Puzzling over the epidemiology of porcine circovirus type 2

Tesi doctoral presentada per Sergio López Soria per accedir al grau de Doctor en Veterinària dins del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Dr. Joaquim Segalés i Coma

Bellaterra, 2014

FACULTAT DE VETERINÀRIA DE BARCELONA

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Certifica:

Que la tesi doctoral titulada "Puzzling over the epidemiology of porcine circovirus type 2" presentada per Sergio López Soria per l'obtenció del grau de Doctor en Veterinària, s'ha realitzat sota la seva direcció a la Universitat Autònoma de Barcelona i al CReSA.

I per tal que consti als efectes oportuns, signem el present certificat a Bellaterra, a 19 de setembre de 2014.

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The work performed in this PhD Thesis was funded by:

- The QLRT-PL-199900307 Project from the European Commission's Fifth Framework Programme 1998/2002 and by the Special Action AGL2002-10252-E from the Spanish Government.
- Merial SAS, Lyon (France).

Dedicada a mis padres Emilia y Dámaso,
mi esposa Blanca y mis hijos Júlia y Adrià.

AGRADECIMIENTOS

La realización de una tesis doctoral supone la dedicación de una serie de años a la formación genérica en investigación y a la específica en el tema que se investiga. En este caso, como los años dedicados no han sido precisamente pocos, tampoco lo han sido la cantidad de personas de las que he podido adquirir y ampliar conocimientos. Así pues, este apartado va dedicado a todas aquellas personas que me han acompañado en este camino y cuya influencia seguro que se verá reflejada en esta tesis doctoral.

En primer lugar quiero agradecer el esfuerzo a mis padres por estar siempre a mi lado y ayudarme a llegar al punto de partida de esta tesis.

También quiero agradecer a Quim Segalés el haberme dado la oportunidad de realizar esta tesis doctoral, haber confiado en mí desde un principio y encontrar siempre un hueco para resolver mis dudas. Es un placer el poder aprender de tu conocimiento y experiencia y contar con tu actitud positiva y dinámica día tras día.

Cómo no, también dar las gracias a todas los compañeros que han hecho posible los estudios de esta tesis participando día a día ya sea diseñando el estudio, como trabajando en granja y laboratorio, o discutiendo los resultados: Albert Rovira, Alberto Allepuz, Almudena Mínguez, André Jestin, Anna Espinal, Antonio Callén, Ayub Darji, Blanca Pérez, Carlos Artigas, Catherine Charreyre, Edgar G. Manzanilla, Eva Huerta, François Joisel, François Madec, Humberto Ramírez, Jaime Maldonado, Jordi Casal, José Manuel Serrano, Llíliane Ganges, Llorenç

Grau, Maria Calsamiglia, María José Viñas, Mariano Domingo, Marina Sibila, Marta Bruguera, Mercè Roca, Merche Mora, Miquel Nofrarías, Mónica Pérez, Nicolas Rose, Nilsa de Deus, Oliver Valero, Óscar Marín, Pere Riera y Philippe Blanchard, así como a los técnicos del Laboratorio de Diagnóstico Veterinario de Laboratorios Hipra. También quiero dar las gracias a Enric Mateu, Ignasi Badiola, Joan Pujols y Lorenzo Fraile que, aunque no han participado directamente en estos estudios, también han sido personas de referencia para mí y han dado respuesta a muchas de las dudas que me han surgido durante estos años y de quienes considero que también he aprendido mucho.

Por último darles las gracias a mi esposa Blanca, mis hijos Adrià y Júlia y a mis suegros Ramona y Roque por haberme facilitado la vida y hacer posible que haya sacado tiempo para poder compatibilizar el trabajo, la elaboración de esta tesis y mi vida personal.

Espero no haberme dejado a nadie en el olvido, en cualquier caso, a todas las personas que han hecho posible esta tesis, de todo corazón, muchísimas gracias!

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SUMMARY

The present thesis aimed to provide information on porcine circovirus type 2 (PCV2) epidemiology, since at the time of starting the present work, in 2002, PCV2 was a recently discovered virus and scarce information about it and its associated diseases was available. The studies included in this PhD Thesis are summarised below:

1. The first study aimed to assess the prevalence of PCV2 and other swine viruses, namely reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), Aujeszky's disease virus (ADV) and porcine parvovirus (PPV) in Spanish pig herds. The serosurvey consisted of two studies. Firstly, a retrospective study assessed the proportion of seropositive boar, sow and fattening pig herds and their seroprevalences to PRRSV, SIV, ADV gE protein (ADV gE) and PPV from 2003 to 2005, and to PCV2 from 2000 to 2005. Such information was obtained from routine serologic analyses from two veterinary diagnostic laboratory services. Secondly, a cross-sectional study in sow and fattening pig herds from 44 farms was performed to provide information on seroprevalences and co-seropositivity to PRRSV, SIV, ADV gE and PCV2 (PPV was excluded due to widespread vaccination), and to elucidate their relationships with farm characteristics, management and productive parameters. For this purpose farms not vaccinating against the studied viruses, or vaccinating with a DIVA (Differentiating Infected from Vaccinated Animals) vaccine were used. Similar seroprevalences were observed in both studies, although some variations were obtained, probably because of vaccination schedules, number of tested sera, sampling age and regional variations. Percentage of PRRSV and SIV seropositive herds was over 85% for sows, around

80% for fatteners and around 50% for boar studs. The proportion of ADV gE seropositive sow herds decreased from 41% to 30% between 2003 and 2005, whereas such decrease was from 41% to 33% in fattening pig herds and from 13% to 4% in boar studs. PCV2 antibodies were widespread as well as those against PPV; in the latter case, if antibodies were elicited by infection and/or vaccination was not assessed. Concurrent presence of PCV2, PRRSV and SIV antibodies was found in 89% and 66% sow and fattening herds, respectively. No statistical associations were obtained between seroprevalences or co-seropositivity and farm characteristics, management or productive parameters.

2. The second work consisted in an exploratory case-control study aimed to assess risk factors that, in association with PCV2 infection, induced porcine circovirus type 2-systemic disease (PCV2-SD) expression. This study was motivated by the situation that PCV2-SD is a multifactorial disease, where the virus was ubiquitous but only some farms suffered from the disease and no clear control and prophylactic measures were available at the moment this study was carried out, in 2002/2003. This study involved 62 Spanish pig farms of different production systems. Two groups of farms selected according to their PCV2-SD status were compared: “cases” (farms with clinical PCV2-SD, n=32) and “controls” (farms without clinical signs compatible with PCV2-SD, n=30). A filled-in questionnaire that included 191 variables and 45 blood samples (15 sows, and two groups of 15 pigs of 12 and 20 weeks of age, respectively) were obtained from each farm. Additionally, two to three diseased pigs were necropsied and relevant tissues to diagnose PCV2-SD collected when the disease was clinically suspected (“case” farms). A statistical analysis to compare ‘‘case’’ versus ‘‘control’’ farms was performed with the variables obtained from the questionnaire (191

variables) and the serologic test results (20 variables). Data were analysed using conditional logistic regression with a nested n:m matched design taking into account the farm size. Three variables were found significant in the final model: two related to the vaccination scheme and one to PCV2 seroprevalence in growing pigs, where “case” farms showed a higher prevalence of PCV2 antibodies in pigs at 12 weeks of age than “control” ones. Vaccination of gilts against PRRSV increased the odds of PCV2-SD expression, and vaccination of sows against atrophic rhinitis was related to decreased odds for disease presence; however, the possibility that those two factors could be spurious effects cannot be ruled out.

3. The third study focused on the pig genetic background, a specific risk factor for PCV2-SD pointed out by veterinary practitioners. In this study, three different boar lines, namely A (100% Pietrain), B (50% Large White x 50% Pietrain) and C (25% Large White x 75% Duroc), were used to inseminate sows from the same genetic line (37.5% Large White x 37.5% Duroc x 25% Landrace) located on two PCV2-SD affected farms (farm-1 and farm-2). The PCV2-SD clinical expression of their offspring was monitored from weaning to slaughter, evaluating three parameters: total post-weaning mortality (PWM), PWM associated to PCV2-SD and body weight (BW) evolution. The effect of other variables potentially related with PCV2-SD, including sow and piglet PCV2 exposure, sow parity, piglet gender and piglet BW at weaning, were also considered in the study design. Overall, a total of 6.5% PWM and 4.3% PCV2-SD associated PWM occurred in the monitored farms. Pigs from boar line C showed the highest PWM (16.3%) and PCV2-SD associated PWM (12.4%), and the lowest BW; pigs from boar line A showed the lowest PWM (1.8%) and the highest BW. Furthermore, PWM was also

higher in piglets from farm-2 and from multiparous sows. In farm-2, PCV2-SD associated PWM was higher in piglets from multiparous sows. Finally, BW was influenced by interactions between genetics and both farm and pig age, and was lower in piglets from farm-2. This study represents a consistent observation of the genetic background effect on PCV2-SD clinical expression under field conditions.

4. Finally, the last study aimed to assess if different PCV2 loads in serum from 3 (weaning) to 21 weeks of age (slaughter) experienced by individual pigs were associated with different growth rates during the postweaning period. A characteristic sign of two PCV2 associated diseases (PCVDs), PCV2-SD and PCV2-subclinical infection (SI), is average daily weight gain (ADWG) reduction. Thus, a subsample of pigs from the previous study was used. One or two pigs per sow were selected (60, 61 and 51 piglets from Pietrain, Pietrain x Large White and Duroc x Large White boar lines, respectively). Pigs were bled at 3, 9, 15 and 21 weeks of age and weighted at 3 and 21 weeks. Area under the curve of the viral load over time (AUCqPCR 3-21) was calculated for each animal according to standard and real time quantitative PCR results; this variable was categorized as “negative or low” ($<10^{4.3}$ PCV2 genome copies/ml of serum), “medium” ($\geq10^{4.3}$ to $\leq10^{5.3}$) and “high” ($>10^{5.3}$). Data regarding sex, PCV2 antibody titre at weaning and sow parity was also collected. A generalized linear model was performed, obtaining that paternal genetic line and AUCqPCR 3-21 were related to ADWG 3-21. ADWG 3-21 (mean±typical error) for “negative or low”, “medium” and “high” AUCqPCR 3-21 were 672±9, 650±12 and 603±16 g/day, respectively, showing significant differences among them. This study describes different ADWG performances in 3 pig populations that suffered from different loads of PCV2 viraemia.

RESUMEN

El objetivo de la tesis aquí presentada fue el de proporcionar información sobre la epidemiología del circovirus porcino tipo 2 (PCV2), debido a que en el momento de iniciar este trabajo, en 2002, PCV2 era un virus de reciente descubrimiento y había escasa información disponible sobre él y sus enfermedades asociadas. Los estudios incluidos en esta Tesis Doctoral se resumen a continuación:

1. El primer estudio fue dirigido a averiguar la prevalencia de PCV2 y otros virus porcinos, en concreto el virus reproductivo y respiratorio porcino (PRRSV), el virus de la influenza porcina (SIV), el virus de la enfermedad de Aujeszky (ADV) y parvovirus porcino (PPV) en granjas porcinas españolas. El sondeo de seroprevalencias se realizó mediante dos estudios. Primero, un estudio retrospectivo evaluó la proporción de granjas de verracos, cerdas y cerdos de engorde seropositivos y las seroprevalencias dentro de cada granja frente a PRRSV, SIV, glicoproteína E de ADV (ADV gE) y PPV desde 2003 hasta 2005, y frente a PCV2 de 2000 a 2005. Esta información se obtuvo de dos servicios de diagnóstico serológico rutinario. Segundo, se realizó un estudio transversal en 44 granjas de cerdas y cerdos de engorde con la intención de proporcionar información sobre seroprevalencias y co-seropositividad frente a PRRSV, SIV y ADV gE (PPV fue excluido debido a la vacunación masiva de la cabaña), y para dilucidar su relación con las características de la granja, manejo y parámetros productivos. Para este propósito, se seleccionaron aquellas granjas que no vacunaban frente a los virus estudiados, o vacunaban con vacunas marcadas (que permiten diferenciar animales infectados de vacunados). Se observaron seroprevalencias similares en ambos estudios, aunque con algunas

variaciones, probablemente debido a protocolos vacunales utilizados, el número de sueros testados, la edad al muestreo y variaciones regionales. El porcentaje de granjas seropositivas frente a PRRSV y SIV estaba por encima del 85% para cerdas, alrededor del 80% para engorde y alrededor del 50% para verracos. El porcentaje de granjas de cerdas seropositivas a ADV gE se redujo del 41% al 30% entre 2003 y 2005, mientras que esta reducción fue del 41% al 33% en engorde y del 13% al 4% en verracos. Anticuerpos frente a PCV2 y PPV se detectaron de forma generalizada; en este último caso, no se averiguó si los anticuerpos se debieron a infección y/o vacunación. Se encontraron de forma concurrente anticuerpos frente a PCV2, PRRSV y SIV en el 89% y 66% de granjas de cerdas y engorde, respectivamente. No se detectaron asociaciones estadísticas entre seroprevalencias o co-seropositividad y características de la granja, manejo o parámetros productivos.

2. El Segundo trabajo consistió en un estudio exploratorio de casos-controles dirigido a encontrar factores de riesgo que, en asociación con la infección por PCV2, inducían la expresión de la enfermedad sistémica por PCV2 (ES-PCV2). Este estudio fue motivado por la multifactorialidad de la ES-PCV2, donde el virus era ubicuo pero únicamente algunas granjas sufrían la enfermedad y no se disponía de medidas claras de control y profilaxis en el momento de iniciar este estudio, en 2002/2003. Este estudio involucró a 62 granjas españolas de diferentes sistemas de producción. Se compararon dos grupos de granjas seleccionadas de acuerdo a su estatus frente a ES-PCV2: “casos” (granjas con clínica de ES-PCV2, n= 32) y “controles” (granjas sin signos clínicos compatibles con ES-PCV2, n=30). De cada granja se obtuvo un cuestionario relleno que incluyó 191 variables y 45 muestras

de sangre (15 cerdas, y dos grupos de 15 cerdos de 12 y 20 semanas de edad, respectivamente). Adicionalmente, dos o tres cerdos enfermos se necropsiaron y se tomaron tejidos relevantes para el diagnóstico de ES-PCV2 cuando se sospechaba la presencia de enfermedad clínica (granjas "caso"). Se realizó un análisis estadístico para comparar granjas "caso" frente a "control" con las variables obtenidas en el cuestionario (191 variables) y los resultados de los tests serológicos (20 variables). Los datos fueron analizados usando una regresión logística condicional con un diseño de datos apareados n:m teniendo en cuenta el tamaño de la granja. En el modelo final se obtuvieron tres variables significativas: dos relacionadas con el esquema vacunal y una con la prevalencia de PCV2 in cerdos en crecimiento, donde las granjas "caso" mostraban una mayor prevalencia de anticuerpos frente a PCV2 en cerdos de 12 semanas de edad que las "control". La vacunación de la reposición frente a PRRSV aumentaba la probabilidad de expresión de ES-PCV2, y la vacunación de cerdas frente a rinitis atrófica estaba relacionada con la reducción de la probabilidad de presencia de enfermedad; sin embargo, la posibilidad de que estos dos factores sean factores espurios no se puede descartar.

3. El tercer estudio se enfocó a los antecedentes genéticos, un factor de riesgo específico para la ES-PCV2 señalado por veterinarios clínicos. En este estudio, tres líneas de verracos diferentes, denominadas A (100% Pietrain), B (50% Large White x 50% Pietrain) y C (25% Large White x 75% Duroc), fueron utilizadas para inseminar cerdas de una misma línea genética (37.5% Large White x 37.5% Duroc x 25% Landrace) localizadas en dos granjas afectadas por ES-PCV2 (granja-1 y granja-2). Se monitorizó la expresión clínica de ES-PCV2 de su descendencia desde el destete hasta la edad de matadero,

evaluando tres parámetros: mortalidad total post-destete (PWM), PWM asociada a ES-PCV2 y evolución del peso corporal (BW). También se tuvo en cuenta en el diseño del estudio el efecto de otras variables potencialmente relacionadas con ES-PCV2, incluyendo exposición de cerda y lechón a PCV2, paridad de la cerda, sexo del lechón y BW del lechón al destete. En global, las granjas monitorizadas presentaron un total de 6.5% de PWM y 4.3% de PWM asociada a ES-PCV2. Los cerdos de la genérica paterna C mostraron la mayor PWM (16.3%) y PMW asociada a ES-PCV2 (12.4%), y el menor BW; los lechones de la línea paterna A mostraron la menor PWM (1.8%) y el mayor BW. Además, PWM también fue superior en lechones de la granja-2 y procedentes de cerdas multíparas. En la granja-2, PWM asociada a ES-PCV2 fue superior en lechones de cerdas multíparas. Finalmente, BW estaba influenciado por interacciones entre genética y ambos granja y edad del cerdo, y era inferior en lechones de la granja-2. Este estudio representa una observación consistente del efecto del antecedente genético en la expresión clínica de la ES-PCV2 bajo condiciones de campo.

