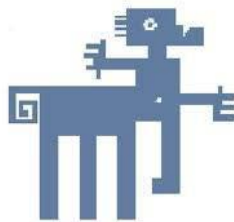


New insights on PCV2 vaccination: thinking out of the box

The thesis submitted by Hua Feng in fulfilment of the requirements
for the PhD degree of Veterinary Sciences at the *Facultat de
Veterinària* of the *Universitat Autònoma de Barcelona*, under the
direction of Dr. Joaquim Segalés and Dr Marina Sibila

Bellaterra, 2015



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CERTIFIQUEN:

Que la Tesi doctoral titulada “*New insights on PCV2 vaccination: thinking out of the box*” presentada por Hua Feng per a l’obtenció del grau de Doctor en Veterinària, s’ha realitzat sota la nostra supervisió al Centre de Recerca en Sanitat Animal.

Per tal de que consti, als efectes oportuns, signen el present certificat a Bellaterra (Barcelona), a 17 de Setembre de 2015.

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The work performed in this PhD Thesis has been funded by:

1. European PCV2-Award 2011 sponsored by Boehringer Ingelheim.
2. European PCV2-Award 2012 sponsored by Boehringer Ingelheim.
3. China Scholarship Council (No. 2011704032).

*Dedicated to my parents,
my girlfriend and my friends
I will go through all the roads full of thorns,
cause I'm full of courage, cause I know you are beside me !*

Acknowledgements

Here come my thanks for the people who helped me during those four years. Once I came into CReSA, the passionate and friendly people let me feel in a big family. I'm very glad to be one of you!

Firstly, I will give my deepest gratitude and foremost to my respectable supervisors: Quim Segalés and Marina Sibila. To Quim, he is a person always full of energy, enthusiasm and optimism, who give me lots of kindly advice and helps on my papers and thesis writing, encouraged me many times, opened my mind on my way of scientific research. To Marina, I should give my special thanks for her constant encouragement, and patient guidance. She has walked through all the stages of my PhD life; without her consistent and illuminating instruction, this thesis could not be completed. Many thanks to all of you!!!

I would also like to express my gratitude to Tuija, who let me learnt lots of knowledge related to my studies and her rigorous attitude to scientific work always encouraged me. Another person need to be thanked, is Lorenzo Fraile; he gave lots of wise advice and assistance on data analysis for my papers.

Besides, there were too many tasks that could not be finished without the help of CReSA fellows. Firstly, I would like to thank Anna Llorens and Eva Huerta. I still remember the scene of the first time you show me how to do IPMA and serum extraction by robot. I cannot really remember how many times we worked together on serum, DNA and other samples. Many thanks for your excellent technical assistance. And thanks to all these people who helped so much on the farms: Rosa López, Diego Pérez, Miquel Nofrarías, Sergio López, Beatriz García and Salvador Oliver.

And also special thanks to Mario Aramouni, Alexandra Jiménez, David Nieto and Mar Costa, who gave me lots of help on my life and research studies.

Of course, I will never forget to give my gratitude to my all other colleagues who helped and listened to me and my problems during the difficult course of the thesis and life in Barcelona: Juliana Gonzalez, Massimiliano Baratelli, Fernando Núñez, Bernardo Bello, Beatriz Vidaña, Guillermo Cantero and Marco Brustolin.

Last, my sincere thanks are for my beloved family, my father Jinxing Feng, my mother Aiqin Wang, for their loving considerations and great confidence in

me all through these years. And for my lovely girlfriend Zhen Wei, for her patience, optimism, kind-heart, which let me to treasure all what I have and to be confident everyday!

SUMMARY / RESUM

SUMMARY

Porcine circovirus type 2 (PCV2) is the etiological agent of PCV2 systemic disease (PCV2-SD), which causes severe clinical signs in nursery and fattening pigs and, in consequence, leads to considerable economic losses. Besides PCV2-SD, this virus is also associated to other diseases, named collectively as porcine circovirus diseases (PCVDs). Nowadays, the control of PCVDs depends on vaccines and, to a lesser degree, on improving management strategies as well as controlling risk factors. Until now, a number of commercial vaccines are available in the swine production industry worldwide. Most of those products are based on PCV2a genotypes, and have been shown to be highly effective in controlling PCV2 infection, PCVDs and improve productive parameters. This thesis aimed to assess two poorly or non-explored aspects related with PCV2 vaccination. On one hand, the putative interference of different maternally derived antibody (MDA) levels at the time of vaccination on the average daily weight gain (ADWG) evolution was studied. On the other hand, the possibility of PCV2 eradication by means of a mass vaccination strategy was tested for the first time. In both studies, the same subunit PCV2 vaccine was used.

The first study aimed to compare the efficacy of a PCV2 commercial vaccine in terms of seroconversion, infection dynamics and ADWG in pigs with different MDA levels. A total of 337 animals from a PCV2 subclinically infected farm were distributed into two groups based on weight and PCV2 antibody levels (high [H] or low [L]) at 2 weeks of age. One week later, these animals were subdivided in four groups according to the treatment received. Vaccinated (V) pigs (H-V and L-V) received 1 mL of a commercial vaccine and non-vaccinated (NV) ones (H-NV and L-NV) received 1 mL of PBS. All piglets were subsequently bled at 7, 12, 18, 22 and 25 weeks of age and weighted at 12 and 25 weeks of age. V animals showed significantly lower PCV2 infection rate and viral load, and higher ELISA S/P ratios and ADWG than NV ones. Compared with H-V piglets, L-V pigs showed a numerically lower PCV2 infection rates, lower area under the curve of viral load, an earlier seroconversion and a numerically higher (but no statistically significant) ADWG. The worst growth rates were observed in the L-NV group. In this study, MDA did not seem to interfere significantly with the effect of PCV2 vaccination on ADWG. However, only when a small subpopulation of pigs with the highest ELISA S/P ratios was considered, an apparent interference of vaccine efficacy on ADWG was noticed. Therefore, the impact of this possible interference under field conditions is probably negligible for most farms.

In the second study, the feasibility to eradicate PCV2 in a conventional PCV2 infected farm by vaccinating both sows and piglets using a commercially subunit vaccine was assessed. Vaccination strategy implied that all sows, boars and gilts of the farm were vaccinated every four months, and all piglets were vaccinated and revaccinated with the same vaccine at 4 and 7 weeks of age, respectively. This vaccination strategy was applied during 12 consecutive months. Blood samples from 15 piglets of 4, 8, 12, 16, 20 and 24 weeks of age and 15 sows were taken monthly PRE, DURING and POST mass vaccination strategy. From all the collected sera ($n = 1796$), a representative proportion of them ($n = 1235$, 69%) were analysed ($n = 1121$ from piglets and $n = 114$ from sows). All these samples were tested by PCV2 ELISA and PCV2 PCR (and quantitative-PCR when PCR positive). All tested sows were negative by PCR but seropositive. ELISA mean OD values of sows decreased throughout the study. Percentages of PCV2 PCR positive samples in piglets were 8% (12/150), 0.9% (6/659) and 3.5% (11/312) PRE, DURING and POST application of the mass vaccination program, respectively. ELISA mean OD values of PCV2 seropositive animals progressively decreased until the end of the mass vaccination period, but a clear seroconversion was observed after stopping such strategy. In conclusion, one year period of mass PCV2 vaccination (without implementing further farm management practices or biosafety measures) was

not able to clear out PCV2 infection. In deed the virus became detectable again when vaccination was stopped.

Resum

El Circovirus porcí tipus 2 (PCV2) és l'agent causal de la circovirosis porcina (CP). Aquesta malaltia causa greus signes clínics en porcs de transició i engreix, i com a conseqüència, comporta importants pèrdues econòmiques. A part de la CP, aquest virus està associat a altres malalties, anomenades en conjunt com a malalties associades al circovirus porcí (PCVDS). Avui en dia, el control de PCVDS depèn de les vacunes i, en menor mesura, de la millora de les estratègies de maneig i del control dels factors de risc. Actualment i a nivell mundial, en el sector de la producció porcina existeixen diverses vacunes comercials. La majoria d'aquests productes estan basats en el genotipus PCV2a i han demostrat ser altament eficaços en controlar l'infecció per PCV2 i en millorar els paràmetres productius.

L'objectiu de la present tesi doctoral era explorar dos conceptes poc coneguts en quan a la vacunació enfront a PCV2. Per una banda, es va estudiar la possible interferència del nivell d'anticossos d'origen matern (AOM) en el moment de la vacunació en l'evolució del guany mig diari de pes (GMDP). Per una altra banda, es va avaluar per primera vegada la possibilitat d'eradicar l'infecció de PCV2 mitjançant una estratègia de vacunació massiva. En ambdós casos, es va utilitzar una vacuna de subunitat.

L'objectiu del primer estudi era comparar l'eficàcia d'una vacuna comercial en quan a la seroconversió, dinàmica d'infecció i GMDP en porcs amb diferents nivells d'AOM. Un total de 337 animals procedents d'una granja infectada subclínicament amb PCV2 van ser distribuïts en dos grups basats en el pes i en els valors S/P (a les dues setmanes de vida) enfront a PCV2 (alts [A] o baixos [B]). Una setmana més tard, aquest animals es van subdividir en quatre grups en funció del tractament rebut. Els animals vacunats (A-V i B-V) van rebre 1 ml de la vacuna comercial i els animals NV (A-NV i B-NV) van rebre 1 ml de PBS. Tots els animals inclosos a l'estudi es van sagnar a les 7, 12, 18, 22 i 25 setmanes d'edat i pesats addicionalment a les 12 i 25 setmanes d'edat. Els animals V van mostrar un nivell d'infecció i una carrega viral menors, uns valors S/P més alts i un GMDP més alts que els NV. Comparat amb els A-V, els animals B-V van mostrar un nivell d'infecció numèricament inferior, una àrea sota la corba de la càrrega viral inferior, una seroconversió més d'hora i un GMDP numèricament (però no estadísticament significatiu) més elevat. El pitjor índex de creixement el van tenir els animals del grup B-NV. En aquest estudi, els AOM semblen no interferir significativament amb l'efecte de la vacunació enfront a PCV2 sobre el GMDP. De totes maneres, quan es va considerar la subpoblació d'animals amb els valors S/P més alts, es va detectar una possible interferència en l'eficàcia de la vacuna sobre el GMDP. Per tant,

l'impacte d'aquesta possible interferència en condicions de camp és probablement negligible en la majoria de les granges.

En el segon estudi, es va avaluar la viabilitat d'eradicar la infecció de PCV2 en una granja convencional infectada subclínicament amb PCV2 mitjançant la vacunació de truges i garrins utilitzant una vacuna comercial de subunitats. L'estratègia de vacunació va implicar que truges, llavors i verros es van vacunar cada quatre mesos, i que tots els garrins van ser vacunats i revacunats a les 4 i 7 setmanes de vida, respectivament. Aquesta estratègia de vacunació es va aplicar durant 12 mesos consecutius. En els períodes PRE, DURANT i POST estratègia vacunal es van treure, mensualment, mostres de sang de 15 garrins de 4, 8, 12, 16, 20 i 24 setmanes d'edat i de 15 truges. De totes les mostres de sang obtingudes (n=1796), se'n va analitzar una part representativa (n=1235 [69%], 1121 i 114 mostres procedent de garrins i de truges, respectivament). Totes aquestes mostres es van analitzar mitjançant una ELISA i una PCR (i per PCR quantitativa en les mostres PCR positives) de PCV2. Totes les mostres de sang de les truges van ser negatives per PCR però seropositives. Els valors migs de densitat òptica (DO) van disminuir al llarg de l'estudi. El percentatge de mostres positives per PCR de PCV2 va ser del 8% (12/150), 0.9% (6/659) i del 3.5% (11/312) pels períodes PRE, DURANT i POST aplicació de la vacunació massiva, respectivament. Els valors serològics

migs dels animals seropositius a PCV2 van disminuir progressivament fins al final del període de la vacunació massiva. Un cop es va aturar la vacunació massiva es va observar una clara seroconversió. En conclusió, l'aplicació durant una any de la vacunació massiva enfront a PCV2 (sense implementar mesures específiques de maneig o de bioseguretat) no va ser capaç d'eliminar l'infecció per PCV2. De fet, un cop la vacunació es va aturar, es va detectar de nou el virus.

The results from the present PhD Thesis have been published or submitted for publication in international scientific peer-reviewed journals:

Feng, H., Blanco, G., Segalés, J., Sibila, M. (2014). Can porcine circovirus type 2 (PCV2) infection be eradicated by mass vaccination? *Veterinary Microbiology* 172 (1-2), 92-99.

Feng, H., Segalés, J., Fraile, L., López-Soria, S., Sibila, M. Effect of maternally derived antibodies on porcine circovirus type 2 infection dynamics and production parameters in PCV2 vaccinated pigs under field conditions (submitted for publication).

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LIST OF ABBREVIATIONS

AA	Amino acid
ADWG	Average daily weight gain
CD	Cluster of differentiation
DC	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
IFN- γ	Interferon- γ
IFN- γ -SC	Interferon- γ -secreting cells
IL	Interleukin
IPMA	Immunoperoxidase monolayer assay
Kb	kilobase
MDA	Maternally derived antibodies
MDI	Maternally derived immunity
mPCV2b	Mutant PCV2b
NA	Neutralizing antibodies
NT	Nucleotides
ORF	Open reading frames
PBMC	Peripheral blood mononuclear cell
PCV1	<i>Porcine circovirus type 1</i>
PCV2	<i>Porcine circovirus type 2</i>
PCV2-ED	PCV2 enteric disease
PCV2-LD	PCV2 lung disease

List of Abbreviation

PCV2-RD	PCV2 reproductive disease
PCV2-SD	PCV2 systemic disease
PCV2-SI	PCV2 subclinical infection
PCVD	Porcine circovirus diseases
PDNS	Porcine dermatitis and nephropathy syndrome
PK-15	Porcine kidney-15 cell line
PMWS	Post-weaning multi-systemic wasting syndrome
PPV	<i>Porcine parvovirus</i>
PRRSV	<i>Porcine reproductive and respiratory syndrome virus</i>
Q-PCR	Quantitative PCR
RF	Replicative form

CHAPTER 1

INTRODUCTION

1.1. Porcine circovirus type 2 (PCV2)

1.1.1. The discovery and origin of PCV2

In 1996, a new, sporadic, multi-systemic disease was described in Canada and named as postweaning multisystemic wasting syndrome (PMWS) (Harding, 1996; Clark, 1997). Affected pigs were clinically characterized by weight loss, skin pallor, respiratory distress and specific lesions in lymphoid organs (Clark, 1997; Rosell et al., 1999; Segalés et al., 2005a). One year later, a *Porcine circovirus* (PCV)-like agent was detected by means of immunohistochemistry in lymphoid tissues (Clark, 1997) of PMWS affected pigs. Subsequently, the virus was isolated from PMWS-affected pigs firstly in North America and Europe (Allan et al., 1998; Ellis et al., 1998; Allan et al., 1999b), and then in Asia (Onuki et al., 1999; Choi et al., 2000). In 1998, sequencing of the whole genome of the virus showed that it was completely different from the previously known PCV derived from porcine kidney-15(PK-15) cells (ATCC-CCL-33) (Tischer et al., 1982; Hamel et al., 1998; Meehan et al., 1998). Therefore, the cell derived non-pathogenic PCV (Tischer et al., 1986) was name as *Porcine circovirus type 1* (PCV1) and the one associated with PMWS as *Porcine circovirus type 2* (PCV2) (Allan et al., 1999b).

The earliest evidence of the correlation between PCV2 and PMWS was

provided by a retrospective study, in which samples from 1962 to 1998 were processed by PCR (Jacobsen et al., 2009). Data obtained in that work indicated that while PCV2 genome was firstly detected in 1962, PMWS did exist since at least 1985.

Nowadays PMWS is denominated as PCV2-systemic disease (PCV2-SD) (Segalés, 2012). Besides PCV2-SD, PCV2 is also associated to other conditions, such as PCV2 subclinical infection (PCV2-SI), PCV2 reproductive disease (PCV2-RD), porcine dermatitis and nephropathy syndrome (PDNS), PCV2 lung disease (PCV2-LD) and PCV2 enteric disease (PCV2-ED) (Segalés, 2012). Whereas in Europe all those diseases attributable to PCV2 are collectively termed as porcine circovirus diseases (PCVD) (Segalés et al., 2005a), in North America they are named as porcine circovirus associated diseases (PCVAD) (Opriessnig et al., 2007). Currently, PCV2-LD and PCV2-ED are considered to be mainly part of the PCV2-SD scope and not as separated entities (Ticó et al., 2013; Baró et al., 2015).

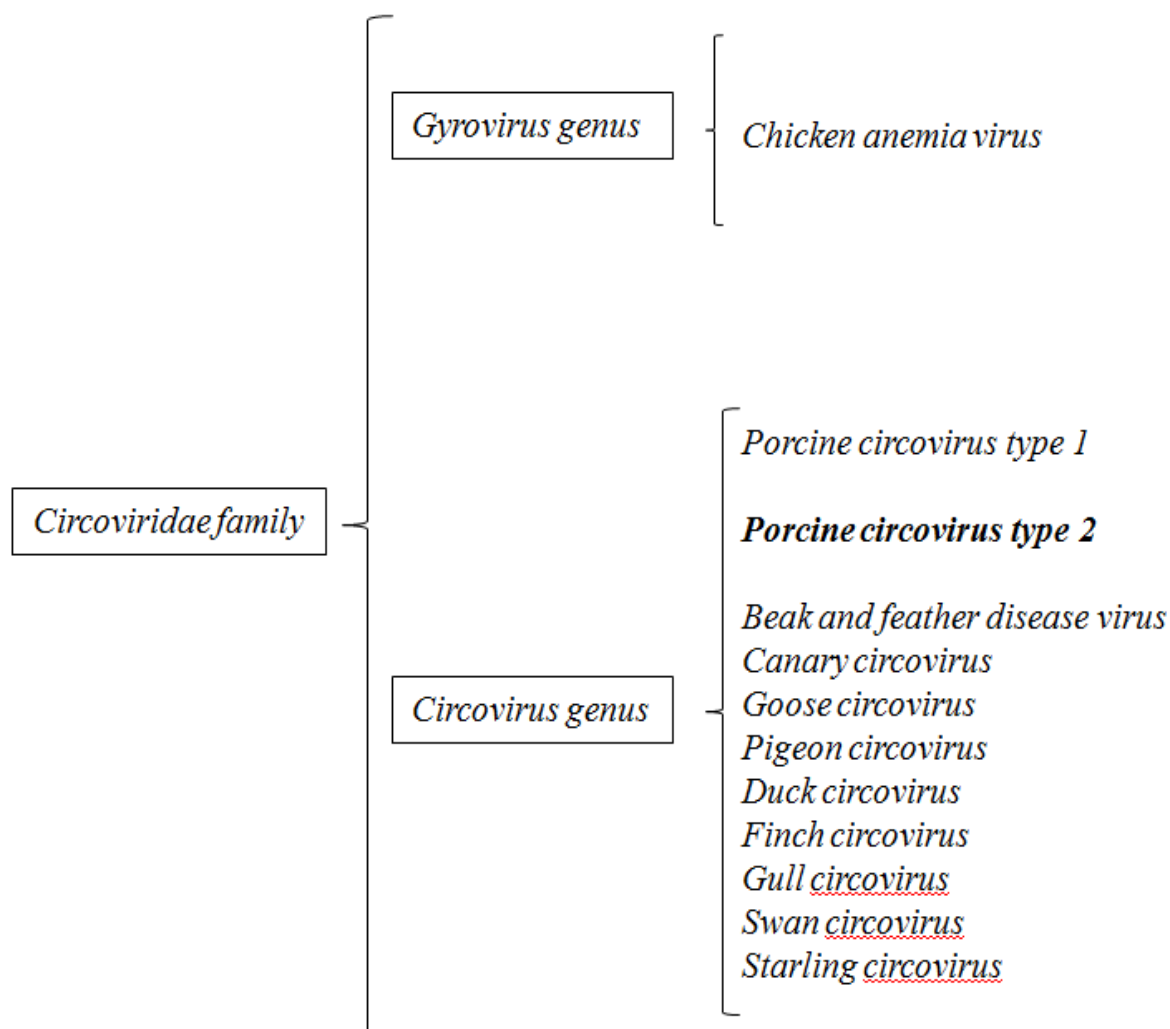
1.1.2. Taxonomy and classification

Both PCV2 and PCV1 are classified within the family *Circoviridae* (Figure 1.1), since their virions are small, icosahedral, non-enveloped and with

closed-circular single-stranded DNA genome (Fauquet and Fargette, 2005; Opriessnig et al., 2007). Within the *Circoviridae* family, two genera are included, namely *Circovirus* and *Gyrovirus*. Based on the International Committee for the Taxonomy of Viruses (ICTV, <http://www.ictvonline.org/>), besides PCV2 and PCV1, genus *Circovirus* also includes viruses affecting birds (*Beak and feather disease virus*, *Canary circovirus*, *Goose circovirus*, *Pigeon circovirus*, *Duck Circovirus*, *Finch circovirus*, *Gull circovirus*, *Swan circovirus* and *Starling circovirus*). On the other hand, *Gyrovirus* genus only comprises one species, *Chicken anaemia virus*. Although not yet included in the list of ICTV, some new species of circoviruses have been reported in ravens (Stewart et al., 2006) and mink (Lian et al., 2014), as well as a new *Gyrovirus* in humans (Cheung, 2012; Maggi et al., 2012; Biagini et al., 2013). The main differences between both genera is that the members of *Circovirus* genus have ambisense genomes (Meng, 2013), while the ones in *Gyrovirus* have a negative-sense genome (the viral proteins are encoded by the complementary strand) (Gelderblom et al., 1989; Sauvage et al., 2011). Moreover, in 2010, a new genus called *Cyclovirus*, characterized by viruses with an ambisense genome able to infect humans and wild chimpanzees, was proposed to be a new member of *Circoviridae* family (Blinkova et al., 2010; Li et al., 2010b).

