Universitat Autònoma de Barcelona

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Tesi doctoral presentada per **Salvador Oliver Ferrando** per accedir al grau de Doctor en el marc del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. **Marina Sibila Vidal**, del Dr. **Joaquim Segalés Coma** i del Dr. **Antonio Callén Mora**.

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La Dra. **Marina Sibila Vidal**, investigadora del Centre de Recerca en Sanitat Animal, de l´Institut de Recerca i Tecnologia Agroalimentàries (CReSA-IRTA); el Dr. **Joaquim Segalés Coma**, professor titular del Departament de Sanitat i Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona (UAB) i investigador del CReSA-IRTA; i el Dr. **Antonio Callén Mora**, veterinari especialista en porcí.

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Que la memòria titulada "**Effect of *Porcine circovirus 2* (PCV2) sow or piglet vaccination in different PCV2 subclinical infection scenarios**" presentada per **Salvador Oliver Ferrando** per a l´obtenció del grau de Doctor en Medicina i Sanitat Animals, s´ha realitzat sota la seva supervisió i tutoria, i autoritzen la seva presentació per a que sigui valorada per la comissió establerta.

I perquè així consti als efectes oportuns, signen la present declaració a Bellaterra (Barcelona), a 13 de novembre del 2017.

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Als meus pares Salvador i Mª Elisa,

Per estar al meu costat sempre que ho he necessitat

LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
AI	Artificial insemination
AUC	Area under the curve
bp	Base pairs
cDC	Conventional DC
cRPMI	Complete RPMI (medium)
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dpi	Days post-infection
dsDNA	Double-stranded DNA
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
GAG	Glycosaminoglycan
HE	Haematoxylin/eosin
ICTV	International Committee on the Taxonomy of Viruses
IFN	Interferon
IFN-γ-SCs	Interferon-γ-secreting cells
Ig	Immunoglobulin

IHC	Immunohistochemistry
IL	Interleukin
IPC	Internal positive control
IPMA	Immunoperoxidase monolayer assay
ISH	*In situ* hybridization
MDA	Maternally derived antibodies
MDI	Maternally derived immunity
MHC	Major histocompatibility complex
mPCV2b	Mutant of PCV2b
NA	Neutralizing antibodies
NCC	Number of completed circles around the enclosure
NK	Natural killer
NV	Non-vaccinated
OD	Optical density
OF	Oral fluid
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV1	*Porcine circovirus 1*
PCV2	*Porcine circovirus 2*
PCV2-RD	PCV2 reproductive disease
PCV2-SD	PCV2 systemic disease

PCV2-SI	PCV2 subclinical infection
PCVAD	Porcine circovirus-associated diseases
PCVD	Porcine circovirus disease
pDC	Plasmacytoid DC
PDNS	Porcine dermatitis and nephropathy syndrome
PK	Porcine kidney
PMWS	Post-weaning multisystemic wasting syndrome
PPV	*Porcine parvovirus*
PRRSV	*Porcine reproductive and respiratory syndrome virus*
qPCR	Quantitative PCR
RPMI	Roswell Park Memorial Institute (medium)
S. suis	*Streptoccocus suis*
S/P	Sample to positive
SD	Standard deviation
SLA	Swine leucocyte antigen
ssDNA	Single-stranded DNA
Th	T helper
TNF	Tumor necrosis factor
U	Udder stimulation
V	Vaccinated
VI	Vitality index
VNT	Viral neutralization test
%VN	Percentage of virus neutralization

ABSTRACT

Porcine circovirus 2 (PCV2) is one of the most prevalent viruses that causes great economic losses to the worldwide pig industry. This virus has a ubiquitous nature in the pig population and is the causative agent of a number of clinical and subclinical conditions named Porcine circovirus diseases (PCVDs). The most important control tool for PCVDs is the vaccination, although it should be applied together with other measures such as proper farm management practices and control of potential risk factors for disease occurrence. During the last decade, commercial PCV2 vaccines have proven to be effective in controlling PCV2 infection, reducing PCV2 systemic disease (PCV2-SD) outbreaks and improving the production parameters. Currently, the use of these vaccines remains widespread, although it is mostly applied under PCV2 subclinical infection (PCV2-SI) scenarios. In this context, the present thesis aimed to investigate some still existing gaps on the potential influence of sow vaccination on reproductive outcomes as well as on the transfer of maternally derived cellular immunity. In addition, it was also intended to expand the knowledge on piglet vaccination by comparing the infection dynamics of different vaccination timings using both serum and oral fluid (OF) samples.

The first study sought to evaluate the effect of sow vaccination against PCV2 on reproductive parameters during two consecutive reproductive cycles. The study was performed in a PCV2 subclinically infected breeding herd (evidence of PCV2 circulation but absence of major reproductive problems). Ninety-four pregnant sows were primo-immunized with a commercial PCV2 vaccine and ninety-seven were injected with phosphate-buffered saline at 6 and 3 weeks before farrowing, and then boosted at 2 weeks before the second one. Blood samples were taken throughout the study to assess PCV2 DNA load and antibodies. At farrowing, main reproductive parameters and piglet vitality index

were registered. In addition, in those litters with more than three mummified or stillborn piglets, microscopic examination and PCV2 antigen detection in foetal myocardium was done. Vaccinated sows showed significantly higher antibody levels compared to the non-vaccinated counterparts. PCV2 DNA was only detected at farrowing in 2 (4.2%) non-vaccinated sows. Vaccinated sows had 1.3 more live-born piglets per litter at the second cycle than non-vaccinated counterparts. Piglets from vaccinated sows had significantly higher (+12.7%) vitality score than the ones born from non-vaccinated sows. No PCV2 compatible lesions neither PCV2 antigen were detected in the tested foetal hearts. The present study represents the first attempt to demonstrate that PCV2 sow vaccination may have a positive influence on prolificacy and vitality of the offspring in a subclinically infected breeding herd.

In the second study, the effect of PCV2 sow vaccination on humoral and cell-mediated immune responses in sows and their progeny was assessed. At 7 weeks before farrowing, fifteen PCV2 PCR negative pregnant sows with medium-low antibody values were selected and randomly distributed in two groups according to the antibody levels. Seven sows were vaccinated with a commercial PCV2 vaccine and eight were injected with phosphate-buffered saline at 6 and 3 weeks before farrowing. Blood samples were taken from sows at farrowing and their offspring at 48-72 hours of life. PCV2 DNA and antibodies were tested in sera (n=90; 6 piglets per litter), and cytokine (IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β, IL-8, IL-4, IL-6 and IL-10) levels were assessed in supernatant (n=30; 2 piglets per litter) from cultured peripheral blood mononuclear cells. All sows and piglets were negative by PCV2 PCR throughout the study. Significantly higher PCV2 antibody levels were detected in vaccinated sows after vaccination and in their offspring after colostrum ingestion compared to the non-vaccinated counterparts. Vaccinated sows did not show significant differences in cytokine secretion levels at farrowing compared to unvaccinated dams. In contrast,

piglets from vaccinated sows had significantly higher levels of cytokines linked to Th1 memory cells (IFN-γ and TNF-α) in comparison to the ones from non-vaccinated dams. In conclusion, PCV2 sow vaccination, apart from triggering a humoral immune response in sows and their progeny, might be associated to an increased transfer of cell-mediated immunity from the dam to the piglet.

The purpose of the third study was to determine the PCV2 serological and virological infection dynamics in piglets vaccinated at different ages in a PCV2-SI scenario. Six hundred and forty-four 2 week-old healthy piglets were selected and distributed into four treatment groups: vaccination at 3, 6 or 10 weeks of age (3W-VAC, 6W-VAC and 10W-VAC groups, respectively) and unvaccinated pigs (NON-VAC group). Blood from a subpopulation of pigs (n=112 animals) and OF (n=40 pens) samples were taken throughout the study to assess PCV2 load, humoral immunity and viral genotyping. Generally, percentage of PCV2-DNA positive sera raised mainly by 10 weeks of age, being maximum at 14 weeks of age, and then started to decrease at 18 and 25 weeks of age. Specifically, PCV2 vaccination at 3 or 6 weeks of age yielded similar results, since they produced an earlier seroconversion and reduced, at different sampling points, the proportion of viraemic animals in comparison to the unvaccinated group. In contrast, PCV2 vaccination at 10 weeks of age only achieved such reduction at 25 weeks of age; in this case, vaccination coincided with the increase of the percentage of viraemic pigs in the population. Both antibody detection techniques used in sera and OF offered similar results with a high and statistically significant correlation. On the other hand, a higher percentage of PCV2 DNA positivity was detected in OF in comparison with sera. In conclusion, under the present study conditions, the optimal time for piglet vaccination to control PCV2 infection was at either 3 or 6 weeks of age. In addition, OF proved to be a useful matrix for the evaluation of seroconversion dynamics, however, PCV2 DNA detection in OF did not show to be an effective

method for the infection control assessment during the studied vaccine programs.

RESUMEN

El *Circovirus porcino tipo 2* (PCV2) es uno de los virus más prevalentes en la cabaña porcina mundial, causando grandes pérdidas económicas. Este virus es de naturaleza ubicua en la población porcina y es el agente causante de una serie de condiciones clínicas y subclínicas llamadas enfermedades asociadas a circovirus porcino (PCVDs). La herramienta de control más importante frente a las PCVDs es la vacunación, aunque debe aplicarse junto con otras medidas, como las prácticas adecuadas de manejo en granja y el control de los posibles factores de riesgo para el desencadenamiento de la enfermedad. Durante la última década, las vacunas comerciales frente a PCV2 han demostrado ser eficaces para controlar la infección por PCV2, reducir los brotes de enfermedad sistémica asociada a PCV2 (PCV2-SD) y mejorar los parámetros productivos. Actualmente, el uso de estas vacunas sigue siendo generalizado, aunque se aplican principalmente en escenarios de infección subclínica por PCV2 (PCV2-SI). En este contexto, la presente tesis tuvo como objetivo principal investigar aspectos todavía desconocidos sobre la posible influencia de la vacunación de cerdas en los resultados reproductivos, así como en la transferencia de la inmunidad maternal celular. Además, también se pretendió ampliar el conocimiento sobre la vacunación de lechones mediante la comparación de la dinámica de infección obtenida después de la aplicación de la vacunación a distintas edades utilizando muestras de suero y fluido oral (OF).

El primer estudio evaluó el efecto de la vacunación de cerdas frente a PCV2 sobre los parámetros reproductivos durante dos ciclos gestacionales consecutivos. El estudio se realizó en una explotación de reproductoras subclínicamente infectada por PCV2 (evidencia de circulación de PCV2, pero ausencia de problemas reproductivos aparentes). Se inmunizaron 94 cerdas gestantes con una vacuna comercial frente a PCV2, y 97 fueron inyectadas con

una solución salina tamponada con fosfato (PBS). En el primer ciclo reproductivo el tratamiento fue aplicado a las 6 y 3 semanas antes del parto, y en el segundo ciclo se aplicó una dosis de recuerdo o PBS a las 2 semanas antes del parto. Se tomaron muestras de sangre en varios momentos del estudio para evaluar la carga de ADN de PCV2 y los anticuerpos específicos frente al virus. Al parto, se registraron los principales parámetros reproductivos, así como el índice de vitalidad de los lechones. Además, en aquellas camadas con más de tres fetos momificados o lechones nacidos muertos, se tomaron muestras de corazón para posteriores exámenes microscópicos y detección del antígeno de PCV2 en el miocardio fetal. Las cerdas vacunadas mostraron niveles de anticuerpos significativamente más altos en comparación con las no vacunadas. El ADN de PCV2 solo se detectó al parto en 2 (4,2%) cerdas no vacunadas. Las cerdas vacunadas tuvieron 1,3 lechones nacidos vivos más por camada en el segundo ciclo que las no vacunadas. Los lechones provenientes de cerdas vacunadas tuvieron una puntuación de vitalidad significativamente más alta (+ 12,7%) que los nacidos de cerdas no vacunadas. No se detectaron lesiones compatibles con PCV2 ni tampoco antígeno viral en los corazones fetales testados. El presente estudio demostró por primera vez que la vacunación de cerdas frente a PCV2 puede tener una influencia positiva sobre la prolificidad y la vitalidad de su descendencia en reproductoras subclínicamente infectadas por PCV2.

En el segundo estudio, se evaluó el efecto de la vacunación de cerdas frente a PCV2 sobre la respuesta inmune humoral y celular en cerdas y su progenie. A las 7 semanas antes del parto, se seleccionaron 15 cerdas negativas a PCV2 por PCR y con valores de anticuerpos medio-bajos. Dichas cerdas fueron distribuidas aleatoriamente en dos grupos de tratamiento según sus niveles de anticuerpos. Siete cerdas fueron vacunadas a las 6 y 3 semanas antes del parto con un producto comercial frente a PCV2 y 8 fueron inyectadas con PBS. Se

tomaron muestras de sangre de las cerdas en el momento del parto y de su descendencia a las 48-72 horas de vida. El ADN de PCV2, así como los anticuerpos específicos frente al virus, fueron analizados en muestras de suero (n = 90; 6 lechones por camada). Por otro lado, los niveles de diferentes citoquinas (IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β, IL-8, IL-4, IL-6 e IL-10) fueron evaluados en el sobrenadante (n = 30; 2 lechones por camada) del cultivo de células mononucleares de sangre periférica. Todas las cerdas y lechones fueron negativos a PCV2 por PCR a lo largo del estudio. Se detectaron niveles de anticuerpos frente a PCV2 significativamente mayores en las cerdas del grupo vacunado y en sus lechones después de la ingestión de calostro en comparación con los del grupo no vacunado. Las cerdas vacunadas no mostraron diferencias significativas en los niveles de secreción de citoquinas al parto en comparación con las hembras no vacunadas. Por el contrario, los lechones nacidos de cerdas vacunadas tenían valores significativamente más altos de citoquinas vinculadas a las células Th1 de memoria (IFN-γ y TNF-α) en comparación con los provenientes de las hembras no vacunadas. En conclusión, la vacunación de cerdas frente a PCV2, además de desencadenar una respuesta inmune humoral en cerdas y su progenie, podría estar asociada a una mayor transferencia de inmunidad mediada por células de la madre al lechón.

El objetivo del tercer estudio fue determinar la dinámica serológica y virológica de la infección por PCV2 en lechones vacunados a diferentes edades en un escenario de PCV2-SI. Se seleccionaron 644 lechones sanos de 2 semanas de edad que se distribuyeron aleatoriamente en cuatro grupos de tratamiento: vacunación frente a PCV2 a las 3, 6 o 10 semanas de edad (grupos 3W-VAC, 6W-VAC y 10W-VAC, respectivamente) y cerdos no vacunados (grupo NON-VAC). Se tomaron muestras de sangre de una subpoblación de cerdos (n = 112 animales) y OF (n = 40 corrales) en distintos momentos del estudio para evaluar la carga de PCV2, la inmunidad humoral y el genotipo viral. En general, el

porcentaje de sueros positivos a PCV2 por PCR incrementó principalmente a las 10 semanas de edad, siendo máximo a las 14 semanas de edad, para luego comenzar a disminuir entre las 18 y 25 semanas de edad. Específicamente, en la vacunación frente a PCV2 a las 3 o 6 semanas de edad se obtuvieron resultados serológicos y virológicos similares, ya que ambas pautas desencadenaron una seroconversión temprana y redujeron, en diferentes puntos del muestreo, la proporción de animales virémicos en comparación con el grupo no vacunado. En contraste, la vacunación frente a PCV2 a las 10 semanas de edad solo logró dicha reducción a las 25 semanas de edad; en este caso, la vacunación coincidió con el aumento del porcentaje de cerdos virémicos en la población. Ambas técnicas de detección de anticuerpos utilizadas en suero y OF ofrecieron resultados similares con una correlación alta y estadísticamente significativa. Por otro lado, se detectó un mayor porcentaje de positividad a PCV2 por PCR en OF en comparación con los sueros. En conclusión, bajo las condiciones del presente estudio, el tiempo óptimo de vacunación de los lechones para controlar la infección por PCV2 fue a las 3 o 6 semanas de edad. Además, los OF demostraron ser una matriz útil para la evaluación de la dinámica de seroconversión, en cambio, la detección del ADN de PCV2 en OF no mostró ser un método efectivo para la evaluación del control de la infección durante los programas vacunales estudiados.

RESUM

El *Circovirus porcí tipus 2* (PCV2) és un dels virus més prevalents a la producció porcina mundial, causant grans pèrdues econòmiques. Aquest virus té una naturalesa ubiqua en la població porcina i és l'agent causant d'una sèrie de condicions clíniques i subclíniques anomenades malalties associades al circovirus porcí (PCVDs). L'eina de control més important per a les PCVDs és la vacunació, tot i que s'ha d'aplicar juntament amb altres mesures, com les pràctiques adequades de maneig en granja i el control dels possibles factors de risc pel desenvolupament de la malaltia. Durant l'última dècada, les vacunes comercials enfront a PCV2 han demostrat ser eficaces en controlar la infecció per PCV2, reduir els brots de malaltia sistèmica associada a PCV2 (PCV2-SD) i millorar els paràmetres productius. Actualment, l'ús d'aquestes vacunes segueix sent generalitzat, tot i que s'apliquen principalment en escenaris d'infecció subclínica per PCV2 (PCV2-SI). En aquest context, l'objectiu principal d'aquesta tesi va ser investigar alguns aspectes encara desconeguts sobre la possible influència de la vacunació de truges en els resultats reproductius, així com en la transferència de la immunitat maternal cel·lular. A més, també es va pretendre ampliar el coneixement sobre la vacunació de garrins mitjançant la comparació de la dinàmica d'infecció obtinguda després de l'aplicació de la vacuna a diferents edats utilitzant mostres de sèrum i fluids orals (OF).

El primer estudi va avaluar l'efecte de la vacunació de truges enfront a PCV2 sobre els paràmetres reproductius durant dos cicles gestacionals consecutius. L'estudi es va realitzar en una explotació de reproductores subclínicament infectades per PCV2 (evidència de circulació de PCV2, però absència de problemes reproductius aparents). Es van immunitzar 94 truges gestants amb una vacuna comercial enfront a PCV2, i 97 van ser injectades amb una solució salina tamponada amb fosfat (PBS). En el primer cicle reproductiu el tractament

va ser aplicat a les 6 i 3 setmanes abans del part, i en el segon cicle es va aplicar una dosi de record o PBS a les 2 setmanes abans del part. Es van prendre mostres de sang en diferents moments de l'estudi per avaluar la càrrega d'ADN de PCV2 i els anticossos específics enfront al virus. En el part, es van registrar els principals paràmetres reproductius, així com l'índex de vitalitat dels garrins. A més, en aquelles camades amb més de tres fetus momificats o garrins nascuts morts, es van agafar mostres de cor pel seu posterior examen microscòpic i de detecció de l'antigen de PCV2 en el miocardi fetal. Les truges vacunades van mostrar nivells d'anticossos significativament més elevats en comparació amb les no vacunades. L'ADN de PCV2 només es va detectar al part en 2 (4,2%) truges no vacunades. Les truges vacunades van tenir 1,3 garrins nascuts vius més per camada en el segon cicle que les no vacunades. Els garrins provinents de truges vacunades van mostrar una puntuació de vitalitat significativament més alta (+ 12,7%) que els nascuts de truges no vacunades. No es van detectar lesions compatibles amb PCV2 ni tampoc antigen viral en els cors fetals testats. El present estudi va demostrar per primera vegada que la vacunació de truges enfront a PCV2 pot tenir una influència positiva sobre la prolificitat i la vitalitat de la seva descendència en una explotació de reproductores subclínicament infectades per PCV2.

En el segon estudi, es va avaluar l'efecte de la vacunació de truges enfront a PCV2 sobre la resposta immune humoral i cel·lular en truges i la seva progènie. A les 7 setmanes abans del part, es van seleccionar 15 truges negatives a PCV2 per PCR i amb valors d'anticossos mitjans-baixos. Aquestes truges es van distribuir aleatòriament en dos grups de tractament segons els seus nivells d'anticossos. A les 6 i 3 setmanes abans del part, 7 truges van ser vacunades amb una vacuna comercial enfront a PCV2 i 8 van ser injectades amb PBS. Es van prendre mostres de sang de les truges en el moment del part i de la seva descendència a les 48-72 hores de vida. L'ADN de PCV2, així com els

anticossos específics enfront al virus, van ser analitzats en mostres de sèrum (n = 90, 6 garrins per camada). Per altra banda, es van avaluar els nivells de diferents citoquines (IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β, IL-8, IL-4, IL-6 i IL-10) en el sobrenedant (n = 30, 2 garrins per camada) del cultiu de cèl·lules mononuclears de sang perifèrica. Totes les truges i garrins van ser negatius a PCV2 per PCR al llarg de l'estudi. Es van detectar nivells d'anticossos enfront a PCV2 significativament més elevats en les truges del grup vacunat i en els seus garrins després de la ingestió de calostre en comparació amb els del grup no vacunat. Les truges vacunades no van mostrar diferències significatives en els nivells de secreció de citoquines al part en comparació amb les femelles no vacunades. Per contra, els garrins nascuts de truges vacunades tenien nivells significativament més alts de citoquines vinculades a les cèl·lules Th1 de memòria (IFN-γ i TNF-α) en comparació amb els provinents de les femelles no vacunades. En conclusió, la vacunació de truges enfront a PCV2, a més de desencadenar una resposta immune humoral en truges i la seva progènie, podria estar associada a una major transferència d'immunitat cel·lular de la mare al garrí.

L'objectiu del tercer estudi va ser determinar la dinàmica serològica i virològica de la infecció per PCV2 en garrins vacunats a diferents edats en un escenari de PCV2-SI. Es van seleccionar 644 garrins sans de 2 setmanes d'edat que foren distribuïts aleatòriament en quatre grups de tractament: vacunació enfront a PCV2 a les 3, 6 o 10 setmanes d'edat (grups 3W-VAC, 6W-VAC i 10W-VAC, respectivament) i porcs no vacunats (grup NON-VAC). Al llarg de l'estudi, i amb l'objectiu d'avaluar la càrrega de PCV2, la immunitat humoral i el genotipus víric, es van prendre mostres de sang d'una subpoblació de porcs (n = 112 animals) i OF (n = 40 corrals). En general, el percentatge de sèrums positius a PCV2 per PCR va incrementar principalment a les 10 setmanes d'edat, sent màxim a les 14 setmanes d'edat, per després començar a disminuir entre les 18 i

25 setmanes d'edat. Específicament, amb la vacunació enfront a PCV2 a les 3 o 6 setmanes d'edat es van obtenir resultats similars, ja que les dues pautes van desencadenar una seroconversió eficient i van reduir, en diferents punts de mostreig, la proporció d'animals virèmics en comparació amb el grup no vacunat. Per altra banda, la vacunació enfront a PCV2 a les 10 setmanes d'edat només va aconseguir aquesta reducció a les 25 setmanes d'edat; en aquest cas, la vacunació va coincidir amb l'augment del percentatge de porcs virèmics. Les dues tècniques de detecció d'anticossos utilitzades en sèrum i OF van oferir resultats similars amb una correlació alta i estadísticament significativa. D'altra banda, es va detectar un major percentatge de positivitat a PCV2 per PCR en OF en comparació amb els sèrums. En conclusió, sota les condicions del present estudi, el temps òptim de vacunació dels garrins per controlar la infecció per PCV2 va ser a les 3 o 6 setmanes d'edat. A més, els OF van demostrar ser una matriu útil per a la avaluació de la dinàmica de seroconversió, en canvi, la detecció del ADN de PCV2 en OF no va mostrar ser un mètode efectiu per a la avaluació del control de la infecció durant els programes vacunals estudiats.

PUBLICATIONS

The results presented in this Thesis have been published or submitted for publication in international scientific peer-reviewed journals:

- Oliver-Ferrando S., Segalés J., López-Soria S., Callén A., Merdy O., Joisel F., Sibila M. Exploratory field study on the effect of *Porcine circovirus 2* (PCV2) sow vaccination on serological, virological and reproductive parameters in a PCV2 subclinically infected sow herd. Submitted for publication.

- Oliver-Ferrando S., Segalés J., Sibila M., Díaz I. Comparison of cytokine profiles in peripheral blood mononuclear cells between piglets born from *Porcine circovirus 2* vaccinated and non-vaccinated sows. Submitted for publication

- Oliver-Ferrando S., Segalés J., López-Soria S., Callén A., Merdy O., Joisel F., Sibila M. Evaluation of natural porcine circovirus type 2 (PCV2) subclinical infection and seroconversion dynamics in piglets vaccinated at different ages. Veterinary Research, 2016; 47:121.

CHAPTER 1

General Introduction

1.1. *PORCINE CIRCOVIRUS 2* (PCV2)

1.1.1. History of PCV2

Porcine circovirus (PCV) was discovered in 1974 (Tischer *et al*., 1974) as a contaminant of porcine kidney-15 cells (PK-15 cells). Further studies determined the presence of this virus in pig serum samples from several countries: Germany (Tischer *et al*., 1986), Canada (Dulac and Afshar, 1989), England (Edwards and Sands, 1994), and the USA (Hines and Lukert, 1995), revealing its ubiquitous nature. PCV was considered a non-pathogenic virus, since the inoculation of pigs with the PK-15 cell contaminant agent did not cause any disease (Tischer *et al*., 1986; Allan *et al*., 1995).

In the early 1990s, a new disease of pigs, clinically characterized by wasting, emerged in Canada and was named post-weaning multisystemic wasting syndrome (PMWS) (Clark, 1997; Harding and Clark, 1997). Electron microscopy, immunohistochemistry (IHC) and *in situ* hybridization (ISH) identified the presence of circovirus in tissues from PMWS-affected pigs (Ellis *et al*., 1998). The genome sequence analyses of the virus isolated from affected pigs determined that it was a distinct PCV from the previously published non-pathogenic one, since they only had 68% of nucleotide identity (Hamel *et al*., 1998). The novel pathogenic virus associated with PMWS was designated as PCV2 and the non-pathogenic virus from PK-15 cells was termed PCV type 1 (PCV1) (Meehan *et al*., 1998).

PMWS was characterized by growth retardation, increased mortality, enlarged lymph nodes, occasional jaundice, and characteristic microscopic lesions in multiple organs (mainly in lymphoid tissues) (Clark, 1997; Rosell *et al*., 1999). This syndrome, since its onset in Canada, spread to the major porcine producing countries around the world (Segalés *et al*., 2005a). PCV2 is nowadays

recognized as one of the most important swine pathogens worldwide, causing severe economic losses in the pig industry (Segalés *et al*., 2013).

Retrospective studies from archived tissues indicated that PCV2 was present years before the description of PMWS. PCV2 DNA sequences have been reported in pig tissues as soon as 1962 in Germany (Jacobsen *et al*., 2009). However, it was not until 1985 when PCV2 was detected in tissues with PMWS-compatible lesions (Jacobsen *et al*., 2009).

Both clinical and pathological findings related to PCV2 infection have evolved since the early 1990s. Apart from PMWS, currently known as PCV2 systemic disease (PCV2-SD) (Segalés, 2012), the virus is involved in other conditions currently encompassed under the name of porcine circovirus diseases (PCVD) in Europe (Segalés *et al*., 2005a) or porcine circovirus-associated diseases (PCVAD) in North America (Opriessnig *et al*., 2007). Currently, PCVD include the following conditions (Segalés, 2012): PCV2 subclinical infection (PCV2-SI), PCV2-SD, PCV2 reproductive disease (PCV2-RD) and porcine dermatitis and nephropathy syndrome (PDNS).

1.1.2. Taxonomy and classification

PCV2 belongs to the family *Circoviridae*, which is characterized by icosahedral, small, non-enveloped and single-stranded DNA (ssDNA) viruses that infect vertebrates (Lukert *et al*., 1995). In recent years, the discovery of new viruses and the re-evaluation of genomic features of members of this family ended up with an update of the *Circoviridae* taxonomy by the International Committee on the Taxonomy of Viruses (ICTV), which was ratified in 2016.

