, different results could have been obtained.

In summary, we have shown that vaccinated pigs had a significant lower duration of viremia compared to NV pigs and that their viral load, measured in terms of AUC, was also reduced compared to NV animals. In addition, the first detection of viremia in V pigs occurred one week later compared to NV. Thus, although our experimental design represents the worst scenario for evaluating the effect of vaccination on viral transmission, vaccination still can have beneficial effects for slowing transmission and reduce the global viral load.

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GENERAL DISCUSSION

Porcine reproductive and respiratory syndrome virus was identified over two decades ago and soon it was evident that control of this infection had to become one of the main goals in the health policies of the swine industry because of the economic losses associated to the disease. Understanding of PRRSV immunity and transmission are essential for the purpose of controlling the disease through vaccination. However, the data available on these topics are scarce and often contradictory.

In infected pigs, the virus is shed in nasal secretions, saliva/oral fluids, feces, milk and colostrum and semen (Christianson *et al.,* 1993; Yoon *et al.,* 1993; Rossow *et al.,* 1994; 1995; Swenson *et al.,* 1994; Christopher-Hennings *et al.,* 1995a; 1995b; Nielsen *et al.,* 1997; Wills *et al.,* 1997b; Christopher-Hennings *et al.,* 2001; Wagstrom *et al.,* 2001; Prickett *et al.,* 2008; Kang *et al.,* 2010; Kittawornrat *et al.,* 2010). It is assumed that transmission can occur by direct contact (Bierk *et al.,* 2001a; Wills *et al.,* 1997a), through the air (Trincado *et al.,* 2004b; Torremorell *et al.,* 1997; Wills *et al.,* 1997a) or by fomites (Dee *et al.,* 2002; Otake *et al.,* 2002c; Pirtle and Beran, 1996) or iatrogenically (Otake et al., 2002d), although direct contact is assumed to be the main way of contagion. Also, since the infectious period lasts several weeks and most infected pigs survive the infection, the risk for introducing the infection or spreading it in a farm is high. According to Corzo *et al.* (2010) commingling of pigs of different PRRSV status represents the most important route for virus spread.

The first step in controlling PRRSV in a farm is to obtain weaned pigs free of the virus. This means that the infection is no longer circulating in late pregnancy sows and that "downstream" infection in nursery and finishing populations will have a lesser impact. Thus, PRRSV control in a farm always begins with the breeding herd. After stabilization of the breeding population, the control of PRRSV in growing pigs is often achieved by solely pig flow strategies, such as all-in/all-out movement of pigs by facility or site along with biosecurity techniques. As a matter of fact, strategies such as partial depopulation or piglet vaccination are not effective unless the farm is stabilized (Dee and Joo, 1994b).

Nevertheless, in endemically infected farrow-to-finish farms sometimes is very difficult to make a distinction between what happens in sows and what happens in nurseries. This is so because management and facilities are often designed in a way that either does not permit to avoid contact between different production phases or does not permit the application of adequate management policies.

One of the measures that can be used to reduce or stop the infection is vaccination. In a general sense, at a population level vaccination is aimed to decrease the proportion of susceptible animals and if animals are eventually infected, to decrease their contagiousness. At present, several PRRSV vaccines, including MLV and IV, are being marketed but in all cases the protection that they confer at an individual level is just partial. This means that vaccinated animals can be infected in a challenge experiment with a heterologous isolate but often show reduced viremia compared to naïve controls (Martelli *et al.,* 2007; Zuckermann *et al.,* 2007). In practical terms, given the genetic diversity of PRRSV all challenge situations in the field can be considered as heterologous (Murtaugh *et al.,* 2010).

However, for the purpose of controlling a disease in a population, a vaccine does not necessarily have to produce sterilizing immunity but to primarily reduce the probability of transmission of the infection within and between herds (Bouma *et al.,* 1997). Herd immunity will be thus the result of reduced virus transmission, which in turn is the result of the combined effect of a decreased infectivity of infected vaccinated individuals (less virus shed during the whole infectious period) and a decreased susceptibility of vaccinated animals (generally by an increase in the minimum infectious dose required to infect the animal). The effectiveness of a vaccine in epidemiological terms related to stop transmission can be evaluated by means of the quantification of the reproduction rate (R).