4. Finalmente, el último estudio fue dirigido a averiguar si diferentes cargas de PCV2 en suero desde las 3 (destete) hasta las 21 semanas (matadero) experimentadas por cerdos individuales estaban asociadas con diferentes índices de crecimiento durante el periodo post-destete. Un signo característico de dos enfermedades asociadas a PCV2 (PCVDs), ES-PCV2 e infección subclínica por PCV2 (IS-PCV2), es la reducción de la media de ganancia de peso diario (ADWG). Así, se utilizó una subpoblación de cerdos del estudio previo. Se seleccionaron uno o dos cerdos por cerda (60, 61 y 51 lechones de verracos Pietrain, Pietrain x Large White y Duroc x Large White, respectivamente). Se

tomó muestra de sangre a los lechones a 3, 9, 15 y 21 semanas de vida y se pesaron a 3 y 21 semanas. Se calculó el área bajo la curva de la carga vírica en el tiempo (AUCqPCR 3-21) para cada animal de acuerdo a los resultados de PCR estándar y PCR cuantitativa; esta variable se categorizó como “negativa o baja” ($<10^{4.3}$ PCV2 copias de genoma/ml de suero), “media” ($\geq10^{4.3}$ a $\leq10^{5.3}$) y “alta” ($>10^{5.3}$). También se recogió información relacionada con el sexo, título de anticuerpos frente a PCV2 al destete y paridad de la cerda. Se realizó un modelo linear generalizado, obteniendo que la genética paterna y la AUCqPCR 3-21 estaban relacionadas con la ADWG 3-21. ADWG 3-21 (media± error típico) para “negativa o baja”, “media” y “alta” AUCqPCR 3-21 fueron 672±9, 650±12 and 603±16 g/día, respectivamente, mostrando diferencias significativas entre ellas. Este estudio describe diferentes rendimientos de ADWG en tres poblaciones de cerdos que sufrieron diferentes cargas de viremia por PCV2.

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

Study I: López-Soria S, Maldonado J, Riera P, Nofrarías M, Espinal A, Valero O, Blanchard P, Jestin A, Casal J, Domingo M, Artigas C, Segalés J. Selected Swine viral pathogens in indoor pigs in Spain. Seroprevalence and farm-level characteristics. *Transbound Emerg Dis.* 2010 Jun;57(3):171-179.

Study II: López-Soria S, Segalés J, Rose N, Viñas MJ, Blanchard P, Madec F, Jestin A, Casal J, Domingo M. An exploratory study on risk factors for postweaning multisystemic wasting syndrome (PMWS) in Spain. *Prev Vet Med.* 2005 Jun 10;69(1-2):97-107.

Study III: López-Soria S, Nofrarías M, Calsamiglia M, Espinal A, Valero O, Ramírez-Mendoza H, Mínguez A, Serrano JM, Marín O, Callén A, Segalés J. Post-weaning multisystemic wasting syndrome (PMWS) clinical expression under field conditions is modulated by the pig genetic background. *Vet Microbiol.* 2011 May 5;149(3-4):352-357.

Study IV: López-Soria S, Sibila M, Nofrarías M, Calsamiglia M, Manzanilla EG, Ramírez-Mendoza H, Mínguez A, Serrano JM, Marín O, Joisel F, Charreyre C, Segalés J. Effect of porcine circovirus type 2 (PCV2) load in serum on average daily weight gain during the postweaning period. *Accepted for publication, Vet Microbiol.*

LIST OF ABBREVIATIONS

ADV	Aujeszky's disease virus
ADV gE	Aujeszky's disease virus glycoprotein E
ADWG	Average daily weight gain
AUC	Area under the curve
AUCqPCR 3-21	Area under the curve drawn by the qPCR results at 3, 9, 15 and 21 weeks of age
BW	Body weight
CV	Coefficient of variation
DIVA	Differentiating Infected from Vaccinated Animals
DU	Duroc
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
INE	*Instituto Nacional de Estadística*
IPMA	Immunoperoxidase monolayer assay
ISH	*In situ* hibridization
LOD	Limit of detection
LW	Large White
MAPA	*Ministerio de Agricultura Pesca y Alimentación*
MDA	Maternally derived antibodies
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV2	Porcine circovirus type 2
PCV2-SD	Porcine circovirus type 2 systemic disease
PCV2-SI	Porcine circovirus type 2 subclinical infection
PCVD	Porcine circovirus diseases
PI	Pietrain
PMWS	Postweaning multisystemic wasting syndrome
PCV2-SD-PWM	Postweaning mortality associated to PCV2-SD
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
PWM	Postweaning mortality
qPCR	Quantitative PCR
SD	Standard deviation
Se	Sensitivity
SIV	Swine influenza virus
Sp	Specificity

CHAPTER 1

INTRODUCTION

1.1 Historical perspective of a novel swine disease: porcine circovirus type 2 systemic disease (PCV2-SD)

In 1991, a veterinary practitioner, Dr. John Harding, and a pathologist, Dr. Edward G. Clark, described a new syndrome in a swine farm free from *Porcine reproductive and respiratory syndrome virus* (PRRSV) infection located in Saskatchewan (Canada). This novel condition showed an increase on post-weaning mortality with pigs showing progressive loss of weight, respiratory distress, skin pallor and very specific microscopic lesions in lymphoid tissues. The disease was formally described in 1996, after several farms reported similar clinical manifestations (Harding, 1996; Clark, 1997). At that moment the name of post-weaning multisystemic wasting syndrome (PMWS) was proposed to designate this new disease. Nowadays, this disease is referred as porcine circovirus type 2-systemic disease (PCV2-SD) (Segalés, 2012).

Abundance of a variant of porcine circovirus (PCV) was detected in lymphoid tissues of affected pigs (Daft et al., 1996; Clark, 1997; Segalés et al., 1997). At that moment, PCV was only known as a contaminant of PK-15 cell lines, obtained from porcine kidney (Tischer et al., 1974, 1982), but no pathogenic properties were attributed to this virus until that moment (Tischer et al., 1986). One year later, the virus present in diseased pigs was isolated (Allan et al., 1998; Ellis et al., 1998), evidencing phylogenetic and antigenic differences with PCV strains from PK-15 cell lines (Hamel et al., 1998; Meehan et al., 1998). Nowadays, the International Committee on Taxonomy of Viruses (ICTV) recognizes them as two different species within the genus *Circovirus*, being *Porcine circovirus type 2* (PCV2) the virus associated to the new syndrome and

Porcine circovirus type 1 (PCV1) the PK-15 cellular lines contaminant virus (http://www.ictvonline.org).

1.2 PCV2 and associated diseases (PCVDs)

1.2.1 Virus properties

PCV2 is a non-enveloped, icosahedral virus of 12-23 nm of diameter (Tischer et al., 1982; Rodríguez-Cariño and Segalés, 2009) with single stranded circular 1.76-1.77 kb DNA (Hamel et al., 1998; Meehan et al., 1998; Mankertz et al., 2000). The virus belongs to the genus *Circovirus* from the family *Circoviridae* (Segalés et al., 2005a). In fact, it is the smallest known virus that affects mammals. PCV2 has 60 capsid protein elements arranged in 12 slightly protruding pentameric units (Crowther et al., 2003). PCV2 genome is composed by 11 open reading frames (ORFs) (Hamel et al., 1998), but protein expression has been only described for ORF1 (Rep gene, encoding for nonstructural replicase proteins, *Rep* and *Rep'*), ORF 2 (Cap gene, encoding for the capsid protein) and ORF 3 (encoding for a nonstructural protein that induces apoptosis in PK-15 cells) (Nawagitgul et al., 2000; Mankertz et al., 2004; Liu et al., 2005).

First sequencing studies revealed a nucleotide identity >93% between PCV2 strains (Mankertz et al., 2000). Differences on PCV2 genome have been assessed in order to associate them with its pathogenicity. In this sense, some studies in Canada (Larochelle et al., 2002, 2003), France (de Boisseson et al., 2004), The Netherlands (Grierson et al., 2004), China (Wen et al., 2005), Ireland (Allan et al., 2007) and Brazil (Martins Gomes de Castro et al., 2007) did not find any

relationship between PCV2-SD and PCV2 strains, clusters, genogroups or genotypes. On the other hand, more subsequent studies in Canada (Gagnon et al., 2007), USA (Cheung et al., 2007), Denmark (Dupont et al., 2008), Sweden (Timmusk et al., 2008) and Switzerland (Wiederkehr et al., 2009) and Spain (Cortey et al., 2011a) found such association with PCV2 genogroups, genotypes or genotype subgroups. These studies described such diversity of classifications of PCV2 strains, generating a confusing terminology within the scientific community; for this reason it was agreed a genotype definition and nomenclature (Segalés et al., 2008). In this sense, PCV2 strains were initially divided into two main groups (Gagnon et al., 2007; Grau-Roma et al., 2008; Olvera et al., 2007) designed as genotype “a” (PCV2a) and “b” (PCV2b). Later, the presence of a third genotype (PCV2c) in pigs from Denmark in the1980’s was described through a retrospective study (Dupont et al., 2008). More recently, a fourth genotype (PCV2d) has been described in China (Guo et al., 2010).

PCV2 biological and physicochemical properties have been partially characterized. The virus is highly resistant in the environment, showing also high resistance to chemical and thermal treatments. In this sense, infectivity decreases in acid buffer, despite PCV2 remains viable even at pH <2. Infectivity is markedly reduced at pH 11-12 (Kim et al., 2009). Complete inactivation was not achieved after pasteurization at 60ºC for 24h or 75ºC for 30 min, dry heat at 80ºC for 72h or 120ºC for 30 min (Welch et al., 2006), as well as wet heat at 75ºC for 15 min (O’Dea et al., 2008). However, PCV2 was inactivated with wet heat at 80ºC for 15 min (O’Dea et al., 2008). These results may vary depending on the matrix and viral load, since PCV2 is more labile in liquid matrices and may show higher loads in pig meat (O’Dea et al., 2008).

Spray dried porcine plasma (SDPP) was suggested as a potential source of infection by PCV2 in pigs. Such hypothesis was confirmed under experimental conditions by inoculating intraperitoneally an experimentally produced SDPP to piglets (Patterson et al., 2010); however, feeding piglets with industrially produced SDPP did not result in infection under experimental conditions (Pujols et al., 2008, 2011).

PCV2 has also shown to be resistant to lipid-dissolving disinfectants based on alcohol, clorhexidine, iodine and phenol (Royer et al., 2001). On the other hand, the virus titre can be reduced with alkaline disinfectants (i.e. sodium hydroxide), oxidizing agents (i.e. sodium hypochlorite) and quaternary ammonium compounds (Martin et al., 2008).

1.2.2 PCVDs and diagnostic criteria

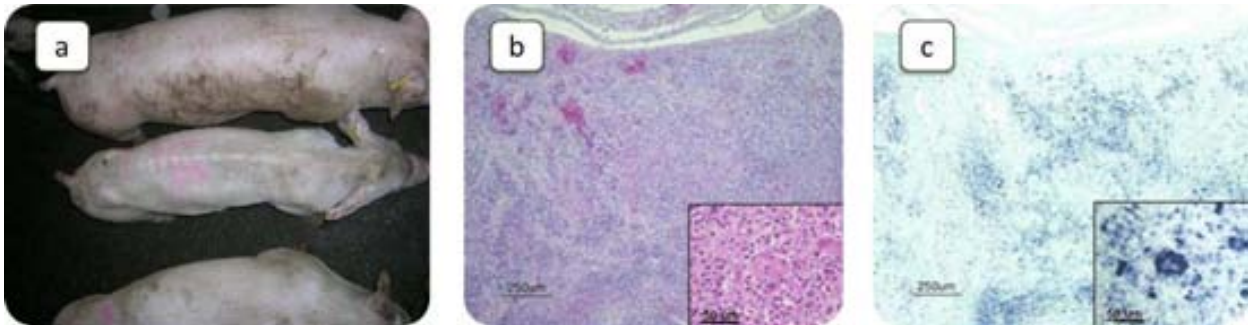
Despite PCV2-SD was the first disease associated to PCV2 and also initially considered the most economically important one, other pathological conditions have also been linked to the virus (Chae, 2005; Segalés et al., 2005a; Opriessnig et al., 2007; Segalés, 2012). They have been collectively named porcine circovirus diseases (PCVDs) in Europe (Allan et al., 2002a) and porcine circovirus associated diseases (PCVADs) in North America (http://www.aasv.org). The name of PCVDs has been recently proposed in order to standardize terminologies globally (Segalés, 2012). In the same line, standardized terminologies to refer to each PCVD have also been proposed as described in Table 1, and they will be used in the present work.

Table 1. New terminology proposed to refer to PCVDs, former terminology and criteria to establish their diagnosis.

PCVD (acronym)	Diagnostic criteria
PCV2-systemic disease (PCV2- SD) Formerly known as: Post-weaning multisystemic wasting syndrome (PMWS), porcine circovirosis, and PCV2-associated systemic infection	1. Wasting, weight loss, clinically evident decrease on average daily weight gain, ill thrift or poor-doer, skin paleness (respiratory and/or digestive signs may also be present). 2. Severe to moderate lymphocyte depletion and granulomatous inflammation of lymphoid tissues (plus granulomatous inflammation in other tissues). 3. Moderate to high amount of PCV2 within lesions. (see figure 1)
PCV2-subclinical infection (PCV2- SI)	1. Decreased average daily weight gain without any evident clinical sign. 2. No or minimal histopathological lesions in tissues (mainly lymphoid). 3. Low PCV2 amount in few (lymphoid) tissues Criteria 2 and 3 can potentially be substituted by PCV2 detection techniques such as standard PCR.
PCV2-reproductive disease (PCV2- RD) Formerly known as: Reproductive failure associated to PCV2	Abortions or mummifications: 1. Reproductive failure at late gestation. 2. Fibrous to necrotizing myocarditis of foetuses. 3 Moderate to severe amount of PCV2 in heart. The use of real time quantitative PCR on foetal tissues might be more sensitive to detect PCV2-RD. Regular return-to-oestrus: 1. Posterior PCV2 seroconversion and/or PCV2 PCR positivity around return-to-oestrus occurrence.
Porcine dermatitis and nephropathy syndrome (PDNS)*	1. Dark red papules and macules on skin, mainly in hind limbs and perineal area. 2. Haemorrhagic and necrotizing skin lesions and/or swollen and pale kidneys with generalized cortical petechia. 3. Systemic necrotizing vasculitis, and necrotizing and fibrinous glomerulonephritis.

* PDNS is considered an immune-complex disease of not yet demonstrated aetiology; link with PCV2 is still circumstantial, and detection of PCV2 is not considered into its diagnostic case definition.

Figure 1. Individual diagnosis of PCV2-SD requires the fulfilment of three criteria: a) compatible clinical signs, b) presence of moderate to severe characteristic lymphoid lesions (haematoxilin/eosin stain), and c) presence of moderate to high amount of PCV2 within lesions (PCV2 *in situ* hybridization).



Some other diseases have been attributed to PCV2 such as PCV2 lung disease (PCV2-LD) and PCV2 enteric disease (PCV2-ED). However, it has been suggested that the first one is negligible in the field, and PCV2 would mainly contribute to porcine respiratory disease complex (PRDC) under a PCV2-SD scenario (Hansen et al., 2010; Kim et al., 2003; Ticó et al., 2013); the same situation seems to apply for PCV2-ED. Finally, PCV2 was initially linked to type AII congenital tremors (Stevenson et al., 2001); however, such association was not corroborated in later studies (Kennedy et al., 2003; Ha et al., 2005).

From all PCVDs, PCV2-subclincial infection (SI) is nowadays considered the most frequent and costly one in commercial farms followed by PCV2-SD (Armstrong and Bishop, 2004; Burch, 2009; Alarcón et al., 2013a). PCV2-RD has been under study lately, where PCV2 has additionally been linked to SMEDI (Stillbirth, Mummification, Embryonic Death and Infertility) in primiparous sows (Meyns et al., 2012), however the impact of this PCVD in the pig industry is globally considered of much lower relevance than PCV2-SI or PCV2-SD.