Currently, the family *Circoviridae* is divided into two genera based on their morphology and genomic organization (Rosario *et al*., 2017):

- Genus *Circovirus*: The viruses included in this genus, commonly known as circoviruses, are the aetiological agents of potentially fatal diseases affecting birds and swine (Todd, 2000). Traditionally, all reported cases of circoviruses belonged to porcine or avian species. However, since 2010, several studies using unconventional hosts have detected the presence of circovirus DNA in fishes (Lorincz *et al*., 2011, 2012), dogs (Li *et al*., 2013), bats (Lima *et al*., 2015; Wu *et al*., 2016), minks (Lian *et al*., 2014), chimpanzees (Li *et al*., 2010) and humans (Li *et al*., 2010) (Table 1-1).
- Genus *Cyclovirus* (new taxon): In 2010, a group of viruses closely related to circoviruses were discovered through viral metagenomics analysis and degenerate polymerase chain reaction (PCR) methodologies in samples from animals and humans (Li *et al*., 2010). The viruses from this new group shared genomic features with circoviruses (Delwart and Li, 2012). Nevertheless, phylogenetic and genomic differences between both groups caused the creation of a second taxonomic unit, the newly established genus *Cyclovirus*, within the family *Circoviridae* (Rosario *et al*., 2017). In contrast to circoviruses, cycloviruses are associated with both vertebrates and invertebrates (Table 1-1).

After the ICTV update of the *Circoviridae* taxonomy in 2016, a novel PCV, named PCV3, was discovered (Phan *et al*., 2016; Palinski *et al*., 2017). The PCV3 genome sequence was not included in the analyses presented in the last report of the ICTV (Table 1-1); however, preliminary analyses indicate that indeed PCV3 represents a new circovirus species containing a putative Cap

protein with 37% nucleotide identity with respect to PCV2 (Phan *et al*., 2016; Palinski *et al*., 2017). Pathogenicity of PCV3 is currently not clarified.

Table 1-1. Member species of the family *Circoviridae* (modified from ICTV, https://talk.ictvonline.org/).

Genus	Virus name	Acronyms	Number of species
Circovirus	*Barbel circovirus*	BarCV	1
	Bat associated circovirus	BatACV-1 to BatACV-8	8
	Beak and feather disease virus	BFDV	1
	Canary circovirus	CaCV	1
	Canine circovirus	CanineCV	1
	Chimpanzee associated circovirus 1	ChimpACV-1	1
	Duck circovirus	DuCV	1
	European catfish circovirus	EcatfishCV	1
	Finch circovirus	FiCV	1
	Goose circovirus	GoCV	1
	Gull circovirus	GuCV	1
	Human associated circovirus 1	HuACV-1	1
	Mink circovirus	MiCV	1
	Pigeon circovirus	PiCV	1
	Porcine circovirus	**PCV1 and PCV2**	**2**
	Raven circovirus	RaCV	1
	Starling circovirus	StCV	1
	Swan circovirus	SwCV	1
	Zebra finch circovirus	ZfiCV	1
Cyclovirus	*Bat associated cyclovirus*	BatACyV-1 to BatACyV-16	16
	Bovine associated cyclovirus 1	BoACyV-1	1
	Chicken associated cyclovirus 1	ChickACyV-1	1
	Chimpanzee associated cyclovirus 1	ChimpACyV-1	1
	Cockroach associated cyclovirus 1	CroACyV-1	1
	Dragonfly associated cyclovirus	DfACyV-1 to DfACyV-8	8
	Feline associated cyclovirus 1	FeACyV-1	1
	Goat associated cyclovirus 1	GoACyV-1	1
	Horse associated cyclovirus 1	HoACyV-1	1
	Human associated cyclovirus	HuACyV-1 to HuACyV-11	11
	Squirrel associated cyclovirus 1	SqACyV-1	1

1.1.3. Morphology and molecular organization

PCV2 is a very small and non-enveloped virus (12 to 23 nm in diameter) that has a covalently closed circular ssDNA of 1.7 kilobases (Meehan *et al*., 1998; Rodríguez-Cariño and Segalés, 2009). The PCV2 genome has an ambisense organisation containing 11 open reading frames (ORFs), ORF1 to ORF11, arranged on different strands of a double-stranded DNA (dsDNA) replicative form (Hamel *et al*., 1998). However, only four ORFs (ORF1 to ORF4) have been recognized as coding for functional proteins:

- The ORF1 (also called *Rep*) gene is the largest ORF of PCV2 (945 base pairs [bp]), which is placed in the viral plus-strand and oriented clockwise. This highly conserved gene encodes DNA replication-associated proteins, being Rep and Rep´ the most significant ones (Cheung, 2003).
- The ORF2 (also named *Cap*) gene is composed of 702 bp and located on the viral complementary strand with counter-clockwise orientation. This gene encodes the Capsid (*Cap*) protein, which is considered: 1) the major viral structural protein (Nawagitgul *et al*., 2000); 2) the most immunogenic one (Nawagitgul *et al*., 2000) and 3) the most suitable phylogenetic and epidemiological marker for PCV2 (Olvera *et al*., 2007).
- The ORF3 gene has approximately 315 bp, completely overlaps the ORF1 gene and is oriented in the opposite direction (Liu *et al*., 2005). The protein encoded by ORF3 is a non-structural protein that can induce apoptosis in virus-infected cells, such as PK-15 cells (Liu *et al*., 2005) and porcine peripheral blood mononuclear cells (PBMCs) (Lin *et al*., 2011). Besides, its apoptotic activity has also been demonstrated *in vivo* (Liu *et al*., 2006).

- The ORF4 gene contains approximately 180 bp, it is located within ORF3 gene and oriented in the same direction. This gene encodes a new protein (He *et al*., 2013) that may play an important role in the suppression of virus-induced apoptosis (Gao *et al*., 2014).

1.1.4. Physico-chemical properties

PCV2 has high environmental stability since it is very resistant under high temperatures and a wide range of pH conditions (Patterson and Opriessnig, 2010). Moreover, PCV2 has been shown to be fairly resistant against several types of disinfectants. Specifically, reduction of PCV2 titres under *in vitro* conditions is most probable when using an oxidizing, halogen, or sodium hydroxide containing product; products containing iodine, alcohol, phenol or formaldehyde seem to be less effective (Royer, 2001; Yilmaz and Kaleta, 2004; Martin *et al*., 2008; Kim *et al*., 2009a,b). However, complete inactivation of PCV2 under *in vitro* conditions is difficult, suggesting that PCV2 is one of the most resistant viruses.

1.1.5. PCV2 genotypes

PCV2 is divided into four major genotypes (PCV2a, PCV2b, PCV2c and PCV2d) according to the phylogenetic analysis of viral sequences (Segalés *et al*., 2008; Guo *et al*., 2010; Franzo *et al*., 2015b). However, other PCV2 genotypes (PCV2e and PCV2f) have been recently described (Davies *et al*., 2016; Bao *et al*., 2017), although its distribution, prevalence and importance is still uncertain.

The *Cap* gene is the most used in these phylogenetic studies, although the whole PCV2 genome is also utilized (Xiao *et al*., 2015). Among genotypes, PCV2a, PCV2b and PCV2d are prevalent worldwide (Wiederkehr *et al*., 2009; Wang *et al*., 2013; Franzo *et al*., 2015b), being PCV2a the most frequent genotype found in pigs until 2003. Nevertheless, around 2001-2004, a change in genotype prevalence (genotype shift) occurred, becoming PCV2b the most widespread and predominant genotype (Allan *et al*., 2012; Segalés *et al*., 2013). This genotype shift coincided with the advent of the most severe outbreaks of PCV2-SD (Carman *et al*., 2006; Cheung *et al*., 2007; Timmusk *et al*., 2008; Wiederkehr *et al*., 2009; Cortey *et al*., 2011). Regarding the PCV2c genotype, it was firstly described in Danish samples collected between 1980 and 1990 (Dupont *et al*., 2008) and recently identified in feral pigs from Brazil (Franzo *et al*., 2015a) and in pig farms from China (Liu *et al*., 2016). On the other hand, the PCV2d (previously known as a mutant of PCV2b [mPCV2b]) genotype, was first identified in 1998 in samples collected in Switzerland. Then, it was detected in China, being later widespread throughout the country (Ge *et al*., 2012). Currently, PCV2d is present worldwide and its prevalence is increasing (Guo *et al*., 2010; Franzo *et al*., 2015b; Xiao *et al*., 2015), suggesting an ongoing genotype shift from PCV2b towards PCV2d (Xiao *et al*., 2015).

1.2. PCV2 AND PORCINE CIRCOVIRUS DISEASES (PCVD)

1.2.1. Epidemiology

1.2.1.1. Susceptibility of other species

Apart from domestic pigs, feral pigs (Franzo *et al*., 2015a), wild boars (Ellis *et al*., 2003; Schulze *et al*., 2004) and peccaries (de Castro *et al*., 2014) are also susceptible to PCV2 infection. In fact, PCV2-SD has been described in wild

boars (Ellis *et al*., 2003; Schulze *et al*., 2004; Lipej *et al*., 2007; Hohloch *et al*., 2015).

Moreover, PCV2 can replicate and can be transmitted in mice (Kiupel *et al*., 2001; Csagola *et al*., 2008). However, clinical signs in mice were not observed and microscopic lesions in PCV2-experimentally infected mice were insignificant and different to those in PCVD-affected pigs (Kiupel *et al*., 2005; Opriessnig *et al*., 2009a). Besides, PCV2 genome was detected in tissues from dead mice and rats taken from two PCV2-infected pig farms (Lorincz *et al*., 2010). Nevertheless, PCV2 DNA was not detected in tissues from rodents collected in areas far from pig farms. These results suggest that rodents may play a role as local reservoirs and vectors of PCV2. In this context, one study (Blunt *et al*., 2011) showed that flies (*Musca domestica*) may also act as vectors for PCV2, since in that study identical sequences were found in pigs and flies associated with them. Interestingly, in the same study, dissected viscera from cockroaches (*Blatta sp*.) in contact with pigs were also collected and analysed, although they were PCV2 PCR negative (Blunt *et al*., 2011).

The experimental inoculation of rabbits (Quintana *et al*., 2002), sheep (Allan *et al*., 2000a) and cattle (Ellis *et al*., 2001) with PCV2 isolates from PCV2-SD affected pigs did not induce clinical signs, viraemia, antibody response and microscopic lesions. However, PCV2 has been detected by PCR from bovine respiratory disease and abortion cases (Nayar *et al*., 1999) and from bovine haemorrhagic diathesis cases (Kappe *et al*., 2010). Consequently, in cattle, the results are inconclusive, requiring additional research in order to clarify the pathogenesis and the potential role of PCV2 in these clinical cases.

With regard to other species such as avian, caprine, equine and human, there is no evidence about their susceptibility to PCV2 infection. Therefore, PCV2 transmission to swine from other species seems to be unlikely.

1.2.1.2. Transmission

In general terms, effective transmission of an infectious agent to its host depends on several factors (Thrusfield, 2005):

- The characteristics of the host: The host must be susceptible
- The characteristics of the pathogen: The pathogen must be virulent, infective and stable in the environment
- The type of exposure: The interaction between the pathogen and host must be favourable for infection (right infectious dose, suitable contact time, etc.)

1.2.1.2.1. Horizontal transmission

1.2.1.2.1.1. Routes of shedding

Several experimental and field studies have demonstrated the PCV2 shedding through a wide variety of routes (Patterson and Opriessnig, 2010). In fact, it has been described that PCV2-SD-affected pigs have greater shedding in comparison to healthy infected controls (Segalés *et al*., 2005b). At farm level, PCV2 is present in large quantities in nasal, oral and faecal excretions for relatively extended periods of time following natural infection (Sibila *et al*., 2004; Patterson *et al*., 2011), representing the most likely routes (Rose *et al*., 2012). In addition, PCV2 can be shed in saliva, urine, colostrum, milk and

semen, as well as in bronchial and ocular secretions (Krakowka *et al*., 2000; Larochelle *et al*., 2000; Shibata *et al*., 2003; Segalés *et al*., 2005b; Shibata *et al*., 2006; Ha *et al*., 2009). Moreover, pork products represent a potential source of introduction of PCV2 isolates into a pig population. This fact was demonstrated in an experimental study in which uncooked PCV2 DNA positive tissues from viraemic pigs were able to infect naïve pigs by the oral route (Opriessnig *et al*., 2009c).

1.2.1.2.1.2. Evaluation of the transmission: direct and indirect contact

Generally, horizontal transmission can occur by: 1) direct contact with an infected host or the infected host's secretions or 2) indirect contact of a virus with a living vector, contaminated inanimate object or aerosol droplets (Thrusfield, 2005). In the specific case of PCV2, horizontal transmission by direct (nose-to-nose) contact has been experimentally demonstrated (Albina *et al*., 2001; Bolin *et al*., 2001). This fact was also observed at farm level, where PCV2-SD affected pigs were able to infect healthy pigs by direct contact (Dupont *et al*., 2009; Kristensen *et al*., 2009). On the other hand, horizontal transmission by indirect contact was also reported in pigs allocated in separate pens (Andraud *et al*., 2008; Kristensen *et al*., 2009), although the effectiveness was lower than the one obtained by direct contact.

Several features of the PCV2 infectious period were evaluated in a time-dependent transmission model (Andraud *et al*., 2009). In this study, six successive transmission trials were conducted using experimentally infected pigs commingled with naïve contact pigs. The values estimated in this work were:

- PCV2 incubation period of 8 days
- basic reproduction ratio of 5.9
- mean disease generation time of 18.4 days

Moreover, the transmission rate decreased gradually between the period from 15 to 55 days post-infection (dpi), even though pigs remained viraemic with loads of 10^3-10^6 PCV2 copies/mL at those later times. These data suggested a correlation between the presence of protective immunity and decreased transmission (Andraud *et al*., 2009).

1.2.1.2.2. Vertical transmission

During fertilization, embryos can be infected by PCV2 contaminated semen. In addition, at any stage of sow pregnancy, PCV2 has the ability to cross the placental barrier and replicate in the embryos and foetuses, causing reproductive disorders in sows (Madson and Opriessnig, 2011). Moreover, after farrowing, PCV2 can also be transmitted to the new-born piglets by an oral route through colostrum or milk as well as by direct contact (Shibata *et al*., 2006; Ha *et al*., 2009; Patterson and Opriessnig, 2010).

At experimental level, embryos has been infected by sow insemination using PCV2-spiked semen, inducing reproductive disorders as well as viraemic live-born piglets (Rose *et al*., 2007; Madson *et al*., 2009b). Additionally, several trials have been conducted to infect the foetuses by *in utero* inoculation (Sánchez *et al*., 2001; Johnson *et al*., 2002; Sánchez *et al*., 2003). Other studies, demonstrated PCV2 trans-placental infection, since the virus was detected in both aborted and live-born piglets from intra-nasally inoculated sows (Park *et al*., 2005; Ha *et al*., 2008).

Under field conditions, different prevalence of intrauterine PCV2 infection has been reported. In a study conducted in several Spanish farms, late-term aborted foetuses with a negative *Porcine reproductive and respiratory syndrome virus* (PRRSV) PCR result were analysed for PCV2, detecting only 1% of PCV2 PCR positive samples (Maldonado *et al*., 2005). This very low prevalence was also described in a Danish field work (Ladekjaer-Mikkelsen *et al*., 2001). In contrast, a retrospective study on abortion cases in Korea reported a 13% PCV2 prevalence (measured by PCR) of the total of aborted foetuses and stillborn piglets (Kim *et al*., 2004). In addition, another study conducted in five commercial breeding herds located in Mexico and the U.S. showed high PCV2 DNA prevalence in sow serum (47.2%), colostrum (40.8%) and in pre-suckling piglet serum (39.9%), suggesting that PCV2 viraemia in sows may be linked to trans-placental infection (Shen *et al*., 2010). Nevertheless, the importance of PCV2 infection in sow reproductive failures under natural conditions still remains uncertain, since clinical and noticeable reproductive problems attributed to PCV2, at field level, are infrequent (Pensaert *et al*., 2004; Madson and Opriessnig, 2011; Karuppannan *et al*., 2016).

1.2.1.3. Factors associated with disease development

Apart from the abovementioned factors in the virus transmission section and due to PCV2-SD multifactorial nature, others parameters may facilitate its development (Segalés, 2012). Currently, the virus is ubiquitous on farms around the world (Segalés *et al*., 2013), therefore, most of the animals get a subclinical infection during their lifespan; however, only some of them will develop PCV2-SD. The main risk factors for triggering the disease can be classified into three groups: 1) virus and host related factors, 2) management and husbandry related

factors, and 3) co-infections. Almost no information is available regarding triggering factors for other PCVD.

1.2.1.3.1. Virus and host related factors

The main factors influencing the PCV2-SD development are the immune status of the sow, timing of PCV2 infection, variations in the virulence of PCV2 genotypes and animal-dependent features (Table 1-2).

Table 1-2. Major risk factors related to the virus and host for the development of PCV2-SD at herd level (information sources: Grau-Roma *et al*., 2011, Rose *et al*., 2012).

	Factors increasing the risk of PCV2-SD
Sows	• Abscesses/injuries at the injection site (neck) • Viraemic sows during gestation • Low antibody levels at farrowing
Pigs	• Gender (male) • Genetics: mainly Landrace • Litter of origin • Colostrum-deprived piglets • Low levels of maternally-derived immunity against PCV2 • Early PCV2 infections • Low birth weight • Low weaning weight • Low weight at the beginning of fattening period
PCV2 genotype	• Infections with PCV2b

1.2.1.3.2. Management and husbandry related factors

Farm housing, management, hygiene, biosecurity, vaccination schedules as well as treatment and nutrition practices can significantly influence the PCV2-SD expression (Grau-Roma *et al*., 2011; Rose *et al*., 2012) (Table 1-3).

Table 1-3. Summary of the most important management and husbandry factors influencing the risk of PCV2-SD development (modified from Grau-Roma *et al*., 2011).

	Factors increasing the risk of PCV2-SD	**Factors decreasing the risk of PCV2-SD**
Farm facilities	• Large number of sows • Large pens in nursery and growing areas • Proximity to other pig farms	• Separate pit for adjacent fattening rooms • Shower facilities
Management practices	• High level of cross-fostering • High density in pens • Short empty periods at weaning and fattening • Large range in age and weight entering to nursery • Continuous flow through nursery • Purchase of replacement gilts • Early weaning (<21 days of age)	• Sorting pigs by sex at nursery stage • Greater minimum weight at weaning • Group housing sows during pregnancy • Visitors with no pig contact for several days before visiting farm • Use of semen from an insemination centre
Vaccination	• Vaccination of gilts against PRRSV • Vaccination of sows against *Escherichia coli* • Use of separate vaccines against *Erysipelothrix rhusiopathiae* and *Porcine parvovirus* on gilts	• Vaccination of sows against atrophic rhinitis
Treatment		• Regular treatment for ectoparasitism • Use of oxytocin during farrowing
Nutrition		• Use of spray-dried plasma in initial nursery ration • Use of antioxidants in the diet

1.2.1.3.3. Co-infections

Several studies have confirmed that PCV2 replication and associated lesions can be enhanced by concurrent infection with other agents, being PRRSV, *Porcine parvovirus* (PPV) and *Mycoplasma hyopneumoniae* the most important ones (Opriessnig and Halbur, 2012). The list of agents that can be concomitantly found in the PCV2-SD affected animals at farm level are detailed in Table 1-4.

Table 1-4. List of co-infecting agents associated with PCV2-SD field cases (modified from Grau-Roma *et al*., 2011).

Agent type	Name of the agent
Viruses	• PRRSV • PPV • *Porcine epidemic diarrhoea virus* • *Aujeszky´s disease virus* • *Hepatitis E virus* • *Torque teno sus viruses* • *Porcine teschovirus* • *Swine influenza virus* • *Porcine bocavirus*
Bacteria	• *Mycoplasma hyopneumoniae* • *Mycoplasma hyorhinis* • *Mycoplasma suis* • *Salmonella cholerasuis* • *Escherichia coli* • *Haemophilus parasuis* • *Pasteurella multocida* • *Streptococcus suis*
Agents considered as opportunistic (secondary infections after immunosuppression caused by PCV2-SD)	• *Pneumocystis carinii* • *Candida albicans* • *Aspergillus spp*. • *Cryptosporidium parvum* • *Chlamydia spp*. • *Zygomycetes spp*.

1.2.2. Pathogenesis

The molecular pathogenesis of PCV2 infection is still not fully understood. It seems that PCV2 has a strong dependency on host cellular enzymes, since it does not code for its own DNA polymerases. Besides, PCV2 replication tends to occur in cells that are in the S phase of the cell cycle (Tischer *et al*., 1987). A conserved heparin-binding motif in the Cap protein of PCV2, glycosaminoglycans (GAGs), heparan sulphate and chondroitin sulphate have been described as molecules that facilitate the attachment of PCV2 to the host cells (Misinzo *et al*., 2006). The GAGs (ubiquitously distributed in animal tissues) may serve as the first point of attachment; however, other fusion and internalization receptors are likely to be involved in the internalization of viral particles. Nevertheless, it is possible that PCV2 does not need a unique receptor for viral entry, since it can infect both immune and epithelial cells in several tissues (Ramamoorthy and Meng, 2009).

PCV2 infections in pigs may occur before birth (at different stages of embryonic and foetal development) and after birth (at different ages throughout their productive life) (Madson and Opriessnig, 2011; Segalés, 2012), resulting in variable outcomes (Figure 1-1):

- Embryos: The embryonic cells are from the very beginning susceptible; however, when they are covered by the *zona pellucida*, embryos are resistant to infection (Mateusen *et al*., 2004). After hatching, the embryos may become infected since this barrier disappears. Once into the embryonic cells, PCV2 replicates extensively causing the death of the embryo and its subsequent reabsorption in the utero (Mateusen *et al*., 2007). When the embryonic mortality is partial, the sow can continue with the gestation, losing only those reabsorbed embryos. Nevertheless, when mortality affects most embryos, the sow returns to oestrus.

- Foetus at 40-70 days of gestation: PCV2 replicates mainly in the heart, and also in liver, lymphoid organs and lungs to a lower degree (Sánchez *et al*., 2001; Saha *et al*., 2010). Therefore, at that stage, the main target cells are cardiomyocytes, hepatocytes and macrophages (Sánchez *et al*., 2003). PCV2 replication in the heart leads to heart failure, foetal death and mummification (Pensaert *et al*., 2004).

- Foetus at 70-115 days of gestation: From this phase, the foetus begins to be immunocompetent, reason why as the age of the foetus increases, the replication of the virus decreases (Madson *et al*., 2009c).

- Post-natal: In this step, a change of PCV2 tropism seems to take place, since the virus is mainly found in macrophages and lymphoblasts. Macrophages are mainly taking up virus particles, although a certain proportion of them may also support PCV2 replication (Pérez-Martín *et al*., 2007). In addition, lymphoblasts are susceptible targets in which the virus replicates (Sánchez *et al*., 2004; Lefebvre *et al*., 2008). In fact, lymphoid tissues contain the highest concentration of PCV2 (Rosell *et al*., 1999; Quintana *et al*., 2001). However, PCV2 replication mainly occurs in endothelial and epithelial cells (Pérez-Martín *et al*., 2007); the virus may also be detected in enterocytes, hepatocytes, smooth muscle cells, and pancreatic acinar and ductal cells (Rosell *et al*., 1999; Segalés *et al*., 2012).

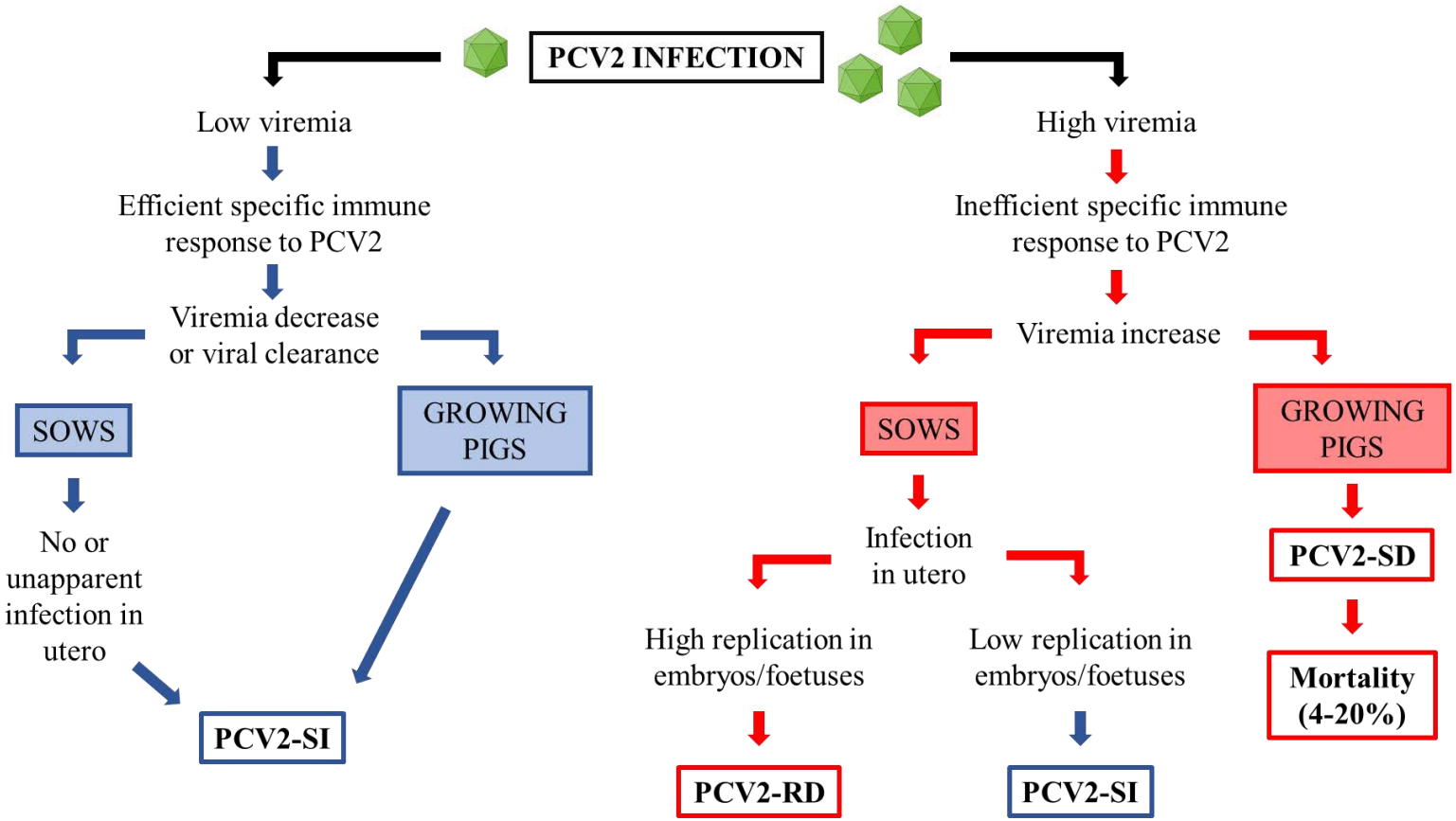

Figure 1-1. Model of the understanding of the progression of PCV2 infection in sows or growing pigs toward PCV2 subclinical infection (PCV2-SI), PCV2 systemic disease (PCV2-SD) or PCV2 reproductive disease (PCV2-RD).

Little is known about PDNS pathogenesis, currently there are a number of circumstances that associate PCV2 with PDNS; however, a causal relationship between the agent and the disease has not been established yet (Segalés *et al*., 2012). The pathogenesis of the characteristic lesions of PDNS is attributed to a type III hypersensitivity reaction; importantly, the presence of PCV2 cannot be confirmed in most of these lesions. Therefore, nowadays, the detection of PCV2 is not a mandatory condition for the diagnosis of PDNS (Segalés *et al*., 2012).