A representative example of how a disease can be eradicated even without having a 100% effective vaccine is what happened with Aujeszky's disease (AD) control campaigns in many countries of Europe. Similarly to PRRSV, the available vaccines against AD virus (ADV) are not 100% efficacious at individual level (Stegeman, 1995) but it was well documented that they produce a substantial reduction in

shedding of the virus and reduce the susceptibility of vaccinated animals (Pensaert *et al.,* 1990; Van Oirschot, 1991; Vannier *et al.,* 1995). As a result, under experimental conditions, R of double ADV-vaccinated pigs was estimated to be between 0.3 and 0.7 (Bouma *et al.,* 1995; De Jong and Kimman, 1994) whereas R=10 in unvaccinated animals (De Jong and Kimman, 1994). In the field, R for ADV transmission was estimated to be 0.7 for multiple-vaccinated breeding animals while was determined to be 3.4 among single-vaccinated finishing pigs, and 1.5 among double-vaccinated finishing pigs (Stegeman *et al.,* 1997). Although R for vaccinated finishing pigs in field conditions was above one, the R for the whole herd in case of vaccination was estimated to be less than one, supporting the fact that eradication of the Aujeszky's disease was even possible by means of mass vaccination of the animals (Stegeman *et al.,* 1997). A similar case could be this of PRRSV. Surprisingly, the number of studies dealing with the quantification of PRRSV transmission is very scarce and, at moment, only one of them (Rose *et al.,* 2015) was able to demonstrate a significant reduction of R below one as a result of the vaccination.

In the present thesis we aimed to preliminary evaluate the potential of vaccination to stop genotype 1 transmission of PRRSV. In the first study we determined the basic reproduction rate of PRRSV infection in two endemic farrow-to-finish farms. To our knowledge, no other field studies assessing R in growing pigs have been published and therefore no previous estimation of R in real conditions was available. Our results show that R, as calculated for weaners and fatteners, ranged between 3.5 and 5.3. This range agrees with the results of experimental studies in piglets (Charpin *et al.,* 2012; Rose *et al.,* 2015) and although is a little higher than the estimate R for sows (3.0 according to Nodelijk *et al.,* 2000), R=5.3 is still compatible with a relative slow transmission of the virus. This is particularly evident if compared for example with an R of 100 and 15 for classical swine fever virus in weaning and finishing animals, respectively (Klinkenberg *et al.,* 2002). Moreover, the R value found in farm 1 for naïve piglets of our first study was quite similar to the R value for single-vaccinated growing pigs in the case of ADV (Stegeman *et al.,* 1997) and therefore, the potential for reducing R substantially by vaccination can be considered high. However, the observational nature of the first study and the lack of control over transmission

conditions made difficult to assess several important parameters related to transmission such as the duration of shedding, the time needed to transmit the infection to naïve contact penmates, etc. Also, it was difficult to predict whether or not those R values can be extrapolated.

Considering this, in the second study of the present thesis, we focused in the assessment of R in vaccinated pigs but also in evaluating viremia and viral shedding as biological indicators of the reduction of the infectious pressure. In this case, we selected a model of transmission by contact with seeder pigs unlike to most vaccine efficacy experiments. The reason for that decision was based on the fact that under natural conditions transmission takes place mostly by direct contact. Moreover, evidences from field cases of use of MLV vaccines in piglets suggested that the efficacy in reducing transmission of the virus could be higher than what could be inferred from experimental models. Although these evidences were not obtained from controlled experiments, we reasoned that they were strong enough to consider the possibility that the inoculation challenge models underestimated vaccine efficacy because they could overwhelm the immune system. Also, we decided to perform a group experiment, namely, to use one seeder pig introduced in a group of five naïve or vaccinated pigs. Again the decision for this model relied in our belief that the model fitted better reality.