It has been widely described that PCV2-SD affected pigs harbour a higher viral load in serum as well as other tissues and secretions compared to infected, healthy pigs (Liu et al., 2000; Brunborg et al., 2004; Sibila et al., 2004; Olvera et al., 2004; Segalés et al., 2005b). Therefore, several studies have suggested the use of real time quantitative PCR (qPCR) as an *in vivo* diagnostic tool for this disease (Brunborg et al., 2004; Olvera et al., 2004; Segalés et al., 2005b; Fort et al., 2007; Harding et al., 2008; Grau-Roma et al., 2009). In this sense, the use of pools of sera (mixing several sera in the same analytical sample) can be an economical choice in PCV2-SD affected farms (Cortey et al., 2011b). However, qPCR in serum does not have specificity and sensitivity enough to substitute the combination of histopathology plus viral load detection in tissues for the PCV2-SD diagnosis of individual animals (Grau-Roma et al., 2009).

1.2.3 Virus and disease: host range and geographical distribution

PCV2 affects suidae species, being domestic pigs and wild boars the natural hosts and the ones that can develop PCV2-SD (Segalés et al., 2005a; Ellis et al., 2003; Vicente et al., 2004; Lipej et al., 2007). The ability of PCV2 to infect other species such as bovines, ovines, goats, equines, canines, felines, rabbits, guinea pigs, poultry or humans has been investigated, showing no apparent susceptibility (Allan et al., 2000; Ellis et al., 2000, 2001; Quintana et al., 2002; Rodríguez-Arrioja et al., 2003a). However, other studies point that PCV2 is able to replicate and transmit between mice (Kiupel et al., 2001; Opriessnig et al., 2009a). It has been suggested a role of mice and rats as alternate hosts or mechanical vectors in swine herds, since PCV2 has been found in these species in pig farms, but not outside them (Lorincz et al., 2010).

PCV2 has been detected in all countries that have searched for it since its first description in the 90's, evidencing its ubiquity. Moreover, PCV2-SD has been diagnosed in all the five continents (Chae, 2005; Segalés et al., 2005a; Opriessnig et al., 2007, Grau-Roma et al., 2011) (Figure 2). The number of diagnosed PCV2-SD cases increased worldwide after its first description in 1996, taking place main epidemic outbreaks in Europe and Asia between 1998 and 2004, and in America between 2004 and 2007.

Retrospective studies in archived serum and tissue samples have revealed the existence of infected pigs at least since 1962, and the presence of PCV2-SD at least since 1985 (Jacobsen et al., 2009). In Spain, the first known cases by retrospective studies date from 1985 and 1986 for PCV2 infected pigs and PCV2-SD affected ones, respectively (Rodríguez-Arrioja et al., 2003b). Very limited information does exist on the other PCVDs, since they are less clinically evident and they have not incited as much research as PCV2-SD.

Figure 2. Countries where PCV2-SD has been diagnosed (highlighted in red).

1.2.4 PCV2 transmission routes and infection dynamics

PCV2 has been detected in all tested excretion/secretion routes, being present in nasal cavity, tonsil, bronchi, saliva, ocular conjunctiva, faeces, urine, milk and semen (Larochelle et al., 2000; Krakowka et al., 2000; Bolin et al., 2001; Shibata et al., 2003, 2006; Sibila et al., 2004; Segalés et al., 2005b; Ha et al., 2009; Park et al., 2009). Oronasal direct contact is considered the most relevant horizontal transmission route, being more efficient with direct contact (Andraud et al., 2008); transmission is also possible among pigs located in adjacent pens (Kristensen et al., 2009). PCV2-SD transmission has also been achieved by mixing affected and healthy pigs (Dupont et al., 2009; Kristensen et al., 2009). Infection by oral route is also possible, as it has been observed after feeding piglets with tissue from viraemic animals (Opriessnig et al., 2009b).

The virus can be detected in semen (Larochelle et al., 2000). Presence of virus was detected in semen from boars experimentally infected with PCV2 trough the intranasal route. Furthermore, it was confirmed that this semen was infectious when it was intraperitoneally inoculated to naïve pigs. However, same semen did not show evidence of infection in sows artificially inseminated with it (Madson et al., 2009a). On the other hand, the use of deliberately spiked semen with PCV2 was able of reproduce PCV2-RD in artificially inseminated sows (Madson et al., 2009b). Nowadays it is unknown the potential risk and the frequency of this transmission route in the field, although the viral load excreted in semen is probably too low to infect sows under natural conditions.

Regarding vertical transmission, transplacentary route from sow to foetus has been described in intranasally infected sows at the end of gestation, 3 weeks before farrowing, detecting infection in liveborn and aborted piglets (Park et al., 2005; Ha et al., 2008). PCV2 impact on reproductive problems is still unknown, since it has been described as a rare event (Ladekjaer-Mikkelsen et al., 2001; Pensaert et al., 2004), although others have reported a 13-46% of infected aborted foetuses and stillbirths (Lyoo et al., 2001; Kim et al., 2004). In Spain, anecdotal cases of PCV2 infected aborted foetuses have been found (Segalés et al., 2002; Maldonado et al., 2005). Globally, it has been speculated that the high antibody titres to PCV2 in sows has minimized its impact worldwide (Pensaert et al., 2004). However, research of PCV2 in the reproductive area is still scarce.

PCV2 infection dynamics may be similar between PCV2-SD affected and non-affected herds (Larochelle et al., 2003), although a higher risk of affection has been described in farms with earlier evidence of infection (Rose et al., 2003a). Piglets are commonly infected by the sow, but maternally derived antibodies (MDA) usually protect them against disease expression until 6-8 weeks of age, approximately. At that moment, the levels of MDA are low enough to allow PCV2 replication and dissemination between animals (Rodríguez-Arrioja et al., 2002). Approximately 2-4 weeks after this viral replication, the immune system responds with a seroconvertion and pigs maintain a high antibody titre until slaughter age. The highest percentage of infected animals, pigs with PCV2-SD and evidence of seroconversion usually occurs from 8 to 16 weeks of age (Harding, 1998, 2004; Segalés and Domingo, 2002; Grau-Roma et al., 2009; Larochelle et al., 2003). Besides this situation, there may also exist viraemic born piglets due to intrauterine infection that may

experience a maximum PCV2 load at 28 days of age (Patterson et al., 2011), even not showing clinical signs (Shen et al., 2010).

1.3 Risk factors for PCV2-SD development

Multifactoriality of PCV2-SD was soon evidenced by the facts that PCV2 was a ubiquitous virus in the porcine livestock, only some pigs developed clinical signs and disease prevalence was variable among farms. To date, several risk factors to develop PCV2-SD have been described for each of the epidemiological triad elements: the host, the virus and the environment. The most relevant ones are described in the following sections.

1.3.1 Facilities, management and biosecurity

Early after discovering PCV2, generic biosecurity and management measures were the unique available tools to fight against PCV2-SD. A good example of this situation were the “20 point plan” proposed by Dr. François Madec (*Agence Nationale de Sécurité Sanitaire de l’Alimentation, de l’Environnement et du Travail*, ANSES) in France, that helped on reducing disease impact (Madec et al., 2000). Multiple epidemiological studies were carried out worldwide to identify those facility, management and biosecurity factors with a higher relevance on disease expression: Canada (Cottrell et al., 1999; Dewey et al., 2006), United Kingdom (Cook et al., 2001; Woodbine et al., 2007), France (Rose et al., 2003a), The Netherlands (Elbers et al., 2006), Denmark (Enoe et al., 2006) and Japan (Kawashima et al., 2007). These studies

included a high number of affected and non-affected farms and tested a wide range of factors; the most relevant ones are shown in Table 2.

Table 2. Factors related to facilities, management and biosecurity that increase or decrease the risk of PCV2-SD expression.

	Increases the risk	Decreases the risk
Facilities	• Herd size (>400 sows) • Large pens in nurseries (≥7.8m^2)	• Separate pits for adjacent fattening rooms • Shower facilities
Management	• On farm semen collection • High level of cross-fostering • Large range in age and weight entering to nursery • Continuous flow in nurseries • Sows with neck injuries due to poor injection technique • Early weaning (<21 days of age) • PRRSV vaccination to gilts/piglets • *Micoplasma hyopneumoniae* vaccination (some vaccines) • Separate vaccination to PPV and Erysipelas in gilts • *Escherichia coli* vaccination in sows • *Actinobacillus pleuropneumoniae* vaccination • Classical swine fever virus vaccination	• Use of semen from an insemination centre • Long empty period (nurseries ≥4 days / parity rooms ≥5 days) • Dry sows in collective pens • Self-replacement scheme for the gilts • Sorting pigs by sex at nursery stage • Higher minimum weight at weaning • Group housing during pregnancy • Regular treatment of external parasites • Sow vaccination to Atrophic Rhinitis • Use of oxytocin during farrowing • Use of spray-dried plasma in initial nursery ration
Biosecurity	• Proximity to PCV2-SD farms (<3 km)	• Quarantine for purchased pigs/gilts • Change of boots/clothes in entrance room • Visitants avoiding pig contact at least 3 days prior visiting the farm

A more recent study described how management measures are able to influence PCV2 infection dynamics; among them, it was demonstrated that avoiding cross-fostering and mixing litters in small pens at weaning reduced the risk of early infections (Andraud et al., 2009).

1.3.2 Co-infections

PCV-SD expression has been scarcely reproduced under experimental conditions by only infecting animals with PCV2. The most successful disease model includes co-infection of piglets with porcine parvovirus (PPV) (Allan et al., 1999), PRRSV (Rovira et al., 2002) or *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004). In fact, the same situation is observed under field conditions, where PCV2-SD affected farms suffer from concurrent diseases more frequently than non-affected ones (Ellis et al., 2004). In fact, an endless number of concomitant infections/diseases have been found together with PCV2-SD: PRRSV, PPV, *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis,* Aujeszky's disease, Glässer's disease, streptococcal meningitis, salmonellosis, post-weaning colibacillosis, non-specific diarrhoea, hepatosis dietetica and bacterial pneumonias (Segalés et al., 2005a). However, in such scenarios, it is difficult to clarify if PCV2-SD is triggering the other pathologies or *vice versa*; nevertheless all pathologies coinciding in the farm should be considered co-responsible of the global clinical situation in the herd.

1.3.3 Stimulation of the immune system

Several experimental and field studies have evidenced the synergism of PCV2 replication and/or the induction of PCV2-SD clinical expression with the stimulation of the immune system. As immunostimulants, these studies used keyhole limpet hemocyanin (KLH) in incomplete Freund Adjuvant (IFA) (Krakowka et al., 2001), or commercial vaccines against *Mycoplasma hyopneumoniae* alone (Allan et al., 2001; Kyriakis et al., 2002) or *Actinobacillus pleuropneumoniae* (Opriessnig et al., 2003). Furthermore, it has been pointed that the type of vaccine adjuvant and the moment of vaccination could play a role on this detrimental effect. In this sense, higher PCV2 loads have been found in pigs when using mineral oil adjuvanted vaccines. On the other hand, plant oil-aqueous antigen emulsions or aluminum salts had none or minimal effects on PCV2 replication (Krakowka et al., 2007). The possible influence of vaccination against *Mycoplasma hyopneumoniae* on disease outcome could be solved *a priori* by adjusting the moment of vaccination instead of deciding to eliminate such vaccine that might be needed to maintain the health status of the farm (Opriessnig et al., 2006a).

Despite some studies did not describe the same results using KLH-IFA (Ladekjaer-Mikkelsen et al., 2002) or commercial vaccine adjuvants (Resendes et al., 2004), nowadays it is considered that immunostimulation is a potential risk factor. However, its real impact under field conditions has not really been assessed and might probably be low.

1.3.4 The host

Certain breeds or genetic lines have shown a higher susceptibility or resistance to disease. Studies carried out under experimental conditions describe more severe lesions and more frequency of PCV2-SD cases in Landrace pigs than Duroc, Large White and Pietrain breeds (Opriessnig et al., 2006b, 2009c). However, the introduction of Pietrain as terminal boar did not produce any difference on disease expression in French farms (Rose et al., 2005).

PCV2-SD has also been mainly described in piglets with lower weight at birth, weaning and entry to fattening (Corrégé et al., 2001) and in castrated males (Corrégé et al., 2001; Rodríguez-Arrioja et al., 2002). The effect of castration could potentially be explained by secondary infections to the procedure or even to either hormonal or genetic factors.

1.3.5 PCV2 infection dynamics

As stated before, despite infection dynamics may be similar between farms with and without PCV2-SD, case-control studies have described a higher risk of disease in those ones with earlier infections (Rose et al., 2003a). The same phenomenon has been described at individual level comparing pigs within PCV2-SD affected farms, where the disease occurred with a higher probability when early infection took place (Rose et al., 2009). However, such association was not observed in another study (Grau-Roma et al., 2009).

The sow has a special relevance on the maintenance of PCV2 in the herd and is usually the infection origin for piglets. At the same time,

the sow is also the source of passive immunity against the virus through colostrum (nowadays piglets can also acquire active immunity through vaccination). In this sense, a study in PCV2-SD affected farms revealed a higher mortality in piglets from viraemic sows or from sows with low antibody titres at farrowing (Calsamiglia et al., 2007). Furthermore, it is considered that PCV2-SD protection through MDA is titre dependent, being usually protected those piglets with higher levels (McKeown et al., 2005).

1.3.6 PCV2 genotypes

Both most frequent genotypes (PCV2a and PCV2b) are able to reproduce PCV2-SD experimentally, but PCV2b has been linked to disease expression to a higher frequency (Grau-Roma et al., 2008; Tomás et al., 2008; Carman et al., 2006, 2008). In fact, several epidemiological studies performed in different countries such as Canada (Gagnon et al., 2007), the United States (Cheung et al., 2007), Denmark (Dupont et al., 2008), Sweden (Timmusk et al., 2008), Switzerland (Wiederkehr et al., 2009) and Spain (Cortey et al., 2011a) have associated the shift of the predominant genotype in their livestock with the epidemic outcome of PCV2-SD.

Nowadays all vaccines against PCV2 in the market are based on PCV2a, but cross-protection between PCV2a and PCV2b has been evidenced (Fort et al., 2008; Opriessnig et al., 2010). However, experimental PCV2b vaccines have apparently shown better results against PCV2b infection than PCV2a ones, suggesting a more effective homologous protection (Beach and Meng, 2012; Opriessnig et al., 2013). Furthermore, differences in mortality reduction associated to different

genetic types of PCV2 have been reported in Japan (Takahagi et al., 2010). A case of suspected PCV2 vaccine failure in PCV2-SD affected pigs from the United States was associated with the presence of a mutant PCV2b (Opriessnig et al., 2013). However, it was later evidenced that PCV2 vaccines were also protective against the experimental infection with this novel strain (Opriessnig et al., 2014).

1.4 Control and prevention measures

As pointed above, first control measures against PCV2-SD were directed to implement management and biosecurity measures to reduce infection transmission, genetic changes, “serum-therapy” (i.e. treatment of disease by the injection of blood serum from immune animals) and controlling other known risk factors.

First commercial vaccine against PCV2 appeared in 2004 in France and Germany, targeted for sows. This vaccine arrived to most European countries in 2007, when most veterinary practitioners thought that it was arriving late since PCV2-SD outbreak prevalence was lowering. PCV2 vaccines for piglets came to the market slightly later, and offered a short term solution in comparison with sow vaccination. All available vaccines nowadays have demonstrated excellent results (Fachinger et al., 2008; Kixmöller et al., 2008; Segalés et al., 2009; Pejsak et al., 2010; Martelli et al., 2011) and have revolutionized the pig industry. In fact, it is estimated that >80% of farms apply this vaccine in Spain, including farms without apparent PCV2-SD clinical signs. These percentages can be very variable among countries worldwide, but some of them are vaccinating almost 100% of their porcine livestock.

The benefit of vaccination in PCV2-SD affected farms has been widely demonstrated, improving feed conversion rate, average daily weight gain, batch homogeneity, and reducing mortality, the percentage of wasting pigs, concomitant infections and veterinary treatments (Horlen et al., 2008; Kixmöller et al., 2008; Segalés et al., 2009; Pejsak et al., 2010; Fraile et al., 2012a). Taking into account that PCV2 vaccination reduces viral load in tissues, it has also a potential benefit in subclinically infected farms (Kurmann et al., 2011).

The long-term benefit of gilt and sow vaccination on reproductive parameters has been also demonstrated, improving the fertility rate, number of piglets born alive and weaned per litter as well as birth body weight and percentage of piglets weighing >1 kg (Pejsak et al., 2012). Furthermore, sow vaccination increases and homogenizes the antibody titres against PCV2 in the sows (Sibila et al., 2013) and reduce their viral excretion, facts that should have a positive effect in piglets' infection dynamics.