Figure 1.1. Taxonomy of *Circoviridae* family members (ICTV,

<http://www.ictvonline.org/>).



1.1.3. Molecular and genomic organization

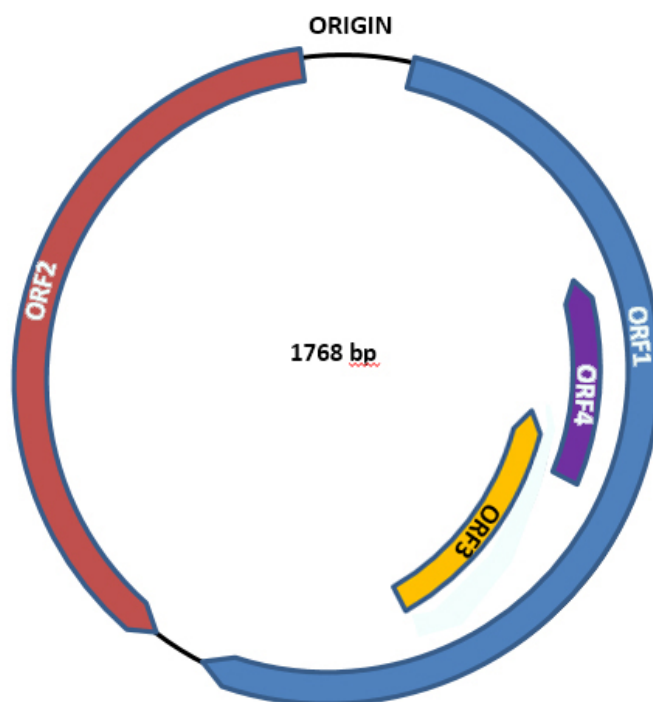
PCV2 has a 1.76-1.77 kilobases (Kb), a single stranded circular DNA, and a virion with 12-23 nm in diameter (Figure 1.2) (Meehan et al., 1998;

Rodríguez-Cariño and Segalés, 2009). PCV2 capsid particle is constituted by 60 capsid protein subunits arranged into 12 slightly protruding pentameric units (Crowther et al., 2003; Khayat et al., 2011).

Once viral replication starts, the ambisense genome is converted into a double-stranded form (also named as replicative form, RF). Viral proteins are encoded by both virus genome and complementary strands; potentially, the PCV2 genome contains about 11 open reading frames (ORFs) (Hamel et al., 1998). Until now, the functions of 4 ORFs have been described. ORF1 and ORF2, the two major ORFs, are essential for virus replication and orientated in opposite directions. ORF1 gene is the largest ORF (approx. 940 nucleotides [NT]) located in the positive strand and encodes for two non-structural replicase proteins (Rep and Rep') (Mankertz et al., 1998; Mankertz and Hillenbrand, 2001; Cheung, 2003). Rep (314 amino acid [AA]) is derived from the whole ORF1, while Rep' (178 AA) is originated from the alternatively spliced ORF1 transcript. ORF2 gene (701 NT) is located in the antisense strand and encodes for the major immunogenic protein (234 AA), the Cap protein (Nawagitgul et al., 2000). Cap is the base unit to construct the whole capsid and play a role in translocating the viral genome into nucleus during replication (Nawagitgul et al., 2000; Liu et al., 2001). Overlapped with ORF1, ORF3 (about 315 NT)

encodes a 105 AA non-structural protein in an antisense way. In addition, this protein is associated to viral pathogenesis since can induce cell apoptosis in PK-15 cells (Nawagitgul et al., 2000; Mankertz et al., 2004; Liu et al., 2005) and it is linked to the viral replication process (Karuppannan et al., 2009). Recently, a new viral protein encoded by the ORF4 gene (180 NT) has been identified. This latter protein has been described as non-essential for PCV2 replication but can suppress the caspase activity and regulate CD4⁺ and CD8⁺ T lymphocytes during PCV2 infection (He et al., 2013).

Figure 1.2. Genomic organization of PCV2



1.1.4. PCV2 genotypes

Phylogenetic analyses based on the sequencing of ORF2 gene showed that PCV2 strains was able to be divided into 2 distinct groups: group 1 (with 3 clusters, 1A-1C) and group 2 (including 5 clusters from 2A to 2E) (Olvera et al., 2007). Afterwards, PCV2 groups were designated as genotypes and were divided into PCV2a (group 2 or North American-like isolates) and PCV2b (group 1 or European-like isolates) (Segalés et al., 2008; Gillespie et al., 2009). Subsequently, a new PCV2 genotype was recovered from tissues archived during 80s in Denmark and was accordingly named as PCV2c (Dupont et al., 2008; Segalés et al., 2008; Franzo et al., 2015a). In 2009, two additional PCV2 genotypes were described in China and named as PCV2d and PCV2e (Wang et al., 2009), but the subsequent analysis failed to support this finding (Cortey et al., 2011a). Then in the following year, another new genotype called PCV2d was found in China (Guo et al., 2010; Ge et al., 2012), which was also known as mutant PCV2b (mPCV2b) for some time (Xiao et al., 2012; Opriessnig et al., 2013b). Recently, PCV2d has also been found in Europe and USA (Franzo et al., 2015b; Xiao et al., 2015).

Nowadays, PCV2a and PCV2b are the major genotypes circulating worldwide in the pig industry (Allan et al., 2012), but PCV2d is rapidly

increasing in its prevalence, apparently (Xiao et al., 2015). Based on the database from GenBank, prior to 2003, PCV2a was endemic in PCV2-SD affected swine herds; whereas since 2003, after severe outbreaks all over the world, PCV2b started to become predominant (Olvera et al., 2007). Such shift from genotype a to b and the dominance of PCV2b were also reported by different studies around the world (Dupont et al., 2008; Takahagi et al., 2008; Timmusk et al., 2008; Chiarelli-Neto et al., 2009; Wang et al., 2009; Wiederkehr et al., 2009; Cortey et al., 2011a; Jantafong et al., 2011).

Recently, under the condition of widely usage of vaccines, the new emerging PCV2d was identified and implied that vaccination pressure may set an evolution force on circulating viruses (Kekarainen et al., 2014; Segalés, 2015). Combining this information with the fact of increasing prevalence of PCV2d, all together, might be driving a potential antigen shift from PCV2b to PCV2d (Xiao et al., 2012). Anyway, those speculations will deserve further investigations.

1.2. PCV2 and PCV associated diseases (PCVDs)

1.2.1. Epidemiology

1.2.1.1. Alternative hosts and geographical distribution

PCV2 is considered a host specific virus, which can only affect *suidae* species (domestic pigs and wild boars, mainly) (Ellis et al., 2003; Vicente et al., 2004; Lipej et al., 2007; Segalés et al., 2013). However, mice and rat have been described as alternative hosts under both experimental and field conditions. Previously, data showed that PCV2 could be detected in lung tissues of rodents after inoculation, implying these animals could be a potential host for PCV2 transmission (Kiupel et al., 2005; Li et al., 2010a). Interestingly, a relatively high prevalence of PCV2 has been detected in mice (65%) and rat (23.8%) from swine farms, but the prevalence in rodents outside pig farms was zero (Lorincz et al., 2010; Pinheiro et al., 2013). All those data may indicate a potential way of PCV2 transmission from rodents to pigs and the possible maintenance of the virus in non-swine reservoirs.

Since the earliest PCV2 detection in 1962 (Jacobsen et al., 2009) up to now, PCV2 infection and PCV2-SD have been described in different countries of all five continents, indicating the ubiquitous nature of this virus (Allan and Ellis, 2000; Grau-Roma et al., 2011). Curiously, although Australia was

considered a country free from PCV2-SD, PCV2 was widely distributed in its swine herds (Raye et al., 2005; Finlaison et al., 2007; Grau-Roma et al., 2011).

1.2.1.2. PCV2 transmission

PCV2 can be easily spread within and between swine herds by means of both horizontal and vertical transmission routes. PCV2 can be shed through excretions/secretions as nasal discharges, saliva, tears, faeces, urine, milk and semen of both clinically and sub-clinically infected pigs (Krakowka et al., 2000; Larochelle et al., 2000; Bolin et al., 2001; Shibata et al., 2003; Sibila et al., 2004; Chung et al., 2005; Segalés et al., 2005b; Shibata et al., 2006; Park et al., 2009; Beach and Meng, 2012). Furthermore, previous studies also showed a higher quantity of virus shed in animals with clinical signs compared to infected animals without clinical signs (Rose et al., 2012; Segalés, 2012).

1.2.1.2.1. Horizontal transmission

Oro-nasal route is considered as the most frequent and efficient way of PCV2 infection and transmission (Andraud et al., 2008; Tomas et al., 2008). Experimentally, control healthy pigs comingled with PCV2 infected or PCV2-SD affected animals got infected (by contact) or developed PCV2-SD clinical signs, respectively (Albina et al., 2001; Bolin et al., 2001; Shibata et al., 2003).

Moreover, a study indicated that PCV2 can be more efficiently transmitted in animals comingled in the same pen than in different pens (Andraud et al., 2008).

The transmission of PCV2-SD between groups has been also demonstrated (Jaros et al., 2006; Kristensen et al., 2006; Kristensen et al., 2007; Dupont et al., 2009). For example, in Dupont et al. (2009), 14 contact pigs from a non-PCVD affected herd developed PCV2-SD after being in contact with clinically affected pigs by different ways: direct contact in same pens, nose-to-nose contact between pens and no-contact pigs in separated pens (Dupont et al., 2009).

1.2.1.2.2. Vertical transmission

PCV2 vertical transmission occurs mainly by means of transplacental infection and infection by colostrum or milk from sows to piglets (Rose et al., 2012).

Data from field studies indicated the existence of PCV2 in aborted foetuses, stillborn or new-born piglets (Kim et al., 2004; Shen et al., 2010), which prompted an association between transplacental infection and PCV2 viremia in dams. Furthermore, this transplacental infection was also described after experimental infection of pregnant sows (Park et al., 2005; Ha et al., 2008).

Although high prevalence of PCV2 has been found in newborn piglets in some studies, its association with reproductive failure was described as rare cases (Ladekjaer-Mikkelsen et al., 2001; Maldonado et al., 2005; Shen et al., 2010; Chae, 2012b; Seo et al., 2014c; Segalés, 2015). Recently, a study indicated that production of PCV2-free offspring was feasible in an infected farm by means of embryo transfer (Bielanski et al., 2013).

Shedding by colostrum or milk is also an important transmission route from sows to piglets, which has been suggested under both field and experimental conditions (Shibata et al., 2006; Ha et al., 2009; Dvorak et al., 2013). The importance of this way of transmission was further supported by the fact that vaccinated sows can decrease, although not eliminate, virus shedding in the colostrum (Gerber et al., 2011).

In contrast, transmission of PCV2 by semen is still under debate. PCV2 has been detected in semen under both field and experimental conditions (Laroche et al., 2000; McIntosh et al., 2006; Schmoll et al., 2008; Madson et al., 2009b). This fact would suggest the possibility of PCV2 transmission from one generation to the next one. The infectiousness of the virus from PCV2 PCR positive semen has been proven after inoculating the semen intraperitoneally in

naïve piglets (Madson et al., 2009b). The semen, however, cannot lead to infection to the sows and their offspring after regular artificial insemination. Only artificial insemination with semen spiked with a sufficient amount of PCV2 (higher load than that resulting from a boar infection) resulted in foetal infection and reproductive failure (Rose et al., 2007; Madson et al., 2009a). Although artificially infected semen can cause viremia in fetuses and produce seropositive animals (Sarli et al., 2012), it is still unknown if semen of naturally infected boars with PCV2 is capable to develop reproductive failure.

1.2.1.2.3. Other routes of PCV2 transmission

PCV2 can be potentially transmitted to swine through other ways. Since PCV2 is highly resistant in the environment, the airborne route was found as another potential way to infect animals (Verreault et al., 2010; Rose et al., 2012). As previously mentioned, rodents inhabited in pig farms can probably be an alternative host to transmit PCV2 (Rose et al., 2012) and some insects, like flies and mosquito, may also serve as a transmission media (Zhai et al., 2014). In addition, some data indicated that PCV2 may also be transmitted by ingesting uncooked meat and using contaminated vaccines (Quintana et al., 2006; Opriessnig et al., 2009c; Victoria et al., 2010). However, those potential

indirect transmission routes cannot be as efficient as horizontal and vertical transmission ways.

In addition, other factors (international trading, growing swine productive scale and the rapid evolution rate of PCV2) may also contribute to the spreading of this virus. A study from Italy indicated that PCV2d strains found in that country may originate from the strains detected in China, probably as a result of international trading (Franzo et al., 2015b).

1.2.2. Clinical signs and pathological findings

1.2.2.1. Clinical signs of PCVDs

As mentioned previously, PCVDs compile a group of PCV2 associated diseases, which include PCV2-SI, PCV2-SD, PCV2-RD, PDNS, PCV2-LD and PCV2-ED (Segalés, 2012).

PCV2 infection is worldwide distributed, implying that the most common form of PCV2 infection is the PCV2-SI; in consequence, the prevalence rate of the clinical condition is relatively low (Young et al., 2011; Alarcon et al., 2013). Decreased average daily weight gain (ADWG) without any evident clinical signs is normally seen in PCV2 subclinically infected farms (Kurmann et al.,

2011; Young et al., 2011; Alarcon et al., 2013). Since the discovery of PCV2-SI by means of the use of vaccines, it has become the most economically important form of PCV2 infection in commercial farms (Kurmann et al., 2011; Segalés, 2012).

PCV2-SD is the main clinical form of PCV2 infection in terms of prevalence and economic impact. It usually affects pigs after weaning and growing animals from 7 to 16 weeks of age (McKeown et al., 2005; Opriessnig et al., 2011). Affected pigs are typically characterized by wasting, weight loss, pallor of the skin and respiratory disease (Rosell et al., 1999; Krakowka et al., 2004). Some other non-specific clinical signs can be seen in affected animals, such as dyspnoea, diarrhoea and jaundice (Harding, 2004). Enlarged lymph nodes were commonly seen in infected pigs (Clark, 1997; Rosell et al., 1999; Harding, 2004). In addition, PCV2-SD normally lead to a 4-30% morbidity and 4-20% mortality depending on the affected farm (Segalés and Domingo, 2002). PCV2-LD and PCV2-ED, clinically characterized by respiratory distress and diarrhoea, respectively, are considered to be into the scope of PCV2-SD (Ticó et al., 2013; Baró et al., 2015).

PCV2-RD is clinically characterized by stillborn, mummification,

embryonic death, infertility and increased pre-weaning mortality in the breeding herd, especially in primiparous sows (West et al., 1999; Brunborg et al., 2007; Madson et al., 2009a; Madson and Opriessnig, 2011; Meyns et al., 2012; Opriessnig and Langohr, 2013). The first reproductive failure linked to PCV2 was reported in 1999 in Canada (West et al., 1999); however, the occurrence of this problem under field conditions is infrequent (Pensaert et al., 2004).

PDNS can affect pigs at different ages, but mainly fattening animals (Drolet et al., 1999). Affected swine are characterized by the onset acute of cutaneous lesions (irregular, red-to-purple macules and papules), and pigs become anorexic and depressed with little or no pyrexia (Segalés et al., 1998; Drolet et al., 1999; Segalés, 2012; Opriessnig and Langohr, 2013). This disease is commonly fatal with a nearly 100% mortality in pigs older than 3 month of age and 50% in younger animals (Segalés, 2012). PDNS has never been experimentally reproduced and PCV2 is the suspected antigen linked to this immunocomplex disease (Segalés, 2012).

In 2009, a novel PCV2 associated syndrome was reported for the first time, although not further described afterwards. It was termed as acute pulmonary oedema (APE) (Cino-Ozuna et al., 2011) and characterized by acute respiratory

distress followed rapidly by death of apparently healthy pigs. Some data indicated that the appearance of this syndrome could be related with a low amount of PCV2 antibodies (maternally derived immunity, MDI) to control PCV2 infection before vaccination (Segalés, 2015).

1.2.2.2. Pathology of PCVDs

Gross lesions of PCVDs may represent a wide range of non-specific findings, while microscopic lesions may be specific enough to determine the diagnosis of the disease (Rosell et al., 1999; Segalés et al., 2004).

- **PCV2-SI:** No apparent gross and microscopic lesions are found in PCV2 subclinically infected animals. Occasionally, mild lymphocyte depletion with granulomatous inflammation of lymphoid tissues can be observed (Segalés, 2012).

- **PCV2-SD:** The most significant gross lesions are non-collapsed lungs, enlargement of lymph nodes and thymus atrophy (Rosell et al., 1999; Harding et al., 2008). In addition, white spots on kidney cortex and discoloration of liver can be seen (Rosell et al., 2000; Kim and Chae, 2002; Segalés et al., 2004; Martínez et al., 2006). Characteristic microscopic lesions comprise systemic

moderate to severe lymphocyte depletion associated with histiocytic infiltration in lymphoid tissues (Darwich et al., 2003b). PCV2 inclusion bodies can also be found in cytoplasm of histiocytes or multinucleate giant cells in lymphoid tissues (Rosell et al., 1999; Segalés et al., 2004). Lymphohistiocytic to granulomatous inflammation can be seen virtually in any tissue in severely PCV2-SD affected pigs (Onuki et al., 1999; Opriessnig et al., 2007; Segalés, 2012). Besides the mentioned findings, some other less frequent lesions have been also described in PCV2-SD, such as necrotizing lymphadenitis, heart failure, cerebellar lymphohistiocytic vasculitis and others. (Rosell et al., 1999; Segalés et al., 2004; Opriessnig et al., 2006b; Segalés, 2012; Resendes and Segalés, 2015).