1.2.3. Clinical signs and pathological findings

Main clinical signs, gross lesions and histopathological findings characteristic of each PCVD, as well as the PCV2 amount detected in these lesions and/or sera from affected animals (Segalés, 2012), are listed in Tables 1-5a and 1-5b.

1.2.3.1. PCV2 subclinical infection (PCV2-SI)

Nowadays, the development of clinical PCVD is controlled by means of vaccination against PCV2 (Segalés, 2012). The widespread use of these vaccines has decreased the occurrence of PCV2-SD outbreaks, but has not achieved the infection eradication (Feng *et al*., 2014). Therefore, currently, PCV2 infection remains still ubiquitous (Segalés *et al*., 2013). This implies that most of the infected pigs have a subclinical presentation, generally resulting in a lower growth without evident clinical signs (Young *et al*., 2011) (Table 1-5).

1.2.3.2. PCV2 systemic disease (PCV2-SD)

- Clinical signs: PCV2-SD is clinically characterized by weight loss (Figure 1-2), skin pallor, respiratory distress, diarrhoea and,

occasionally, jaundice (Harding and Clark, 1997). This disease can be observed in pigs between 30 and 180 days of age, although the most common occurrence is between 60 and 90 days. Morbidity observed in affected farms is usually between 4-30% (occasionally 50-60%) and mortality ranges from 4 to 20% (Segalés and Domingo, 2002).

Figure 1-2. PCV2-SD affected pig compared with an age-matched healthy one. A severe growth retardation is observed in the affected animal. Source: Joaquim Segalés, CReSA-IRTA and UAB, Spain.

- Pathological findings: Lesions are found mainly in lymphoid tissues. In the early phase of PCV2-SD, lymph nodes are enlarged (Figure 1-3), whereas in more advanced stages of the disease, they are usually of normal size or even atrophied (Clark, 1997; Segalés *et al*., 2004). Thymus atrophy is also commonly observed in affected animals (Darwich *et al*., 2003a; Hansen *et al*., 2013). At microscopic level, these findings correspond to lymphocyte depletion with infiltration of

histiocytes and multinucleated giant cells (Figure 1-4) (Clark, 1997; Rosell *et al*., 1999), as well as cortical atrophy of the thymus (Darwich *et al*., 2003a). The lungs may be tan-mottled, non-collapsed and with elastic consistency. Microscopically, interstitial pneumonia is observed and, in more advanced cases, there is also peribronchiolar fibrosis and fibrous bronchiolitis (Clark, 1997; Segalés *et al*., 2004). The liver may also be atrophic, pale, firm and with a rough surface. Microscopic lesions in the liver may range from mild lymphohistiocytic hepatitis to massive inflammation with disruption of hepatic cords (Rosell *et al*., 2000a). White spots on renal cortex that correspond to non-purulent interstitial nephritis can sometimes be observed. Lymphohistiocytic inflammation can be detected in most of the tissues from PCV2-SD affected animals (Segalés *et al*., 2004) (Table 1-5).

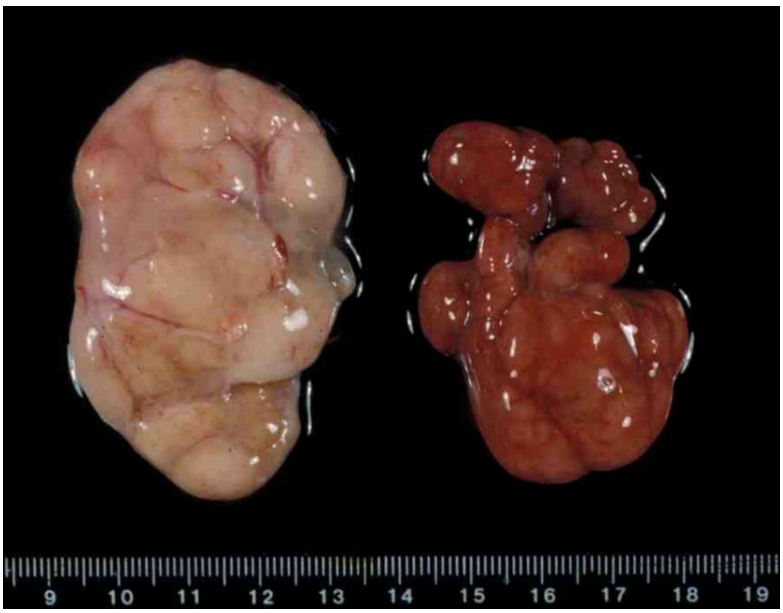

Figure 1-3. Macroscopic appearance of superficial inguinal (left) and submandibular (right) lymph nodes from a PCV2-SD affected animal. A marked increase in size is observed in both lymph nodes. Source: Joaquim Segalés, CReSA-IRTA and UAB, Spain.

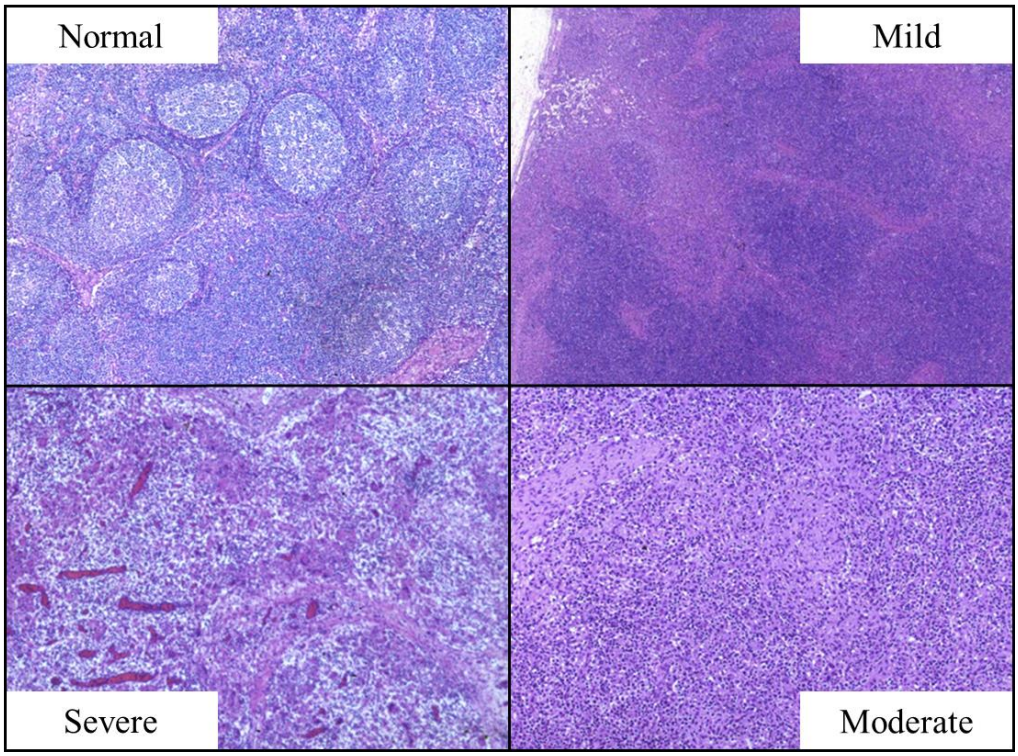

Figure 1-4. Histology of lymph nodes from a healthy pig (normal) and from PCV2-SD affected animals with different lesional severity (mild, moderate and severe). Moderate to severe lymphocyte depletion with histiocytic infiltration is associated with PCV2-SD. Haematoxylin and eosin stain. Source: Joaquim Segalés, CReSA-IRTA and UAB, Spain.

Table 1-5. Summary of clinical signs and main lesions frequently found in PCV2 subclinical infection (PCV2-SI) and PCV2 systemic disease (PCV2-SD) as well as PCV2 amount usually detected in tissues and/or sera of affected animals (modified from Segalés, 2012).

PCVD	Clinical signs	Macroscopic lesions	Microscopic lesions	PCV2 amount (ISH/IHC/qPCR) in tissues and/or sera
PCV2 subclinical infection (PCV2-SI)	Decreased average daily weight gain (ADWG) without any evident clinical sign	Absence	No or minimal histologic lesions in lymphoid tissue (lymphocytic depletion with granulomatous inflammation)*	Low viral load in (lymphoid) tissues*
PCV2 systemic disease (PCV2-SD)	Wasting, weight loss, mortality, paleness of skin (respiratory or digestive clinical signs may be present as well)	Enlargement of lymph nodes, absence of pulmonary collapse, white spots on kidney´s cortices, hepatic atrophy and liver paleness, rough hepatic surface, catarrhal enteritis	Moderate to severe lymphocytic depletion with granulomatous inflammation of lymphoid tissues, lymphohistiocytic to granulomatous inflammation in multiple tissues (lung, kidney, liver and intestine, mainly)	Moderate to high amount of PCV2 in tissues and serum**

* For the diagnosis of PCV2-SI, the presence of microscopic lesions as well as PCV2 amount detected in tissues can be replaced by PCV2 detection techniques in serum samples, such as conventional PCR. If qPCR is used, the viral loads detected in serum samples should be less than 10^6 PCV2 copies/mL.

** For the diagnosis of PCV2-SD by using qPCR from serum samples, the viral loads detected in the affected animals should be greater than 10^7 PCV2 copies/mL.

1.2.3.3. PCV2 reproductive disease (PCV2-RD)

- Clinical signs: The clinical manifestation of PCV2 infection during gestation is variable, since it depends on the viral infection timing, immune response and duration of viraemia in the sow. Table 1-6 summarizes the different clinical presentations of reproductive failure associated with PCV2 with respect to the time of embryonic-foetal infection during gestation (Madson and Opriessnig, 2011). In sows, clinical signs related to PCV2-RD are generally null or unapparent. Nevertheless, there may be a low percentage of abortions due to a systemic process, showing fever and anorexia (Cariolet *et al*., 2002; Park *et al*., 2005). The most common clinical presentation of PCV2-RD is the increased number of mummified and stillborn piglets per litter (O'Connor *et al*., 2001). In addition, return to oestrus (Mateusen *et al*., 2007), delayed farrowing (>118 days of gestation) and pseudo-gestation have also been associated with PCV2-RD, although the two latter ones have been rarely reported (Madson and Opriessnig, 2011). In conclusion, clinical signs of PCV2 infection in the dam are highly variable (Table 1-7). Therefore, PCV2-RD should be included in the differential diagnosis of any reproductive problem observed in the breeding herd.

- Pathological findings: Macroscopic foetal lesions are not always present in PCV2-RD (Madson *et al*., 2009a, c). Often, the unique indication of *in utero* infection is the increased number of stillborns and mummified foetuses at birth. When present, the lesions include dilated cardiomyopathy with pale regions (usually due to fibrous or necrotizing myocarditis), pneumonia with pulmonary oedema, hepatomegaly with an accentuated lobular pattern, hydrothorax, ascites and subcutaneous oedema (O'Connor *et al*., 2001; Madson *et al*., 2009b). Other infrequent lesions such as lymphadenopathy, thymic atrophy, perirenal edema and

cerebral and splenic petechiation can also be observed (Madson *et al*., 2009b).

1.2.3.4. Porcine dermatitis and nephropathy syndrome (PDNS)

- Clinical signs: This condition may affect pigs in nursery or fattening periods and sows (Drolet *et al*., 1999), although their prevalence is usually very low (Segalés *et al*., 1998). Severely affected animals (especially at kidney level) usually die within a few days after the onset of clinical signs. Surviving pigs tend to recover, gaining weight 7-10 days after the onset of the syndrome (Segalés *et al*., 1998). The affected pigs are depressed and reluctant to move; therefore, they are usually lying down. In chronic cases, these animals may be cachectic (Drolet *et al*., 1999). The characteristic clinical finding of PDNS is the presence of macules and dark red papules irregularly distributed on the skin, mainly in hind limbs and perineal area, although they may also be generally distributed. If the animal survives over time, these lesions become dark crusts and gradually disappear, sometimes leaving scars (Drolet *et al*., 1999).

- Pathological findings: Microscopically, macules and papules are observed as haemorrhagic and necrotic skin associated with necrotizing vasculitis (Segalés *et al*., 1998). Pigs severely affected by PDNS have bilaterally enlarged kidneys with red cortical petechiae and oedema of the renal pelvis (Segalés *et al*., 2004). These lesions correspond to necrotizing or fibrinous glomerulonephritis with non-purulent interstitial nephritis (Segalés *et al*., 1998). PDNS, apart from skin and kidney lesions, may also cause enlarged lymph nodes and splenic infarcts (Segalés *et al*., 1998). Histopathologically, lymphoid lesions similar to

those of PCV2-SD (but less severe) can be observed (Rosell *et al*., 2000c).

Table 1-6. Clinical presentation of PCV2 reproductive disease (PCV2-RD) with regards to the infection time of the foetus during gestation (modified from Madson and Opriessnig, 2011).

Stage of gestation	Clinical presentation
Early (1-35 days)	Embryonic death
	Irregular return to oestrus
	Pseudo-pregnancy
	Small litter sizes
Mid (35-70 days)	Mummified foetuses
	Abortion
Late (70-115 days)	Mummified foetuses
	Abortion
	Stillborn piglets
	Weak-born piglets
	Delayed farrowing

Table 1-7. Summary of clinical signs and main lesions frequently found in PCV2 reproductive disease (PCV2-RD) and porcine dermatitis and nephropathy syndrome (PDNS) as well as PCV2 amount usually detected in tissues of affected animals (modified from Segalés, 2012).

PCVD	Clinical signs	Macroscopic lesions	Microscopic lesions	PCV2 amount (ISH/IHC) in tissues
PCV2 reproductive disease (PCV2-RD)	Return to oestrus, reproductive failure (abortions, mummifications, stillborn piglets) and delayed farrowing	Mummified or oedematous foetuses, foetal hepatic congestion and enlargement, foetal cardiac hypertrophy, foetus with ascites, hydrothorax and hydropericardium	Fibrous or necrotizing myocarditis and mild pneumonia in foetuses	Moderate to high amount of PCV2 in foetal myocardium
Porcine dermatitis and nephropathy syndrome (PDNS)	Dark red papules and macules on skin, mainly in hind limbs and perineal area	Haemorrhagic and necrotizing cutaneous lesions and/or enlarged and pale kidneys with generalized cortical petechiae	Systemic necrotizing vasculitis, necrotizing or fibrinous glomerulonephritis and, usually, lymphocytic depletion with granulomatous inflammation	Absence or low amount of PCV2 in lymphoid tissues (not considered as a diagnostic element so far)

1.2.4. Laboratory diagnosis

Due to the ubiquitous nature of the PCV2 infection, diagnosis cannot be based only on the detection of the agent or antibodies against it. The diagnosis of PCVD must be based on three criteria: 1) presence of clinical signs, 2) presence of PCVD compatible microscopic lesions and 3) virus detection in these lesions (Segalés *et al*., 2005a).

In order to detect PCV2 genome or antigen in different tissues, several techniques have been developed, being ISH, IHC and PCR the most commonly used (Rosell *et al*., 1999). The main difference between animals with clinical and subclinical infections is the amount of PCV2 in affected tissues/sera (Olvera *et al*., 2004; Segalés, 2012).

ISH and IHC assays performed on formalin-fixed paraffin-embedded tissues are semi-quantified by a pathologist´s visual scoring of staining. Positive tissues are usually classified into three categories: low, moderate or high; according to the amount of PCV2 nucleic acid or antigen present in them (Figure 1-5). In fact, a direct correlation between the amount of PCV2 detected in tissues and the severity of microscopic lymphoid lesions in PCV2-SD has been established (Rosell *et al*., 1999).

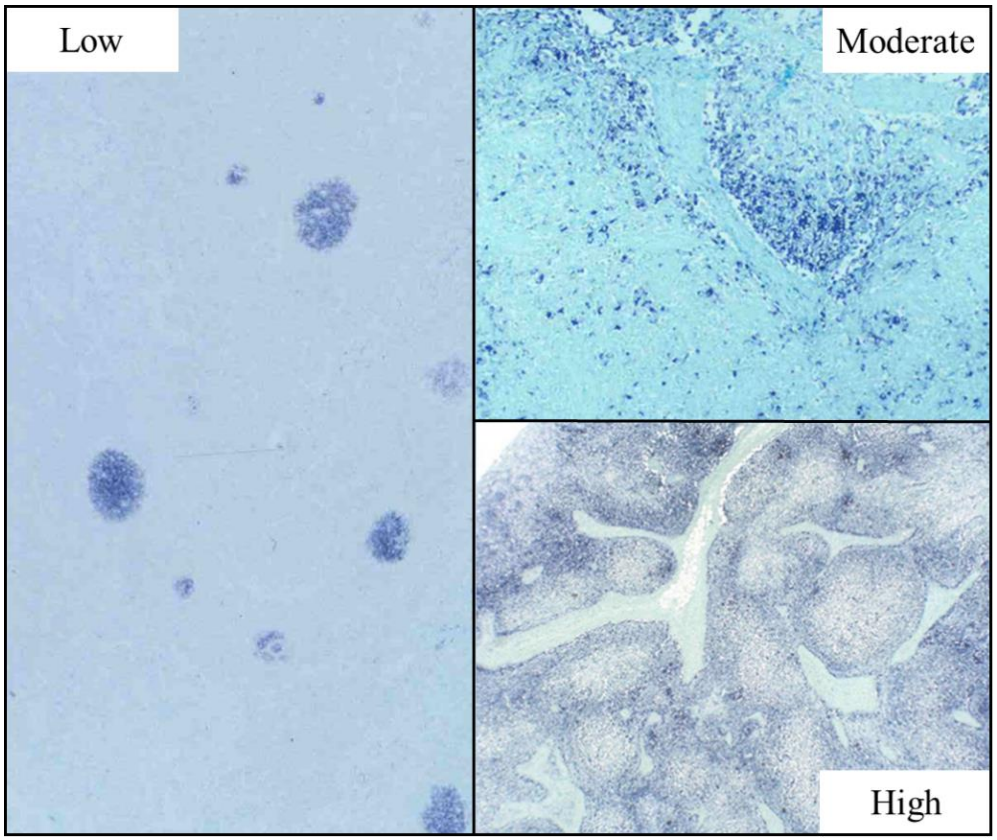

Figure 1-5. *In situ* hybridization (ISH) for the detection of PCV2 in lymph nodes. Low, moderate or high quantity of PCV2 nucleic acid in the cytoplasm of multinucleate giant cells and macrophages (dark stained cells) from animals affected by PCV2-SD. Moderate and high amounts are associated with PCV2-SD. Fast green counterstain. Source: Joaquim Segalés, CReSA-IRTA and UAB, Spain.

In addition, PCV2 load can be quantified by means of qPCR techniques. Indeed, it has been described that viral loads around or higher than 10^7 PCV2 copies/mL are associated with PCV2-SD (Brunborg *et al*., 2004; Olvera *et al*., 2004; Fort *et al*., 2007). Nevertheless, at individual level, it is recommended to combine genome quantification with pathological criteria (Grau-Roma *et al*., 2009).

Several techniques for the detection of antibodies against PCV2 in serum are available (Segalés and Domingo, 2002). However, the use of these techniques is not specifically focused on the diagnosis of PCVD, since most of the seropositive animals are clinically healthy. These tests are frequently used in other scenarios such as 1) epidemiologic studies (Sibila *et al*., 2004), 2) antibody dynamics assessment during experimental infection trials (McKeown *et al*., 2005), 3) vaccine program monitoring (Fachinger *et al*., 2008), and 4) evaluation of the potential interference of maternally derived immunity (MDI) with vaccination (Fort *et al*., 2009b).

1.2.5. Immunity developed upon PCV2 infection

1.2.5.1. Innate immunity and immunomodulatory activity of PCV2

The interaction of PCV2 with the host activates the innate immune response, which represents the first line of recognition and defence against pathogens. The innate immunity has two major functions: 1) a direct effector response and 2) efficient activation of the specific immunity (Chase and Lunney, 2012). The necessary mechanisms to carry out these two functions are the following: 1) quick activation of tissue cells (epithelial cells and resident tissue cells); 2) early production of pro-inflammatory cytokines, mainly interleukin (IL)-1β, IL-8, tumour necrosis factor (TNF)-α, interferon (IFN)-α and IFN-β; and 3) recruitment and activation of innate immune cells (mainly natural killer [NK] cells, neutrophils, macrophages and dendritic cells [DCs]) (Chase and Lunney, 2012; Darwich and Mateu, 2012). DCs and macrophages are first immune cells to encounter pathogens. They internalize and process pathogens, and then present the antigen fragments associated with major histocompatibility complex (MHC) II (in swine are called swine leucocyte antigen II or SLA II) to T helper

(Th) lymphocytes, triggering the adaptive immune response (Chase and Lunney, 2012; Darwich and Mateu, 2012).

DCs are divided in two main types: conventional DC (cDC) with the main role of presenting antigens, and plasmacytoid DC (pDC) that produce type I interferons (IFN-α and IFN-β) (Summerfield *et al*., 2003; Guzylack-Piriou *et al*., 2004). The presence of PCV2 in DCs did not significantly affect the functionality of cDCs; however, the virus exerts an immunomodulatory effect on pDCs (Vincent *et al*., 2005; Vincent *et al*., 2007).

PCV2 causes both immunostimulatory and inhibitory effects on the IFN-α secretion (Hasslung *et al*., 2003; Kekarainen *et al*., 2008a; Baumann *et al*., 2013). The main function of IFN-α (secreted by pDCs) is the induction of an antiviral state. Different studies conducted in cultured pDCs/monocytic cells have demonstrated that PCV2 down-regulate the induction of IFN-α, even in the presence of potent IFN-α stimulators (Vincent *et al*., 2007; Kekarainen *et al*., 2008b). In contrast, *in vivo* infection studies have shown that PCV2 induces IFN-α secretion (Stevenson *et al*., 2006; Fort *et al*., 2009b). This double function of PCV2 could be explained by the characteristics of its genome (Kekarainen and Segalés, 2015). PCV2 genome can modulate cytokine responses, probably via inhibitory/stimulatory CpG motifs interacting with cytosolic or endosomal receptors in the cells (Hasslung *et al*., 2003; Kekarainen *et al*., 2008a). Additionally, the balance between the levels of encapsulated genomic ssDNA (stimulatory effect) and free dsDNA (inhibitory effect) replicative form of PCV2 seems to determine the immunomodulatory features of PCV2 infection (Baumann *et al*., 2013; Kekarainen and Segalés, 2015).

The expression of IL-10 can also be altered by PCV2 infection both *in vitro* and *in vivo*. This cytokine is produced by both innate (macrophages, DCs) and

adaptive cells (B cells and $CD4^+$ and $CD8^+$ lymphocytes) and may inhibit the activity of Th1 cells, macrophages and NK cells (involved in pathogen clearance). Some *in vitro* studies have demonstrated that PCV2 infection in cultured PBMCs, especially in monocyte/DC/macrophage populations, induces IL-10 expression (Darwich *et al*., 2003a; Kekarainen *et al*., 2008a). Moreover, the secretion of this cytokine after PCV2 infection in cultured PBMCs cause the inhibition of IFN-γ, IFN-α and IL-12 stimulated by recall antigen of another virus (Kekarainen *et al*., 2008a). Curiously, in a previously published study (Fort *et al*., 2010), the stimulation of PBMCs with the whole PCV2 virion induced the secretion of IL-10; however, this fact was not observed in PBMCs stimulated with Cap or Rep recombinant proteins. In addition, several *in vivo* studies have shown that IL-10 secretion is closely linked to PCV2-SD for the following reasons: 1) systemic secretion of IL-10 has been associated with animals suffering from PCV2-SD (Sipos *et al*., 2004; Stevenson *et al*., 2006); 2) the levels of IL-10 in the thymus of PCV2-SD affected pigs are increased, and they have been associated with thymic depletion and atrophy (Darwich *et al*., 2003b); and 3) systemic IL-10 levels have been correlated with PCV2 viral load in blood at 21 days post-infection (Darwich *et al*., 2008).

1.2.5.2. Adaptive immunity

1.2.5.2.1. Humoral immune response

Humoral immunity against PCV2 infection has been extensively studied, both in experimental and natural conditions (Kekarainen *et al*., 2010). Moreover, it has been evaluated in foetal stages as well as throughout the productive life of the animals:

- Foetus at 40-70 days of gestation: The antibody transfer from sow to piglet occurs (almost completely) via colostrum (Shibata *et al*., 2006). Therefore, at this gestation stage, there should be no presence of antibodies in the foetuses, since, although they may be infected, they are still unable to mount an immune response (Sánchez *et al*., 2001).
- Foetus at 70-115 days of gestation: In this stage, *in utero* infected foetuses can mount humoral immune responses against PCV2 (Sánchez *et al*., 2001; Saha *et al*., 2010). In consequence, the presence of PCV2 antibodies in aborted foetuses or stillborn piglets is generally considered an evidence of intra-uterine PCV2 infection. Nevertheless, a previous study (Saha *et al*., 2014) has suggested that small amounts of maternal antibodies may leak through the placenta, since low antibody titres where found in foetuses coming with sows with very high antibody titres without evidence of PCV2 infection during gestation. Besides, in that study, antibody levels from colostrum-deprived piglets were also tested, finding a correlation with those obtained in their dams. The results of this study suggested that care needs to be taken when diagnosing an intra-uterine PCV2 infection based solely on antibody detection (Saha *et al*., 2014).
- Post-natal: At farm level, maternally derived antibodies (MDA) decline during the lactation and nursery periods (Rodríguez-Arrioja *et al*., 2002). This passive immunity confers protection to the offspring against disease development although does not prevent early PCV2 infection and viraemia in new-born piglets (McKeown *et al*., 2005; Ostanello *et al*., 2005; Gerber *et al*., 2012). Anyway, the percentage of viraemic new-born piglets found during lactation is usually low (Eddicks *et al*., 2016). Generally, PCV2 viraemia is first detected around 7 dpi, showing the

maximum values at 14-21 dpi (Allan *et al*., 1999; Rovira *et al*., 2002; Opriessnig *et al*., 2008). When pigs are infected, they are able to mount an effective humoral immune response (Rodríguez-Arrioja *et al*., 2002; Larochelle *et al*., 2003; Grau-Roma *et al*., 2009). Under field conditions, this active seroconversion usually occurs at 7-15 weeks of age, depending on the farm, and antibodies may last at least until 28 weeks of age (Rodríguez-Arrioja *et al*., 2002). Neutralizing antibodies (NA) are directly correlated with protection against PCV2 infection. This fact has been demonstrated in several studies in which NA were able to clear the PCV2 from circulation. Indeed, low levels of NA have been associated with increased PCV2 replication and PCV2-SD development (Meerts *et al*., 2006; Fort *et al*., 2007). Curiously enough, one study suggested that differences between PCV2 isolates from different farms at sequence level may cause functional antigenic differences in viral neutralization (Kurtz *et al*., 2014).

1.2.5.2.2. Cellular immune response

The passive transfer of the PCV2-specific cellular immune response to the new-born piglets was demonstrated in one study in which maternally derived colostral lymphocytes were transferred to the offspring (Oh *et al*., 2012).

The active cell-mediated immune response developed upon PCV2 infection is a key element in the protection against PCV2-SD, since anti-PCV2 antibodies are not always fully protective (Rodríguez-Arrioja *et al*., 2002; Sibila *et al*., 2004). The cellular response is commonly measured by the assessment of PCV2 specific IFN-γ-secreting cells (IFN-γ-SCs), which are Th1 cells able to produce IFN-γ upon stimulation with a recall antigen, mainly for the following reasons:

1) levels of PCV2 specific IFN-γ-SCs increase after PCV2 infection and vaccination (Fort *et al*., 2009b; Koinig *et al*., 2015); 2) IFN-γ-SCs are inversely correlated with PCV2 viral loads in serum (Seo *et al*., 2012a, b); and 3) these T cells are specific for both PCV2 Cap and Rep proteins (Fort *et al*., 2010).