It has also been raised the interest of the so-called "global or continuous protection", what consists on vaccination of both sows and piglets (Pejsak et al., 2010; Fraile et al., 2012b). The aim of this program is to obtain the benefit of both vaccination schedules. In any case, to choose one or another vaccine program, including or not both pigs and sows, it is important to perform a cost-benefit analysis. In all cases, the challenge for the veterinarians resides on designing the vaccine schedule that offers the maximum performance taking into account the presence of PCVDs in the farm and the moment of application of PCV2 and other vaccines according to the moment when diseases take place.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

At the beginning of this PhD Thesis, in 2002, there were many unanswered questions about PCV2 and the most important disease that was associated to this virus, PCV2-SD (at that moment known as PMWS). In fact, at that time, part of the professionals of the swine sector did not support the idea of PCV2 as the aetiology of such disease.

The first case of PCV2-SD in Spain was described in 1997 (Segalés et al., 1997), and the studies herein presented were conducted in selected farms from 2002 to 2005. These studies were designed starting with the knowledge available at that moment and aiming to assess some relevant epidemiological aspects that were unknown at that moment. For instance, the fact that PCV2 was considered a ubiquitous virus, but only some farms experienced PCV2-SD was a confusing situation. Furthermore, the lack of awareness of what factors triggered such situation complicated the approach of the problem. Generic management practices to reduce the infection dissemination were implemented to fight against the disease at that time, since PCV2 vaccines were not available.

With the premises of this situation, the present PhD Thesis is based on epidemiological studies that were carried out in order to determine PCV2 prevalence in Spain, to identify risk factors for PCV2-SD occurrence, and to focus in two specific risk factors (pig genetics and PCV2 viral load). Specifically, the objectives of the studies that define this thesis were:

1. To assess the prevalence of PCV2 in the Spanish swine livestock and its potential relationship with other pathogens' prevalences including

porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), Aujeszky's disease virus (ADV) and porcine parvovirus (PPV).

2. To elucidate potential risk factors related to farm characteristics, management, biosecurity measures, vaccination schedules, co-infections to trigger PCV2-SD within the framework of a case-control study involving 62 Spanish farms.

3. To compare the effect of 3 different genetic boar lines on the expression of PCV2-SD in their offspring.

4. To assess whether different PCV2 loads in serum of pigs from weaning to slaughter were associated with different ADWG values.

CHAPTER 3

STUDY I:

Selected swine viral pathogens in indoor pigs in Spain. Seroprevalence and farm-level characteristics

3.1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine circovirus type 2 (PCV2) and Aujeszky's disease virus (ADV) are some of the most frequent viral pathogens responsible for respiratory and systemic disorders in fattening pigs (Choi et al., 2003). Some of these viruses, i.e. PRRSV, ADV and PCV2, can also produce reproductive disorders in sows (Almond et al., 2006).

The occurrence of pathogen co-infections in pig herds is one of the most concerning problems in the current swine industry worldwide due to the difficulty to establish a precise diagnosis and to implement reliable control measures (Gutiérrez-Martín et al., 2000; Thacker and Thacker, 2000). A good example of such situation is porcine respiratory disease complex (PRDC), which is caused by concurrent viral, bacterial and mycoplasmal infections in combination with unbalanced environmental factors (Dee, 1996). The above mentioned agents (PRRSV, SIV, PCV2 and ADV) are considered some of the most relevant viral contributors to PRDC (Halbur, 1998; Thacker and Thacker, 2000).

Furthermore, the expression of some swine diseases may be influenced by the presence of other viral infections, as it is the case of the infection by PRRSV or porcine parvovirus (PPV) on the expression of PCV2-SD when pigs are co-infected with PCV2 (Allan et al., 1999; Harms et al., 2001; Rovira et al., 2002). Besides its co-factor role in PCV2-SD, PPV is mainly relevant in pig production for causing reproductive failure in non-immune pregnant sows. Such reproductive

problems are characterized by embryonic and foetal infection and death, usually without apparent clinical signs in sows (Mengeling, 2006).

In Spain, PRRSV, SIV, PCV2 and ADV are considered important pig pathogens due to their widespread occurrence and associated economical losses. PRRSV infection was reported to be widely spread in the Spanish porcine livestock between 1998 and 2001 showing a range of 57–72% of seropositive pigs, across all ages (Segalés, 2003). A recent nation-wide survey reported SIV as highly prevalent in sows in Spain since it was found that antibodies to this virus were present in sows from 83 out of 100 unvaccinated herds and in 76% of the tested animals (Maldonado et al., 2006). Previous serological surveys showed PCV2 ubiquity in Spanish commercial swine farms affected or not by PCV2-SD (Segalés et al., 2003a,b; Sibila et al., 2004). Since 1995, Aujeszky's disease has been a major objective for eradication by the Spanish animal health authorities (RD 245/1995; RD 206/2005). This fact has entailed an exhaustive serological survey for antibodies against ADV glycoprotein E (gE) in the country, providing information on proportion of seropositive breeding herds stratified by territory areas that can be consulted in the government webpage (http://rasve.mapa.es/). Finally, although PPV infection is assumed to be widespread in the Spanish swine population, no detailed prevalence studies are yet available.

Furthermore, despite many studies have been done in pig producing countries to assess risk factors associated to prevalence of single infection of the mentioned agents (Ewald et al., 1994; Boelaert et al., 1999; Mortensen et al., 2002; Rose et al., 2003a; Oravainen et al., 2005), only few have focused on multiple infections (Maes et al., 2000). In the Spanish territory, the situation is very similar, since, as above

mentioned, several studies have been performed on seroprevalences; however, minimal studies exist on co-seropositivity or risk factors associated to seroprevalences (Gutiérrez-Martín et al., 2000). Therefore, the present study aims to assess three different objectives related to swine pathogen seroprevalences to provide more information on this context. The first objective is to provide detailed and homogeneous information on the seroprevalence against PRRSV, SIV, PCV2, ADV and PPV in boar, sow and fattening pig herds from indoor pig farms from Spain. The second objective is to provide information on seroprevalences and co-seropositivity in Spanish herds avoiding the measurement of those antibodies elicited by vaccination. Finally, the third objective is to assess the influence of farm characteristics and management on seroprevalences and co-seropositivity as well as the influence of seroprevalences and co-seropositivity on productive parameters.

3.2 Materials and methods

3.2.1 Seroprevalence to PRRSV, SIV, PCV2, ADV and PPV in the Spanish swine livestock

A retrospective study using information provided by two veterinary diagnostic laboratory services was performed to achieve the first objective. Both laboratory services provided information on serological results for each virus classified by year of analysis, farm of origin and animal category (boar, sow and fattening pig) to assess the seroprevalences to the selected viruses in the Spanish livestock. The two laboratories involved in this study were the Veterinary Diagnostic

Laboratory at *Laboratorios HIPRA, S.A.* (Girona, Spain) and the Pathology Diagnostic Service at the Veterinary School of the *Universitat Autònoma de Barcelona*.

Information provided by the Veterinary Diagnostic Laboratory at *Laboratorios HIPRA, S.A.* corresponded to serologic results on PRRSV, SIV, ADV gE and PPV, from boars, sows and 14-week-old or older fattening pigs that had been submitted between 2003 and 2005. These sera had been tested for the presence of specific antibodies to one or several of the following viruses: PRRSV (European variant; cut off for positivity set at percentage relative index >20; expected sensitivity (Se) and specificity (Sp) of 96 and 100%, respectively), SIV (cut off for positivity set at percentage relative index >20; expected Se and Sp of 94 and 100%, respectively), ADV (cut off for positivity set at inhibition percentage >45; expected Se and Sp of 99% for both parameters) or PPV (cut off for positivity set at OD>0.3; expected Se and Sp of 99.5 and 100%, respectively). Indirect ELISA was performed for PRRSV, SIV and PPV and blocking ELISA for ADV gE using commercially available ELISA kits (CIVTEST™ Laboratorios HIPRA S.A., Girona, Spain for PRRSV, SIV and ADV gE; INGEZIM®, INGENASA, Madrid, Spain for PPV). Test procedures and interpretation of results followed manufacturer's instructions. Range of herds and sera tested in each animal category (boars, sows and fattening pigs) during the evaluated years are shown in Table 3 for each virus.

Table 3. PRRSV, SIV, ADV gE and PPV seropositive herds and their seroprevalence in Spanish pig breeding stock and fatteners (2003-2005).

		Percentage of seropositive herds (CI 95%) No. tested herds - No. tested sera			Pigs' seroprevalence in seropositive herds (CI 95%) No. tested sera in seropositive herds		
		2003	**2004**	**2005**	**2003**	**2004**	**2005**
Boars	PRRSV	48 (35-60) 65 - 633	45 (30-61) 40 - 317	51 (37-64) 57 - 846	73 (67-78) 245	71 (64-77) 202	33 (29-37) 607
	SIV	53 (30-75) 19 - 160	67 (35-89) 12 - 70	53 (30-75) 19 - 247	66 (58-74) 141	55 (42-68) 58	50 (42-58) 155
	ADV gE	13 (7-25) 67 - 615	8 (2-23) 38 - 298	4 (1-15) 50 - 428	62 (48-74) 60	24 (13-40) 45	67 (13-98) 3
	PPV	89 (70-97) 27 - 193	65 (39-85) 17 - 96	94 (69-100) 17 - 165	83 (77-88) 184	82 (71-90) 78	67 (59-74) 161
Sows	PRRSV	89 (86-91) 604 – 16,093	86 (83-89) 519 – 13,062	85 (82-88) 572 – 16,349	75 (74-76) 14,416	72 (71-73) 11,758	76 (75-77) 14,309
	SIV	87 (81-91) 191 – 5,175	95 (91-97) 244 – 5,681	92 (87-95) 221 – 6,282	50 (49-51) 4,787	74 (73-75) 5,506	79 (78-80) 5,910
	ADV gE	41 (37-45) 576 – 18,213	34 (30-38) 522 – 16,795	30 (26-34) 527 – 23,135	25 (24-26) 9,360	30 (29-31) 7,650	26 (25-27) 13,054
	PPV	99 (97-100) 307 – 7,483	99 (97-100) 246 – 5,726	99 (97-100) 267 – 6,709	90 (89-91) 7,476	95 (94-96) 5,690	94 (93-95) 6,661
Fattening pigs	PRRSV	78 (74-81) 541 – 14,147	79 (75-83) 507 – 12,389	83 (80-86) 565 – 15,959	58 (57-59) 12,158	59 (58-60) 10,279	58 (57-59) 13,936
	SIV	68 (60-75) 152 – 4,802	78 (72-83) 223 – 5,121	82 (76-87) 217 – 7,089	34 (33-36) 4,048	44 (43-46) 4,258	45 (44-46) 6,087
	ADV gE	41 (37-45) 532 – 14,905	33 (29-37) 459 – 11,186	33 (29-37) 501 – 13,482	33 (32-34) 8,033	38 (37-40) 4,318	31 (30-32) 5,308
	PPV	94 (69-100) 17 - 296	80 (30-99) 5 - 215	95 (73-100) 20 - 830	75 (70-80) 288	38 (32-46) 185	45 (42-49) 815

Information provided by the Pathology Diagnostic Service at the Veterinary School of the *Universitat Autònoma de Barcelona* corresponded to serologic results on PCV2 from boars, sows and 10-week-old or older fattening pigs that had been submitted between 2000 and 2005. Antibody titres against PCV2 in these sera had been determined by a previously described immunoperoxidase monolayer assay (IPMA) (Rodríguez-Arrioja et al., 2000). Results were grouped either as negative or low antibody titres (below 1:320) and moderate or high titres (1:320 or higher) on the IPMA assay (Rodríguez-Arrioja et al., 2000). The number of tested sow and fattening pig herds ranged 1-3 and 2-9 per year, respectively; the number of tested sera for these two animal groups ranged 10-57 and 51-525 per year, repectively. On the other hand, only 28 sera from one boar stud were tested in year 2000.

A bias in the results was expected mainly due to four different reasons: a) laboratory diagnostic services were selected by convenience, although considering that they had to offer their services to all the Spanish territory; b) sera were sent voluntarily for routine analyses; c) different number of sera were analysed among farms and animal categories for each viral agent, what should be related with the epidemiologic situation of each virus and their productive relevance; and, d) some sera could result positive due to vaccination.

3.2.2 Pathogen seroprevalences and co-seropositivity (avoiding vaccination interferences)

For the second objective, 66 indoor pig farms (with sow and fattening pig herds) were selected to perform a cross-sectional study during 2002 and 2003. Selection process was performed by

convenience, contacting veterinary practitioners that agreed to contribute in the present study. These farms were located at the densest pig rearing areas in Spain (MAPA, 2003). Farms that applied vaccination to the studied viruses were excluded to avoid measuring antibodies elicited by vaccination (at the time of this survey, vaccination to ADV with glycoprotein E deleted vaccines was compulsory in Spain and vaccines to PCV2 were not available yet in the country). Six out of the 66 studied farms vaccinated sows to SIV, 19 to PRRSV (13 to sows, 3 to fatteners and 3 to both) and all of them vaccinated sows to PPV. Since the use of PPV vaccines was widespread, antibodies to this agent were not assessed in this part of the study. Therefore, 44 farms without vaccine application to PRRSV and SIV were finally included for PRRSV, SIV, PCV2 and ADV gE antibody analyses (these farms were provided by 22 veterinarians, who supplied a range of 1 to 6 farms/veterinarian). The number of studied farms was limited by economic funds.

To assess seroprevalences, thirty pig serum samples were collected from the 44 studied farms: 15 from sows and 15 from pigs at 20-weeks of age (samples of boars were not collected since most farms purchased semen for artificial insemination). All collected serum samples were analysed for presence of antibodies to PRRSV (indirect ELISA, HerdCheck® PRRS Virus Antibody Test Kit 2XR, IDEXX Laboratories Inc., Maine, USA; cut off for positivity set at Sample/Positive ratio ≥0.40; expected Se and Sp of 97.4 and 99.6%, respectively), SIV (indirect ELISA, CIVTEST™ SUIS Influenza Laboratorios Hipra S.A. Girona, Spain; cut off for positivity set at percentage relative index >20; expected Se and Sp of 94 and 100%, respectively), PCV2 (ORF2-based ELISA test described by Blanchard and colleagues in 2003; cut off for positivity set at OD ratio ≥1.5; expected Se and Sp of 98.2 and 94.5%,

respectively) and ADV gE protein (blocking ELISA, HerdCheck® Anti-PRV gpI, IDEXX Laboratories Inc., Maine, USA; cut off for positivity set at Sample/Negative ratio ≤0.6; expected Se and Sp of 99% for both parameters, approximately, according to manufacturer information). Taking into account the studied sample size for each animal category (n=15) and the sensitivity and specificity of the performed serological tests (information provided by manufacturers), a minimum seroprevalence of 23, 20, 35 and 28% for PRRSV, SIV, PCV2 and ADV gE, respectively, was expected to be detected with a 95% of confidence interval (Survey Toolbox version 1.0).

A new variable named co-seropositivity was created for each animal category (sows and fattening pigs) to determine the number of viral agents that elicited antibodies in the herds. The variable was dichotomized as presence of antibodies to 1-3 or 4 viral agents in sow herds, and 1-2 or 3-4 in fattening pig herds.

3.2.3 Associations to production parameters

For this third objective, the same subsample of 44 farms described above was used (PPV was excluded from the analyses due to extensive vaccination). A filled-in questionnaire was obtained from each farm concerning general farm characteristics, farm management and productive parameters. Once data from all the questionnaires were obtained, farms were divided into three size categories: less than 250 sows, between 250 and 500 sows and more than 500 sows. Data from the questionnaire were comprised in a total of 14 variables: 6 discrete (Table 4) and 8 quantitative ones (Table 5).

Table 4. Distribution of the 44 studied pig farms without vaccine interferences on serological results according to the discrete variables assessed in the questionnaire.

General farm characteristics		**No. of farms**
Geographical location	Barcelona	18
	Girona	6
	Lleida	6
	Aragón	4
	Murcia	6
	Other[a]	4
Farm size	<250 sows	15
	250-500 sows	11
	>500 sows	18
Production system	Farrow-to-Finish	24
	Multi-site[b]	20
Pen partition in nursery	Open	32
	Solid	12
Pen partition in fatteners	Open	26
	Solid	18
Farm management		
Animal flow	Continuous flow	22
	All in/All out	22

[a]These farms were located in Cuenca (n=1) and Burgos (n=3) and were not included in the statistical analyses due to their lower representation.