- **PCV2-RD:** Gross lesions are normally seen as foetal mummification or oedematous foetuses; also, foetal hepatic enlargement and congestion and cardiac hypertrophy with multifocal areas of myocardial discoloration can be seen (Opriessnig et al., 2007). Ascites, hydrothorax and hydropericardium may also be present in foetuses (Segalés, 2012) The main hallmark of microscopic foetal lesion is a non-suppurative to necrotizing or fibrosing myocarditis (West et al., 1999; O'Connor et al., 2001).

- **PDNS:** Gross lesions of PDNS are characterized by irregular, red-to-purple macules and papules in the skin, accompanied by bilaterally enlargement of kidneys, small cortical petechiae and oedema of the renal pelvis (Segalés, 2012, 2015). Histopathologically, lesions are featured by systemic necrotizing vasculitis, and fibrinous-necrotizing glomerulonephritis (Segalés et al., 2005a; Opriessnig et al., 2007).

1.2.3. Co-infections

The fact that PCV2 infection can appear in both diseased and healthy pig indicates that PCV2 is the primary and essential agent to cause PCVDs, but other associated factors are needed to trigger disease (Ellis et al., 1999; Bolin et al., 2001; Ladekjaer-Mikkelsen et al., 2002; Rose et al., 2012). Furthermore, successful PCV2-SD reproduction by the single inoculation of PCV2 is really rare (Ellis et al., 2004; Tomas et al., 2008; Opriessnig and Langohr, 2013). Under field conditions, co-infections can be frequently observed in PCV2-SD affected pigs. It was speculated that concomitant bacterial and other viral agents could enhance the effect of PCV2 infection and increase the likelihood to trigger PCV2-SD (Opriessnig and Halbur, 2012; Segalés et al., 2013). In fact, experimental infections with *Porcine parvovirus* (PPV) (Allan et al., 1999a), *Porcine reproductive and respiratory syndrome virus* (PRRS) (Rovira et al.,

2002) or *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004a) demonstrated the capability of these agents to trigger PCV2-SD in PCV2 infected pigs. Other swine pathogens may also play a role in PCV2-SD development, such as *Torque teno sus virus*, *Swine influenza virus*, *Aujeszky's disease (Pseudorabies) virus* and *Lawsonia intracellularis* (Opriessnig and Halbur, 2012). However, specific mechanisms by which co-infecting pathogens can trigger PCV2-SD in PCV2 infected pigs are still unknown.

1.2.4. Diagnosis

Diagnosis of PCVDs can be suspected by clinical signs, but need to be confirmed by means of laboratory tests (Segalés, 2012). There are a number of laboratory techniques to detect PCV2 genome or antibodies, as well as to assess histological lesions. Such list includes standard PCR, real-time quantitative PCR (Q-PCR), immunohistochemistry, *in situ* hybridization, immunoperoxidase monolayer assay (IPMA), immunofluorescence assay and ELISA (Cottrell et al., 1999; Allan and Ellis, 2000; Rodríguez-Arrioja et al., 2000; Wu et al., 2008; Grau-Roma et al., 2009).

In 2005, the diagnostic criteria of three major PCVDs (PCV2-SD, PDNS and PCV2-RD) was described in a review (Segalés et al., 2005a). In 2012,

diagnostic criteria for PCVDs were expanded, including PCV2-SI, PCV2-SD, PCV2-LD, PCV2-ED, PCV2-RD and PDNS (Segalés, 2012). However, in Segalés et al. (2015), PCV2-LD and PCV2-ED were considered as overlapping entities with PCV2-SD, so those two conditions have been excluded from Table 1.1, which summarize diagnostic criteria for each PCVD.

For PCV2-SD, a farm level diagnosis was also defined (www.pcvd.eu) (Segalés, 2012). Accordingly, a farm should be considered as affected by PCV2-SD if fulfilling two criteria included in the herd case definition: a) significant increase in mortality with clinical signs compatible with PCV2-SD compared to historical data, and b) individual diagnostic criteria for PCV2-SD in 1 out of 3-5 studied animals (Grau-Roma et al., 2012). The individual case definition, in turn, is based on three main criteria, as indicated in Table 1.1.

Table 1.1. Diagnostic criteria for PCVDs (modified from Segalés, 2012).

PCVDs	Criteria for diagnosis
PCV2-SI	<ol style="list-style-type: none"> 1. Decreased body weight and ADWG. 2. No apparent histopathological lesions in lymphoid tissues (or mild lymphocyte depletion and granulomatous inflammation) 3. Low PCV2 viral load in few (lymphoid) tissues. <p>Criteria 2 and 3 can potentially be substituted by PCV2 detection by techniques such as standard PCR</p>
PCV2-SD	<ol style="list-style-type: none"> 1. Wasting, weight loss, decreased ADWG and paleness of skin (respiratory and/or digestive clinical signs may be present as well) 2. Moderate to severe lymphocyte depletion with granulomatous inflammation of lymphoid tissues (plus granulomatous inflammation in other tissues) 3. Moderate to high amount of PCV2 in damaged tissues
PCV2-RD	<p>Regular return-to-estrus:</p> <ol style="list-style-type: none"> 1. PCV2 seroconversion following the return-to-estrus and/or PCV2 PCR positivity around return-to-estrus occurrence <p>Abortions or mummifications:</p> <p>Reproductive failure at late gestation</p> <ol style="list-style-type: none"> 2. Fibrous to necrotizing myocarditis of fetuses 3. Moderate to high amount of PCV2 in heart
PDNS	<ol style="list-style-type: none"> 1. Haemorrhagic and necrotizing skin lesions and/or swollen and pale kidneys with generalized cortical petechial 2. Systemic necrotizing vasculitis, and necrotizing and fibrinous glomerulonephritis

1.2.5. PCV2 pathogenesis and immunity

1.2.5.1. PCV2 pathogenesis

Until now, little *in vivo* information is available regarding to the early events taking place at the very beginning of infection, and the target cells for initial PCV2 replication. Since PCV2 cannot generate its own DNA polymerase and depend on the ones of host, the cells with high mitotic rate were presumed as the place for PCV2 replication (Gilpin et al., 2003; Vincent et al., 2003). Although high concentration of virus was detected in the cytoplasm of monocytes and macrophages in infected animals (Rosell et al., 1999; Sanchez et al., 2004), the *in vitro* data suggested that no efficient PCV2 replication in macrophage and monocyte cultured cells was proven (Gilpin et al., 2003; Chang et al., 2006a; Chang et al., 2006b). Although **monocytes** did not act as the primary target cell, it was suggested that their function was to **disseminate PCV2 within the host** (Vincent et al., 2003). In addition, it has been demonstrated that PCV2 can be internalized and persist in **dendritic cells** (DC), without alteration of the immune functions of those cells (Vincent et al., 2003; Vincent et al., 2005). Since DCs act as antigen presenting cells, persistence in those cells may facilitate the transmission of PCV2 within the host as well as compromise its immune responses (Vincent et al., 2005).

PCV2 viremia firstly appears around 7 days post inoculation and viral load reaches a peak after 14 to 21 days post inoculation (Resendes et al., 2004; Opriessnig et al., 2008b). In PCV2 infected pigs, the virus can be detected in multi-organs, especially in lymphoid tissues (Quintana et al., 2001). By characterizing PCV2-infected leukocyte subpopulations, it has been proven that mainly circulating T lymphocytes (CD4⁺ and CD8⁺) and, to a lesser extent, B lymphocytes support PCV2 replication, but not PBMC-derived monocytes (Yu et al., 2007; Lefebvre et al., 2008; Lin et al., 2008). This data suggests that B cells may be a target cell for PCV2 infection. However, replication in lymphocytes is rather limited (Pérez-Martín et al., 2007), so, this cannot explain the lymphocyte depletion observed in PCV2-SD affected pigs. Therefore, it is expected that indirect mechanisms would impair the function of dendritic cells and reduce the number of B-cells, natural killer cells, $\gamma\delta$ T cells and CD4⁺ and CD8⁺ T lymphocytes of the circulation and lymphoid tissues. The loss of the immune effective cells would harm the development of innate and adaptive immunity, causing immunosuppression (Darwich et al., 2002; Grierson et al., 2007; Darwich and Mateu, 2012). This impairment of the immune system would explain why high PCV2 viremia is associated to the development of PCVDs.

PCV2 has been also detected in epithelial cells from kidney and the respiratory tract, smooth muscle cells, endothelial cells, enterocytes, hepatocytes, pancreatic acinar and ductular cells (Rosell et al., 1999; Shibahara et al., 2000; Sanchez et al., 2004). Nowadays it is considered that epithelial/endothelial cells are probably the most important ones supporting PCV2 replication (Pérez-Martín et al., 2007).

Up to now, and after more than fifteen years of research, the mechanisms of PCV2 pathogenesis are still poorly understood. During these years, many attempts to experimentally reproduce the disease by the sole inoculation of PCV2 have been unsuccessful (Fenaux et al., 2002; Opriessnig et al., 2007). Indeed, the disease has been mainly reproduced experimentally or observed under field conditions when other co-factors, (such as co-infection with other pathogens and poor housing, farm management and husbandry practices, etc...) have been considered (Beach and Meng, 2012; Rose et al., 2012; Meng, 2013).

1.2.5.2. Immunity induced by infection

The protection of piglets against PCV2 depends on MDI from sows and activation of their adaptive immunity. Generally speaking, the balance between the ability of piglets to mount a proper humoral and cellular mediated antiviral

responses and the virus ability to counteract it might be important to induce PCVDs.

MDI can normally last about 6-10 weeks of age, which can explain why PCV2-SD is not usually observed in piglets younger than 4 weeks of age (Grau-Roma et al., 2009; Kekarainen et al., 2010). Under field conditions, seroconversion due to an active infection normally happens between 7 and 15 weeks of age (Rodríguez-Arrioja et al., 2002). Experimentally induced seroconversion occurs around 10-20 days post-inoculation, depending on the serological technique used (Kekarainen et al., 2010). However, total PCV2 antibodies are not able to fully prevent animals from getting infected with PCV2, because several studies have shown the co-existence of PCV2 in serum with high titres of antibodies (Okuda et al., 2003; Meerts et al., 2006; Tribble et al., 2012). In consequence, the humoral immune response cannot be used as a parameter to measure protective immunity. Further studies indicated that PCV2-specific neutralizing antibodies (NA) are the responsible of clearing the virus from blood and decrease the viral burden (Seo et al., 2012; Ferrari et al., 2014; Seo et al., 2014b). Indeed, the level of NA seems positively correlated with the level of total antibodies (TA) (Fort et al., 2007). Under experimental conditions, low titres of NA were associated with increase of PCV2 viral load

and the start of clinical signs (Meerts et al., 2006). Another study also showed that an efficient NA response results in a reduction of PCV2 associated lesions and PCV2 viremia (Opriessnig et al., 2008a). It is considered that an insufficient TA and NA responses are linked to the development of PCV2-SD in PCV2 infected pigs.

Naturally PCV2-SD affected pigs show an impaired T lymphocyte cell response, as well as lymphocyte depletion and histiocytic infiltration (Kekarainen et al., 2010). In addition, in such clinically affected animals, high PCV2 load is linked to an increase of monocytes and neutrophils in blood, virus-specific **interleukin-10 (IL-10)** secreting cells (SC) in peripheral blood mononuclear cells (PBMC) and lymphoid organs, and reduction of blood **leukocytes** (specifically lymphocytes) (Kekarainen et al., 2010; Ferrari et al., 2014). PCV2 is also able to modulate cytokine profiles by increasing interferon- α in blood and decreasing IL-2, IL-4, IL-12 and interferon- γ (IFN- γ) levels in tissues and blood (Darwich et al., 2003a; Sipos et al., 2004; Kim et al., 2006; Vincent et al., 2007; Seo et al., 2012; Borghetti et al., 2013). IL-10 is well documented to have immunosuppressive and anti-inflammatory capabilities (Borghetti et al., 2013), and elevated amounts of its expression are linked to PCV2-SD expression (Stevenson et al., 2006). Moreover, IL-10 can suppress

the secretion of other cytokines, including IFN- γ , IL-4, IL-2, TNF- α and GM-CSF (Darwich et al., 2008; Crisci et al., 2010). IFN- γ is involved in CD4⁺ cells anti-viral response regulation by controlling the differentiation of naïve CD4⁺ into CD4⁺ (Seo et al., 2012; Ferrari et al., 2014). During PCV2 infection, suppression of IFN- γ secretion results in the loss of CD4⁺ cells, which would impair the immune system in the pig and may facilitate co-infection with other viral or bacterial pathogens, which could trigger PCV2-SD (Oh et al., 2012). Interestingly, a recent study indicated that after PCV2 natural exposure, a high frequency of IFN- γ -SC can be present; however, a low productivity of IFN- γ by those cells may not allow an efficient control of the virus (Ferrari et al., 2014).

1.3. Control and prevention measures

1.3.1. Controlling PCVDs by non-vaccination methods

Traditionally, before the wide use of vaccines, the prevention of PCV2 infection and PCVDs mainly depended on “Madec’s 20–point plan” (Madec et al., 2000; Madec et al., 2001). Such plan included measures based on management, environment and pig health. Briefly, this guide recommends all-in-and-all-out procedures, disinfection, limitation of pig-to-pig contact, to avoid mixing batches and cross-fostering practices, isolation or euthanasia of diseased

pigs, maintenance of appropriate temperature airflow and space conditions in pens, to apply recommended de-worming, anti-parasite treatments and vaccination schedules, and herd beneficial nutrition. Implementation of this plan can efficiently reduce PCV2 infection and PCVD occurrence and it is now accepted by the pig productive industry as a basic guide of management (Zhai et al., 2014). Besides, some studies have shown that improving housing, farm management and husbandry practices are able to affect the course of PCV2 infection and prevent to certain degree from the impact of PCVD (López-Soria et al., 2005; Dewey, 2008; Andraud et al., 2009; Rose et al., 2009). So the following points have been suggested by Rose et al. (2012) as risk factors for PCV2-SD occurrence:

- a) Housing conditions: large pens in weaning facilities, proximity to other pig farms, and a common pit between different fattening rooms.
- b) Vaccination schedules: vaccination of gilts against PRRSV and the use of separate vaccines against *Erysipelothrix rhusiopathiae* and PPV on gilts were found to be risk factors whereas vaccination of sows against *Escherichia coli* and atrophic rhinitis were protective.
- c) Hygiene and husbandry practices: short empty periods in nursery and farrowing sectors, intensity of pig mixing, early weaning (<21 days), purchase of replacement gilts, use of farm-boars for semen collection,

sows in poor conditions induced by poor injection techniques and rare treatments against ectoparasites in sows.

- d) Biosecurity: lack of shower facilities and visitors in contact with pigs before visiting the farm.
- e) Controlling PCV2 co-infection with other pathogens is also a crucial step to prevent PCVDs, since diseases are normally induced by the presence of concurrent infections.

In addition, genetic background or breed of animals have been reported as important in order to prevent PCVDs, since genetic susceptibility and resistance have been described (Rose et al., 2012). Under experimental conditions, Landrace pigs were more sensitive to PCV2 infection compared with Duroc, Large White and Pietrain animals, because a higher score of PCV2-associated microscopic lesions was observed in Landrace pigs (Opriessnig et al., 2006a; Opriessnig et al., 2009b). Under field conditions, pure or cross-bred Pietrain pigs had lower mortality compared to Large White–Duroc cross-bred pigs (López-Soria et al., 2011). In contrast, Rose et al. (Rose et al., 2005) showed no protective effect by means of introducing the Pietrain breed in a PCV2-SD affected farm. All in all, the mechanisms lying under genetic susceptibility or resistance to PCVDs remain unknown.

1.3.2. Controlling PCVDs by vaccination

1.3.2.1. Vaccines and vaccination

1.3.2.1.1. PCV2 vaccines

Nowadays, a total of 4 commercial vaccines are available in main swine producing industries worldwide. Since these vaccines were commercialized in different countries, their names might vary from country to country; however, the vaccines from the same pharmaceutical company have the same basic design (Table 1.2). All those vaccines were different in terms of antigen and adjuvant type, licensing (sow/piglets or both) and recommended usage (Beach and Meng, 2012; Chae, 2012a; Meng, 2013). The number of PCV2 vaccines in some Asian countries is much higher than those most widely used, mainly due to local laboratories commercializing their products in few or one single country (Zhai et al., 2014).

The first commercial vaccine against PCV2 (Circovac[®], Merial, Inc.), which is an inactivated PCV2a vaccine adjuvanted with light paraffin oil, appeared in France and Germany in 2004 (Charreyre et al., 2005). Afterwards (2007), this vaccine was available in other European countries and North America. Initially, Circovac[®] was licensed to be applied on sows and gilts 2-4 weeks prior to farrowing; later, it was licensed for piglets older than 3 weeks of

age by using a reduced dosage (Beach and Meng, 2012; Chae, 2012a; Fraile et al., 2012a; Meng, 2013). The other three vaccine products are licensed for growing pigs older than 2 or 3 weeks of age. FosterTM PCV (called Suvaxyn PCV2 One Dose[®] in Europe, Zoetis Inc.) is an attenuated chimeric viral vaccine with an adjuvant of sulpholipo-cyclodextrin in squalene-in-water. The chimeric virus was constructed by inserting the ORF2 gene of PCV2a into the non-pathogenic PCV1 backbone (Fenaux et al., 2003; Fenaux et al., 2004). Vaccines from Boehringer Ingelheim Vetmedica, Inc. (Ingelvac CircoFLEX[®]) and Merck. Inc. (Porcilis PCV[®] and Circumvent[®] in Europe and USA, respectively) are subunit vaccines based on PCV2a capsid protein (ORF2 protein) expressed in baculovirus systems (Chae, 2012a). The adjuvant of Boehringer Ingelheim product contains an aqueous polymer, while the Merck vaccine contains D1- α -tocopherol plus liquid paraffin (Chae, 2012a).

Currently, PCV2 vaccination is considered the most efficient tool to control PCV2 infection and PCVDs. The efficient control of PCV2 vaccines has been well demonstrated under both experimental and field conditions. Such vaccines are able to decrease or reduce mortality, clinical signs, viremia burdens, co-infection rate and PCV2 associated lesions. In addition, they are able to improve PCV2-specific immune responses, ADWG, body weight at

slaughter, reproductive parameters and finally increase the profits of the swine industry (Fort et al., 2008; Thacker et al., 2008; Pejsak et al., 2010; Martelli et al., 2013).

Although all those vaccines are based on PCV2a strains, it has been proved that all of them are capable to cross protect against PCV2b infection under field and experimental condition (Fort et al., 2008; Tribble and Rowland, 2012; Ellis, 2014; Opriessnig et al., 2014; Seo et al., 2014c; Zhai et al., 2014).