On the other hand, PCV2 infection may also induce B and T cell lymphopenia, but only in animals that subsequently develop PCV2-SD. In fact, the depletion of B and memory/activated T lymphocytes has been only reported in pigs suffering from PCV2-SD (Nielsen *et al*., 2003).

1.3. CONTROL AND PREVENTION MEASURES

1.3.1. Non-vaccination methods

Apart from PCV2 vaccination methods, PCVD (mainly PCV2-SD) can be controlled (and prevented) by avoiding all the potential risk factors for disease occurrence mentioned in section 1.2.1.3. These control measures would be included within the following points:

- Management practices: A 20-point plan was proposed (Madec *et al*., 2000) to help producers identifying management practices that can control the disease. Important recommendations include: following all-in/all-out management system with thorough disinfection, limiting animal contact, avoiding mixing of batches and cross-fostering, maintaining appropriate temperature, space and airflow conditions in pens and following recommended anti-parasitic treatments.
- Stimulation of the immune system: Non-specific stimulation of the immune system by different vaccines might potentiate PCV2 replication, triggering the development of PCV2-SD (Krakowka *et al*., 2001;

Kyriakis *et al*., 2002; Krakowka *et al*., 2007). Therefore, vaccine programs on farms should be adapted in order to avoid (when possible) the vaccination of PCV2 viraemic animals.

- Co-infection´s control: These measures should be mainly focused on the control of the concomitant pathogens found in a farm suffering from PCV2-SD. It is recommended to pay major attention to PRRSV, PPV and *Mycoplasma hyopneumoniae*, since they have been considered as significant pathogens worsening the clinical picture (Opriessnig and Halbur, 2012).

- Breeding: Some breeds may be more susceptible to the development of clinical PCVD than others (López-Soria *et al*., 2004; Opriessnig *et al*., 2006; Opriessnig *et al*., 2009b). Therefore, in those farms where PCV2 problems are detected, the genetic line of both sows and boars should be reviewed and changed when necessary.

- Serum therapy: It was utilized by some European practitioners to control PCV2-SD before the availability of commercial vaccines. It consists of injecting convalescent pig serum into naïve piglets by either subcutaneous or intraperitoneal routes (Waddilove and Marco, 2002). However, the effectivity of this method is uncertain, and more importantly, may not be safe (Madec *et al*., 2008).

In conclusion, although nowadays the best method to control PCV2 infection is by means of vaccination, abovementioned measures (with the exception of serum therapy) remain very important control aids and should continue to be applied together with PCV2 vaccination for a better control of the disease.

1.3.2. Autogenous vaccines

Prior to the availability of commercial vaccines, some practitioners from the United States proved to use autogenous vaccines in farms with severe PCV2-SD problems. Autogenous vaccines were prepared from lung or lymphoid tissue homogenates obtained from PCV2-SD pigs and then inactivated with 2% formaldehyde (Opriessnig, 2008). After the application of these vaccines, those practitioners reported marked reduction of mortality and limited to no adverse side effects (Wagner, 2007). These vaccines were subsequently tested under experimental conditions, finding that PCV2 was not sufficiently inactivated by the formalin treatment (Opriessnig, 2008). Therefore, as commented in the serum therapy section, autogenous vaccines also represented a risk in terms of biosecurity and are no longer used since commercial vaccines are available (Blanchard *et al*., 2003; Fenaux *et al*., 2004; Kamstrup *et al*., 2004).

1.3.3. Current commercial PCV2 vaccines and vaccination strategies

1.3.3.1. Types of vaccines

Nowadays, there are several commercial PCV2 vaccines available in the world for the prevention of PCVD in pig herds (Beach and Meng, 2012; Chae, 2012; Afghah *et al*., 2017). In Europe, there are two commercial vaccines licensed to be used in breeding dams and piglets and another two to be used exclusively in piglets. All of them are based on the PCV2a genotype; nevertheless, vaccines have been shown to be effective against the major circulating genotypes worldwide (PCV2a, PCV2b and PCV2d) due to the apparent cross-protection between them (Fort *et al*., 2008; Kurtz *et al*., 2014; Opriessnig *et al*., 2014). The characteristics of these vaccines as well as the recommended vaccination schedules are detailed in Table 1-8. In America, similar vaccine types are also

commercialized by the same pharmaceutical companies. In this case, the name of some of the vaccine products is different, although the design and characteristics of the vaccines from the same company are similar to the European ones (Segalés, 2015). In Asia, the number of available vaccines is greater, especially in China, where at least 16 products are marketed (Zhai *et al*., 2014). Interestingly, there are vaccines based on the three main genotypes (PCV2a, PCV2b and PCV2d) in this country. In addition, among the four vaccines sold in Europe and America, only one of them (Ingelvac CircoFLEX®) is available in China (Zhai *et al*., 2014).

During the last years, the manufacturing companies of Circovac®, Porcilis® PCV and Suvaxyn® PCV have changed due to mergers between companies (with the consequent change of the company name) or to vaccine sales from one company to another. Therefore, the current pharmaceutical company to which each PCV2 vaccine belongs is specified in Table 1-8. In addition, combined vaccines including *Mycoplasma hyopneumoniae* and PCV2 have been marketed (http://www.ema.europa.eu).

Table 1-8. Summary of product characteristics and recommended vaccination strategies from the current commercial PCV2 vaccines in Europe (Source: http://www.ema.europa.eu).

Pharmaceutical company	Product's name	Antigen	Adjuvant	Licensed for	Administration dosage	Vaccination strategy recommended by the manufacturer
	**Circovac®**	Inactivated PCV2a	Light paraffin oil	Gilts	2 mL, IM	One injection, followed 3 to 4 weeks later by a second injection, at least 2 weeks before mating. One further injection must be given, at least 2 weeks before farrowing
				Sows	2 mL, IM	• Primo-vaccination: one injection, followed 3 to 4 weeks later by a second injection, at least 2 weeks before farrowing
						• Re-vaccination: one injection at each gestation, at least 2 to 4 weeks before farrowing
				Pigs	0.5 mL, IM	One injection, from 3 weeks of age onwards
	**Ingelvac CircoFLEX®**	PCV2a Cap protein	Aqueous polymer (carbomer)	Sows	1 mL, IM	One injection, during pregnancy or lactation
				Pigs	1 mL, IM	One injection, from 2 weeks of age onwards
	**Porcilis® PCV***	PCV2a Cap protein	dl-α-tocopheryl acetate + light paraffin oil	Pigs	2 mL, IM	One injection, from 3 weeks of age onwards
	**Suvaxyn® PCV****	Inactivated recombinant PCV1 expressing the PCV2a Cap protein	Sulfolipo-cyclodextrin + squalane	Pigs	2 mL, IM	One injection, from 3 weeks of age onwards

1.3.3.2. Vaccination programs

1.3.3.2.1. Gilts/sows

PCV2 vaccination in gilts/sows is mainly focused on the protection of the offspring by antibody transfer through the colostrum. This strategy has been tested in both experimental and field studies, resulting in prevention of PCV2-SD (Pejsak *et al*., 2010), and decrease of viraemia (Fraile *et al*., 2012b; O'Neill *et al*., 2012; Dvorak *et al*., 2017) and PCV2 tissue load (Opriessnig *et al*., 2010) in their progeny, and improvement of the ADWG in subclinically infected finisher pigs (Kurmann *et al*., 2011). Curiously enough, one field study also showed a reduction of ear necrosis syndrome prevalence in nursery pigs after sow vaccination (Pejsak *et al*., 2011). Despite these facts, it has been demonstrated that sow vaccination does not fully prevent PCV2 vertical transmission (Madson *et al*., 2009a,c; Gerber *et al*., 2012; Hemann *et al*., 2014).

On the other hand, the use of this vaccination strategy may also control PCV2-RD or potentially improve the reproductive parameters, although these issues have been described to a lesser extent:

- Vaccination of gilts during the acclimatization: This strategy avoids the presence of seronegative populations at farm entry. In fact, these populations are a risk factor for the development of the PCV2-RD, since this pathologic condition has typically been reported in new facilities or farms (likely related to seronegative populations) (West *et al*., 1999; Ladekjaer-Mikkelsen *et al*., 2001; O'Connor *et al*., 2001; Brunborg *et al*., 2007; Hansen *et al*., 2010).
- Vaccination of gilts and sows: There is little information in peer-reviewed literature about the influence of gilt/sow immunization on reproductive parameters. In one study conducted in a farm with apparent

PCV2-RD problems (Pejsak *et al*., 2012), long-term vaccination of boars, gilts (pre-mating) and sows (pre-farrowing) caused an improvement of insemination rate, number of live-born and weaned piglets per litter and birth weight. In contrast, in another study carried out in two farms without reproductive problems (PCV2-SI breeding herd), a similar immunization protocol was applied during one reproductive cycle (Kurmann *et al*., 2011). In this last study, no significant differences on reproductive parameters were observed between vaccinated and non-vaccinated sows after vaccine application. Therefore, it seems that vaccination of breeding stock only triggers reproductive benefits in PCV2-RD affected farms.

1.3.3.2.2. Piglets

The active immunization of piglets against PCV2 is a widespread practice in most farms around the world. This strategy is usually applied around weaning (3-4 weeks of age) to protect the piglet from subsequent PCV2 infection (Segalés, 2015).

Efficacy of PCV2 commercial vaccines in piglets has been widely demonstrated under experimental and field conditions (Beach and Meng, 2012; Chae, 2012; Segalés, 2015; Karuppannan and Opriessnig, 2017). Major effects implied reduction of the impact of PCV2-SD by improvement of production parameters, decrease of co-infections, and reduction of PCV2 viraemia and shedding (Cline *et al*., 2008; Fachinger *et al*., 2008; Horlen *et al*., 2008; Kixmoller *et al*., 2008; Desrosiers *et al*., 2009; Segalés *et al*., 2009; Pejsak *et al*., 2010; Martelli *et al*., 2011; Martelli *et al*., 2013; Oh *et al*., 2014). Moreover, most of the pigs from PCV2-SD affected farms but not displaying this condition also suffer from a

PCV2-SI. Furthermore, vaccination against PCV2 has been shown to be economically worthy even in PCV2-SI scenarios (Young *et al*., 2011; Fraile *et al*., 2012a; Alarcón *et al*., 2013; Feng *et al*., 2016).

Several experimental and field studies have been carried out to compare the efficacy of different PCV2 commercial vaccines under the same conditions. These studies have shown benefits in all vaccinated groups, regardless of the vaccine used (Kristensen *et al*., 2011; Lyoo *et al*., 2011; O'Neill *et al*., 2011; Opriessnig *et al*., 2014; Seo *et al*., 2014a).

1.3.3.2.3. Sows and piglets

Several studies have demonstrated greater efficacy of double (sow and piglet) with respect to a single (sow or piglet) vaccination strategy in terms of serological, virological or productive parameters (Opriessnig *et al*., 2010; Pejsak *et al*., 2010; Fraile *et al*., 2012b; Haake *et al*., 2014; Oh *et al*., 2014; Martelli *et al*., 2016).

Vaccination of piglets at weaning (3-4 weeks of age) in the presence of high MDA levels (typically under a sow vaccination scenario) is able to reduce PCV2 viraemia but seems to interfere with the active humoral immune response development elicited by the vaccine (Fort *et al*., 2009b; Fraile *et al*., 2012a,b; Haake *et al*., 2014; Feng *et al*., 2016). However, despite this lower antibody response, most of the pigs are able to overcome this situation, since piglet vaccination in presence of high MDA levels has shown to improve the ADWG in a non-significantly different magnitude as animals vaccinated in presence of low MDA levels (Fraile *et al*., 2012b; Feng *et al*., 2016).

In order to evaluate the possibility of avoid the interference of high MDA with the seroconversion after immunization, two studies were conducted comparing vaccination at weaning and after weaning (Oh *et al*., 2014; Martelli *et al*., 2016). These trials demonstrated that, if sows are previously vaccinated, piglet vaccination at 6-7 weeks of age may offer a higher efficacy in terms of PCV2 infection control than piglet vaccination at weaning (3-4 weeks of age), pointing out to some interference of high MDA levels with the induction of an efficient immune response in the last group (Oh *et al*., 2014; Martelli *et al*., 2016).

1.3.3.3. Immunity conferred by PCV2 vaccination

Commercial vaccines induce, actively or passively, both humoral and cellular immune responses (Fort *et al*., 2009b; Kekarainen *et al*., 2010; Martelli *et al*., 2011; Seo *et al*., 2014b). Several studies have demonstrated that all licensed vaccines trigger a humoral response in terms of total antibodies in immunized sows and/or piglets (Gerber *et al*., 2011; Sibila *et al*., 2013; Segalés, 2015). Nevertheless, humoral protection is linked to the NA, since the induction of NA is inversely correlated with the PCV2 load (Meerts *et al*., 2006; Fort *et al*., 2007; Seo *et al*., 2012a). The amount of NA elicited by each of the four major vaccines available on the market is apparently different (Seo *et al*., 2014b). Moreover, the absence of seroconversion or the presence of low antibody levels after the immunization with some vaccines (mainly subunit vaccines) does not always rule out protection (Fenaux *et al*., 2004; Pérez-Martín *et al*., 2010). In fact, cellular immunity, and in particular IFN-γ-SCs, play a significant role in the immunological protection. This cell-mediated response has also been evaluated under the same conditions for all commercial vaccines, observing a positive induction after application of all vaccine products but, again, with apparent quantitative differences between them (Seo *et al*., 2014b).

In sows, PCV2 immunization has been linked to increased transference of functional PCV2-specific lymphocytes to new-born piglets. Indeed, one single study showed that this transfer played a critical role in protecting new-born piglets after PCV2 challenge at 3 weeks of age (Oh *et al*., 2012).

In piglets, several experimental and field studies have described the development of PCV2-specific IFN-γ-SCs after active immunization with any of the commercial products (Fort *et al*., 2009b; Martelli *et al*., 2011; Fort *et al*., 2012; Borghetti *et al*., 2013; Ferrari *et al*., 2014; Koinig *et al*., 2015).

CHAPTER 2

Hypothesis and Objectives

Since the advent of the first commercial PCV2 vaccine in 2004, numerous studies have shown positive results in terms of efficacy using any commercial vaccine (Beach and Meng, 2012; Chae, 2012; Segalés, 2015; Afghah *et al*., 2017). The application, over the years, of these vaccines to control PCV2-SD problems has drastically reduced the prevalence of this disease (Segalés, 2012). In fact, PCV2-SI is nowadays the most prevalent PCVD, representing the highest economic impact at farm level in comparison to the other PCVD (Alarcón *et al*., 2013). This is the reason for the wide use of these vaccines all over the world (Segalés, 2015). Although the advent of PCV2 vaccines represented a great advance of swine health globally, there are still question marks related with its use under field conditions and in a PCV2-SI context. When this PhD Thesis started, the effect of dam vaccination on reproductive performance as well as on the transfer of maternally derived cellular immunity to the offspring in a PCV2-SI context was not sufficiently documented in the peer-reviewed literature. Moreover, little information was available on whether the commonly used vaccine strategy in piglets (at weaning) gets the best profit in terms of efficacy. On the other hand, oral fluid samples have been shown to be good candidates for the assessment of PCV2 infection (natural or experimental) and seroconversion dynamics (Kim, 2010; Prickett *et al*., 2011; Ramirez *et al*., 2012). However, information on the use of oral fluids for the monitoring of vaccine programs was scarce.

Considering the abovementioned rationales, a PCV2 inactivated vaccine was used in the studies of the present Thesis with the general aim to evaluate the effect of PCV2 vaccination in different PCV2 subclinically scenarios both in breeding and growing pigs. The specific objectives of this PhD Thesis were:

- To assess the potential influence of sow vaccination against PCV2 on reproductive outcomes during two consecutive reproductive cycles (*Chapter 3*).

- To evaluate the effect of sow vaccination against PCV2 on antibody and cytokine levels; and assessment of the same parameters in their colostrum-fed offspring (*Chapter 4*).

- To determine which age for piglet vaccination against PCV2 offers the best serological and virological performance under a no-sow vaccination scenario (*Chapter 5*).

- To assess the value of oral fluid to monitor PCV2 infection and antibody dynamics during different pig vaccination schedules in comparison with serum sampling (*Chapter 5*).

CHAPTER 3

Study I

Exploratory field study on the effect of *Porcine circovirus 2* (PCV2) sow vaccination on serological, virological and reproductive parameters in a PCV2 subclinically infected sow herd

3.1. INTRODUCTION

PCV2 has been shown to be involved in reproductive disorders in sows such as return-to-oestrus, late term abortions and increased number of mummified, stillborn and non-viable piglets at birth (West *et al*., 1999; Ladekjaer-Mikkelsen *et al*., 2001; Brunborg *et al*., 2007; Mateusen *et al*., 2007; Madson *et al*., 2009b; Madson and Opriessnig, 2011), as well as early embryo mortality (Kim *et al*., 2004; Mateusen *et al*., 2007). In this context, PCV2 can be transmitted to the embryos as soon as they get rid of the *zona pellucida* (Mateusen *et al*., 2004), to the foetus through the placenta (Pensaert *et al*., 2004) and to the new-born piglets by colostrum (Shibata *et al*., 2006). Under experimental conditions, some studies have demonstrated that infection of sows by both artificial insemination (AI) with PCV2-spiked semen or PCV2 inoculation can cause foetal infection and reproductive disorders (Park *et al*., 2005; Madson *et al*., 2009b). Moreover, foetal infections and reproductive abnormalities have been reproduced by intrauterine inoculation of foetuses or foetal liquids (Sánchez *et al*., 2001; Johnson *et al*., 2002; Yoon *et al*., 2004). However, at farm level, it is feasible that a proportion of PCV2 effects on reproduction are linked to embryonic death (Mateusen *et al*., 2004; Mateusen *et al*., 2007), which nowadays is still beyond the diagnostic capacity. This overall situation leads to the fact that clinical and noticeable reproductive disease attributed to PCV2 (PCV2-RD), at field level, is infrequent (Pensaert *et al*., 2004; Madson and Opriessnig, 2011; Karuppannan *et al*., 2016).

The benefits of dam vaccination on their progeny have been demonstrated in terms of reduction of PCV2-SD prevalence, viraemia and PCV2 load in tissues (Opriessnig *et al*., 2010; Pejsak *et al*., 2010; Fraile *et al*., 2012b; O'Neill *et al*., 2012). However, this strategy does not provide full protection against PCV2

infection in the offspring, as PCV2 vertical transmission in vaccinated sows can occur (Madson *et al*., 2009a, c; Hemann *et al*., 2014).

In contrast, influence of PCV2 sow vaccination on reproductive parameters has been poorly investigated. As for example, this type of problems has been tackled as a secondary goal and in a very limited number of sows (1-3 sows per group) in some experimental studies (Madson *et al*., 2009a,c; Hemann *et al*., 2014). At farm level, this issue has only been assessed in two peer-reviewed studies: firstly in one farm suffering from PCV2-SD in growing pigs but without evident reproductive problems (PCV2-SI scenario in the sow herd) (Kurmann *et al*., 2011) and, secondly, in one farm with PCV2-SD among weaners and serious reproductive problems in sows (Pejsak *et al*., 2012). Whereas in the first study no significant differences on the main reproductive parameters between treatments were detected, in the second one an improvement of all measured reproductive parameters were recorded. However, information about the effect of this vaccination strategy on reproductive parameters in a farm with neither reproductive nor PCV2-SD problems (scenario resembling a situation of most breeding herds) is missing in the peer-reviewed literature. Thus, the current work was aimed to evaluate the potential effects of sow vaccination against PCV2 on serological (ELISA), virological (qPCR) and reproductive parameters during two consecutive reproductive cycles in a PCV2-SI scenario (PCV2 circulation in the breeding herd but absence of clinical reproductive problems).

3.2. MATERIAL AND METHODS

3.2.1. Farm selection

The study was conducted in a 1,900-sow farm located in Catalonia (Spain). This farm worked with weekly farrowing batches in all-in/all-out management

system. The vaccination program applied by routine included sow immunization against PRRSV, PPV, *Aujeszky's disease virus*, *Swine influenza virus*, *Erysipelothrix rhusiopathiae*, *Escherichia coli* and *Clostridium perfringens*. Piglets were vaccinated against *Mycoplasma hyopneumoniae* and PCV2 at 5 days pre-weaning and at weaning, respectively. Weaning was performed at 3 weeks of age. Moreover, no signs of any major pig diseases were present and herd immunity status against PRRSV was deemed as "positive-stable" (II-A) (Holtkamp *et al*., 2011).

This farm had average reproductive parameter performance in line with the average Spanish national records (www.bdporc.irta.es) and did not suffer from PCVD clinical signs. Prior to the start of the study, PCV2 antibody detection was confirmed (Ingezim Circo IgG 11.PCV.K1®, Ingenasa, Madrid, Spain) in 7 out of 7 (100%) gilts and in 33 out of 38 (87%) sows of different parity that have never been vaccinated before against PCV2 (Figure 3-1). In addition, antibody levels were heterogeneous (ranging from 0.20 to 1.49 ELISA S/P values), with a decreasing value trend in older sows, suggesting circulation of the virus in younger sows.

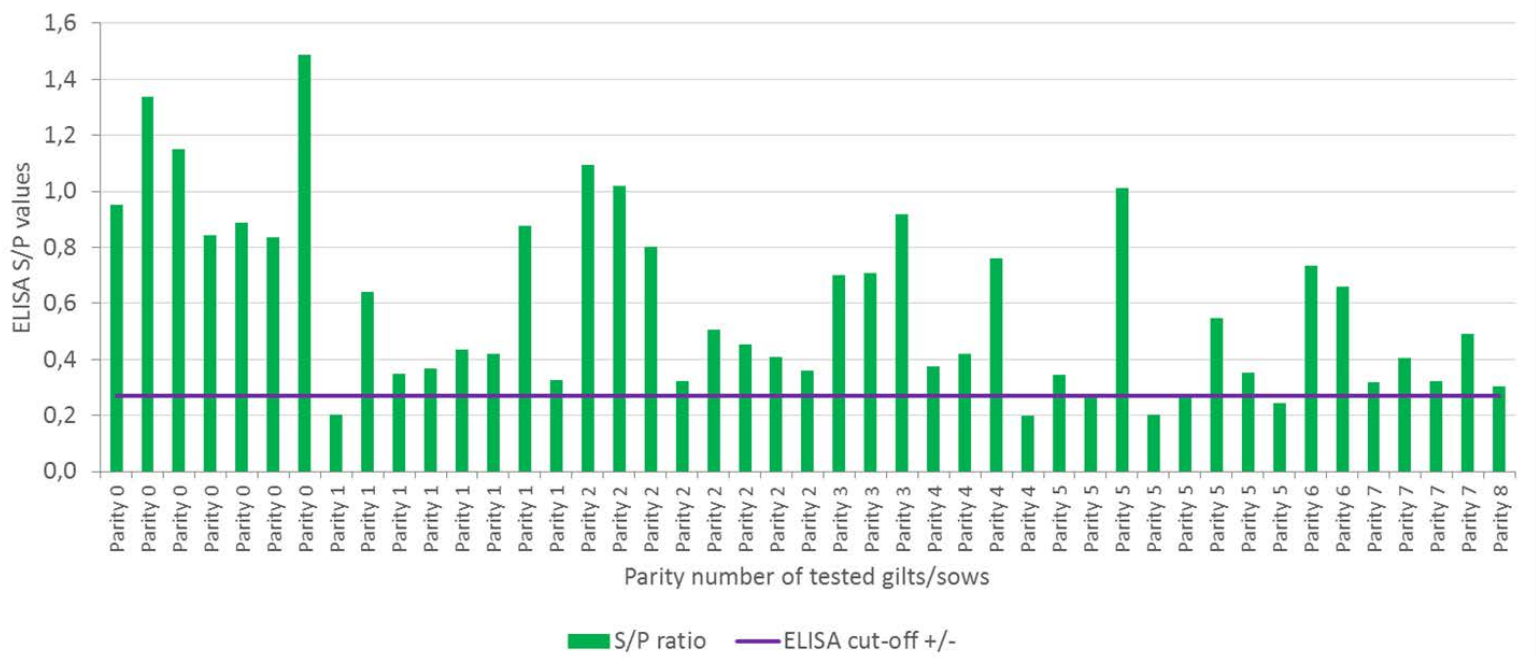

Figure 3-1. Individual PCV2 ELISA S/P results in serum samples from gilts and sows with different parity number prior to the start of the study (farm screening).

3.2.2. Study design

One hundred and ninety-one healthy sows were selected in three consecutive farrowing batches at 6 weeks pre-farrowing. These animals were individually ear-tagged and randomly distributed in two treatment groups (Table 3-1) according to batch number, parity (from 1 to 8) and number of total-born, live-born and weaned piglets in the previous farrowing. The study was conducted in two consecutive reproductive cycles. Sows were vaccinated by intramuscular injection with 2 mL of a commercial inactivated PCV2 vaccine (CIRCOVAC®, Ceva) at time points indicated in Table 3-1. Non-vaccinated sows received 2 ml of phosphate buffer saline (PBS) at the same time points and by the same route. Animals with different treatments were located comingled in the same gestation pens as well as in the same farrowing unit rooms.

Table 3-1. Treatment distribution of sows and vaccination schedule in both gestational cycles.

Population	N*	Group	Treatment	Volume and doses		Number of sows bled
				First gestational cycle	Second gestational cycle	
Sows	94 (75)	V	PCV2 vaccine[a]	2 ml at 6 and 3 weeks pre-farrowing	2 ml at 2 weeks pre-farrowing	48
	97 (75)	NV	PBS			48

[a] Animals were vaccinated with CIRCOVAC®
V= vaccinated, NV= non-vaccinated
*In parentheses, number of sows remaining for the second gestational cycle in each group

Study design is represented in Table 3-2. Any abnormality related to general state, condition of the skin, hair and mucosa, respiratory, digestive and nervous signs, and locomotive problems was registered at different time points. At the end of the first experimental reproductive cycle, as part of routine breeder management, sows with major pathologies (lameness, injuries, etc.) and high parity (older sows) were excluded from the study. In addition, those sows showing return-to-oestrus (non-pregnant ones) in regards their counterparts, were registered and removed from the second cycle. Furthermore, sow mortality was also recorded.

Blood samples from a randomly selected subpopulation of sows (n=48 per treatment group) were taken at different time points throughout the first (at vaccination, farrowing and weaning) and second (at farrowing) gestational cycle (Table 3-2). Once in the laboratory, these samples were allowed clotting, and were centrifuged at 3200 rpm during 20 min at 4°C. All sera were aliquoted and stored at -20ºC until testing.

Table 3-2. Study design schedule.

	First gestational cycle				Second gestational cycle			
Population	**6 weeks pre-farrowing**	**3 weeks pre-farrowing**	**Farrowing**	**Weaning**	**AI**	**2 weeks pre-farrowing**	**Farrowing**	**Weaning**
Sows	CS T BL	CS T	CS BL RP	CS BL		CS T	CS BL RP	CS
Piglets			S CS VI	CS			S CS VI	CS

AI= artificial insemination, CS= clinical signs, T= treatment application, BL= blood sampling, RP= reproductive parameters assessment, S= sex recording, VI= vitality index

In each reproductive cycle, the following reproductive parameters were registered: return-to-oestrus, abortion rate, interval between expected and real farrowing date, weaning-to-fertile mating interval and number of live-born, mummified, stillborn, crushed and weaned piglets per litter.