[b]Eight of these farms were three site and 12 were two site (breeding-postweaning independent from the fattening unit).

Table 5. Descriptive statistics of the continuous variables assessed in the 44 studied Spanish pig farms without vaccine interferences on serological results.

	Median	Percentiles (5^{th}-95^{th})
Farm management		
Pig density in nursery unit (pigs/m^2)*	4.8	1.8-6.7
Pig density in fattening unit (pigs/m^2)*	1.4	1.1-1.9
Productive parameters		
No. of litters/sow/year	2.3	2.1-2.5
Liveborn piglets/sow/farrow	10.4	9.3-11.9
Weaned piglets/sow/year	21.8	18.5-23.9
Nursery mortality	2.8	1.0-9.7
Fattening mortality	4.9	1.5-11.5
Overall postweaning mortality	8.0	4.6-16.6

*Data not available in 3 farms for the nursery unit and 5 farms for the fattening unit.

The influence of farm characteristics and management on sow and fattening pig herd seroprevalence to each individual viral agent was assessed using Poisson regression models (Cameron and Trivedi, 1998). The count response variables for these models were the number of seropositive sows and the number of seropositive fattening pigs in each farm, respectively. The established models were written as:

$$E(Y_i / \boldsymbol{x}_i) = \exp(\boldsymbol{x}_i \beta) \qquad i=1,\ldots,44$$

where Y_i is the number of seropositive sows or fattening pigs for each viral agent, $\boldsymbol{x}_i$ is the vector of covariates (geographic location, production

system, farm size, animal flow, and type of pen partition and pig density in nursery and fattening periods) and β the parameter of interest.

The effect of farm characteristics and management on co-seropositivity was analysed by means of a logistic regression model (Hosmer and Lemeshow, 2000), where the dependent variable was the number of viral agents that elicited antibodies in the herd (co-seropositivity). One model was built for sow herds and other for fattening pig herds. The established model was written as:

$$\ln \frac{\pi(\boldsymbol{x}_i)}{1-\pi(\boldsymbol{x}_i)} = \boldsymbol{x}_i \beta \qquad \text{i=1,…,44}$$

where $\pi(\boldsymbol{x}_i)=P(Yi/\ \boldsymbol{x}_i)$ is the probability of having co-seropositivity to 4 viral agents in sow herds or to 3 or 4 agents in fattening pig herds, $\boldsymbol{x}_i$ is the vector of covariates (geographic location, production system, farm size, animal flow, and type of pen partition and pig density in nursery and fattening periods) and β the parameter of interest.

Both, the influence of sow and fattening pig herd seroprevalences and the influence of co-seropositivity on productive parameters were modelled using Linear Mixed models (Verbeke and Molenberghs, 1997). The average of liveborn piglets/sow/farrow, farrowings/sow/year and weaned piglets/sow/year were used as response variables for the reproductive parameters associations on sow herds. Nursery, fattening and total (nursery plus fattening) mortalities were used as response variables for the productive parameters associations on fattening pig herds. Covariates included in the models were either pathogen seroprevalences or co-seropositivity for sow and fattening pig herds.

Seroprevalences to each agent were introduced as dichotomic variables using the following thresholds: presence or absence (ADV), <100% or 100% (PCV2 in fattening pigs) and ≤80% or > 80% (PCV2 in sows, PRRSV and SIV).

Analyses were performed using the SAS System V.9.1 (SAS Institute Inc.). Significance level was fixed at 5%.

3.3 Results

3.3.1 Seroprevalence to PRRSV, SIV, PCV2, ADV and PPV in Spanish swine livestock

The proportion of seropositive herds and their seroprevalences to PRRSV, SIV, ADV gE and PPV are displayed in Table 3, stratified by animal category (boars, sows and fattening pigs). Regarding PCV2, all tested sow and boar herds had pigs with medium to high titres; this situation also applied for almost all fattening pig herds, since the 13 analysed pigs from one herd in year 2004 had negative or low PCV2 antibody titres. Seroprevalence to PCV2 in sow herds ranged 93-100%, whereas it was 92-98% in fattening pig herds. All tested boars had PCV2 medium to high titres.

3.3.2 Pathogen seroprevalences and co-seropositivity (avoiding vaccination interferences)

A summary of descriptive statistics for general farm characteristics, farm management and productive parameters from the 44 studied farms is shown in Tables 4 and 5.

Descriptive information on the proportion of seropositive herds and their seroprevalences to PRRSV, SIV, PCV2 and ADV gE are displayed by animal category in Table 6 for the 44 studied farms. Table 7 shows the frequency of concurrent presence of antibodies to the different viruses. Twenty-three out of the 44 farms were seropositive to all four studied viral pathogens whereas 31 fattening pig herds showed antibodies to 3 or 4 studied agents.

Table 6. Percentage of seropositive herds and their seroprevalence in sow and fattening pig herds to selected swine viral pathogens in 44 studied Spanish farms without vaccine interferences on serological results.

		% of seropositive herds (95% CI)	Pigs' seroprevalence in seropositive herds	
			Median	Percentiles (5^{th}-95^{th})
PRRSV	**Sows**	91 (78-97)	80	40-100
	Fattening pigs	86 (72-94)	100	7-100
SIV	**Sows**	96 (84-99)	87	41-100
	Fattening pigs	73 (57-85)	60	7-100
PCV2	**Sows**	100 (90-100)	80	44-100
	Fattening pigs	100 (90-100)	100	80-100
ADV gE	**Sows**	55 (39-70)	37	9-87
	Fattening pigs	25 (14-41)	100	7-100

PRRSV: porcine reproductive and respiratory syndrome virus; SIV: swine influenza virus; PCV2: porcine circovirus type 2; ADV gE: Aujeszky's disease virus glycoprotein E.

Table 7. Concurrent presence of antibodies to selected swine viral pathogens in sow and fattening pig herds from 44 Spanish farms without vaccine interferences on serological results. Results on productive parameters are also included for each co-seropositivity situation.

Co-seropositivity situations in Sow herds	**No. of farms**	**No. Litters/sow/year**		**No. Liveborn pigs/sow/farrow**		**No. Weaned pigs/sow/year**	
		Median	**Percentiles (5^{th}–95^{th})**	**Median**	**Percentiles (5^{th}–95^{th})**	**Median**	**Percentiles (5^{th}–95^{th})**
PCV2 + SIV + PRRSV + ADV gE	23	2.3	2.1-2.4	10.5	9.3-12.3	21.8	17.8-23.9
PCV2 + SIV + PRRSV	16	2.3	2.1-2.5	10.4	8.7-12.0	21.7	18.5-23.5
PCV2 + SIV	2	2.5	2.4-2.5	10.1	10.0-10.2	23.5	22.5-24.5
PCV2 + SIV + ADV gE	1	2.3	-	10.8	-	21.0	-
PCV2 + PRRSV	1	2.4	-	9.9	-	21.6	-
PCV2	1	2.4	-	10.0	-	22.0	-

Co-seropositivity situations in Fattening pig herds	**No. of farms**	**Weaning mortality (%)**		**Fattening Mortality (%)**		**Total mortality (%)**	
		Median	**Percentiles (5^{th}–95^{th})**	**Median**	**Percentiles (5^{th}–95^{th})**	**Median**	**Percentiles (5^{th}–95^{th})**
PCV2 + SIV + PRRSV	20	2.7	1.0-8.9	5.8	2.1-14.7	7.8	4.6-16.7
PCV2 + SIV + PRRSV + ADV gE	9	2.5	1.0-10.0	5.5	3.0-10.0	9.0	6.5-16.0
PCV2 + PRRSV	8	3.5	1.0-14.2	3.8	2.5-6.0	6.8	5.0-17.2
PCV2	3	3.5	0.8-8.7	1.3	0.8-9.0	10.0	1.6-12.5
PCV2 + SIV	2	4.3	2.0-6.5	7.3	2.5-12.0	11.5	9.0-14.0
PCV2 + SIV + ADV gE	1	2.8	-	3.0	-	5.8	-
PCV2 + PRRSV + ADV gE	1	8.0	-	4.0	-	12.0	-

3.3.3 Associations to production parameters

None of the studied seroprevalences or co-seropositivity resulted influenced by farm characteristics according to the performed statistical models.

None of the evaluated productive parameters resulted significantly influenced by seroprevalences or co-seropositivity according to results from statistical model analyses.

3.4 Discussion

A seroprevalence study was firstly performed on PRRSV, SIV, PCV2, ADV gE and PPV in boar, sow and fattening pig herds to obtain data on the occurrence of these viral infections in Spain, one of the major pig producing countries in Europe (FAO, Statistics Division). This first study used data from laboratory diagnostic services and provided us a broad descriptive view of the relevance of the five studied viruses in the different Spanish porcine production stages. Despite the already mentioned expected biases, this study managed data from a high number of farms and animals that would not be feasible to obtain under more controlled conditions and without a considerable economic support. Additionally, a second complementary study was carried out in 44 pig farms in which the measure of antibodies elicited by vaccination was avoided; the extensively practiced PPV vaccination entailed, therefore, the exclusion of PPV from these analyses. This study allowed us not only to assess herd seroprevalences not due to vaccination to PRRSV, SIV, ADV gE and PCV2 but also co-seropositivity to the mentioned viruses

and the analyses of their relationships with farm characteristics, management and productive parameters. Taking into account that a low sample size was used (n=44) and that farms were located in the most populated Spanish pig rearing regions, a bias was expected. The bias due to the farm selection criteria was diminished by the participation of a relatively high number of veterinary practitioners.

Seroprevalences obtained in the first and second studies to PRRSV, SIV, ADV gE and PCV2 were similar in sow herds. Results in fattening pig herds from the second study showed wider confidence intervals due to a smaller sample size and higher PRRSV, SIV and ADV gE seroprevalences due to the age of the tested animals (20 weeks versus >10-14 weeks in the first study), fact that increased the probability of being infected with the virus at some time point previous to the tested age. Minimal bias due to vaccination was expected in the first study except for PPV, since vaccination was uncommon for PRRSV and SIV, discriminative for ADV gE and null for PCV2. PPV seropositive sow herds were ubiquitous; however, vaccination to this agent was extensively applied and, therefore, presence of antibodies was expected. In this sense, despite antibody titre elicited by vaccination is assumed to be lower than by infection (Straw et al., 2006), titration was not performed in the studied samples. Seroprevalence to PPV in the fattening units was mainly attributed to the long-lasting maternal immunity for this agent (Mengeling, 2006). The occasional vaccination to PRRSV in the Spanish territory was also observed in previously published reports (Segalés, 2003); furthermore, the PRRSV vaccination schedule in farms from the present study was highly variable, including farms with breeding stock and/or postweaning pig vaccination. Moreover, 80% of sow herds and 100% postweaning pig herds that performed

PRRSV vaccination used modified (attenuated) live vaccines. Both studies reported a high proportion of PRRSV seropositive sow and fattening pig herds with a high dissemination among their pigs; the overall proportion of positive sera is in accordance to that reported by Segalés from 1998 to 2001 (Segalés, 2003), indicating an endemic situation for this virus. Presence of SIV antibodies also showed a wide distribution in Spanish sow and fattening pig herds. A high proportion of sows from SIV seropositive herds had elicited antibodies, whereas it was only close to the half of the animals in the fattening units; sow records were in accordance to those reported by Maldonado et al. in the same period (Maldonado et al., 2006). Seropositivity to ADV gE was less relevant, showing a decrease in boar, sow and fattening pig herds with lower dissemination in sows than fattening pigs from those affected farms. This evolution of ADV should be attributed to the eradication programme for this infection. The present study also reports ubiquity of PCV2, in accordance to previously published studies (Segalés et al., 2003a,b; Sibila et al., 2004). The number of boars tested was much lower to that of sows and fattening pigs due to its lower representativity in the swine population. This fact implied lower precision in the seroprevalences. The proportion of seropositive boar studs to PRRSV, SIV and ADV gE were lower to those of sows and fattening pig herds; this could be explained by the fact that boars are usually reared under high health conditions to avoid infection transmission by contaminated semen since artificial insemination is widely practiced (Maes et al., 2008). Co-seropositivity with PRRSV, SIV and PCV2 seemed to be very common in Spanish sow and fattening pig herds. The obtained co-seropositivity situation was in accordance with the fact that these three viruses were the most frequently found in the studied herds.

Taking into account that multi-site production systems have been theoretically designed to obtain lower infection levels (Amass and Baysinger, 2006), differences on seroprevalences or co-seropositivities between production systems were expected; however, no statistical differences were observed. In the same sense, the practise of all in/all out management did not seem to be an effective measure to diminish the viral transmission of the studied agents in the fattening pigs, as it should be expected as well (Amass and Baysinger, 2006). This absence of relationships also occurred regarding the effect of seroprevalences or co-seropositivities to the studied viruses on productive parameters. The long antibody persistence in breeding herds could explain the lack of associations between co-seropositivity and reproductive parameters since such problems could have occurred much earlier than the survey visit. It must be pointed out that the role of PCV2 is difficult to assess since it was present in all fattening and sow herds. Nevertheless, despite statistical models have not detected significant associations, we can not rule out the possibility of a weak effect of any of the studied variables since a relatively low number of farms have been studied.

To the authors' knowledge, this study represents the most complete epidemiological description of the occurrence of the five selected swine viral pathogens in the Spanish territory. Results obtained not only update previous information on the relevance of these agents in the country, but also expands it. Ubiquity of PPV and PCV2 seropositive pigs was observed. High seroprevalences were observed for PRRSV and SIV in sow and fattening pig herds. Lower seroprevalences were observed in boar studs, since they represent a strategic point of infection transmission within the swine population. Concurrent presence of antibodies to PCV2, PRRSV and SIV was present in most breeding and

fattening herds, respectively. A decrease in ADV gE seroprevalence has been observed from 2003 to 2005. Furthermore, associations between seroprevalences and production parameters in Spain were assessed. The fact that the studied parameters resulted non-influential suggests that their effect, if any, would not be of high relevance on the control of the transmission of these viruses.

CHAPTER 4

STUDY II:

An exploratory study on risk factors for porcine circovirus systemic disease (PCV2-SD) in Spain

4.1 Introduction

Porcine circovirus type 2 (PCV2) is considered the causal agent of PCV2-SD (Allan et al., 1999; Albina et al., 2001; Bolin et al., 2001; Harms et al., 2001). PCV2-SD is a worldwide disease associated with a considerable economic impact in the pig industry due to the increase in mortality and production of non-marketable pigs (Segalés and Domingo, 2002).

PCV2-SD has been described in almost all types of farms, including farrow-to-finish and multi-site operations, and herd sizes varying from 30 to 10,000-sows (Segalés et al., 2003a,b). Morbidity and case-fatality risks are variable depending on the farm and the batch of animals, but can be as high as 4-30% and 70-80%, respectively (Segalés and Domingo, 2002).

PCV2 is a necessary component but other factors to trigger the disease are needed. In that sense, the identification of risk factors for PCV2-SD expression can be achieved by epidemiological studies, such as case/control or cross-sectional studies comparing affected versus non-affected farms. These results should give clues to reduce the clinical impact of PCV2-SD. However, minimal studies have focused on the assessment of risk factors on PCV2-SD expression (Cottrell et al., 1999; Cook et al., 2001; Rose et al., 2003a). Therefore, the objective of the present study was to assess, through an explorative case-control study in Spanish farms, those factors that, in association with PCV2 infection, might trigger clinical PCV2-SD.

4.2 Materials and methods

4.2.1 Survey design

A case-control study was carried out in Spain in 2002 and 2003 involving 72 farms of different production systems mainly located in the North-eastern and Eastern Spanish areas, some of the most populated pig rearing regions where approximately 2/3 of the country pig production is located (MAPA-INE, 1999). In this study we compared two types of farms obtained by a convenient sampling: farms were selected by veterinary practitioners according to their PCV2-SD status as “cases” or “controls”. The farms were further confirmed as “cases” if they fulfilled all the three following criteria: 1) ≥10% of post-weaning pigs showing PCV2-SD compatible clinical signs (including wasting, respiratory distress and paleness of the skin), 2) mortality increase more than 2% from weaning to market age since the appearance of the PCV2-SD compatible clinical picture, or mortality higher than 7% during the acute phase of the disease, and 3) confirmation of PCV2-SD presence in the farm based on the fulfilment of previously described diagnostic criteria (Segalés, 2002), in at least one pig showing the mentioned clinical signs (2 to 3 pigs were necropsied per farm for this purpose). The accepted diagnostic criteria for PCV2-SD include: 1) clinical picture whose major clinical sign is wasting, 2) moderate to severe lymphocyte depletion and granulomatous inflammation of lymphoid tissues, and 3) presence of moderate to high amount of PCV2 in these lesions (Segalés, 2002). “Controls” were farms selected from the same geographical regions, based on the lack of clinical history of PCV2-SD and, therefore, the lack of accomplishment of the two first previous mentioned criteria to define “cases”.

