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Efficacy assessment of different vaccination strategies against Porcine circovirus 2 (PCV-2) based on clinical trials

Patricia Pleguezuelos García

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PhD Thesis

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Tesis doctoral presentada por Patricia Pleguezuelos García para acceder al grado de Doctora en el marco del programa de *Doctorat en Medicina i Sanitat Animals* de la *Facultat de Veterinària* de la *Universitat Autònoma de Barcelona*, bajo la dirección de Dr. Joaquim Segalés Coma, Dra. Marina Sibila Vidal y Dr. Sergio López Soria.

Bellaterra, 2023

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Certifican:

Que la memoria titulada "**Efficacy assessment of different vaccination strategies against *Porcine circovirus 2* (PCV-2) based on clinical trials**" presentada por **Patricia Pleguezuelos García** para la obtención del grado de Doctora en Medicina y Sanidad Animals, se ha realizado bajo su supervisión y tutoría, y autorizan su presentación para que sea valorada por la comisión establecida.

Y para que así conste a los efectos oportunos, firman la presente declaración en Bellaterra (Barcelona), a 14 de Junio del 2023.

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A mis padres y a mi hermana,
por darme la motivación necesaria para hacer cada uno de mis sueños realidad,
y a Fidel, por acompañarme en este camino y estar siempre a mi lado

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LIST OF ABBREVIATIONS

ADV	Aujeszky's disease virus
ADWG	Average daily weight gain
AEMPS	*Agencia Española de Medicamentos y Productos Sanitarios*
AI	Artificial insemination
APP	*Actinobacillus pleuropneumoniae*
bp	Base pair
Cap	Capsid
CI	Confidence interval
CLA	Conjugative linoleic acid
CVMP	Committee for Medicinal Products for Veterinary Use
cPCV-1/2a	PCV-1/PCV-2a chimera
cPCV-1/2b	PCV-1/PCV-2b chimera
DEPC	Diethylpyrocarbonate
dsDNA	Double-stranded DNA
ELISA	Enzyme linked immunosorbent assay
EMA	European Medicines Agency
EP	Enzootic pneumonia
EU	European Union
GL	Guideline
HE	Haematoxylin-eosin
HR	Histiocytic replacement
IFN	Interferon
IHC	Immunohistochemistry
IM	Intramuscular
IPMA	Immunoperoxidase monolayer assay
ISH	*In situ* hybridization
KLH–ICFA	Keyhole limpet hemocyanin in Freund’s incomplete adjuvant
LD	Lymphocyte depletion
LOD	Limit of detection
LOQ	Limit of quantification

MDA	Maternally derived antibodies
MDI	Maternally derived immunity
M. hyopneumoniae	*Mycoplasma hyopneumoniae*
NA	Neutralizing antibodies
NV	Non-vaccinated group
OD	Optical density
ORFs	Open reading frames
PBS	Phosphate buffered saline
Ph. Eur.	European Pharmacopoeia
PCR	Polymerase chain reaction
PCV	*Porcine circovirus*
PCVAD	Porcine circovirus associated diseases
PCVD	Porcine circovirus disease
PCV-1	*Porcine circovirus 1*
PCV-2	*Porcine circovirus 2*
PCV-3	*Porcine circovirus 3*
PCV-4	*Porcine circovirus 4*
PCV-2-RD	PCV-2-reproductive disease
PCV-2-SD	PCV-2-systemic disease
PCV-2-SI	PCV-2-subclinical infection
PDNS	Porcine dermatitis and nephropathy syndrome
PK	Porcine kidney
PMWS	Postweaning multisystemic wasting syndrome
PPV	*Porcine parvovirus*
PRDC	Porcine respiratory disease complex
PRRSV	*Porcine reproductive and respiratory syndrome virus*
PUC	Porcine umbilical cord
qPCR	Real-time quantitative PCR
SD	Standard deviation
SE	Standard error
SIV	*Swine influenza virus*
SC	Secreting cells

S/P	Sample/positive control
ssDNA	Single-stranded DNA
TMB	tetramethylbenzidine
TTSuV1a	*Torque teno sus virus 1a*
VICH	Veterinary International Conference on Harmonization
V	Vaccinated group
V LG	Vaccinated at late gestation
V MG	Vaccinated at mid gestation
V PM	Vaccinated at pre-mating
WOA	Weeks of age

ABSTRACT

Porcine Circovirus 2 (PCV-2) is a ubiquitous swine virus and the causative agent of clinical and subclinical manifestations comprised under the term of porcine circovirus diseases (PCVDs). PCV-2 vaccination is the most effective tool for controlling infection. There are several commercial vaccines to be administered in piglets, but only two of them are also authorized for sows. Therefore, existing products allow different vaccination regimes that may combine vaccination of piglets and/or sows.

In this context, the objective of this thesis was the implementation of different vaccine strategies against PCV-2 in sows and piglets for the evaluation of their efficacy through clinical trials.

Study I explored the benefits of PCV-2 vaccination in sows (n=188) in terms of productive, virological and serological parameters. Immunization was carried out at different stages of the production cycle (before insemination, mid or late gestation) mimicking a blanket vaccination fashion. The negative control group received phosphate-buffered saline at the same vaccination days. Reproductive parameters and offspring weight at birth and weaning were recorded. Blood samples were taken from sows at the time of vaccination and from piglets at 3 weeks of age (WOA) to analyse the detection of the genome and antibodies by qPCR and/or ELISA, respectively. Additionally, placental umbilical cords (PUC) were taken for qPCR analysis. The overall results indicated that vaccinated sows had heavier piglets at birth and weaning, less adoptions, lower viral load at birth and in PUCs, and higher levels of antibodies at birth, than non-vaccinated sows. When comparison was performed between the four groups, sows vaccinated at mid- or late-gestation had heavier piglets at birth and a lower proportion of positive PCV-2 qPCR PUC than non-vaccinated sows. In addition, adoptions were lower in sows vaccinated before artificial insemination and mid-gestation than in non-vaccinated sows. Therefore, the present study showed benefits of sow vaccination against PCV-2 mimicking a blanket vaccination at the productive, serological and virological levels.

Studies II and III evaluated the efficacy of a trivalent vaccine based on inactivated PCV-2a and PCV-2b chimeric viruses together with a *Mycoplasma hyopneumoniae* bacterin. This

product was administered in two different regimens: one piglet dose at 3 WOA (Study II, n=4,076) and two doses, at 3 days of age and 3 weeks later (Study III, n=3,973). Blood samples were taken prior to (first) vaccination, as well as blood samples and faecal swabs at 7, 11, 16, 20 and 25 WOA for analysis by qPCR and/or ELISA. Weight was recorded before vaccination, at 16 WOA and before slaughter. Animals dead or euthanized during the study were necropsied and sampled (superficial inguinal, tracheobronchial, and mesenteric lymph nodes plus tonsil). Samples were analysed by PCV-2 histopathology and immunohistochemistry (IHC) for the diagnosis of PCVD. Upon detection of the first case of PCVD, 60 animals were selected for necropsy and sampling to perform histopathology and IHC analysis of PCV-2. The efficacy of this trivalent vaccine was demonstrated in both regimens in terms of reduction of lymphoid lesions and detection of PCV-2 in tissues, viraemia, and faecal excretion, as well as improvement of productive parameters (body weight and average daily weight gain) in vaccinated animals compared to non-vaccinated animals. Even so, it should be noted that interference of maternal antibodies with seroconversion of the vaccine was detected.

RESUMEN

El *Circovirus porcino 2* (PCV-2) es un virus porcino ubicuo a nivel mundial y el agente causal de manifestaciones clínicas y subclínicas (en inglés, *porcine circovirus diseases*, PCVDs). La vacunación frente a PCV-2 es la herramienta más eficaz que existe para controlar la infección. Existen varias vacunas comerciales para ser administradas en lechones, pero solo dos de ellas están autorizadas también para cerdas. Por tanto, los productos existentes permiten diferentes regímenes de vacunación que pueden combinar la vacunación de lechones y/o cerdas.

En este contexto, el objetivo de la presente Tesis fue la implementación de diferentes estrategias vacunales frente a PCV-2 en cerdas y lechones para la evaluación de su eficacia mediante ensayos clínicos.

El estudio I exploró los beneficios de la vacunación de PCV-2 en cerdas (n=188) en cuanto a parámetros productivos, virológicos y serológicos. La inmunización se realizó en diferentes etapas del ciclo productivo (antes de la inseminación, a mitad o a final de gestación) simulando la vacunación en sabana. El grupo control negativo recibió solución salina tamponada con fosfato. Se registraron los parámetros reproductivos y el peso de la descendencia al nacimiento y al destete. Se tomaron muestras de sangre de cerdas en los momentos de vacunación y de los lechones a 3 semanas de vida (SDV) para analizar la detección del genoma y anticuerpos mediante qPCR y/o ELISA, respectivamente. Adicionalmente, se tomaron muestras de cordón umbilical (PUC) para analizar por qPCR. Los resultados globales indicaron que las cerdas vacunadas tuvieron lechones más pesados al nacimiento y destete, menos adopciones, menor carga viral en el parto y en los PUC, y mayores niveles de anticuerpos en el parto, que las no vacunadas. Al comparar los cuatro grupos, las cerdas vacunadas a mitad o finales de gestación tuvieron lechones más pesados al nacimiento y una menor proporción de PUC qPCR PCV-2 positivos que las cerdas no vacunadas. Además, las adopciones fueron menores en las cerdas vacunadas antes de la inseminación artificial y a mitad de gestación que en las no vacunadas. Por tanto, el presente estudio mostró beneficios a nivel productivo, serológico y virológico de la vacunación en sabana de cerdas frente a PCV-2.

En los estudios II y III se evaluó la eficacia de una vacuna trivalente basada en virus quiméricos inactivados de PCV-2a, PCV-2b juntamente con una bacterina de *Mycoplasma hyopneumoniae*. Este producto se administró en dos regímenes diferentes: una dosis a lechones de 3 SDV (estudio II, n=4.076) y dos dosis, a los 3 días de vida y 3 semanas después (estudio III, n=3.973). Se tomaron muestras de sangre antes de la (primera) vacunación, así como muestras de sangre e hisopo fecal a las 7, 11, 16, 20 y 25 SDV para analizar por qPCR y/o ELISA. El peso se registró antes de la (primera) vacunación, a 16 SDV y antes de matadero. Los animales muertos/sacrificados durante el estudio fueron necropsiados y muestreados (linfonodos inguinales superficiales, traqueobronquiales, mesentéricos y tonsila). Las muestras se analizaron por histopatología e inmunohistoquímica (IHC) de PCV-2 para el diagnóstico de circovirosis porcina (CP). Al detectar el primer caso de CP, se seleccionaron 60 animales para necropsia y muestreo (para estudios de histopatología y IHC de PCV-2). La eficacia de esta vacuna trivalente se demostró en los dos regímenes en términos de reducción de lesiones linfoides y detección de PCV-2 en tejidos, viremia y excreción fecal, así como la mejora de los parámetros productivos (peso corporal y ganancia media diaria) en los animales vacunados en comparación con los no vacunados. Aun así, cabe destacar que se detectó interferencia de los anticuerpos maternales con la seroconversión a la vacunación.

RESUM

El *Circovirus porcí 2* (PCV-2) és un virus porcí ubicu a nivell mundial i l'agent causal de varies manifestacions clíniques i subclíniques (en anglès, *porcine circovirus diseases*, PCVDs). La vacunació enfront a PCV-2 és l'eina més eficaç que existeix per controlar la infecció. Existeixen diverses vacunes comercials per a l'administració en garrins, però només dues estan autoritzades també per a truges. Per tant, els productes existents permeten diferents règims de vacunació que poden combinar la vacunació de garrins i/o truges.

En aquest context, l'objectiu d'aquesta Tesi va ser la implementació de diferents estratègies vacunals enfront a PCV-2 en truges i garrins per l'avaluació de la eficàcia mitjançant assajos clínics.

L'estudi I va explorar els beneficis de la vacunació enfront PCV-2 en truges (n=188) en relació a paràmetres productius, virològics i serològics. La immunització es va realitzar en diferents etapes del cicle productiu (abans de la inseminació, a meitat o a finals de gestació) simulant la vacunació en llençol. El grup control negatiu va rebre, en els mateixos moments, una solució salina tamponada amb fosfat. Es van registrar els paràmetres reproductius i el pes de la descendència al naixement i al deslletament. Es van prendre mostres de sang de truges en els moments de vacunació i dels garrins a 3 setmanes de vida (SDV) per analitzar la detecció del genoma i anticossos mitjançant qPCR i/o ELISA, respectivament. Addicionalment, es van prendre mostres de cordó umbilical (PUC) per analitzar mitjançant qPCR. Els resultats van indicar que les truges vacunades van tenir garrins més pesats al naixement i deslletament, menys adopcions, menor càrrega viral en el part i en els PUC, i majors nivells d'anticossos al part, que les no vacunades. Al comparar els quatre grups, les truges vacunades a meitat o finals de gestació van tenir garrins més pesats al naixement i una menor proporció de PUC qPCR PCV-2 positius que les truges no vacunades. A més, les adopcions van ser menors en les truges vacunades abans de la inseminació artificial i a meitat de gestació que en les no vacunades. Per tant, aquest estudi va mostrar beneficis a nivell productiu, serològic i virològic de la vacunació en llençol de truges enfront a PCV-2.

Els estudis II i III van permetre avaluar l'eficàcia d'una vacuna trivalent basada en virus quimèrics inactivats de PCV-2a, PCV-2b juntament amb una bacterina de *Mycoplasma*

hyopneumoniae. Aquest producte es va administrar en dos règims diferents: una dosi a garrins de 3 SDV (estudi II, n=4.076) i dues dosis als 3 dies de vida i 3 setmanes després (estudi III, n=3.973). Es van prendre mostres de sang abans de la vacunació, i mostres de sang i hisop fecal a les 7, 11, 16, 20 i 25 SDV per analitzar mitjançant qPCR i/o ELISA. El pes es va registrar abans de la (primera) vacunació, a 16 SDV i abans d'escorxador. Els animals morts/sacrificats durant l'estudi van ser necropsiats i mostrejats (linfonodes inguinals superficials, traqueobronquials, mesentèrics i tonsil·la). Les mostres es van analitzar per histopatologia i immunohistoquímica (IHC) de PCV-2 per al diagnòstic de circovirosi porcina (CP). En detectar el primer cas de CP es van seleccionar 60 animals per a necròpsia i mostreig (per realitzar histopatologia i IHC de PCV-2). L'eficàcia d'aquesta vacuna es va demostrar en els dos règims en termes de reducció de lesions limfoides i detecció de PCV-2 en teixits, virèmia i excreció fecal, així com la millora dels paràmetres productius (pes corporal i guany mig diari) en els animals vacunats en comparació amb els no vacunats. Tot i així, cal destacar que es va detectar interferència dels anticossos maternals amb la seroconversió a la vacunació.

PUBLICATIONS

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

- **Study I:** Pleguezuelos, P., Sibila, M., Cuadrado, R., López-Jiménez, R., Pérez, D., Huerta, E., Llorens, A. M., Núñez, J. I., Segalés, J., López-Soria, S., 2021. Exploratory field study on the effects of *Porcine circovirus 2* (PCV-2) sow vaccination at different physiological stages mimicking blanket vaccination. Porcine health management 7, 35. doi:10.1186/s40813-021-00213-2.

- **Study II:** Pleguezuelos, P., Sibila, M., Ramírez, C., López-Jiménez, R., Pérez, D., Huerta, E., Llorens, A. M., Pérez, M., Correa-Fiz, F., Mancera Gracia, J. C., Taylor, L. P., Smith, J., Bandrick, M., Borowski, S., Saunders, G., Segalés, J., López-Soria, S., Fort, M., Balasch, M., 2022. Efficacy studies against PCV-2 of a new trivalent vaccine including PCV-2a and PCV-2b genotypes and *Mycoplasma hyopneumoniae* when administered at 3 weeks of age. Vaccines 10, 2108. doi:10.3390/vaccines10122108.

- **Study III:** Pleguezuelos, P., Sibila, M., Cuadrado-Matías, R., López-Jiménez, R., Pérez, D., Huerta, E., Pérez, M., Correa-Fiz, F., Mancera-Gracia, J. C., Taylor, L. P., Borowski, S., Saunders, G., Segalés, J., López-Soria, S., Balasch, M., 2022. Efficacy studies of a trivalent vaccine containing PCV-2a, PCV-2b genotypes and *Mycoplasma hyopneumoniae* when administered at 3 days of age and 3 weeks later against *Porcine circovirus 2* (PCV-2) Infection. Vaccines 10, 1234. doi:10.3390/vaccines10081234.

CHAPTER 1

General Introduction

1.1 PORCINE CIRCOVIRUS 2 (PCV-2)

1.1.1 History of PCV-2

A non-cytopathic, picornavirus-like contaminant of porcine kidney (PK) cell line PK-15 was first described in 1974 (Tischer *et al.,* 1974). This very small DNA virus was subsequently called as *Porcine circovirus* (PCV) (Tischer et al., 1982). This cell contaminant virus was ubiquitous in the nature since different serological surveys detected a high prevalence of anti-PCV antibodies in swine populations from several countries all over the world, including Germany, Canada, England, Ireland, and USA (Tischer et al., 1986; Dulac and Afshar, 1989; Allan et al., 1994; Edwards and Sands, 1994). However, this virus was not associated to any disease neither under experimental conditions nor on commercial farms. Hence, it was considered a non-pathogenic virus (Tischer et al., 1986; Allan et al., 1995; Tischer et al., 1995).

Few years later, in 1991 and 1994, Canadian farmers reported an increase in nursery mortality about 12-15% and affected pigs showing jaundice, diarrhoea, respiratory disease, icterus, sudden death and characteristic microscopic lesions mainly in lymphoid organs (Harding et al., 1997). These clinicopathological findings were considered a new disease referred as post-weaning multisystemic wasting syndrome (PMWS). Nucleotide sequence analysis of the associated PCV revealed that it had a 68% of homology with the previously known cell-contaminant PCV, indicating these two viruses were distinct (Allan et al., 1998; Hamel et al., 1998; Meehan et al., 1998). Therefore, the PK-15 originated PCV was named as *Porcine circovirus type 1* (PCV-1) while the new DNA-virus associated with PMWS was designated as *Porcine circovirus type 2* (PCV-2) (Meehan et al., 1998; Allan et al., 1999a).

Although the first description of the disease in Canada was performed during 1990s, PCV-2 and PMWS were already present in swine population many years before (Jacobsen et al., 2009). Retrospective polymerase chain reaction (PCR) analyses demonstrated PCV-2 genome in pig tissues from 1962; the oldest description of PCV-2 detection in tissues with PMWS-compatible lesions is from 1985 (Jacobsen et al., 2009). Since then, PMWS has spread worldwide becoming an economically devastating swine disease (Armstrong and Bishop, 2004).

Currently, PMWS is known as PCV-2-systemic disease (PCV-2-SD) (Segalés, 2012) and it is included within the group of diseases collectively known as porcine circovirus disease (PCVD) (Segalés et al., 2005a) in Europe or porcine circovirus associated diseases (PCVAD) in North America (Opriessnig et al., 2007). PCVDs include three other conditions, apart from PCV-2-SD named as PCV-2-subclinical infection (PCV-2-SI), PCV-2-reproductive disease (PCV-2-RD) and porcine dermatitis and nephropathy syndrome (PDNS) (Segalés, 2012).

1.1.2 The virus

PCV-2 is a member of the genus Circovirus in the family *Circoviridae* (Franzo et al., 2015; Rosario et al., 2017) and has a close circular ssDNA surrounded by an icosahedral protein-based capsid. This capsid has no envelope and measures approximately 17nm in diameter (Tischer et al., 1982). The ssDNA consists of 1766-1769 nucleotides that encodes for proteins in both senses, in a clockwise and counterclockwise manner resulting in an ambisense organization of the genome (Hamel et al., 1998; Meehan et al., 1998; Mankertz et al., 2004).

The PCV-2 genome contains 11 potential open reading frames (ORFs), from ORF1 to ORF11 (Hamel et al., 1998). However, only 4 ORFs have been functionally characterized: ORF1 (essential for replication proteins, Rep and Rep′), ORF2 (encodes the structural and immunogenic capsid [Cap] protein), ORF3 (encoding for a non-structural protein that induces host cell apoptosis *in vitro*) and ORF4 (encodes for a protein involved in apoptosis suppression) (Mankertz et al., 1998; Cheung, 2003; Blanchard et al., 2003; Shang et al., 2009; Liu et al., 2005; Liu et al., 2006; Lin et al., 2011; Gao et al., 2014).

PCV-2 is a ubiquitous virus in the domestic pig population and wild boar (Ellis et al., 2003; Vicente et al., 2004; Segalés et al., 2005a) since the virus has been reported on the five continents and a minimal number of PCV-2 antibody free farms have been detected (Larochelle et al., 2003; Rose et al., 2003; López-Soria et al., 2005).

Although PCV-2 infects exclusively suidae species (Allan et al., 2000a; Ellis et al., 2001; Ellis et al., 2000), PCV-2 DNA has been detected in mice (65%) and rats (24%) inhabiting

pig farms. This finding indicates a possible role of rodents as reservoirs and mechanical vectors of PCV-2 transmission (Lorincz et al., 2010; Pinheiro et al., 2013).

1.1.3 PCV-2 genotypes

Nowadays, nine genotypes are described; from PCV-2a to PCV-2i (Franzo and Segalés, 2018; Wang et al., 2020). This increase in the number of PCV-2 genotype definitions is probably due to its high nucleotide substitution rate (Firth et al., 2009).

Since the first PCV-2 detection in 1996 until now, two global genotype shifts have occurred from an epidemiological point of view. The first one happened around 2000-2003 when a shift from PCV-2a to PCV-2b took place (Patterson and Opriessnig, 2010), coinciding with the transition from sporadic to epidemic cases of PCV-2-SD outbreaks in America, Asia, and Europe (Carman et al., 2006; Dupont et al., 2008; Cortey et al., 2011a). Moreover, PCV-2d, although retrospectively and firstly detected in Swiss samples collected in 1998, its detection increased (Wiederkehr et al., 2009) from 2012 onwards, inducing a second globally genotype shift from PCV-2b to PCV-2d, probably associated to the worldwide PCV-2 vaccination pressure (Franzo and Segalés, 2018). Importantly, genotype co-infection with PCV-2a, PCV-2b and/or PCV-2d has been reported within the same farm and the same pig (Grau-Roma et al., 2008; Hesse et al., 2008; Correa-Fiz et al., 2018).

1.2 PORCINE CIRCOVIRUS DISEASES (PCVD)

PCVDs comprise four main conditions (Table 1.1): PCV-2-SD, PCV-2-SI, PCV-2-RD and PDNS (Segalés, 2012).

PCV-2-SD mainly affects pigs between 30 and 180 days of age, being the ones from 60 to 90 days of age the most commonly affected. Morbidity in affected non-vaccinated farms usually ranges between 4–30% (reaching 50–60% in some cases), and mortality between 4–20% (Segalés and Domingo, 2002).

PCV-2 infection is mainly controlled using vaccines, resulting in a reduction of PCV-2-SD outbreaks worldwide. However, these vaccines are not sterilizing, and the virus is still

considered ubiquitous (Segalés et al., 2013; Feng et al., 2014), implying that the most common presentation nowadays is the PCV-2-SI (Young et al., 2011).

PCV-2-RD is infrequently detected under field conditions (Pensaert et al., 2004), probably due to the high seroprevalence of PCV-2 in adult pigs, preventing clinical disease development (Segalés, 2012). This disease is usually observed in herds with a high range of gilts or seronegative sows (West et al., 1999; Opriessnig et al., 2007; Togashi et al., 2011). Moreover, porcine embryonic cells have a susceptibility to PCV-2 infection that increases with the development stage (Mateusen et al., 2004). When PCV-2 infection occurs intrauterine, the infection may happen at different embryonic or foetal stages (Madson and Opriessnig, 2011; Segalés, 2012):

- At the early gestation stage, PCV-2 replicates in zona-pellucida-free morulae and blastocysts (Mateusen et al., 2004; Mateusen et al., 2007) causing death of the embryo and resulting in the reabsorption in the utero (Mateusen et al., 2007). When a total embryonic mortality occurs, the sow returns to oestrus. In case of a partial embryonic death, gestation continue with the surviving embryos.

- At mid gestation (between 40 and 70 days of gestation), main PCV-2 susceptible cells are myocardiocytes, hepatocytes, and monocyte-macrophage lineage cells (Sánchez et al., 2003). Cardiac tissues contain the highest viral load. In most cases, foetuses die in the uterus and mummification may occur (Pensaert et al., 2004).

- At late gestation (between 70 and 115 days of gestation), PCV-2 replication decreases due to immunocompetence of the foetus (Madson et al., 2009c).

PDNS is an immune-mediated disorder with a very low prevalence in non-PCV-2 vaccinated herds (<1%) and affecting late weaning, and growing/ finishing animals and, in some cases, sows (Drolet et al., 1999). The specific etiological agent of PDNS is still unknown (Segalés and Sibila, 2022; Drolet et al., 1999). Immunocomplex diseases can theoretically be triggered by a variety of factors, but circumstantial evidence involved PCV-2 and high antibody titres against this virus as the most likely risk factors (Wellenberg et al., 2004). Although never demonstrated under experimental conditions, the extensive use of PCV-2

vaccination across the world practically eliminated the occurrence of this pathological condition. It has been claimed that co-infection by *Torque teno sus virus 1a* (TTSuV1a) and *Porcine reproductive and respiratory syndrome virus* (PRRSV) generated a PDNS-like condition (Krakowka et al., 2008). Also, a single experimental infection of weaning pigs with *Porcine circovirus 3* (PCV-3) caused lesions that were suggested being compatible with PDNS (Jiang et al., 2019). In both cases, macroscopic and microscopic lesions described did not fit with the original reports of PDNS (Segalés and Sibila, 2022). Moreover, although pathogenesis of *Porcine circovirus 4* (PCV-4) is not yet well established, co-infections of PCV-4 with PCV-2 have been reported in pigs with clinical signs of PDNS (Tian et al., 2021; Wang et al., 2022).

Table 1.1. Summary of major clinical signs, macroscopic and microscopic findings, as well as the individual case definition of described PCVDs (adapted from Segalés, 2012, and Segalés and Sibila, 2022).

	PCV-2-SI	PCV-2-SD	PCV-2-RD	PDNS
Main clinical signs	Slower growth with no evidence of clinical signs	Wasting, weight loss, rough hair coat, respiratory distress, diarrhoea	Abortions, mummification, return-to-oestrus, stillbirth, and embryonic death	Red to purple macules and papules on the skin, mostly on the hind limbs and perineal area
Main macroscopic findings	No evidence of macroscopic lesions	Enlargement of lymph nodes. Thymus atrophy. Non-collapsed and tan-mottled lungs. Pale and atrophic liver with granular surface. White spots in the kidney's cortex and catarrhal enteritis	Foetal hepatic enlargement and congestion, plus cardiac hypertrophy with multifocal pale areas in myocardial area. Oedematous foetus, hydrothorax and ascites may be also detected in foetus.	Subcutaneous haemorrhages and oedematous affected tissues. Lymph node enlargement and renal petechiae with oedema of the renal pelvis
Main microscopic findings	None or mild lymphoid depletion and granulomatous infiltration in lymphoid tissues	Lymphocyte depletion (LD) with granulomatous infiltration. Interstitial pneumonia, interstitial nephritis and lymphohistiocytic hepatitis. Granulomatous enteritis. Possibility of lymphohistiocytic inflammatory infiltrates in many tissues	Non-suppurative, fibrotic or necrotic myocarditis in foetuses. Hepatic congestion and mild pneumonia in foetuses.	Systemic necrotizing vasculitis. Fibrino-necrotizing glomerulitis with non-purulent interstitial nephritis. Mild/moderate LD with mild granulomatous inflammation of lymphoid tissues
Diagnosis	1.Lack of evident clinical signs 2.No or mild histopathological lesions, mainly in lymphoid tissues 3.Low PCV-2 load in few (lymphoid) tissues, usually in follicular areas Criteria 2 and 3 can be substituted by PCV-2 detection techniques such as standard PCR.	1. Weight loss and paleness of skin. Possibility of respiratory and/or digestive clinical signs 2.Moderate/severe LD with granulomatous inflammation of lymphoid tissues, and granulomatous inflammation in other tissues. 3. Moderate/high PCV-2 load in lymphoid tissues. Variable amount in the rest of affected tissues	<u>Abortions or mummies:</u> 1.Reproductive failure at late gestation or SMEDI-like condition * 2.Fibrous and/or necrotizing myocarditis of foetuses 3.Moderate/high PCV-2 load in the foetal heart <u>Regular return-to-oestrus:</u> 1.Regular return-to oestrus/infertility 2.PCV-2 seroconversion following the return-to oestrus and/or PCV-2 PCR/ qPCR positivity around the return-to oestrus	1. Haemorrhagic and necrotizing skin lesions and/or swollen and pale kidneys with generalized cortical petechiae. 2. Systemic necrotizing vasculitis, and necrotizing and fibrinous glomerulonephritis PDNS does not currently include the detection of PCV-2

*SMEDI-like condition: stillbirth, mummification, embryonic death and infertility (this last condition apply to return-to-oestrus scenario).

1.2.1 PCV-2 Transmission

In general terms, PCV-2 can be transmitted through different routes (Patterson and Opriessnig, 2010) mainly divided in horizontal and vertical ones:

- **Horizontal transmission**

Faecal-oral and nose-to-nose contact are considered the most frequent PCV-2 transmission routes (Rose et al., 2012). In fact, it has been observed a large amount of the virus in nasal, oral, and faecal secretions (Sibila et al., 2004; Patterson et al., 2011). However, viral infection may also occur indirectly by contaminated fomites, aerosol droplets or infected living vectors (Segalés et al., 2005b; Madson and Opriessnig, 2011). Indeed, when animals are in direct contact, the virus is more efficiently transmitted than when pigs are allocated in different pens (Andraud et al., 2008; Kristensen et al., 2009).

In the lactation period, sows and piglets may be viraemic (Grau-Roma et al., 2009; Shen et al., 2010), indicating the possibility of transmission from sow to piglet by direct contact or by colostrum and milk (Shibata et al., 2006; Ha et al., 2009; Ha et al., 2010; Patterson and Opriessnig, 2010; Rose et al., 2012).