In agreement with Rose *et al.* (2015) the result of our study showed that the use of MLV was effective in reduce R below one. Also, vaccination reduced significantly viremia and viral shedding, the biological parameters related to transmission. Besides this, 3/4 sentinels close to the pens of vaccinated-challenged pigs and sharing the same air space were not infected while the totality (10/10) of those pigs housed close to naïve-infected pigs became infected. This was a clear indication that vaccination reduced viral shedding enough to significantly reduce aerogenous transmission. It can be argued that the model used was somewhat biased by the fact that animals were not exposed to the virus during the first month after vaccination, which could be not the case in the field. The argument is right but in order to assess the potential of

vaccination to stop transmission it was necessary to establish first a model with well-immunized animals, at least at the full efficacy that vaccine could induce.

The following step in the thesis was to assess transmission from vaccinated-infected pigs to other vaccinated pigs. In this case the use of a transmission model in groups was quite difficult for several reasons. First of all because of the difficulty to differentiate between vaccinated and infected animals. Secondly, because given the shorter viremias that usually suffer vaccinated pigs that became infected, the precise infectious period was difficult to assess. Finally, because it was necessary to perform a three-step experiment: vaccination, contact with seeders and comingling of vaccinated-infected with new vaccinated pigs and, for logistic reasons, to set up such an experiment in groups was deemed to be almost impossible. For these reasons, as well as to better assess the individual participation of each animal, a one-to-one transmission experiment was performed.

In this third study, vaccinated pigs exposed to seeders also needed a bigger time of exposure to become infected but, contrarily to the previous group experiment, all animals became infected. This was somehow expectable since in the present case vaccinees only could have contact with a seeder, increasing thus the likelihood of an effective contact to occur. Interestingly, a delayed transmission as well as a significant reduction of the viral load and duration of viremia were also observed, indicating that even in this worst-case scenario vaccination was able to reduce viral shedding. In the second phase of exposure, that is a vaccinated pig exposed to a vaccinated-infected pig, only in one replica transmission did not occur. Interestingly, vaccinated pigs became infected sooner that in the previous phase. It is worth to discuss here the possible explanations for this. In our opinion, the selected model, in which animals were moved twice and mixing of vaccinated-infected animals with vaccinated pigs occurred just after the starting of viremia, created the optimal situation for transmission both in terms of the potential effects of stress as well as by the amount of virus shed. Nevertheless, even in this case, the area under the curve for viremia in vaccinated pigs infected from other vaccinated pigs was reduced, indicating that

probably, under more natural conditions the reduction of transmission from infected-vaccinated pigs to other vaccinated pigs would have been substantial.

The use of mass vaccination to stop PRRSV transmission is not well document for genotype 1 isolates. However, in the case of genotype 2, several case-reports from North America suggested the effectiveness of this strategy in eliminating PRRSV from growers or even the whole production system when combined with other management or pig flow measures (Dee and Philips, 1998; Gillespie and Carroll, 2003; Philips and Dee, 2003). For example, Dee and Philips (1998) obtained a cease in viral circulation in a multi-site farm of 800 sows by combining vaccination with a unidirectional flow system. Likewise, Philips and Dee (2003) obtained a complete PRRSV elimination from the finishing population of four multi-site farms utilizing double mass vaccination of pigs in combination with closure of facilities and unidirectional pig flow. In four additional farms that employed the same protocol described above but without mass vaccination, all the attempts to eliminate PRRSV from the finishing population failed. This indicated that, at least in these cases, the immunization of pigs with MLV vaccines was an essential component for achieving the PRRSV elimination.

In summary, in the present thesis we gathered scientific evidences enough to suggest that mass vaccination of piglets could be an effective tool for stopping PRRSV transmission in weaners and fatteners, although the precise limitations of this use in terms of timing of vaccination, interference with maternal immunity and vaccination regimes have to be determined yet.

CONCLUSIONS

1. In the follow-up study, the basic reproduction rate (R) of genotype 1 PRRSV from weaning to slaughter ranged from 3.5 to 5.3. These values are compatible with a relative slow transmission of the virus and suggest that relatively low vaccine efficacies could result in a reduction of R<1.