Initially, 72 farms were involved in the study, 42 were potential "cases" and 30 "controls". Ten farms from the "cases" group were discarded since not all the criteria were finally met (lack of available necropsied pigs, animal sera or fulfilment of the third classification criteria). All selected "control" farms were included in the study.

4.2.2 Data collection

A filled-in questionnaire and 45 blood samples were obtained from each farm. Additionally, when PCV2-SD was clinically suspected, two to three pigs were necropsied and relevant tissues to diagnose PCV2-SD were collected.

The questionnaire, which was designed based on a previous epidemiological survey performed in France (Rose et al., 2003a), included the following issues grouped by topic:

1. General farm characteristics (production system, farm size, type of batch rearing, production parameters, cross-fostering, castration practices, geographical proximity of other swine herds, presence of other production animals in the farm). A total of 24 variables were included in this group.

2. Breeding and replacement practices (artificial insemination practices, genetic types, origin of replacement gilts and boars, self-replacement practices, acclimatisation housing and management for replacement stock, quarantine practices, pregnancy and farrowing facilities). A total of 21 variables were included in this group.

3. Weaning and fattening facilities description (number of rooms per building, pens per room, stocking density, and contact between pigs of different litters, pens or ages). A total of 36 variables were included in this group.

4. Hygiene practices (cleansing, disinfection and disinsectation protocols). A total of 25 variables were included in this group.

5. Vaccination schedule for all pigs present in the farms (vaccination versus no vaccination, attenuated versus inactivated vaccine, and vaccination timing). A total of 74 variables were included in this group.

6. Biosecurity conditions (measures for visitors, foot-bath pool, carcass storage, hospital facilities for sick animals). A total of 11 variables were included in this group.

4.2.3 Serology

Collected blood samples (n=45) from each farm were divided into 3 groups (calculated to detect the disease in case of prevalence higher than 10% for each age group and for each pathogen). The first group comprised 15 sows and the second and third groups of blood samples corresponded to 15 pigs of 12 and 20 weeks of age, respectively. The presence of antibodies to PCV2 was investigated in all groups of animals using a previously published ORF2-based ELISA test (Blanchard et al., 2003; cut off for positivity set at OD ratio ≥1.5; sensitivity and specificity of the test were 98.2 and 94.5%, respectively). Antibodies to ADV gE (HerdCheck® Anti-PRV gpI, IDEXX Laboratories Inc., Maine, USA; cut

off for positivity set at Sample/Negative ratio ≤0.6; sensitivity and specificity of the test were 99%, approximately, for both values), PRRSV (HerdCheck® PRRS Virus Antibody Test Kit 2XR, IDEXX Laboratories Inc., Maine, USA; cut off for positivity set at Sample/Positive ratio ≥0.40; sensitivity and specificity of the test were 97.4 and 99.6%, respectively) and PPV (INGEZIM PPV®, INGENASA, Madrid, Spain; cut off for positivity set at titration ≥1:200; sensitivity and specificity of the text were 99.5 and 100%, respectively) were determined in serum samples of sows and 20-week-old pigs, just to assess if there were infection/vaccination with those pathogens. The information obtained from serological tests was summarised in a total of 20 variables.

4.2.4 Histopathology

To assess the presence of PCV2-SD in farms where the disease was clinically suspected, two to three pigs were necropsied by the participating veterinarian. Samples of inguinal superficial and mesenteric lymph nodes, tonsil and lung were collected and fixed by immersion in 10% buffered neutral formalin. Fixed samples were dehydrated, embedded in paraffin wax, sectioned at 4 µm, and stained with haematoxylin and eosin. Correlative sections were also placed on silane-coated (3-[trietoxysilil]-propilamine) slides and PCV2 nucleic acid presence was determined by a previously described *in situ* hybridisation (ISH) technique (Rosell et al., 1999). A pig was considered as suffering from PCV2-SD when moderate to severe lymphocyte depletion and histiocytic infiltration, and moderate to high amount of PCV2 genome were found in lymphoid tissues (Segalés, 2002).

4.2.5 Statistical analyses

According to the results obtained from an initial statistical analysis performed on the data from the questionnaire (191 variables) and the serological studies (20 variables) and the experience of previous studies (Rose et al. 2003a), farm size was considered to be a confounding variable rather than a possible risk factor. Therefore, the variable number of sows in the farm was categorized in three homogenised groups: equal or less than 250 sows, between 251 and 500 sows and more than 500 sows. Data were analysed as a nested matched case-control design stratified according to the farm size (Rose et al., 2003b). Numeric variables were categorized (0: lower than the median value; 1: equal or higher than median value). A univariable analysis was performed between the PCV2-SD status ("case" or "control") and each variable obtained from the filled-in questionnaire and the serological tests, using the Likelihood ratio chi square test (taking into account the stratification).

Variables under study with p-values lower than 0.20 were selected in a preliminary step. All bivariable relationships between possible explanatory variables belonging to the same group (the six groups of the questionnaire) were tested using the chi square test. For relationships that presented strong collinearity ($P<0.05$) only the variable with the "a priori" stronger biological association with PCV2-SD outcome was retained. Once performed, 7 factors with P values lower than 0.20 were retained for further logistic regression analysis. The data set corresponding to the variables with P values lower than 0.20 was analysed as a nested n:m matched case-control design related to the farm size (Rose et al., 2003b) using the PHREG procedure (SAS Institute Inc., 2000). Finally, a stepwise procedure was used to include

the variables in the model. The model was rerun until all remaining variables presented statistically significant values (P<0.05).

4.3 Results

The “case” group included 17 farrow-to-finish and 15 multi-site farms, while the “control” one had 19 and 11, respectively. Farms were of larger size in the “case” group and of medium size in the “control” one (P<0.05). Production data of farms included in the study (n=62) are displayed in Table 8. Mortalities were higher (P<0.05) in the “case” than in the “control” groups of farms (6.9% versus 2.9% in weaners and 7.7% and 4.0% in feeders, respectively).

All studied farms had PCV2 antibodies in all tested age groups. Seroprevalences to PCV2, PRRSV, ADV and PPV per production system and pig age group from “case” and “control” farms are displayed in Table 9. After the bivariable analysis and the collinearity study, 7 variables with P-values <0.20 were offered to the multivariable model (Table 10). Only three variables were found significant after the stepwise procedure (Table 11). Vaccination of gilts against PRRSV increased the odds ratio for PCV2-SD (P=0.04; OR=5.1), while farms having sows vaccinated against atrophic rhinitis presented a lower odds ratio for disease presentation (P=0.03; OR=0.19). The odds ratio for clinical PCV2-SD was significantly increased when more than 90% of 12 week-old pigs tested positive for PCV2 antibodies (P=0.02; OR=5.99).

Table 8. Production data from 62 Spanish pig herds included in a case-control study of risk factors for PCV2-SD, 2002-2003.

	CASE farms	CONTROL farms
Average number of live-born piglets / sow	10.43 ±0.51	10.59 ±0.74
Average number of weaned piglets / year / productive sow	21.34 ±1.49	21.67 ±1.58
Average daily weight gain from weaning to finishing (g)	637 ±99	650 ±100
Average feed conversion indices (from weaning to finishing)	2.60 ±0.27	2.69 ±0.17
Mean age at weaning (days)	22.24 ±2.14	22.79 ±2.78

Table 9. Distribution of antibodies PCV2, ADV, PRRSV and PPV, in the different age-groups of animals from 62 Spanish pig farms included in a case-control study on PCV2-SD, 2002-2003. A Chi-square test was performed for the animal groups to compare seroprevalence to different pathogens.

			Percentage with antibodies (%)						
			CASES			CONTROLS			
		Production system	Sows	12 week-old	20 week-old	Sows	12 week-old	20 week-old	P-value
PCV2	Farms	F-to-F	100	100	100	100	100	100	NS
		Multi-site	100	100	100	100	100	100	NS
	Animals	F-to-F	83.1	91.0*	98.0**	76.8	76.1*	93.7**	* P<0.0001 **P=0.022
		Multi-site	77.8	78.6	96.4	81.3	83.0	97.6	NS
ADV	Farms	F-to-F	71	-	29	63%	-	26	NS
		Multi-site	60	-	20	55	-	37	NS
	Animals	F-to-F	36.4	-	23.9	28.4	-	21.4	NS
		Multi-site	24.4	-	15.5*	30.3	-	27.2*	*P=0.005
PRRSV	Farms	F-to-F	100	-	88	89	-	89	NS
		Multi-site	93	-	93	91	-	100	NS
	Animals	F-to-F	80.7	-	81.5*	75.0	-	88.0*	*P=0.040
		Multi-site	75.1	-	75.5	67.8	-	71.5	NS
PPV	Farms	F-to-F	100	-	88	100	-	37	NS
		Multi-site	100	-	40%	100	-	45	NS
	Animals	F-to-F	98.4	-	49.4*	97.5	-	27.7*	*P<0.0001
		Multi-site	92.4	-	22.2*	91.5	-	41.2*	*P<0.0001

NS: non-significant (P>0.05); F-to-F: farrow-to-finish operations; Multi-site: Multi-site production operations

Table 10. Distribution of selected risk factors (P-value<0.2 in the bivariable analysis) for PCV2-SD in 62 Spanish pig herds included in a case-control study, 2002-2003.

	Case farms		Control farms	
	No.	**%**	**No.**	**%**
General farm characteristics				
Number of sows				
≤250	10	31	11	37
251-500	4	13	15	50
>500	18	56	4	13
Animal flow in fattening				
AI/AO	9	28	16	53
Continuous	23	72	14	47
*Feed conversion */***				
<2.68 kg feed/kg of weight gain	12	67	5	29
≥2.68 kg feed/kg of weight gain	6	33	12	71
Breeding and replacement stock practices:				
*Pietrain genes in paternal line **				
Yes	16	52	23	82
No	15	48	5	18
Vaccination schedule:				
Vaccination of sows to atrophic rhinitis				
Yes	9	28	15	50
No	23	72	15	50
Vaccination of gilts to PRRSV				
Yes	13	41	6	20
No	19	59	24	80
Serology Resuts:				
Number of positive pigs to PCV2 by ELISA at 12 weeks of age				
≤13 (<90%)	11	34	17	57
>13 (≥90%)	21	66	13	43

* Data from all the farms were not available for this variable

** Median value for this variable in the studied population

Table 11. Results of final multivariable conditional* logistic regression analysis of risk factors for PCV2-SD in 62 Spanish pig farms, 2002-2003.

Variables	β	se (β)	Odds Ratio	Confidence interval (95%)
Vaccination of gilts to PRRSV				
No	0	0	1	-
Yes	1.63	0.80	5.10	1.06-24.4
Vaccination of sows to atrophic rhinitis				
No	0	0	1	-
Yes	-1.68	0.74	0.19	0.04-0.79
Number of positive pigs to PCV2 by ELISA at 12 weeks of age				
≤13 (<90%)	0	0	1	-
>13 (≥90%)	1.79	0.76	5.99	1.4-26.4

*Nested m:n matched case-control, according to farm size

Model: Intercept=-1.88, model deviance=43.9, model df.=3 (P=0.001)

4.4 Discussion

The present case-control study involved 62 farms distributed in the Spanish areas where pig production is mostly concentrated (MAPA-INE, 1999). The limited set of general production record we gathered indicated that the farms were representative of the current situation in the country (e-Bdporc, 2002). Significant differences in mortality between case and control farms were observed. This was expected since

mortality was one of the selection criteria for classification. However, no differences were observed in average daily weight gain and average feed conversion rates (from wean to finish). This fact may be explained by the high case-fatality risk of PCV2-SD (Segalés and Domingo, 2002) that would have increased the average food intake of the surviving pigs, being most of them PCV2-SD non-affected animals with normal growth and feed consumption rates. A similar situation has already been described by Cook et al. (2001) in a case-control study of PCV2-SD performed in the United Kingdom.

The number of “case” and “control” farms included in the study was considered balanced, and the different production systems were clearly represented in both groups. As previously described (Cook et al., 2001; Rose et al., 2003a), PCV2-SD was strongly related to farm size, since larger farms had a higher probability of PCV2-SD than other farms. However, farm size is related to several variables and could be considered as potential confounder (Rose et al. 2003b). For this reason and also because of the limited sample size, a nested matched analysis was performed to determine the crude effect of the variables without any confusing relation due to farm size.

After the stepwise procedure, only three variables of the whole study, two related to the vaccination schedule and one to the PCV2 infection dynamics, were found to be statistically related to PCV2-SD expression.

Vaccination of replacement stock against PRRSV could be interpreted as a type I error (due to the high number of vaccination variables in our database), or a spurious effect since it can be speculated that vaccination took place because of having problems with that

particular disease. However, other concomitant infections such as PPV or PRRSV were not finally confirmed as risk factors for PCV2-SD in this study (although certain statistical significant differences were observed when using a Chi-square test to compare seroprevalence to these pathogens in “cases” to “controls”, by production system, as it has been suggested from experimental and epidemiological points of view (Pogranichniy et al., 2002; Segalés and Domingo 2002; Rose et al., 2003a). The highly widespread nature of PRRSV infection (Segalés, 2003) and PPV vaccination (Maldonado et al., 2005) in our country and the influence of other potential factors in PCV2-SD development when the co-infection occurs may be pointed out as the reasons for these findings. As far as immunostimulation could be concerned with the use of vaccines (Krakowka et al., 2001, Kyriakis et al., 2002) as a potential triggering factor for PCV2-SD, there is no apparent relationship between PCV2-SD in growing pigs and vaccination of the gilts against PRRSV. Therefore, the hypothesis of immunostimulation of PCV2-infected pigs on subsequent PCV2-SD in the offspring expression does not apply here.

Regarding vaccination of sows against atrophic rhinitis, it is probable that this variable was a type I error or a spurious effect and no clear reasons seem to explain the protective effect on PCV2-SD expression. Otherwise, it could be argued that farms using this vaccine are farms with a better homogeneity of the immune status of the breeding herd regarding atrophic rhinitis causative agents. A decrease of toxigenic *Pasteurella multocida* colonization of the offspring would diminish partly the general microbiological load in piglets and, therefore, potential co-infections with PCV2, as it has been suggested by Rose et

al. (2003a) for a similar result obtained with the *Escherichia coli* vaccination in sows.

The third significant variable suggests that farms where animals become infected earlier with PCV2 have a higher risk of developing PCV2-SD than farms with a later infection. This result was also observed in a previous study in France (Rose et al., 2003a). Therefore, it could be speculated that all those factors that favour longer maternal immunity to PCV2 (such as the potential use of vaccines to PCV2 in sows) or delay PCV2 infection in pigs would be of benefit to control PCV2-SD.

The number of farms (n=62), might have not been large enough in order to identify significant differences for most of the studied variables (especially for co-infection variables for which the seroprevalence is very high). A higher number of studied farms would probably have identified a higher number of risk factors that explain PCV2-SD triggering in certain PCV2 infected farms.

Additionally, from the seven significant variables obtained in the French study (Rose et al, 2003a), only three (two predisposing for PCV2-SD: percentage of fattening pigs seropositive to PRRSV and PPV, on-farm semen collection; and one protective for the disease: longer empty period in farrowing and weaning facilities) were included in our database. From the other four variables (all of them considered as risk factors for PCV2-SD: large pens in postweaning stage, individual housing for sows during pregnancy, separated vaccination to Erysipelas and PPV, treatment of sows against ectoparasites), it would have been of interest to include the variable related to pen size but not the other three, since there are almost no differences on sow housing practice during

pregnancy in Spanish farms and the other two variables were not apparently, at least in advance, very likely to be considered as risk factors for PCV2-SD. Further investigations in the epidemiological field are necessary to elucidate those and other potential risk factors that might have an influence in PCV2-SD expression.

CHAPTER 5

STUDY III:

Porcine circovirus systemic disease (PCV2-SD) clinical expression under field conditions is modulated by the pig genetic background

5.1 Introduction

Porcine circovirus type 2 is found worldwide in pigs and has been linked to several pathological conditions collectively named porcine circovirus diseases (PCVD) (Segalés et al., 2005a). The most economically important PCVD is PCV2-SD, which results in losses of €900 million per year in the European Union (Armstrong and Bishop, 2004).