- **Vertical transmission**

Vertical transmission, understood as transmission from one generation to the subsequent one via infection of the embryo or foetus *in utero,* can occur in the following scenarios:

- **PCV-2 infected sows**: Transplacental transmission has been proven after *in utero* PCV-2 inoculation of foetuses (Johnson et al., 2002; Sanchez et al., 2001) and in foetuses of sows experimentally infected three weeks before farrowing by intranasal route (Park et al., 2005; Ha et al., 2008). PCV-2 can pass over the placental barrier and replicate in embryos and foetuses, resulting in reproductive failures such as abortion, increased rates of mummified, stillborn and weak-born piglets (O'Connor et al., 2001; Madson and Opriessnig, 2011) and probably early embryonic death (Kim et al., 2004; Mateusen et al., 2007; Madson and Opriessnig, 2011). Moreover, the virus has been associated with myocarditis in aborted foetuses and in stillborns (West et al., 1999; Brunborg et al., 2007). However, PCV-2 infection associated to reproductive failure has been rarely reported in Europe (Pensaert et al., 2004; Maldonado et al., 2005). Thus, the relevance of PCV-2

infection in reproductive failure under field conditions is probably limited, since clinical and noticeable reproductive disorders associated to PCV-2 are infrequent (Pensaert et al., 2004; Madson and Opriessnig, 2011; Karuppannan et al., 2016). On the other hand, the impact of PCV-2-SI on reproduction is poorly known.

- **PCV-2 infected boars**: PCV-2 has been detected in semen of naturally and experimentally infected boars (Larochelle et al., 2000; Kim et al., 2003a; McIntosh et al., 2006; Schmoll et al., 2008; Madson et al., 2009a). Moreover, sows artificial inseminated with PCV-2-spiked semen showed reproductive failures and infected foetus (Rose et al., 2007; Madson et al., 2009b; Sarli et al., 2012). However, these results are questionable under field conditions, since the viral load excreted on semen is probably too low to infect the foetuses as reported in experimental studies (Rose et al., 2012).

1.2.2 Risk factors for PCV-2-systemic disease development

Factors detected as risk factors to promote the PCV-2-SD development are described below:

- **Immune status:** PCV-2 maternally derived antibodies (MDA) are considered protective against PCVD development (Rodríguez-Arrioja et al., 2002; López-Soria et al., 2005; Grau-Roma et al., 2009; Rose et al., 2009). In fact, a link between piglet PCV-2 immune status and the age of infection has been reported as piglets with MDA showed a significant decrease of early PCV-2 infection risk (Rose et al., 2009).

- **Timing of infection:** Some epidemiological studies have described that the earlier PCV-2 infection occurs, the higher the probability of PCV-2-SD development (Rose et al., 2003; López-Soria et al., 2005; Rose et al., 2009).

- **PCV-2 virulence:** Potential differences in virulence of PCV-2a, PCV-2b and PCV-2d genotypes has been suggested, although not unequivocally demonstrated (Guo et al., 2012, Cho et al., 2020). To date, experimental studies analysing differences in virulence showed controversial results. Whereas Opriessnig et al. (2014a) showed that infection with a PCV-2d strain induced more severe disease than PCV-2a or PCV-2b infections, these results were not supported by the findings of two other experimental inoculations.

(Seo et al., 2014; Cho et al., 2020). Besides, Suh et al. (2023) described a higher virulence when a co-infection of PCV-2d with PRRSV in terms of lymphoid lesions severity and clinical signs, compared to other 3 genotypes (2a, 2b, and 2e) also in combination with PRRSV. Therefore, the putative differences in virulence among different PCV-2 genotypes deserves further investigation.

- **Pig genetics:** Some studies reported breed genetic susceptibility to PCV-2-SD (López-Soria et al., 2004, Opriessnig et al., 2006; Lopez-Soria et al., 2011). One field study showed a high mortality due to PCV-2-SD in pure or crossbreed Pietrain in comparison with Large White–Duroc cross pigs (Lopez-Soria et al., 2011). Besides, an experimental infection demonstrated more susceptibility to PCV-2-SD and lesions in Landrace pigs than Large White and Duroc ones (Opriessnig et al., 2006). In contrast, a cohort field study did not find breed-related differences in mortality between piglets from sows inseminated with Pietrain semen and piglets from sows inseminated with the semen used in the commercial farm as per routine (Rose et al., 2005). Later, in another experimental study, it was observed that Landrace pigs infected with PCV-2 showed more severe lymphoid lesions than Pietrain ones (Opriessnig et al., 2009b). Although, differences observed in these studies can be breed-related or correlated to genes that might contribute to a better resistance or susceptibility, the relation between breed/genetic line and PCV-2 susceptibility remains poorly known and more studies are needed.

- **Management and husbandry factors:** The impact of PCV-2-SD can be significantly modified by farm biosafety, management, hygiene, housing, vaccination schedule and husbandry practices (Grau-Roma et al., 2011; Rose et al., 2012). In fact, the appropriate implementation of management measures showed a significant reduction of mortality in PCV-2-SD affected farms (Madec et al., 2000).

- **Nutrition:** Antioxidant feed additives like bioflavonoids, anthrocyanins and essential oils may decrease PCV-2-SD clinical expression and reduce the mortality (Donadeu et al., 2003). The addition of spray-dried plasma to the feed at weaning period can reduce PCV-2-SD incidence probably due to its capacity to provide immunoglobulins at intestinal mucosa level (Dewey et al., 2006). Moreover, conjugated linoleic acid (CLA) has important immunomodulatory properties, promoting the reduction of the microscopic

lesions and improvement of the cellular response after PCV-2 experimental infection (Bassaganya-Riera et al., 2003), Besides, a beneficial effect in PCV-2-SD of selenium supplementation in the diet has been observed at *in vitro* level inducing an inhibition of PCV-2 replication in PK-15 cells (Pan et al., 2008), but it was not confirmed *in vivo*. In any case, the specific effect of nutrition on PCV-2 infection outcome has been poorly explored.

- **Co-infections:** PCV-2 associated lesions and the incidence of PCV-2-SD under experimental and field conditions can be exacerbated by concomitant viral and bacterial infections (Opriessnig and Halbur, 2012). The most significant infectious agents considered as potential PCV-2-SD triggers are *Porcine parvovirus* (PPV), PRRSV, and *Mycoplasma hyopneumoniae (M. hyopneumoniae)* (Opriessnig and Halbur 2012; Rose et al., 2012).

  Co-infection of PCV-2 with PRRSV was one of the most common co-infections associated with swine disease under field conditions (Park et al 2014a) when no PCV-2 vaccines were used extensively. Noteworthy, it has been shown that PCV-2 and PRRSV experimental co-infection increased diseased impact and microscopic lesions (Allan et al., 2000b: Harms et al., 2001) and PRRSV infection potentiated PCV-2 replication (Allan et al., 2000b; Rovira et al., 2002). Similarly, PPV and PCV-2 co-infection induced more severe lesions and clinical signs than non-coinfected piglets (Allan et al., 1999b; Kennedy et al., 2000). Besides, PCV-2 and *M. hyopneumoniae* are two of the major players in porcine respiratory disease complex (PRDC) (Fraile and Sibila et al., 2012a; Kim et al., 2003b) causing a slower growth, lethargy, coughing, dyspnoea, fever and increasing of mortality in pigs between 16 and 22 weeks of age (Gerber et al., 2009; Jeong et al., 2016; Park et al., 2016). Some experimental trials have shown that incidence of PCV-2-SD and PCV-2 associated lesions can be triggered by concurrent infection with *M. hyopneumoniae* (Opriessnig et al., 2004b; Opriessnig et al., 2011a; Opriessnig and Halbur, 2012).

  However, in another study where a concurrent inoculation with *M. hyopneumoniae* and PCV-2 was performed, no synergistic clinical outcome was observed (Sibila et al., 2012). Controversial results can be probably explained by differences in timing of infection

among pathogens, number of animals per group, the source of animals (seronegative animals instead of seropositive) or the strain (and its virulence) used for the experimental inoculations.

- **Stimulation of the immune system:** Several research have revealed a synergy between the non-specific stimulation of the immune system and the development of PCV-2-SD clinical expression (Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). In these studies, keyhole limpet hemocyanin in Freund's incomplete adjuvant (KLH–ICFA) (Krakowka et al., 2001), as well as *M. hyopneumoniae* (Allan et al., 2001; Kyriakis et al., 2002) and *Actinobacillus pleuropneumoniae* (APP) bacterines (Opriessnig et al., 2003) were used as immunostimulants. However, other studies using the same immunostimulants did not report such association (Ladekjaer-Mikkelsen et al., 2002; Resendes et al., 2004). Therefore, according to the results obtained in these studies, the impact under field conditions of the immunomodulators might probably be low or inconsistent.

1.2.3 PCV-2 detection methods

Different laboratory methods have been developed to detect PCV-2 genome (PCR and ISH) or antigen (IHC) detection (Rosell et al., 1999; Segalés et al., 2004; Segalés and Sibila, 2022). ISH and IHC are used to detect PCV-2 genome and antigen, respectively, in tissues and to correlate its detection with the presence of histological lesions (Rosell et al., 1999; Segalés et al., 2004; Segalés and Sibila, 2022). However, one of the most commonly techniques used to detect PCV-2 genome is the PCR, and especially the real time quantitative PCR (qPCR) method (Segalés and Sibila, 2022).

The qPCR technique allows PCV-2 nucleic acid detection in a wide range of samples, such as blood, tissues, colostrum, semen, saliva as well as nasal, faecal and urinary secretions (Larochelle et al., 2000; Segalés et al., 2005a; Shibata et al., 2006; Ramirez et al., 2012), environment (air, manure, water, sow and piglet axillary skin surfaces, gestation crate floor and bar surfaces) (Verreault et al., 2010; Dvorak et al., 2013; Viancelli et al., 2012;) and also in non-porcine species inhabiting in swine farms (rodents, invertebrates) (Lorincz et al., 2010; Blunt et al., 2011). The mere detection of PCV-2 (by conventional PCR) does not

allow diagnosing PCVDs since the virus is ubiquitous and positive results with no clinical disease are common (Segalés and Sibila, 2022). This technique may be used for monitoring and surveillance, not only in serum, but also in oral fluids, placental umbilical cords, processing fluids or tissues and environmental samples (Segalés and Sibila, 2022).

A positive correlation between the amount of PCV-2 in tissues and the severity of microscopic lymphoid lesions was found (Rosell et al., 1999). In fact, the amount of PCV-2 in affected tissues is the main difference between PCV-2-SD and PCV-2-SI (Segalés and Sibila, 2022; Segalés, 2012). Hence, although the combination of histopathology of lymphoid tissues with ISH or IHC is considered the gold-standard for a confirmatory diagnostic approach to PCVD, the use of qPCR combined with microscopic lesions can also be used as a proxy (Segalés and Sibila, 2022).

PCV-2 antibody detection techniques such as immunoperoxidase monolayer assay (IPMA) and immunofluorescence assay and enzyme linked immunosorbent assay (ELISA) (Allan and Ellis, 2000; Rodriguez-Arrioja et al., 2000) cannot be used to diagnose PCVDs, since PCV-2 antibody detection may be derived from maternal transfer through colostrum, vaccination or from natural infection (Segalés and Sibila, 2022). However, these techniques are very useful for monitoring or surveillance purposes (Sibila et al., 2004; Grau-Roma et al., 2009; Ramirez et al., 2012; Turlewicz-Podbielska et al., 2020).

1.2.4 Immunity developed upon PCV-2 infection

The earlier line of defence against pathogen infections, including PCV-2, is the innate, non-adaptative immune response (Kekarainen and Segalés, 2015). This first barrier induces phagocytic cells, cytokines, chemokines, and proteins that provide antimicrobial protection, promotes the inflammatory process, and activates the adaptive immune response (Chase and Lunney, 2019).

Adaptative immune responses are divided in humoral and cellular immune response:

- **Humoral immune response**

Humoral immune responses may be developed in both *in utero* and after birth (Kekarainen and Segalés, 2015). In fact, it has been observed that immunocompetent foetuses (>70 days of gestation) are capable to develop antibodies against PCV-2 (Sanchez et al., 2001; Saha et al., 2010) and clear PCV-2 infection prior to parturition (Madson et al., 2009c). Moreover, maternally antibody transfer from sow to foetus throughout placenta, can occur in a limited manner. When happens, it seems that the higher the dam anti-PCV-2 antibody titres, the higher the probability of offspring antibody detection, probably by placental barrier leakage. Consequently, antibody detection in newborns that did not take yet the colostrum can be of maternal origin or from a PCV-2 intrauterine infection. For this reason, serological test results are not considered an accurate diagnostic method for intrauterine infections (Saha et al., 2014).

After birth, piglets are protected due to maternally derived immunity (MDI) obtained from sow via colostrum and milk (McKeown et al., 2005; Ostanello et al., 2005; Martinez-Boixaderas et al., 2022). Although we usually measure only antibodies (MDA), such transfer also includes a cell component. These antibodies and immune cells may cross the intestinal barrier and reach the peripheral blood (Martinez-Boixaderas et al., 2022). The highest antibody transference is observed immediately after birth. Hence, it is very important to ensure that the piglets suckle at least the first 6 hours of life from their biological mother (Bandrick et al., 2011).

The MDA duration (age at which MDA levels fall below the limit of detection of the test) (Opriessnig et al., 2004a; Martinez-Boixaderas et al., 2021) may vary between 4 and 12 weeks of age (Fachinger et al. 2008; Opriessnig et al., 2008a; Martelli et al., 2011; Feng et al., 2016; Martelli et al., 2016; Kiss et al., 2021), but would depend on the initial MDA levels and the threshold of the serologic test used (Morris et al., 1994). The MDA rate of decay, also called half-life, indicates the time for MDA levels reduction to 50% and it may vary from 16 to 45 days depending on the serological test and the method used for its calculation (Opriessnig et al., 2008a; Fort et al., 2009a; Polo et al., 2012; Kiss et al., 2021). However,

the specific PCV-2 antibody half-life in piglets born to sows vaccinated against PCV-2 is still unknown (Sibila et al., 2022).

Once pigs are infected, they can mount an effective humoral immune response (Rodríguez-Arrioja et al., 2002; Larochelle et al., 2003; Grau-Roma et al., 2009). This active seroconversion normally occurs at 7-15 weeks of age (Rodríguez-Arrioja et al., 2002). However, several studies have observed a co-existence of PCV-2 infection in serum with a high antibody titers, indicating that PCV-2 antibodies are not able to fully prevent or neutralize PCV-2 infection (Okuda et al, 2003; Meerts et al., 2006; Trible and Rowland, 2012). On the other hand, further studies showed that neutralizing antibodies (NA) are the responsible of virus clearance from circulation reducing PCV-2 load. Indeed, low NA titres have been directly correlated with high PCV-2 load, severe lymphoid lesions and PCV-2-SD development (Meerts et al., 2006; Fort et al., 2007).

- **Cellular immune response**

The cellular immune response is considered of great importance on PCV-2 control since, as indicated above, PCV-2 antibodies are not able to confer full protection against PCV-2 (Meerts et al., 2006; Trible and Rowland, 2012). In general terms, PCV-2 infection/vaccination adaptive immune response including both cell mediated response (measured by IFN-γ-secreting cells, IFN-γ-SC) and NA, is responsible for PCV-2 clearance in infected animals (Fort et al., 2009b; Kekarainen and Segalés 2015). It has been suggested that a failure in one or both responses could facilitate PCV-2-SD development (Kekarainen et al., 2010).

1.3 CONTROL AND PREVENTION MEASURES

Prior to PCV-2 vaccines availability in the market, the control and prevention measures against PCVD were focused on avoiding the potential risk factors for disease development.

1.3.1 Non-vaccination methods

The most important tools to minimize the impact of PCVD would be the following:

- **Management practices:** The "Madec's 20-point plan" (Madec et al., 2000) provided some recommendations to reduce the impact of infectious diseases, and more specifically PCV-2-SD. This plan includes all-in–all-out procedures, disinfection, reduction of pig-to pig contact, mixing of batches and cross-fostering, separation or euthanasia of diseased pigs, preservation an appropriate temperature, airflow and space within pens, and establishment of an appropriate anti-parasitic and vaccination schedules.

- **Control of co-infections:** The control of concurrent pathogens is a significant factor to decrease the incidence of PCV-2-SD in absence of PCV-2 vaccination. Hence, the measures recommended is control the concomitant pathogens detected in a farm suffering from PCV, with major attention to PRRSV, PPV and *M. hyopneumoniae*, since they are considered the most relevant contributors' factors of PCV-2-SD development (Thacker and Thacker, 2000; Opriessnig and Halbur, 2012).

- **Genetic pig background:** Some genetic breeds or genetic lines seem to be more susceptible to develop PCV-2-SD (López-Soria et al., 2004; Opriessnig et al., 2006; Opriessnig et al., 2009a) than others as described in section 1.2.2. Hence, changing the genetic line of sows and boards would be recommended when PCV-2 problems are detected in the farm. Again, this measure was especially used before the advent of PCV-2 vaccines.

- **Stimulation of the immune system:** Immune system stimulation was occasionally observed when the use of non-specific stimulation of the immune system as a triggering factor for PCVD (Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). These findings caused a certain concern about the vaccination against different pathogens, suggesting an adaptation of the vaccine programme to avoid this synergy. However, other investigations did not describe this relationship between the non-specific stimulation of the immune system and the PCVD development suggesting a low/null impact under field conditions.

1.3.2 Vaccination

PCV-2 vaccines became available in 2004 in Europe in a couple of countries and then from 2007 onwards in the rest of the continent (Franzo and Segalés, 2020), in 2006 in North America (Franzo and Segalés, 2020) and in 2009 in China (Zhai et al., 2014). Such products caused a significant reduction of PCV-2 circulation and of its impact worldwide (Dvorak et al., 2016; Eddicks et al., 2016). More specifically, the main effects of PCV-2 vaccination were reduction of PCV-2-SD clinical signs and lymphoid lesions (Chae, 2012), improvement of the average daily weight gain (ADWG), higher percentage of lean meat production and feed conversion plus a reduction of medication charges (Horlen et al., 2008; Kixmöller et al., 2008).

1.3.2.1 Types of PCV-2 vaccines

Commercial PCV-2 vaccines differ in their antigen and adjuvant types, the age-group where they are applied (sow, piglet or both) and in their dosage (one or two doses) (Chae, 2012). The characteristics of the commercial vaccines available in Europe as well as their application schedules are detailed in Table 1.2. Marketed PCV-2 vaccines can be classified into two main categories considering the antigen used: inactivated and subunit vaccines (Guo et al., 2022).

The first commercial vaccine introduced in the market (Circovac®, Ceva Inc.) was an inactivated PCV-2a vaccine with light paraffin oil as an adjuvant. Originally, this vaccine was licenced to be applied in sows, although later it was registered to be used in piglets older than 3 weeks of age (EMA, 2021a). Since then, other inactivated vaccines have been licenced such as Suvaxyn® Circo (Zoetis Inc.) or Circomax® (Zoetis Inc.). Suvaxyn® Circo is an inactivated recombinant vaccine based on a chimeric virus based on PCV-1 backbone and the PCV-2 Cap gene, with poloxamer and polysorbate in squalene-in-water emulsion (EMA, 2020). Circomax® is a recombinant chimeric inactivated virus that includes the PCV-1 backbone and the PCV-2b and PCV-2a Cap genes with poloxamer and polysorbate in squalane-in-water emulsion as an adjuvant named MetaStim® (EMA, 2021b).

The PCV-2 subunit vaccines commercialized so far are Ingelvac CircoFLEX® (Boehringer Ingelheim Inc.) (EMA, 2021c) and Porcilis® PCV (Merck/MSD Animal Health Inc.) (EMA,

2021d). These two commercial PCV-2 subunit vaccines contain the Cap protein of PCV-2a genotype.

In addition, combined vaccines including *M. hyopneumoniae* and PCV-2 are also marketed. Both pathogens can be associated with PRDC (Chae, 2016) and, although other pathogens may also participate in this complex (Opriessnig et al., 2011b), prevention and control of PCV-2 and *M. hyopneumoniae* infections are fundamental to deal with this clinical condition, since a synergistic effect of both infections has been demonstrated in some assays (Opriessnig et al., 2004b; Opriessnig et al., 2011a). Moreover, the coincidence in terms of vaccination timepoint implies that this combined vaccine strategy is frequently preferred by producers as it reduces pig stress and decrease labour cost (Sibila et al., 2020). Nowadays, several combined vaccines against PCV-2 and *M. hyopneumoniae* are commercialized, being all of them inactivated recombinant vaccines:

- Porcilis® PCV *M Hyo (*MSD Animal Health Inc.) is a ready-to-use bivalent vaccine that contains an inactivated *M. hyopneumoniae* bacterium and a baculovirus-expressed PCV-2 ORF2 antigen adjuvanted with aluminium in light mineral oil (EMA, 2021e).

- FLEXCombo® is a combination of CircoFLEX® and MycoFLEX® as a ready-to-mix product (Kaalberg et al., 2017; Yang et al., 2022).

- Suvaxyn® CIRCO+MH RTU (Zoetis Inc.) is a ready-to-use vaccine that comprises a recombinant vaccine based in a chimeric PCV-1 and PCV-2, plus *M. hyopneumoniae* bacteria with poloxamer and polysorbate in squalane-in-water as an adjuvant (EMA, 2021f).

- CircoMax Myco® (Zoetis Inc.) is a ready-to-use trivalent vaccine containing a recombinant vaccine based in a chimeric PCV-1 plus PCV-2a ORF2 protein, chimeric PCV-1 plus PCV-2b ORF2 protein and *M. hyopneumoniae* bacterin, adjuvanted with poloxamer and polysorbate in squalane-in-water (EMA, 2021g).

- Mhyosphere® PCV ID (Hipra Inc.) is a ready-to-use intradermal vaccine that consists of an inactivated recombinant *M. hyopneumoniae* expressing the PCV-2 capsid protein adjuvanted with light mineral oil (EMA, 2022a).

Table 1.2. Main characteristics of PCV-2 vaccines commercialized in Europe (http://www.ema.europa.eu).

Pharmaceutical company	Vaccine name	Licenced for	Dosage	Schedule
Ceva	Circovac®	Gilts /Sows	2 mL, IM	Two doses before farrowing. Re-vaccination at each gestation
		Pigs	0.5 mL, IM	One dose, 3 weeks of age or older
Boehringer Ingelheim	Ingelvac CircoFLEX®	Sows	1 mL, IM	One dose during pregnancy or lactation
		Pigs	1 mL, IM	One dose, 2 weeks of age or older
	FLEXcombo®	Pigs	2 mL, IM	One dose, 3 weeks of age or older
MSD	Porcilis® PCV	Pigs	2 mL, IM	One dose, 3 weeks of age or older
	Porcilis® PCV M Hyo	Pigs	2 mL, IM	One dose, 3 weeks of age or older
			1 mL, IM	Two doses, 3 days of age or older and at least 18 days after first injection
Zoetis	Suvaxyn® Circo	Pigs	2 mL, IM	One dose, 3 weeks of age or older
	CircoMax®	Pigs	2 mL, IM	One dose, 3 weeks of age or older
	Suvaxyn® CIRCO+MH RTU	Pigs	2 mL, IM	One dose, 3 weeks of age or older
	CircoMax Myco®	Pigs	2 mL, IM	One dose, 3 weeks of age or older
			1 mL, IM	Two doses, 3 days of age or older and 3 weeks later
HIPRA	Mhyosphere® PCV ID	Pigs	0.2 mL, ID	One dose, 3 weeks of age or older

1.3.2.2 Immunity conferred by PCV-2 vaccination

The current vaccines are efficient in inducing humoral and cell-mediated immunity against PCV-2 (Fort 2007; Fort et al., 2009b; Fort et al., 2012; Kekarainen et al., 2010), although the level of humoral response induced may vary depending on the vaccine used (Segalés, 2015). However, the absence of seroconversion or low antibody levels after PCV-2 vaccination do not imply lack of protection as cell-mediated immune response also plays an important role in vaccine-induce protection (Fenaux et al., 2004; Pérez-Martín et al., 2010). Specifically, both experimental and field reports indicate PCV-2-specific IFN-γ-SCs production after PCV-2 vaccination (Fort et al., 2009a; Martelli et al., 2011; Fort et al., 2012; Borghetti et al., 2013; Ferrari et al., 2014; Koinig et al., 2015).

PCV-2 antibodies elicited by sow vaccination are transferred to its offspring (Kurmann et al., 2011; Fraile et al., 2012a; Sibila et al., 2013; Hemann et al., 2014; Oh et al., 2014). However, the levels of MDA transferred will depending on two major factors (Martinez-Boixaderas et al., 2022; Sibila et al., 2022):

1) The immunological status of the sow: This factor in turn will depend on sow vaccination schedule applied in the herd and the PCV-2 infection pressure in the farm. Moreover, the higher the sow herd immunity acquired by infection or vaccination, the more delayed the infection of the piglet, since the antibodies provided throughout colostrum are crucial for piglet protection until immunity system of piglet is competent to respond (Poonsuk and Zimmerman, 2018).

2) The time and volume of colostrum intake by the piglet (Klobasa et al., 1981), since MDA are present in the colostrum (Fachinger et al., 2008; Opriessnig et al., 2008a; Martelli et al., 2011; Rose et al., 2012; Martelli et al., 2016; Kiss et al., 2021).

The PCV-2 commercial vaccines most used in Europe and North-America are based on PCV-2a (Opriessnig et al., 2007; Karuppannan and Opriessnig, 2017) and cross-protection against different genotypes has been reported under experimental (Fort et al., 2008; Opriessnig et al., 2008b; Opriessnig et al., 2014a, Rose et al., 2016; Park et al., 2019) and field conditions (Chae, 2012; Jeong et al., 2015; Opriessnig et al., 2017). These results indicates that current available vaccines in the market may prevent clinical disease in most

of the cases, independently of the genotype circulating in the farm (Karuppannan and Opriessnig, 2017). However, protection acquired by PCV-2 vaccination is attributed to the common epitopes between vaccine and PCV-2 viruses (Bandrick et al., 2020). Consequently, protection of PCV-2 vaccines might be increased if multiple genotypes are included in the vaccine, or when the genotype of the vaccine and PCV-2 infection are the same (Opriessnig et al., 2013; Bandrick et al., 2020). Considering this factor and the fact that nowadays the two major genotypes circulating are PCV-2b and PCV-2d (Franzo and Segalés, 2020), more studies with homologous vs heterologous PCV-2 vaccines and even with multi-genotype vaccines would be desirable (Karuppannan and Opriessnig, 2017; Bandrick et al., 2020).

1.3.2.3 Vaccination schedules

- **Sow**

The main purpose of breeding stock vaccination is to generate passive immunity through the MDI (Segalés, 2015), providing protection of piglets against PCV-2 infection from the very beginning of their life (Pejsak et al., 2010).

Sow vaccination can prevent PCV-2-SD development in the offspring (Pejsak et al., 2010) by inducing cellular and humoral response (Madson et al., 2009c; Sibila et al., 2013; Lopez-Rodriguez et al., 2016). Moreover, presence of colostral anti-PCV-2 antibodies have been confirmed after vaccination of sows (Madson et al., 2009c; Sibila et al., 2013). However, early PCV-2 infections, presence of PCV-2 DNA in colostral samples, peri-partum maternal viraemia and virus excretion in lacteal secretions may not be prevented in some cases (Madson et al., 2009c; Gerber et la., 2011; Gerber et al., 2012).

Two different vaccination strategies are mainly used in dams:

1) Sow vaccination before mating can homogenize breeding stock PCV-2 immune status during gestation period (Gerber et al., 2011; Kurmann et al., 2011; O'Neill et al., 2012; Sibila et al., 2013), therefore, preventing potential effects of PCV-2 infection during gestation.

2) Sow vaccination before farrowing confers PCV-2 protection to the offspring. This strategy has been studied under field conditions observing a reduction in viraemia (Fraile et al., 2012a) and macroscopic lesions, microscopic lymphoid lesions, and viral load in lymphoid tissues under experimental conditions (Opriessnig et al., 2010).

Interestingly, one study where breeding stock vaccination was boosted during 3 cycles for 3 years, detected an improvement of the reproduction rate, number of piglets born alive, birth weight of piglets and number of piglets weaned per a litter (Pejsak et al., 2012). Besides, in another study where the boost was applied for two consecutive cycles of sow production, a higher number of live-born piglets per litter at the second cycle and higher vitality score in piglets already at the first cycle were achieved (Oliver-Ferrando et al., 2018a).

- **Piglets**

The principal purpose of piglet vaccination is the induction of active immunity to protect them against PCV-2 infection. This vaccination schedule is usually administered at weaning (3-4 weeks of age) and its efficacy has been broadly demonstrated by both experimental and field studies (Segalés, 2015). The main effects observed are the reduction in viraemia, shedding, viral load in tissues, microscopic lymphoid lesions, co-infections and, the improvement of production parameters (Fachinger et al., 2008; Horlen et al., 2008; Kixmöller et al., 2008; Segalés et al., 2009; Pejsak et al., 2010; Kristensen et al., 2011; Martelli et al., 2011; Heißenberger et al., 2013; Martelli et al., 2013; da Silva et al., 2014; Oh et al., 2014; Opriessnig et al., 2017; Do et al., 2021). Moreover, vaccine benefits have also been reported in pigs without evident clinical signs. An improvement of ADWG, a decreasing of PCV-2 infection prevalence and PCV-2 load in serum and faeces have been demonstrated in a PCV-2-SI scenario (Fraile et al., 2012b; Alarcon et al., 2013; Oliver-Ferrando et al., 2016).

In general terms, pig vaccination is applied in a single dose around weaning, although two PCV-2 and *M. hyopneumoniae* combined vaccines (Porcilis® PCV M Hyo, MSD Animal Health Inc., and CircoMax Myco®, Zoetis Inc.) give the option of a split-dose administration at 3 days of age and then around weaning.

- **Sow and piglets**

There are several reports on the benefit of both sow and piglet vaccination in productive and virological parameters (Opriessnig et al., 2010; Pejsak et al., 2010; Fraile et al., 2012a; Haake et al., 2014; Oh et al., 2014; Feng et al., 2016; Villa-Mancera et al., 2016; Martelli et al., 2016). Specifically, it has been observed a reduction of viraemic piglets (Opriessnig et al., 2010; Fraile et al., 2012a; Haake et al., 2014; Oh et al., 2014; Martelli et al., 2016; Feng et al., 2016; Villa-Mancera et al., 2016), viral load in blood (Fraile et al., 2012a; Haake et al., 2014; Feng et al., 2016; Martelli et al., 2016; Villa-Mancera et al., 2016), in lymphoid tissues (Opriessnig et al., 2010; Oh et al., 2014), and an improvement of piglets' morbidity (Martelli et al., 2016), mortality (Pjsak et al., 2010; Fraile et al., 2012a; Martelli et al., 2016; Villa-Mancera et al., 2016) and ADWG (Pejsak et al., 2010; Fraile et al., 2012a; Haake et al., 2014; Martelli et al., 2016; Feng et al., 2016; Villa-Mancera et al., 2016).