All piglets issued from the studied sows (first and second gestational cycle) were ear-tagged, gender recorded and assigned a vitality index (VI) (see section 2.3). Cross-fostering was only allowed within sows of the same treatment group and, when possible, within the same parity group (1^{st} parity; 2^{nd}-4^{th} parity; 5^{th}-8^{th} parity). Moreover, from those litters with more than three mummified or stillborn foetuses, heart samples were taken and fixed in 10% neutral-buffered formalin for further histopathological analysis and PCV2 antigen detection by IHC (see section 2.4). All piglets included in the study were monitored for clinical signs and mortality during lactation.

Housing conditions, feeding system, feed characteristics and health management remained consistent along the course of the trial, and were the same for both experimental groups. The present study was approved by the Ethics Committee for Animal Experimentation from the *Universitat Autònoma de Barcelona* and the Animal Experimentation Commission from the local government (*Dpt. de Medi Ambient i Habitatge from the Generalitat de Catalunya*; Reference 5796).

3.2.3. Vitality index scoring method

The vitality of the piglets (within the first 3 hours of life, approximately) was assessed according to a previously published index (Muns *et al*., 2013). The behavioral variables evaluated were the following:

- Udder stimulation (U): 0= no head movements, no emulating udder stimulation movements or no searching behavior within 30 sec; 1= head movements, emulating udder stimulation movements or searching behavior within 30 sec.
- Number of completed circles around the enclosure (NCC): 0= Not able to turn piglet body axis 360° from its initial orientation nor able to walk along the limits of the bucket; 1= Able to turn piglet body axis 360° from its initial orientation or walk along the limits of the bucket once within 30 sec; 2= Able to turn piglet body axis 360° from its initial orientation or walk along the limits of the bucket at least twice within 30 sec.

3.2.4. Histopathology and IHC in heart of foetuses

After fixation by immersion in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were processed routinely for haematoxylin/eosin (HE) staining. These samples were examined under a light microscope for the evaluation of potential microscopic lesions.

For IHC technique, serial sections (3 μm) were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase was blocked with 3% of H_2O_2 (30%) in methanol during 30 min, followed by a 5-min wash in distilled water. Sections were then subjected to proteolytic enzyme digestion with protease type XIV (Sigma, Madrid, Spain) at a concentration of 0.1% in PBS for 8 min at 37°C. After digestion, the sections were washed three times during 5 min in PBS. Then, these sections were incubated for 1 hour at room temperature with 2% of bovine serum albumin (Sigma, Madrid, Spain) in PBS. A specific monoclonal antibody (isotype IgG2a) (Ingenasa, Madrid, Spain) against PCV2 Cap protein was applied at a 1 in 1000 dilution in 2% of bovine

serum albumin in PBS (pH 7.4) to tissue sections and incubated overnight at 4ºC. Sections were then washed three times in PBS for 5 min. After washing, sections were incubated for 45 min with peroxidase-labelled polymer-horseradish peroxidase conjugated to goat anti-mouse immunoglobulins (Envision 1 System-HRP-DAB; Dako, Barcelona, Spain). Staining was completed by incubation with 3,3′diaminobenzidine chromogen solution (Envision 1 System-HRP-DAB; Dako, Barcelona, Spain). Sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted. Samples were examined under a light microscope for the analysis of immunostaining.

3.2.5. DNA extraction and real-time quantitative PCR

DNA was extracted from 200 µl of serum by using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems) following the manufacturer´s instructions. DNA obtained was suspended in 90 µl of elution solution. To quantify PCV2 DNA in serum samples, a real-time qPCR assay (LSI VetMAX™ Porcine Circovirus Type 2-Quantification, Life Technologies) was performed. Each extraction and qPCR plate included negative controls where DNA was substituted for diethylpyrocarbonate (DEPC)-treated water. In addition, each sample reaction had an internal positive control (IPC) to monitor DNA extraction and amplification procedures. Viral concentrations were expressed as the mean $\log_{10}$ PCV2 genome copies/mL ± standard deviation (SD).

3.2.6. Indirect ELISA for detecting anti-PCV2 IgG antibodies

All serum samples were tested by Ingezim Circo IgG 11.PCV.K1® assay (Ingenasa, Madrid, Spain). Optical density (OD) was measured at 450 nm by PowerWave XS reader (BioTek). Mean positive cut-off was established at 0.3

OD (± SD) following kit´s instructions (positive cut-off = OD of negative control + 0.25). ELISA results were expressed as mean S/P ratio (OD of sample/OD of positive control for each ELISA plate) ± SD.

3.2.7. Statistical analyses

Statistical analyses were carried out using SAS v9.4, SAS Institute Inc., Cary, NC, USA. Significance level was set at $p<0.05$. Proportion of animal mortality and exclusion rates between vaccinated and non-vaccinated sows was compared using Fisher exact test and Chi-square test, respectively. Reproductive parameters from all sows included in the study were analyzed and compared between experimental groups within each reproductive cycle (intra-cycle comparison) using the non-parametric Wilcoxon test. In addition, reproductive parameters from those sows farrowing in both reproductive cycles were compared between cycles (inter-cycle comparison) using generalized linear mixed models. Treatment, cycle and their interaction were considered as a fixed effects and sow as a random effect. Percentage of positive qPCR serum samples and mean $\log_{10}$ PCV2 copies/mL among those qPCR positive samples were compared at each sampling point using Fisher exact tests and Wilcoxon tests, respectively. ELISA S/P values were analyzed using a linear mixed model. For the analysis of VI (U and NCC), clinical signs in piglets and piglet mortality during lactation generalized linear mixed models were used. Treatment was considered as a fixed effect and sow as a random effect.

3.3. RESULTS

3.3.1. Clinical signs in sows

No evident clinical signs were observed in any of the sows, but one, throughout the study. The particular affected sow was from the vaccinated group and developed severe lameness at the end of the first reproductive cycle, when it was excluded from the study.

3.3.2. Mortality and exclusion in sows

Mortality and exclusion rates and reason of exclusion are detailed in Table 3-3. In both reproductive cycles, non-vaccinated sows showed numerically higher (but not significant) mortality than the vaccinated ones. Moreover, no statistically significant differences among treatment groups were observed between the two reproductive cycles with regards to the number of excluded females and the exclusion reason.

Table 3-3. Number and percentage (in brackets) of dead and excluded sows and reason of exclusion.

Treatment		V (n=94)	NV (n=97)
Mortality in first reproductive cycle		2 (2.13%)	5 (5.15%)
Exclusion at the end of the first reproductive cycle	Return to oestrus	13 (13.83%)	14 (14.43%)
	Culling. Old sow	2 (2.13%)	1 (1.03%)
	Culling. Lameness	1 (1.06%)	0 (0%)
	Human mistakes (vaccination, misplaced sows…)	1 (1.06%)	2 (2.06%)
	TOTAL excluded animals	17 (18.09%)	17 (17.53%)
Mortality in second reproductive cycle		0 (0%)	2 (2.67%)

No statistically significant differences ($p<0.05$) among experimental groups were observed
V= vaccinated, NV= non-vaccinated

3.3.3. Reproductive parameters

3.3.3.1. Comparison between experimental groups within each reproductive cycle (Intra-cycle comparison)

Main reproductive parameters for each treatment group at first and second reproductive cycles are listed in Table 3-4. Number of live-born piglets at the second cycle was significantly ($p<0.05$) higher (+1.3 piglets/sow) in the

vaccinated group. In addition, a tendency of higher number of weaned piglets (+0.8) in vaccinated sows was observed ($p<0.1$). Moreover, most reproductive parameters (return-to-oestrus [-0.6%], interval between expected and real farrowing date [-0.3 days] and number of mummified [-0.1] piglets per litter) were numerically better for the vaccinated group in the second cycle. Whereas at first reproductive cycle number of stillborn piglets per litter was significantly higher in the vaccinated group, at the second period this parameter was numerically higher in the non-vaccinated one. Besides, number of crushed piglets at birth was numerically higher in the vaccinated group at first and second cycles. Regarding the abortions, there was only one abortion in each cycle for each treatment group. In addition, piglets from vaccinated sows had significantly higher vitality than the ones derived from non-vaccinated sows in both reproductive cycles. Specifically, piglets from vaccinated group showed higher percentage of head movements emulating the udder searching (statistically significant at first cycle) and greater mobility (statistically significant in both cycles) during the 30 tested seconds than the ones from non-vaccinated group.

Table 3-4. Reproductive parameters (mean ± SD) in PCV2 vaccinated (V) and non-vaccinated (NV) sows during both cycles.

	First gestational cycle		Second gestational cycle	
	V (n=94)	**NV (n=97)**	**V (n=75)**	**NV (n=75)**
Interval between expected and real farrowing date (days)	0.45 ± 2.07[a]	0.73 ± 1.36[a]	0.72 ± 1.57[a]	1.00 ± 1.33[a]
Live-born*/litter	14.34 ± 3.24[a]	13.61 ± 3.12[a]	15.42 ± 3.43[a]	14.16 ± 3.45[b]
Crushed/litter	0.53 ± 0.84[a]	0.43 ± 0.66[a]	0.95 ± 1.16[a]	0.73 ± 1.13[a]
Mummified/litter	0.35 ± 0.62[a]	0.44 ± 0.87[a]	0.38 ± 0.70[a]	0.47 ± 0.74[a]
Stillborn/litter	1.45 ± 1.72[a]	0.88 ± 1.36[b]	0.88 ± 1.05[a]	1.24 ± 1.35[a]
Weaned/litter	12.35 ± 2.89[a]	12.06 ± 2.71[a]	12.82 ± 2.75[a]	12.01 ± 3.31[a]
Vitality index (%)**-U				
○ 1 (positive score)	91.91[a]	79.87[b]	97.14[a]	89.35[a]
Vitality index (%)**-NCC				
○ ≥ 1 (positive score)	23.40[a]	10.69[b]	22.86[a]	10.19[b]
Weaning to fertile mating interval (days)	4.51 ± 2.53[a]	4.49 ± 2.67[a]	NA	NA
Abortion (%)	0[a]	1.0[a]	1.3[a]	0[a]

Different letters in superscript mean statistically significant differences ($p<0.05$) among experimental groups within each reproductive cycle. NA =not available
* including crushed piglets at birth
** This index was calculated only from piglets of three or less hours of life

3.3.3.2. Comparison of each experimental group between reproductive cycles (inter-cycle comparison)

In the second cycle, vaccinated sows had significantly different number of live-born (+1.17), crushed (+0.34) and stillborn (-0.55) piglets per litter in comparison to the same sows (n=75) in the first reproductive cycle. Moreover, non-vaccinated sows (n=75) showed at their second cycle significantly higher number of crushed (+0.32) and stillborn (+0.36) piglets per litter than in their previous cycle. No statistically significant differences between cycles were observed for the rest of the parameters.

3.3.4. Clinical signs and mortality in suckling piglets

In the first lactation period, 9 out of 93 (9.7%) and 10 out of 96 (10.4%) litters from vaccinated and non-vaccinated sows, respectively, evidenced diarrhoea. Besides, 65 out of 1333 (4.9%) and 53 out of 1307 (4.1%) piglets from vaccinated and non-vaccinated sows, respectively, showed neurological signs clinically attributed to *Streptoccocus suis (S. suis)* infection. In the second suckling period, 8 out of 74 (10.8%) and 9 out of 75 (12%) litters from vaccinated and non-vaccinated sows, respectively, suffered from diarrhoea. In addition, prevalence of *S.suis*-like infections was reduced, recording only 3 out of 1141 (0.3%) and 2 out of 1062 (0.2%) clinical cases in piglets from vaccinated and non-vaccinated sows, respectively. No statistically significant differences between treatments in terms of diarrhoea and neurological signs were observed for any of the two lactation periods.

Piglet mortality rate (including crushed piglets around birth) in lactation was 13.9% and 16.8% for piglets from vaccinated sows at first and second cycles, respectively. Similarly, pre-weaning mortality rate in piglets from non-

vaccinated sows was 11.4% and 15.2% at first and second suckling periods, respectively. However, no statistically significant differences among treatment groups were observed in any of the two lactation periods.

3.3.5. Microscopic evaluation and PCV2 antigen detection in foetal heart tissues

Although, 14 litters (7.33%) from the first farrowing cycle had more than three mummified or stillborn piglets, only 2 of them were sampled. On the contrary, at second farrowing cycle, foetal heart samples from all the litters (n=11, 7.33%) presenting high number of mummified or stillborn piglets were taken and evaluated. No microscopic lesions associated to PCV2 infection or PCV2 antigen were observed in myocardium of mummified or stillborn piglets from all the tested litters.

3.3.6. Quantification of PCV2 DNA in sow serum samples

All vaccinated sows were qPCR negative (48 out of 48) throughout the study, whilst 2 out of 48 (4.17%) non-vaccinated sows were qPCR positive (mean viral load: 4.15 $\log_{10}$ PCV2 copies/mL) at farrowing sampling of the first reproductive cycle. No statistically significant differences between treatment groups were observed.

3.3.7. Anti-PCV2 IgG antibody levels in sow serum samples

The course of antibodies against PCV2 for sows of the two treatment groups is shown in Figure 3-2. From 6 weeks before farrowing to sampling at delivering of first gestational cycle, vaccinated group showed an increase of ELISA S/P

values, resulting in significantly higher (p<0.05) antibody levels compared to the ones from the non-vaccinated group at farrowing and weaning. In the second gestational cycle, ELISA S/P values from vaccinated sows increased again, reaching the maximum difference with the antibody levels from the non-vaccinated counterparts.

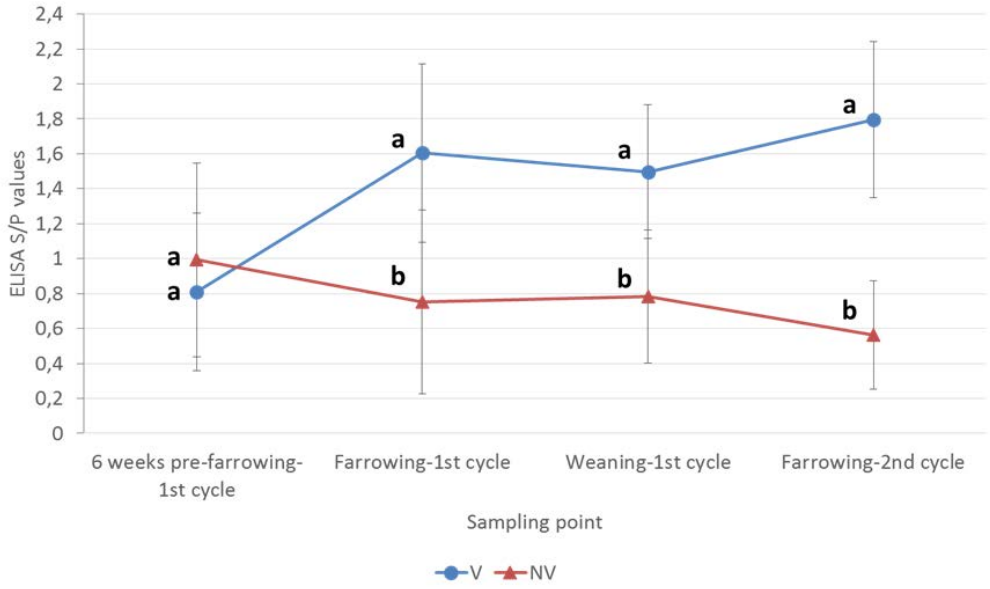

Figure 3-2. PCV2 ELISA S/P results (mean±SD) in serum samples taken from the sows included in the study at different time points. Different letters in superscript mean statistically significant differences (p<0.05) among experimental groups at each sampling point.

3.4. DISCUSSION

The objective of the present study was to evaluate the potential effect of sow PCV2 vaccination in a PCV2 subclinically infected breeding herd (PCV2

circulation but absence of overt reproductive problems). The supporting evidence of a subclinically infection was the presence of seropositive gilts and sows before starting the trial together with the low percentage of viraemic animals (less than 5%) detected within the studied sow population. This low prevalence would resemble the situation of other PCV2-SI farms (Sibila *et al*., 2013; Feng *et al*., 2014; Eddicks *et al*., 2016). In parallel, in order to have additional information about the PCV2 infection status at the time when the study was carried out, blood samples from 12 gilts at acclimatization were taken and tested by qPCR. Indeed, PCV2 DNA was detected in 2 out of those 12 gilts (16.7%, data not shown), corroborating the PCV2-SI in the studied scenario.

Three criteria have to be fulfilled to diagnose a clinical case of PCV2-RD during late gestation (Madson and Opriessnig, 2011): 1) presence of clinical signs associated to late reproductive disorders (abortions, increased number of mummified, stillborn piglets at birth, etc.), 2) microscopic lesions in foetal heart or lymphoid tissues, and 3) detection of PCV2 antigen or DNA in those foetal tissues. In addition, return-to-oestrus problems have also been associated to PCV2-RD at early gestation (Segalés, 2012). The negative results of histopathology and IHC in all tested foetuses from litters with high number of mummies and stillbirths indicated that these findings were not apparently related to PCV2. Therefore, as expected, the present farm was not suffering from PCV2-RD (late reproductive failures or return-to-oestrus), since the average of all reproductive parameters were within normal ranges according to the Spanish national records.

In the present farm conditions, the PCV2 vaccination strategy applied (primo-immunization at 6 and 3 weeks pre-partum and a booster at 2 weeks before the subsequent farrowing) led to a significantly higher antibody response throughout the study period with regard to their non-vaccinated counterparts. This fact

tallies with other studies where sow vaccination before mating or farrowing elicited a high antibody response (Sibila *et al*., 2013), including NA (Gerber *et al*., 2011), in serum. Besides, the booster vaccination at the second cycle resulted in higher antibody levels than the ones observed after first cycle vaccination, suggesting greater protection for both sows and piglets. At reproductive level, sows immunized with PCV2 vaccine showed a significantly ($p<0.05$) higher number of live-born piglets and tend ($p<0.1$) to have higher number of weaned piglets per litter at the second gestational cycle. In this study, sow vaccination at first cycle was applied at a relatively late time during pregnancy when the litter size is already established; therefore, the potential impact of PCV2 vaccination on litter size was only expected after second cycle vaccination. This effect on reproductive parameters is in line with those observed by Pejsak et al. (Pejsak *et al*., 2012), but in disagreement with the ones reported by Kurmann et al. (Kurmann *et al*., 2011). The first trial (Pejsak *et al*., 2012) was conducted in a farm with important reproductive problems (most likely related to PCV2-RD) and sporadic PCV2-SD cases. In that study, the application of a 3-year PCV2 vaccination in boars, gilts (at acclimatization) and sows (before farrowing) resulted in the improvement of all measured reproductive parameters (insemination rate, number of live-born and weaned piglets per litter and birth weight). These positive effects were more evident after several months of vaccine application, resembling the findings of the present study. On the contrary, in the other study (Kurmann *et al*., 2011), the application of PCV2 vaccine at 4 and 2 weeks before AI and 4 weeks pre-partum during 14 months in two farms with a history of recurrent PCV2-SD in growing pigs but with no apparent reproductive problems in sows did not culminate in better reproductive parameters. Therefore, to the authors' knowledge, the current study represents the first approach in the peer-reviewed literature to show the potential benefits of PCV2 sow vaccination on

reproductive parameters in a subclinically infected sow herd. It must be kept in mind, however, that reproductive performance may be influenced by other factors at farm level (Koketsu *et al*., 2017); in consequence, although the number of studied sows is relatively high, the present study should be considered of exploratory nature and a higher number of sows and production cycles would be needed to validate obtained results.

Curiously enough, at the first farrowing post-vaccination, the number of stillborns per litter was significantly higher in the vaccinated group. The cause of this result is unknown, as there was no evidence of any factor that could adversely affect this parameter. Nevertheless, at the second gestational cycle this situation was reversed, since vaccinated and non-vaccinated groups significantly reduced and increased the number of stillborn piglets, respectively. Generally, 10 to 15% of piglets are born dead in pig farms (Baxter *et al*., 2008); therefore, the stillborn rate reported in the present study falls within regular values in both reproductive cycles. This parameter might be related to hypoxia during farrowing since the number of stillbirths increases in cases of high litter size, prolonged farrowing time and high birth weight (Canario *et al*., 2006; Wittenburg *et al*., 2011). Besides, stillbirth can be associated with other factors such as environmental temperature, sow parity, farrowing induction, infectious diseases, mycotoxins and uterine capacity (Vanderhaeghe *et al*., 2013).

Moreover, piglets from vaccinated sows had higher vitality (in the first three hours of life) than the animals issued from non-vaccinated ones in both reproductive cycles. This finding was subjectively reported in a farm with major reproductive problems, most probably related to PCV2, using the same vaccine than the present study (Pejsak *et al*., 2012). Besides, since VI was higher in piglets coming from vaccinated sows, one would expect to have less crushed piglets in that group. However, vaccinated sows showed higher (but non-

significantly) number of crushed piglets. Most probably, this was a fortuitous event and not associated to piglet vitality, since crushing is related to other factors such as sow behaviour (depends of the sow genetics), design of farrowing crates and management practices (Kirkden *et al*., 2013).

In conclusion, after two reproductive cycles, sows vaccinated against PCV2 experienced significantly higher antibody levels, prolificacy and vitality of their offspring. However, as reproductive performance may be influenced by multiple factors, the present study represents a further investigation of the PCV2 sow vaccination effects on reproductive parameters under a PCV2 subclinical infection scenario.

CHAPTER 4

Study II

Comparison of cytokine profiles in peripheral blood mononuclear cells between piglets born from *Porcine circovirus 2* vaccinated and non-vaccinated sows

4.1. INTRODUCTION

PCV2 vaccination elicits both humoral and cellular immune responses against PCV2 (Fort *et al*., 2009b; Martelli *et al*., 2011; Seo *et al*., 2014b). In sows, the goal of vaccination before farrowing, is the protection of the offspring by means of maternal immunity transfer through colostrum. Several studies have shown the maternal antibody transfer from sows to piglets (Kurmann *et al*., 2011; Fraile *et al*., 2012b; Sibila *et al*., 2013; Oh *et al*., 2014; Dvorak *et al*., 2017). Nevertheless, the passive transfer of the PCV2-specific cellular immune response to the offspring has hardly been investigated. To our knowledge, only one peer-reviewed study has demonstrated that maternally derived colostral lymphocytes from PCV2 immunized sows may be transferred to the progeny (Oh *et al*., 2012). In that study, the participation of these lymphocytes in the adaptive immune response was measured by *in vivo* delayed type hypersensitivity (DTH) responses, *in vitro* lymphocyte proliferation and the presence of PCV2-specific IFN-γ-SCs in new-born piglets. However, in this context, information on cytokine profiles in piglets after colostrum intake and the influence of sow vaccination on these profiles is not available. Therefore, the objective of the present work was to assess the effect of sow vaccination against PCV2 on humoral and cell-mediated immunity in sows and their offspring.

4.2. MATERIAL AND METHODS

4.2.1. Farm selection

The study was conducted in a commercial farm with 1,060 sows (Large White x Landrace) located in Spain. This farm was a two-site herd with all-in/all-out management and 4-week batch farrowing system. PCV2 vaccination in sows and piglets had never been applied in the studied herd. Sows were routinely

vaccinated against *Porcine reproductive and respiratory syndrome virus*, *Aujeszky's disease virus*, *Swine influenza virus*, *Porcine parvovirus*, *Erysipelothrix rhusiopathiae*, *Escherichia coli* and *Clostridium perfringens*. Piglets were vaccinated against *Mycoplasma hyopneumoniae* at 2 days pre-weaning. Weaning was performed at 3 weeks of age. Besides, PCVD clinical problems were never detected in this herd and the average of the reproductive parameters was within the Spanish one (www.bdporc.irta.es). Furthermore, no clinical signs of any other significant diseases were observed.

In order to evaluate the PCV2 antibody levels in the sow herd, blood samples from 30 sows from parity 2nd to 7th were taken and processed by ELISA (Ingezim Circo IgG 11.PCV.K1®, Ingenasa, Madrid, Spain). PCV2 antibodies were detected in 29 out of 30 (96.7%) sows, observing the highest ELISA S/P values in 5th parity sows (Figure 4-1).

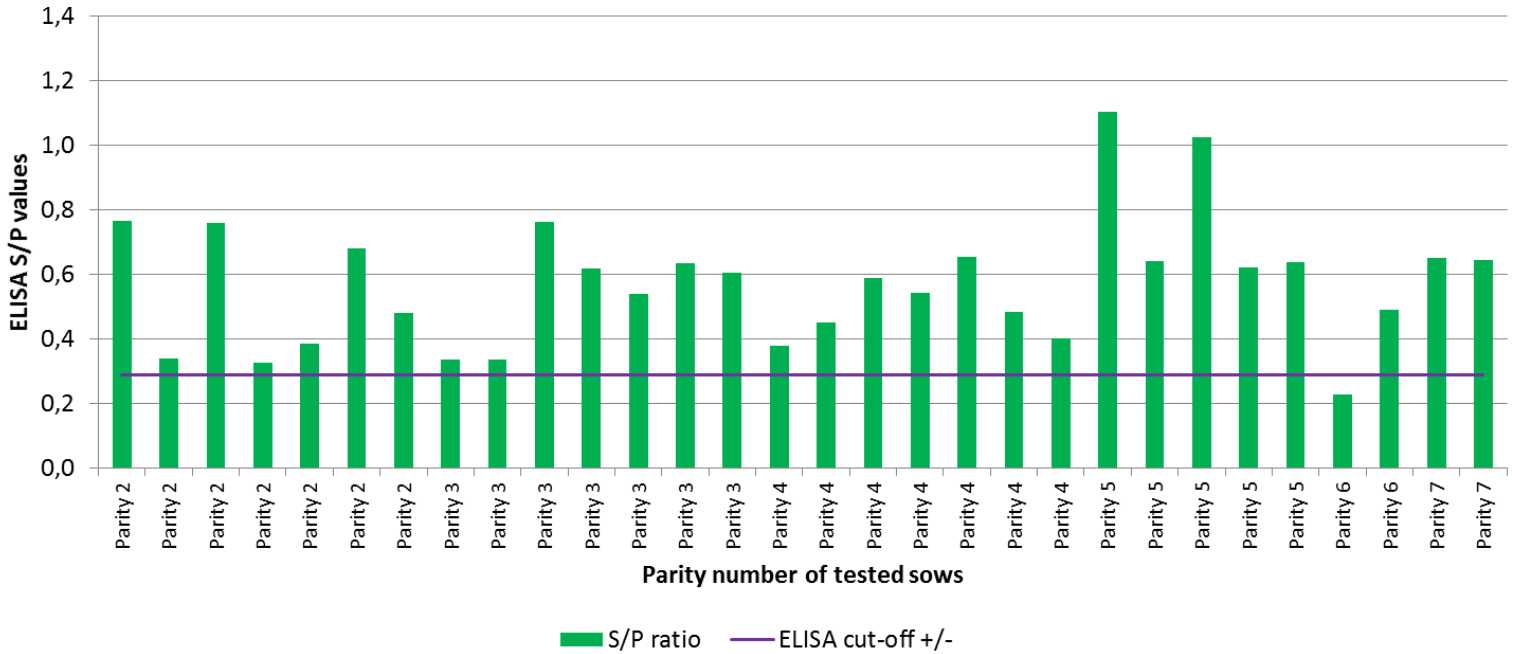

Figure 4-1. Individual PCV2 ELISA S/P results in serum samples from sows with different parity number prior to the start of the study (farm screening).

4.2.2. Study design

Fifteen healthy sows (parity 3-4th) with the same expected farrowing day were selected from the screened farm at 7 weeks pre-farrowing. These animals were individually ear-tagged and bled. Blood samples were tested by conventional PCV2 PCR (Quintana *et al*., 2002) and ELISA (Ingezim Circo IgG 11.PCV.K1®). All sows were PCR negative and showed low-medium (ranging from 0.27 to 0.85) ELISA S/P values. At 6 weeks pre-farrowing, sows were randomly distributed in two treatment groups according to S/P values. Seven sows were vaccinated by intramuscular injection with 2 mL of a commercial inactivated PCV2 vaccine (CIRCOVAC®, Ceva) at 6 and 3 weeks pre-farrowing. In parallel, eight non-vaccinated sows received 2 mL of PBS at the same time points and by the same route. Animals with different treatments were located mixed in the same gestation pens as well as in the same farrowing unit rooms. In sows, blood samples were taken in vacuum tubes by jugular venepuncture at 6 weeks pre-farrowing and at the farrowing week (Table 4-1).