Recently, some potential “vaccine failure” cases have been reported in Brazil, Korea, some European countries and USA, and data indicated a potential link between PCV2d (usually referred as mutant PCV2b in the literature) infection and those cases (Xiao et al., 2012; Salgado et al., 2014; Seo et al., 2014d; Segalés, 2015). The fact raised concern about whether PCV2a based vaccines cannot cross-protect against PCV2d infection. However, Opriessnig et al. (2014) demonstrated such cross-protection. Very recently, a field study showed that commercial PCV2a vaccines reduced the PCV2d load with the appearance of both PCV2d-specific NA and IFN- γ -SC, which indicated that the current available PCV2a vaccines really cross-protect against PCV2d infection under commercial production conditions (Jeong et al., 2015).

Table 1.2.Current commercial PCV2 vaccines

PCV2 vaccine	Circovac	Ingelvac CircofLEX	Circum vent	Porcilis PCV	Fostera PCV	Suvaxyn PCV2
Company						
Antigen	Inactivated PCV2	PCV2 capsid protein	PCV2 capsid protein	PCV2 capsid protein	Inactivated chimaeric PCV1/2 virus	
Recommended animals	sow & piglet	Piglet	Piglet	Piglet	Piglet	
Admin istration	2 doses for sows at breeding age; 1 dose for piglets > 3 weeks of age	1 dose >2 weeks of age	2 dose at 3 and 6 weeks of age	1 dose >3 weeks of age	1 dose >3 weeks of age	

PCV2a based vaccines can control PCVDs and reduce the prevalence of PCV2a, but it has been suggested that the control of PCV2b infection may be not as efficiently as the one provided against PCV2a (Opriessnig et al., 2013a). Therefore, it has been speculated that a vaccine based on PCV2b might be more specific and efficient in controlling PCV2b, the current most prevalent genotype. A recent study has compared the efficacy of two experimental live-attenuated chimeric vaccines based on PCV2b and PCV2a, respectively, against PCV2b infection. The PCV2b product showed higher efficacy in controlling PCV2 infection than the PCV2a one (Opriessnig et al., 2013a).

Besides PCV2b based vaccines, some other new experimental vaccines are still under testing, such as modified live-attenuated PCV2, DNA-based, vectored and marker vaccines (Beach and Meng, 2012).

1.3.2.1.2. Vaccination strategies

Data available in the literature indicate that all commercial PCV2 vaccines are able to induce both humoral and cellular mediated immunity in sows and piglets (Fort et al., 2009; Chae, 2012a; Martelli et al., 2013; Park et al., 2014; Seo et al., 2014a). Three different strategies to vaccinate against PCV2 are being

used in the swine industry: sow, piglet or both. The purpose of all three strategies is to prevent PCVDs and reduce PCV2 infection pressure in the herd.

1.3.2.1.2.1. Sow vaccination

To prevent the appearance of PCVDs in piglets, one strategy is to vaccinate the breeding herd. From those vaccines mentioned above, only Circovac® was licensed for breeding animals. With this product, gilts and sows are recommended to be vaccinated 4-6 weeks before farrowing and a booster dose 2-3 weeks afterwards; in subsequent gestations only a booster dose at 2-3 weeks before farrowing is recommended (Beach and Meng, 2012; Fraile et al., 2012a). Circovac® vaccinated sows can transfer MDA and PCV2-specific immune cells to piglets (Goubier et al., 2008).

Although PCV2-RD is seldom reported, some data showed the sow and gilts vaccination can also increase the number of live born, birth weight and decrease the rate of abortion, mummies per sow (Vila, 2004; Pejsak et al., 2012). However, an earlier study showed there had no difference in those production parameters, even a significantly higher PCV2 antibody level was seen in vaccinated sows than non-vaccinated ones (Kurmann et al., 2011).

Under both experimental and field conditions, sow and gilt vaccination is able to reduce PCV2 viremia, systemic viral load, lymphoid lesion in piglets and improving the ADWG in subclinically infected offspring (Segalés, 2015). It is well documented that protection generated by sow and gilt vaccination comes from MDI (Allan et al., 2002; McKeown et al., 2005; Fraile et al., 2012b). Sow vaccination can increase PCV2 antibody in secretions, especially in colostrum (Gerber et al., 2011, 2012; Sibila et al., 2013). Besides humoral immunity, it is known that cell mediated immune response (measured as PCV2-specific IFN- γ -SC) can also be transferred from sows to piglets via colostrum (Goubier et al., 2008). These maternally derived PCV2-specific IFN- γ -SC may help protecting piglets from infection (Oh et al., 2012). However, duration of this maternally derived adaptive cellular immunity is still unclear (Kekarainen et al., 2010; Oh et al., 2012). Some studies have suggested that vaccination of sows with piglet vaccines (FosteraTM PCV, Ingelvac[®] CircoFLEXTM) does not induce MDA levels as high as those elicited by the sow licensed vaccine (Circovac[®]) (Opriessnig et al., 2010; Oh et al., 2014).

1.3.2.1.2.2. Piglet vaccination

The objective of piglet vaccination is to prevent them to get diseased by means of an early and significant antibody and IFN- γ -SC response.

Piglet vaccination is normally applied at around 2 to 4 weeks of age, and can efficiently reduce mortality, viremia, viral load in tissues, microscopic lymphoid lesions and increase the productive parameters in piglets (Segalés et al., 2009; Pejsak et al., 2010; Takahagi et al., 2010; Martelli et al., 2011; Haake et al., 2014; Segalés, 2015). After vaccination, an early seroconversion is usually observed around 3 weeks post-vaccination (Opriessnig et al., 2010; Fraile et al., 2012b; Tribble et al., 2012). However, not all vaccines display an evident seroconversion in spite of being efficient to control PCVDs, which indicates that TA elicited by vaccines are not a measure of protection.

Duration of immunity conferred by piglet vaccination might not allow reaching the slaughter age fully protected. One study indicated that early vaccinated animals may have a risk of re-infection in the late finishing phase (Opriessnig et al., 2009a). However, another study indicated that animals from vaccinated sows vaccinated at 7 weeks of age resulted in lower mortality and a significantly higher productive parameters compared with the animals from only vaccinated sows and the ones only vaccinated at 4 weeks of age (Pejsak et al., 2010). The results indirectly implied that the proper vaccination timing that can cover the whole production period still has room for improvement.

1.3.2.1.2.3. Sow and piglet vaccination

Until now, there are some data available evaluating the efficacy of combination of sow and piglet vaccination (Opriessnig et al., 2010; Pejsak et al., 2010; Fraile et al., 2012b; Oh et al., 2014). Under experimental conditions, vaccinating both sows and piglets could significantly reduce viremia and the viral load in lymphoid tissues (Opriessnig et al., 2010; Oh et al., 2014). In Oh et al. (2014), a stronger humoral as well as cell mediated immune responses and milder lymph node lesion scores were observed in the combination of sow and piglet compared with animals from single sow or piglet vaccination. Data from field studies showed this strategy could also result in a higher ADWG than single vaccination of sows or piglets (Pejsak et al., 2010; Fraile et al., 2012b).

However, the combined vaccination strategy has led to a concern about the potential MDI interference on piglet vaccination.

1.3.2.2. Maternally derived immunity (MDI) and vaccination

Although commercial PCV2 vaccine efficacy has been extensively proven in controlling PCV2 infection and PCVDs (Meng, 2013; Segalés, 2015), a potential interference of MDI on vaccine efficacy is still under debate. Some early studies concluded that high MDA titres did not have interference on the

seroconversion to vaccination (Opriessnig et al., 2010; Martelli et al., 2011). However, other experimental (Fort et al., 2009; Oh et al., 2014) and field (Fraile et al., 2012a; Fraile et al., 2012b) studies proved that the seroconversion due to vaccination is impaired only when the immunization is done in presence of high levels ($IPMA \geq 10\text{Log}_2$) of MDA. Therefore, it was hypothesized that vaccine induced seroconversion interference is MDA titre-dependent.

Basically, the ideal vaccination timing is recommended when MDA are minimal and before the animals become naturally infected (Fort et al., 2009; Seo et al., 2014c). Some studies have tackled this issue (Haake et al., 2014; Oh et al., 2014). In Haake et al. (2014), piglets with relatively high MDA titres from two farms were vaccinated at 1 or 3 weeks of age. Results of this study showed a stronger MDA interference on activation of humoral immunity in animals vaccinated at 1 weeks of age than vaccinated 3 weeks of age. In the other study (Oh et al., 2014), three vaccination regimens (vaccination of sow, piglet at 3 or 7 weeks of age, or combine sow and piglet at 3 or 7 weeks of age) were explored. Obtained data suggested that a significant negative correlation between MDA at the day of vaccination and the increase of antibody titres after vaccination of sows and piglets at 3 weeks of age occurred. In contrast, no interference was observed in piglets vaccinated at 7 weeks of age coming from

vaccinated sows. The lower viremia and the most effective humoral and cellular immune responses were observed in this latter group as well (Oh et al., 2014).

As it has been previously mentioned, high levels of MDA are able to affect the vaccine induced seroconversion. However, the effect of such high antibody level to the efficacy of the vaccine in terms of productive parameters is not so well characterized. Following the same premise, one would expect a negative correlation between high level MDA at the time of vaccination and ADWG. This putative interference has only been assessed, for the moment, in three studies (Fachinger et al., 2008; Fraile et al., 2012b; Haake et al., 2014). All these studies finally concluded that the MDA level at the time of vaccination cannot interfere the growth of ADWG. Therefore, although seroconversion to vaccination seems to be interfered by MDA, vaccines seem to overcome such MDA in terms of efficacy. However, Fraile et al. (2012b) described a negative correlation between MDA levels at the time of vaccination and ADWG, but this correlation was not significant. Furthermore, data from Haake et al. (2014) showed a higher ADWG ($P>0.05$) in animals vaccinated at 3 weeks of age (with relatively low MDA titres at vaccination) compared to those vaccinated at 1 weeks of age (with high MDA titres at vaccination). So, the correlation between the level MDA at the time of vaccination and ADWG is still unclear.

Overcoming such MDI is probably related with the vaccine-induced cellular immunity (Fort et al., 2009; Chae, 2012a; Martelli et al., 2013; Park et al., 2014; Seo et al., 2014a). However, it seems that the cellular immune response is not interfered by the level of MDI (Martelli et al., 2013; Oh et al., 2014).

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

Nowadays, PCV2 vaccines are widely available in the pig industry worldwide. All of them have been proven efficient in controlling PCV2 infection and PCVDs. Different regimes of vaccine application have been described, combining or not sow and piglet vaccination. Importantly, vaccinating piglets with the existence of high levels of MDA may result in jeopardizing the humoral immune response elicited by the vaccine. Besides, previous data also showed a lower ADWG in vaccinated animals with high MDA level compared with the counterpart with lower MDA (Haake et al., 2014). Therefore, there is still the controversy about the potential interference of vaccination in presence of high MDA values on the productive efficacy of the vaccine product.

PCV2 sole infection rarely results in clinical disease. Previous data indicated high PCV2 viral load in newborn piglets is associated with the development of PCV2-SD (Meerts et al., 2006; Seo et al., 2014c). Nowadays, the measure of viral load becomes an important parameter to evaluate the efficacy of commercial vaccines. Few experimental data concluded that one or two doses of a PCV2 vaccine may result in a complete clearance of PCV2 from blood (Fort et al., 2008; Hemann et al., 2012). Therefore, taking into account the well-known one dose vaccine efficacy to control PCVDs, these latter results

may allow hypothesizing the possibility to eradicate or eliminate PCV2 from pig herds by massive and long-term PCV2 vaccination.

Considering the abovementioned rationales and hypotheses, the present thesis aimed to characterize the potential interference of different MDA levels on virological parameters and productive data, as well as to assess the feasibility to eradicate PCV2 from a conventional farm. The specific objectives of this thesis were the following ones:

- To assess PCV2 vaccine efficacy in terms ADWG in purposely selected animals with high and low PCV2 ELISA S/P levels at the time of PCV2 vaccination.
- To evaluate the feasibility to eradicate PCV2 in a conventional farm by mass vaccination in both sows and piglets during a 1-year period

CHAPTER 3

Study 1:

*Effect of maternally derived antibodies on Porcine
circovirus type 2 (PCV2) infection dynamics and production
parameters in PCV2 vaccinated pigs under field conditions*

Submitted for publication

3.1. Introduction

PCV2 is the essential causative agent of a series of diseases known as PCVD (Segalés, 2012; Meng, 2013). PCV2-SD is one of the most economically important PCVD, since increases the mortality rate and reduces production parameters (Darwich and Mateu, 2012; Meng, 2013). The effects derived from PCV2-SD have been drastically reduced by the use of different available commercial vaccines at the worldwide swine production market (Segalés, 2015).

Besides the contrasted efficacy of PCV2 vaccines, some field and experimental studies have indicated that vaccination in face of high MDA levels may affect such efficacy. This potential interference has been studied at two different levels: vaccine-elicited humoral response and ADWG. In terms of humoral response, it has been proven that high antibody levels at the moment of vaccination jeopardize the seroconversion elicited by vaccination (Fort et al., 2009; Fraile et al., 2012a; Fraile et al., 2012b, Oh et al., 2014). The effect of high MDA level on ADWG is still not clear. So far, there are only three studies in which this effect has been assessed (Fachinger et al., 2008; Fraile et al., 2012b; Haake et al., 2014). In Fachinger et al. (2008), animals included in the study were selected and separated in two groups based on the level of MDA at

the moment of vaccination ($>1:1000$ and $<1:1000$ Indirect Fluorescence Antibody Titration [IFAT] titres). Both groups of animals had similar ($P>0.05$) ADWG and in consequence it was concluded that this parameter was not affected by MDA level. However, the average titre for both groups of animals was not provided in the paper, and apparently they were not sharply different. Similarly, Fraile et al (2012b) did not find statistically significant differences in terms of ADWG between 4-week-old vaccinated piglets derived from vaccinated and non-vaccinated sows. However, the correlation between initial MDA and ADWG (in the double vaccinated ones) showed a negative slope. These results suggested a potential negative effect when higher MDA titres were present at vaccination time. In Haake et al. (2014), pigs were vaccinated at 1 or 3 weeks of age, which rendered different MDA levels at the moment of vaccination. In that study, animals vaccinated at 3 weeks of age had a higher ADWG than the ones vaccinated at 1 week of age. When compared, antibody titres of the pigs at 1 week of age were higher than those at 3 weeks of age

In summary, data available up to now on the effect of the MDA titres at the moment of vaccination on the ADWG are not conclusive and, therefore, further analyses were required. In consequence, the present study aimed to assess PCV2 vaccine efficacy in terms ADWG in purposely selected animals

with high and low PCV2 ELISA S/P levels at the time of PCV2 vaccination. In addition, antibody and infection dynamics and viral loads of these animals were studied.

3.2. Materials and methods

3.2.1. Farm selection

The present study was conducted in a conventional Spanish multi-site production system in which PCV2 vaccination at 3 week-old piglets (Porcilis® PCV, MSD) was applied routinely since 2 years before starting this study. An all-in-all-out strategy was used in both nursery and fattening units.

In order to assess PCV2 infection before the start of the study, blood samples from 10 animals of different ages (5, 9, 14, 18 and 24 weeks of age) were taken. These blood samples were processed by standard PCR (Quintana et al., 2002). PCV2 genome was detected in 30% (3 out of 10) and 40% (4 out of 10) of pigs at 14 and 18 weeks of age, respectively. All tested samples from 5, 9 and 24 weeks of age were negative by PCR.

3.2.2. Study design

To ensure the presence of different levels (from very low to very high) of PCV2 MDA titres at the moment of vaccination, a proportion of sows were vaccinated before farrowing. Thus, from 64 randomly selected sows, 33 (52%) were vaccinated with 1 dose of 2 mL of Circovac[®] (Merial) at 3 and 6 weeks pre-farrowing (V sows). The remaining 31 sows were left non-vaccinated (NV sows).

At 2 weeks of age, all healthy piglets (n = 572) born from these 64 sows were ear-tagged, weighted and bled. Levels of PCV2 antibodies were measured by means of an indirect ELISA (detailed in PCV2 antibody detection section). The ELISA S/P ratios obtained in these 572 animals ranged from 0.14 to 2.68 (mean \pm standard deviation [SD] = 1.25 ± 0.70). From all tested animals, those piglets with the highest (>1.44 , n = 169) and the lowest (<0.96 , n = 168) PCV2 ELISA S/P ratios were selected. Animals with medium (>0.96 and <1.44) PCV2 ELISA S/P ratios were removed from the study. Afterwards, selected animals were distributed based on their weight in 4 treatments groups according to the levels of MDA (H = High, L = Low) and vaccination status (V = vaccinated; NV = Non-vaccinated), as detailed in Table 3.1. At 3 weeks of age, V piglets (n = 171) were injected IM with 1 mL of Ingelvac[®] Circoflex (Boehringer Ingelheim), in the right side of neck. NV animals (n = 166) received the same

dose of PBS at the same anatomic location. Animals from different treatments were comingled in the same pens, both in nurseries and fattening units. Mortality was recorded through the study.

Table 3.1. Piglet distribution according to PCV2 MDA level at 2 weeks of age, PCV2 vaccination (V = vaccinated; NV = Non-vaccinated) and sow treatment (sow treatment).

PIGLETS		Sow treatment		
Level of S/P ratio at 2 weeks of age	Treatment	NV	V	Total
High S/P ratio (> 1.44)	NV	6	70	76
	V	13	80	93
Low S/P ratio (< 0.96)	NV	75	15	90
	V	59	19	78
Total		153	184	337

During the study period, blood samples from all monitored pigs were subsequently taken at 7, 12, 18, 22 and 25 weeks of age. Once in the laboratory, blood samples were allowed to clot and centrifuged at 1500 g for 10 min.

Additionally, animals were weighted at 12 and 25 weeks of age. ADWG was calculated for the following periods: 2-12, 12-25 and 2-25 weeks of age. ADWG was calculated as the weight at the last studied time point minus the

weight at first selected time point divided by the days lapsed between both time points.

Treatments, housing, and husbandry procedures were conducted in accordance with the guidelines of Good Experimental Practices, under the approval of the Ethical and Animal Welfare Committee of the *Universitat Autònoma of Barcelona* and Government of Catalunya (Protocol #DMAH-5796).

3.2.3. DNA extraction, PCR and Q-PCR

DNA extraction from serum samples was done using BioSprint 96 DNA Blood kit (Qiagen, GmbH). All DNA samples were processed by standard PCV2 PCR and those yielding positive results were subsequently tested by a Q-PCR commercial kit (LSI VetMAX Porcine Circovirus Type 2 - Quantification). Standard PCR results were expressed as percentage of positive animals. QPCR results and area under the curve (AUC) of viremia (López-Soria et al., 2014) were expressed as \log_{10} PCV2 DNA copies/mL (\pm SD) for Q-PCR positive samples.

3.2.4. PCV2 antibody detection

Serum samples were tested by an indirect ELISA commercial (INGEZIM, Circo IgG 1.1. PCV. K.1). Mean S/P ratio cut-off for this ELISA test was set at 0.33 (0.4 OD). Results of ELISA were expressed as mean S/P ratio (\pm SD) and percentage of seropositive pigs.

3.2.5. Statistical analyses

All statistical analyses were done by SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). All data (body weight, ADWG, ELISA S/P values, percentage of ELISA and PCR positive pigs as well as mean PCV2 viral load and AUCs) were compared at two different levels: 1) between V and NV piglets, and 2) among H-V, L-V, H-NV and L-NV groups. Descriptive statistics were used to summarize categorical and quantitative variables. Normality of distribution of the examined quantitative variables was evaluated by Shapiro Wilk's and Levene tests. Body weight and ADWG were compared using an unpaired T-test. The Chi-square or Fischer exact test was applied to evaluate the proportion of positive and negative animals by ELISA, PCR and the mortality among these four groups. Data on ELISA S/P ratios, PCV2 viral load and AUCs were assessed with a non-parametric Mann–Whitney test. The significance level was set at 0.05.