However, in the scenario of sow and piglet vaccination, the level of MDA at the moment of vaccination may be an important point to consider. In some studies, a negative MDA effect on vaccine-induced humoral immune response has been detected (Fort et al., 2009a; Fraile et al., 2012a, b; Haake et al., 2014; Feng et al., 2016). Such interference seems to not affect the cellular immune response elicited by the vaccine (Martelli et al., 2013; Oh et al., 2014). Therefore, according to the results obtained in some studies, the lack of seroconversion after vaccination in the presence of MDA should not be considered as a negative indicator for the vaccine effectiveness (Fort et al., 2009a; Fraile et al., 2012b; Tzika et al., 2015; Tassis et al., 2017; Figueras-Gourgues et al., 2019). On the other hand, in few studies (Feng et al., 2016; Haake et al., 2014), MDA interference on productive parameters were noticed. Feng et al. (2016) detected lower production parameters only when piglets had extremely high MDA titres at vaccination. Noteworthy, such high MDA titres are unusual under field conditions. On the other hand, Haake et al. (2014) showed lower productive parameters in pigs immunized at 1 week of age compared to those immunized at 3 weeks of age, detecting a higher antibody level in animals at 1 week than at 3 weeks of age.

1.4 CLINICAL TRIALS

Clinical trials are those studies that are developed under field conditions with the objective to ensure the safety and efficacy of a test product in the target population and under the

context of intended use (Francis et al., 2020; EMA, 2000). In the development process of medicines, these studies may be included in the final phase of the regulatory approval process and in the post-authorization phase (Francis et al., 2020; AEMPS, 2019).

Clinical trials are motivated to test the medicine under field conditions where the variability is very high, and the conditions are not as controlled as in experimental conditions. In addition, the population used to perform clinical trials is much numerous than the one used in preclinical studies. This high sample size helps to detect unlikely events not detected within a smaller population.

The development of a vaccine is a long process (up from 3 to 6 years) (Hunter et al., 2011; Artaud et al., 2019; Francis et al., 2020) and it is divided in 4 stages: discovery/feasibility phase, preclinical, clinical and post-authorization phases.

The discovery/feasibility and preclinical phases are performed before the clinical phase and they are carried out under experimental conditions (Francis et al., 2020). In these phases, the potential vaccine candidates are tested for proof-of-concept studies (Artaud et al., 2013; Francis et al., 2020). In case of the preclinical phase, the vaccine candidate is studied in a reduced number of animals from the target specie/s to demonstrate its safety and efficacy (Francis et al., 2020). These preclinical studies are also important to determine the parameters that will be considered in the future clinical trials performed under “real” conditions (Francis et al., 2020).

Once the clinical phase is performed and vaccine is registered and commercialized, a post-authorisation phase starts, in which pharmacovigilance is needed to detect potential adverse reactions that occur infrequently in large populations and would not be identified in pre-authorisation safety studies (Jones et al., 2007). Moreover, other post-approval studies may be conducted such as 1) the determination of the effectiveness of the vaccine in those conditions in which the vaccine have not been studied; for example, in a therapeutic non-compliance of the label, polymedication, the interaction with other medicines commonly used in routine practice and so on; 2) obtention of new information of the vaccine for potential new claims (dose, duration of treatment, appropriate use) and, 3) pharmacoeconomic analyses to determine the cost-benefit of the vaccine (AEMPS, 2019).

Clinical trials carried out with the purpose of a regulatory approval process must follow specific regulations, being the main ones in the European territory: the European pharmacopeia, European Medicine Agency (EMA) guidelines (GLs) and the VICH guideline (GL) 9 created for the conduction of clinical trials according to the good clinical practices (GCP). Besides, European and regional legislation of the country must be followed to assure the execution of clinical trials in accordance with the legal regulation. In Spain, there is the Spanish Agency for Medicines and Medical Devices (*Agencia Española de Medicamentos y Productos Sanitarios* [AEMPS]), who regulate the veterinary medicine products, including the development of veterinary vaccines based on the European requirements (https://www.aemps.gob.es/). These requirements are described in section 1.4.3.

1.4.1 Parameters used in clinical trials to assess PCV-2 vaccine efficacy and safety

Vaccine efficacy field studies may be performed to confirm safety and efficacy preclinical results or to demonstrate efficacy when the viability of vaccination–challenge studies are not possible due to the epidemiological status of the country (World Health Organization, 2022).

Commonly, safety parameters assessed in a PCV-2 clinical trial are body temperature before and after product administration, size and duration of local reaction plus the proportion of animals showing local or systemic reaction. Other measurements such as mortality can be also included (European Pharmacopeia 04/2013:50206).

In general terms, efficacy parameters used in field studies must be clearly defined and derived from the main diseases' outcomes such as clinical signs, lesions, or mortality (EMA, 2001). Moreover, the parameters defined for the vaccine efficacy acceptance should be linked to the immune response elicited to protect the target species against the studied disease (EMA, 2001). In case of PCV-2 clinical trials, the main efficacy parameters used for assessment of protection against the infection are usually clinical signs, ADWG and/or mortality, antibody levels, viraemia, PCV-2 detection in lymphoid tissues and/or lymphoid microscopic lesions. In those studies where sows are vaccinated, reproductive parameters such as number of liveborn, mummified, stillborn, weak piglets and crushed, litter weight,

weaned piglets per litter, number of piglets lost during the nursing period, return-to-oestrus, abortion rate, interval between expected and real farrowing date and /or weaning-to-fertile mating interval are also included (Kurmann et al., 2011; Oliver-Ferrando 2018a; Cybulski et al., 2020).

All peer-reviewed studies in which the efficacy of PCV-2 commercial vaccines have been assessed are compiled in Table 1.3. The articles included in this table were retrieved from the PubMed database, written in English, and identified using the terms “trial”, “porcine circovirus” and “clinical”. The information retrieved from each study, includes: PCV-2 vaccine, target animal to which the vaccine was administered (piglet, sow or both) and parameters with a statistically significant result used to confirm the efficacy of the vaccine. Some studies included more than one PCV-2 vaccine.

The most frequently efficacy parameters found statistically significant between vaccinated and non-vaccinated animals in PCV-2 efficacy clinical trials were PCV-2 antibody levels (59/66, 89.39%), followed by PCV-2 positive cells in lymphoid tissues (10/12, 83.33%), ADWG (57/69, 82.60%), PCV-2 viraemia (55/68, 80.88%), lymphoid lesions (14/20, 70.00%) and finally, mortality (27/50, 54.00%).

Table 1.3. Proportion of randomized controlled trials in which the main efficacy parameters showed statistically significant differences ($p \leq 0.05$) between PCV-2 vaccinated and non-vaccinated group.

PCV-2 vaccine	Target animal (number of studies using the target animal)	Proportion of studies which reported statistically significant differences between groups in the efficacy parameters						References
		ADWG	PCV-2 viraemia	PCV-2 antibody levels	Mortality	Lymphoid lesions	PCV-2-positive cells in lymphoid tissues	
Ingelvac CircoFLEX®	Piglet (12)	7/10	5/7	7/9	6/7	1/2	1/2	Desrosiers et al., 2008 Fachinger et al., 2008 Kixmöller et al.,2008 Lyoo et al., 2011 Young et al., 2011 Weibel et al., 2012 Jeong et al., 2015 Choi et al., 2019 Figueras-Gourgues et al., 2019 Ellegaard et al., 2021 Kim and Hahn, 2021 Puig et al., 2022
	Sows and piglets (2)	2/2	2/2	2/2	1/2	0/0	0/0	Feng et al., 2016 Villa-Mancera et al., 2016
FLEXcombo®	Piglet (n=3)	3/3	3/3	1/1	0/2	0/1	0/0	Kaalberg et al., 2017 Pagot et al., 2017 Yang et al., 2022
Circo/MycoGard ®	Piglet (n=2)	2/2	2/2	2/2	0/2	2/2	2/2	Yang et al., 2021 Cho et al., 2022 a
CircoPrime	Piglet (n=1)	1/1	1/1	1/1	1/1	1/1	1/1	Han et al., 2013

PCV-2 vaccine	Target animal (number of studies using the target animal)	Proportion of studies which reported statistically significant differences between groups in the efficacy parameters						References
		ADWG	PCV-2 viraemia	PCV-2 antibody levels	Mortality	Lymphoid lesions	PCV-2-positive cells in lymphoid tissues	
CIRCOQ PCV2	Piglet (n=1)	1/1	1/1	1/1	1/1	0/0	0/0	Do et al., 2021
Circovac®	Piglet (n=5)	3 / 4	4 / 5	5 / 5	1 / 5	1/1	1/1	Fraile et al., 2012b Heibenberger et al., 2013 Jeong et al., 2015 Oliver-Ferrando et al., 2016 Boulbria et al., 2021
	Sows (n=4)	2/3	1/2	4/4	1/1	0/0	0/0	Kurmann et al., 2011 Villa-Mancera et al., 2016 Oliver-Ferrando et al., 2018a Oliver-Ferrando et al., 2018b
	Sows and piglets (n=5)	5/5	5/5	5/5	4/5	0/0	0/0	Feng et al., 2016 Villa-Mancera et al., 2016
Circumvent® PCV	Piglet (n=2)	2/2	2/2	1/2	1/1	0/0	0/0	Horlen et al. 2008 Lyoo et al., 2011
	Sows and piglets (n=1)	1/1	1/1	1/1	1/1	0/0	0/0	Villa-Mancera et al., 2016
Fostera® PCV	Piglet (n=1)	1/1	1/1	1/1	0 / 1	1/1	1/1	Jeong et al., 2015
	Sows and piglets (n=1)	1/1	1/1	1/1	1/1	0/0	0/0	Villa-Mancera et al., 2016
Fostera® PCV MH	Piglet (n=1)	1/1	1/1	1/1	1/1	1/1	0/0	Jeong et al., 2016

PCV-2 vaccine	Target animal (number of studies using the target animal)	Proportion of studies which reported statistically significant differences between groups in the efficacy parameters						References
		ADWG	PCV-2 viraemia	PCV-2 antibody levels	Mortality	Lymphoid lesions	PCV-2-positive cells in lymphoid tissues	
Fostera® Gold PCV MH	Piglet (n=4)	4/4	4/4	4/4	0/2	2/4	1/1	Um et al., 2021 Yang et al., 2022 Cho et al., 2022b Um et al., 2022
MHYOSPHERE ® PCV ID	Piglet (n=1)	1/1	0/0	0/0	0/0	0/0	0/0	Puig et al., 2022
Porcilis® PCV	Piglet (n=8)	6/9	8/8	4/6	5/6	0/0	0/0	Martelli et al., 2011 Borghetti et al., 2013 Martelli et al., 2013 Haake et al., 2014 Sno et al., 2016 Nielsen et al., 2017 Ellegaard et al., 2021 Puig et al., 2022
	Sows(n=2)	0/0	0/2	2/2	0/0	0/0	0/0	Sibila et al., 2013 Martelli et al., 2015
	Sows and piglets (n=2)	1/2	0/2	2/2	1/2	0/0	0/0	Fraile et al., 2012a Martelli et al., 2016

PCV-2 vaccine	Target animal (number of studies using the target animal)	Proportion of studies which reported statistically significant differences between groups in the efficacy parameters						References
		ADWG	PCV-2 viraemia	PCV-2 antibody levels	Mortality	Lymphoid lesions	PCV-2-positive cells in lymphoid tissues	
Porcilis® PCV Mhyo	Piglet (n=10)	9/10	9/10	7/7	0/7	1/3	1/1	Tzika et al., 2015 Witvliet et al., 2015 Kaalberg et al., 2017 Pagot et al., 2017 Tassis et al.,2017 Duivon et al., 2018 Lopez-Lorenzo et al., 2021 Um et al., 2021 Cho et al., 2022a Yang et al., 2022
SuiShot Circo-ONE®	Piglet (n=1)	1/1	0/0	1/1	0/0	0/0	0/0	Choi et al., 2019
Suvaxyn® PCV	Piglet (n=4)	3/4	3/4	3/4	2/2	4/4	2/3	Paphavasit et al., 2009 Segalés et al., 2009 Lyoo et al., 2011 Seo et al., 2012
	Sows(n=1)	0/0	1/1	1/1	0/0	0/0	0/0	O'Neill et al., 2012
Suvaxyn® Circo+MH RTU	Piglet (n=1)	0/1	0/1	1/1	0/1	0/0	0/0	Lopez-Lorenzo et al., 2021
Commercial subunit vaccine A	Piglet (n=1)	0/0	0/1	0/1	0/0	0/0	0/0	Vargas- Bermudez et al., 2018
Commercial subunit vaccine B	Piglet (n=1)	0/0	0/1	1/1	0/0	0/0	0/0	Vargas- Bermudez et al., 2018
TOTAL		57/69	55/68	59/66	27/50	14/20	10/12	

1.4.2 EU legal bases and guidelines

The regulatory requirements needed for a vaccine registration guarantees that a licensed vaccine is safe, of quality, that works in the target animal for the indication designed and considers a positive benefit/risk balance for each product (Jones et al., 2007).

The vaccine production regulation is complex and may vary between countries and regions (Edwards, 2007).

This current section is focused on PCV-2 clinical trials developed according to the requirement in the European regulatory environment. Clinical trials performed for a licence authorization or intended to be submitted to regulatory authorities follow the EU criteria for veterinary vaccine regulation described below:

Guidelines

- **International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) GL9**: VICH is a collaborative programme between a regulatory authority and the animal health industries of three geographical regions founded on 1996: European Union (EU), Japan, and the United States of America. Besides there are two other observer regions: Canada and Australia/New Zealand (Holmes and Hill, 2007). Its objective is to harmonise technical requirements for veterinary product registration to guarantee the quality, safety and efficacy standards and reduce the use of test animals and cost of product development standards (Holmes and Hill, 2007). Several GLs of different topics related to quality, safety, efficacy and pharmacovigilance have been approved to provide a basis for wider international harmonization of registration requirements (vichsec.org). Specifically, in case of clinical trials intended to be submitted to regulatory authorities, it was created a VICH GL9 (EMA, 2000). This GL represents an international ethical and scientific quality standard with the objective to provide a guide on the design, conduction, monitoring, recording, and analysing data generated during the study, auditing, and reporting of clinical trials to ensure the GCP compliance. It is not mandatory use this GL since it is not an in-force law. Hence, it can be applied an alternative approach after discussion with the regulatory authorities (EMA, 2000).

- **European pharmacopea:** It is a source of official quality standards for medicines and the ingredients used in the production of medicines in Europe (https://www.ema.europa.eu/en.). It provides a scientific basis for the quality control of a product throughout its life cycle, supporting the pharmaceutical industry and healthcare systems. The European pharmacopeia covers, but is not limited, to synthetic chemical active substances, natural products, biological products and biotechnology derived products, vaccines for human use, veterinary vaccines, mineral products, radiopharmaceutical products, excipients, dosage forms, homoeopathic preparations, standard terms on dosage forms, routes of administration and containers. Some requirements are covered by a general monograph and some others by specific monographic documents dedicated to a particular substance/preparation. Hence, these monographs are complementary instruments to ensure the quality and medical products.

  In case of efficacy and safety evaluation there are the European pharmacopeia. 5.2.7: 04/2013:50207 (monograph on the evaluation of efficacy of veterinary vaccines and immunosera) and European pharmacopeia. 5.2.6: 04/2013:50206 (monograph on the evaluation of safety of veterinary vaccines and immunosera), respectively, to comply with the EMA regulations (EMA, 2008).

Regulatory authorities and laws

The regulatory entities are the medicine agencies that regulate the veterinary medicine products. There are regulatory agencies in each country, being the AEMPS the regulatory authority from Spain (https://www.ema.europa.eu/en.), and regional regulatory agencies, being EMA the regional regulatory agency which comprise European countries. Laws established by regularity authorities from each country are based on the European requirements (https://www.ema.europa.eu/en.).

- **Direct EU laws from European Commission:** Directive 2001/82/EC and Regulation 726/2004 of the European Parliament and the Council, respectively, constitute the Union regulatory framework for the placing on the market, manufacturing, import, export, supply, distribution, pharmacovigilance, control and the use of veterinary medicinal products.

Regulation (EU) 2019/5 amended the EU pharmaceutical legal framework set out by Regulation (EU) 726/2004 and created a legal framework specific to veterinary products. Moreover, the Regulation (EU) 2019/6 on veterinary medicinal products updated the rules on the authorisation and use of veterinary medicines replacing the Directive 2001/82/EC (https://www.ema.europa.eu/en.).

- **European regulatory agency (EMA):** It is a decentralised agency of EU with the objective of evaluate, supervise, and monitor medicines in EU. Its objective is to contribute to the protection of public and animal health by ensuring that medicinal products for human and veterinary use are safe, effective and of high quality (https://www.ema.europa.eu/en.). Specifically, the Veterinary Medicines Division of EMA provides guidance and advice during medicine development, the authorisation process and the safety monitoring of medicines on the market. (https://www.ema.europa.eu/en.). GLs are redacted for harmonisation purposes, although they do not represent legal requirements (except when are imposed by law). For this reason, alternative methods may be considered with the appropriate justification (EMA, 2008).

  EMEA/CVMP/852/99 (Note for Guidance on field trials with veterinary vaccines) was approved in 2000 as a guidance that includes advises on how to perform field trials with veterinary vaccines, what criteria should be considered, what data are expected and how the data should be analysed (EMA, 2001). However, since January 2022, GL EMA/CVMP/IWP/260956/2021 (GL on clinical trials with immunological veterinary medicinal products) replaces the EMEA/CVMP/852/99 guidance (EMA, 2022b).

  The main GLs are those mentioned above, although there are other guides and documents referred on how to deal with specific and more complex situations within clinical trials that can be found on the EMA website (Guidance documents | European Medicines Agency (europa.eu)).

- **Spanish regulatory agency (AEMPS):** It is a Spanish health authority responsible for ensuring compliance of quality, safety, efficacy and correct information of medicines for human and veterinary use, as well as medical devices, biocides, cosmetics and personal

hygiene products, in accordance with Spanish and European Union legislation (https://www.ema.europa.eu/en.). A specific information and normative regarding the clinical investigation on veterinary medicine can be found on the AEMPS website (Investigación clínica con medicamentos veterinarios | Agencia Española de Medicamentos y Productos Sanitarios (https://www.aemps.gob.es), such as the Royal Decree 1157/2021, of 28 December, that regulates the industrially manufactured veterinary medicinal products. This Royal Decree complements the European Regulation (EU) 2019/6 on veterinary medicinal products.

CHAPTER 2

Hypothesis and Objectives

PCV-2 has been recognized as one of the most important pathogens of the pig population worldwide (Opriessnig et al., 2007). The first commercial vaccine was available in 2004, and PCV-2a vaccines developed since then have reduced the prevalence and severity of PCVDs, mainly PCV-2-SD (Bandrick et al., 2022). In fact, thanks to the widespread use of PCV-2 vaccines, the impact of PCV-2-SD shifted from severe clinical outbreaks observed from 1997 until 2007 to a PCV-2-SI as the most prevalent PCVD with occasional overt outbreaks (Poulsen et al., 2021). Although PCV-2-SD was already well-controlled at the beginning of this PhD Thesis (in 2018) by means of vaccination, some questions linked with optimising the potential benefits of vaccines under field conditions were still unanswered. At that time, the herd vaccination effect on reproductive parameters was poorly investigated and the proper use of PCV-2 vaccines in the breeding stock was still in its infancy.

Moreover, several PCV-2 genotype shifts have been identified from 1997 until now, being PCV-2d the most prevalent genotype nowadays (Guo et al., 2012; Xiao et al., 2016; Sibila and Rocco et al., 2021). However, most PCV-2 vaccines available in the market are still PCV-2a-based vaccines in which the effectiveness in avoiding clinical disease produced by heterologous strains has been globally proved; however, doubts about their ability to prevent viral replication or transmission have always been on the table (Madson et al., 2009c; Opriessnig et al., 2014a). On the other hand, information about the best vaccination schedule in terms of age of application to obtain the major benefit is still being debated, since vaccinating piglets with too high levels of MDA may jeopardize the effect of PCV-2 vaccines.

Considering the rationales mentioned above, the main objective of the present PhD thesis was to evaluate the implementation of different vaccination strategies against PCV-2 in sows and piglets for efficacy assessment based on clinical trials. The specific objectives of this Thesis were the following ones:

- To assess the effects of PCV-2 sow vaccination at different stages of the production cycle (before mating, mid gestation and late gestation) mimicking blanket vaccination in terms of reproductive, productive, virological and serological parameters in a PCV-2-SI scenario (Study I).

- To evaluate the efficacy in terms of antibody, viraemia, faecal shedding, lymphoid lesions and production levels using a novel trivalent vaccine including PCV-2a and PCV-2b genotypes and *M. hyopneumoniae* administered as one dose at 3 weeks of age (Study II) or a split dose at 3 days of age and 3 weeks later (Study III).

- To assess the PCV-2 MDA interference in the vaccine-elicited humoral response and the efficacy of a trivalent PCV-2a/b plus *M. hyopneumoniae* vaccine when administered at 3 weeks of age (Study II) or in a split regime at 3 days of age and 3 weeks later (Study III).

CHAPTER 3

Study I

Exploratory field study on the effects of *Porcine circovirus 2* (PCV-2) sow vaccination at different physiological stages mimicking blanket vaccination

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3.1 INTRODUCTION

Intrauterine PCV-2 infections at different stages of pregnancy may cause different reproductive disorders depending on the foetal immunological competence (Eddicks et al., 2016), the so-called PCV-2-RD. Intrauterine PCV-2 infection of foetuses, via insemination (with PCV-2 spiked semen) or transplacental, may cause late-term abortions, mummified, stillborn and weak born piglets (Madson et al., 2009c; Gerber et al., 2012; Nauwynck et al., 2012; Hemann et al., 2014; López-Rodríguez et al., 2016; Oliver-Ferrando et al., 2018a). Furthermore, pigs may be born PCV-2 viraemic due to transplacental infection that may subsequently act as an infection focus for their pen mates. All these situations imply that infected sows have a very important role in PCV-2 infection maintenance and dissemination in the herd due to horizontal and vertical transmission.

Nowadays, vaccination is a very effective tool to control PCV-2 infection. From 2007 to present, four major PCV-2 vaccines have been marketed worldwide (Segalés, 2015), but only two are licensed in several countries for their use in sows to protect the gestation and/or their progeny (see Chapter 1, Table 1.2). Therefore, current products allow applying different vaccination regimes combining piglet and/or sow vaccination (Fraile et al., 2012a; Oh et al., 2014). These abovementioned vaccines are inactivated PCV-2a products, a recombinant vaccine based on an inactivated PCV-1/PCV-2a chimeric virus or subunit vaccines based on a PCV-2a Cap protein. Specifically, the vaccine tested in the present study, Ingelvac Circoflex®, is a PCV-2a subunit vaccine based on the product of the ORF2 gene expressed in a baculovirus system (Segalés, 2015). However, cross-protection between the major genotypes worldwide (PCV-2a, PCV-2b and PCV-2d) has been observed in experimental trials and field studies (Fort et al., 2008; Opriessnig et al., 2012; Franzo and Segalés, 2020; Yu et al., 2023).

Different studies have shown the capacity of the sow vaccination to induce an immune response and the transfer of passive immunity to the offspring. Sow vaccination before mating stabilizes and homogenizes the PCV-2 immune status of the sow population during gestation (Gerber et al., 2011; Kurmann et al., 2011; O'Neill et al., 2012; Sibila et al., 2013). Sow vaccination administered before farrowing can confer protection through MDI to their offspring against PCV-2-SD by reducing viraemia, lesions and viral load in tissues (Opriessnig et al., 2010) as well as increasing their ADWG gain in PCV-2-SI scenarios

(Kurmann et al., 2011). When this vaccination strategy is boosted in the following reproductive cycles at 3 weeks before farrowing (for 3 years), an improvement of the reproduction rate, number of piglets born alive, birth weight of piglets and number of piglets weaned per a litter was achieved (Pejsak et al., 2012). Another study, where the boost was applied on the second cycle at 2 weeks before farrowing, reported higher number of live-born piglets per litter at the second cycle and higher vitality score in the first and second cycles (Oliver-Ferrando et al., 2018a) compared to the non-vaccinated (NV) group. Nevertheless, the effect of PCV-2 sow vaccination strategies on reproductive parameters has been, up to now, scarcely studied (Pejsak et al., 2012; Oliver-Ferrando et al., 2018a). Furthermore, practices in the field have explored the option of sow blanket vaccination (personal communication of field veterinarians); however, no contrasted data has been scientifically described.

Hence, the objective of the present work was to evaluate the effects of sow vaccination against PCV-2 applied at different stages of the production cycle (before mating, mid gestation, and late gestation), mimicking a blanket vaccination fashion, on productive parameters as well as on virological and serological parameters in sows and their progeny.

3.2 MATERIALS AND METHODS

3.2.1 Farm selection

The inclusion criteria for farm selection were: a) sows/gilts housed in a single conventional farm (site I), b) no PCV-2 vaccination schedule in gilts/sows, c) allowance of controlled cross-fostering practices, and d) evidence of PCV-2 infection by means of viral genome detection in sows and porcine umbilical cords (PUCs) in site I. Indeed, prior to the start of the study, PCV-2 DNA was detected in serum samples from 18 out of 30 (60%, viral load range $10^{3.50}$ and $10^{7.91}$ genome copies/mL) clinically healthy sows from different parity number and in 24 out of 30 (80%, viral load range $10^{3.50}$ and $10^{7.06.}$ genome copies/ml) PUCs from the same sows, indicating a PCV-2-SI scenario. Inclusion criteria for gilts/sows selection were: a) healthy animals, b) not pregnant, c) from the same genetic line, d) of all parities (gilts, primiparous and multiparous).

The study was conducted in a two-site commercial farm located in Catalonia (Spain). The farm had 1,400 sows (including gilts) and used a weekly farrowing batch system; piglet weaning was performed at 25 days of age approximately. The farm was *M. hyopneumoniae* positive, PRRSV positive stable, and negative for *Aujeszky's disease virus* (ADV). Gilts and sows were crossbred (Duroc x Landrace) and were artificially inseminated with Pietrain boar semen. The vaccination routine of the farm included gilt and sow immunization against PRRSV, PPV, *Erysipelothrix rhusiopathiae, Escherichia coli* and *Clostridium perfringens*. Piglets were vaccinated against PCV-2 and *M. hyopneumoniae* before weaning.

3.2.2 Study design

The present study was a parallel group, randomised and controlled trial. The study was unmasked for personnel involved in study design, monitoring, vaccine dispensation and for personnel involved in body weight and sample collection. On the other hand, it was masked for farm personnel (routine management, daily observation, and routine reproductive data recording) as well as for laboratory technicians. Study design is summarized in Figure 3.1.

Sows were bled before mating and tested for PCV-2 IgG and IgM ELISAs. Animals were randomly allocated into 4 different treatment groups blocking by parity (from 0 to 8) and optical density (OD) values for PCV-2 IgG and PCV-2 IgM. A total of 288 healthy sows were selected through six consecutive breeding batches and distributed in the following treatments groups: vaccination of 73 at pre-mating (PM), 72 at MG, 73 at LG and 70 were kept as NV sows. These three specific immunization times were chosen to mimic a blanket vaccination fashion, which is performed in sows at all physiological status at a given time point. Gestation was monitored until farrowing, and then their piglets were followed up until weaning. Sows from all treatment groups were housed comingled during the study (in same pens when they were housed in groups, and in same room when they were individualized). Cross-fostering of piglets was only allowed among sows of the same experimental group.

All sows included in the study were injected (with phosphate buffered saline [PBS] or PCV-2 vaccine) three times before farrowing. Sows were vaccinated by intramuscular (IM) route (neck muscle) with 1 mL of PCV-2 Ingelvac Circoflex® or PBS. Blood samples from sows were collected at the three vaccine/PBS application times as well as at farrowing. Obtained

sera were tested for PCV-2 IgG and IgM antibodies using a commercial ELISA assay and to quantify virus using a qPCR assay. At pre-mating sampling point, PCV-2 IgG and IgM ELISA assay was performed in 288 sow sera to randomize the sows in 4 experimental groups. ELISA assay from mid gestation, late gestation and farrowing sampling points and qPCR assay from the fourth sampling points were performed only in those artificially inseminated sows (Table 3.1).

Table 3.1. Number of animals analysed at each sampling point.

Group	Sows at:									Piglets at weaning
	Pre-mating*		Mid gestation		Late gestation		Farrowing			
	ELISA	qPCR	ELISA	qPCR	ELISA	qPCR	ELISA	qPCR	qPCR PUC Pools**	ELISA***
V PM	73	63	60	60	60	60	58	58	50	294
V MG	72	61	57	57	56	56	54	54	47	272
V LG	73	62	61	61	61	61	60	60	45	300
NV	70	60	52	52	51	51	49	49	42	139
Total	**288**	**246**	**230**	**230**	**228**	**228**	**221**	**221**	**184**	**1005**

*The number of samples tested by ELISA referred to the animals sampled to be distributed within the groups (pre-screening); the qPCR results referred to those sows with confirmed pregnancy. The number of samples tested by ELISA and qPCR at the remaining sampling points referred to the number of sows available at each time point.

**Pools with 2-3 PUC per sow were constructed. Two sows with 4 PUC were analysed in 2 pools of 2 PUC each pool.

*** Sera samples from 4 to 6 randomly selected piglets per each sow were taken and used to detect PCV-2 antibodies using an ELISA test.

Blood from 3 (ranging 1 to 4) PUCs per sow were individually collected at farrowing to quantify virus in serum samples using the qPCR assay. To minimize PCV-2 environmental contamination, gloves were changed, and scissors were disinfected with ethanol for each PUC sampling. Additionally, productive parameters from gilts/sows and piglets were recorded. Moreover, at weaning, sera samples from 4 to 6 randomly selected piglets per each sow were taken and used to detect PCV-2 antibodies using an ELISA test.

Once in the laboratory, blood samples were centrifuged at 2500 rpm (1300 g) during 10 min at 4ºC to obtain sera. All sera were stored at -20ºC until testing.