2. In a genotype 1 subtype 1 model of infection by contact in groups, vaccination significantly delayed PRRSV transmission and reduced the basic reproduction rate (R) of vaccinated pigs to <1. Also, vaccination reduced shedding and viremia. These results indicate that there is a potential for the use of mass vaccination in control programs aimed to stop PRRSV transmission in piglets.

3. In the one-to-one experiment of transmission by contact, vaccination did not prevent the infection of vaccinated animals from naïve seeders. Transmission from infected vaccinees to vaccinated pigs also occurred. However, vaccination was effective in terms of reducing most of the biological parameters that can be related to transmission such as the length of viremia. These results indicate that even in the worst-case scenario, vaccination contributes to reduce the infectious load in the herd.

4. With the present results, it can be concluded that slight increases in the efficacy of current genotype 1 vaccines in terms of reduction of transmission, could be enough to think of eradication through mass vaccination without the need of developing vaccines inducing sterilizing immunity.

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ANNEX

In this annex, a compilation of articles and international congress contributions published during the course of the present thesis is shown.

Publications about PRRSV:

1. Pileri, E., Gibert, E., Soldevila, F., García-Saenz, A., Pujols, J., Diaz, I., Darwich, L., Casal., J., Martín, M., Mateu, E., 2015. Vaccination with a genotype 1 modified live vaccine against porcine reproductive and respiratory syndrome virus significantly reduces viremia, viral shedding and transmission of the virus in a quasi-natural experimental model. Veterinary Microbiology, 175, 7–16.

Congress contributions about PRRSV:

1. Pileri, E., Martín-Valls, G.E., Díaz, I., Simon-Grifé, M., García-Saenz, A., Casal, J., Mateu, E.: Estimation of the reproduction rate (R) of Porcine reproductive and respiratory syndrome virus in two endemic farrow-to-finish farms. Abstract selected for poster presentation at the International Society for Veterinary Epidemiology and Economics, November 3-7, 2015 (Mérida, Mexico).

2. Pileri, E., Gibert, E., Li, Y., Alarcón, L.V., Martín-Valls, G., Díaz, I., Casal, J., Martín, M., Darwich, L,, Mateu, E.: Vaccination of piglets with a genotype 1 modified live PRRS virus vaccine delays transmission between pigs and decreases viral load in a one-to-one transmission experiment. European Symposium of Porcine Health and Management, April 22-24, 2015. Nantes, France. Poster.

3. Pileri, E., Gibert, E., Soldevila, F., López-Soria, S., García, A., Díaz, I., Pujols, J., Darwich, L., Casal, J., Martín, M., Mateu, E.: Quantification of PRRSV transmission: effect of pig vaccination. International PRRS Symposium and PCVAD (PCV2), May 20-22, 2013. Beijing, China. Oral presentation.

4. Gibert, E., Pileri, E., Martín-Valls, G.E., Cano, E., Pérez, D., López, R., Nofrarias, M., López, S., Mateu, E.: Porcine reproductive and respiratory syndrome virus (PRRSV) oral fluid excretion is modified according to animal status (is reduced in vaccinated animals). International PRRS Congress, June 3-5, 2015. Ghent, Belgium. Oral presentation.

5. Gibert, E., Pileri, E., Cano, E., Martín-Valls, G.E., Mateu, E.: In vitro comparison of several matrices for the individual or collective sampling of oral fluids in pigs for PRRSV detection by quantitative RT-PCR.

6. Gibert, E., Pileri, E., Cano, E., López, R.M., López-Soria, S., Martín-Valls, G.E., Mateu, E.: Experimental evaluation of individual and collective oral fluid sampling for the early detection of PRRSV- infected piglets. 23rd International Pig Veterinary Society (IPVS) Congress, June 8-11, 2014. Cancún, México. Poster.

7. Crenshaw, J., Pujols, J., Polo, J., Campbell, J., Rodríguez, C., Navarro, N., Pileri, E., Ciacci-Zanella, J.,Rangel, J.: Analysis of spray dried porcine plasma indicates absence of PRRSV infection in Brazilian pigs. 23rd International Pig Veterinary Society (IPVS) Congress, June 8-11, 2014. Cancún, México. Poster.

Publications not related to PRRSV:

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