3.3. Results

3.3.1. Clinical findings

No PCV2-SD-like clinical signs were observed throughout the trial. Percentage of dead pigs was 3.2% (3 out of 93), 2.5% (2 out of 78), 2.6% (2 out of 76) and 4.4% (4 out of 90) for H-V, L-V, H-NV, L-NV animals, respectively ($P>0.05$). Specific causes for such mortality were not investigated. In addition, 10 animals were excluded from the study because of losing ear tags.

3.3.2. Comparisons between vaccinated and non-vaccinated pigs

3.3.2.1. PCR and Q-PCR

PCV2 was firstly detected in both treatments at 18 weeks of age (Figure 3.1). Percentage of PCV2 PCR positive pigs as well as mean PCV2 load in serum was significantly lower at 18, 22 and 25 weeks of age in V than NV pigs. PCV2 load AUC was significantly higher ($P<0.05$) in NV ($6.0 \pm 1.3 \log_{10}$ PCV2 DNA copies/mL) than in V ($4.8 \pm 1.1 \log_{10}$ PCV2 DNA copies/mL) animals.

3.3.2.2. Antibody dynamics

At 7, 12 and 18 weeks of age, percentage of seropositive pigs was significantly higher in V group than in their NV counterparts (Figure 3.2). Mean ELISA S/P values were significantly higher ($P < 0.05$) in V compared to NV pigs from 7 to 18 weeks of age. From that moment onwards, the ELISA S/P ratios from V pigs were significantly lower ($P < 0.05$) than those of NV animals.

3.3.2.3. Body weight and ADWG

No statistical differences were found in the body weight between V and NV piglets from the beginning to the end of the study (Table 3.2). ADWG was significantly higher ($P < 0.05$) in V compared to NV during the 12-25 and 2-25 week-periods; specifically, V animals gained 33 and 17g per day more than NV pigs, in the respective periods.

3.3.3. Comparisons among vaccinated and non-vaccinated pigs with low and high ELISA S/P values

3.3.3.1. PCR and Q-PCR

A significantly ($P < 0.05$) lower number of PCV2 PCR positive pigs was observed in L-V compared to NV groups at 18, 22 and 25 weeks of age and in H-V group compared to NV groups at 22 and 25 weeks of age (Figure 3.3).

Between the two V groups, statistical significant differences were only found at 22 weeks of age (higher in the H-V group).

A significantly ($P < 0.05$) lower PCV2 viral load was observed in L-V compared to the both NV groups at 18 and 22 weeks of age and in H-V pigs compared to the NV groups at 22 and 25 weeks of age. No statistical differences were found between L-V and H-V groups throughout the study.

The AUC of viral load in H-V ($5.1 \pm 1.3 \log_{10}$ PCV2 DNA copies/mL) and L-V ($4.5 \pm 1.0 \log_{10}$ PCV2 DNA copies/mL) groups was significantly lower ($P < 0.05$) than in H-NV ($5.8 \pm 1.3 \log_{10}$ PCV2 DNA copies/mL) and L-NV ($6.2 \pm 1.3 \log_{10}$ PCV2 DNA copies/mL). However, no statistical differences were found between H-V and L-V ($P = 0.09$) and between H-NV and L-NV ($P = 0.11$).

3.3.3.2. Antibody dynamics

Statistically significant differences in percentage of ELISA positive animals among the 4 groups were observed at 7, 12 and 18 weeks of age (Figure 3.4). At 7 weeks of age, the lowest ($P < 0.05$) percentage of seropositive pigs was observed in L-NV, followed by the one in L-V group. Five weeks later, L-NV

group showed still a significantly lower ($P<0.05$) percentage of ELISA positive pigs than the other three groups. At that point, while L-V and H-NV had similar percentage of seropositive pigs, H-V group showed the highest rate of ELISA positive pigs. At 18 weeks of age, the dynamic changed since the highest ($P<0.05$) percentage of ELISA positive animals was observed in L-V animals.

A sharp decrease (up to 12 weeks of age) of ELISA S/P values was observed in both H groups (Figure 3.5). On the contrary, in the L groups the decrease in S/P values was seen until 7 weeks of age. At that point, whereas L-V pigs showed a progressive increase of ELISA S/P values, a flat line from 7 to 18 weeks of age was observed in L-NV ones. Afterwards, all groups experienced an increase of ELISA S/P ratios being significantly higher ($P<0.05$) in both NV groups than their V counterparts. Interestingly, at the two latter sampling points, L-V pigs had significantly lower ($P<0.05$) ELISA S/P ratios than H-V ones.

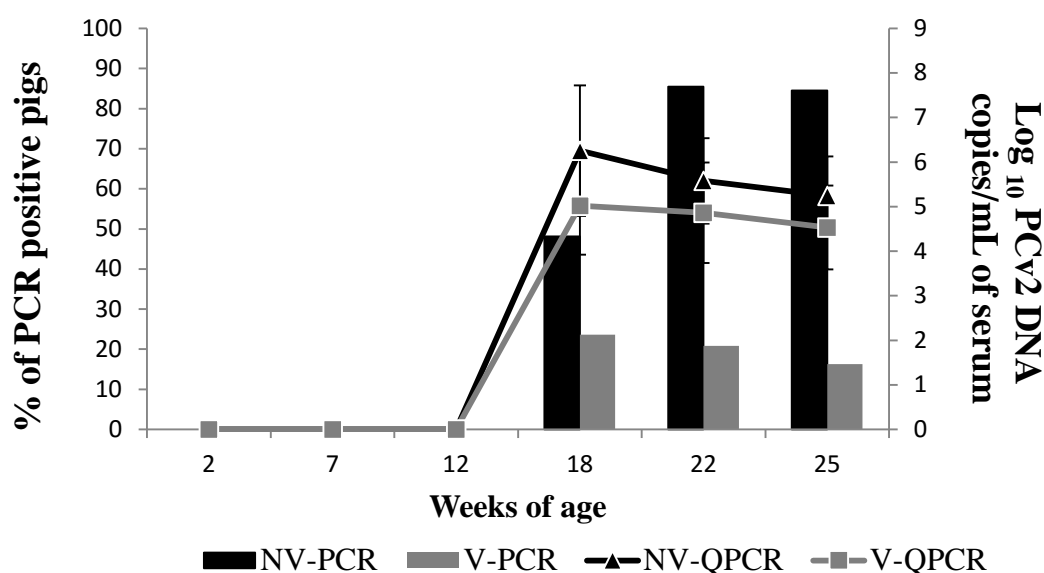
3.3.3.3. Body weight and ADWG

At 2 and 12 weeks of age, no significant differences were observed in body weight among the 4 groups (Table 3.2). At 25 weeks of age, L-NV showed the

lowest body weight, being significantly lower ($P<0.05$) when compared to V pigs.

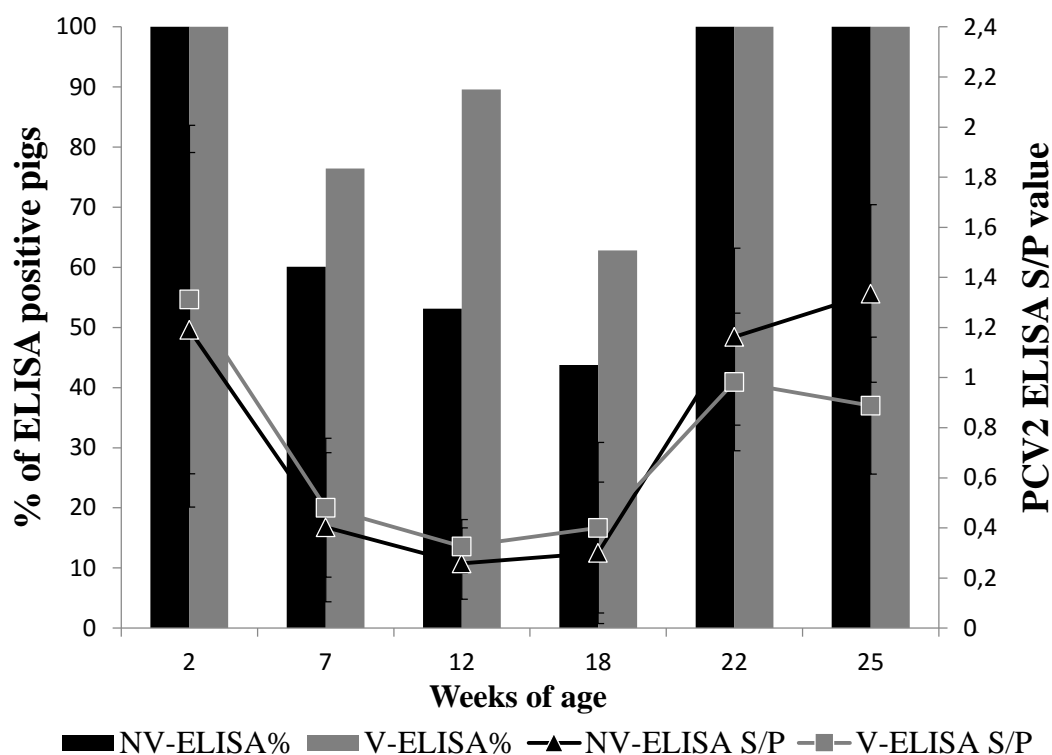
L-V and L-NV pigs showed the highest and the lowest ADWG, respectively, in both periods 12-25 and 2-25 weeks. Statistically significant differences were observed between L-V and NV groups for the period 12-25 weeks and between V and L-NV for the period 2-25 weeks.

Figure 3.1. Percentage of PCV2 PCR positive pigs (bars and left Y axis) and log₁₀ PCV2 DNA viral loads (mean± SD) (lines and right Y axis) of PCR positive pigs in V and NV groups at the six sampling time points, respectively. In the table, different low-case letters within a sampling point mean statistically significant differences in the percentage of PCR positivity between V and NV pigs ($P<0.05$); different capital letters within a sampling point mean statistically significant differences in PCV2 DNA load in serum between V and NV pigs ($P<0.05$).



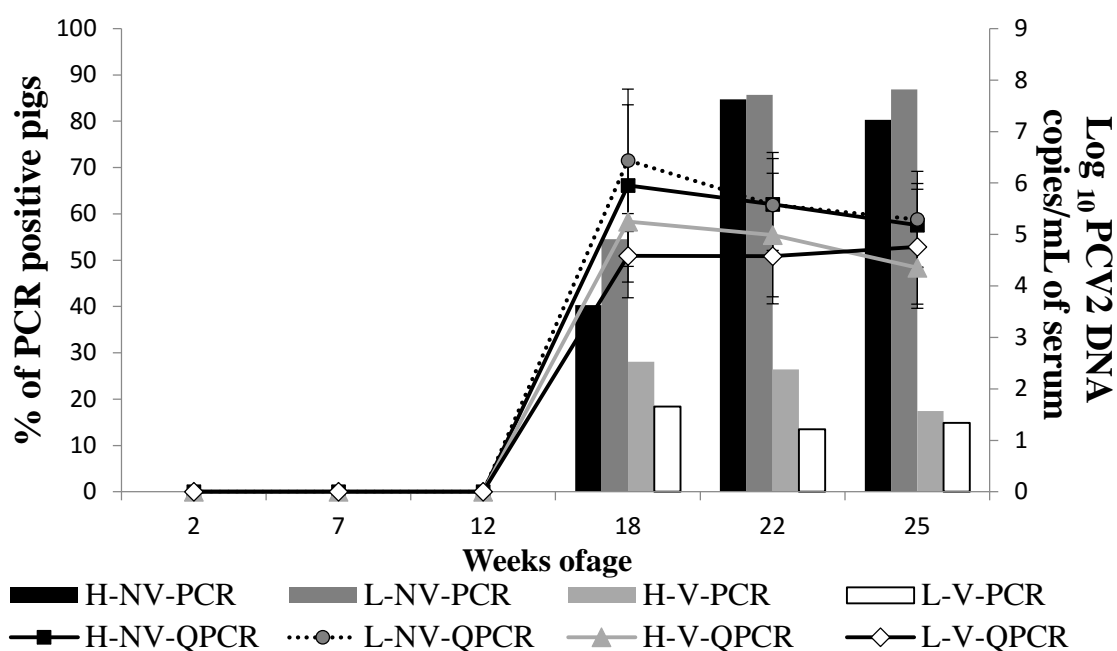
	2 weeks		7 weeks		12 weeks		18 weeks		22 weeks		25 weeks	
	%	load	%	load	%	load	%	load	%	load	%	load
NV	a	A	a	A	a	A	a	A	a	A	a	A
V	a	A	a	A	a	A	b	B	b	B	b	B

Figure 3.2. Percentage of ELISA positive pigs (bars and left Y axis) and PCV2 ELISA S/P ratio (mean \pm SD) (lines and right Y axis) values and in the six sampling points for both V and NV pigs, respectively. Different low-case letters in the table within a sampling point mean statistically significant ($P<0.05$) differences in percentage of ELISA positivity between V and NV animals; different capital letters within a sampling point mean statistically significant differences in ELISA S/P values among the 4 groups ($P<0.05$).



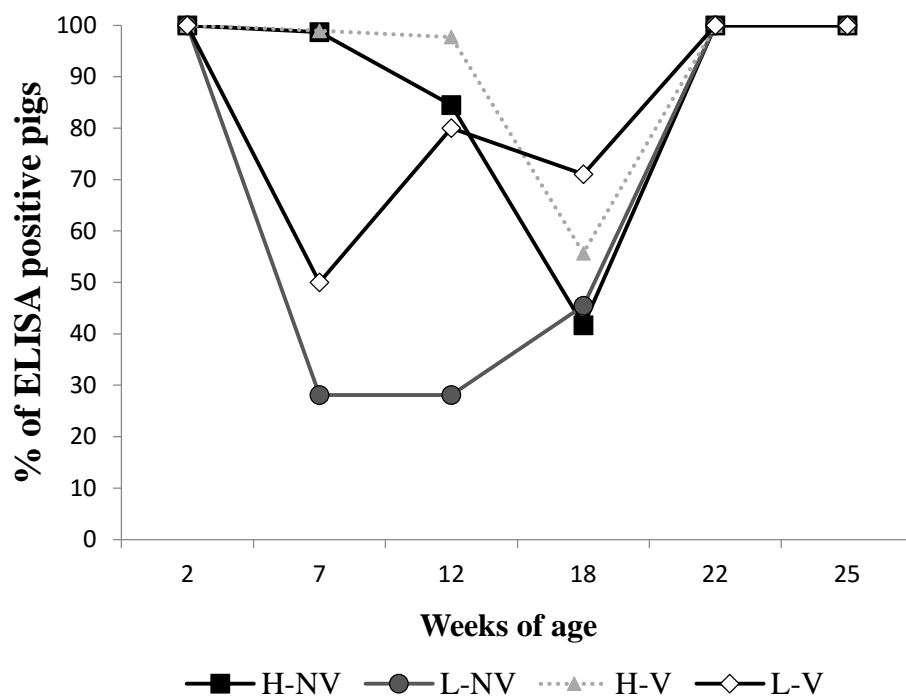
	2 weeks		7 weeks		12 weeks		18 weeks		22 weeks		25 weeks	
	%	S/P	%	S/P	%	S/P	%	S/P	%	S/P	%	S/P
NV	a	A	a	A	a	A	a	A	a	A	a	A
V	a	A	b	B	b	B	b	B	a	B	a	B

Figure 3.3. Percentage of PCV2 PCR positive pigs (bars and left Y axis) and log₁₀ PCV2 DNA loads (mean± SD) (lines and right Y axis) of PCR positive pigs in H-NV, L-NV, H-V and L-V groups at the six sampling times, respectively. In the table, different low-case letters within a sampling point mean statistically significant differences in the percentage of PCR positivity among the 4 groups ($P<0.05$); different capital letters within a sampling point mean statistically significant differences in PCV2 DNA load in serum among the 4 groups ($P<0.05$).



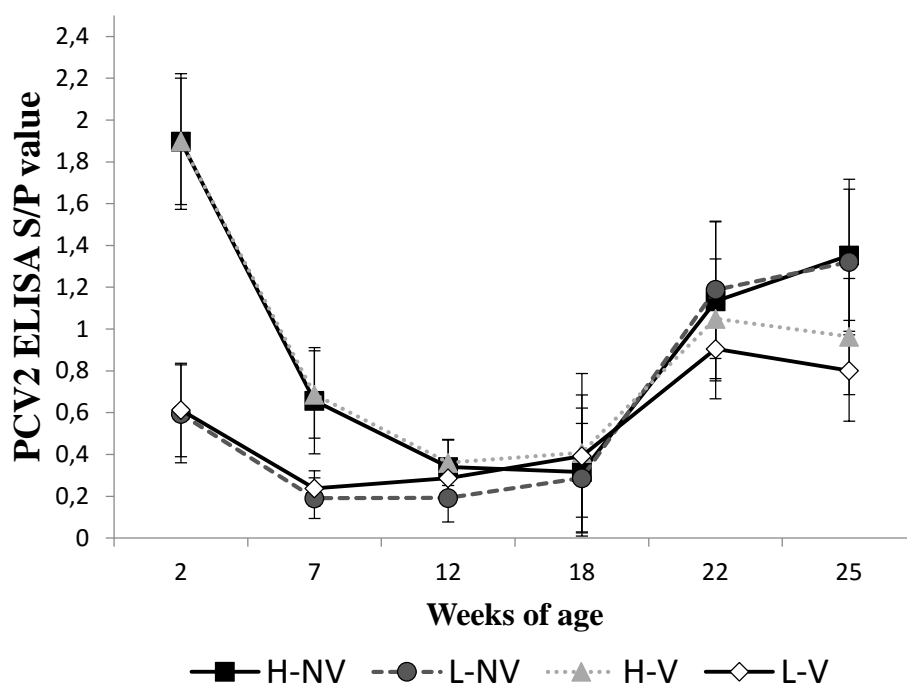
	2 weeks		7 weeks		12 weeks		18 weeks		22 weeks		25 weeks	
	%	load	%	load	%	load	%	load	%	load	%	load
H-NV	a	A	a	A	a	A	ab	AB	a	A	a	A
L-NV	a	A	a	A	a	A	a	A	a	A	a	A
H-V	a	A	a	A	a	A	bc	BC	b	B	b	B
L-V	a	A	a	A	a	A	c	C	c	B	b	AB

Figure 3.4. Percentage of ELISA positive pigs at the six sampling points for H-NV, L-NV, H-V and L-V pigs. Different low-case letters in the table within a sampling point mean statistically significant ($P < 0.05$) differences in percentage of ELISA positive pigs among the 4 groups.



	2 weeks	7 weeks	12 weeks	18 weeks	22 weeks	25 weeks
H-NV	a	a	a	a	a	a
L-NV	a	b	b	a	a	a
H-V	a	a	c	a	a	a
L-V	a	c	a	b	a	a

Figure 3.5. PCV2 ELISA S/P ratio (mean \pm SD) values at the six sampling points for H-NV, L-NV, H-V and L-V pigs. Different low-case letters in the table within a sampling point mean statistically significant ($P<0.05$) differences in ELISA S/P values among the 4 groups.



	2 weeks	7 weeks	12 weeks	18 weeks	22 weeks	25 weeks
H-NV	a	a	a	a	ab	a
L-NV	b	b	c	a	a	a
H-V	a	a	a	b	b	b
L-V	b	b	b	b	c	c

Table 3.2 Body weight (mean kg \pm SD) at different weeks of age and average daily weight gain (ADWG, [g \pm SD]) for different week intervals. Different letters within a sampling point mean statistically significant differences ($p < 0.05$).