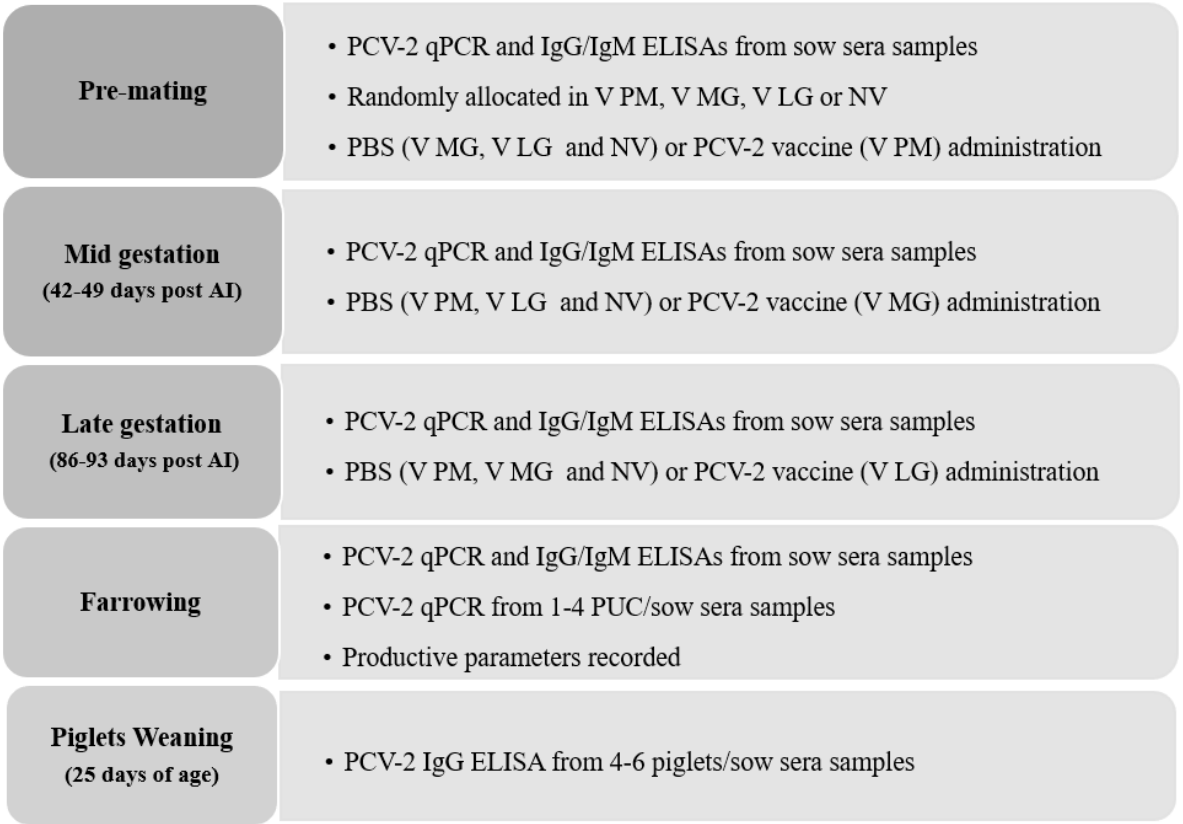

Figure 3.1. Experimental study design, including sampling time points and PCV-2 vaccine/PBS application timing. V PM: vaccinated at pre-mating; V MG: vaccinated at mid gestation; V LG: vaccinated at late gestation; NV: non-vaccinated; AI: artificial insemination; PCV-2 vaccine: 1 mL of PCV-2 Ingelvac Circoflex®; PBS: phosphate buffer saline.

3.2.3 DNA extraction and qPCR

Presence of PCV-2 DNA by qPCR was assessed in the serum samples from sows and PUCs. All these samples were processed by pools. Indeed, pools from 2-3 sow serum samples at each sampling point and pools of 2-3 PUC sera per sow at farrowing (except when only one PUC was collected) were created. When a pool from sow sera was qPCR positive, individual serum samples were tested by qPCR following the same protocol.

DNA was extracted from 200 µL of serum (from sows) or pool by using the MagMAX™ Pathogen RNA/DNA Kit (Thermo Fischer Scientific Baltics. Vilnius, Lithuania) following the manufacturer´s instructions. To quantify the PCV-2 DNA in serum samples, a qPCR assay (LSI VetMAX™ Porcine Circovirus Type 2-Quantification, Applied Biosystems, Lisseu, France) was performed. Each extraction and qPCR plate included negative controls (diethylpyrocarbonate [DEPC]-treated water) and each sample reaction had an internal positive control to monitor DNA extraction and amplification procedures.

PCV-2 qPCR results were transformed as follows:

- Undetermined results and those below limit od detection (LOD) (LOD = $10^{3.50}$ PCV-2 genome copies/mL) were transformed as $\log_{10}(0+1)$.

- Results between LOD and limit of quantification (LOQ) (LOQ = $10^{4.00}$ PCV-2 genome copies/mL) were transformed following the method proposed by Croghan et al., 2003 (Croghan et al., 2003), where the result was calculated as $\log_{10}((LOQ/\sqrt{2})+1)$. Therefore, the imputed value was $\log_{10}(7071+1)$.

- Results over LOQ were transformed as $\log_{10}$(PCV-2 genome copies/mL+1).

3.2.4 PCV-2 antibody detection by ELISA

PCV-2 antibodies in sows were detected at the three vaccine/PBS application times as well as at farrowing using the ELISA kit Ingezim Circo IgG/IgM 11.PCV.K2® assay (Ingenasa,

Madrid, Spain). ELISA results were expressed as mean OD (± Standard Deviation [SD]) according to the kit instructions.

PCV-2 antibodies in piglets at weaning were detected using the ELISA kit Ingezim Circo IgG 11.PCV.K1® assay (Ingenasa, Madrid, Spain). Mean positive cut-off was established as OD of negative control + 0.25). ELISA results from piglets were expressed as sample/positive control (S/P) ratio (OD of sample / OD of positive control for each ELISA plate) following the manufacturer's recommendations.

3.2.5 PCV-2 ORF2 amplification and sequencing

Capsid protein gene (ORF2) was sequenced from PCV-2 qPCR positive sow serum and PUC samples to determine the PCV-2 genotype/s circulating in the farm. DNA was extracted from serum samples by using the MagMax^{TM} Pathogen RNA/DNA Kit (Thermo Fischer Scientific Baltics. Vilnius, Lithuania) following the manufacturer´s instructions.

PCV-2 Cap gene was amplified from nucleotide 1050 to 1735 (PCV-2 genome; GenBank Accession Number: AY181948) using the primers PCV-2all_F (5′ GGGTCTTTAAGATTAAATYC 3′) and PCV-2all_R (5′ ATGACGTATCCAAGGAG 3′) (Oliver-Ferrando et al., 2016). PCR was performed in a 25 µL reaction containing 5 µL of PCR Promega buffer, 2.5 µL of 25Mm MgCl2, 1.25 µL of each primer at 10 pmol/µL, 1 µL of 5 mM dNTPs, 0.15 U of Taq DNA polymerase, 11.35 µL of DEPC-treated water and 2.5 µL of extracted DNA. The PCR was performed with the following program: denaturation of 5 minutes at 94°C, 40 cycles of 30 seconds at 95 °C for denaturation, 30 seconds at 53°C for primer annealing and 40 seconds at 72°C for elongation, with a final elongation of 7 minutes at 72°C. Amplified DNA was confirmed by electrophoresis gel with 2% agarose.

NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel, GmbH & Co. KG, Dueren, Germany) was used to purify the PCR product. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and 3130 × l Genetic Analyser (Applied Biosystems, Ohio, USA) was used to perform the sequencing reaction and the analysis, respectively (Oliver-Ferrando et al., 2016). Sequences were edited and assembled by using

ChromasPro Version 2.1.8 (Technelysium). The sequences obtained were submitted to the GenBank with the following accession numbers MT572494-MT572497).

3.2.6 PCV-2 capsid protein (ORF2) phylogenetic and sequence analysis

To genotype the PCV-2 sequences obtained, an alignment with eighteen representative sequences from genotypes PCV-2a, b, c and d was carried out with Clustal Omega (EMBL-EBI). The phylogenetic tree was created by using the Maximum Likelihood method included in Mega-X software (Kumar et al., 2018). The best substitution model according with the Bayesian information criterion was the Hasegawa-Kishino-Yano model, with a discrete Gamma distribution. Bootstrap resampling test was carried out with 1000 replicates. Bootstrap values higher than 70 were indicated in the constructed phylogenetic tree.

3.2.7 Statistical analyses

Statistical analyses were carried out using the software NCSS (Kaysville, Utah, USA). Comparisons were performed in two ways: 1) all V groups (mimicking a blanket fashion PCV-2 vaccination) versus the NV group, and 2) all experimental groups among them.

When comparison was performed between V and NV groups Mann-Whitney U test was used to total born piglets per sow, live born piglets per sow, weaned piglets per sow, weaning-to-mate interval, weaning-to-fecundation interval, PCV-2 viral load in sera of sows as well as viral load in PUC. Chi-square test was used to compare proportions of abortions, mummified foetuses, stillbirth piglets, dead suckling piglets, cross-fostered piglets, as well as of viraemic sows and PUC. The T –test was used to analyse birth and weaning body weights. Besides, when comparison was performed among all experimental groups, Kruskal Wallis test (including Bonferroni test for multiple comparison) was used to analyse total born piglets per sow, live born piglets per sow, weaned piglets per sow, weaning-to-mate interval, weaning-to-fecundation interval, PCV-2 ELISA antibody values in sow and piglets' serum samples and viral load in sera of sows and PUCs. Serological parameters from sows were analysed performing only the comparison among experimental groups to assess the serological effect of each vaccination. Chi-square or Fisher's Exact test was used to compare proportion of abortions, mummified piglets, stillbirth piglets, dead suckling piglets, cross-

fostered piglets, as well as of viraemic sows and PUCs. Moreover, ANOVA (including Tukey-Kramer multiple comparison test) was used to compare birth and weaning body weights. The significance level (α) was set at $p \leq 0.05$, whereas statistical tendencies were reported when $P \leq 0.10$.

3.3 RESULTS

3.3.1 Productive parameters

Productive parameters from gilts/sows and piglets from the three V groups taken together and the NV group are shown in Table 3.2. The comparison of these parameters among each treatment group are detailed in Table 3.3. In both tables, statistically significant differences and tendencies are indicated.

Table 3.2. Productive parameters (mean± SD or proportion [percentage] plus confidence interval [CI]) of V and NV sows.

	V	NV
Total born piglets /sow	14.1±3.0 [a]	13.6±3.6 [a]
Live born piglets/sow	13.4±3.0 [a]	12.9±3.7 [a]
Weaning-to-mate interval (days)	4.6±5.1 [a]	4.6±5.0 [a]
Weaning-to-fecundation interval (days)	30.7±7.0 [a]	30.2±5.3 [a]
Weaned piglets/sow	12.1±2.7 [a]	11.8±3.7 [a]
Abortions	4/180 (2.2%) [a] CI: 0.1-4.4%	2/53 (3.8%) [a] CI: -1.4–8,9%
Mummies	34/2424 (1.4%) [a] CI: 0.9-1,9%	15/695 (2.2%) [a] CI: 1.1-3.2%
Stillbirth	105/2424 (4.3%) [a] CI: 3.5-5.1%	24/695 (3.5%) [a] CI: 2.1-4.8%
Dead suckling piglets	213/2295 (9.3%) [a] CI: 8.1-10.5%	58/658 (8.8%) [a] CI: 6.6-11.0%
Cross-fostered piglets	**127/2079 (6.1%) [a] CI: 5.1-7.1%**	**53/600 (8.8%) [b] CI: 6.6-11.1%**
Piglets birth weight (Kg)	**1.64±0.39 [a]**	**1.58±0.38 [b]**
Weaned piglet weight (Kg)	**6.51±1.48 [a]**	**6.37±1.48 [b]**

V: Vaccinated; NV: Non-vaccinated. Different letters in superscript in a row indicate significant differences ($p \leq 0.05$) between V and NV groups (highlighted in bold).

Table 3.3. Productive parameters (mean± SD or proportion [percentage] plus CI) of the four studied experimental groups.

	V PM	V MG	V LG	NV
Total born piglets /sow	14.1±2.8 [a]	13.9±2.5 [a]	14.3±3.5 [a]	13.6±3.6 [a]
Live born piglets/sow	13.3±2.7 [a]	13.5±2.6 [a]	13.4±3.6 [a]	12.9±3.7 [a]
Weaning-to-mate interval (days)	4.2±3.6 [a]	4.7±5.9 [a]	4.8±5.7 [a]	4.6±5.0 [a]
Weaning-to-fecundation interval (days)	30.7±7.7 [a]	30.6±6.7 [a]	30.7±6.6 [a]	30.2±5.3 [a]
Weaned piglets/sow	12.0±2.5 [a]	12.2±2.4 [a]	12.1±3.1 [a]	11.8±3.7 [a]
Abortions	1/60 (1.7%) [a] CI: -1.6-4.9%	3/59 (5.1%) [a] CI: -0.5-10.7%	0/61 (0.0%) [a] CI: 0.0%	2/53 (3.8%) [a] CI: -1.4–8,9%
Mummies	**9/815 (1.1%) [A]** **CI: 0.4-1.8%**	**10/753 (1.3%) [A,B]** **CI: 0.5-2.1%**	**15/856 (1.8%) [A,B]** **CI: 0.9-2.6%**	**15/695 (2.2%)[B]** **CI: 1.1-3.2%**
Stillbirth	37/815 (4.5%) [a] CI: 3.1-6.0%	27/753 (3.6%) [a] CI: 2.3-4.9%	41/856 (4.8%) [a] CI: 3.4-6.2%	24/695 (3.5%) [a] CI: 2.1-4.8%
Dead suckling piglets	74/769 (9.6%) [a] CI: 7.5-11.7%	67/727 (9.2%) [a] CI: 7.1-11.3%	72/799 (9.0%) [a] CI: 7.0-11.0%	58/658 (8.8%) [a] CI: 6.6-11.0%
Cross-fostered piglets	**40/694 (5.8%) [a,A,B]** **CI: 4.0-7.5%**	**33/659 (5.0%) [a, A]** **CI: 3.3-6.7%**	**54/726 (7.4%) [a,b,B]** **CI: 5.5-9.3%**	**53/600 (8.8%) [b,A,B]** **CI: 6.6-11.1%**
Piglets birth weight (Kg)	**1.62±0.40 [a,b]**	**1.66±0.38 [a]**	**1.64±0.39 [a]**	**1.58±0.38 [b]**
Weaned piglet weight (Kg)	6.49±1.50 [a]	6.52±1.48 [a]	6.52±1.45 [a]	6.37±1.48 [a]

V PM: vaccinated pre-mating; V MG: vaccinated at mid gestation; V LG: vaccinated at late gestation; NV: non-vaccinated. Different letters in superscript in a row indicate significant differences ($p \leq 0.05$ for lower case letters) or tendency ($p \leq 0.10$ for capital letters) among experimental groups (highlighted in bold).

3.3.2 PCV-2 antibody values in serum samples of sows and piglets

3.3.2.1 PCV-2 IgG and IgM ELISA OD values of sows

Sow blood samples collected at the three vaccine/PBS application times as well as at farrowing were used to determine the dynamics of IgG antibodies against PCV-2 (Figure 3.2). Each of the different V treatments showed significantly higher IgG values than the NV group from the sampling after their vaccine application until farrowing. At this point, no statistically significant differences between sows vaccinated at mid (V MG) and late (V LG) nor between sows vaccinated at pre-mating (V PM) and V MG group were observed.

In general terms, the PCV-2 IgM OD values of the four groups were very low (mean OD values between 0.40±0.14 and 0.51±0.20 at the different sampling points, data not shown) and no statistical differences in OD values between each experimental group at any time point were found.

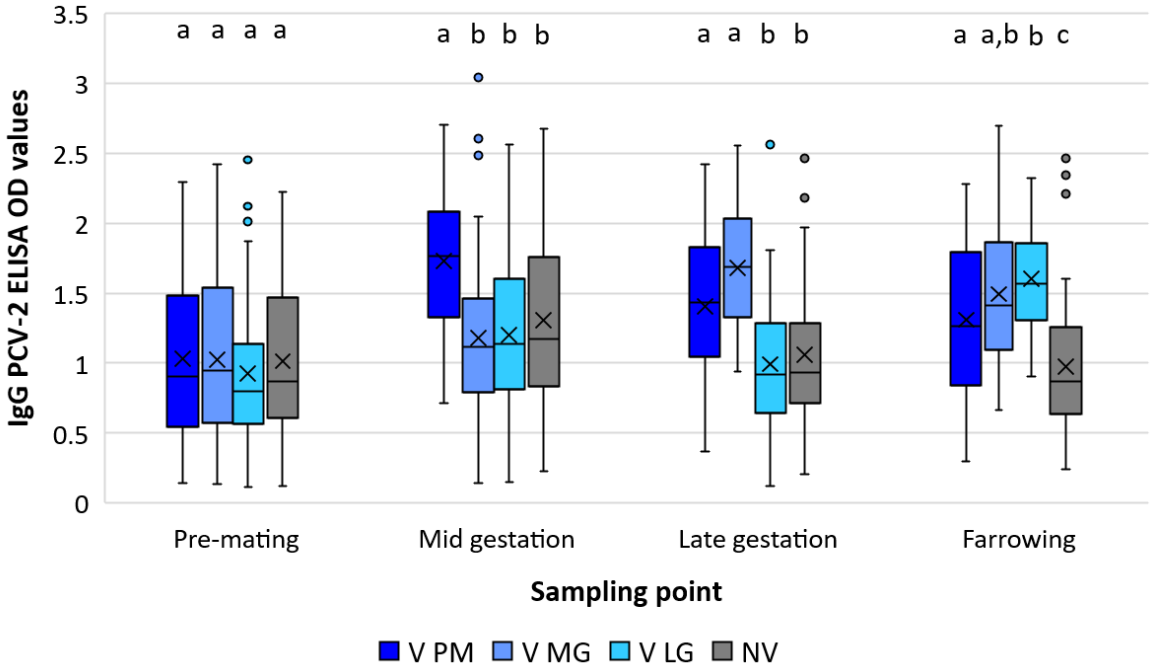

Figure 3.2. PCV-2 IgG OD results (mean ± SD) form sows' serum samples of the four experimental groups. V PM: vaccinated pre-mating; V MG: vaccinated at mid gestation; V LG: vaccinated at late gestation; NV: non-vaccinated. Different letters indicate statistically significant differences ($p \leq 0.05$) among experimental groups for a given sampling point.

3.3.2.2 PCV-2 IgG ELISA S/P values of weaned pigs

Blood samples from 4 to 6 randomly selected piglets per each sow were taken at weaning and used to detect PCV-2 antibodies. Mean PCV-2 S/P values per treatment groups are represented in Figure 3.3. Piglets from V sows showed significantly higher PCV-2 S/P values than piglets from NV sows. The highest values were obtained in piglets from V MG and V LG sows.

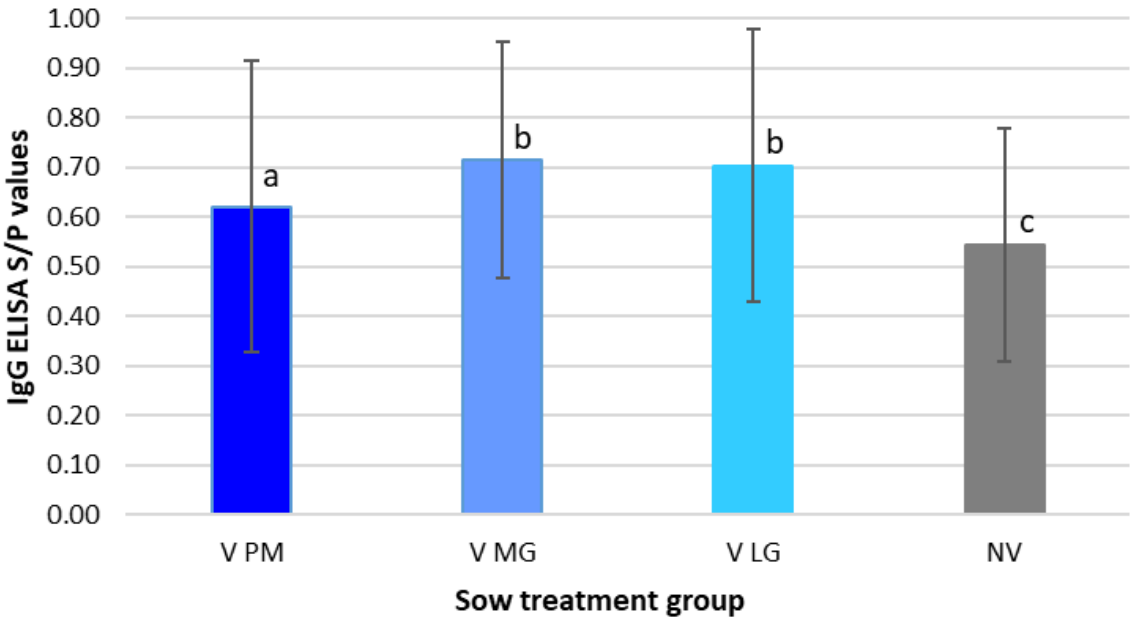

Figure 3.3. PCV-2 IgG S/P values (mean± SD) from weaned piglets' serum samples of each experimental group. V PM: vaccinated pre-mating; V MG: vaccinated at mid gestation; V LG: vaccinated at late gestation; NV: non-vaccinated. Different letters indicate significant differences among experimental groups ($p \leq 0.05$).

3.3.3 qPCR to detect PCV-2

3.3.3.1 PCV-2 DNA in sow serum samples

Blood samples from sows taken at the three vaccine/PBS application times as well as at farrowing were used to assess the proportion of PCV-2 qPCR positive sera from V and NV animals (Table 3.4).

Table 3.4. Proportion of PCV-2 qPCR positive sera in sows and CI from V and NV groups.

	Sampling point			
	Pre-mating	**Mid gestation**	**Late gestation**	**Farrowing**
V	0/186 (0.0%) CI: 0.0%	1/178 (0.6%) CI: -0.5-1.7%	0/177 (0.0%) CI: 0.0%	0/172 (0.0%) [a] CI: 0.0%
NV	0/60 (0.0%) CI: 0.0%	0/52 (0.0%) CI: 0.0%	0/51 (0.0%) CI: 0.0%	2/49 (4.1%) [b] CI: -1.5-9.6%

V: vaccinated; NV: non-vaccinated Different letters in superscript indicate significant differences ($p \leq 0.05$) among experimental groups.

Only three sows were positive to qPCR through the study: One V animal from V PM group was positive at mid gestation sampling point ($10^{4.2}$ PCV-2 copies/mL), and two NV sows were positive at farrowing (one with $10^{5.3}$ PCV2 copies/mL and another one below LOQ ($10^{4.00}$ PCV-2 genome copies/mL) with $10^{3.9}$ PCV2 copies/ml). At farrowing sampling point, a lower ($p=0.01$) proportion of positive PCV-2 qPCR in serum was detected in V sows (0 out of 172) compared to NV ones (2 out of 49).

3.3.3.2 PCV-2 DNA in serum samples from placental umbilical cord pools

Blood from 3 (ranging 1 to 4) PUCs per sow were individually collected at farrowing to quantify virus in serum samples. All these samples were processed as a pool of 2-3 PUC sera/sow at farrowing (n=171), except when only one PUC was collected (n=13). The number of PCV-2 qPCR positive pools of PUCs was significantly lower ($p=0.01$) in V sow groups (12/142 pools, 9% [CI: 3.9-13.0%]) compared to NV group (10/42 pools, 24% [CI: 10.9-36.7%]). When the comparison between each experimental group was performed, animals from V LG group had a significantly lower ($p=0.01$) proportion of PCV-2 qPCR positive pools of PUCs (2/45 pools, 4% [CI: -1.6-10.5%]) compared to NV sows (10/42 pools, 24% [CI: 10.9-36.7%]). Additionally, a tendency ($p=0.08$) in the proportion of PCV-2 qPCR positive pools of PUCs were noted in dams from V MG group (4/47 pools, 9% [CI: 0.5-16.5%]) when compared to NV sows (10/42 pools, 24% [CI: 10.9-36.7%]). Moreover, a lower ($p=0.01$) PCV-2 load in PUC pools was observed in sows from the V groups (below LOD [$10^{0.42} \pm 10^{1.43}$ PCV-2 genome copies/mL]) compared to NV ones (below LOD

[$10^{1.12}$±$10^{2.11}$ PCV-2 genome copies/mL]). No statistically significant differences were observed in PCV-2 load in PUC pools when the comparison was done between each experimental group by separate.

3.3.4 PCV-2 genotyping

PCV-2 ORF2 from serum and PUC samples with some of the highest PCV-2 loads (between $10^{4.19}$ and $10^{8.06}$ PCV-2 genome copies/mL) were sequenced to ascertain the main PCV-2 genotype/s circulating in the farm. Specifically, one positive serum sample from the V PM group collected at mid gestation sampling point and 7 individual PUC samples recovered from 3 pools (three from the NV, two from the V MG and two from the V PM sow groups). The phylogenetic tree including the relationships among the ORF2 sequences determined in this study and reference strains is presented in Figure 3.4. Genotype PCV-2b was found in the V PM sow serum sample from the mid gestation sampling point (MT572494 S 4525) and in 3 PUC from the same NV sow, all three with identical sequence (MT572495 PUC 3978). These two sequences showed 99,54% of nucleotide identity between them. Genotype PCV-2d was found in two PUC from a V PM sow (both with identical sequence, MT572497 PUC 4182) and in other two PUC from a V MG sow (also with the same sequence, MT572496 PUC 4035). These two sequences were identical between them as well.

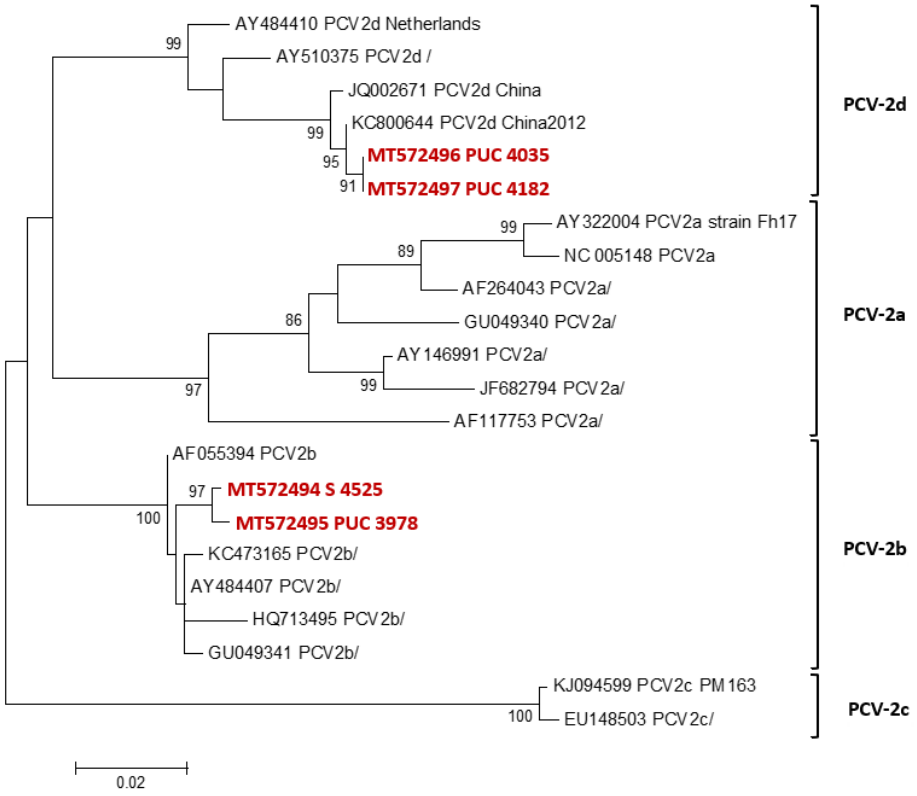

Figure 3.4. Phylogenetic tree derived from PCV-2 capsid protein (ORF2) sequences. The tree was constructed by using Maximum-Likelihood method with 1000 bootstrap replicates. Bootstrap values higher than 70 are indicated. Sequences from this study are highlighted in red and labelled with the accession number + PUC (for PUC samples) or S (for sow sample) + sow number.

3.4 DISCUSSION

The present study deals with a poorly described topic such as the effects of sow vaccination against PCV-2 on productive and infectious parameters of sows and their progeny. To accomplish this task, sows and gilts were vaccinated at three different reproductive periods (before mating, mid gestation and late gestation). When all the vaccinated sow groups were joined (independently of the moment of vaccine application), mimicking a blanket fashion vaccination strategy, significant improvement of productive parameters in terms of piglets' weight at birth and at weaning and cross-fostering practice reduction were achieved. Moreover, a significant reduction in the proportion of PCV-2 infected sows and their viral loads at farrowing and in PUC were also observed when compared to NV sows, although a low PCV-2 infection pressure was detected. When the period of vaccine application was considered, a tendency in reduction of mummies in the V PM group compared to NV one was observed.

Few published studies describe the benefits of sow vaccination on reproductive parameters under field conditions. This is probably due to the low frequency of reproductive disorders associated with PCV-2 (Pejsak et al., 2012), and their rather unknown impact. In one study (Kurmann et al., 2011), dams were vaccinated against PCV-2 at 4 and 2 weeks before AI and 4 weeks pre-partum. In another one (Pejsak et al., 2012), PCV-2 sow vaccination was implemented for 3 years; dams were vaccinated at 6 and 3 weeks before farrowing in the first reproductive cycle and boosted in the following reproductive cycles at 3 weeks before farrowing. In the third study (Oliver-Ferrando et al., 2018a), dams were immunized against PCV-2 at 6 and 3 weeks pre-farrowing on the first cycle and sows received a boost 2 weeks pre-farrowing on the second cycle. Finally, in the last one (Cybulski et al., 2020), three groups of sows were PCV-2 vaccinated one day after weaning, 28 days after weaning and non-vaccinated, respectively. In the present study, in comparison with the abovementioned works, the sow vaccination scheme considered PCV-2 vaccination at different reproductive time points in the same herd, resembling a blanket fashion vaccination strategy.

In the present study, piglets from V sows showed a significantly higher mean birth weight. Among all vaccination groups, the highest body weights were obtained in piglets coming from V MG and V LG groups. Considering the short period between vaccination at late gestation and the delivery, the difference in weight between piglets from V LG and NV groups was unexpected. This result, however, would be similar to the one obtained in one study in which sows were also vaccinated at late gestation (Pejsak et al., 2012). Moreover, the weight of weaned piglets (at 3 weeks of age approximately) coming from V sows was higher compared to those from NV dams. These results are in contrast with the ones of Fraile et al. (Fraile et al., 2012a), where sows were vaccinated pre-mating and no differences were observed in weaned piglet weight at 4 weeks of age. Reasons for such differences may be attributable to the different vaccine product or schedule used and/or lack of power due to the limited sample size of the study. Moreover, there are many inter-farm factors (animal genetics, farm facilities, management practices, treatments, nutrition and vaccination schedule) that may influence the vaccination outcome.