Table 4-1. Study design.

Population	**Sampling points**				
	7 weeks pre-farrowing	**6 weeks pre-farrowing**	**3 weeks pre-farrowing**	**Farrowing**	**48-72 hours after farrowing**
Sows	Clinical signs	Clinical signs	Clinical signs	Clinical signs	Clinical signs
		Treatment	Treatment		
	Blood sampling*	Blood sampling†		Blood sampling*†	
Piglets				Clinical signs	Clinical signs
					Blood sampling*†

*Blood in tubes without anticoagulant; †Blood in tubes with heparin

At birth, all piglets from litters of studied sows were ear-tagged and registered. Cross-fostering was not allowed for the sows included in the study. At 48-72 hours after birth, blood samples from six healthy and medium-sized piglets per litter were taken in tubes without anticoagulant (n=90). In addition, from two of these six piglets selected per litter, blood samples were also taken in heparinized vacuum tubes (n=30). Once in the laboratory, blood samples in heparin tubes were immediately processed to obtain PBMCs, while the ones in tubes without anticoagulant were centrifuged at 750 g during 20 min to extract the sera. Sera were aliquoted and stored at -20ºC until testing.

Any abnormality related to general state, condition of the skin, hair and mucosa, respiratory, digestive and nervous signs, and locomotive problems was registered at different time points (Table 4-1) in both sows and piglets. Housing conditions, feeding system, feed characteristics and health management remained consistent along the course of the trial, and were the same for both experimental groups. The present study was approved by the Ethics Committee for Animal Experimentation from the *Universitat Autònoma de Barcelona* and the Animal Experimentation Commission from the local government (*Dpt. de Medi Ambient i Habitatge* from the *Generalitat de Catalunya*; Reference 9402).

4.2.3. DNA extraction and conventional PCR

DNA was extracted from 200 µL of serum by using the MagMAX^{TM} Pathogen RNA/DNA Kit (Applied Biosystems) following the manufacturer´s instructions. DNA obtained was suspended in 90 µL of elution solution. Then, PCV2 genome was detected by standard PCR (Quintana *et al*., 2002). Each extraction and PCR plate included negative and positive controls, where samples were substituted for DEPC-treated water or known PCV2 infected sample, respectively.

4.2.4. Indirect ELISA for measuring anti-PCV2 IgG antibodies

All serum samples were tested by Ingezim Circo IgG 11.PCV.K1® assay (Ingenasa, Madrid, Spain). OD was measured at 450 nm by PowerWave XS reader (BioTek). Cut-off was established at 0.3 OD (±SD) following kit´s instructions. ELISA results were expressed as mean S/P ratio (OD of sample/OD of positive control for each ELISA plate) ± SD.

4.2.5. Peripheral blood mononuclear cells (PBMCs) isolation and stimulation

PBMCs were isolated from blood collected in heparinized tubes by density gradient centrifugation using Histopaque® 1.077 (Sigma, Madrid, Spain). PBMCs were washed and suspended in complete RPMI-1640 (Lonza, Barcelona, Spain) (cRPMI) plus 10% foetal bovine serum (FBS) (Sigma, Madrid, Spain); cell viability was assessed with Trypan blue staining. Then, PBMCs were seeded into 96-well plates (1 x 10^6 cells/well) and incubated with baculovirus-expressed PCV2 Cap protein (final concentration per well: 0.6 µg/mL); phytohemagglutinin (Sigma, Madrid, Spain) (final concentration per well: 10 µg/mL) as a positive control; or cRPMI plus 10% FBS as a negative control for 24 h at 37°C in a 5% humidified CO_2 atmosphere. After incubation, plates were centrifuged and cell culture supernatants were collected and stored at -80ºC until further examination.

4.2.6. Multiplex immunoassay for the quantification of cytokines

PBMC supernatant samples were analysed using ProcartaPlex Porcine Cytokine & Chemokine Panel 1 (Affymetrix, eBioscience, Vienna, Austria) according to the manufacturer's instructions. This multiplex immunoassay uses Luminex®

xMAP technology for the quantification of 9 cytokines: IFN-α, IFN-γ, IL-12p40, TNF-α, IL-1β, IL-8, IL-4, IL-6 and IL-10. The plates were read by a MAGPIX® analyser (Luminex Corporation) and the cytokine levels were determined according to standard curves using xPONENT® 4.2 software (Luminex Corporation). Then, for the final calculation of PCV2-specific cytokine secretion (pg/mL), cytokine levels in supernatants from PBMCs with medium (background) were subtracted from cytokine levels in supernatants from PBMCs stimulated with PCV2 Cap protein.

4.2.7. Statistical analyses

Statistical analyses were carried out using StatsDirect v3.1.1. Kruskal–Wallis test was used for comparisons of ELISA S/P values and cytokine levels between groups and between sampling points. Significance level was set at $p \leq 0.05$.

4.3. RESULTS

4.3.1. Clinical signs in sows and piglets

No evident clinical signs were observed in sows or piglets throughout the study.

4.3.2. Detection of PCV2 DNA in serum samples from sows and piglets

All sows (15 out of 15) and piglets (90 out of 90) were PCR negative during all the study duration.

4.3.3. Anti-PCV2 IgG antibody levels in sow and piglet serum samples

Mean S/P levels (± SD) in sows and their offspring for both treatment groups are represented in Figure 4-2. From 7 weeks before farrowing to farrowing week, vaccinated sows showed an increase of ELISA S/P values, resulting in significantly higher (p<0.05) antibody levels compared to the ones from the non-vaccinated sows at farrowing. Moreover, piglets from vaccinated sows also had significantly higher S/P values than the ones from non-vaccinated counterparts at 48-72 hours of life.

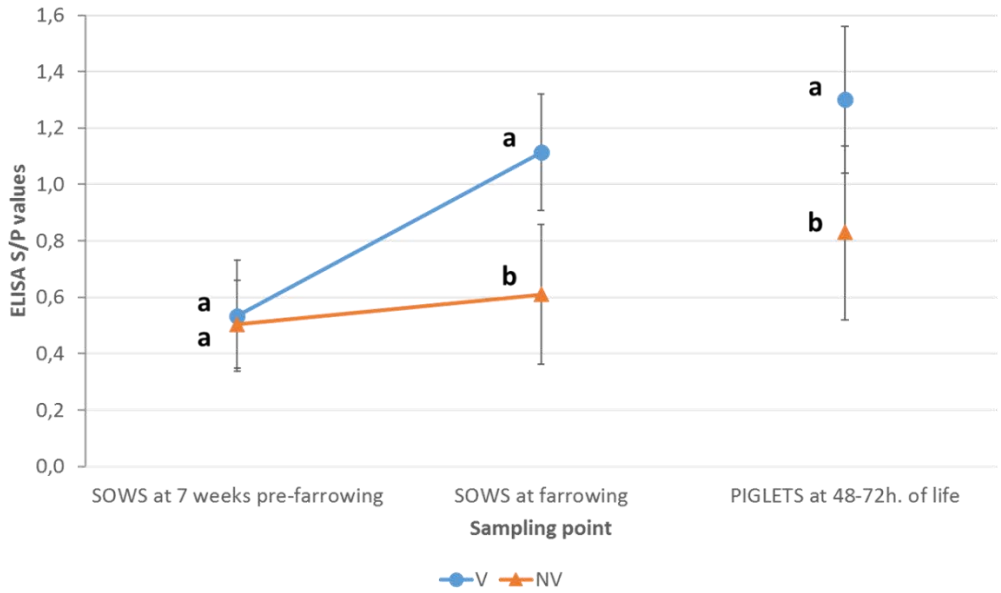

Figure 4-2. PCV2 ELISA S/P results (mean±SD) in serum samples taken from the sows included in the study and their offspring at different time points. Different letters in superscript mean statistically significant differences among experimental groups at each sampling point (a>b; p<0.05).

4.3.4. Cytokine levels

4.3.4.1. PBMC supernatant samples in sows

PCV2-specific cytokine concentrations for sows of the two treatment groups are shown in Figure 4-3. No statistically significant differences were observed when comparing vaccinated and non-vaccinated groups at each sampling point (pre- and post- treatment injection) in any of the tested cytokines.

4.3.4.2. PBMC supernatant samples in piglets

PCV2-specific cytokine values in piglets at 48-72 hours after birth are summarized in Figure 4-4. Piglets born from vaccinated sows had significantly ($p \leq 0.05$) higher levels of IFN-α, IFN-γ, TNF-α and IL-1β than the ones from control group. Regarding the IL-8, very high values close to the upper detection limit of the technique were found in both groups (without significant differences). In the rest of the cytokines (IL-12p40, IL-4, IL-6 and IL-10), no significant differences were found between groups.

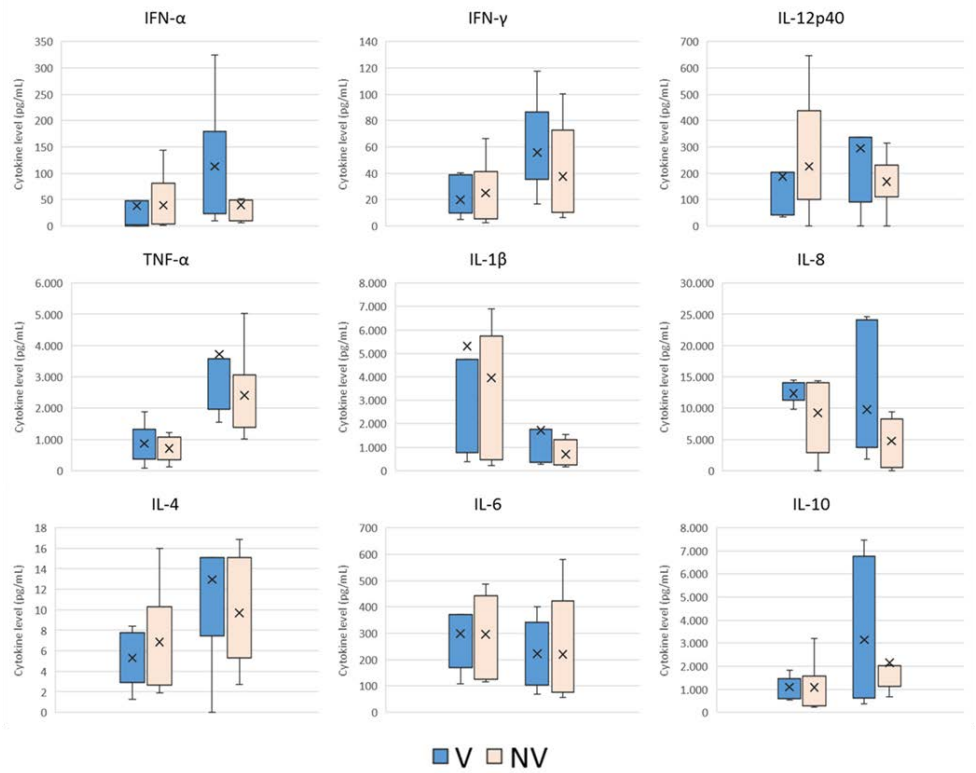

Figure 4-3. PCV2-specific cytokine secretion (pg/mL) in PBMC supernatant samples from sows. In each graphic, the two boxes from the left correspond to the first sampling point (6 weeks pre-farrowing); the two boxes from the right correspond to the second sampling point (farrowing). The "x" symbol indicates the mean. Outliers were considered for the statistical analysis but are not represented in this graph. No statistically significant differences were observed among vaccinated (V) and non-vaccinated (NV) sows at each time point

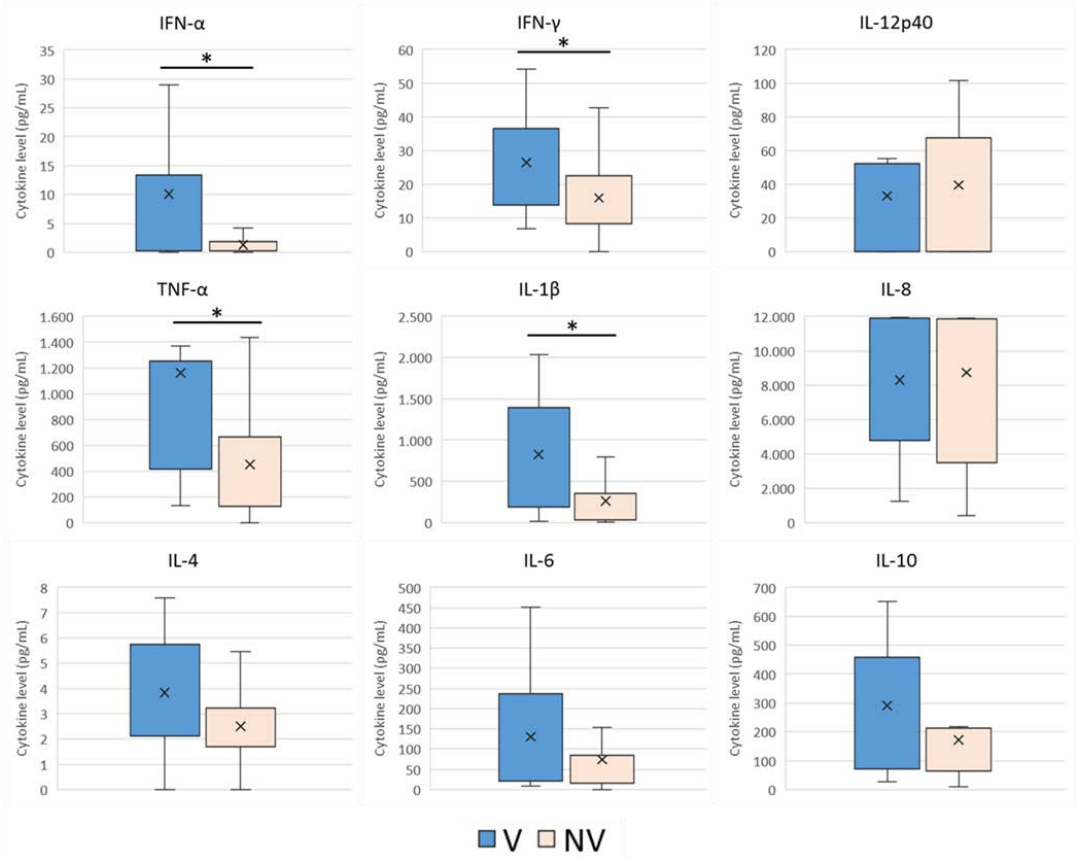

Figure 4-4. PCV2-specific cytokine secretion (pg/mL) in PBMC supernatant samples from piglets at 48-72 hours after birth. The "x" symbol indicates the mean. Outliers were considered for the statistical analysis but are not represented in this graph. *Statistically significant differences ($p \leq 0.05$) among piglets born from vaccinated (V) and non-vaccinated (NV) sows.

4.4. DISCUSSION

The main goal of the present work was to describe the cytokine profiles in piglets born from vaccinated sows in comparison to the ones from unvaccinated sows. In this context, in the present study, piglets from vaccinated sows had significantly higher levels of IFN-γ, suggesting that these animals had memory T cells able to produce IFN-γ upon stimulation with a PCV2 antigen. These findings were correlated with a previously study (Oh *et al*., 2012), where significantly higher levels of IFN-γ-SCs were observed in piglets from vaccinated sows with regard to the ones from unvaccinated sows after colostrum ingestion. The assessment of IFN-γ-SCs is commonly used to measure the cell-mediated immunity conferred by immunization; in fact, PCV2 vaccination induces a long-lasting immunity sustained by memory T cells and IFN-γ secreting cells that might participate in the prevention of PCV2 infection (Ferrari *et al*., 2014). However, to the authors' knowledge, information about general cytokine profiles in piglets after colostrum ingestion is missing in the peer-reviewed literature.

In the present study, piglets from vaccinated sows also had significantly higher levels of TNF-α, IFN-α and IL-1β. Especially relevant is the case of TNF-α, since the production of TNF-α simultaneously with that of IFN-γ by T cells after PCV2 vaccination has been potentially correlated with protection (Koinig *et al*., 2015). In that study, the induction of PCV2-specific antibodies after PCV2 piglet vaccination was only observed in five out of 12 animals. However, at this time point, all vaccinated pigs showed IFN-γ/TNF-α co-producing T cells and all vaccinated piglets were fully protected against viraemia after subsequent challenge (Koinig *et al*., 2015). Regarding the other two cytokines (IFN-α and IL-1β), these are not linked to memory T lymphocytes, since they are produced by other cell types (mainly macrophages and dendritic cells) primarily involved

in innate immunity response (Chase and Lunney, 2012). Therefore, these significant differences observed between groups could be due to differences in the proportion of cell populations in PBMCs from each group, although these cellular subpopulations have not been tested in the present trial.

On the other hand, the same cytokine profiles were tested in sows before and after treatment application. The cell-mediated immune response after PCV2 vaccination has been minimally investigated in sows. In a previously published trial (Oh *et al*., 2012), vaccinated sows showed an increase in the proportions of T lymphocytes ($CD4^+$, $CD8^+$ and $CD4^+CD8^+$) at 1 day post-partum, attributing these changes to PCV2 vaccination. This higher proportion of immunological T cells in vaccinated sows might be related to a higher excretion of cytokines linked to memory response; however, in the present study, no statistically significant differences in any of the tested cytokines were detected between sow groups.

In order to complement the cellular immunity results, active (sows) and passive (piglets) humoral immunity was also evaluated in this study. In sows, PCV2 vaccination twice before farrowing produced an increase of S/P values in comparison to the non-vaccinated counterparts. This antibody response after immunization was observed in other studies when sows were vaccinated before mating or farrowing (Gerber *et al*., 2011; Sibila *et al*., 2013). In this way, in the present work, vaccinated sows had higher levels of antibodies in blood at farrowing. This fact triggered a greater transfer of maternal antibodies to the piglets, which were evident after colostrum ingestion, as was also detected in an earlier study (Kurmann *et al*., 2011).

In conclusion, PCV2 vaccination at 6 and 3 weeks pre-farrowing elicited high antibody values in sows at farrowing and in their offspring. Moreover, piglets

from vaccinated sows had significantly higher levels of cytokines potentially linked to Th1 memory response (IFN-γ and TNF-α), suggesting that this vaccination strategy may confer PCV2 specific cell-mediated passive immunity to the progeny.

CHAPTER 5

Study III

Evaluation of natural *Porcine circovirus 2* (PCV2) subclinical infection and seroconversion dynamics in piglets vaccinated at different ages

5.1. INTRODUCTION

PCV2 commercial vaccines have been shown to be effective in terms of reduction of the PCV2-SD and co-infection occurrence, improvement of production parameters and decrease of PCV2 load (Segalés, 2015). Indeed, the use of these vaccines in animals subclinically infected by PCV2 also showed a significant effect, mainly measured on ADWG improvement (Young *et al*., 2011; Fraile *et al*., 2012a; Alarcón *et al*., 2013; Feng *et al*., 2016).

The most common age of piglet vaccination against PCV2 is at 3-4 weeks of age (around weaning). However, when a sow and piglet vaccination strategy is planned, a delayed piglet vaccination should be considered in order to achieve higher vaccine efficacy (Oh *et al*., 2014; Martelli *et al*., 2016). Under no vaccinated sow scenario, little information is available whether the 3-4 week vaccination-age offers the best profit. Although PCV2 vaccines are routinely used in most of the worldwide porcine production systems, peer-reviewed studies comparing the efficacy of PCV2 vaccination at different ages are scarce in experimental (O'Neill *et al*., 2011; Oh *et al*., 2014) and, particularly, under field conditions (Cline *et al*., 2008).

Serum is the most commonly used sample to assess PCV2 antibody and genome detection (Segalés, 2012). However, blood sampling is an individual and invasive method. Oral fluid (OF) is an economic and easy-to-take sample for detecting antibodies and pathogens in a pig population (Prickett *et al*., 2008; Kittawornrat *et al*., 2010; Prickett and Zimmerman, 2010). This fact allows a more frequent herd monitoring and a greater representativeness of the animal group. During last years, PCV2 dynamics after natural (Kim, 2010; Ramirez *et al*., 2012) or experimental (Prickett *et al*., 2011) infections has been efficiently monitored by OF samples, using both ELISA and PCR techniques. However,

information regarding PCV2 assessment in terms of viral load and antibody levels in OF after immunization is limited (Zanotti *et al*., 2015).

The objectives of the present study were: a) to determine the optimal time for PCV2 vaccination, in terms of serological and virological parameters, in pigs vaccinated at 3, 6 or 10 weeks of age in a PCV2-SI scenario under common PCV2 circulation timings, and b) to expand the knowledge on the use of OF samples to detect PCV2 DNA and antibodies.

5.2. MATERIAL AND METHODS

5.2.1. Farm selection

The study was conducted in a conventional pig farm, located in Catalonia (Spain). In order to assess PCV2 infection status before the start of the study, a cross-sectional seroprofiling was performed including 10 pigs per batch of 6 age groups (3, 7, 11, 15, 19 and 23 weeks of age). Blood samples were processed by standard PCR (Quintana *et al*., 2002) and ELISA (Ingezim Circo IgG 11.PCV.K1®, Ingenasa, Madrid, Spain) to detect viral nucleic acid and antibodies (IgGs), respectively. PCV2 genome was detected in 50%, 30%, 20% and 10% of the sampled pigs at 11, 15, 19 and 23 weeks of age, respectively. All the 3 and 7 weeks tested samples were negative by PCR. Seroconversion was detected from the eleventh week of age onwards. Therefore, as no PCVDs clinical signs were evident in the farm, PCV2-SI was confirmed.

This farm was a two-site herd with 800 sows with all-in/all-out management and 4-week batch farrowing system. PCV2 vaccination in sows and piglets had never been applied in the studied herd. Sows were routinely vaccinated against PRRSV, PPV, *Aujeszky's disease virus*, *Swine influenza virus*, *Erysipelothrix rhusiopathiae*, *Escherichia coli* and *Clostridium perfringens*. Piglets were

vaccinated against *Mycoplasma hyopneumoniae* 3 days pre-weaning. Weaning was performed at 3 weeks of age and pigs were moved to fattening units at 10 weeks of age. Moreover, no signs of any major pig diseases were present and herd immunity status against PRRSV was determined as "positive-stable" (II-A) according to the previously described classification (Holtkamp *et al*., 2011).

5.2.2. Study design

Six-hundred and forty-four 2 week-old healthy crossbred piglets were selected in one single farrowing batch. These piglets came from 59 PCV2 non-vaccinated sows with low number of weak and cross-fostered piglets in their litters. Piglets were individually identified (ear-tagged), bled and their gender was recorded. Blood samples were tested by ELISA (Ingezim Circo IgG 11.PCV.K1®, Ingenasa, Madrid, Spain). Cross-fostered piglets were not included in the trial. At 3 weeks of age, animals were randomly allocated in four treatment groups (Table 5-1). Groups were randomized according to PCV2 ELISA S/P values, sex and litter. Animals from different treatment groups were housed in different pens (32 pens in nursery and 56 pens in fattening units) following a chessboard pattern. Pigs were vaccinated by intramuscular injection with 0.5 mL (single dose) of a commercial inactivated PCV2 vaccine (CIRCOVAC®, Ceva) at either 3, 6 or 10 weeks of age (3W-VAC, 6W-VAC and 10W-VAC groups, respectively), and another group of pigs was kept unvaccinated (NON-VAC group).

Table 5-1. Experimental design.

Group	Total number of pigs	Number of bleeding pigs	Number of pens tested by OF samples		Treatment		
			Nursery unit[a]	Fattening unit[b]	3 weeks of age	6 weeks of age	10 weeks of age
3W-VAC	161	28	6	10	Vaccination[c]	-	-
6W-VAC	161	28	6	10	-	Vaccination[c]	-
10W-VAC	161	28	6	10	-	-	Vaccination[c]
NON-VAC	161	28	6	10	-	-	-

[a] Approx. twenty-three pigs were allocated in each nursery pen
[b] Approx. eleven pigs were allocated in each fattening pen
[c] Animals were vaccinated with a single dose (0.5 ml) of CIRCOVAC® (Ceva)

Among all animals included in the study, 28 animals per group (14 males and 14 females) with a medium antibody titre (ranging from 0.07 to 1.24 ELISA S/P values at 2 weeks of age) and equally distributed in all pens (2 or 4 piglets per pen in nursery and 2 piglets per pen in fattening units) were randomly selected to be bled. From these animals, a blood sample was taken at 6, 10, 14, 18 and 25 weeks of age. Whole blood samples were allowed clotting, and centrifuged at 3200 rpm during 20 min at 4°C. All sera were aliquoted and stored at -20ºC until testing.

OF samples were collected from a representative number of pens (24 nursery and 40 fattening pens) located at the entrance, middle and final area of the nursery and fattening units. OF were taken simultaneously to blood collection by suspending a non-treated, 3-strand, 100% cotton rope in each pen for 30 min (Seddon *et al*., 2012). Each rope was manually squeezed inside a single-use plastic bag for OF extraction; then, the corner of the bag was cut and the sample was poured into a sterile tube. To avoid cross-contaminations, all materials (bag, globes, tube) were changed or disinfected (scissors) between pens. Once in the laboratory, samples were centrifuged at 1000 × g during 10 min at 4ºC for clearing the sample (Kittawornrat *et al*., 2010); then, the supernatant was aliquoted and frozen at -80ºC until use.

From 2 to 25 weeks of age (at each vaccination or bleeding point), all pigs included in the study were monitored for clinical signs and mortality. Animals with major pathologies (hernia, lameness, injuries, etc.) were excluded from the study. Housing conditions, feeding system, feed characteristics and health management remained consistent along the course of the trial, and were the same among all experimental groups. The present study was approved by the Ethics Committee for Animal Experimentation from the *Universitat Autònoma de Barcelona* and the Animal Experimentation Commission from the local

government (*Dpt*. *de Medi Ambient i Habitatge* from the *Generalitat de Catalunya*; Reference 5796).

5.2.3. DNA extraction and real-time quantitative PCR

DNA was extracted from 200 µl of serum or 300 µl of OF samples, by using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems) following the manufacturer´s instructions. The DNA obtained was suspended in 90 µl of elution solution.

To quantify the PCV2 DNA in serum and OF samples, a real-time qPCR assay (LSI VetMAX™ Porcine Circovirus Type 2-Quantification, Life Technologies) was performed. Each extraction and qPCR plate included negative controls (DEPC-treated water) and each sample reaction had an IPC to monitor DNA extraction and amplification procedures. Viral concentrations were expressed as the mean $\log_{10}$ PCV2 genome copies/mL. Area under the curve (AUC) of viral load in serum samples from 2 to 25 weeks of age was calculated according to the trapezoidal method as previously described (López-Soria *et al*., 2014).

5.2.4. Serology

5.2.4.1. Indirect ELISA for detecting anti-PCV2 IgG antibodies in serum samples

All serum samples were tested by the Ingezim Circo IgG 11.PCV.K1® assay (Ingenasa, Madrid, Spain). The OD was measured at 450 nm by the PowerWave XS reader (BioTek). Mean positive cut-off was established at 0.3 OD (± SD) following the kit´s instructions (positive cut-off = OD of negative control +

0.25). ELISA results were expressed as mean S/P ratio (OD of sample / OD of positive control for each ELISA plate).

5.2.4.2. Semi-quantitative ELISA for detecting anti-PCV2 antibodies in OF samples

All OF samples were processed by the SERELISA® PCV2 Ab Mono Blocking kit (Synbiotics, Lyon, France) with some modifications (protocol used at Labocea, Ploufragan - personal communication). The analysis of the samples by this technique led to a semi-quantitative result expressed as 1 (+), 2 (++), 3 (+++) or 4 (++++).