Body weight (kg)				ADWG (g)			
Weeks of age	2	12	25	Period (weeks)	2-12	12-25	2-25
Groups	NV	2.4±0.5 ^a	27.1±5.8 ^a	98.3±13.9 ^a	324.5±72.1 ^a	774.5±111.7 ^a	570.9±81.5 ^a
	V	2.4±0.5 ^a	26.8±4.9 ^a	101.0±12.8 ^a	321.4±60.9 ^a	807.4±107.6 ^b	587.5±75.3 ^b
Groups	H-NV	2.5±0.5 ^a	28.0±5.8 ^a	100.9±12.8 ^{ab}	336.5±72.3 ^a	792.3±90.9 ^a	586.1±74.7 ^{ab}
	L-NV	2.4±0.5 ^a	26.3±5.7 ^a	96.2±14.6 ^a	314.4±70.8 ^a	759.7±125.2 ^a	558.2±85.0 ^a
	H-V	2.4±0.5 ^a	27.5±5.2 ^a	101.3±13.0 ^b	329.9±65.3 ^a	799.0±112.7 ^{ab}	586.8±81.7 ^b
	L-V	2.4±0.6 ^a	26.1±4.4 ^a	100.5±12.6 ^b	311.7±54.1 ^a	816.8±101.5 ^b	588.3±67.8 ^b

3.4. Discussion

The present field study assessed the effect of PCV2 vaccination on productive, serological and virological parameters in piglets with different MDA levels at the time of vaccination in a conventional farm with PCV2-SI scenario. Globally and under the conditions of this study, vaccination significantly reduced PCV2 infection rate and load, increased antibody response and improved ADWG in 17 g/day within the 2-25 week period. These results are in agreement with those published studies in which efficacy of different PCV2 vaccine products under PCV2-subclinical infection conditions were assessed (Fort et al., 2009; Fraile et al., 2012b; Martelli et al., 2013; Ferrari et al., 2014).

The effect of MDA levels at vaccination age was assessed on ADWG as primary outcome. The initial hypothesis was that the higher the MDA at vaccination timing, the lower the ADWG. However, such hypothesis was not confirmed since a potential detrimental effect of MDA on ADWG was not evident. Although L-V animals grew 2 and 18 g per day more than H-V ones in the 2-25 and 12-25 week periods, such differences were not statistically significant. Besides, virological and serological parameters were also studied. In the present study, pigs vaccinated with low MDA seemed to take more

benefit of the treatment than their counterparts with high MDA, since they had lower PCV2 infection rate (at 22 weeks of age), lower AUC of viral load and showed an earlier seroconversion (evident at 12 weeks of age). These latter results would be in accordance with those previously published studies (Fraile et al., 2012a; Fraile et al., 2012b; Oh et al., 2014) in which the interference of high MDA titres at the moment of vaccination with the humoral response elicited by the vaccine was demonstrated. It is worthy to highlight, however, that vaccination was able to overcome such interference since statistically significant differences were seen between H-V vs H-NV animals in terms of infection rate at 22 and 25 weeks of age and mean ELISA S/P ratios at 18 weeks of age.

The specific reason by which MDA affected PCV2 virological and serological parameters but not ADWG remains unknown. Recent data in non-vaccinated pigs has demonstrated that the higher the AUC of viral load, the lower the ADWG (López-Soria et al., 2014). This situation applied in the present work when comparing the V and NV groups, but the scenario is more complex when studying existing subpopulations in terms of low and high MDA at vaccination. Under the scenario of low MDA levels, V animals had significantly lower AUC and significantly higher ADWG than their NV

counterparts; on the contrary, in a high MDA level context, V animals had significantly lower AUC but similar ADWG than NV ones. In addition, the non-significant, numeric ADWG differences between L-V and H-V may suggest that, if occurring, interference of MDA with ADWG would be seen only in those animals with extremely high MDA levels. This hypothesis would be supported by the fact that in the present and in Haake et al. (2014) studies, the best (although no significantly different) productive performances were seen when vaccination was applied in presence of low MDA titres. Indeed, in the present study, the 10 animals with the highest MDA titres (>2.4 ELISA S/P titres) at the moment of vaccination, coming all of them from vaccinated sows, grew 52 g/day less than the rest of the vaccinated animals ($n=151$, with average ELISA S/P value of 1.23 ± 0.65) (data not shown). According to Pileri et al. (2014), these >2.4 S/P values would be equivalent to $>17 \log_2$ IPMA titres. In fact, the MDA titres interference on humoral response to vaccination has been established around $8-10 \log_2$ IPMA titres (Fort et al., 2009), being $14 \log_2$ IPMA the result of the highest dilution of the IPMA test routinely performed (Rodríguez-Arrioja et al., 2000). In consequence, $17 \log_2$ IPMA titres would be an extremely high MDA titre, probably not very frequently found under field conditions. Therefore, if these high MDA titres are present in a very small

proportion of animals, the economic relevance of such putative interference would be presumably low or negligible in most of the cases.

These very high antibody titres were “artificially” created by means of vaccinating a proportion of the sows. This action was aligned with the need of a sufficient number of piglets with the highest MDA levels possible to achieve the objective of this study. It cannot be ruled out that both humoral and cellular immunity linked to the colostrum intake from these sows might have exerted certain effect on the obtained results. However, such effect is difficult to establish. In this study, MDA levels reached the lowest S/P ratios around 12 weeks of age, while evidence of PCV2 infection started at 18 weeks of age. In consequence, it is difficult to believe that MDA exerted an effect on virus dynamics. Moreover, the antibody evolution of piglets with high antibody values coming from vaccinated and non-vaccinated sows were very similar (data not shown), reinforcing the notion that sow vaccination did not apparently bias the obtained results. In addition, and although not measured in the present work, there is controversial data regarding the duration of piglet cellular immunity provided by sow vaccination (Goubier et al., 2008; Oh et al., 2012). In the same line, it is noteworthy that the benefit of piglet vaccination also derives from the protection conferred by the cellular immune response elicited

by vaccines (Fort et al., 2009; Martelli et al., 2011). Also, it should be considered whether this vaccine-derived cellular immunity might also be affected by the MDA level. However, according to the results obtained in a field and an experimental studies (Martelli et al., 2013; Oh et al., 2014), this does not seem to be the case.

CHAPTER 4

Study 2:

*Can Porcine circovirus type 2 (PCV2)
infection be eradicated by mass vaccination?*

Feng H., Blanco, G., Segalés, J., Sibila, M., 2014. Can Porcine circovirus type 2 (PCV2) infection be eradicated by mass vaccination?. Veterinary Microbiology. 172(1-2):92-99.

4.1 Introduction

PCV2, a circular single-strand DNA virus of the Circoviridae family, is the etiologic agent of a number of swine diseases collectively named as PCVD (Segalés, 2012). The most significant conditions included as PCVDs are the PCV2-SD, PDNS and PCV2-RD (Rose et al., 2012; Segalés, 2012). PCV2-SD is considered the most economically significant condition within PCVDs (Segalés et al., 2012).

Traditionally, PCV2-SD control was based on preventing risks or triggering factors by means of management improvement, control of co-infections and changes of the boar genetic background (Fraile et al., 2012a). Nowadays, the disease control is mainly based on vaccination. The vaccines currently available in the international market have shown to be very effective in controlling PCV2 infection and PCV2-SD under both experimental and field conditions (Cline et al., 2008; Opriessnig et al., 2010; Fachinger et al., 2008). Such vaccines are able to improve production parameters (mortality and average daily gain) and reduce viremia, viral shedding, PCV2-SD associated microscopic lesions and the likelihood of co-infection with other viruses (Fort et al., 2008; Fraile et al., 2012b; Gerber et al., 2011; Kixmoller et al., 2008; Martelli et al., 2011; Pejsak et al., 2010; Segalés et al., 2009).

Viral load reduction and lower percentage of infected pigs are perceived as corner-stones in order to control the clinical outcome of the infection. In such respect, results of several PCV2 experimental studies have shown a complete elimination or clearance of PCV2 infection by using two (Fort et al., 2008) and one doses of different PCV2 commercial vaccines (Hemann et al., 2012; O'Neill et al., 2011). These results opened the question whether the use of vaccination may be an efficient way to eventually eliminate or eradicate PCV2 infection. Considering that with the normal vaccination strategy (one dose for most of the commercial vaccines), PCV2 infectious pressure is reduced (Fort et al., 2009; Opriessnig et al., 2009a), one might speculate that a more extensive vaccination program could be used to potentially eradicate the infection.

In the worldwide pig production, there are some examples of pathogen eradication programs based on the combination of vaccination and management strategies. Among them, the eradication program of ADV is one of the most successfully and extended programs. Its strategy is mainly based on a mass vaccination program (vaccination of the entire population) combined with animal movement restrictions. In Spain, ADV eradication program included compulsory vaccination of breeding sows (at least three times per year done in a blanket fashion), fatteners (at least two times separated by 3–4 weeks) and

gilts (three times before entering the reproductive cycle) (Allepuz et al., 2009). Such intense efforts to control this disease concluded with the classification of Spain in 2011 as an ADV officially free country (Vicente-Rubiano et al., 2012).

In the present study, the feasibility to eradicate PCV2 infection in a conventional farm by vaccinating both sows and piglets in a 12 consecutive month period was explored. Besides, the humoral immunological response after vaccination of sows and piglets in different batches of the same farm was monitored.

4.2. Materials and methods

4.2.1. Farm selection

The present study was conducted in a 390-sow, two-site Spanish farm without previous history of PCVD. Site one was composed by breeding, lactating and nurseries units. Site two was located 40.5 km far from site one and was composed by two finishing units (700 animals/unit). Nursery (in site one) was managed all-in-all-out by room (without mixing animals of different ages), whereas site two was managed in continuous flow. The tested farm was 2.5 km away from the nearest pig farm. This herd was conveniently selected mainly due to the willingness of the producer and the practitioner to participate in the

project and the previous evidence of PCV2 infection. The farm used a self-replacement (sows) strategy and at the moment of the study there were 2 boars in the farm. No animals (sows or boars) coming from outside sources entered into the facilities during the study.

Before starting mass vaccination strategy, a PCV2 vaccination program was already in place: sows were vaccinated twice with 2 ml of an inactivated vaccine (Circovac[®], Merial) (at 6 and 3 weeks before farrowing) at the first gestation cycle and once (at 3 weeks before farrowing) in the following cycles. Piglets were vaccinated off-label (0.5 ml/piglet) at 3 weeks of age with a subunit vaccine (Ingelvac[®] Circoflex, Boehringer Ingelheim).

4.2.2. Study design

Study design is summarized in Table 4.1. The initial PCV2 infection and serological status of the farm was assessed by PCR and ELISA, respectively, on serum samples of pigs and sows in two consecutive months before starting (December 2010 and January 2011) the mass vaccination program (February 2011 to January 2012). These two months prior the mass vaccination strategy was named as the “PRE” period for the purpose of the study.

The mass vaccination strategy consisted of the vaccination of all sows, boars and gilts of the farm with 1 ml of Ingelvac® Circoflex every four months (3 doses/animal/year) in a blanket fashion (all animals were vaccinated at the same day, irrespectively of their physiological status). In addition, all piglets were vaccinated with 1 ml of the same vaccine at 4 and 7 weeks of age. The first-dose of piglet PCV2 vaccine was administrated later than the usually recommended age (3 weeks) to avoid a putative interference of maternal immunity resulting from vaccinated sows (Fort et al., 2009; Fraile et al., 2012a,b). This strategy was applied during 12 consecutive months (from February 2011 to January 2012). This 12-month period was named as the “DURING” period for the purpose of the study. To evaluate whether the eradication of PCV2 infection by mass vaccination was successful, the program was stopped after 12 months (February 2012). Subsequently, piglets and sows from the six following monthly batches were followed up (July 2012). This six month period was named as the “POST” period.

During the study period (2 + 12 + 6 months), blood samples from 15 (5 gilts, 5 from 2nd to 5th parity and 5 older than 5th parity, respectively) sows and from 90 piglets (15 of each age-group; 4, 8, 12, 16, 20 and in some cases at 24 weeks of age) were taken monthly (during the third week of each month).

This study design implied that samples from the same animal were taken longitudinally at 4, 8, 12, 16, 20 and 24 weeks of age. Farm boars ($n = 2$) were not monitored in the present study.

Once in the laboratory, blood samples were allowed to clot and were centrifuged at 3400 rpm for 10 min at 4°C. All samples were frozen at -80°C until testing.

Animal care and study procedures were conducted in accordance with the guidelines of Good Experimental Practice, under the approval of the Ethical and Animal Welfare Committee of the *Universitat Autònoma of Barcelona* (Reference 665M2). Treatments, housing, and husbandry conformed to the European Union Guidelines and Good Clinical Practices.

2.3. DNA extraction, PCR and Q-PCR

DNA was extracted from 200 µl of serum using BioSprint® 96 DNA Blood kit (Qiagen, GmbH, D-40724 Hilden) on the Bio Sprint 96 system (Qiagen). Obtained DNA was eluted into 200 µl DNA elution buffer. All the samples were processed by standard PCV2 PCR (Quintana et al., 2002) and those yielding positive results were subsequently tested by a Q-PCR method (Olvera et al.,

2004). Results of the Q-PCR were expressed as the mean PCV2 DNA copy numbers per ml (\pm SD).

4.2.4. PCV2 serology

Serum samples were tested by ELISA using a commercial kit (INGEZIM, Circo IgG 1.1. PCV. K.1, Spain). Results of the ELISA were expressed as mean OD (\pm SD). Mean positive OD cut-off was set at 0.4.

4.2.5. Statistical analyses

From all sera received in the laboratory ($n = 1796$), only a representative part of them (69%) were analysed ($n = 1121$ from piglets and $n = 114$ from sows [3 sows were sampled twice and one sow was sampled three times]) (Table 4.1). Selection of tested sera was based on the feasibility to analyse the infectious/serological status against PCV2 before, during and after the mass vaccination program in two different fashions: cross-sectional (including animals of different ages sampled at the same moment) and longitudinal (including all the samples taken during the lifespan of a given animal). For the cross-sectional follow up in the DURING period, sample selection included sera collected at the beginning, in the middle and at the end of this period. In total, 7 cross-sectional groups (C1 to C7) and 9 longitudinal groups (L1 to L9) were

monitored. The vaccination status of the animals included in the L groups was defined at the age of vaccination. The analysed samples from monthly sampled sows (S) were numbered from S1 to S7.

Moreover, and in order to analyse the overall obtained information, results from C, L and S groups were collectively divided in three periods: PRE (n = 180), DURING (n = 703) and POST (n = 352) mass vaccination periods (Table 4.1). On the other hand, the results of sows were also analysed by different parities, named gilts (G1, n = 46), 2nd to 5th parity (G2, n = 49) and 6th to 8th parity (G3, n = 19).

Table 4.1. Analysed samples from cross-sectional (C) and longitudinal (L) groups of piglets and sows (S) during the three different periods of the study (PRE, DURING and POST mass vaccination).

Year	2010	2011												2012						
Month	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7
Vaccination	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No
Sow	S1	S2						S3					S4	S5	S6			S7	S8	
Piglets 4w*	L1(C1)	L2(C2)	L3				L4	L5(C3)	L6				L7(C4)	L8(C5)	L9			C6	C7	
Piglets 8w	C1	L1(C2)	L2	L3				L4(C3)	L5	L6			C4	L7(C5)	L8	L9		C6	C7	
Piglets 12w	C1	C2	L1	L2	L3			C3	L4	L5	L6		C4	C5	L7	L8	L9	C6	C7	
Piglets 16w	C1	C2		L1	L2	L3		C3		L4	L5	L6	C4	C5		L7	L8	L9(C6)	C7	
Piglets 20w		C2			L1	L2	L3	C3			L4	L5	L6(C4)	C5			L7	L8(C6)	L9(C7)	
Piglets 24w	C1				L1	L2	L3	L3(C3)				L4	L5(C4)						L8(C7)	L9
Period	PRE	DURING												POST						

*w= weeks of age

All statistical analyses were carried out using the SPSS v.20.0 software. The significance level was set at 0.05. Descriptive statistics were used to summarize categorical and quantitative variables. Shapiro Wilk' and Levene tests were used to evaluate the normality of the distribution of the examined quantitative variables and the homogeneity of variances, respectively. The Chi-square or Fischer exact test was applied to evaluate the proportion of positive and negative animals by PCR between the three periods of the study. One way analysis of variance (ANOVA) test was used to compare the ELISA mean OD values and mean viral load between the different study periods.

4.3 Results

4.3.1. Clinical findings

No significant clinical signs were observed during the PRE and DURING periods, besides eventual cases of polyarthritis and unspecific chronic diarrhoea in lactating and nursery pigs. In the POST mass vaccination period, weight loss and diarrhoea were observed in nursery piglets; moreover, the farmer and the veterinarian noticed loss of weight homogeneity and slight increase in mortality in finishing pigs (data not recorded). No clinical PCV2-SD associated problems were observed during the whole study period.

4.3.2. PCV2 infection and seroconversion in sows

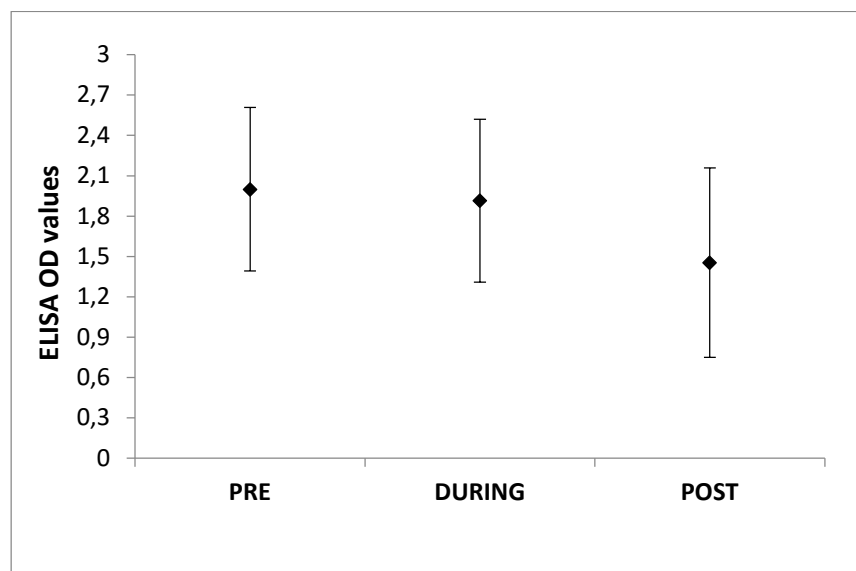
All tested serum samples from sows ($n = 114$) were PCR negative through the whole production period, but all of them were seropositive against PCV2. The mean ELISA OD values of the sows decreased progressively through the three periods of the study (Figure 4.1). The mean ELISA OD values was significantly lower ($P < 0.05$) in POST compared with PRE and DURING periods.

Among the 114 samples, mean OD (\pm SD) values were significantly ($P < 0.05$) higher in G2 (2.0 ± 0.63) and G3 (1.93 ± 0.45) sow parity groups than in G1 (1.47 ± 0.71). No statistical significant differences between G2 and G3 were found.

4.3.3. PCV2 PCR and Q-PCR detection in pigs

Percentage of PCR positive piglet sera is shown in Figure 4.2 (C groups) and Figure 4.3 (L groups), respectively.