Piglets coming from V sows were significantly less cross-fostered compared to the NV group; more specifically, significantly less cross-fostering was performed in V PM and V MG dams compared to the NV group. Also, a tendency to practice less cross-fostering in

piglets from V MG dams was noted compared to that in V LG sows. Cross-fostering is a practice frequently used to increase piglet survival and to organize litters with uniform body weight (Wattanaphansak et al., 2002). However, biosecurity procedures recommend minimizing the number of cross-fostered animals (Rose et al., 2012); therefore, reduction of this practice may help diminishing PCV-2 transmission among piglets.

A tendency of a lower proportion of mummies was observed in the V PM sow group compared with the NV group. Considering that mummification is an outcome of late reproductive problems (Madson et al., 2009a), the potential benefits of PCV-2 vaccination at pre-mating on mummies reduction was expected. This result is in contrast with Kurmann et al. (2011) and Sibila et al. (2013), where sows were vaccinated pre-mating, and with Oliver-Ferrando et al. (2018a), where sows were vaccinated at mid-late gestation. Further studies with a higher number of tested sows would be needed to confirm the data obtained herein.

In relation to stillborn, no statistical differences were observed in the current study, in accordance with Cybulski et al. study (2020). Oliver-Ferrando et al. (2018a) found an inconsistent situation, where a higher number of stillbirths per litter in the V group was observed in the first reproductive cycle but not in the second one. Nevertheless, proportions of stillborn detected in the present study and in Oliver-Ferrando et al. (2018a) are aligned with regular values expected in the average Spanish pig farm (8.6%) according to national records (www.bdporc.irta.es).

Although Oliver-Ferrando et al. (2018a) and Pejsak et al. (2012) reported an improvement in number of liveborn piglets, in the present case only a positive (numerically, but non-significant) effect was obtained similar to the study of Kurmann et al. (2011) and in 28 days after weaning vaccination group from Cybulski et al. study (2020).

In addition, no significant differences in the proportion of abortions were detected in the present study similarly to results obtained by Kurmann et al. (2011). Considering the low frequency of abortions detected in the present study, a larger sample size would be necessary to analyse this parameter and determine their effect. Nevertheless, the design of our study

was not within the scope of detecting the presence of significant differences for this latter parameter.

Similarly, Pejsak et al. (2012) and Oliver-Ferrando et al. (2018a) detected a higher number of weaned piglets/litter (being only statistically significant in the former study) in V dams compared to NV dams, respectively. These two latter studies indicated that the repeated use of PCV-2 sow vaccination can improve reproductive parameters. So, these positive effects (piglets born alive and weaned piglets/litter) may probably be more evident after several gestational cycles vaccinating the sows against PCV-2 rather than one single vaccination of sows as in the present study.

The antibody profile from sows immunized against PCV-2 revealed seroconversion after each vaccination, conferring a stronger herd immunity against PCV-2 compared to the NV (Kurmann et al., 2011; Sibila et al., 2013; Hemann et al., 2014; Oliver-Ferrando et al., 2018a). Besides, the NV group maintained low ELISA OD values during all gestation periods. In this sense, despite sow infection remained low or non-detectable in all studied groups during the gestation period, no V sows were found infected at farrowing while 4% of NV ones were qPCR positive. Although significant differences at the farrowing period in terms of percentage of viraemic sows was detected, the number of PCV-2 qPCR positive sera in sows was low. These results, however, should not be considered surprising since the number of infected sows tend to be low in most farms where subclinical infection is taking place (Eddicks et al., 2016; Dieste-Pérez et al., 2018; Oliver-Ferrando et al., 2018a; Eddicks et al., 2019); moreover, batch differences may also account for certain variability. Therefore, further studies would be desirable to further confirm this finding.

The virological results obtained in the study were in fairly contrast with those obtained in the screening prior to the start of the study, where PCV-2 DNA was detected in serum samples from 18 out of 30 clinically healthy sows. Moreover, PCV-2 genome was also detected in 24 out of 30 PUCs from the same sows. These initial results would fit well with those observed by other research groups in some PCV-2-SI farms in absence of vaccination (Shen et al., 2010). It is very likely that differences on percentage of infected sows among batches and the PCV-2 vaccination in ¾ of the studied groups could explain the variability and reduction of the PCV-2 infection pressure within the same farm when the large study

was carried out. It is speculated that the reduction of the PCV-2 infection pressure probably caused a reduction of transplacental transmission of PCV-2 to foetuses, evidenced by the lower proportion of PUC PCV-2 qPCR positive samples and viral load detected compared to the farm previous screening.

The higher antibody levels of V sows than NV ones at farrowing suggests a higher transfer of maternally derived antibodies (MDA) to piglets of V sows via colostrum as also described (Fraile et al., 2012a; Kurmann et al., 2011; Saha et al., 2014). This scenario should place piglets from V sows in a better immune position to counteract PCV-2 infection at early ages (Fraile et al., 2012a). As expected, the highest MDA transfer was observed in weaned piglets from V MG and V LG groups, followed by V PM. On the other hand, considering that all piglets were vaccinated at 3 weeks of age against PCV-2, certain concerns regarding MDA interference with vaccination could arise. Although in this study MDA interference with pig vaccination was not explored, several studies have reported evidence of such interference with vaccine humoral immune response (Fort et al., 2009a; Fraile et al., 2012a; Fraile et al., 2012b; Haake et al., 2014; Feng et al., 2016). However, the efficacy of the same vaccine used in this study has not shown to be jeopardized by high values of MDA (Figueras-Gourgues et al., 2019).

Two PCV-2 genotypes, PCV-2b and PCV-2d, circulated in the herd, being the most prevalent ones in the field worldwide (Kekarainen et al., 2015; Xiao et al., 2015). The co-circulation of two or more PCV-2 genotypes in the same farm is not unusual (Saporiti et al., 2020; Correa-Fiz et al., 2018; Hesse et al., 2008). Current vaccines based on PCV-2a strains appear to be able to cope with major circulating strains worldwide due to cross-protection among genotypes (Fort et al., 2008; Segalés et al., 2013; Opriessnig et al., 2014a; Franzo and Segalés, 2020).

PCV2 vaccines are the most sold preventive product in the porcine industry and one of the biological products in swine with highest return of inversion (Segalés, 2015). Besides, the combined vaccination of sows and piglets is an increasing practice since provides the optimal performance in animals (Pejsak et al., 2010; Fraile et al., 2012a), and although there is little information about gilt/sow immunization, vaccination of pigs and sows has been estimated

cost-efficient (Segalés, 2015). However, each farm should be analysed case by case to study the cost /benefit of PCV-2 vaccination (Alarcon et al., 2013).

In summary, vaccination of the breeding herd (gilts and sows) against PCV-2, mimicking a blanket fashion schedule in a subclinical infection scenario, improved immune status of dams and the progeny against the virus and reduced virus circulation at farrowing in sows and vertical infection to foetuses. However, a low infectious pressure was detected during the study. PCV-2 sow vaccination also improved piglets' weight at birth and at weaning and reduced cross-fostering practice. Also, a tendency in reduction of mummies from V PM group compared to NV was observed. Due to the limited PCV-2 circulation detected in the dams during the present exploratory study, the impact of PCV-2 vaccination on the productivity was considered low. Further studies should be needed to confirm the currently obtained results.

CHAPTER 4

Study II

Efficacy studies against PCV-2 of a new trivalent vaccine including PCV-2a and PCV-2b genotypes and *Mycoplasma hyopneumoniae* when administered at 3 weeks of age

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4.1 INTRODUCTION

PCV-2-SD is characterized by loss of weight, digestive signs, paleness of skin and dyspnoea in pigs mainly between six and eleven weeks of age. PCVDs represented one of the most severe outcomes causing significant economic impact in the swine industry worldwide during late 1990s and early 2000s (Harding et al., 1997; Rosell et al., 1999).

As other ssDNA viruses, PCV-2 has a high mutation rate, being around 10^3–10^4 substitutions/site/year (Mancera-Gracia et al., 2021). Besides, PCV-2 can also evolve by means of recombination (Gerber et al., 2013; Franzo et al., 2016; Franzo and Segalés, 2018). These two factors result in genetic changes and a wide diversity within PCV-2 (Correa-Fiz et al., 2018; Mancera-Gracia et al., 2021). In fact, nowadays, nine genotypes of PCV-2 are recognized (a-i) based on ORF2 gene sequencing (Wang et al., 2020) being three of them (a, b and d) the most frequently associated with clinical PCV2-SD (Franzo and Segalés, 2018).

Nowadays, PCV-2 vaccines in Europe are based on the PCV-2a genotype or a combination of PCV-2a and PCV-2b genotypes (Fort et al., 2008; Segalés, 2015; Opriessnig et al., 2019). Interestingly, some studies showed a closer genetic and antigenic relation between PCV-2b and PCV-2d sequences compared to PCV-2a and PCV-2d ones (Xiao et al., 2015; Opriessnig et al., 2019). Hence, a bivalent vaccine containing PCV-2a and PCV-2b genotypes may be a relevant option to face against the existing PCV-2 genotypes that are circulating under field conditions (Bandrick et al., 2020). In addition to PCV-2, *M. hyopneumoniae* is also an important pathogen that usually circulates in pigs during the postweaning period. *M. hyopneumoniae* is the main causative agent of enzootic pneumonia (EP) and one of the main contributors of PRDC, a polymicrobial and multifactorial condition in which different bacterial and viral agents are involved, including PCV-2 (Sibila et al., 2007; Maes et al., 2008; Opriessnig et al., 2011b; Thacker and Minion, 2012). Therefore, combination of PCV-2 and *M. hyopneumoniae* in one ready-to-use vaccine is a relevant option to reduce the management of the animals and, consequently, reduce the stress and the management associated costs (Park et al., 2016; Oh et al., 2019; Sibila et al., 2020). In fact, the number of combined vaccines including PCV-2 and *M. hyopneumoniae* has increased in the last few years.

The current work aimed to elucidate the efficacy against PCV-2 naturally infected pigs of a new trivalent vaccine containing inactivated PCV-1/PCV-2a and PCV-1/PCV-2b chimeras (cPCV-1/2a, cPCV-1/2b) as well as *M. hyopneumoniae* (CircoMax Myco®) administered in a single dose at three weeks of age.

4.2 MATERIALS AND METHODS

4.2.1 Farm selection

A total of two field trials were conducted in two different Spanish commercial farms. Criteria for farm selection were the existence of problems with PCVD or history of PCVD in the last two-and a-half years.

Farm A was a two-site commercial farm (breeding and gestation plus nursery) with 2,660 sows and a weekly farrowing batch system. Piglet weaning was carried out around four weeks of age. The sow farm was seropositive against *M. hyopneumoniae*, PRRSV and seronegative to ADV. Gilts and sows were crossbred (Duroc x Landrace). Sow and gilt vaccination farm program included PRRSV, Porcine parvovirus, *Erysipelothrix rhusiopathiae, Swine influenza virus* (SIV), APP and PCV-2 (the piglets at weaning, the gilts at 6 months of age and the sows post-partum) immunizations. At fattening facilities, pigs were vaccinated twice against ADV.

Farm B was a farrow-to-finish commercial farm with 10,500 sows with a weekly farrowing batch system. Piglet weaning was done at approximately 25 days of age. The sow farm was seropositive against *M. hyopneumoniae* and PRRSV, and seronegative to ADV. Gilts and sows were of Pietrain breed. Sow and gilt vaccination farm program included immunization against PRRSV, SIV, Porcine parvovirus, *Erysipelothrix rhusiopathiae*, *Escherichia coli, Clostridium perfringens type C*, atrophic rhinitis, ADV, *M. hyopneumoniae* and PCV-2 (at 3 and 6 weeks of age). Gilts were also vaccinated against PCV-2 at two and a half, six and seven months of age. Piglets were vaccinated against PRRSV before weaning and against ADV, PRRSV and SIV at fattening.

4.2.2 Study design

The design of these field studies was blinded, randomised, and controlled. A total of 4,076 male and female pigs (2,037 V and 2,039 NV) were enrolled in two trials: A and B (Table 4.1).

Table 4.1. Experimental study design and vaccination schedule of clinical studies.

Field trial	Farm	Treatment	Num. of animals	Doses and Volume	Age at vaccination (days)
Field trial A	**Farm A**	V	1,013	2 mL	19-27
		NV	1,011	2 mL	
Field trial B	**Farm B**	V	1,024	2 mL	18-24
		NV	1,028	2 mL	

V: Vaccinated; NV: Non-vaccinated

The sample size used for each variable was calculated by a biometrician using data from field safety and efficacy studies previously performed (Segalés et al., 2009).

The number of animals in each batch was determined by the number of clinically healthy pigs available on the particular week of study initiation. Thus, field trial A required recruitment of pigs from three different batches while for the field trial B, one batch was enough. Selection of pigs included in the study and distribution (blocked by gender) in V and NV groups were done between SD-3 and SD0, being SD0 the vaccination day.

Study animals were clinically observed daily throughout the study. A single vaccination was performed at three weeks of age approximately with two mL of a trivalent vaccine containing inactivated cPCV-1/2a, cPCV-1/2b and *M. hyopneumoniae* bacterin (CircoMax Myco®, Zoetis Inc., Lincoln, NE, USA) by IM route in the neck. NV pigs received two mL of PBS. Pigs from each treatment group were housed comingled in maternity and nursery phase, but male and females were separated by pen at fattening (in each pen there were V and NV animals from the same gender).

Blood samples and faecal swabs from piglets were collected at 7, 11, 16, 20 and 25 weeks of age approximately. Blood samples were also collected at three weeks of age before

vaccination. Sera samples were analysed by a validated in-house PCV-2 antibody ELISA and by a qPCR assay and faecal swabs were analysed by qPCR. Moreover, the body weight was registered before vaccination, at 16 weeks of age and before slaughter for 400 animals approximately per each treatment group (a minimum of 328 animals and a maximum of 438 as indicated in Table 4.2). Animals weighed during the study were not the same at each timepoint due to deviations occurring during the study (animal deaths or animals not found at the weighing moment) as indicated in Table 4.2. Thus, extra-animals from the same treatment group were selected for weighting when any animal selected for this action was missing.

Dead animals or pigs euthanized for welfare reasons from weaning until the slaughterhouse were examined post-mortem to determine the cause of death. Tissue samples collected at each necropsy (tracheobronchial, mesenteric and superficial inguinal lymph nodes, and tonsil) were processed for histopathology and PCV-2 IHC for PCVD diagnosis performed by a pathologist blinded to the treatment status. Moderate and severe histological lesions together with moderate or high amount of PCV-2 antigen in lymphoid tissues were diagnosed as PCV-2-SD (Segalés, 2015). When a PCV-2-SD diagnosis was confirmed in the studied herd, 60 animals (30 per treatment group) were selected and necropsied to obtain the above-mentioned lymphoid tissue samples. These samples were analysed by histopathology and PCV-2 IHC.

The Cap gene (ORF2) from 20 serum samples with the highest PCV-2 viral load (6.3-8.3 $\log^{10}$ DNA copies/mL) belonging to NV groups were sequenced to determine the PCV-2 genotype/s circulating in the farms.

Clinical studies were approved by the Olot Animal Welfare Committee (ID PJ023) and performed according to Guidelines on Good Clinical Practices (EMA, 2000).

Table 4.2. Number of animals enrolled per each action and timepoint performed in clinical studies.

Weeks of age of study animals	Action performed	Num. of animals			
		Field trial A		Field trial B	
		V	NV	V	NV
3	Body weight	328	335	392	394
	Blood sampling	50	52	51	51
7	Faecal swabs	48	51	48	49
	Blood sampling	48	51	48	49
11	Faecal swabs	47	49	39	39
	Blood sampling	47	49	39	39
16	Body weight	349	356	438	404
	Faecal swabs	45	46	59	62
	Blood sampling	45	46	58*	61*
20	Faecal swabs	47	47	57	58
	Blood sampling	47	47	58	59
24-27	Body weight	400	413	393	395
	Faecal swabs	45	46	57	58
	Blood sampling	45	46	57	58

V: Vaccinated; NV: Non-vaccinated
The number of animals weighed and sampled were not the same at each timepoint due to deviations occurring during the study (dead animals or animals not found at the moment).
*Blood sample from 2 animals were wrongly recorded in study form and it is not possible to determine correspondence with animal. Therefore, laboratory results corresponding to these two animals were excluded from analysis.

4.2.3 PCV-2 ORF2 amplification and sequencing

To determine the PCV-2 genotype/s circulating in the farms, Cap gene (ORF2) from 20 serum samples with the highest PCV-2 viral load (6.3–8.3 $\log_{10}$ DNA copies/mL) belonging to NV groups were sequenced. Total DNA was extracted from serum samples using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems) following the manufacturer's instructions. PCV-2 Cap gene was amplified using the primers PCV-2all_F (50 GGGTCTTTAAGATTAAATYC 30) and PCV-2all_R (50 ATGACGTATCCAAGGAG 30), and the procedure described by Oliver-Ferrando et al. (2016) was followed. PCV-2 amplicons were purified with ExoSAP-IT™ (Thermo Fisher Scientific, Vilnius, Lithuania) kit and sequenced by the Sanger method (BigDye® Terminator v3.1 Cycle Sequencing Kit,

Foster City, CA, USA) with the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®, Foster City, CA, USA) at Servei de Genòmica, Universitat Autònoma de Barcelona (Spain). The quality of the sequences was checked using the Finch TV program and trimmed with BioEdit software 7.2.6 (BioEdit, Manchester, UK) (Hall et al., 1999).

The phylogenetic analysis of the PCV-2 amplicon sequences obtained followed the proposed classification by Franzo and Segalés (2018). The amplicons of the PCV-2 ORF2 gene obtained herein were aligned against the representative strains of the proposed PCV-2 genotypes using MAFFT software (Katoh et al., 2019). A neighbour-joining method using the p-distance model was used to build the phylogenetic tree with 1000 bootstraps. The phylogenetic tree was further edited using the iTOL software (Letunic and Bork, 2019) where bootstrap values higher than 70% were maintained.

4.2.4 DNA extraction and qPCR

DNA from serum and faecal samples were extracted and qPCR analysed with a commercial kit LSI VetMAX^{TM} Porcine Circovirus Type 2-Quantification Applied Biosystems, Lisseu, France). The LOD of the technique in serum samples was $4x10^3$ DNA copies/mL and in faecal swabs was $1x10^4$ DNA copies/mL. The LOQ in serum sample and faecal swabs was $1x10^4$ DNA copies/mL. Log_{10} transformation of qPCR results was done, and result were interpretated as follows:

- Negative results or values below LOD were given a value equal to half of the LOD (log_{10} 3.3 copies/mL for serum samples and log_{10} 3.7 copies/mL for faecal swabs).

- Values between LOD and LOQ were considered positive and were given a value equal to LOQ (log_{10} 4.0 for serum and faecal swabs).

- Values over LOQ were considered positive and were given the log_{10} qPCR result obtained.

4.2.5 PCV-2 antibody detection by ELISA

PCV-2 antibodies were detected using a validated in-house PCV-2 antibody ELISA. The in-house ELISA test procedure consisted of a modified indirect ELISA based on recombinant baculovirus-expressed PCV-2 capsid protein (Nawagitgul et al., 2002). The PCV-2 antigen-coated plate was washed three times using a PBST washing buffer (0.1 M PBS-pH7.2 and 0.3% Tween 20). The sera were diluted 1:6000 in 5% milk diluent, and 100 µL of each diluted serum was incubated with positive and negative antigens at 36 ±2 ºC for 1 h. Excess antibodies were removed by washing 3 times with PBST buffer. Then, 100 µL of diluted peroxidase-labelled anti-pig IgG was added to each well and incubated at 36 ±2 ºC for 1 h. After 3 washings, 100 µL of 3,30,5,50 TMB substrate was added and incubated for 20 min at 36 ±2 ºC. The OD value was measured at 650 nm and 490 nm using a microplate reader and their difference per tested serum was reported as the S/P ratio (OD sample–OD negative control/OD positive control– OD negative control). Sera samples with S/P ratio (OD sample–OD negative control/OD positive control–OD negative control) values ≥ 0.5 were considered positive.

4.2.6 Histopathology and PCV-2 IHC

Lymphoid samples collected at necropsy (tracheobronchial lymph node, mesenteric lymph node, superficial inguinal lymph node and tonsil) were fixed by immersion in 10% buffered formalin and examined for lesions compatible with PCV-2, including LD and histiocytic replacement [HR]). Moreover, another section was cut for PCV-2 antigen detection by IHC (Rosell et al., 1999). LD, HR and the amount of PCV-2 antigen were scored from 0 (no lesions/no staining) to 3 (severe lesions/widespread antigen distribution) for each lymphoid tissue collected.

In field trials, naturally dead or euthanized pigs from weaning age were classified as PCV-2-SD or PCV-2-SI, following the diagnostic criteria indicated below:

1. Presence of at least one of the following clinical signs: wasting, weight loss, paleness of the skin, dyspnoea, diarrhoea, jaundice and/or inguinal superficial lymphadenopathy (only applicable to PCV-2-SD cases).

2. LD and/or HR of lymphoid tissues (PCV-2-SI: LD and HR ≤ 1; PCV-2-SD: LD and HR > 1).

3. PCV-2 in lymphoid tissues (PCV-2-SI: IHC ≤ 1; PCV-2-SD: IHC > 1).

4.2.7 Statistical analyses

Statistical analyses were carried out using the software SAS/STAT (User's Version 9.4, or higher, SAS Institute, Cary, NC). A logarithm transformation, where appropriate, was applied to the data before statistical analyses were done when needed. Comparisons were performed between treatment groups (V vs. NV) from each trial.

A general linear repeated measures mixed model was performed to analyse the following variables in each study: sera and faecal qPCR results, ELISA S/P values and body weight. Linear functions of the least squares mean for body weights were used to calculate estimates of the ADWG for each period. Moreover, a Pearson Correlation Coefficient was also calculated to evaluate the correlation between PCV-2 antibodies before vaccination and the ADWG during the whole study. A generalized linear mixed model was performed to analyse the following variables in each study: ever positive (detected positive on at least one sampling point) for viraemia/shedding, mortality, LD, HR and IHC results, and diagnosis of PCV-2-SD or PCV-SI. When the mixed model did not converge, Fisher's Exact test was used for analysis. Besides, MDA effect on seroconversion of V piglets was evaluated by calculating a Pearson Correlation Coefficient between PCV-2 antibodies before vaccination and the increase of PCV-2 antibodies at seven weeks of age (Delta value).

The significance level (α) was set at $p \leq 0.05$ for all statistical analyses.

4.3 RESULTS

4.3.1 Clinical evaluation

Body weight results and the ADWG are represented in Table 4.3. No significant differences in terms of body weight and ADWG were observed among V and NV groups of the field

study A at any time. In field study B, a significantly higher ($p \leq 0.05$) body weight was observed in the V group at 16 and 24-27 weeks of age (1-5 days before the slaughterhouse) compared to the NV one. Besides, in study B, ADWG from V animals was significantly higher ($p \leq 0.05$) in the three periods (from three weeks of age until 16 weeks of age, from 16 weeks of age until 24-27 weeks of age and from three weeks of age until 24-27 weeks of age) than NV group. No significant differences were detected in mortality between treatment groups in either field trials. Besides, no significant correlation was observed between PCV-2 antibody levels before vaccination and ADWG were detected in V groups of either field trials, indicating that ADWG of V pigs was independent of ELISA S/P titres at vaccination.

Table 4.3. Mean body weight (kg± Standard error [SE]), average daily weight gain (ADWG, kg/day) and mortality for each filed trial. Different letters indicate significant differences among experimental groups ($p \leq 0.05$) for each field trial.

Study	Group	Body weight (Kg±SE)			ADWG (Kg/day)			Mortality	
		3WOA (vac)	16WOA	24-27WOA	3WOA to 16WOA	16WOA to 24-27WOA	3WOA to 24-27WOA	Each treatment group	Total
Field trial A	V	5.9±0.29 [a]	51.1±0.56 [a]	100.7±0.78 [a]	0.49 [a]	0.62 [a]	0.78 [a]	89/953 (9.3%) [a]	181/1910 (9.5%)
	NV	5.9±0.29 [a]	50.3±0.56 [a]	99.1±0.70 [a]	0.48 [a]	0.60 [a]	0.76 [a]	92/957 (9.6%) [a]	
Field trial B	V	5.7±0.07 [a]	45.6±0.45 [a]	105.0±0.70 [a]	0.43 [a]	0.59 [a]	0.76 [a]	194/899 (21.6%) [a]	402/1797 (22.4%)
	NV	5.7±0.07 [a]	44.2±0.45 [b]	99.6±0.70 [b]	0.42 [b]	0.56 [b]	0.71 [b]	208/898 (23.2%) [a]	

V: Vaccinated; NV: Non-vaccinated; WOA: Weeks of age

4.3.2 PCV-2 antibody values

No significant differences between treatment groups in mean PCV-2 S/P ratios before the time of vaccine/placebo administration were found in any of both field trials (Fig 4.1A and Fig 4.2A).

In field trial A, piglets from the V group had a significantly higher ($p \leq 0.05$) mean PCV-2 antibodies from 7 to 16 weeks of age compared to those of the NV one (Figure 4.1A). In field trial B, piglets from the V group had a significantly higher ($p \leq 0.05$) mean S/P values at 11 and 16 weeks of age. In contrast, a significantly lower ($p \leq 0.05$) mean PCV-2 S/P ratio was detected at 25 weeks of age compared to NV group (Figure 4.2A).

The correlations between PCV-2 ELISA S/P values of V animals before immunization and their increase at seven weeks of age (Delta value) are represented in Figures 4.3A and 4.3B. A significantly negative ($p \leq 0.05$) correlation between IgG ELISA S/P values and PCV-2 antibody levels at seven weeks of age was detected in V groups from both field studies, indicating that the higher the PCV-2 S/P of maternal before vaccination the lower increase in PCV-2 S/P values were observed at seven weeks of age. Moreover, no significant correlation was obtained for the NV groups in both field trials (data not shown).

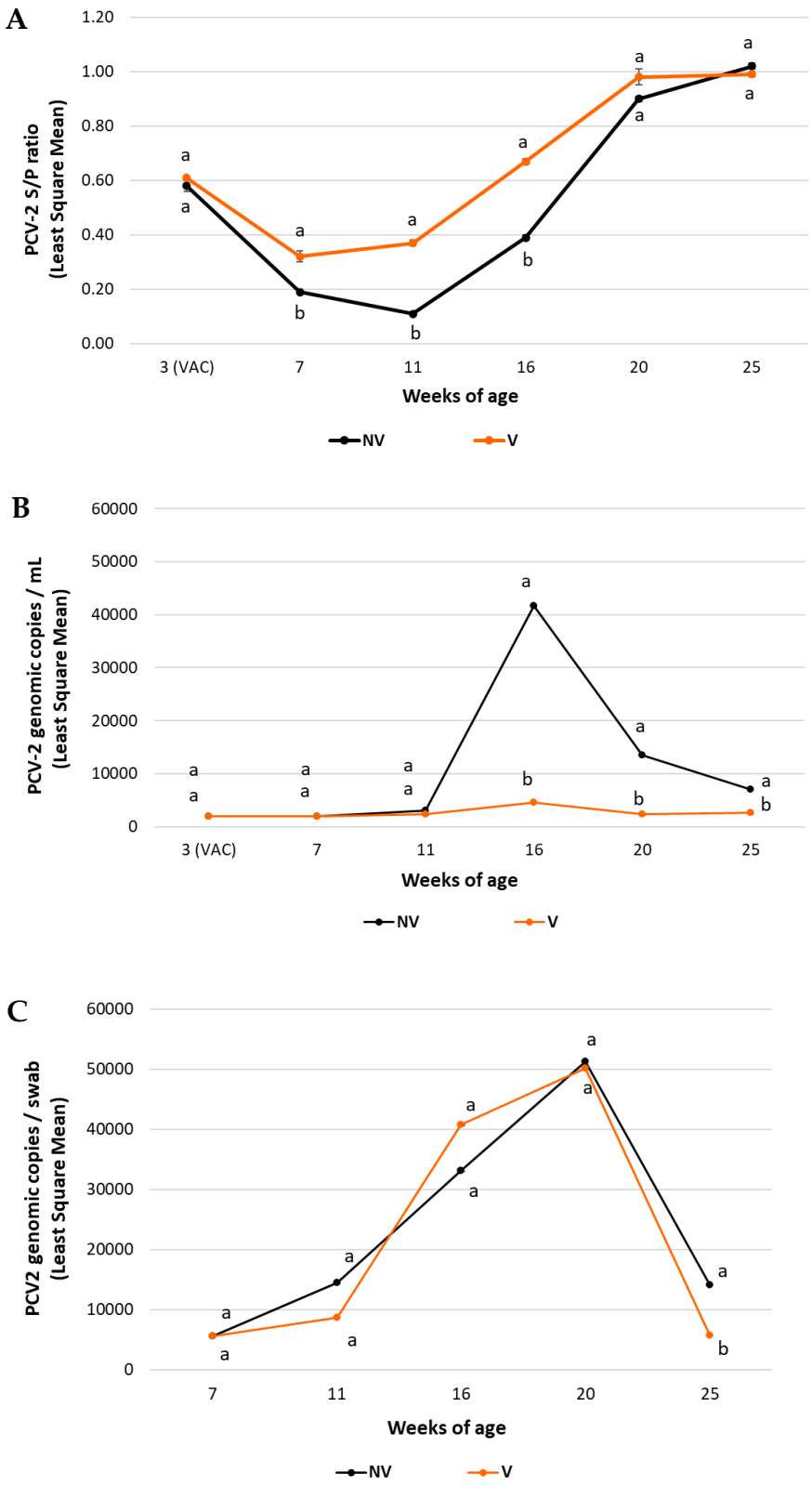

Figure 4.1. Field trial A results: PCV-2 IgG ELISA S/P results (mean ± SE) in serum samples (panel A), PCV-2 viraemia evolution (mean $\log_{10}$ genomic copies/mL±SE) (panel B) and PCV-2 qPCR results (mean $\log_{10}$ genomic copies/swab ±SE) in faecal samples (panel C) at different time points. Different letters indicate significant differences among experimental groups ($p \leq 0.05$).