Infection of pigs with PCV2 and other infectious/non-infectious triggers are required for PCV2-SD to occur (Segalés et al., 2005a). Evaluation of the role of these other triggers is essential in order to understand the pathogenesis of PCV2-SD. Several studies have linked PCV2-SD expression to management measures, presence of concurrent viral infections, stimulation of the immune system, PCV2 viraemia and low serological titres to PCV2 of the sow at farrowing, nutrition, male castration and lower piglet weight at weaning (Segalés et al., 2005a).

Farmers and veterinarians in the field have suggested that different pig breeds or genetic lines have different susceptibilities to PCV2-SD. Similar effects have been previously demonstrated for other diseases, including bacterial (Michaels et al., 1994; Wigley, 2004), parasitic (Reiner et al., 2002a) and viral (Depner et al., 1997; Reiner et al., 2002b; Vincent et al., 2006) diseases. Preliminary experimental studies have suggested a higher susceptibility to PCV2-associated lesions and even on PCV2-SD development of Landrace pigs compared to Duroc, Large White and Pietrain pigs (Opriessnig et al., 2006b, 2009c). Experiences in the field have reported a significantly less often occurrence of clinical signs

resembling PCV2-SD in pure bred conventional Hampshire boars than in pure bred conventional Yorkshire or Landrace boars (Wallgren et al., 2009). However, the introduction of a terminal Pietrain boar onto affected farms showed no effect on PCV2-SD expression (Rose et al., 2005). Besides these preliminary studies minimal scientific work has so far been focused on the relationship between genetics and PCV2-SD development. The objective of the present study was to compare the effect of 3 different genetic boar lines on the expression of PCV2-SD in their offspring.

5.2 Materials and methods

Animal care and use conformed to the European Union guidelines and Good Clinical Practice.

The study was carried out in 2 almost identical 5000-sow farms (farm-1 and farm-2) separated by 300 m. Farm-1 and farm-2 belonged to the same producer company, which had a multi-site production system that used the same sow genetic line (37.5% Large White x 37.5% Duroc x 25% Landrace) across all sites. Farms were seronegative to PRRSV and ADV, and no evidence of PPV circulation was found in a subsample of 90 piglets from the studied batches (data not shown). Post-weaning mortality in the year before study start ranged from 4 to 8% in both farms and was mainly attributed to PCV2-SD, according to previous diagnostic analyses performed in the farms.

Boars from three genetic lines, A (100% Pietrain), B (50% Large White x 50% Pietrain) and C (25% Large White x 75% Duroc), were used

for artificial insemination. Fifteen A, eight B, and four C boars were used. Sow insemination with each boar line was assigned randomly. Each sow was not always inseminated with semen from the same boar in repeated inseminations, but the boars were always from the same genetic line. A total of 63 pregnant sows was obtained on farm-1 (24 pregnant from A boars, 20 from B boars and 19 from C boars) On farm-2 the figures were 67 pregnant sows (29 A, 23 B and 15 C). Sows were randomly placed in the farrowing facilities in both farms taking into account that approximately one third of sows inseminated with each boar line had to be present in each room (there were three rooms per farm). No cross-fostering was allowed.

Semen (diluted for insemination and non-diluted from direct extraction) and serum samples were collected from boars at the moment of the semen extraction. Serum samples from sows were collected 1 week before farrowing.

A total of 1062 piglets (517 from farm-1 and 545 from farm-2) were obtained and ear-tagged before entering the nursery period (3 weeks of age) and monitored until the end of the fattening period (21 weeks of age). Piglet distribution by farm, genetic background, gender and sow parity is shown in Table 12. Sow parity was categorized as 1 or >1 farrowings (Calsamiglia et al., 2007). Nursery pigs (3 to 10 weeks of age) from farm-1 and farm-2 were placed in the same nursery building but in separate rooms (two rooms per group). Fattening pigs (from 10 weeks of age to slaughter) from farm-1 were placed in pens located in front of those of farm-2 from the same room and farm. All pens in both the nursery and fattening units contained pigs from all three genetic backgrounds.

Mortality was monitored during the post-weaning period, from 3 to 21 weeks of age; all dead pigs during this period were necropsied and tissue samples (mesenteric lymph node and lung) collected. A subpopulation of 207 piglets was randomly selected, blocked by genetic background, farm, sow of origin and sex (33 to 36 pigs for each genetic background and farm); these pigs were bled at 3 weeks of age and weighed at 3, 9, 15 and 21 weeks of age.

5.2.1 Laboratory Analyses

A PCR method to detect PCV2 (Quintana et al., 2002) was performed on serum samples from sows and piglets and on serum, diluted semen and non-diluted semen from boars. An IPMA was used to detect PCV2 antibodies (Rodríguez-Arrioja et al., 2000) in sera from boars, sows and piglets. Antibody titres were classified as negative or low titre (<1:320) or medium to high titre (≥1:320) (Rodríguez-Arrioja et al., 2000). Mesenteric lymph node and lung samples collected from necropsied pigs were fixed by immersion in 10% buffered neutral formalin and routinely processed for histopathology. The presence of PCV2 in these tissues was detected using an *in situ* hybridization technique (Rosell et al., 1999). A pig was considered as suffering from PCV2-SD when moderate to severe lymphocyte depletion and histiocytic infiltration were found in lymphoid tissues associated with moderate to high amount of PCV2 (Segalés, 2002).

Table 12. Descriptive statistics on explanatory and response variables from piglets included in the study.

Variable	Farm-1			Farm-2		
	A	B	C	A	B	C
No. of boars	13	7	4	10	6	4
No. of sows	24	20	19	29	23	15
No. of piglets	196	169	152	243	203	99
Piglets' male : female ratio	50 : 50	54 : 46	55 : 45	51 : 49	46 : 54	63 : 37
Piglets from first parity sows, %	20	22	26	33	37	27
Sows with medium to high PCV2 IPMA antibody titres, %	79	85	89	100	100	100
Piglets from sows with medium to high PCV2 IPMA antibody titres, %	82	75	87	100	100	100
PWM, % (n)	1.5 (3)	4.7 (8)	9.9 (15)	2.1 (5)	5.9 (12)	26.3 (26)
PCV2-SD-PWM, % (n)	1.5 (3)	2.4 (4)	6.6 (10)	0.8 (2)	3.0 (6)	21.2 (21)
PCV2-SD-PWM in piglets from primiparous sows – multiparous sows, %	2.6 – 1.3	0 – 3.1	7.7 – 6.2	0 – 1.2	1.3 – 3.9	3.7 – 27.8
PCV2-SD-PWM (Males – Females), %	1.0 – 2.0	3.3 – 1.3	7.2 – 5.8	0.8 – 0.8	2.1 – 3.7	18.0 – 26.3
Piglets with medium to high PCV2 IPMA antibody titres at 3 weeks of age, % [a]	9	70	26	47	37	23
BW at 3 weeks of age (μ ± SD), Kg [a]	7.0 ± 1.5	6.8 ± 1.4	5.9 ± 1.3	6.9 ± 1.4	6.0 ± 1.2	6.2 ± 1.7
BW at 9 weeks of age (μ ± SD), Kg [a]	24.7 ± 3.7	22.8 ± 3.3	21.7 ± 3.2	22.6 ± 4.0	20.6 ± 2.9	21.9 ± 3.6
BW at 15 weeks of age (μ ± SD), Kg [a]	55.7 ± 7.7	53.2 ± 8.3	50.0 ± 9.6	53.1 ± 6.7	50.3 ± 5.8	48.1 ± 7.2
BW at 21 weeks of age (μ ± SD), Kg [a]	88.2 ± 10.4	82.9 ± 12.8	78.4 ± 15.2	85.2 ± 10.5	80.2 ± 10	80.2 ± 5.6

IPMA: immunoperoxidase monolayer assay; PWM: post-weaning mortality; PCV2-SD-PWM: post-weaning mortality associated to PCV2-SD. [a]Statistics were obtained using the subpopulation of 207 pigs.

5.2.2 Statistical Analysis

PCV2-SD expression was evaluated using 3 parameters: body weight (BW) at 3, 9, 15 and 21 weeks of age, total post-weaning mortality (PWM), and PWM due to PCV2-SD (PCV2-SD-PWM). Total PWM, which includes non-PCV2-SD-related mortality, was considered as a relevant production parameter because of the reported synergism between PCV2-SD and other causes of mortality (Segalés et al., 2005a).

Two logistic regression models (Hosmer and Lemeshow, 2000) were established to evaluate both PWM and PCV2-SD-PWM, including the sow as a random effect. A longitudinal linear mixed model with repeated measures (Verbeke and Molenberghs, 1997) was performed to analyze BW. Covariates included in all models were genetic background, farm, sow presence of PCV2 viraemia and antibody titre at farrowing, sow parity, piglet's sex and BW, PCV2 infectious status and PCV2 antibodies at 3 weeks of age. Interactions with farm were also included. The sow was included as a random effect.

All results were obtained using GLIMMIX and MIXED procedures of the SAS System V.9.1 (SAS Institute Inc.). Significance level was fixed at 5%.

5.3 Results

PCV2 was not detected in any boar and sow serum sample, or any semen sample. The proportion of sows with medium to high PCV2

antibody titres as well as the proportion of piglets that came from them is shown in Table 12.

PCV2-SD-PWM occurred on farm-1 from 10 to 15 weeks of age (except for one pig at 20 weeks), and from 9 to 15 weeks of age on farm-2. Further information on PWM and PCV2-SD-PWM expression by farm and genetic background is shown in Table 12. The antibody titres and BW changes of the 207 monitored piglets are also shown in Table 12.

Logistic regression models showed a significant effect of genetic background ($P<0.001$), farm ($P=0.001$) and parity ($P=0.038$) on total PWM. Specifically, PWM was more likely in piglets from C boars than both A (OR=11.95, CI_{OR}=5.10 to 28.02) and B (OR=4.42, CI_{OR}=2.29 to 8.55). Also, PWM was more likely for B than for A (OR=2.70, CI_{OR}=1.08 to 6.77) backgrounds. In addition, PWM was more likely in piglets from farm-2 than farm-1 (OR=2.88, CI_{OR}=1.56 to 5.33). Finally, PWM was more likely in piglets from multiparous than primiparous sows (OR=2.18, CI_{OR}=1.04 to 4.54).

A significant effect of genetic background ($P<0.001$) and a farm and parity interaction ($P=0.048$) on PCV2-SD-PWM was obtained. PCV2-SD-PWM was more likely for piglets from C boars than both A (OR=15.55, CI_{OR}=4.92 to 43.06) and B (OR=8.26, CI_{OR}=3.19 to 21.39). An effect of sow parity was only observed on farm-2, where PCV2-SD-PWM was more likely in piglets from multiparous than primiparous sows (OR=6.86, CI_{OR}=1.35 to 34.94).

Significant covariates for BW in the longitudinal mixed models were genetic background ($P<0.001$), farm ($P=0.003$), age ($P<0.001$), genetics and farm interaction ($P=0.044$) and genetics and age interaction

(P=0.004). Piglets from farm-2 showed lower weights than those from farm-1. Detailed results from the longitudinal mixed model are shown in Table 13.

Table 13. Longitudinal mixed model estimates for BW evolution.

Covariate/s	Estimate	Confidence Interval [a]		P [a]
		Lower	Upper	
Genetic background				
A vs B	2.300	0.893	3.708	<0.001
A vs C	4.009	2.563	5.455	<0.001
C vs B	-1.708	-3.159	-0.258	0.016
Genetic background and farm interaction				
Farm-1: A vs C	5.168	2.665	7.670	<0.001
C vs B	-3.185	-5.685	-0.686	0.004
Farm-2: A vs B	2.618	0.257	4.979	0.020
A vs C	2.850	0.373	5.326	0.014
B farm-1 vs B farm-2	2.711	0.280	5.142	0.019
Genetic background and age interaction				
15 weeks of age[b]: A vs C	5.601	1.489	9.713	<0.001
21 weeks of age[c]: A vs B	4.479	0.499	8.460	0.013
A vs C	7.493	3.271	11.715	<0.001

[a]Confidence intervals and P-values adjusted with the Tukey correction.

[b]Mean weight±standard deviation for piglets with A and C background were 54.6±7.2Kg and 49.1±8.6Kg, respectively.

[c]Mean weight±standard deviation for piglets with A, B and C background were 86.6±10.5Kg, 81.5±11.4Kg and 79.1±12.1Kg, respectively.

5.4 Discussion

Most evidence of the effect of genetic background on PCV2-SD susceptibility has come from the experimental side (Opriessnig et al., 2006b, 2009c). These studies included a limited number of pigs and used pure-bred animals, which is unusual under field conditions. Furthermore, few studies have been performed in the field in regards genetics and PCV2-SD occurrence, where no apparent relationship between boar genetic background and disease expression was observed in the offspring (Rose et al., 2005) or minimal information was available when genetic background susceptibility was observed (Wallgren et al., 2009). This study aimed to assess whether clinical expression of PCV2-SD was influenced by genetic background. Three different boar lines that swine producers had suggested as having different PCV2-SD outcome severity in their offspring were used. Using BW, PCV2-SD-PWM and total PWM as measures of PCV2-SD expression, this study confirmed those suggestions. It also confirmed that other factors, such as farm of origin, sow parity and piglet age, were associated with the effects of PCV2-SD.

Several measures to diminish potential biases that may occur under field conditions were adopted for this study. Two sow farms which were located together, with the same farm management and characteristics and sow genetic background were used. Piglets were reared on the same nursery and fattening farms, so were kept under the most similar environmental conditions. Furthermore, any potential biases due to concurrent diseases are unlikely in this study as PPV, PRRSV and ADV, three viruses reported to be related to clinical expression of PCV2-SD (Ellis et al., 2004), did not circulate in the studied batches. In

addition, total PWM was mainly associated to PCV2-SD based on pathological findings. Finally, other factors, i.e. sow and pig PCV2 infectious status and antibody titre, sow parity, piglet gender and weaning BW, that could influence PCV2-SD expression were also studied.

PCV2 infectious status of sows, boars and weaned piglets was assessed as PCV2 status has a significant effect on litter mortality in PCV2-SD-affected farms (Calsamiglia et al., 2007), and the earlier the pig is infected with PCV2, the higher the risk of suffering from PCV2-SD (Rose et al., 2003a; Study II of the present PhD thesis). The fact that no sample demonstrated PCV2 infection reinforces the role of the genetic background as a risk factor for PCV2-SD, and suggests that it was probably the most significant one on the farms in this study.

Significant differences between genetic backgrounds were observed in the present study. Piglets from C boars were the most affected, showing lower BW and higher rates of PWM and PCV2-SD-PWM than those from A and B boars. Piglets from A boars had higher BW at slaughter age and lower PWM compared to those from the other two studied boar lines. These findings are in agreement with the experimental study by Opriessnig et al., (2009c) who reported that Landrace piglets developed more severe PCV2-SD-like lesions than Pietrain. However they contradict a previous French field study which did not find any difference when the usual terminal boar breed was replaced by Pietrain (Rose et al., 2005). These results may seem contradictory; however, differences in study design may be the cause of these inconsistencies. Moreover, such differences could be attributed to the fact that different Pietrain genetic lines were used in different studies,

and it is possible that PCV2-SD genetic susceptibility/resistance might be related to particular genetic lines rather than breeds or potential allele variants within lines.

The critical period for BW differences between lines was between 15 and 21 weeks of age, just after the PCV2-SD outbreak. The significantly higher weights found in piglets from A boars at 21 weeks of age was noteworthy because Duroc and Large White breeds are expected to show better growth rates than Pietrain (Whittemore, 1998). This result thus supports the idea of a significant impact of PCV2-SD on average daily gain, and is consistent with the improvement of growth rate in pigs vaccinated against PCV2 (Fachinger, et al., 2008; Horlen et al., 2008; Kixmöller et al., 2008).

The link reported here (farm-2) between sow parity and PCV2-SD-associated mortality, and also reported in another study (Armstrong and Bishop, 2004), seems to be a controversial point since others did not find it (Madec et al., 2000; Calsamiglia et al., 2007). In fact, piglets from multiparous sows showed higher total PWM on both farms, but it was only higher for PCV2-SD-PWM on farm-2. There are no apparent consistent explanations for these discrepancies and they may be attributable to the multifactorial nature of PCV2-SD, in which unknown triggering factors may account for specific farm differences.