5.2.4.3. Viral neutralization test (VNT)

The ability to neutralize PCV2 was assessed by VNT in 14 randomly selected serum samples per group (half of collected serum samples). This assay was performed as previously described (Fort *et al*., 2007), with the following modifications: 1) serum was tested in four-fold dilutions (from 1:4 to 1:4096) using supplemented DMEM (Dulbecco's Modified Eagle Medium) in 96-well plates (plates were read using a microscope at 10x magnifications), and 2) number of PCV2 infected cells (nuclear and/or cytoplasmic staining) per well in each sample replica was counted. Percentage of virus neutralization (%VN) at each serum dilution was calculated as follows: % VN = [1 - (mean number of infected cells of the two replicas of each serum dilution / mean number of infected cells in negative control wells)] x 100. Then, VNT50 (i.e. reciprocal of the last dilution of the serum sample in which the number of PCV2 infected cells was reduced to a 50%) was calculated and designated as the NA titre. Results were expressed as $\log_2$ NA titre.

5.2.5. PCV2 amplification and sequencing

With the aim of determining the main PCV2 genotype circulating in the farm, the capsid protein gene (ORF2) was sequenced from two PCV2 qPCR positive samples per treatment group. Amplification was done from nucleotide 1050 to 1735 (PCV2 genome; GenBank Accession Number: AY181948) using primers PCV2all_F (5' GGGTCTTTAAGATTAAATYC 3´) and PCV2all_R (5' ATGACGTATCCAAGGAG 3´). PCR was developed in a 25 µl reaction containing 1.25 µl of each mentioned primer at 10 pmol/µL, 5 µl of 5x PCR buffer, 2.5 µl of $MgCl_2$ at 25 mM, 0.75 U of Taq DNA polymerase, 1 µl of dNTP stock solution at 5 mM, 11.35 µl of DEPC-treated water and 2.5 µl of extracted DNA. The PCR was started with an initial denaturation step of 5 min at 94 ºC. The temperature profile of the following 40 cycles consisted of 30 sec at 95 ºC for denaturation, 30 sec at 53 ºC for primer annealing and 40 sec at 72 ºC for elongation. The reaction was terminated by a final elongation step of 7 min at 72 ºC. Amplified PCR product was run in an electrophoresis gel with 1.8% agarose. The band was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, GmbH & Co. KG, Germany) according to the manufacturer´s instruction. Sequencing reactions were performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analysed using a 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

5.2.6. PCV2 capsid protein (ORF2) phylogenetic and sequence analysis

Nucleotide sequences of the PCV2 capsid protein were analysed using Bioedit v7.0.9.0 (Hall, 1999). Sequences were aligned using the Clustal W multiple alignment method included in the Bioedit package (Thompson *et al*., 1994).

Fifteen strains of different PCV2 genotypes retrieved from the GenBank database were included in the comparison. The phylogenetic tree was constructed according to the Neighbor-Joining method with 1000 bootstrap replicates using MEGA version 4 (Tamura *et al*., 2007).

5.2.7. Statistical analyses

Animal mortality and exclusion rates between groups were compared using the likelihood ratio test. Generalized linear mixed models for longitudinal binary data were performed to analyse the evolution between groups for PCV2 qPCR (positive/negative) values in pigs (serum samples) and pens (OF samples). Treatment group, sampling point and their interaction were considered as fixed effects, and piglet and pen as random effects. Whenever differences between groups were detected, they were further evaluated by pairwise comparisons. P-values were corrected using Tukey's method. Generalized linear mixed models were applied for longitudinal continuous data such as mean $\log_{10}$ PCV2 copies/mL in qPCR positive serum and OF samples, mean ELISA S/P IgG values in sera, mean ELISA semi-quantitative values in OF and $\log_2$ NA titre in sera. The comparison of PCV2 AUC load in serum samples between groups was analysed by a non-parametric test (Kruskall-Wallis statistic). Pearson's correlation coefficient was used to assess the relationship between serum and OF results (ELISA and qPCR), as well as between ELISA values from both serum and OF samples in comparison to NA titres in serum samples. Statistical analyses were carried out using SAS v9.4, SAS Institute Inc., Cary, NC, USA. The significance level was set at $p<0.05$.

5.3. RESULTS

5.3.1. Clinical signs and mortality

No clinical signs related to PCV2-SD were observed during the course of the study. No statistically significant differences in terms of mortality rate and animal exclusion (ranging from 2.5 to 7.5% in all groups) were observed among treatment groups during the whole experimental period (data not shown).

5.3.2. Quantification of PCV2 DNA

5.3.2.1. Serum samples

While very few pigs were qPCR positive at 3 and 6 weeks of age, the percentage of PCV2-DNA positive pig sera raised at 10 weeks of age, was maximum at 14 weeks of age and then started to decrease by 18 and 25 weeks of age. Particularly, animals from 3W-VAC and 6W-VAC groups had a significantly lower ($p<0.05$) percentage of viraemic animals (Figure 5-1A) compared to the NON-VAC group at 14, 18 and 25 weeks of age (in 3W-VAC group) and at 14 and 18 weeks of age (in 6W-VAC group). In contrast, the 10W-VAC group showed a higher percentage of viraemic pigs than 3W-VAC and 6W-VAC groups at 10, 14 and 18 weeks of age (only significantly different at 18 weeks of age), but lower than that of control group at 14, 18 and 25 weeks of age (only significant at 25 weeks of age). At the peak of infection (14 weeks of age), the 3W-VAC group showed a significantly lower ($p<0.05$) PCV2 load (Figure 5-1B) than the NON-VAC group. The 6W-VAC and 10W-VAC groups also showed the same trend with lower viral loads than the NON-VAC group but these differences were not statistically significant. The 3W-VAC and 6W-VAC groups experienced a significantly lower AUC of viral load than the NON-VAC

group (p<0.05) (Table 5-2). However, PCV2 AUC of 10W-VAC group was only numerically lower than that of the NON-VAC group.

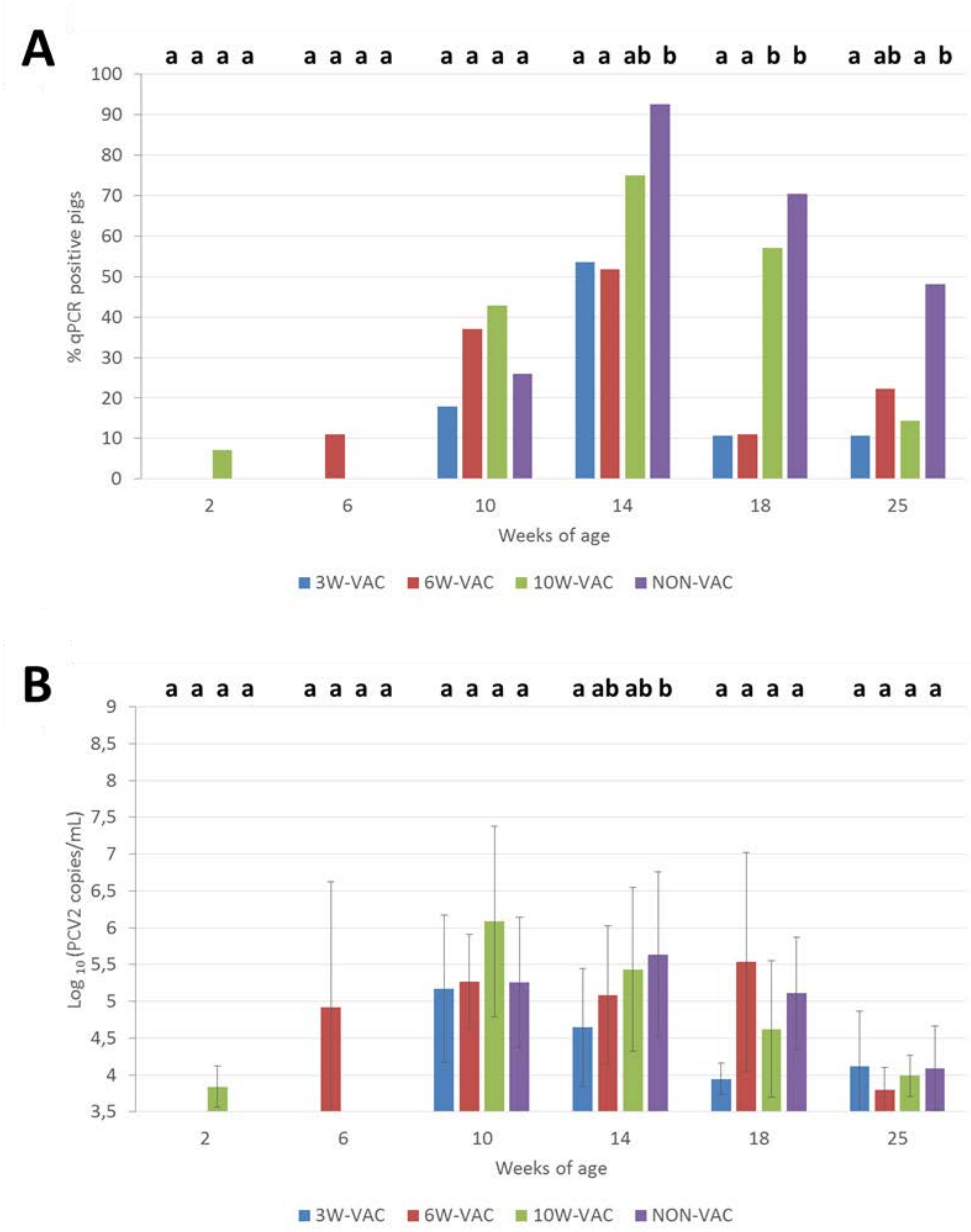

Figure 5-1. Percentage of PCV2 qPCR positive pigs (A) and mean viral load (±SD) of qPCR positive serum samples (B). Different letters in superscript mean statistically significant differences (p<0.05) among experimental groups at each sampling point.

Table 5-2. Area under the curve (AUC) of PCV2 load (log_{10} PCV2 copies/mL) in serum samples from 2 to 25 weeks of age.

Group	Mean AUC of viral load
3W-VAC	3.25^{a} (Min.=0.00 / Max.=6.48)
6W-VAC	3.79^{a} (Min.=0.00 / Max.=7.41)
10W-VAC	4.92^{ab} (Min.=0.00 / Max.=7.75)
NON-VAC	5.65^{b} (Min.=0.00 / Max.=7.90)

Different letters in superscript mean statistically significant differences ($p<0.05$) among experimental groups

5.3.2.2. OF samples

At 3 weeks of age, OF collection was not possible since piglets did not chew the ropes. Percentage of PCV2 qPCR positive pens (Figure 5-2A) was high at all sampling points and no statistically significant differences among treatment groups were observed. At the peak of infection (14 weeks of age), 100% of positivity was observed in all groups. At this time point, viral load (Figure 5-2B) was numerically lower in all the vaccinated groups compared to the control group.

Virological results obtained from serum and OF samples showed positive but non-significant correlations: percentage of PCV2 qPCR positive samples (r=0.86; p=0.06) and viral load (r=0.76; p=0.13).

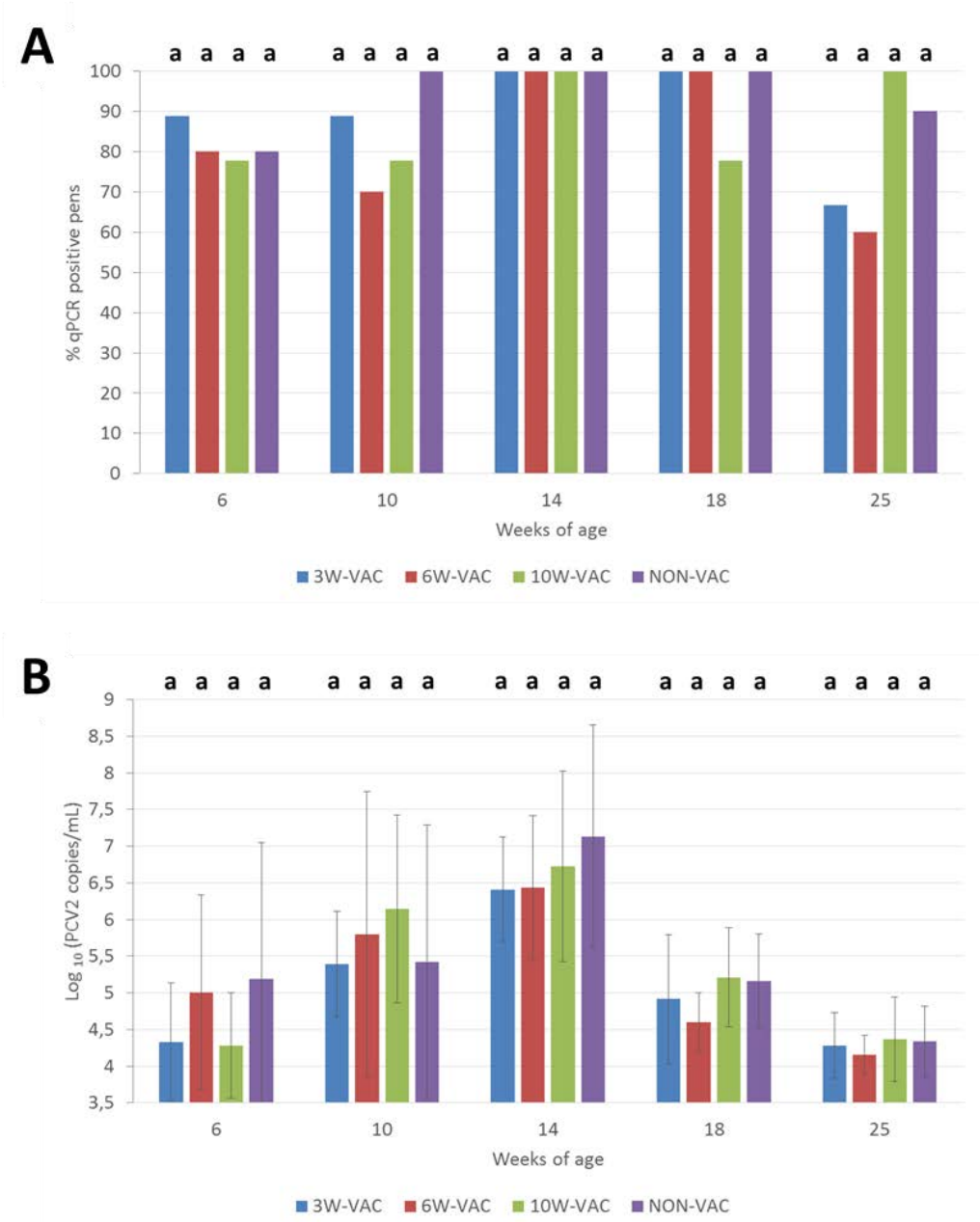

Figure 5-2. Percentage of PCV2 qPCR positive pens (A) and mean viral load (±SD) of qPCR positive oral fluids samples (B). Different letters in superscript mean statistically significant differences (p<0.05) among experimental groups at each sampling point.

5.3.3. Serology

5.3.3.1. Anti-PCV2 IgG antibody levels in serum samples

The course of antibodies against PCV2 in the four treatment groups is shown in Figure 5-3. From 2 to 6 weeks of age, all groups presented a decrease of ELISA S/P values and no differences between groups were observed. At 10 weeks of age, 3W-VAC and 6W-VAC groups showed significantly higher (p<0.05) S/P values than 10W-VAC and NON-VAC groups. The 10W-VAC group seroconverted by 14 weeks of age, reaching significantly higher antibody levels at 18 weeks of age compared to the other groups. The NON-VAC group seroconverted by 14 to 18 weeks of age. From this time point onwards, S/P values of the vaccinated groups began to decrease whereas the ones of the control group remained stable.

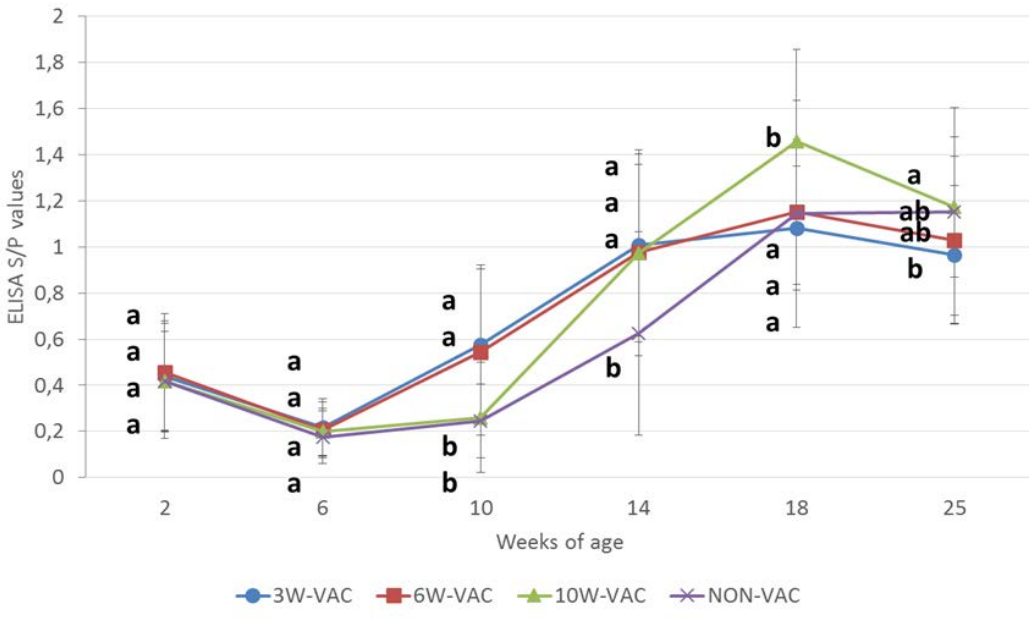

Figure 5-3. PCV2 ELISA S/P results (mean±SD) in serum samples. Different letters in superscript mean statistically significant differences (p<0.05) among experimental groups at each sampling point.

5.3.3.2. Anti-PCV2 antibody levels in OF samples

Mean semi-quantitative antibody values in OF are summarized in Figure 5-4. At 10 weeks of age, the 3W-VAC group displayed a statistically significant increase of antibody response compared to all other groups. At the same time point, the other groups experienced a decrease of antibody levels, being the ones of the 6W-VAC group significantly higher than the ones of the NON-VAC group. Four weeks later, i.e. at the peak of infection, all vaccinated groups showed higher antibody values than the NON-VAC group. From 18 weeks of age onwards, antibody levels from all groups remained high and no significant differences were observed between them.

A high and statistically significant correlation (r=0.95, p=0.015) between serum and OF ELISA results was observed.

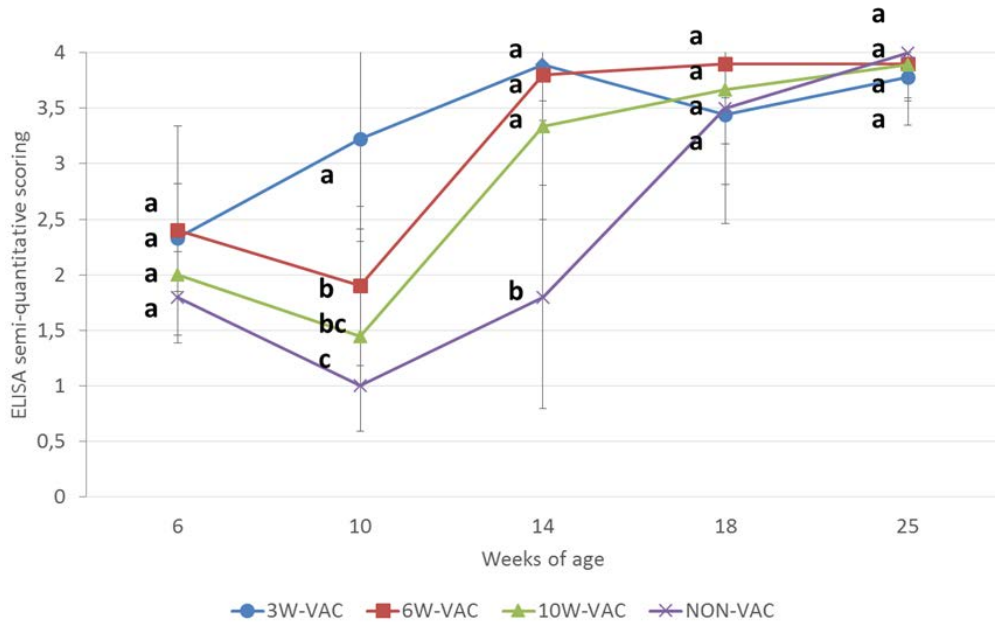

Figure 5-4. PCV2 ELISA semi-quantitative values (mean±SD) in oral fluid samples. Different letters in superscript mean statistically significant differences (p<0.05) among experimental groups at each sampling point.

5.3.3.3. Neutralizing antibodies titres in serum samples

Mean NA titres (±SD) dynamics for each treatment group is depicted in Figure 5-5. From 2 to 6 weeks of age, all groups showed a decrease of NA titres and no differences between groups were observed. Subsequently, pigs from groups 3W-VAC and 6W-VAC had significantly higher NA levels compared to the 10W-VAC (at 10 weeks of age) and NON-VAC (at 10 and 14 weeks of age) pigs. In the 10W-VAC group, the increase of NA titres was observed 4 weeks after vaccination, i.e. 14 weeks of age, being significantly higher than the ones in NON-VAC pigs. The NA response for animals of the NON-VAC group was detected at 14 weeks of age, reaching maximum levels at 18 weeks of age. After this sampling point, NA levels from all groups began to decrease.

High and statistically significant correlations were found between NA titres tested in serum samples in comparison to ELISA values detected in serum (r=0.97, p=0.001) and OF (r=0.90, p=0.038) samples.

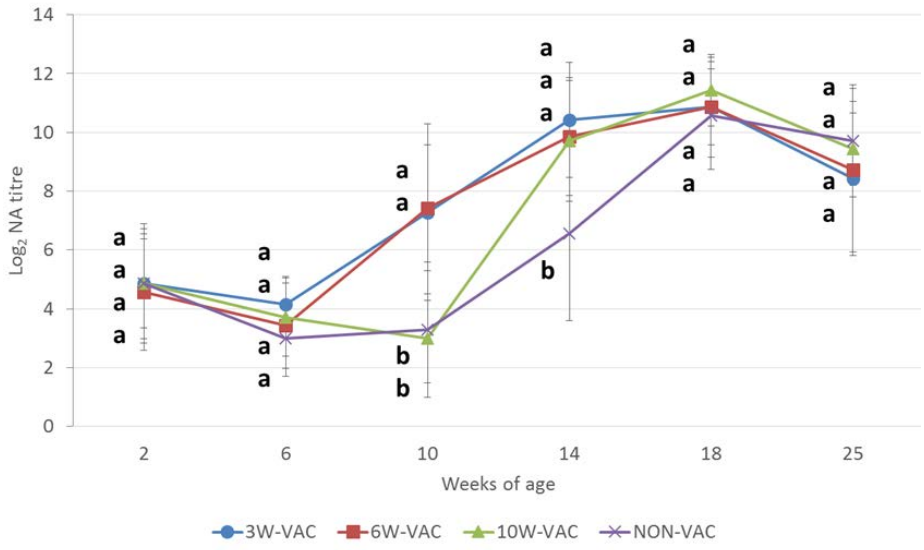

Figure 5-5. PCV2 NA titres (mean±SD) in serum samples. Different letters in superscript mean statistically significant differences (p<0.05) among experimental groups at each sampling point.

5.3.4. PCV2 genotyping

A Neighbor-Joining phylogenetic tree including the relationships among the PCV2 isolates sequenced in this study (two per experimental group) and reference strains is represented in Figure 5-6. All serum samples sequenced (GenBank accession numbers: KX670778-KX670785) were genetically closely related and clustered within PCV2a genotype.

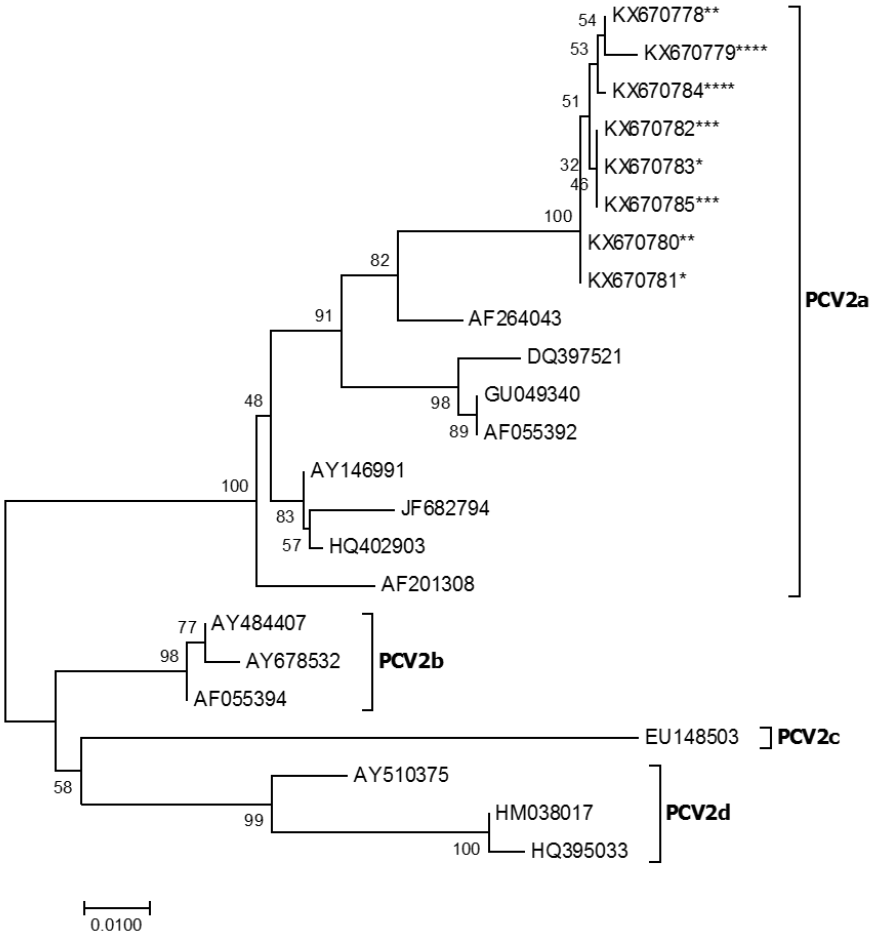

Figure 5-6. Neighbor-Joining phylogenetic tree with 1000 bootstrap replicates showing the relationships among the nucleotide sequences of the PCV2 capsid protein. PCV2 strains sequenced in this study from 3W-VAC (*), 6W-VAC (**), 10W-VAC (***) and NON-VAC (****) groups are compared to PCV2 types a, b, c and d strains. Horizontal branches indicate the sequence distance (number of base differences per site).

5.4. DISCUSSION

Several studies have shown that PCV2 piglet vaccination at weaning age (3-4 weeks of age) is effective in most farms regardless of the PCVD farm status (PCV2-SD or PCV2-SI) and the brand of commercial vaccine used (Segalés, 2015). Vaccination schedules at earlier ages with one single dose are rarely applied, since high levels of MDA at the vaccination time may cause a lower humoral response (interference with the seroconversion) elicited by the vaccine (Fort *et al*., 2009b; Fraile *et al*., 2012a,b; Oh *et al*., 2014) and may eventually jeopardize the efficacy of vaccination (Haake *et al*., 2014; Feng *et al*., 2016).