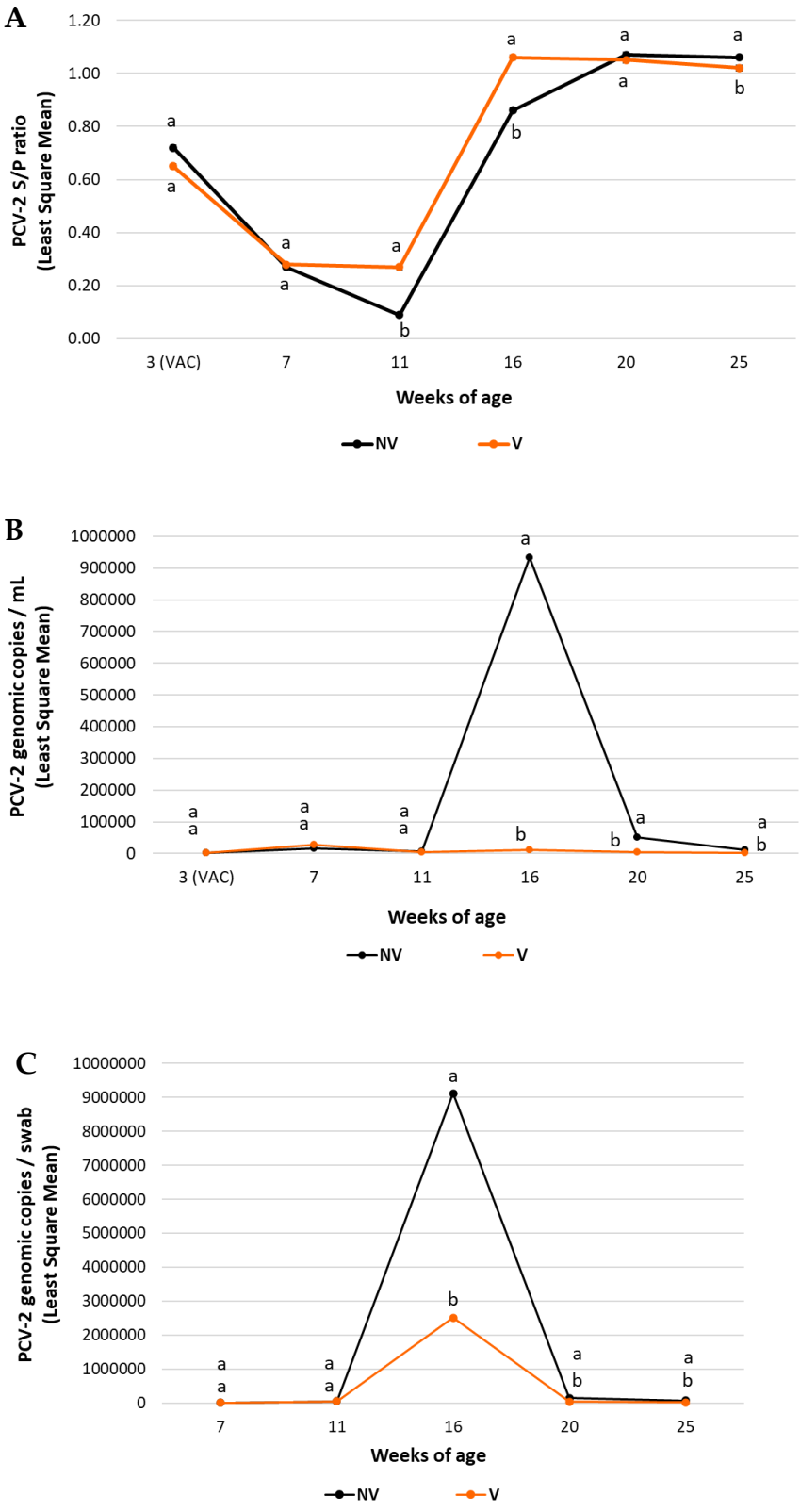

Figure 4.2. Field trial B results: PCV-2 IgG ELISA S/P results (mean ± SE) in serum samples at different time points (panel A), PCV-2 viraemia evolution (mean $\log_{10}$ genomic copies/mL±SE) (panel B) and PCV-2 qPCR results (mean $\log_{10}$ genomic copies/swab ±SE) in faecal samples (panel C) at different time points. Different letters indicate significant differences among experimental groups ($p \leq 0.05$).

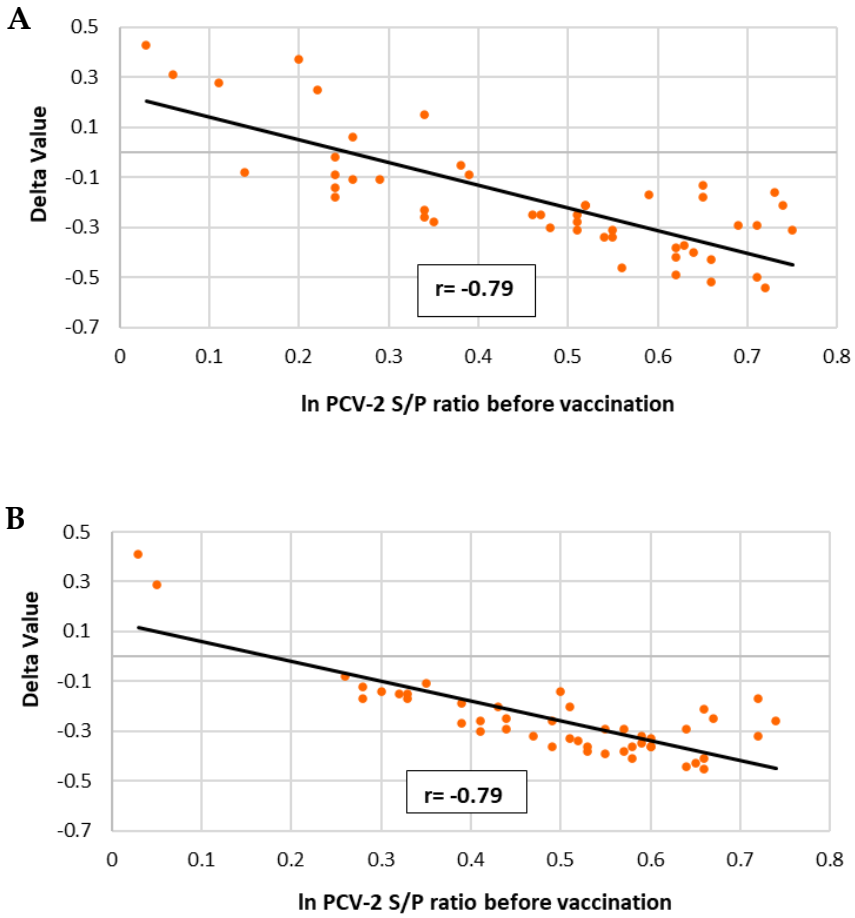

Figure 4.3. Linear regression and Pearson correlation coefficient between PCV-2 ELISA S/P ratios at vaccination and the increase of these titres until seven weeks of age approximately (Delta Value) in V piglets of field trial A (figure A) and field trial B (figure B).

4.3.3 PCV-2 viraemia

All tested pigs (n=204) were PCV-2 qPCR negative before vaccination. Significantly lower ($p \leq 0.05$) PCV-2 load and percentage of viraemic pigs was observed in V pigs from both field trials from 16 to 25 weeks of age compared to the NV groups (Figures 4.1B and 4.2B and Table 4.4).

In field trial A, the percentage of positive pigs peaked at 20 weeks of age in the NV group (36/47 [76.6%]), and at 16 weeks of age in the V one (13/45 [28.9%]). The peak of viraemia (maximum viral load in serum) was observed at 16 weeks of age for both groups.

In field trial B, the percentage of positive pigs increased to a maximum of 100% (61/61) at 16 weeks of age in the NV group. In the V group, it was obtained at seven weeks of age (28/48 [58.3%]) and

decreased afterwards. The peak of viraemia was observed at seven and at 16 weeks of age in the V and the NV groups, respectively. Besides, the percentage of pigs ever viraemic (detected positive at least at one sampling point) of both field trials were also significantly lower ($p \leq 0.05$) in the V group compared to the NV one (Table 4.4).

Table 4.4. Proportion and percentage of PCV-2 qPCR positive pigs (> 3.3 log_{10} DNA copies/mL) at least in one sample point for each experimental group and field trial. Different letters indicate significant differences among experimental groups ($p \leq 0.05$) for each field trial.

Study	Group	Proportion (%) of pigs detected viraemic per sampling point						Total Proportion (%) of ever viraemic pigs*
		3 WOA (vac)	7 WOA	11 WOA	16 WOA	20 WOA	25 WOA	
Field trial A	V	0/50 (0.0%) [a]	0/48 (0.0%) [a]	3/47 (6.4%) [a]	13/45 (28.9%) [a]	6/47 (12.8%) [a]	4/45 (8.9%) [a]	22/45 (48.9%) [a]
	NV	0/52 (0.0%) [a]	0/51 (0.0%) [a]	5/49 (10.2%) [a]	30/46 (65.2%) [b]	36/47 (76.6%) [b]	22/46 (47.8%) [b]	44/46 (95.7%) [b]
Field trial B	V	0/51 (0.0%) [a]	28/48 (58.3%) [a]	5/39 (12.8%) [a]	26/58 (44.8%) [a]	18/58 (31.0%) [a]	12/57 (21.1%) [a]	45/64 (70.3%) [a]
	NV	0/51 (0.0%) [a]	23/ 49 (46.9%) [a]	8/ 39 (20.5%) [a]	61/ 61 (100%) [b]	57/ 59 (96.6%) [b]	39/ 58 (67.2%) [b]	65/65 (100%) [b]

V: Vaccinated; NV: Non-vaccinated; WOA: Weeks of age
*Negative animals with a missing value in any of the time points were excluded from the analysis.

4.3.4 PCV-2 faecal shedding

PCV-2 faecal shedding results from field trial A and B are summarized in Figures 4.1C and 4.2C, respectively. In field trial A, statistical significantly lower ($p \leq 0.05$) PCV-2 faecal shedding was observed in V pigs at 25 weeks of age compared to NV pigs. In field trial B, statistically lower ($p \leq 0.05$) PCV-2 load in faecal swabs was also detected in V pigs at 16 and 20 weeks of age than in NV ones.

In field trial A, the peak of faecal shedding (maximum viral load in faeces) was observed at 20 weeks of age for both groups. In case of field trial B, peak faecal shedding was observed at 16 weeks of age for both groups.

Regarding the percentage of positive faecal swabs detected at least in one sampling point, no statistical differences were detected in any of the two studies between the V pigs (45/46 [97.8%] and 63/63 [100.0%] from field trials A and B, respectively) and the NV ones (45/45 [100.0%] and 61/61 [100.0%] from field trials A and B, respectively).

4.3.5 PCV-2 genotyping

To determine the main PCV-2 genotype/s circulating in the farms during the study periods, a total of 20 PCV-2 qPCR positive samples with the highest viral load (6.3-8.3 $\log_{10}$ DNA copies/mL), 10 per each field trial and belonging to NV groups were sequenced. A phylogenetic tree relating the ORF2 sequences obtained in these studies together with reference strains was built to determine the predominant genotypes present (Figure 4.4). In field trial A, genotype PCV-2b was found in nine out of 10 serum samples. One serum sample failed to be sequenced. In field trial B, genotype PCV-2a was found in two serum samples, PCV-2d in four samples and no sequence was obtained from the remaining four sera.

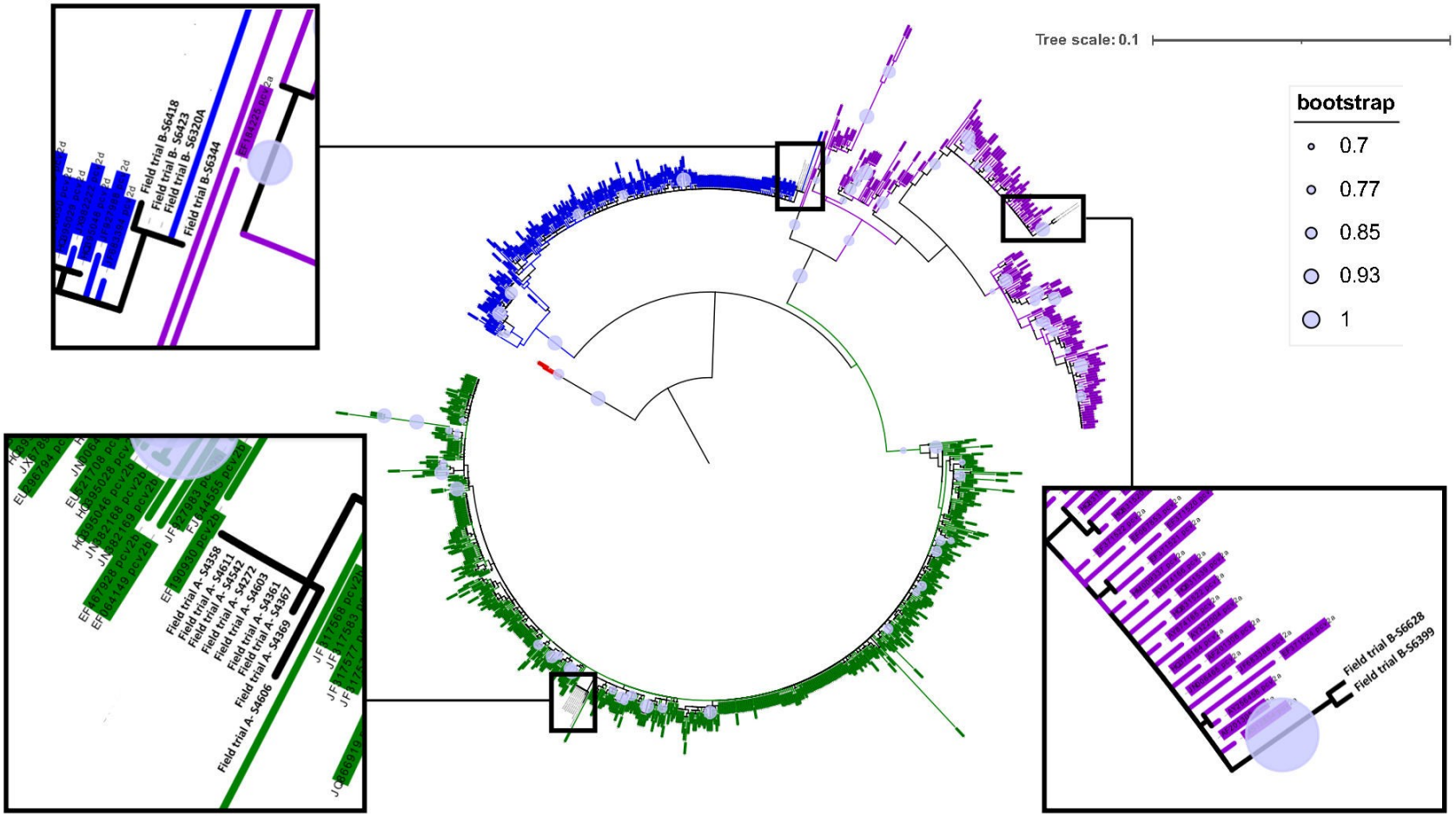

Figure 4.4. Phylogenetic tree derived from PCV-2 capsid protein (ORF2) sequences. Sequences from this study are indicated as Filed trial A or B plus the sample identification. The phylogenetic tree includes the relationships among the ORF2 sequences indicated with circles.

4.3.6 Histopathology and PCV-2 IHC

Histopathology and IHC results of the field trials are summarized in Table 4.5.

NV animals from both field studies showed a significantly higher ($p \leq 0.05$) HR and positive PCV-2 IHC compared to V ones. Moreover, in field trial A, a significantly higher ($p \leq 0.05$) incidence of PCV-2-associated lymphoid lesions (HR and LD together) was detected in NV pigs than in V ones.

The number of cases diagnosed as PCVD-SD was 0.9% (1/116) and 2.4% (6/245) in NV groups from field trials A and B, respectively. In V groups, a 0.0% (0/111) and a 0.5% (1/218) of PCVD-SD cases were diagnoses in field trial A and B, respectively. Besides, the number of PCVD-SI cases in NV groups showed statistically higher ($p \leq 0.05$) values (field trial A: 21 out of 116 [18.3%] and field trial B: 26 out of 245 [10.9%]) compared to those in V animals (field trial A: 4 out of 111 [3.6%] and field trial B: four out of 218 [1.8%]).

Table 4.5. Proportion of animals with histopathology (HR and LD) and IHC results scores >0 in at least one of the four lymphoid tissues evaluated (mesenteric lymph node, superficial inguinal lymph node, tracheobronchial lymph node and tonsil) corresponding to pigs which died or were euthanized during the study. Different letters indicate significant differences among experimental groups ($p \leq 0.05$) within each field trial.

Study	Group	HR	LD	HR or LD	IHC
Field trial A	V	3/111 (2.7%) [a]	6/111 (5.4%) [a]	7/111 (6.3%) [a]	4/110 (3.6%) [a]
	NV	12/116 (10.3%) [b]	15/116 (12.9%) [a]	19/116 (16.4%) [b]	22/116 (19.0%) [b]
Field trial B	V	4/218 (1.8%) [a]	30/218 (13.8%) [a]	30/218 (13.8%) [a]	9/228 (3.9%) [a]
	NV	21/245 (8.6%) [b]	39/245 (15.9%) [a]	39/245 (15.9%) [a]	43/ 253 (17.0%) [b]

4.4 DISCUSSION

PCVDs are causing great economical losses to the swine industry (Segalés, 2012). Vaccination of piglets against PCV-2 is the main control method to prevent PCVD in swine farms worldwide (Park et al., 2014b). In general, combined vaccination of PCV-2 and *M.*

hyopneumoniae around three weeks of age is one of the main strategies to reduce the impact of these two diseases (Park et al., 2016; Oh et al., 2019; Sibila et al., 2020).

PCV-2 vaccine benefits have been reported in terms of reduction in mortality (Segalés et al., 2009), PCV-2 viraemia and lymphoid lesions (Fachinger et al., 2008; Horlen et al., 2008), frequency of co-infections and improvement of the ADWG (Cline et al., 2008; Fachinger et al., 2008; Horlen et al., 2008; Kixmöller et al., 2008; Desrosiers et al., 2009) in PCV-2-SD scenarios. Moreover, an improvement of ADWG, percentage of runts, body condition and carcass weight has been also detected in case of PCV-2-SI (Young et al., 2011).

Interestingly, most PCV-2 vaccines in the market are based on the PCV-2a genotype. This is because the high degree of cross-protection between the major circulating genotypes worldwide (PCV-2b and PCV-2d) (Fort et al., 2008; Kurtz et al., 2014; Opriessnig et al., 2014a; Rose et al., 2016; Opriessnig et al., 2017; Park et al., 2019). However, PCV-2 vaccines do not eliminate virus replication and transmission, and it has been speculated that a broader-spectrum genotype-based vaccines may help in controlling better the infection under field conditions (Bandrick et al., 2022). Hence, the aim of the present study was to evaluate the efficacy against PCV-2 of a new trivalent vaccine containing inactivated cPCV-2a, cPCV-2b and *M. hyopneumoniae*, administered in piglets around three weeks of age, in a randomized controlled trial.

Improvement of clinical variables such as signs compatible with PCVDs, body weight evolution, ADWG or mortality are usual claims of PCV-2 vaccines. However, these differences are unlikely to be detected under experimental settings with a limited number of animals and the fact that PCV-2 infection outcome is usually subclinical. Therefore, these claims are mostly demonstrated under field conditions, by means of large trials. In the present case, a significantly greater body weight at 16 and 24-27 weeks of age (one-five days before going to the slaughterhouse) and higher ADWG at the three periods (3-16 weeks of age, 16-slaughter, and three weeks of age -slaughter) were observed in V pigs compared to NV ones in field trial B. These differences on body weight were not statistically significant in field trial A. However, they showed a remarkable tendency for improvement of approximately 0.8 kg live weight at 16 weeks of age and 1.7 kg, at slaughter, being an interesting improvement from an economical perspective (Alarcon et al., 2013). These

results are similar to those of several studies where a bivalent vaccine against *M. hyopneumoniae* and PCV-2 was evaluated in pigs V at three weeks of age, showing a greater ADWG during the finishing period (Tzika et al., 2015; Witvliet et al., 2015; Jeong et al., 2016; Pagot et al., 2017; Tassis et al., 2017; Duivon et al., 2018) or from vaccination until slaughter period (Tzika et al., 2015; Witvliet et al., 2015). Remarkably, no correlation between MDA and ADWG was observed in V animals, evoking that ADWG was independent of the MDA present at the time of vaccination as already observed in other studies (Fachinger et al., 2008; Fraile et al., 2012a), and indicating no evidence of interference of vaccine efficacy by MDA levels of the pigs from the tested herds.

A high mortality was detected in field trial B compared to the historical mortality in the farm, probably related to an outbreak of *Streptococcus suis* or *Glaesserella parasuis* infection, since gross lesions associated with these pathogens (fibrinous polyserositis, fibrinous pericarditis and /or polyarthritis) were observed in a high number of necropsied pigs. However, no significant effect of the vaccine on mortality was found in any of the studies in agreement with some studies where PCV-2-*M. hyopneumoniae* combined vaccine was administered at three-week-old pigs (Fraile et al., 2012a; Tzika et al., 2015; Pagot et al., 2017; Tassis et al., 2017; Duivon et al., 2018), but in contrast to other studies where a lower statistically significant difference in mortality was observed in V animals (Jeong et al., 2016; Nielsen et al., 2018) compared to NV ones. Noteworthy, the present field studies were designed with V and NV commingled within the same pens, so, globally, V pig benefits could be worsened, and NV detriments be ameliorated due to an overall increase of infectious pressure for V animals and a lower one for NV ones (Nielsen et al., 2018).

Vaccination of pigs with one dose at three weeks of age with the trivalent vaccine reduced significantly (PCV-2b pre-clinical an both clinical trials) or numerically (PCV-2a pre-clinical trial) the IHC scorings in V animals. Besides, a significantly lower percent of pigs with lymphoid lesions (when HR+LD were analysed together and when HR alone) were detected in the field trials. In the study of Park et al. (2016) where a PCV-2-*M. hyopneumoniae* combined vaccine was administered at three weeks of age and a challenge three weeks later with PCV-2 and *M. hyopneumoniae* was performed, it was demonstrated the reduction of the percentage of animals with lymphoid lesions and the PCV-2 positive cells in their lymph nodes in pigs V compared to NV ones.

Besides, incidences of PCVD-SD and PCV-2-SI from both field studies (A and B) were numerically and statistically higher, respectively in NV groups compared to those of V ones, further indicating that vaccination reduces the clinical and subclinical impact of PCV-2 infection.

Vaccination generated a higher level of IgG antibodies after a natural infection resulting in a faster humoral immune response upon infection. Such response paralleled with a reduction of PCV-2 loads in serum, faecal excretion, percentage of PCV-2 viraemic pigs and percentages of ever viraemic animals. The results agree with several studies under experimental and field conditions where piglets were injected with a combined PCV-2-*M. hyopneumoniae* vaccine or placebo at different ages (three days of age plus three weeks later, three weeks of age or four weeks of age) (Tzika et al., 2015; Jeong et al., 2016; Park et al., 2016; Kaalberg et al., 2017; Pagot et al., 2017; Tassis et al., 2017; Sibila et al., 2020) and PCV-2 viraemia and/or faecal excretion were significantly reduced in the V group compared to the placebo group.

Levels of MDA are very important for piglet immune response success upon vaccination (Oh et al., 2012) and the potential MDA interference on vaccine efficacy has not been yet demonstrated under field conditions (Poulsen et al., 2021), except in very particular situations with extremely high antibody values at vaccination (Haake et al., 2014; Feng et al., 2016). In both field trials, a statistically significant negative correlation was detected between PCV-2 IgGs before vaccination and antibody values at seven weeks of age in all V animals, indicating a PCV-2 elicited antibody response of the vaccine dependent on MDA titres. These results indicate that a lower PCV-2 S/P ratio levels should, ideally, ensure a seroconversion response after vaccination. Nevertheless, it has been widely demonstrated that MDA do interfere with vaccine seroconversion (Fort et al., 2009a; Fraile et al., 2012a; Fraile et al., 2012b; Feng et al., 2016; Oh et al., 2014), although in not all studies (Fachinger et al., 2008; Kixmöller, et al., 2008). Importantly, such negative MDA effect on vaccine-elicited humoral immune response is not apparently related with a reduction of vaccine efficacy as observed in the present and other studies (Fort et al., 2009a; Tzika et al., 2015; Poulsen et al., 2021). However, it is also evident that vaccine efficacy cannot be measured by vaccine seroconversion since not only humoral response, but cell-mediated response is

involved in the protection against PCV-2 (Fort et al., 2009a; Tzika et al., 2015; Feng et al., 2016; Tassis et al., 2017; Figueras-Gourgues et al., 2019).

PCV-2 genotype co-infection (PCV-2a and PCV-2d) was found within the same farm in field trial B, fact that has been described elsewhere (Hesse et al., 2008; Correa- Fiz et al., 2018; Saporiti et al., 2020). In contrast, only PCV-2b was found in field trial A. The new trivalent vaccine assayed in the present studies contains cPCV-2a and cPCV-2b genotypes. However, several experimental works have shown cross-protection between the major genotypes worldwide (PCV-2a, PCV-2b and PCV-2d) (Fort et al., 2008; Kurtz et al., 2014; Opriessnig et al., 2014a; Rose et al., 2016; Opriessnig et al., 2017; Park et al., 2019) and a closer relation between PCV-2b and PCV-2d compared to PCV-2a and PCV-2d genotypes (Xiao et al., 2015; Opriessnig et al., 2019). Nevertheless, further studies would be necessary to corroborate it.

In summary, and according to the results obtained globally in the two presented field studies, a single immunization at three weeks of age approximately with the novel PCV-2a/PCV-2b/*M. hyopneumoniae* vaccine was effective against PCV-2 infection (PCV-2a or mixed PCV-a/PCV-2d) by reducing productive losses, viral load and shedding and histopathological lymphoid lesions.

CHAPTER 5

Study III

Efficacy studies of a trivalent vaccine containing PCV-2a, PCV-2b genotypes and *Mycoplasma hyopneumoniae* when administered at 3 days of age and 3 weeks later against Porcine Circovirus 2 (PCV-2) infection

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5.1 INTRODUCTION

Vaccination is a very successful and efficacious tool in controlling PCV-2 infections, and there are numerous commercial PCV-2 vaccines available worldwide (Opriessnig et al., 2020). In Europe, all of them are based on inactivated virus or recombinant subunits based on PCV-2a alone or a combination of PCV-2a and PCV-2b (Fort et al., 2008; Segalés, 2015; Opriessnig et al., 2019), being in some cases combined with a *M. hyopneumoniae* bacterin. This combined vaccine strategy is frequently preferred as it reduces pig stress and decreases labour cost (Sibila, et al., 2020).

PCV-2 vaccine efficacy in piglets has also been demonstrated in the face of MDA against PCV-2 (Kekarainen et al., 2010). However, the potential interference on vaccine efficacy produced by MDA has not been demonstrated under normal field conditions (Haake et al., 2014; Feng et al., 2016; Pousen et al., 2021). Interestingly, some studies have reported MDA interference with the development of a humoral response after vaccination (Fort et al., 2008; Opriessnig et al., 2008; Fort et al., 2009a; Martelli et al., 2011; Fraile et al., 2012b), while others not (Fachinger et al., 2008; Kixmöller et al., 2008).

The present work aimed to elucidate the efficacy of a novel trivalent vaccine containing inactivated cPCV-1/2a, cPCV-1/2b and *M. hyopneumoniae* bacterin administered in pigs in a two-dose regime at 3 days of age and 3 weeks later.

5.2 MATERIALS AND METHODS

5.2.1 Farm selection

Two different field trials were conducted in two commercial farms located in North-Eastern Spain, the same ones mentioned in Study II. These farms were selected based on the existence of problems with PCVD or a history of PCVD in the previous two and a half years.

5.2.2 Study design

These clinical studies were blinded, randomized, and controlled trials. A total of 3,973 male and female pigs (1,983 V and 1,990 NV) were enrolled in these studies (Table 5.1).

Table 5.1. Experimental study design and vaccination schedule of clinical studies.

Field trial	Farm	Treatment	Num. of animals	Doses and Volume	Age at vaccination (days)
Field trial A	**Farm A**	V	1,017	2; 1 mL	2-4 and 23-25
		NV	1,021	2; 1 mL	
Field trial B	**Farm B**	V	966	2; 1 mL	2-5 and 23-25
		NV	969	2; 1 mL	

V: Vaccinated; NV: Non-vaccinated

Animals from Farm A (field trial A) came from three different batches and animals from Farm B (field trial B) came from one single batch. The studied pigs were selected within each batch during the first three days of life and were randomly distributed (blocked by gender) in two groups: V and NV.

The pigs were vaccinated twice (two doses) by IM injection (neck muscle) with Circo-Max Myco® (Zoetis Inc., Lincoln, NE, USA) at 2–5 days and at 23–25 days of age. NV pigs received 1 mL of PBS IM at each vaccine administration timing. The pigs from each treatment group were housed comingled within the same pens and barns during the study. Males and females were comingled in the maternity and nursery phase, but genders were separated by pen at fattening.

General health observation of the animals was carried out daily throughout the study. Moreover, blood samples from the piglets were collected at six different time points (before first vaccination and at 7, 11, 16, 20 and 25 weeks of age, approximately) for PCV-2 antibody testing by ELISA and to quantify virus levels by qPCR. Faecal swabs were collected at the same time points (but before vaccination) and tested by qPCR. Body weight was recorded 3 times during the study: before the first vaccination, at 16 weeks of age approximately and before going to the slaughterhouse (around 25 weeks of age). The number of animals weighed was not the same at each timepoint due to deviations occurring during the study (death of animals or animals not found at the weighing moment) (Table 5.2); therefore, extra animals not selected at the beginning but from the same treatment group were weighed and included in the study.

Dead animals or pigs euthanized for welfare reasons from weaning until the slaughterhouse were necropsied to determine the cause of death. Lymphoid samples (tracheobronchial, mesenteric, superficial inguinal lymph nodes and tonsil) for monitoring PCV-2 associated lesions and antigens were collected at each necropsy and fixed by immersion in 10% buffered formalin and processed for histopathology and PCV-2 IHC as indicated in Section 5.2.6. Moderate to severe histological lesions together with a moderate or high amount of PCV-2 antigens in lymphoid tissues were diagnosed as PCV-2-SD. Pathological analyses were performed in real time, so, when the first PCV-2-SD case was diagnosed, 60 animals (30 animals per treatment group) were randomly selected and necropsied to obtain lymphoid tissues to assess PCV-2 associated lesions and antigen detection by IHQ. These clinical studies were approved by the Olot Animal Welfare Committee (ID PJ023) and carried out according to the Guidelines on Good Clinical Practices (EMA, 2000).

Table 5.2. Number of animals enrolled per each action and timepoint performed in clinical studies.