Figure 4.1. Mean PCV2 ELISA OD (\pm SD) values of tested sows in PRE (n = 30), DURING (n = 44) and POST (n = 40) mass vaccination periods.



4.3.3.1. Cross-sectional groups

PCV2 was detected in animals of different ages from the two groups sampled before the mass vaccination strategy (PRE period; 6/15 and 6/15 animals in C1 and C2, respectively) and in the first group of pigs (3/15 animals in C3) exposed to the mass vaccination application (DURING period). No PCV2 infection was detected in C4 and C5. When the vaccination strategy was stopped, a number of PCV2 infected pigs were detected again in finishing pigs of C6 (n = 3/15) and C7 (n = 8/15) groups (POST period).

Figure 4.2. Percentage of PCR positive pigs (bars and right Y axes) and mean ELISA OD (\pm SD) ELISA values (black triangle and left Y axes) in the seven cross-sectional analysed groups PRE, DURING and POST mass vaccination periods. The mean cut-off level of the ELISA test was 0.4. Different letters within a sampling point means statistically significant differences ($P < 0.05$).

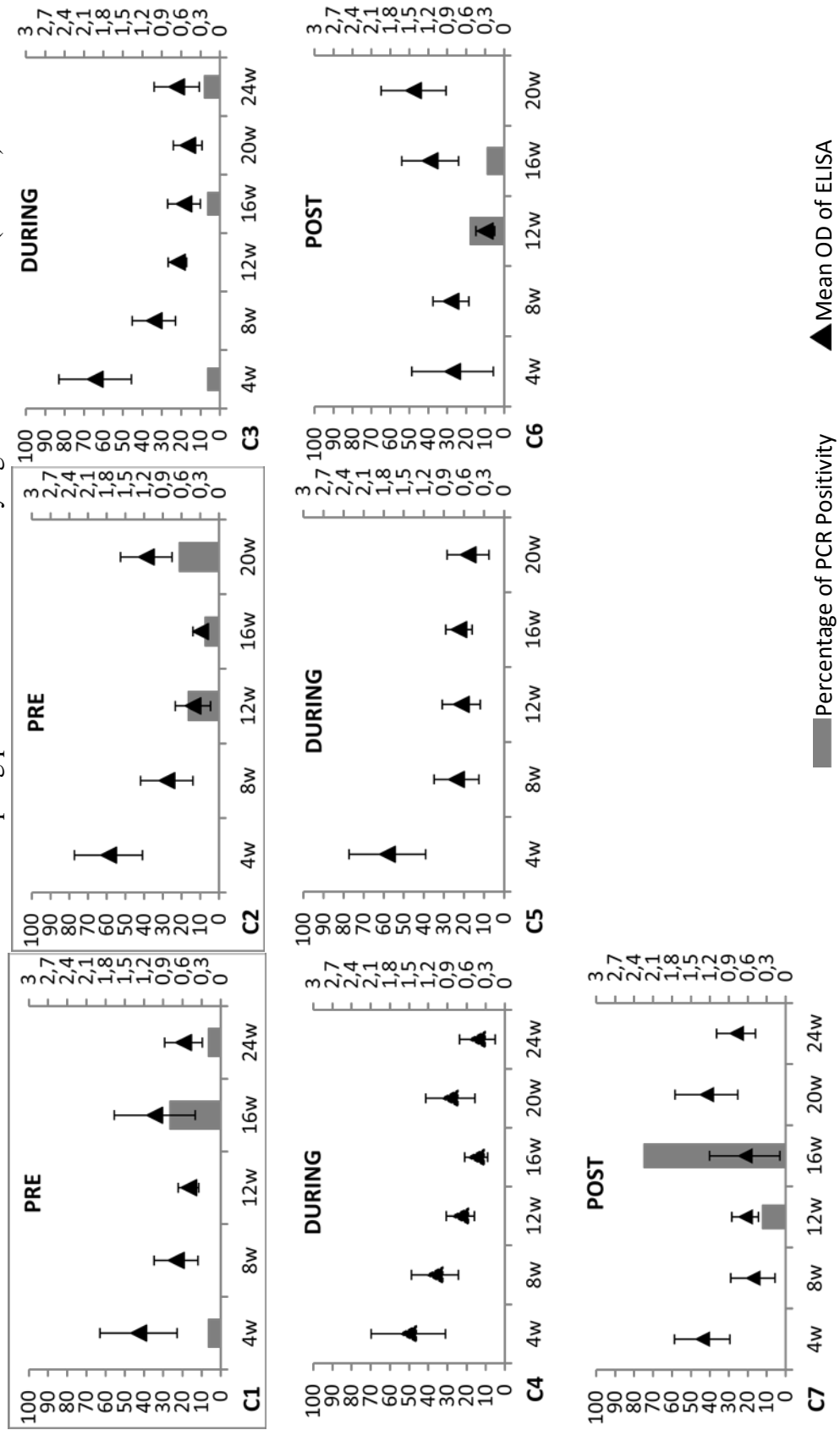
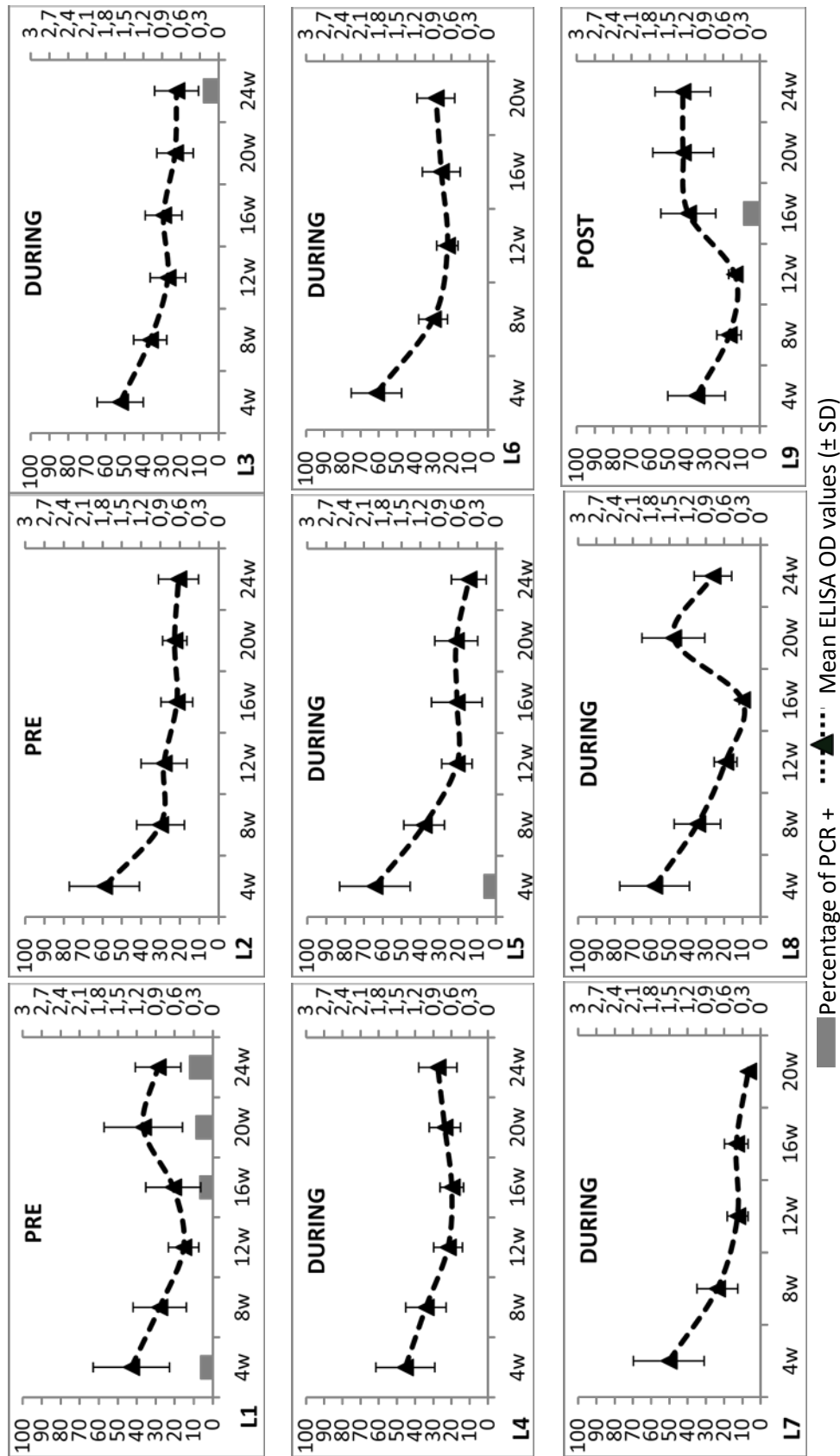


Figure 4.3 Percentage of PCR (bars and right Y axes) positive pigs and mean OD (\pm SD) ELISA values (discontinuous lines and left Y axes) in the nine longitudinal analyzed groups PRE, DURING and POST mass vaccination periods. The mean cut-off level of the ELISA test was 0.4. Different letters within a sampling point means statistically significant differences ($p < 0.05$).



4.3.3.2. Longitudinal groups

PCV2 was detected in L1 pigs (4/15) at different time points, and only two samples yielded a PCR band in animals from L3 (1/15) and L5 (1/15). In three consecutive longitudinal batches (L6, L7, and L8), PCV2 was not detected in any of the samples taken during the lifespan of the tested piglets. After stopping this vaccination strategy, PCV2 virus was detected again in L9 (1/15).

4.3.3.3. Global analysis of cross-sectional and longitudinal groups

Overall, PCV2 PCR detection in the three periods of the study is represented in Figure 4.4-A. Percentage of PCV2 PCR positive pigs was significantly ($P < 0.05$) higher in the PRE and POST versus DURING (at 12 and 16 weeks of age) periods and in the PRE versus DURING and POST (20 weeks of age) mass vaccination periods.

Only 11 out of the 29 positive samples were PCV2 QPCR positive (2 in PRE, 2 in DURING and 7 in POST mass vaccination periods). The mean viral load in the three study periods was $2.7 \times 10^4 \pm 2.0 \times 10^4$, $2.6 \times 10^6 \pm 2.4 \times 10^6$ and $1.2 \times 10^6 \pm 2.9 \times 10^6$ PCV2 genome copies/ml in the PRE, DURING and POST periods, respectively. No statistical differences in the number of Q-PCR

PCV2 positive samples as well as in the mean PCV2 viral load were found among the three periods of the study.

4.3.4. PCV2 serology in pigs

Mean ELISA OD values are shown in Figure 4.2 (C groups) and Figure 4.3 (L groups), respectively.

4.3.4.1. Cross-sectional groups

In the C1 and C2 groups, a decline of the PCV2 antibody ELISA OD (probably due to MDA waning) was followed by an active seroconversion by 16 (C1) and 20 (C2) weeks of age. During the mass vaccination strategy, mean ELISA OD values were high in young animals but decreased progressively with age (C3–C5). Indeed, an apparent seroconversion was not observed in any group (although slight increase of OD values was observed in C4 at 20 weeks of age). When vaccination was stopped, the OD values-profile in C6 and C7 changed completely since the decline of seropositive pigs was followed by an active seroconversion (16–20 weeks of age).

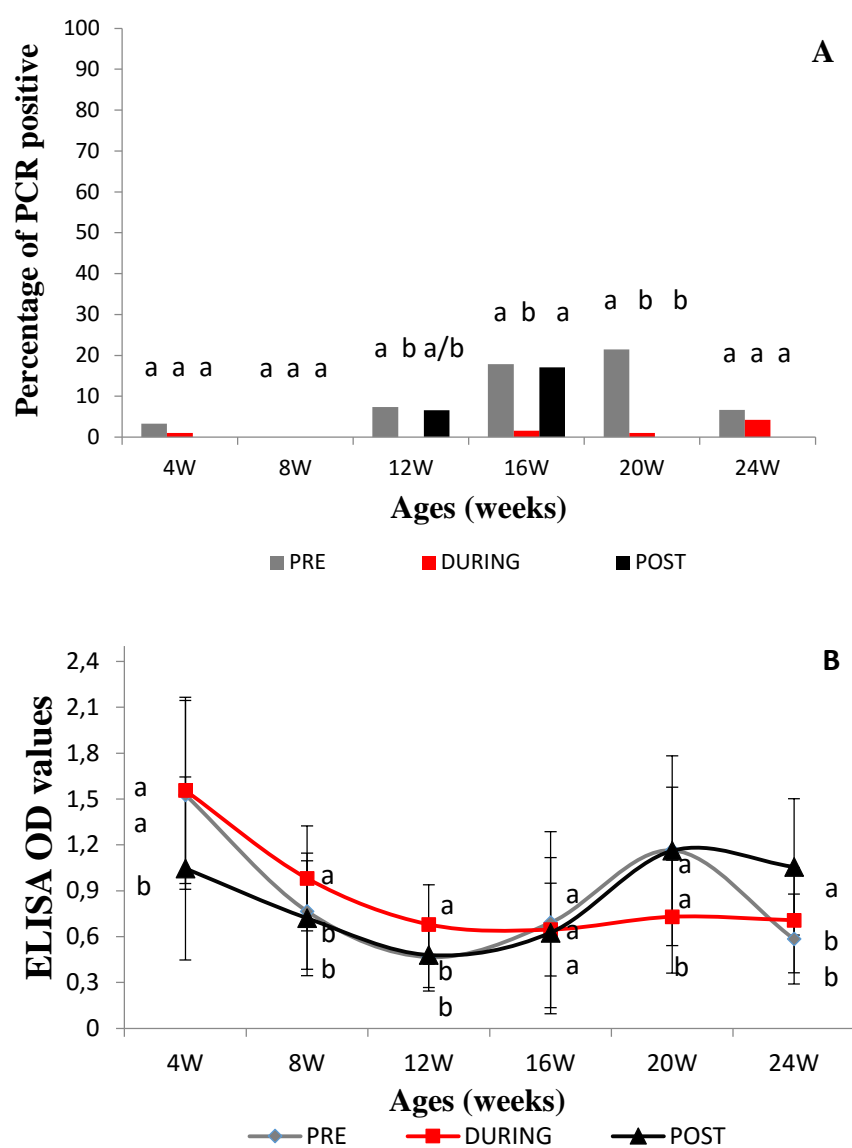
4.3.4.2. Longitudinal groups

In L1, a decline followed by a slight increase (at 16–20 weeks of age) of the ELISA OD values was observed. From L2 to L7, the mean OD values were high at early ages but decreased over the time, showing no evident seroconversion. In L8 and L9 (POST mass vaccination), after MDA waned, an active seroconversion by 16–20 weeks of age was observed again.

4.3.4.3. Global analysis of cross-sectional and longitudinal groups

Overall, mean ELISA OD values was significantly higher in PRE and DURING than in POST mass vaccination periods at 4 weeks of age. At 8 and 12 weeks of age, mean ELISA OD values was significantly higher in the DURING than in PRE and POST mass vaccination periods (Figure 4.4-B). At 16 weeks of age, the mean ELISA OD values were similar in the three periods of the study. After that age, the DURING mean ELISA OD values were significantly lower than the ones in the PRE (20 and 24 weeks of age) and POST (20 weeks of age) periods.

Figure 4.4. A: Percentage of PCR (bars) positive pigs in the PRE (n = 150), DURING (n = 659), and POST (n = 312) massive vaccination periods. **B:** Mean ELISA OD (\pm SD) values of samples included in PRE, DURING and POST mass vaccination periods. The mean cut-off level of the ELISA test was 0.4. Different letters within a sampling point means statistically significant differences ($P < 0.05$).



4.4. Discussion

In the present study, a PCV2 eradication program based on the mass vaccination concept in a conventional farm was applied. Such strategy implied sow, gilt, boar and piglet vaccination. To date, this study is the first attempt to use such intensive vaccination program to get rid of PCV2 infection.

As a consequence of the mass vaccination program, PCV2 infectious pressure was significantly reduced, reaching undetectable levels by the end of the period of applying this vaccination strategy. Indeed, in two cross-sectional (C4, C5) as well as in four longitudinal groups (L4, L6, L7 and L8), no PCV2 infected animals were detected by means of standard PCR. During the first half of mass vaccination program, a low number of pigs were PCR positive, and only two of these PCR positive samples had Q-PCR detectable viral load. No positivity was detected in the second half of the whole mass vaccination timing, suggesting that PCV2 vaccine can effectively reduce the percentage of infected pigs (Fachinger et al., 2008; Opriessnig et al., 2010). However, evidence of infection after stopping the vaccination program might indicate either re-infection or that the virus was never cleared out from the farm. Taking into account that no external animals were introduced in the herd during the whole

study period and the continuous flow management system used in the growing units, the second hypothesis might be more likely.

A different antibody profile was observed in the three study periods. While a progressive wane of the MDA was observed in all groups at all periods, evident seroconversion in the finishers was only observed in the PRE and POST ones. An interesting antibody profile is the one observed for the L8 group. In that group, although no PCR positive animals were detected, seroconversion of finishers was observed. These results might be explained by the fact that although these pigs were double vaccinated (so, belonging to the DURING period), they spend most of their productive life with animals included in the POST period (non-vaccinated animals). These results would be in agreement with the conclusion of Opriessnig et al. (2009), which indicated that early-age vaccinated piglets exposed to PCV2 in late finishing phase can be infected. On the other hand, the antibody-profile of sows indicated an overall decrease of ELISA OD values during the whole study period. Low global infectious pressure, mainly, and the removal of PCV2 vaccination in sows in the POST period may have accounted for these results.

The overall decrease of OD values in both sow and pig population together with the lack of seroconversion DURING the mass vaccination period supports the reduction of the infectious pressure elicited by the vaccination program (Opriessnig et al., 2004b). Therefore, such intensive vaccination program might be apparently able to potentially eradicate this viral infection. However, the removal of the mass vaccination program led to the reappearance of detectable infection together with an unequivocal seroconversion.

It is important to highlight that PCV2 vaccination was already implemented before the mass vaccination program started, which may account for the already existing low PCV2 infectious pressure. It is speculated that the effect of the mass vaccination program in a farm without previous PCV2 vaccination would have probably been more evident. Noteworthy, and on purpose, the study was performed in a farm suffering from a subclinical PCV2 infection (no PCVD cases were reported in the year previous to the start of the study). It would be expectable as well, that benefits of a mass vaccination strategy in terms of protection would have also been much more noticeable under a PCVD scenario.

The mass vaccination strategy was only applied during 12 consecutive months, in only one specific farm and without implementing any change in the farm management practices. Disease eradication programs are usually based on the application of intense vaccination programs (in combination with management strategies or animal moving policies) at county, state or country levels (Grosse Beilage et al., 1997; Ketusing et al., 2014; Komaromi and Szabo, 2005; Motha et al., 1994). Considering the efficacy of the commercial PCV2 vaccines in terms of infection reduction, the effect of the strategy used in the present work at a region/state level in a continuous manner should not be underestimated. This continuous larger-scale vaccination strategy might be an efficient way to eradicate the virus. Such scenario would be comparable with the A3 status in ADV eradication programs, in which a serological ADV negative status co-exist with vaccination (Vicente-Rubiano et al., 2012). Obviously, a key point to guarantee the success of PCV2 eradication by mass vaccination at a farm/region level would be to avoid the re-infections (De Smet et al., 1992; Hemann et al., 2012).