In the peer-reviewed literature, little information does exist on PCV2 vaccine efficacy obtained by comparing vaccination of piglets (coming from non-vaccinated sows) at different post-weaning ages (Cline *et al*., 2008; Oh *et al*., 2014). In the present study, PCV2 vaccination in piglets at 3 or 6 weeks of age yielded similar virological and serological results, producing a relatively early humoral immune response and reducing the proportion of viraemic animals in comparison to the unvaccinated group. These results are in accordance with two previously published trials, where no statistically significant differences in terms of PCV2 viraemia and/or humoral and cellular immunity were found between pigs vaccinated at three and six weeks of age (Cline *et al*., 2008), and at three and seven weeks of age (Oh *et al*., 2014). In addition, it has also been demonstrated that vaccination of older animals (8.5 weeks of age) with a subunit vaccine and under a PCV2-SD scenario resulted in a significantly lower mortality in vaccinates than in controls (Desrosiers *et al*., 2009). Although in this latter study, serological and virological parameters from pigs vaccinated at this time point were not tested, the vaccination took place, most probably, when a proportion of pigs were already infected. As far as the authors knowledge, no more information is available on the efficacy of piglet vaccination at older ages.

Thus, the present study represents the first time comparing the use of vaccination at 10 weeks of age (entering to the fattening facilities) with earlier ages. Under the conditions of the present farm trial, PCV2 vaccination at 10 weeks of age was probably done too late for an optimal performance as it coincided with the increase of the percentage of viraemic pigs in all the treatment groups. Under field conditions, PCV2 viraemia usually starts at the end of the nursery or at the beginning of fattening periods (Segalés *et al*., 2012). In the present farm scenario, vaccination at 10 weeks of age was able to numerically reduce the percentage of viraemic animals at 14 (peak of infection), 18 and 25 weeks of age in comparison with the control group, being statistically significant at the latter time point. This evidence is in agreement with a previous experimental trial (Seo *et al*., 2014c), showing that vaccination of PCV2 viraemic and seropositive piglets leads to a humoral and cellular immune response able to reduce PCV2 viraemia. Therefore, although not optimal, vaccination of viraemic pigs seems to exert a positive effect compared to viraemic, non-vaccinated ones.

The current work further demonstrated the ability of an inactivated vaccine to produce a NA response after piglet immunization at different ages. This response led to a significantly greater protection (in terms of PCV2 viraemia) of groups vaccinated before natural infection compared to the group vaccinated after the onset of infection, i.e. at 10 weeks of age, and the control group. The inverse dynamics between NA titres and PCV2 load in serum found in the present study had previously been described (Meerts *et al*., 2006; Fort *et al*., 2007; Seo *et al*., 2012a). In addition, an immune response analysis of the four major vaccines available on the market was performed in a recent study (Seo *et al*., 2014b), confirming the ability of the inactivated vaccine used in this study to induce a NA response after vaccination, producing higher NA levels than the ones from subunit vaccines. Moreover, in the current work, high and statistically

significant correlations were found between ELISA values from both serum and OF samples in comparison to NA titres in sera. This finding suggests that antibody levels tested by the used ELISA kits might be used as potential predictor of NA titres.

Both serological techniques used in serum and OF samples offered similar results with a high and statistically significant correlation among them. These results would suggest that OF samples can be an alternative to serum for studying PCV2 antibody dynamics. This outcome is in agreement with a reported trial (Kim, 2010) in which positive correlation between OF and pooled sera in terms of antibody detection was found. In contrast, a higher PCV2 qPCR positivity was detected in the present study from OF in comparison with sera at all sampling points, and no significant differences between treatment groups were observed by using OF. Moreover, PCV2 circulation was detected earlier in OF (from 6 weeks of age) compared to sera (from 10 weeks of age) in all groups of pigs. These findings are in accordance with a previous study (Kim, 2010) and support the fact that the starting time of PCV2 circulation and viraemia might be different. However, in terms of PCV2 load, whereas similar levels of PCV2 DNA in OF and serum samples with a significant correlation were described (Kim, 2010), higher mean viral loads in OF (over one logarithm) with no significant correlation to PCV2 loads in sera were detected in the current study at infection peak (14 weeks of age). The higher qPCR positivity percentages and PCV2 load in OF compared to sera may be explained by a number of reasons. First, serum samples were obtained from only two or four pigs per pen, but OF sample was a collective sample representing around 23 or 11 pigs per nursery or fattening pen, respectively. Therefore, there is a reasonable probability that some viraemic/shedder animals were not bled or alternatively that the bled subjects were not the ones with the highest viral loads. Second, since PCV2 replicates firstly in the tonsil (Rosell *et al*., 1999; Allan *et al*., 2000b), it might

be probably detected at an earlier stage and with a greater concentration in OF with regards to sera as has been previously suggested (Kim, 2010). Finally, PCV2 is an endemic and very stable virus (Opriessnig *et al*., 2007) that might be ever-present in pens (Dvorak *et al*., 2013). In fact, it has been demonstrated that PCV2 subclinically infected pigs may excrete medium to high viral loads in faeces (McIntosh *et al*., 2009; López-Rodríguez *et al*., 2016). Therefore, it should be taken into account that ropes might be spoiled by the traces of faeces present in the mouth/skin of the pigs.

In all sequenced samples (n=8), PCV2a genotype was identified. Although this genotype has a worldwide distribution (Franzo *et al*., 2016), the most current prevalent genotype in the pig population is PCV2b (Segalés *et al*., 2013; Franzo *et al*., 2015c). Indeed, it has been proposed that PCV2b is more prevalent than PCV2a in PCV2-SD cases and in vaccinated farms (Shen *et al*., 2012). The PCV2-SI scenario in the studied farm and the fact that no PCVD compatible clinical signs had ever been observed before the start of the study (and in consequence, vaccination had never been applied before this trial) might be related with the detection of PCV2a genotype in the farm. The apparent sole presence of PCV2a genotype was not enough to produce overt disease in this farm. In fact, the speculation that PCV2a might not be as efficient as PCV2b to trigger clinically evident disease came from the demonstration of a worldwide genotype shift from PCV2a to PCV2b coinciding with major outbreaks of PCV2-SD around the globe (Segalés *et al*., 2013).

In conclusion, under the conditions of this study, the optimal age for piglet vaccination was at 3 or 6 weeks of age, since it was applied when the percentage of viraemic pigs was minimal, triggering an effective humoral immune response before the peak of infection. These strategies were able to reduce, at different sampling points, the proportion of viraemic animals in comparison to the

unvaccinated group. In contrast, PCV2 vaccination at 10 weeks of age (coinciding with the increase of the percentage of viraemic pigs in the population) only achieved such reduction at 25 weeks of age. Therefore, age at PCV2 vaccination should be adapted according to the viral infection dynamics present in the studied farm. Moreover, both serological techniques used in sera and OF were useful to study PCV2 antibody dynamics. In contrast, viral detection in OF might be useful to have an idea of the infection dynamics at population level but should remain only as a raw indicative method.

CHAPTER 6

General Discussion

Before the emergence of PCV2 vaccines, PCV2 infection was mainly associated to PCV2-SD (Harding and Clark, 1997; Segalés and Domingo, 2002) and, to a lesser extent, PDNS (Rosell *et al*., 2000b). In addition, several studies also reported late-term reproductive failures associated with PCV2 in breeding herds, mainly related to increased numbers of mummies, stillborns and non-viable piglets at farrowing (O'Connor *et al*., 2001).

Currently, it is unlikely to detect PCV2-RD problems at farm level probably due to the widespread seropositivity usually found in gilts and sows (Segalés, 2012). This circumstance, together with the fact that there was only one licensed vaccine for being used in sows, implied that effectiveness of commercial vaccines in this scenario has been minimally tested. In one of these few studies, repeated vaccination of the breeding herd suffering from apparent PCV2-RD problems resulted in a significant improvement of the reproductive performance (Pejsak *et al*., 2012).

On the other hand, PCV2-SI is nowadays the most prevalent PCVD (Segalés *et al*., 2013). The detrimental effects produced by this condition in growing pigs were discovered as a result of the use of PCV2 vaccines in PCV2-SD scenarios. In fact, most of the producers observed benefits not only in diseased animals, but also in non-diseased ones. Further testing under field conditions demonstrated that vaccinated animals grew more than unvaccinated ones even in absence of overt clinical disease (Young *et al*., 2011; Fraile *et al*., 2012a; Alarcón *et al*., 2013). Benefits of PCV2 vaccination under a subclinical infection context in the breeding stock have been poorly explored. In consequence one of the aims of this Thesis was to investigate such scenario. In *Chapter 3,* a significant improvement of prolificacy and vitality of new-born piglets from vaccinated sows in their second gestational cycle was recorded. This difference in prolificacy between vaccinated and non-vaccinated sows

might be explained by a protective effect of PCV2 vaccination on the embryos during early gestation. In fact, *Chapters 3 and 4* showed that PCV2 sow vaccination at 6 and 3 weeks before parturition triggered high antibody levels at farrowing. In addition, these levels remain high at the end of lactation (*Chapter 3*). In consequence, vaccinated sows should be protected against PCV2 infection around fertilization time (about 1 week after weaning). Furthermore, the vitality results obtained in *Chapter 3* are novel. Piglets born from PCV2 vaccinated sows have shown to have a consistent vitality during the first hours of life. These piglets displayed a high mobility after birth (evaluated by the NCC parameter), as well as a constant udder search behaviour (evaluated by the U parameter). These facts are crucial to ensure a suitable colostrum intake of the piglet. Moreover, although it is known that sow vaccination does not fully prevent PCV2 vertical transmission (Madson *et al*., 2009a,c; Gerber *et al*., 2012; Hemann *et al*., 2014), it would be interesting to carry out future trials to study the correlation between the vitality index and the level of infection in new-born piglets (not sampled in *Chapter 3*) derived from vaccinated and non-vaccinated dams.

Under field conditions, reproductive parameters may be influenced by many factors (Koketsu *et al*., 2017). In the present Thesis, with the aim to diminish the number of influencing elements, sows were randomly distributed in two experimental groups according to the reproductive outcomes of the previous farrowing and, additionally, both groups were allocated under the same environmental conditions (same farrowing rooms). Therefore, study conditions were controlled enough to consider that benefits observed in the vaccinated group were truly due to vaccination. Nevertheless, it would be interesting to gain new knowledge on the vaccination of sows under PCV2-SI scenarios, and potential trial/s may include:

- More reproductive cycles. As described by Pejsak et al. (2012), the longer the period of vaccine application, the better the reproductive results observed in the vaccinated group.
- Increased number of subclinically infected farms. Since reproductive performance may depend on many factors (Koketsu *et al*., 2017), repeating the study described in *Chapter 3* on several farms, more contrasted results might be obtained. In addition, since PCV2 infection can affect any stage of gestation, there is a possibility that differences between vaccinated and non-vaccinated groups could also be observed in additional reproductive parameters (abortions, increased number of mummies or stillborns, etc.). In all cases, the higher the PCV2 infectious pressure of the farm, the more benefits achieved by vaccination should be expected.
- Testing different vaccine schedules. In this Thesis, vaccination before farrowing was performed following the specific characteristics of the product (Circovac®). Taking into account that the first immunization was applied when foetuses were already formed, the potential benefits on reproductive parameters were expected as early as at the second gestational cycle. Nevertheless, pre-farrowing vaccination is focused on protection of the offspring by means of MDI transfer (*Chapter 4*). Therefore, if the vaccine would have been applied before mating (off-label use for Circovac®), the embryos and foetuses might have been protected during the subsequent pregnancy, presumably enabling to observe reproductive benefits from the first cycle of application.

As mentioned before, in *Chapter 4*, PCV2 immunization of sows pre-parturition produced significantly higher antibody levels detected at farrowing compared with non-vaccinated ones. These statistically significant differences, however,

were not observed when cytokine levels were compared between sow treatment groups. This fact may be explained by the following hypotheses:

- The PCV2 specific cytokine secretion is variable among animals (Darwich and Mateu, 2012). In addition, the older the sow at the moment of vaccination, the higher the likelihood of having been exposed to different unspecific (co-infections, vaccinations, stress, etc.) and specific (previous PCV2 circulations) external factors that may influence the immune system (Darwich and Mateu, 2012; Lee *et al*., 2016). Therefore, in this multifactorial context, the immune response intensity in terms of cytokine secretion levels of the sows may not depend solely on the PCV2 vaccine administered. This is probably the reason why information on cellular immune response developed after vaccination in sows is very scarce. In fact, most studies evaluating the cell-mediated immunity conferred by vaccination have been carried out in piglets under controlled environmental conditions (Fort *et al*., 2009b; Fort *et al*., 2012; Seo *et al*., 2014a; Koinig *et al*., 2015). In *Chapter 4*, although the tested piglets were located in the farm where many influencing factors may be present, blood samples were obtained few days after birth. Therefore, it may be assumed that the obtained sample was fairly specific for passive immunity because: 1) the piglets still had an immature immune system, 2) they had not been injected with any treatment or vaccine, and 3) probably, they had not been exposed to common pathogens on the farm.
- In spite of the facts commented in the previous point, PCV2-specific levels of cytokines related to protection after vaccination (IFN-γ and TNF-α) (Koinig *et al*., 2015) were numerically higher in vaccinated sows; however, these differences were not significant. This situation was

most probably due to the low number of tested sows (only 7-8 sows per group).

High PCV2 specific antibody levels in sows have also been linked to a higher transfer of MDA to the progeny (Kurmann *et al*., 2011; Fraile *et al*., 2012b; Sibila *et al*., 2013; Oh *et al*., 2014; Dvorak *et al*., 2017); however, the knowledge about transfer of maternally derived cellular immunity is scarce. The findings observed in *Chapter 4* support and complement the only previously described peer-reviewed study that showed that, apart from MDA, there is also a transfer of PCV2 specific lymphocytes from the immunized sow to the piglet (Oh *et al*., 2012). The most relevant and novel result of *Chapter 4* was the significantly higher double secretion of IFN-γ/TNF-α by PBMCs of piglets from vaccinated sows compared to their counterparts. This fact acquires great importance since it has been demonstrated that the induction of IFN-γ/TNF-α co-producing T cells (in a PCV2 piglet vaccination scenario) is potentially correlated with protection (Koinig *et al*., 2015). Although in *Chapter 4* the protection conferred by maternal derived lymphocytes was not evaluated, Oh et al. (2012) reported that the maternally derived adaptive cellular immune responses in the new-born piglets play an important role in protection after challenge (Oh *et al*., 2012). Therefore, the results of *Chapter 4* might be interpreted as indirect indicators of passive protection.

Another more direct way to evaluate the transfer of immune cells would have been to analyse the colostrum, testing PCV2-specific cell subpopulations or cytokine (mainly IFN-γ) secretion. However, given the difficulty of isolating colostral cells, the study described in *Chapter 4* offers a more practical (PBMC isolation and stimulation) and quick (Multiplex immunoassay for the quantification of different cytokines at the same time) method to assess indirectly but apparently reliably (significantly higher MDA and cytokines

potentially linked to Th1 memory response in piglets from vaccinated sows) the potential transfer of cellular immunity to the offspring.

The high levels of PCV2 specific MDA transferred from sows vaccinated before farrowing to the piglets (*Chapter 4*) have also been described in other studies using the same sow vaccination strategy (Kurmann *et al*., 2011; Oh *et al*., 2014). Additionally, PCV2 sow vaccination before mating has also been shown to confer higher levels of MDA in colostrum compared to a control group (O'Neill *et al*., 2012; Sibila *et al*., 2013). In fact, these levels remain high in the offspring serum for several weeks and may cause a more limited humoral response elicited by the piglet vaccine (interference with seroconversion) when applied at 3-4 weeks of age. The interference with vaccine-elicited humoral immune response has been reported in piglets coming from pre-farrowing vaccinated sows (Oh *et al*., 2014; Feng *et al*., 2016) as well as in those from pre-mating vaccinated sows (Fraile *et al*., 2012b; Martelli *et al*., 2016). Therefore, the time at which the piglet is vaccinated becomes very important. In scenarios with high MDA levels, piglet vaccination at 6-7 weeks of age is recommended, since this strategy is able to control the PCV2 infection in a more effective way than piglet vaccination at weaning (3-4 weeks of age) (Oh *et al*., 2014; Martelli *et al*., 2016). On the other hand, when sow PCV2 vaccination is not applied in the herd, piglets are usually vaccinated at 3-4 weeks of age (weaning age). In this sense, the third study (*Chapter 5*) was conducted to assess if the most common piglet age of vaccination (at weaning) is the one that really offers the best serological and virological performance. In consequence, this age was compared with later vaccination times (at 6 and 10 weeks of age) using piglets born from PCV2 non-vaccinated sows.

Curiously enough, in the group of piglets vaccinated at 3 weeks of age, a partial interference with the development of the humoral (total IgGs and NA) response

was observed (*Chapter 5*). This fact was unexpected, since such interference is usually linked with fairly high MDA levels (above 1.2 ELISA S/P values) at the time of vaccination (Fort *et al*., 2009b; Pileri *et al*., 2014), in contrast with the low MDA levels (around 0.2-0.4 ELISA S/P values) detected at treatment application time in all piglet groups from *Chapter 5*. Despite this evidence, a PCV2 immunization scheme at 3 weeks of age was able to overcome this initial interference with vaccine-elicited antibodies, triggering a seroconversion pattern almost equal to that of the group vaccinated at 6 weeks of age throughout the study. In addition, both groups also offered very similar virological (PCV2 infection control) results, without significant differences between them. Consequently, both vaccination schemes proved to be the most effective ones. In contrast, the latest vaccination time (at 10 weeks of age), which was administered in presence of a large percentage of PCV2 infected animals, offered less efficacy from a virological point of view throughout the study.

The optimal time of PCV2 vaccination can vary from one farm to another, since it is known that each herd has different characteristics. In general terms, the factors to be considered to establish the optimal time of vaccination against PCV2 are the following:

- MDI: As a reference, the interference of high MDA levels at vaccination time with the seroconversion elicited by the vaccine may be observed when antibody titres are higher than 10 $\log_2$ IPMA (immunoperoxidase monolayer assay) (Fort *et al*., 2009b) or 1.2 ELISA S/P values (Pileri *et al*., 2014). However, the cell-mediated immune response induced by the vaccines seems not to be negatively affected by the presence of high MDA levels and contributes to a long-lasting protection (Fort *et al*., 2009a,b; Martelli *et al*., 2011; Ferrari *et al*., 2014; Martelli *et al*., 2016).

- Maturity of the immune system of the piglet: The maturity degree of the immune system of a new-born piglet is still not clearly defined (Chase and Lunney, 2012). However, in an experimental study it was found that PCV2 vaccination of seronegative piglets at 5 days of age induced protective immunity against subsequent experimental PCV2 challenge (O'Neill *et al*., 2011). This indicates that, at least at that age, the piglet immune system is already mature enough to produce a protective immune response. Nevertheless, at the farm level it is unusual to find seronegative piglets, mainly due to the seropositivity of the sows. This last scenario was evaluated in another study (Haake *et al*., 2014), where piglet vaccination was applied at one week of age in presence of high MDA levels. As expected, this vaccination was rather ineffective in serological and virological terms, most probably due to the immune interference caused by high MDA.

- PCV2 infection dynamics: Under field conditions, PCV2 viraemia usually starts at second half of the nursery or at the beginning of fattening periods, although it varies from one farm to another and even between production batches (Segalés *et al*., 2012). Very early infection dynamics can also occur when the sow herd is not stable. Therefore, sow vaccination should be applied to achieve an early protective immunity in the piglets. Although high prevalence of PCV2 viraemia in new-born piglets has been reported in some studies (Shen *et al*., 2010; Dvorak *et al*., 2013), generally, this percentage of infection is usually low (Eddicks *et al*., 2016). As explained in *Chapter 1*, horizontal transmission is very efficient in susceptible piglets. Therefore, it is important to protect the animals (via sow vaccination) prior to the infection. Furthermore, it has been experimentally demonstrated that immunization of PCV2 viraemic piglets in presence of MDA is effective, since this protocol achieved a

reduction of PCV2 viraemia and PCV2 DNA in lymphoid tissues after experimental inoculation (Seo *et al*., 2014c). Nevertheless, it is very likely that the efficacy of vaccinating already viraemic pigs is not as good as vaccinating non-viraemic pigs with sufficient anticipation to the infection timing, as was also observed in *Chapter 5*.

- Immunosuppression: Any factor that may cause immunosuppression or modulation of the immune system at the time of vaccination can reduce vaccination efficacy. Among these factors, secondary infections or concurrent diseases, stress and malnutrition are included (Chase and Lunney, 2012; Lee *et al*., 2016).

The impact of all these variables on the vaccine efficacy will depend on the age at which the piglet PCV2 vaccination is performed. Therefore, taking into account all these factors, it can be concluded that the optimal age for piglet vaccination would be the one that combines the presence of low values of MDA and the development of a protective immunity against PCV2 before the occurrence of natural infection, as observed in *Chapter 5* (Figure 6-1).

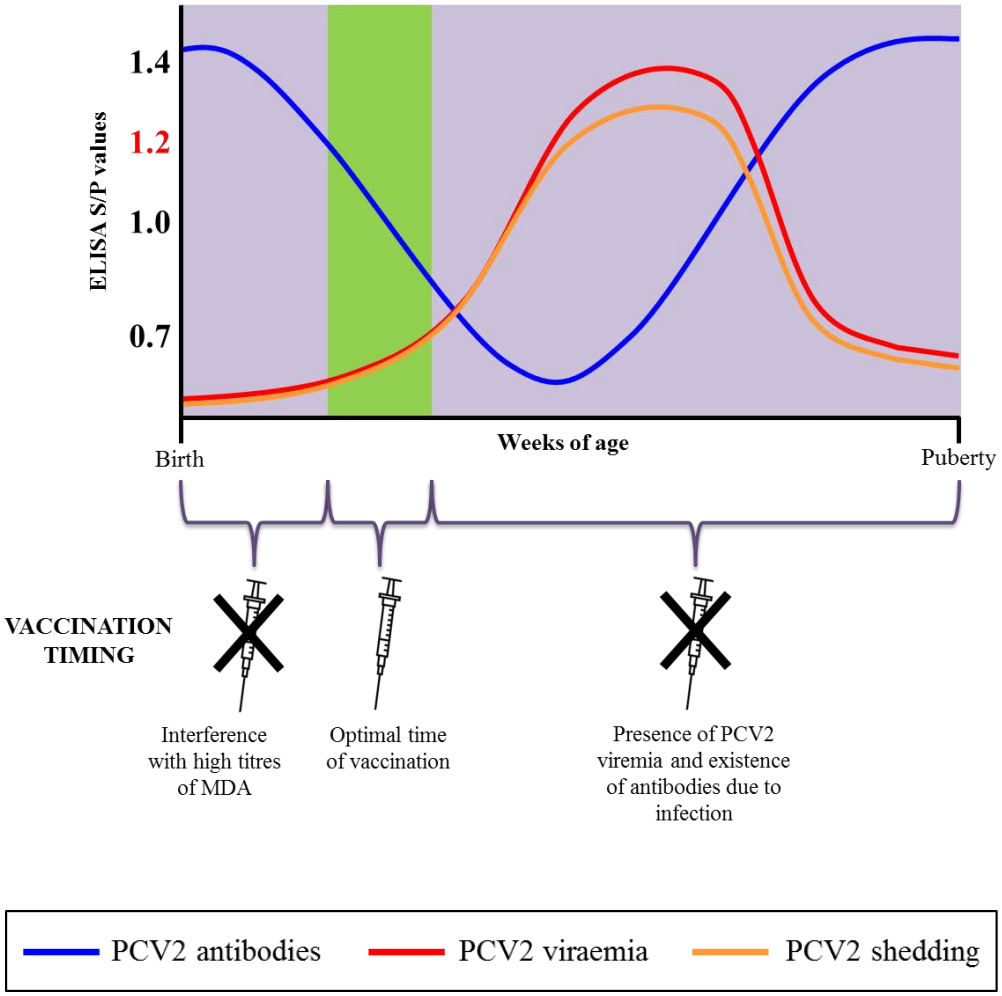

Figure 6-1. Determining the optimal time for PCV2 piglet vaccination.

The second purpose of *Chapter 5* was to compare seroconversion and infection dynamics obtained in serum with respect to OF during all the study duration. Although OF could not be collected at the first sampling point (2 weeks of age) since suckling piglets did not chew the OF ropes, antibody patterns detected in sera from the second sampling point (6 weeks of age) onward were also effectively assessed through the OF use for each treatment group. In fact, a high and significant correlation in PCV2 antibody detection was observed between

sera and OF, suggesting that OF sampling can be a useful alternative method to sera for antibody dynamics monitoring after vaccination. These results agree with those previously described on the evaluation of humoral responses in OF after PCV2 natural infection (Kim, 2010). In contrast, the high percentage of PCV2 PCR positive pens detected in OF throughout the study was one of the reasons for not obtaining a significant correlation between sera and OF in virological terms. These PCR results seem reasonable since PCV2 is considered to be rather ubiquitous in the environment (Opriessnig *et al*., 2007; Dvorak *et al*., 2013), being able to contaminate the ropes in contact with floor and walls of the pen, secretions, faeces, etc. In addition, OF is a collective sample representing all animals in the pen, while blood samples were individually collected from only 2-4 pigs per pen. Therefore, to achieve a greater association between both matrixes, it would have been interesting to compare individual samples of serum and OF taken from the same animal. This could be performed using, for instance, an individual OF collection device (such as Salivette®, Sarstedt AG & Co). Besides, this method would also reduce the possibility of OF contamination.

As a summary, in farms with low infection pressure or without early PCV2 circulation during lactation or at the beginning of the nursery period, PCV2 sow vaccination would not be recommended, as the cost would be probably higher than the benefits. However, the results of this Thesis further support the potential use of sow vaccination, since it may improve reproductive parameters (*Chapter 3*). In addition, the second study (*Chapter 4*) has also shown a greater adaptive cellular immune responses in new-born piglets from immunized sows, suggesting that these high IFN-γ/TNF-α levels in the progeny may be related to protection. The third study (*Chapter 5*) represents the first time in which, under a scenario of no vaccinated sows, three different PCV2 vaccination timings in piglets were compared. These results have a very practical application for

veterinary practitioners, since the selected farm had viral characteristics similar to many of current farms worldwide (PCV2-SI scenario and usual circulations timings). The other practical application obtained from this study is the ability to reliably monitor the antibody dynamics during a vaccination program by using an easy-sampling, economic, non-aggressive and non-stressful method such as OF.

CHAPTER 7

General Conclusions

1. Immunization of the sow with a PCV2 vaccine at 6 and 3 weeks before farrowing significantly increases the antibody levels at farrowing. A single booster dose in the next gestational cycle around 2 weeks pre-farrowing maintains high antibody levels at delivery.

2. PCV2 vaccination of sows before farrowing during two consecutive gestations in a PCV2 infected farm without overt reproductive problems has a positive effect on the number of live-born piglets per litter at the second delivery.

3. Piglets born from sows vaccinated against PCV2 before farrowing have higher vitality scores during the first hours of life compared to those coming from non-vaccinated ones.

4. Peripheral blood mononuclear cells isolated from piglets born from pre-farrowing PCV2 vaccinated sows release higher levels of IFN-γ/TNF-α after stimulation with a PCV2 Cap protein compared to those coming from non-vaccinated sows. This fact suggests a greater adaptive cellular immune response in piglets coming from vaccinated sows at 48-72 hours after birth.

5. Piglet vaccination against PCV2 at 3 or 6 weeks of age offers the best efficacy in terms of viraemia reduction, since these strategies induce an early seroconversion capable of controlling the subsequent PCV2 natural infection. In contrast, a vaccination scheme at 10 weeks of age is less effective, since it is not able to control the proportion of viraemic pigs until the end of fattening period.

6. Oral fluid (OF) collected at pen level is a useful sample for the evaluation of antibody dynamics during a vaccine monitoring program. However, since PCV2 is likely to be present in the pig environment, viral detection in OF should remain only as a raw indicative method.

CHAPTER 8

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