Weeks of age of study animals	Action performed	Num. of animals			
		Field trial A		Field trial B	
		V	NV	V	NV
<1	Body weight**	399	400	337	360
	Blood sampling	47	50	43	48
7	Faecal swabs	42	44	30	31
	Blood sampling	42	44	30	31
11	Faecal swabs	43*	43	42	46
	Blood sampling	44	43	42	46
16	Body weight**	325	323	378	389
	Faecal swabs	44	41	40	42
	Blood sampling	44	41	40	42
20	Faecal swabs	42	39	41	37
	Blood sampling	42	39	41	37
24-27	Body weight**	395	404	417	404
	Faecal swabs	39	40	48	53
	Blood sampling	39	40	48	53

*One missing faecal swab
** The number of animals weighed was not the same at each timepoint due to deviations occurring during the study (dead animals or animals not found at the weighing moment)

5.2.3 PCV-2 ORF2 amplification and sequencing

To ascertain the PCV-2 genotype/s circulating in the farms, Cap gene (ORF2) from 19 serum samples with the highest PCV-2 viral load (6.6–8.3 $\log_{10}$ DNA copies/mL) belonging to NV groups was sequenced. Total DNA was extracted from serum samples using the MagMAXTM Pathogen RNA/DNA Kit (Applied Biosystems) following the manufacturer's instructions. PCV-2 Cap gene was amplified using the primers PCV-2all_F (50 GGGTCTTTAAGATTAAATYC 30) and PCV-2all_R (50 ATGACGTATCCAAGGAG 30), and the procedure described by Oliver-Ferrando et al. (2016) was followed. PCV-2 amplicons were purified with ExoSAP-IT™ (Thermo Fisher Scientific, Vilnius, Lithuania) kit and sequenced by the Sanger method (BigDye® Terminator v3.1 Cycle Sequencing Kit, Foster City, CA, USA) with the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem®, Foster City, CA, USA) at Servei de Genòmica, Universitat Autònoma de Barcelona (Spain). The quality of the sequences was checked using the Finch TV program and trimmed with BioEdit software 7.2.6 (BioEdit, Manchester, UK) (Hall et al., 1999).

The phylogenetic analysis of the PCV-2 amplicon sequences obtained followed the proposed classification by Franzo and Segalés (2018). The amplicons of the PCV-2 ORF2 gene obtained herein were aligned against the representative strains of the proposed PCV-2 genotypes using MAFFT software (Katoh et al., 2019). A neighbour-joining method using the p-distance model was used to build the phylogenetic tree with 1000 bootstraps. The phylogenetic tree was further edited using the iTOL software (Letunic and Bork, 2019) where bootstrap values higher than 70% were maintained.

5.2.4 DNA extraction and qPCR

DNA from serum and faecal samples collected from clinical studies was extracted by using the BioSprint 96 DNA Blood Kit following the manufacturer´s instructions. PCV-2 DNA quantification was performed as described in Oliver-Ferrando et al. (2018a) using a commercial kit (LSI VetMAX Porcine Circovirus Type 2, Life Technologies, Lissieu, France). The LOD of the technique in serum samples was $4x10^3$ DNA copies/mL and in faecal swabs was $1x10^4$ DNA copies/mL. The LOQ in serum samples and faecal swabs was $1x10^4$ DNA copies/mL. qPCR results were $\log_{10}$ transformed and interpreted as described in Study II.

5.2.5 PCV-2 antibody detection by ELISA

PCV-2 antibodies were detected using a validated in-house PCV-2 antibody ELISA. The in-house ELISA test procedure consisted of a modified indirect ELISA based on recombinant baculovirus-expressed PCV-2 capsid protein (Nawagitgul et al., 2002). The PCV-2 antigen-coated plate was washed three times using a PBST washing buffer (0.1 M PBS-pH7.2 and 0.3% Tween 20). The sera were diluted 1:6000 in 5% milk diluent, and 100 µL of each diluted serum was incubated with positive and negative antigens at 36 ±2 ºC for 1 h. Excess antibodies were removed by washing 3 times with PBST buffer. Then, 100 µL of diluted peroxidase-labelled anti-pig IgG was added to each well and incubated at 36 ±2 ºC for 1 h. After 3 washings, 100 µL of 3,30,5,50 tetramethylbenzidine (TMB) substrate was added and incubated for 20 min at 36 ±2 ºC. The OD value was measured at 650 nm and 490 nm using a microplate reader and their difference per tested serum was reported as the S/P ratio (OD sample–OD negative control/OD positive control– OD negative control). Sera samples with S/P ratio values ≥ 0.5 were considered positive.

5.2.6 Histopathology and PCV-2 IHC

Tissue samples collected at each necropsy (tracheobronchial lymph node, mesenteric lymph node, superficial inguinal lymph node and tonsil) were fixed by immersion in 10% buffered formalin. Then, the fixed tissue samples were dehydrated and embedded in paraffin blocks. From each paraffin block, two consecutive 4 µm thick sections were cut. One section was stained with haematoxylin-eosin (HE) stain and examined for lesions compatible with PCV-2, including LD and HR. The other section was processed by IHC for PCV-2 antigen detection. These lymphoid samples were scored for microscopic lesions associated to PCV-2 (LD and HR) and the presence of PCV-2 antigens by IHC (Rosell et al., 1999). Briefly, LD, HR and the amount of PCV-2 antigen were scored from 0 (no lesions/no staining) to 3 (severe lesions/widespread antigen distribution) for each lymphoid tissue collected.

Besides, any pig that died or was euthanized beyond weaning age was classified as PCV-2-SD or PCV-2-SI, if they complied with the following diagnostic criteria:

1. Presence of at least one of the following clinical signs: wasting, weight loss, paleness of the skin, dyspnoea, diarrhoea, jaundice and/or inguinal superficial lymphadenopathy (only applicable to PCV-2-SD cases).

2. LD and/or HR of lymphoid tissues (PCV-2-SI: LD and HR ≤ 1; PCV-2-SD: LD and HR > 1).

3. PCV-2 in lymphoid tissues (PCV-2-SI: IHC ≤ 1; PCV-2-SD: IHC > 1).

5.2.7 Statistical analyses

Statistical analyses were carried out using the software SAS/STAT (User's Version 9.4, or higher, SAS Institute, Cary, NC, USA). When needed, a logarithm transformation was applied to the data before statistical analyses were carried out. Comparisons were performed between the treatment groups (V vs. NV) from each field trial.

A generalized linear repeated measures mixed model was performed to analyse the following variables after the corresponding data transformation in each study: sera and faecal qPCR results, serology and body weight. When the mixed model did not converge, Fisher's Exact test was used for analysis. Linear functions of the least-squares mean for body weights were used to calculate estimates of the ADWG for each period. Moreover, a Pearson Correlation Coefficient was also calculated to evaluate the correlation between PCV-2 antibodies before vaccination and the ADWG during the whole study.

A generalized linear mixed model was performed to analyze the following variables after the corresponding data transformation in each study: ever positive (detected positive on at least one sampling point) for viraemia/shedding, mortality, LD, HR and IHC results and diagnosis of PCV-2-SD or PCV-SI. When the mixed model did not converge, Fisher's Exact test was used for analysis.

The MDA effect on seroconversion due to vaccination in piglets from field trials was evaluated by calculating a Pearson Correlation Coefficient for the correlation between PCV-

2 antibodies before vaccination and the increase in PCV-2 antibodies at 7 weeks of age (Delta value) after natural logarithm data transformation.

The significance level (α) was set at $p \leq 0.05$ for all statistical analyses.

5.3 RESULTS

5.3.1 Clinical evaluation

Body weight results, ADWG and mortality are represented in Table 5.3. In field trial A, a significantly higher ($p \leq 0.05$) body weight was observed in the V group at 16 and 24-27 weeks of age compared to the NV group. Moreover, the ADWG was significantly higher ($p=0.02$) in V animals compared to NV ones during the whole study period. In field trial B, no statistically significant differences in body weight nor in ADWG were detected.

It is worth noting that no significant correlation between PCV-2 ELISA S/P ratios before vaccination and ADWG were detected in the V and NV groups of both field trials.

Moreover, no statistically significant differences were detected in mortality between treatment groups from each field trial.

According to the macroscopic lesions detected in the necropsy of animals from field trial B, the high mortality was likely related to an outbreak of *Streptococcus suis* or *Glaesserella parasuis* (no bacteriological investigations were conducted, but those are the most likely agents for cases of fibrinous polyserositis, fibrinous pericarditis and polyarthritis, as we observed in a significant number of necropsies).

Table 5.3. Mean body weight (kg±SE), ADWG (kg/day) and mortality for each filed trial. Different letters indicate significant differences among experimental groups ($p \leq 0.05$) for each field trial.

Study	Group	Body weight (Kg±SE)			ADWG (Kg/day)			Mortality	
		<1WOA (vac)	16WOA	24-27WOA	<1WOA to 16WOA	16WOA to 24-27WOA	<1WOA to 24-27WOA	Each treatment group	Total
Field trial A	V	2.2±1.73 [a]	56.4±1.73 [a]	114.3±1.73 [a]	0.47 [a]	0.90 [a]	0.63 [a]	108/896 (12.1%)	221/1801 (12.3%)
	NV	2.1±1.74 [a]	55.0±1.74 [b]	112.2±1.73 [b]	0.46 [a]	0.89 [a]	0.62 [b]	113/905 (12.5%)	
Field trial B	V	1.5±0.52 [a]	45.6±0.48 [a]	103.4±0.47 [a]	0.39 [a]	0.72 [a]	0.53 [a]	259/806 (32.1%)	565/1652 (34.2%)
	NV	1.5±0.48 [a]	44.7±0.45 [a]	102.4±0.45 [a]	0.39 [a]	0.72 [a]	0.53 [a]	306/846 (36.2%)	

V: Vaccinated; NV: Non-vaccinated; WOA: Weeks of age

5.3.2 PCV-2 antibody values

No statistically significant differences between treatment groups in mean PCV-2 ELISA S/P ratios before the time of treatment administration were found in both studies.

In field trial A, piglets from the V group showed higher ($p < 0.05$) mean PCV-2 ELISA S/P ratios from 7 until 20 weeks of age compared with those from the NV group (Figure 5.1A). In field trial B, the gender had a significant treatment interaction effect on serological results; therefore, treatment comparisons for each gender were performed. The vaccinated female pigs showed higher ($p \leq 0.01$) PCV-2 ELISA S/P ratios at 16 weeks of age compared to the NV ones. In contrast, the V group male pigs had significantly lower ($p \leq 0.05$) mean PCV-2 ELISA S/P ratios at 20 and 25 weeks of age compared to the NV group (Figure 5.2A).

The correlation between PCV-2 ELISA S/P ratios of the V group animals before first immunization and the increase in PCV-2 antibody titres at 7 weeks of age (Delta value) is represented in Figure 5.3. A significantly ($p \leq 0.01$) negative correlation between the PCV-2 ELISA S/P ratios at first vaccination timing and 7 weeks of age was detected in the V groups from both field trials, indicating that the higher the MDA at vaccination time, the lower the PCV-2 antibody at 7 weeks of age. No significant correlation was obtained for the NV groups in both field trials (data not shown).

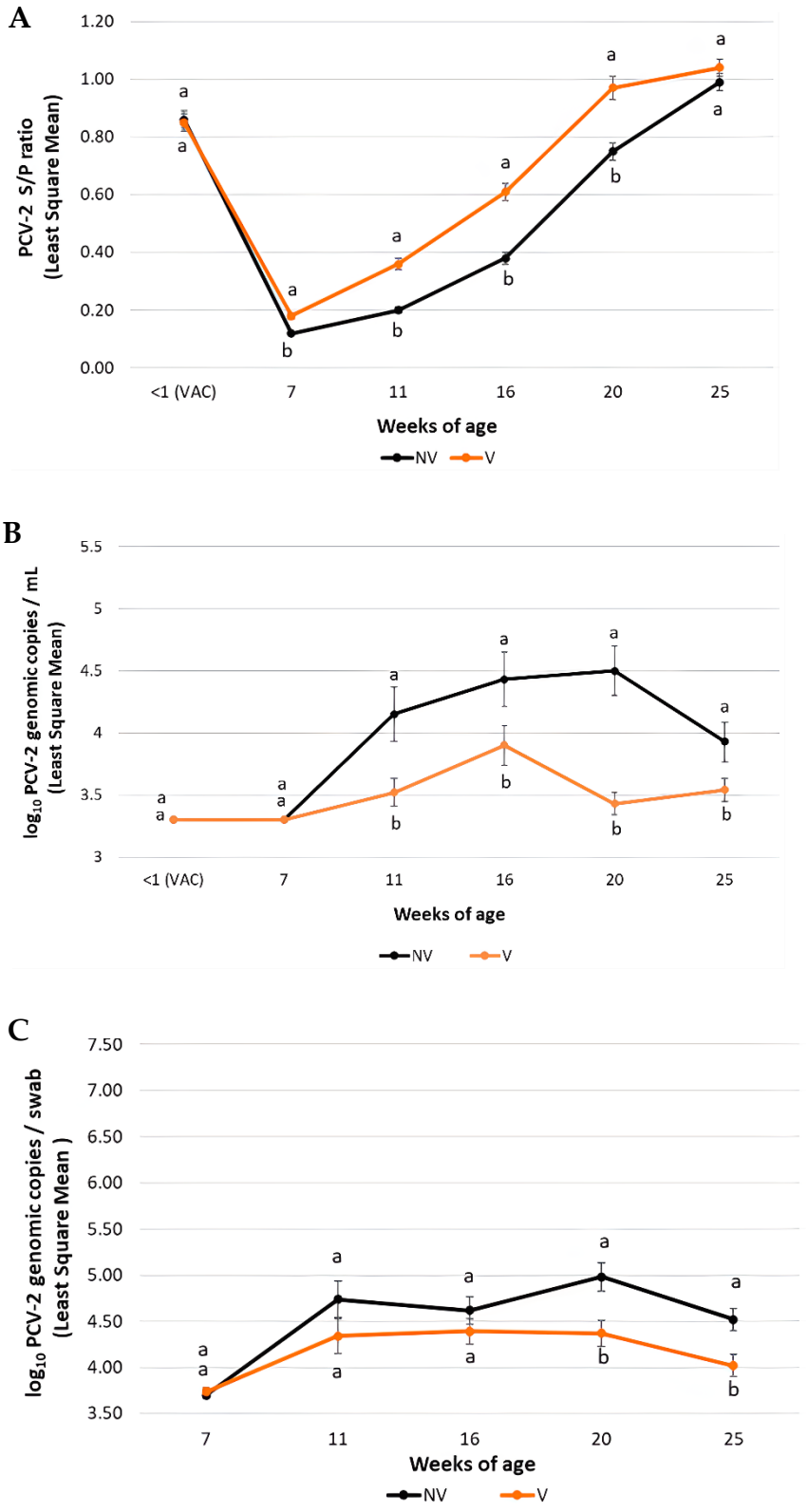

Figure 5.1. Field trial A results: PCV-2 IgG ELISA S/P results (mean ± SE) in serum samples (panel A), PCV-2 viraemia evolution (mean $\log_{10}$ genomic copies/mL±SE) (panel B) and PCV-2 qPCR results (mean $\log_{10}$ genomic copies/swab ±SE) in faecal samples (panel C) at different time points. Different letters indicate significant differences among experimental groups ($p \leq 0.05$).

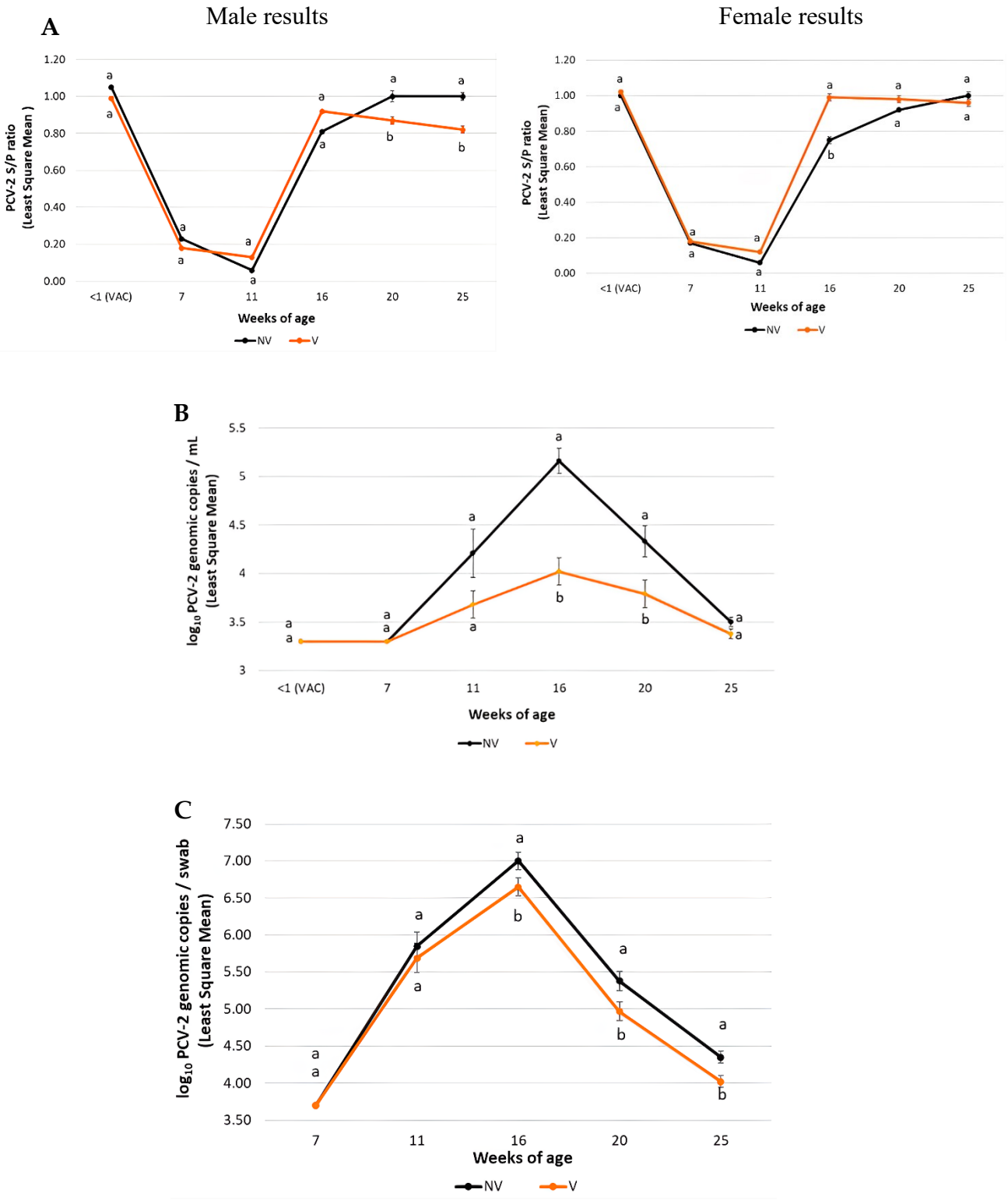

Figure 5.2. Field trial B results: PCV-2 IgG ELISA S/P results (mean ± SE) in serum samples at different time points (panel A). Treatment comparisons for each gender were performed due to a significant treatment interaction effect on serological results. Moreover, PCV-2 viraemia evolution (mean $\log_{10}$ genomic copies/mL±SE) (panel B) and PCV-2 qPCR results (mean $\log_{10}$ genomic copies/swab ±SE) in faecal samples (panel C) at different time points. Different letters indicate significant differences among experimental groups ($p \leq 0.05$).

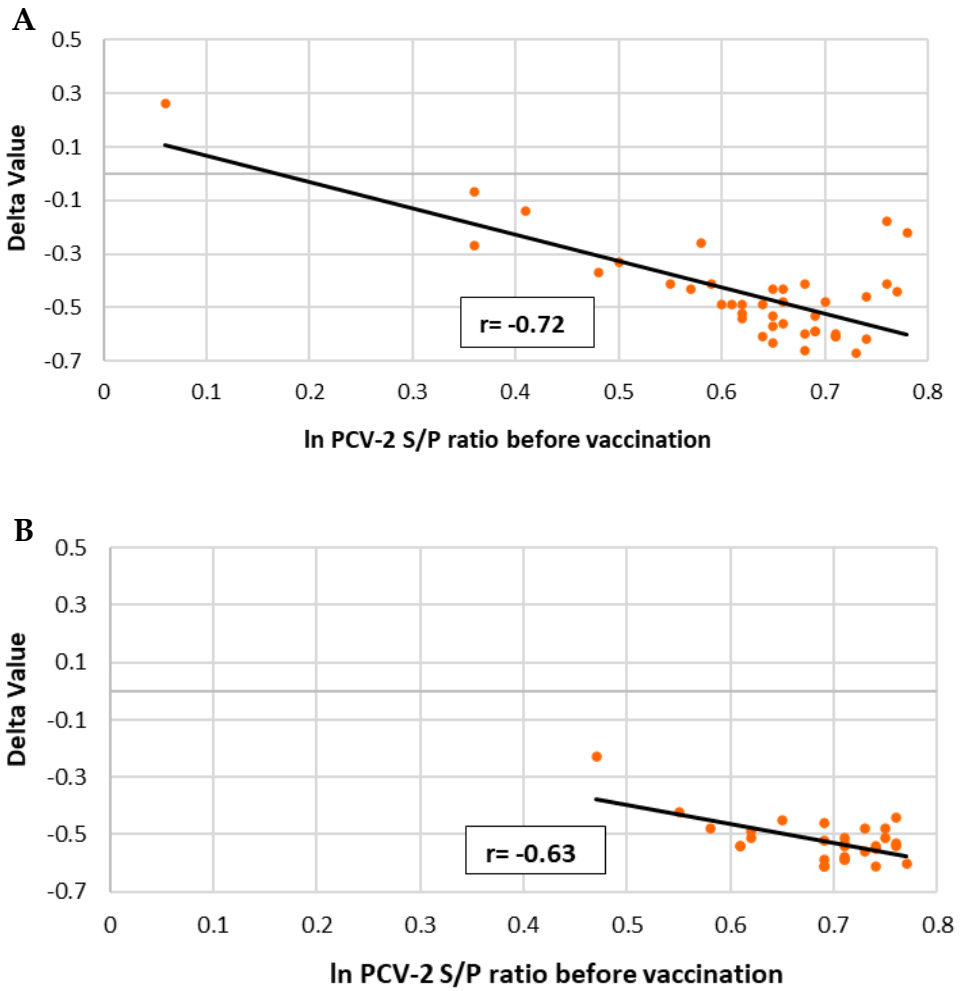

Figure 5.3. Linear regression and Pearson correlation coefficient between PCV-2 ELISA S/P ratios at vaccination and the increase of these titres until 7 weeks of age approximately (Delta Value) in vaccinated piglets of field trial A (figure A) and field trial B (figure B).

5.3.3 PCV-2 viraemia

All tested pigs from both trials (n = 188) were PCV-2 qPCR negative before vaccination.

In field trial A, significantly lower ($p \leq 0.05$) viral loads in serum were detected in the V group pigs from 11 to 25 weeks of age compared to the NV group ones. In addition, a significantly lower ($p \leq 0.01$) percentage of PCV-2 viraemic pigs was detected in the V group animals at 20 and 25 weeks of age compared to the NV group ones (Figure 5.1B).

Regarding field trial B, a statistically significant lower ($p < 0.01$) PCV-2 load in serum was observed in the V group pigs at 16 and 20 weeks of age compared to the NV group

pigs (Figure 5.2B). In addition, the percentage of pigs ever-viraemic (detected positive at least at one sampling point) in both studies were significantly lower ($p < 0.05$) in the V group than in the NV one (Table 5.4).

Table 5.4. Proportion and percentage of PCV-2 qPCR positive pigs (> 3.3 log_{10} DNA copies/mL) at least in one sample point for each experimental group and field trial. Different letters indicate significant differences among experimental groups ($p \leq 0.05$) for each field trial.

Study	Group	Proportion (%) of pigs detected viraemic per sampling point						Total Proportion (%) of ever viraemic pigs*
		<1WOA (vac)	7 WOA	11 WOA	16 WOA	20 WOA	25 WOA	
Field trial A	V	0/47 (0.0%) [a]	0/42 (0.0%) [a]	7/44 (15.9%) [a]	22/44 (50.0%) [a]	12/42 (28.6%) [a]	3/39 (7.7%) [a]	30/ 43 (69.8%) [a]
	NV	0/50 (0.0%) [a]	0/44 (0.0%) [a]	13/43 (30.2%) [a]	23/ 41 (56.1%) [a]	27/39 (69.2%) [b]	21/40 (52.5%) [b]	39/43 (90.7%) [b]
Field trial B	V	0/43 (0.0%) [a]	0/30 (0.0%) [a]	14/42 (33.3%) [a]	25/40 (62.5%) [a]	17/41 (41.5%) [a]	5/48 (10.4%) [a]	33/52 (63.5%) [a]
	NV	0/48 (0.0%) [a]	0/31 (0.0%) [a]	15/46 (32.6%) [a]	42/42 (100%) [a]	27/37 (73.0%) [a]	20/53 (37.7%) [a]	51/65 (78.5%) [b]

V: Vaccinated; NV: Non-vaccinated; WOA: weeks of age.
*Negative animals with a missing value in any of the time points were excluded from the analysis.

5.3.4 PCV-2 faecal shedding

In field trial A, statistical significantly lower ($p<0.01$) PCV-2 loads in faecal swabs was observed in V group animals from both studies at 20 and 25 weeks of age compared to the NV group (Figure 5.1C). In field trial B, a statistically lower ($p=0.04$) PCV-2 faecal shedding in V group pigs was also detected at 16 weeks of age compared to the NV group (Figure 5.2C).

Regarding the percentage of positive faecal swabs detected at least in one sampling point, no statistical differences were detected in any of the two studies between V group pigs (41/44 [93.2%] and 51/57 [89.5%] from field trials A and B, respectively) and NV group animals (42/43 [97.7%] and 56/66 [84.8%] from field trials A and B, respectively).

5.3.5 PCV-2 genotyping

The 19 PCV-2 qPCR-positive samples with the highest viral load (6.6-8.3 $\log_{10}$ DNA copies/mL), 10 and 9 from field trials A and B, respectively and belonging to NV groups from both field trials were sequenced to elucidate the main PCV-2 genotype/s circulating in the farms during the study periods (Figure 5.4). In field trial A, the PCV-2b genotype was found in 6 out of 10 sera analysed, while PCV-2d was detected in 2 sera; no sequences were obtained in 2 more samples. In addition, in field trial B, genotype PCV-2a was found in 8 out of 9 serum samples, and no sequence was obtained in 1 serum sample. One single genotype was found per sequenced serum.

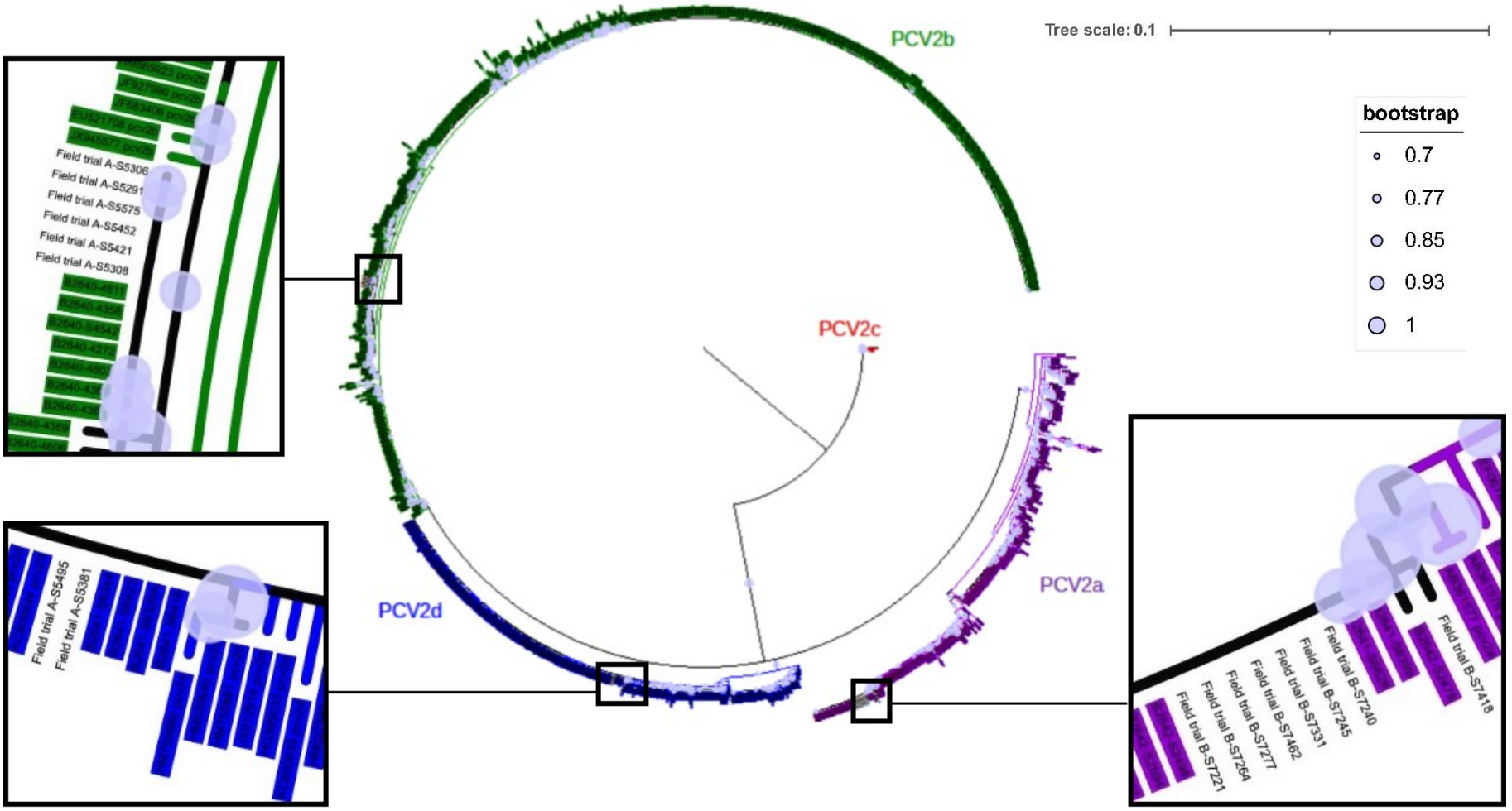

Figure 5.4. Phylogenetic tree derived from PCV-2 capsid protein (ORF2) sequences. Sequences from this study are indicated as Filed trial A or B plus the sample identification. The phylogenetic tree includes the relationships among the ORF2 sequences indicated with circles.

5.3.6 Histopathology and PCV-2 IHC

Table 5.5 summarizes the histopathology and IHC results of studied lymphoid tissues in died or euthanized pigs during the field trials' duration.