In summary, under the current study conditions, one year of PCV2 mass vaccination was able to reduce PCV2 infectious pressure at the level of no viral detection by means of PCR and lack of seroconversion. However, around 4

months after stopping the intensive vaccination program, evidence of PCV2 infection by means of seroconversion and PCR positivity was detected again. Therefore, although PCV2 eradication seemed feasible after one year of mass vaccination, the eventual application of such control measure should be extended over an undetermined period.

CHAPTER 5

GENERAL DISCUSSION

Since PCV2 was discovered in 1998, the epidemiology of the diseases derived from its infection (PCVD) has evolved from severe clinical outbreaks to a subclinical infection (PCV2-SI) scenario. This change can be mainly attributed to the constant (since 2007) and widespread use of PCV2 commercial vaccines, combined with the ubiquitously, persistence and high frequency of transmission of this virus (Grau-Roma et al., 2011; Rose et al., 2012; Alarcon et al., 2013; Meng, 2013; Segalés et al., 2013). PCV2-SI, however, has also a negative impact on the productive parameters (Alarcón et al., 2013). Nowadays, the economic losses attributed to PCV2 are mainly caused by the subclinical infection rather than by clinical outbreaks (Segalés, 2012; López-Soria et al., 2014). Based on Alarcon et al. (2013) calculations, the total cost due to PCV2-SI in UK in 2008 was around 28.5 million euros before implementation of PCV2 vaccination. Therefore, it is meaningful to evaluate the efficacy of PCV2 vaccines under the conditions of PCV2-SI, as well as to identify the more profitable vaccination schedules.

Nowadays, PCV2 vaccines are widely used worldwide and their high efficacy on controlling PCV2 infection is very well documented. However, in terms of vaccination against PCV2, there are still some issues that deserve to be tackled. Some questions are still to be answered and some strategies to improve

vaccine efficacy or to expand their benefits have not been assessed yet. On one hand, although the interference of MDA at the moment of vaccination on humoral immunity response to vaccines was well proven, the effect of such high titres on the efficacy in terms of productive parameters is still not clear (study 1). On the other hand, the high efficacy of PCV2 vaccines on controlling PCV2 viral load and PCVDs may suggest that PCV2 infection could be eradicated by means of an intensive and long-term vaccination program (study 2). The current thesis will try to give an answer to these two open questions.

The two field studies presented in the current PhD Thesis were also conducted in PCV2 subclinically infected farms. Therefore, those two field studies were conducted with the aim to gain knowledge on the efficacy of PCV2 vaccination in subclinically infected farms in two different scenarios:

1. To vaccinate in presence of high and low MDA titres in piglets
2. To continuously protect sows and piglets to the point of potential eradication

Overall and as expected, all vaccinated piglets were efficiently protected in these two studies from serological and virological (studies 1 and 2) and production (study 1) points of view, while non-vaccinated animals (study 1)

showed a poorer performance on serological, virological and productive outcomes.

As mentioned in the introduction, nowadays PCV2 vaccination strategies most commonly used in commercial farms include immunization in the piglet, sow or both (sow and piglet). From these three strategies, it seems that the benefits of vaccinating both are higher in terms of virological, immunological, pathological and productive parameters than when only sows or piglets are treated (Pejsak et al., 2010; Fraile et al., 2012b; Oh et al., 2014). However, it is known that high levels of MDA ($>10 \log_2$ IPMA titres, derived from sow vaccination and/or infection) at the moment of piglet vaccination may jeopardize the vaccine-induced humoral response in the own piglet (Fort et al., 2009; Fraile et al., 2012b; Haake et al., 2014; Oh et al., 2014). However, this situation did not show a direct parallelism with an impairment of ADWG of piglets, since there were no published studies in which animals with a clear different level of MDA at the moment of vaccination were contemporaneously studied. Therefore, that was the objective of study 1, in which animals with low and high MDA levels at the moment of vaccination were selected in purpose. Our hypothesis was that the higher the levels of MDA at the moment of vaccination, the lower the ADWG. This hypothesis was supported by two facts.

The first one was based on the higher the MDA levels the lower seroconversion rate and reduction of viral load (O'Neill et al., 2011; Fraile et al., 2012b; Haake et al., 2014). The second one was based on the existing negative correlation between viral load and ADWG (López-Soria et al., 2014). The combination of all these data suggested that the level of MDA at the time of vaccination could potentially affect the ADWG.

Results obtained in study 1 corroborated the first point of our hypothesis: high levels of MDA at the moment vaccination implied lower percentage of vaccine-induced seropositive animals as well as high PCV2 loads and percentage of infected pigs. However, the second point of our hypothesis was not demonstrated; although those ten animals with the highest MDA titres (S/P ratio: 2.46 ± 0.1) at the moment of vaccination grew 52g/day less than the remaining vaccinated ones, no statistically significant differences in ADWG were observed when considering all pigs with high and low MDA levels.

Therefore, results of this study opened the following question: why does the presence of high MDA at the moment vaccination affect the efficacy of the vaccine in terms of the humoral immune response but does not in terms of

productive parameters (ADWG)? The exact answer to this question is still unknown. However, some explanations are given:

- 1) Cellular immunity: All PCV2 commercial vaccines are able to elicit a humoral but also cellular immune response (Park et al., 2014; Seo et al., 2014a). In a clear contrast with the vaccine derived humoral response, cellular immunity induced by the vaccine (Ferrari et al., 2014; Oh et al., 2014), seems not to be affected by the MDI levels (Martelli et al., 2013; Oh et al., 2014). In addition, although still unclear, a study suggested that duration of cellular immunity derived from the sow is probably short (Oh et al., 2014), and this might explain why no interference on vaccine-induced cellular immunity.
- 2) Origin of the MDA: In this study, high antibody levels were achieved by means of sow vaccination. It would be interesting to know if the MDA conferred by natural infection would be somewhat different from the one derived from sow vaccination (probable lower antigenic repertoire, especially if using subunit vaccines). Indeed, further analysis of the data obtained from vaccinated (v) pigs (coming from V or NV sows, [s]) in study 1 showed that the ADWG of Hv-NVs animals was higher than Hv-

Vs, Lv-NVs and Lv-Vs groups (Table 5.1). Although speculative at this point, and with lack of significant differences probably due to low numbers of animals, it looks like that vaccination of piglets with high MDA coming from NV sows offers the best productive results. The lower ADWG of Hv-Vs compared with Lv-Vs piglets (Table 5.1) suggests that high levels of MDA coming from vaccinated sows may jeopardize ADWG. The same analysis was done using Fraile et al. (2012b) database, but these differences were not observed (data not shown). Indeed, in that study, double vaccinated animals (Hv-Vs) showed the highest productive parameters. Therefore, further studies would be needed to confirm that the origin of MDA may affect the ADWGs of vaccinated piglets.

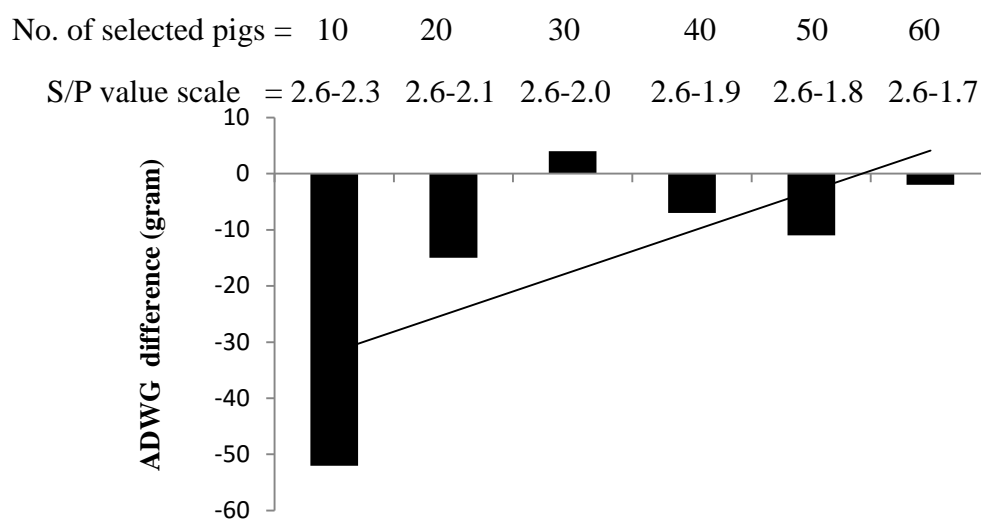
Although not significantly different, H-V pigs showed a numerically lower ADWG compared to L-V ones. Such results could indicate that an interference on ADWG exist only in a subpopulation of H-V piglets. In order to know to which extent this effect could be important under field conditions, the evolution of the ADWG in some subpopulations of vaccinated animals with the highest MDA values was analyzed. Again, although no statistical differences were found, ADWG differences between the 10, 20, 30, 40, 50 or 60 vaccinated

animals with the highest antibody values at vaccination and the rest of V pigs showed a negative correlation (Figure 5.1).

Table 5.1. ADWG and number of vaccinated (v) piglets with high (H) and low (L) MDA coming from non-vaccinated (NV) and vaccinated (V) sows (s). Different letters within a column means statistically significant differences ($p < 0.05$).

	No. of piglets	ADWG \pm SD(g)
Hv-NVs	13	618.1 \pm 48.1 ^a
Hv-Vs	80	581.4 \pm 84.6 ^a
Lv-NVs	59	582.3 \pm 71.7 ^a
Lv-Vs	19	605.9 \pm 52.4 ^a

Figure 5.1. ADWG difference between the 10, 20, 30, 40, 50 or 60 vaccinated piglets with S/P titre values within the V group compared to the rest of V piglets.



Under field conditions, these differences might be of importance depending on the number of animals showing these high titers. However, there were no significant statistical differences between the selected groups of animals and the rest of V piglets. In the present study, those 10 vaccinated animals with very high titers grew 52 g. less than the rest of vaccinated counterparts. If these high titers would have been seen in a high number of animals, then these 52 g. less would have represented a significant economic importance. In consequence, study 1 cannot really conclude that MDI does not truly affect the ADWG upon PCV2 vaccination, but it suggests that the impact of high level MDA would be presumably low or negligible under most of the field conditions observed worldwide.

Taking all available information together, it looks like that there is still room to improve optimal piglet vaccination strategy, fitting with the quote of avoiding interference with MDI and taking place before natural infection. Some studies indicated that a later vaccination strategy could avoid the abovementioned interference and results in a better vaccine efficacy (Fraile et al., 2012b; Haake et al., 2014; Oh et al., 2014). However, MDA interference with vaccine elicited seroconversion was also observed when vaccinating at 4 weeks of age (Fraile et al., 2012b). In addition, since PCVDs mainly affect

piglets varying 7-16 weeks of age (Meng, 2013), a too late vaccination strategy (7 weeks of age) would not be appropriate in a number of cases. Of course, PCV2 infection timing would basically depend on each particular farm, but in most of them infection takes place mainly after week 10 of life. In fact, to talk about age might be risky, since the driving force is the window between MDA and natural infection, independent from the age.

Multiple studies have shown that the level of viremia is an essential factor in developing PCV2-SD. In fact, high viremia levels ($>10^6$ - 10^7 DNA copies/ml of serum) have been associated to the appearance of the clinical disease (Rosell et al., 1999; Olvera et al., 2004; Meerts et al., 2006; Seo et al., 2012; Martelli et al., 2013; Seo et al., 2014c). In addition, all PCV2 vaccines have demonstrated to be really efficient (using one or two doses) in reducing PCV2 viral load (Fort et al., 2008; O'Neill et al., 2011; Hemann et al., 2012). Therefore, the combination of these two premises may suggest that a continuous, long-lasting, intensive and exhaustive vaccination programme may be used to diminish significantly the PCV2 infectious pressure and, eventually, to eradicate the infection.

It is true that eradication of a ubiquitous agent looked poorly feasible from the very beginning, but examples on disease and infection eradication, such as bovine brucellosis, virulent footrot, ADV and smallpox by means of mass vaccination have been published elsewhere (Millar et al., 1971; Egerton et al., 2002; Blasco and Moriyon, 2010; Ketusing et al., 2012). In consequence, it was interesting to explore the possibility of PCV2 eradication by the so-called mass vaccination strategy. In order to accomplish such objective, all sows, gilts and boars were vaccinated 3 times per year and all piglets were vaccinated twice at 4 and 7 weeks of age with a commercial subunit vaccine. It must be clarified that this study was the first attempt to explore the effect of a mass vaccination program in a farm with subclinically infected by PCV2. In addition, this pilot vaccination strategy was applied only in one farm during 12 consecutive months and without changing any management and husbandry settings.

Obviously, such study design had several limitations that should be considered:

- Disease eradication programmes are based not solely on an intense vaccination programme, but also on restriction of animal moving policies,

environmental hygienic measures and specific management and husbandry strategies

- These eradication programmes are applied during long periods of time (years), in multiple herds covering the pig population at state, county or country level (Dowdle and Cochi, 2011). For example, the ADV eradication program applied in Spain (Allepuz et al., 2008; Allepuz et al., 2009), for many years before declaring the territory free of infection.

- Sensitivity of the diagnostic techniques used and the capability of the vaccine to eliminate the infection can influence the success of the eradication programme

In the present study, and after several months of application of such vaccination strategy, the virus and the level of antibodies against it became undetectable by means of PCR and ELISA techniques, respectively. However, when the strategy was stopped, the infection appeared again. This re-appearance might associated to two potential reasons (not mutually excluded):

- *Partial elimination of the virus:* the vaccine used in this study was able to reduce the viral load, but not to completely eliminate the infection. This would imply that the virus was not completely cleared out from the farm and was kept within the population in a very low level of infection. In consequence, the virus was not able to be detected by the PCR technique used, at least during the mass vaccination period.

- *New entrance of the virus to the farm:* in order guarantee the success of an eradication program, especial caution should be applied to avoid the reappearance of the infection (De Smet et al., 1992; Hemann et al., 2012). To avoid PCV2 re-infection, more attention should be paid on controlling the routes of PCV2 transmission by non-animal (such as reducing airborne transmission, adequate management of herd visitors) (Rose et al., 2012) and/or animal (such as rodent (Pinheiro et al., 2013) and wild boar (Boadella et al., 2012; Ketusing et al., 2012)) contacts. In fact, international trading has been shown as the most important way to spread different PCV2 genotypes all over the world (Segalés et al., 2013).

Considering all these limitations, results obtained in study 2 provided interesting information on PCV2 infection and seroconversion dynamics after a

long, intensive vaccination strategy. Therefore, and although the first attempt to eradicate PCV2 failed (the infection was found in the POST period), eradication of PCV2 by means of a national program including all the variables stated before could be feasible. Obviously, the first step to consider before establishing such program would be to analyse if this program would be economically worthy.

Based on the data from 2014 (https://www.3tres3.com/estadisticas_porcino/graficos/#14), 43.2 million of pigs were slaughtered in Spain, and the breeding stock census was 2.35 million. The cost (considering the cost of one dose as one Euro) of applying a one-year eradication program (2 dose for piglet and 3 dose for sow/year) applied to the whole Spanish pig population would be 93.45 million euros. On the other hand, the numbers of slaughtered pigs (9.42 million) and sows (0.48 million) in the UK in 2008 were about 4.5 less times than in Spain. Considering the calculation on the economic cost of PCV2-SI infection in the UK in that year (28.5 million euros), an estimation of the cost of PCV2-SI infection in 2014 in Spain would be around 128 (28.5×4.5) million euros. Therefore, if one year eradication campaign is applied in Spain in 2014, more than 34.55 million euros would be saved. Certainly, whatever economic estimation would probably have a significant variation, not only among

countries (UK versus Spain), but also on the particular clinical situation of each country. Besides these considerations, which are not minor, one may speculate that a wide scale vaccination campaign for eradicating PCV2 would be worthy from an economic point of view.

Currently, the main PCV2 genotype circulating worldwide is PCV2b (Cortey et al., 2011b; Segalés et al., 2013). This was also the situation in the farm where the study 2 was conducted (data not shown). However, all PCV2 commercially available vaccines nowadays in Europe are based on PCV2a. Although the cross-protection of PCV2a vaccine on PCV2b infection has been proven (Fort et al., 2008; Tribble and Rowland, 2012; Ellis, 2014; Opriessnig et al., 2014; Seo et al., 2014c; Zhai et al., 2014), the question whether a PCV2b vaccine based eradication program would have worked better compared with a PCV2a vaccine based one is open. A recent experimental study, comparing two live attenuated chimeric vaccines based on PCV2a and PCV2b showed that the PCV2b based vaccine was able to induce a significantly higher PCV2 antibody levels and lower PCV2 viremia than the PCV2a based vaccine (Opriessnig et al., 2013a). Assuming that this situation would be systematically true, it would be expectable that a PCV2 eradication programme based on a PCV2b vaccine would be even more efficient than a one based on a PCV2a vaccine.

The wide use of PCV2 vaccines seems to prevent PCVDs very efficiently, but do not totally prevent the infection, which results in the circulation of PCV2 in vaccinated farms. This scenario implies that vaccination pressure may set, as it has been already suggested (Kekarainen et al., 2014), the evolution of PCV2. Hence, an intensive vaccination scheme such as the one proposed to eradicate PCV2 in study 2 would probably have important effects on PCV2 evolution. In consequence, it is not deniable to think that a wide scale application of a potential PCV2 eradication program would result in the shift from the most prevalent PCV2 genotype to another one, as it has happened already from PCV2a to PCV2b, and might be ongoing from PCV2b to PCV2d (Xiao et al., 2012). This genotype shift may also have some consequence on the vaccine efficacy, for example escaping from vaccine-induced protection and resulting in a potential vaccine failure. Indeed, the detection of PCV2d in PCV2 vaccinated animals showing PCV2-SD has been suggested as potential vaccination failures (Xiao et al., 2012; Segalés, 2015). One may speculate that current PCV2a based vaccines are not efficiently enough to control PCV2b (and PCV2d) infections. However, so far, most of these potential “vaccination failures” have been attributed to failure in vaccine application (dose, timing, etc.), rather than the generation of escape mutants.

In summary, the first study of this thesis tried to clarify the correlation between ADWG and the MDA level at vaccination by comparing animals with high and low MDA levels. The data obtained showed no correlation between those two factors and implied this kind of interference may exist in a negligible population of animals under field conditions. The second study was the first attempt of PCV2 eradication on one farm by one year of intensive vaccination. The results showed PCV2 could not be eradicated with only one year's vaccination, but the decreasing antibody levels and the lack of viral detection during the second half of the vaccination period shed a light on eradicating this virus by applying a longer term vaccination in a wider area. These two studies complement the current knowledge on PCV2 vaccination efficacy under subclinical infection conditions and give new creative concepts (“thinking out of the box”) for future related studies.

CHAPTER 6

CONCLUSIONS

1. PCV2 vaccination was able to reduce PCV2 viral load by means of piglet and/or sow plus piglet vaccination (studies 1 and 2) as well as to increase ADWG (study 1) in PCV2 subclinical infection scenarios.
2. Pigs with the best growth performance measured as ADWG were those with low ELISA S/P values at the moment of vaccination; however, presence of high MDA values at that moment did not interfere in the ADWG of pigs.
3. Evident detrimental effects of MDA on ADWG were exclusively observed in a minimal number of pigs with extremely high MDA value at the moment of vaccination, which probably represents a negligible population of animals under field conditions.
4. One year of PCV2 mass vaccination in sows and piglets (without implementing further farm management practices or other biosafety measures) in a PCV2 subclinically infected farm was able to reduce PCV2 infectious pressure at the level of no viral detection by means of PCR and lack of seroconversion.

5. However, four months after stopping the mass vaccination program, evidence of PCV2 infection by means of seroconversion and PCR positivity was detected again.

CHAPTER 7

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