In field trial A, the percent of animals diagnosed as PCV-2-SD was 2.2% (2 out of 91 pigs) in NV group and 0.0% (0 out of 81 pigs) in V group. In contrast, a significantly higher (p=0.03) proportion of NV group animals were diagnosed as PCV-2-SI (20 out of 89 [22.5%]) compared to the V group (8 out of 81 [9.9%]). Regarding pathological findings, the NV group animals had a significantly higher (p=0.02) positive PCV-2 IHC scoring compared to that of the V group ones, but no significant differences for the rest of variables among both studied groups were found.

In field trial B, no PCV-2-SD was detected in any of the studied animals, and no statistical differences in cases of PCV-2-SI were detected between V and NV groups (NV: PCV-2-SI was detected in 4 pigs out of 220 died/euthanized pigs [1.8%] and V: PCV-2-SI was detected in 3 pigs out of 171 died/euthanized pigs [1.8%]). Regarding histopathological findings, a significantly higher (p=0.01) incidence of LD was detected in NV pigs compared to V pigs, but no significant differences for the rest of variables among both studied groups were found.

Table 5.5. Proportion of animals with histopathology (HR and LD) and IHC results scores >0 in at least one of the four lymphoid tissues evaluated (mesenteric lymph node, superficial inguinal lymph node, tracheobronchial lymph node and tonsil) corresponding to pigs which died or were euthanized during the study. Different letters indicate significant differences among experimental groups ($p \leq 0.05$) within each field trial.

Study	Group	HR	LD	HR or LD	IHC
Field trial A	V	6/81 (7.4%) [a]	13/81 (16.0%) [a]	13/81 (16.0%) [a]	8/81 (9.9%) [a]
	NV	10/91 (11.0 %) [a]	16/91 (17.6%) [a]	16/91 (17.6%) [a]	22/91 (24.2%) [b]
Field trial B*	V	0/172 (0.0%) [a]	24/171 (14.0%) [a]	24/172 (14.0%) [a]	3/192 (1.6%) [a]
	NV	1/220 (1.0%) [a]	55/221 (24.9%) [b]	55/221 (24.9%) [b]	4/241 (1.7%) [a]

V: Vaccinated; NV: Non-vaccinated
*Some tissue samples were not scored by histopathology because the samples were not evaluable.

5.4 DISCUSSION

PCVDs are important diseases in swine production worldwide and, since the last decade, vaccination is the main tool for disease prevention (Opriessnig et al., 2014b). Although PCV-2 vaccines are responsible for PCVD reduction in pig herds, they do not confer full protection and do not eliminate virus replication and transmission (Opriessnig et al., 2019). Hence, the present work reports the efficacy of the results against PCV-2 infection of a new trivalent vaccine containing inactivated cPCV-1/2a, cPCV-1/2b and *M. hyopneumoniae* bacterin (see Study II), administered in a two-dose regimen, and assayed in a randomized controlled trial under farm conditions.

In field trial A, a statistically significant greater body weight and ADWG was observed. Although these differences on body weight were not statistically significant in field trial B, they show a remarkable tendency for improvement of approximately 0.9 kg live weight at 16 weeks of age and 1.0 kg at slaughter, which is notably important from a financial viewpoint (Alarcon et al. 2013). Besides, no correlation between MDA and ADWG was observed in V animals. This result suggested that ADWG was independent of the MDA present at the time of vaccination as described in other studies (Fachinger et al., 2008;

Fraile et al., 2012a). Moreover, the global mortality rate from field studies were lower in V piglets than in NV ones, although they were not statistically significant. This could be related to the fact that V and NV animals were comingled in the same pen/room. In such scenario (not frequent under field conditions), the infectious pressure of NV piglets may have hindered the vaccine efficacy (Nielsen et al., 2018).

Vaccinated pigs with the trivalent vaccine had a lower percentage (although non-significant) of animals with lymphoid tissue lesions (HR and LD), and a significantly lower amount of PCV-2 positive cells by IHC, compared to NV pigs. Based on these pathological results, the incidence of PCV-2-SD and PCV-2-SI were higher in the NV group, only significantly for PCV-2-SI. These findings confirmed previous studies, which have indicated that vaccination reduces microscopic PCV-2-associated lesions and reduces the amount of PCV-2 antigen (Fenaux et al., 2003; Fenaux et al., 2004; Jeong et al., 2016; Park et al., 2016). In case of field trial B, the percentage of animals with LD was significantly higher in NV group although no statistically significant differences were observed in PCV-2-SD nor PCV-2-SI. These subtle differences are probably due to the low PCV-2 pressure detected at the time of the study performance showing no PCVD compatible clinical signs during this study. However, these results would be in agreement with those studies reporting that PCV-2 piglet vaccination is effective despite the PCVD farm status (PCV-2-SD or-PCV-2-SI) (Segalés, 2015).

The current work demonstrates the ability of a vaccination in a two-dose regimen to stimulate the development of IgG in presence of MDA subsequently after PCV-2 natural infection (in case of field trial A). Such immunization would result in a reduction of the PCV-2 loads in serum, faecal excretion, percentage of PCV-2 viraemic pigs (stand only for field trial A) and percentages of ever viraemic. These results are in concordance with several published experimental studies where animals were infected with PCV-2 after vaccination at different ages (5 days of age, 10 days of age, 3 and /or 6-7 weeks of age), where a higher PCV-2 antibody response plus a reduction of PCV-2 viral load (Fort et al., 2008; Opriessnig et al., 2008; Fort et al., 2009a; Opriessnig et al., 2010; O'Neill et al., 2011; Witvliet et al., 2015; Park et al., 2016; Sibila et al., 2020; Ahn et al., 2021) and faecal excretion (Fort et al., 2008; Fort et al., 2009a) were also observed. As indicated above, no significant differences were detected in the percentage of PCV-2 viraemic pigs at each of the sampling time points in field trial B (although significant differences were

detected in ever viraemic at any time point analysis). This result can be explained by the low PCV-2 nature infection, since in this study no PCV-2-SD was detected and only PCV-2-SI was observed.

MDA are essential for the neonate's immune response, and it is also an important component that can have an impact on the success of immunization (Oh et al., 2012). In the present work, a PCV-2 antibody response of the vaccine dependent on MDA titres was suggested in field studies, since a statistically significant negative correlation was detected between PCV-2 IgG antibodies before vaccination and PCV-2 IgG antibody evolution up to 7 weeks of age in V animals from both field studies. These results are in line with several studies in which a clear interference of MDA in vaccine efficacy in terms of seroconversion have been shown (Fort et al., 2009a; Fraile et al., 2012a; Fraile et al., 2012b; Oh et al., 2014; Feng et al., 2016). However, a negative MDA effect on humoral immune response of piglets is suggested to be not related to a negative impact on vaccine efficacy except for those cases where MDA titres are high (≥8 log2 IPMA antibodies) (Poulsen et al., 2021). Besides, PCV-2 vaccines induce not only humoral immunity, but also cellular immune response (Martelli et al., 2011; Segalés et al., 2015). Therefore, PCVD protection in the absence of a specific serologic response can be due to cellular immunity (Lin et al., 2020) and consequently, the absence of seroconversion after vaccination in the presence of MDA should not be assessed as any negative indicator for the effectiveness as it can be observed in some studies (Fort et al., 2009a; Tzika et al., 2015; Feng et al., 2016; Tassis et al., 2017; Figueras-Gourgues et al., 2019).

Different PCV-2 genotypes were detected (PCV-2a, PCV-2b and PCV-2d) in the two commercial farms where field studies were performed. In fact, co-infection of several PCV-2 genotypes in the same farm is not rare (Hesse et al., 2008; Correa-Fiz et al., 2018; Saporiti et al., 2020). Although several experimental studies have shown cross-protection between the major genotypes worldwide (PCV-2a, PCV-2b and PCV-2d) (Fort et al., 2008; Kurtz et al., 2014; Opriessnig et al., 2014a; Rose et al., 2016; Opriessnig et al., 2017; Park et al., 2019), a closer epitopic relationship between PCV-2b and PCV-2d than between PCV-2a and PCV-2d genotypes (Kurtz et al., 2014; Xiao et al., 2015; Opriessnig et al., 2019) has been detected. Although to be demonstrated at an efficacy level, these data may suggest that PCV2b-based vaccines could offer better protection against PCV-2d compared to a PCV-2a-based vaccines (Opriessnig et al., 2013). In fact, in a recent

study of Bandrick et al. (2022), animals vaccinated with a cPCV-1/2a, cPCV-1/2b bivalent vaccine showed higher levels of protection compared to PCV-2a and PCV-2b monovalent vaccines against PCV-2a and PCV-2b challenges. Animals treated with the bivalent vaccine showed less (although non-significant) PCV-2 shedding in faeces, ever shed PCV-2 in their faeces, viraemia and ever viraemic pigs compared to animals treated with the monovalent vaccine. These results are in concordance with the new vaccine used in the present study containing PCV-2a and PCV-2b genotypes, therefore expanding the epitopic repertoire of the vaccine product and potentially inducing a wider protection than monovalent vaccines against heterologous PCV-2.

In summary and according to the results of the present field studies, a double immunization at 3 days of age and 3 weeks later with the novel trivalent PCV-2a/PCV-2b/*M. hyopneumoniae* vaccine was effective against PCV-2 infection by reducing the histopathological lymphoid tissue lesions and PCV-2 detection in tissues (IHC), serum and faeces (qPCR), as well as reducing losses in productive parameters (BW and ADWG).

CHAPTER 6

General Discussion

PCV-2 has been one of the most studied pathogens of swine species since 1997, when the first peer-reviewed papers were published on PCV-2-SD, named as PMWS at that time (LeCann et al., 1997; Segalés et al., 1997). Economic losses due to the clinical outcome of PCVDs was the major reason of scientists and manufacturer companies to investigate on vaccine development (Segalés, 2015). Although PCV-2 vaccines are not sterilizing (Segalés et al., 2013; Feng et al., 2014), PCV-2 infection outcome has been mainly controlled using these vaccines, resulting in a reduction of PCV-2-SD outbreaks worldwide. In fact, PCV-2-SI has been always the dominant form, but this situation became evident once vaccines were commercialized and the prevalence of PCV-2-SD decreased (Segalés and Sibila, 2022). Nevertheless, PCV-2-SI is still causing a negative economic impact in the swine industry as PCV-2 subclinically infected pigs have a lower ADWG and might be more susceptible to other pathogens infection (Opriessnig et al., 2007; Segalés, 2012), contributing to post-weaning mortality.

The capability of PCV-2 vaccination in preventing the disease impact has been shown by multiple studies (Fachinger et al., 2008; Fort et al., 2008; Segalés et al., 2009; Martelli et al., 2011; Fraile et al., 2012a; Chae, 2012; Seo et al., 2012; Feng et al., 2014; Dvorak et al., 2016; Afghah et al., 2017; Karuppannan et al., 2017; Czyżewska-Dors et al., 2018; Park et al., 2019; Woźniak et al., 2019a; Woźniak et al., 2019b). Moreover, vaccination is also economically justified in subclinical infected herds, since the improvement of ADWG has been demonstrated under field conditions in vaccinated animals in absence of clinical disease (Young et al., 2011; Fraile et al., 2012b; Alarcón et al., 2013). Therefore, piglet vaccination against PCV-2 has become imperative in the swine industry.

In contrast, the role of the PCV-2 vaccination in breeding animals has been less explored and only few published studies have reported the benefits of sow vaccination. Nowadays, only two vaccines (Circovac® and Ingelvac CircoFLEX®) are specifically licenced for the breeding stock. This could be explained by the fact that the detection of PCV-2-RD is rare under field conditions, probably due to the relatively high seroprevalence in adult pigs (Segalés, 2012). Nevertheless, field veterinarians claim that PCV-2 sow vaccination may result in an improvement of reproductive parameters even in PCV-2-SI scenarios (Pejsak et al., 2012; Oliver-Ferrando et al., 2018a). However, others reported no evident reproductive parameters improvement (Kurmann et al., 2011; Cybulski et al., 2020). Therefore, in light

of such controversial data, one of the objectives of this Thesis was to further investigate the PCV-2 sow vaccination effect on a PCV-2-SI scenario. More specifically, the vaccination in a blanket fashion was explored (Study I) since no literature investigating this particular strategy and its effects had been described when this Thesis started. One of the objectives of vaccinating the sows is to transfer a more homogeneous humoral and cellular immunity to their offspring, which in turn causes an increase of the MDA levels at the moment of piglet vaccination (Sibila et al, 2022). In fact, several studies have shown putative interference of MDA on the humoral immune response elicited by piglet vaccination. However, such interference on the efficacy in terms of productive parameters is considered to be very limited. Nevertheless, considering the role of the MDA levels on the vaccine efficacy, the optimal time of vaccination it is still a matter of debate. Therefore, the other major objective of this Thesis was to evaluate the efficacy of PCV-2 piglet vaccination using two different dose regimes at two different ages (Studies II and III).

Two different types of clinical assays were performed in the present PhD Thesis to evaluate efficacy of PCV-2 vaccines licensed for their use in piglets and/or sows:

- One post-authorization trial conducted to explore the benefits of an already commercialized vaccine when used in a blanket vaccination strategy of breeding stock (Study I). Information on productive parameters, PCV-2 infection and immune status in sows and their progeny were obtained. This study was performed to explore if an already existing vaccine in the market licensed for its use in piglets was efficient in another swine population (sows), since they play an important role in PCV-2 epidemiology.

- Two clinical trials performed to evaluate the efficacy of a newly developed trivalent vaccine against PCV-2 (including two genotypes as well as a *M. hyopneumoniae* bacterin) administered to piglets in a two-dose regime (3 days of age and 3 weeks later) or in one-dose regime (at 3 weeks of age). These two studies were carried out for a licence permission process of the new vaccine (Studies II and III).

The Study I addressed a poorly studied topic such as the effect of PCV-2 blanket vaccination on production and virological parameters of sows and their offspring. Considering the three vaccinated groups altogether (regardless of the timing of vaccination, and therefore

mimicking a blanket vaccination), production parameters such as piglet body weight at birth and weaning were significantly improved; in addition, cross-fostering was reduced. Also, the proportion of PCV-2-infected sows and the viral load at farrowing and in PUC were significantly lower compared to NV sows. When the three vaccinated groups were considered separately, the number of mummies tended to be lower when vaccination was applied pre-mating.

In this study, PCV-2 vaccination took place in 75% of all sows under study (three groups vaccinated while only one non-vaccinated). This fact probably caused an overall reduction of PCV-2 infection pressure and transmission (horizontal and vertical) affecting also the non-vaccinated sows (all groups were comingled in the same facilities). Indeed, the decreased frequency of PCV-2 transplacental transmission was revealed by a lower proportion of PUC PCV-2 qPCR positive samples and viral loads compared to the results obtained during the farm screening.

Although these results gave light on the benefits of PCV-2 sow vaccination under a PCV-2-SI scenario, the study design revealed several limitations that may be considered for future studies:

- **Study sample size:** Some productive parameters such as total born piglets, live born piglets, weaned piglets, abortions and stillborn resulted numerically better in V groups than in NV ones, although no statistically significant differences were detected. Besides, V groups had a trend towards less number of mummies and need of cross-fostering. Therefore, a larger sample size of sows would have probably been necessary to confirm unequivocally the effect of PCV-2 sow vaccination on these parameters. However, it must be highlighted the intrinsic difficulty to work with a large number of pregnant sows and their piglets, especially for a demanding study like the one performed in this Thesis.

- **Vaccination in consecutive gestational cycles:** In this Thesis, the possible benefits in productive parameters of PCV-2 vaccination of dams mimicking a blanket vaccination were monitored in only one batch of animals. However, as reported by Pejsak et al. (2012) and Oliver-Ferrando et al. (2018a) studies, vaccination during several consecutive gestational cycles can lead to an improvement of reproductive parameters in a more

consistent form. Hence, the continued use PCV-2 sow vaccination through the production life of the sows may probably evidence better reproductive results.

- **PCV-2 circulation in the farm:** In the Study I, the frequency of PCV-2 detection in sow sera and PUCs was low. These results are aligned with the situation observed in most of commercial farms worldwide where the number of infected sows tends to be low (Eddicks et al., 2016; Dieste-Pérez et al., 2018; Oliver-Ferrando et al., 2018a; Eddicks et al., 2019; Cybulski et al., 2020). A higher PCV-2 infectious pressure in the farm when the study was done would have probably provided better conditions to detect differences between V and NV groups.

- **PCV-2 MDA levels:** The antibody response induced by sow immunization indicated that the closer the vaccination to the farrowing, the higher humoral immunity transfer levels to the piglets. Nevertheless, considering that all piglets were vaccinated around weaning against PCV-2, certain concerns regarding MDA interference with vaccination efficacy may occur.

MDA transfer to the piglets from sows vaccinated pre-farrowing (Kurmann et al., 2011; Oh et al., 2014) or pre-mating (O'Neill et al., 2012; Sibila et al., 2013) has been described in some studies. Importantly, piglet vaccination can generate a humoral response against the vaccine in spite of certain levels of MDA (Fraile et al., 2012a; Oh et al., 2014; Feng et al., 2016; Martelli et al., 2016, Oliver-Ferrando et al., 2016; Sibila et al., 2022). However, a too early piglet immunization might imply that piglets still have MDI, leading to a potential interference between the immunity of maternal origin and the vaccine uptake (Martinez-Boixaderas et al., 2022). This interference would be translated as a reduction or lack of seroconversion upon vaccination (Opriessnig et al., 2008; Fort et al., 2009a; Martelli et al., 2011; Fraile et al., 2012a; Fraile et al., 2012b; Haake et al., 2014; Oh et al., 2014; Feng et al., 2016; Martelli et al., 2016). In any case, the negative MDI effect on vaccine-elicited humoral immune response has not a direct correlation with reduction of vaccine efficacy, since PCV-2 vaccination efficacy (in terms of ADWG) in presence of MDA has been widely demonstrated in the literature (Kixmöller et al., 2008; Opriessnig et al., 2008; Fort et al., 2009a; Opriessnig et al., 2010; Fraile et al., 2012a; Tzika et al., 2015; Feng et al., 2016; Figueras-Gourgues et al., 2019; Do et al., 2021; Poulsen et al., 2021) as well as in this Thesis

(Studies II and III). In this regard, results obtained in Studies II and III corroborated two major points:

- The higher the MDA levels at piglet vaccination, the lower the increase of humoral response seven weeks later. However, no statistically significant correlation was observed between MDA and ADWG (the most widely used parameter of measuring vaccination effectiveness at production level). In Study III (split-dose vaccination at 3 days of age and 3 weeks later), the animals received the first doses when antibody values were very high (at 3 days of age) and, in consequence, the negative correlation detected between PCV-2 antibodies before vaccination and the increase in PCV-2 antibodies at 7 weeks of age (Delta value) was more noticeable than those from Study II, in which the animals were vaccinated at 3 weeks of age.

- The efficacy of piglet vaccination was confirmed in terms of productive parameters, viral load and shedding as well as histopathological lymphoid lesions and PCV-2 detection in tissues, independently of the presence of MDA at vaccination timepoint.

It is worthy to remark that efficacy can be measured by vaccine seroconversion (NA) but also by the cell-mediated response, since both components are involved in the protection against PCV-2 (Fort et al., 2009a; Tzika et al., 2015; Martelli et al., 2016). PCV-2 commercial vaccines are capable to produce cellular immune response, which is not apparently affected by MDA levels (Martelli et al., 2013; Oh et al., 2014; Martelli et al., 2016; Choi et al., 2019; Do et al., 2021). Nevertheless, Oh et al. (2014) suggested that the duration of the cellular immune component of the MDI in the piglet may be shorter than the humoral immune response. This idea would suggest that MDI basically would interfere with the humoral immune response produced by PCV-2 vaccination of piglets rather than the cellular one when piglet vaccination is applied at weaning age. However, the cellular immune response was not investigated in any of the three studies described in this Thesis. Considering the importance of the protection conferred by the cellular immunity, it would be interesting to obtain more information on the transfer of PCV-2-specific immune cells from sows to the offspring and the putative interference produced (if any) upon piglet PCV-2 vaccination (at different ages and with different doses, and probably with different vaccine trademarks).

On the other hand, the optimal time of PCV-2 vaccination for piglet protection has been widely tackled in the peer-reviewed scientific articles. PCV-2 vaccine strategies comprise the immunization of piglets, dams or both. Although the selection of the best time of vaccination may differ between farms, the following factors should be considered:

- **Dynamics of PCV-2 infection in the farm**: PCV-2 infection timing in commercial farms may vary considerably, especially between 30 and 180 days of age (Segalés and Domingo, 2002). Therefore, if PCV-2 infection is detected at early stages of life (lactating or early nursery periods) or the herd immunity is poor, sow vaccination (Study I) or a split-dose regime (vaccination of piglets at 3 days of age and 3 weeks later, Study III) would be advisable to confer PCV-2 immunity as early as possible via MDI or active immune response. On the other hand, in those farms with later detection of PCV-2 infection, a single immunization around weaning should be enough to prevent or reduce the likelihood of PCV-2-SD, as proven in Study II.

- **Herd immunological status**: The sow vaccination schedule is one factor that modifies the dam immunological status. Depending on the strategy used to vaccinate the herd, the MDA levels of the offspring also differ (Sibila et al., 2022). In fact, in Study I, weaned piglets coming from sows V MG and V LG had significantly higher antibody levels than those coming from sows V PM. Besides, high levels of herd immunity acquired by infection or vaccination usually causes infection delay, since the MDA provided throughout colostrum are crucial for piglet protection at early stages of life (Poonsuk and Zimmerman, 2018).

- **Immunosuppression and/or immunomodulation**: A reduction of vaccine efficacy can be observed when a modulation/suppression of the immune system occurs at vaccination timing. Some factors involved in the immune response disfunction are stress, deficiencies in nutrition or secondary infections (Chase and Lunney, 2019; Lee et al., 2016). The most evident demonstration of such effect is the suppression of the cellular immune response upon PCV-2 vaccination in piglets already infected with PRRSV (Canelli et al., 2016). Therefore, the assessment of the infection dynamics of other pathogens could help deciding the best moment to apply the targeted vaccine.

- **Maturity of the piglet immune system:** Pig foetuses are immunocompetent from around day 70 of gestation approximately (Madson et al., 2009c). Moreover, it has been shown that seronegative piglets vaccinated at 5 days of age may generate a protective immune response (O'Neill et al., 2011). In Study III of this Thesis, a split-dose regimen (vaccination at 3 days of age and 3 weeks later) was applied, resulting in a reduction of PCV-2 detection in tissues, serum and faeces, histopathological lymphoid tissue lesions, as well as losses in productive parameters. However, in this scenario, it was not possible to dissect the specific effect of vaccination at 3 days of age compared to that at 3 weeks of age. In fact, it was not the purpose of this Thesis to investigate the effects of newborn vaccination. This situation was explored by Haake et al. (2014) in which piglets were vaccinated at 1 week of age (exclusively) and no effect on serological values was detected since levels were comparable to those from the control (NV) group. Besides, results of that study also indicated a lower reduction in virological parameters of piglets vaccinated at 1 week of age compared to those vaccinated at 3 weeks of age. In fact, the ADWG during the fattening period of vaccinated animals at 1 week of age was comparable to that of the non-immunized group. Hence, the single vaccination at 1 week of age did not work probably due to the MDA interference.

Taking into account all these data, the moment of PCV-2 vaccine application should be decided considering the different scenarios depicted in Figure 6.1.

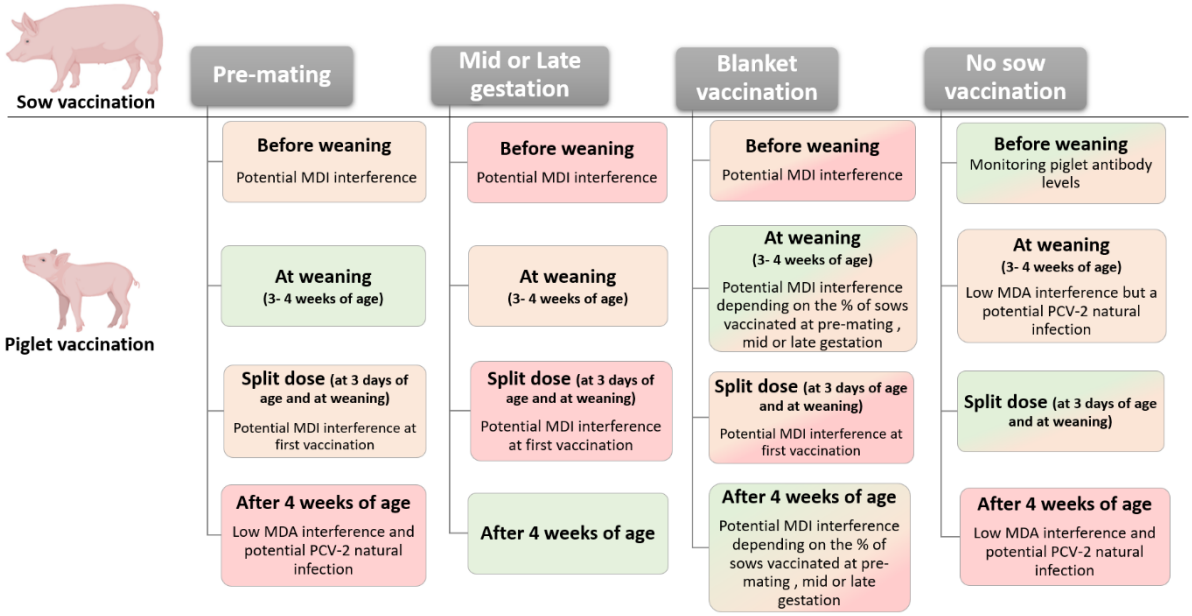

Figure 6.1. Piglet PCV-2 vaccination scenarios according to the immunization timing of the sow. Depending on the MDI levels, the likelihood of the most effective vaccine uptake by the piglet is depicted in a colour grading. Green colour indicates the most optimal timing, the red one might compromise seriously the vaccine efficacy, and the orange one would represent an intermediate situation of uncertainty.

In Studies II and III, a trivalent PCV-2 vaccine including *M. hyopneumoniae* bacterin was used for piglet vaccination. The strategy of including *M. hyopneumoniae* and PCV-2 in the same vaccine product has been explored in the last 10 years (Sibila et al., 2020), since co-infection of PCV-2 and *M. hyopneumoniae* plays an important role in the PRDC (Fraile et al., 2012a; Kim et al., 2003b). Moreover, some studies showed an increase of PCV-2-SD and PCV-2 associated lesions when *M. hyopneumoniae* was involved (Opriessnig et al., 2004b; Opriessnig et al., 2011a; Opriessnig and Halbur, 2012). For this reason, to avoid concomitant infections and considering the similar infection dynamics of both pathogens (the peak of infection usually occurs during the postweaning period), the use of a PCV-2 - *M. hyopneumoniae* combined vaccine is interesting for the swine industry (Sibila et al., 2020). Therefore, the concept of a combined application allows reducing the handling labour and, consequently, reducing the stress and the management associated costs (Park et al., 2016; Oh et al., 2019; Sibila et al., 2020; Boulbria et al., 2021; Cho et al., 2022). In Studies II and III, a low infection pressure of *M. hyopneumoniae* was detected in both commercial farms studied (data not shown), implying a lack of *M. hyopneumoniae* seroconversion and

preventing the potential assessment of vaccine efficacy of this pathogen. Consequently, efficacy of the trivalent vaccine against *M. hyopneumoniae* was not possible to be evaluated.

Since PCV-2a was initially identified as the most common genotype during 1990s, there have been two major genotype shifts resulting in a switch of predominant circulating virus from PCV-2a to PCV-2b around 2000 and a later shift to PCV-2d in 2014-2015 (Xiao et al., 2015; Xiao et al., 2016; Yang et al., 2019; Opriessnig et al., 2019; Saporiti et al., 2020; Sibila et al., 2021). In this Thesis, the three main circulating genotypes worldwide were identified during the conducted studies (Study I: PCV-2b and PCV-2d; Studies II and III: PCV-2a, PCV-2b and PCV-2d). Although nowadays the predominant genotype worldwide is PCV-2d, most commercial vaccines are still based on PCV-2a viruses (Segalés, 2015; Afghah et al., 2017). Cross-protection among genotypes has been demonstrated (Fort et al., 2008; Opriessnig et al., 2014a; Seo et al., 2014; Opriessnig et al., 2017; Park et al., 2019), but there is still a limited concern on the full capability to prevent viral replication and transmission of the virus when heterologous genotype vaccines are used (Madson et al., 2009c; Opriessnig et al., 2014a). In fact, there are also evidences that vaccines based on the same genotype of the challenge strain are apparently better at reducing viraemia compared to a heterologous challenge (Karuppannan et al., 2017). As a result, some companies decided to update PCV-2 vaccines to improve their efficacy (Segalés, 2015). In Studies II and III, a chimeric vaccine based on PCV-2a and PCV-2b was used with the hypothesis that a combined vaccine with two different PCV-2 genotypes would offer a wider genotype protection (Bandrick et al., 2022). The PCV-2a and PCV-2b chimeric vaccine tested in the present thesis was able to protect against PCV-2a, PCV-2b and PCV-2d infection. However, a direct comparison with a commercial vaccine based only on PCV-2a vaccine was not performed. Recently, a meta-analysis with the objective of comparing the efficacy of different combined vaccines, including the trivalent PCV-2 vaccine used in Studies II and III, has been published (Poulsen Nautrup et al., 2023). This meta-analysis determined that the trivalent vaccine tested in the present Thesis had at least the same efficacy, in terms of ADWG and mortality, as other PCV-2a based vaccines in the market.

CHAPTER 7

Conclusions

1. PCV-2 vaccination of sows under field conditions reduced vertical transmission of PCV-2 infection corroborated by a significantly lower proportion of infected PUCs and with lower viral load. In addition, this vaccine scheme improved body weight of piglets at birth and at weaning and reduced the cross-fostering practice in the farrowing crates.

2. The closer the PCV-2 sow vaccination to the farrowing date, the higher the transfer of antibody levels to the piglets.

3. Pre-mating sow vaccination was the best strategy to confer moderate to high antibody levels during gestation as well as to provide not too high maternally derived antibodies to the offspring.

4. Both piglet vaccination regimes (one dose at 3 weeks of age or split dose at 3 days of age and 3 weeks later) were efficacious against PCV-2 infection in terms of controlling productive losses, viral load, viral shedding, histopathological lymphoid lesions, and PCV-2 presence in tissues.

5. A trivalent vaccine containing PCV-2a, PCV-2b and *M. hyopneumoniae* bacterin applied to piglets was able to protect against the three major PCV-2 genotypes circulating worldwide, namely PCV-2a, PCV-2b and PCV-2d infection.

6. The higher the MDA levels at piglet vaccination timing, the lower the increase of humoral response seven weeks later. However, the maternally derived antibody interference on vaccine-elicited humoral immune response did not correlate with a reduction of vaccine efficacy.

CHAPTER 8

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