Several studies have shown that humoral immunity plays a major role on PCV2-SD development. PCV2 maternally derived antibodies seem to protect against piglet mortality based on field (Allan et al., 2002b; Calsamiglia et al., 2007; Grau-Roma et al., 2009) and experimental (Allan et al., 2002b; McKeown et al., 2005; Ostanello et al.,

2005) data. However, no effect of sow IPMA antibody titre at farrowing or piglet titre at 3 weeks of age in PCV2-SD-PWM were observed in the present study, probably because of the very high percentage of piglets coming from sows with medium to high PCV2 antibody titres. Curiously, piglets from A boars experienced lower total PWM and PCV2-SD-PWM despite showing the lowest proportion of piglets with medium to high PCV2 antibody titres in farm-1 and the highest in farm-2. Again, PCV2 antibody titres in sows and piglets should be considered as elements that modulate PCV2-SD expression within the context of a multifactorial disease. Therefore, not all the risk factors so far identified for PCV2-SD have an equal on different farms with different production scenarios.

PCV2-SD-PWM records in the present study were very similar between females and males, being 4.3 and 4.4%, respectively. Previous studies have found that castrated males had higher PCV2-SD-associated mortality than females (Corrégé et al., 2001; Rodríguez-Arrioja et al., 2002). In the present study males were not castrated, suggesting that physiological and behavioural changes or surgical complications due to male castration (Prunier et al., 2006) may be a risk factor for PCV2-SD occurrence.

Susceptibility of lighter weanling pigs to clinically compatible PCV2-SD has been reported (Corrégé et al., 2001). This association between BW and PCV2-SD has not been observed in the present study, although disease has been measured with different parameters in both studies.

This study represents a consistent observation of the genetic background effect on PCV2-SD clinical expression under field conditions. However, such study cannot be used to determine if the observed effect

was an effect of breed (such as a protective effect for Pietrain or susceptibility in Duroc), particular boar lines or individuals (only 4 boars from genetic line C were used in the study, so the effect of each individual was higher than in the other genetic lines). Furthermore, it cannot be concluded that this effect would appear if a different sow genetic line was used. Further studies are needed to elucidate the exact role by which porcine genetics affect susceptibility/resistance to PCV2-SD.

CHAPTER 6

STUDY IV:

Effect of porcine circovirus type 2 (PCV2) load in serum on average daily weight gain during the postweaning period

6.1 Introduction

Porcine circovirus type 2 has been aetiologically linked to several pathological conditions collectively named porcine circovirus diseases (PCVDs) (Segalés, 2012). Among PCVDs, PCV2-SD and PCV2-SI are those associated to major economic losses for pig producers.

It has been widely reported that PCV2-SD affected pigs harbour and excrete higher PCV2 loads than non-affected ones (Olvera et al., 2004; Segalés et al., 2005b). Furthermore, since it is a multifactorial disease, numerous infectious and non-infectious factors have been associated with disease triggering. Some of these factors are related to PCV2 infection, such as higher mortality in piglets from PCV2 viraemic sows and from sows with low antibody titres (Calsamiglia et al., 2007) or higher probability of PCV2-SD in piglets infected with the genotype “b” of the virus than the genotype “a” (Tomás et al., 2008). Other factors are associated with a higher risk of disease in presence of concomitant infections such as PRRSV (Rovira et al., 2002) and PPV (Allan et al., 1999). Facilities and management practices also play a role in PCV2-SD development as it has been observed after applying a set of measures known as the “Madec’s 20 point plan” (Madec et al., 2000) and those that have been reported in multiple epidemiological studies (Cottrell et al., 1999; Rose et al., 2003a, Elbers et al., 2006; Andraud et al., 2009) as well as in Study II of the present PhD thesis. Finally, factors related with the animals such as castration in males, lower weight at birth, weaning or beginning of fattening (Corrégé et al., 2001; Rodríguez-Arrioja et al., 2002) or genetics (Opriessnig et al., 2006b, 2009c; Study III of the present PhD thesis) may contribute to disease expression as well.

The relevance of PCV2-SI has been recently highlighted since it is the most common situation in pig farms. Despite pigs with PCV2-SI show no evidence of clinical signs, they harbour low systemic loads of PCV2 and such condition seems to be related with a decrease of average daily weight gain (ADWG) (Segalés, 2012). Its impact on ADWG has been evidenced through the use of vaccines in PCV2-SD non-affected farms (Kurmann et al., 2011; Fraile et al., 2012a). In fact, vaccines against PCV2 have demonstrated reduction of the percentage of infected animals and viral load as well as excellent results on productive parameter improvement in all production stages (Kixmöller et al., 2008; Segalés et al., 2009; Pejsak et al., 2010; Fraile et al., 2012a; Pejsak et al., 2012; Heißenberger et al., 2013).

The objective of the present study was to determine the effect of PCV2 load in serum of non-vaccinated pigs on ADWG during the weaning to slaughter period. Furthermore, other factors that may influence ADWG and PCV2-SD expression were also assessed.

6.2 Material and methods

Animal care and use conformed to the European Union guidelines.

The study design of the present work is summarized below and further information can be found in Study III of the present PhD thesis where a larger pig population of this field study was used.

6.2.1 Animal selection

The study was carried out with 172 piglets from two almost identical 5000-sow farms (1 and 2) separated by 300 m. Farms 1 and 2 had a multi-site production system and used the same sow genetic line (37.5% Large White x 37.5% Duroc x 25% Landrace). Farms had a previous diagnosis of PCV2-SD, and were seronegative against PRRSV and ADV, and no evidence of PPV circulation was found. Furthermore, these farms did not apply any PCV2 vaccination schedule since such vaccines were not available in the market at the moment of the study.

Three different genetic boar lines, PI (100% Pietrain), PIxLW (50% Large White x 50% Pietrain) and DUxLW (75% Duroc x 25% Large White), were used for artificial insemination. Sows were randomly placed in the farrowing facilities in both farms taking into account that approximately one third of sows inseminated with each boar line had to be present in each room (there were three rooms per farm) and no cross-fostering was allowed. Serum samples from sows were collected 1 week before farrowing.

One or two piglets per sow (Farm 1=63 sows; Farm 2=67) were followed up, obtaining a total of 207 piglets; however, only those that arrived to slaughter age were selected for the present study. Therefore, 58 and 59 sows from Farms 1 and 2, respectively, and 172 piglets were finally included in the study (Farm 1: 29 piglets from each boar line; Farm 2: 31, 32 and 22 piglets from boar lines PI, PIxLW and DUxLW, respectively). Pigs from both origin farms (1 and 2) were placed in the same nursery and fattening farms. Piglets from all three genetic lines

were intermingled in all nursery and fattening pens. Piglets were bled at 3, 9, 15 and 21 weeks of age and weighted at 3 and 21 weeks of age.

6.2.2 Laboratory analyses

6.2.2.1 Immunoperoxidase monolayer assay

PCV2 MDA in sera from 3 week-old piglets were measured using an IPMA (Rodríguez-Arrioja et al., 2000). Antibody titres were classified as negative or low titre (<1:320) or medium to high titre (≥1:320) (Rodríguez-Arrioja et al., 2000).

6.2.2.2 Quantitative real time PCR

PCV2 viral load in sera from sows and piglets was measured at all sampling points. This quantification was performed in an up to two steps procedure. Firstly, a standard PCR technique to detect PCV2 (Quintana et al., 2002) was performed. Subsequently, only those sera that tested positive to PCR were further analysed by means of quantitative real-time PCR (qPCR); this latter technique had a limit of detection (LOD) of 10^4 PCV2 genome copies/mL (Olvera et al., 2004).

6.2.3 Statistical analyses

6.2.3.1 Calculation of the area under the curve of PCV2 load

A new variable was constructed in order to measure the PCV2 load experienced by each pig from weaning to slaughter in terms of duration

and quantity. Therefore, the values of this variable were the area under the curve (AUC) drawn by the viral load results at 3, 9, 15 and 21 weeks of age for each animal calculated following the trapezoidal rule. This variable was named AUCqPCR 3-21.

The differentiation among PCVD clinical conditions (mainly PCV2-SD and PCV2-SI), besides lymphoid lesion severity, resides on the systemic PCV2 load (Olvera et al., 2004). In fact, previous studies have reported qPCR thresholds to discriminate between these situations (Segalés, 2012). All together, these data indicate that the gradient of PCV2 amount in serum of pigs can vary substantially within a batch of production, including also non-infected animals. Therefore, in order to represent these different viral loads and their potential effect on ADWG, three categories of pigs regarding viral load were created following these two criteria: a) animals negative to PCR or positive to PCR but negative to qPCR at all sampling times were included in the first category, named “negative or low” AUCqPCR 3-21, and would include non-infected pigs and animals with very low amount of PCV2 in serum (lower than the LOD of the qPCR), and b) taking into account that the analysis of ADWG requires a given number of animals and it was relatively limited in the present study, the 50% of the cases not included in the first category were included in each of the two remaining categories in order to maximize their sample size and, therefore, obtaining the maximum affordable statistical power; these two categories were named “medium” and “high” AUCqPCR 3-21.

For AUCqPCR 3-21 calculation, the method described by Croghan and Egeghy (2003) for imputing values to results below the LOD of a technique was used in those cases with positive results to standard PCR

but negative to qPCR; therefore, LOD/$\sqrt{2}$ imputation was used in these cases (i.e. 7071 PCV2 genome copies/ml since LOD of the qPCR is 10^4 as stated above).

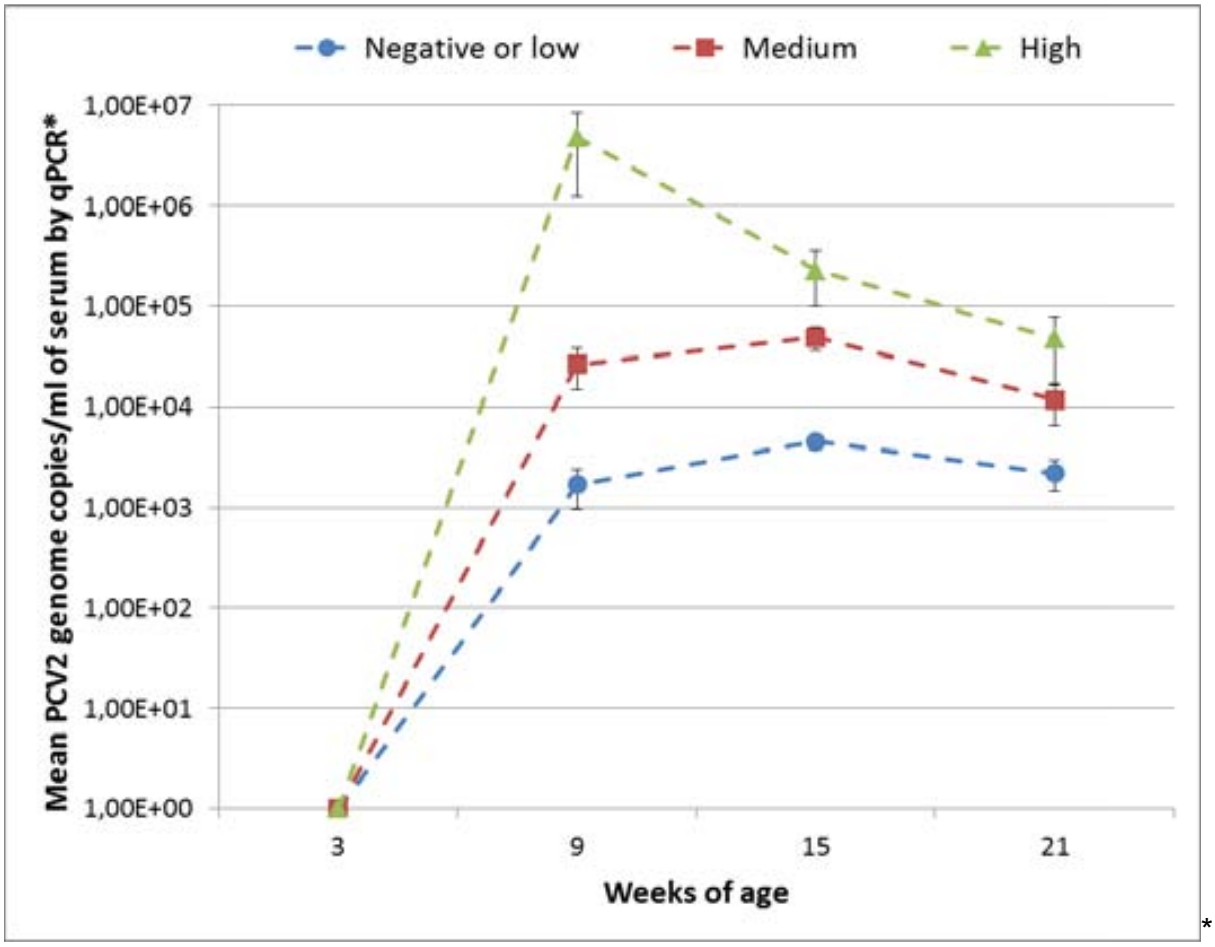
6.2.3.2 Statistical model construction

All data analyses were carried out using SAS 9.2. (Cary, NC, US). Candidate predictor variables to be included in the statistical model were: farm, sex, paternal genetic line, IPMA titre at 3 weeks of age, maternal parity and AUCqPCR 3-21. Initially, a univariable analysis was performed by means of Mann-Whitney U or Kruskal Wallis tests where each predictor variable was included as a single fixed factor of prediction. Variables that had P<0.25 were selected to build the multivariable model (Dohoo et al., 1996). Before entering the variables into a multivariable model, bivariate Pearson´s and Spearman´s correlations were performed among all independent variables in order to avoid multicollinearity problems between variables. Dummy variables were created for those variables with more than 2 categories. The model was built using a manual backward generalized linear model procedure using ADWG 3-21 as the dependent variable and including all possible two way interactions; all factors with a P<0.05 were retained in the final model. After fitting the model, both normality and homoscedasticity of the residuals were evaluated by plotting the standardized residuals versus the predicted values and by plotting a Q-Q plot of the residuals.

6.3 Results

None of the studied animals were viraemic at one week before farrowing (sows) or 3 weeks of age (piglets). Cut-off points for AUCqPCR 3-21 categories were: "negative or low" = $<10^{4.3}$ PCV2 genome copies/ml of serum; "medium" = $\geq 10^{4.3}$ to $\leq 10^{5.3}$; and "high" = $>10^{5.3}$. Descriptive statistics for the studied variables are shown in Table 14. Evolution of PCV2 load in serum at each sampling time is shown in Figure 3 for each category of the variable AUCqPCR 3-21.

Figure 3. Dynamics of PCV2 load in serum (mean ± confidence interval) by AUCqPCR categories.



*Viral load calculated by means of a real-time quantitative PCR (qPCR)
AUCqPCR 3-21: area under the curve of real time quantitative PCR at 3, 9, 15 and 21 weeks of age (Negative or low: negative or $<10^{4.3}$ PCV2 genome copies/ml; Medium: $\geq 10^{4.3}$ to $\leq 10^{5.3}$; High: $>10^{5.3}$).

Table 14. Descriptive statistics of explanatory and response variables studied in piglets included in the study.

Variable	N (%)	Mean	SD
Piglets by farm of origin:			
Farm 1	87 (51%)	-	-
Farm 2	85 (49%)	-	-
Piglets by paternal genetic line:			
PI	60 (35%)	-	-
PIxLW	61 (35%)	-	-
DUxLW	51 (30%)	-	-
Piglets by gender:			
Male	92 (53%)	-	-
Female	80 (47%)	-	-
Piglets by their maternal parity:			
Primiparous	45 (26%)	-	-
Multiparous	127 (74%)	-	-
Piglets by PCV2 IPMA antibody titres at 3 weeks of age:			
Negative or low (<1:320)	109 (63%)	-	-
Medium to high (≥1:320)	63 (37%)	-	-
Piglets by AUCqPCR 3-21 (PCV2 genome copies/ml serum):			
Negative or low (negative or $<10^{4.3}$)	72 (42%)	-	-
Medium ($\geq 10^{4.3}$ to $\leq 10^{5.3}$)	50 (29%)	-	-
High ($>10^{5.3}$)	50 (29%)	-	-
ADWG from 3 to 21 weeks of age (g/day)	172 (100%)	645	±94

SD: Standard deviation; PCV2: Porcine circovirus type 2; IPMA: immunoperoxidase monolayer assay; AUCqPCR 3-21: area under the curve of real time quantitative PCR at 3, 9, 15 and 21 weeks of age; ADWG: average daily weight gain.

Two variables (sex and sow parity) were not included in the multivariable model because they had a P>0.25 in the univariable analysis. No collinearity (r<0.60) was detected between variables entered in the model. Only two variables remained significant after the manual backward generalized linear model procedure, being ADWG 3-21 explained by AUCqPCR 3-21 (P<0.001) and the paternal genetic line (P=0.001). Interaction between these two variables was not statistically significant (P=0.332). The estimation of the parameters of the model is shown in Table 15.

Table 15. Parameters estimation of the generalized linear model with ADWG 3-21 as response variable.

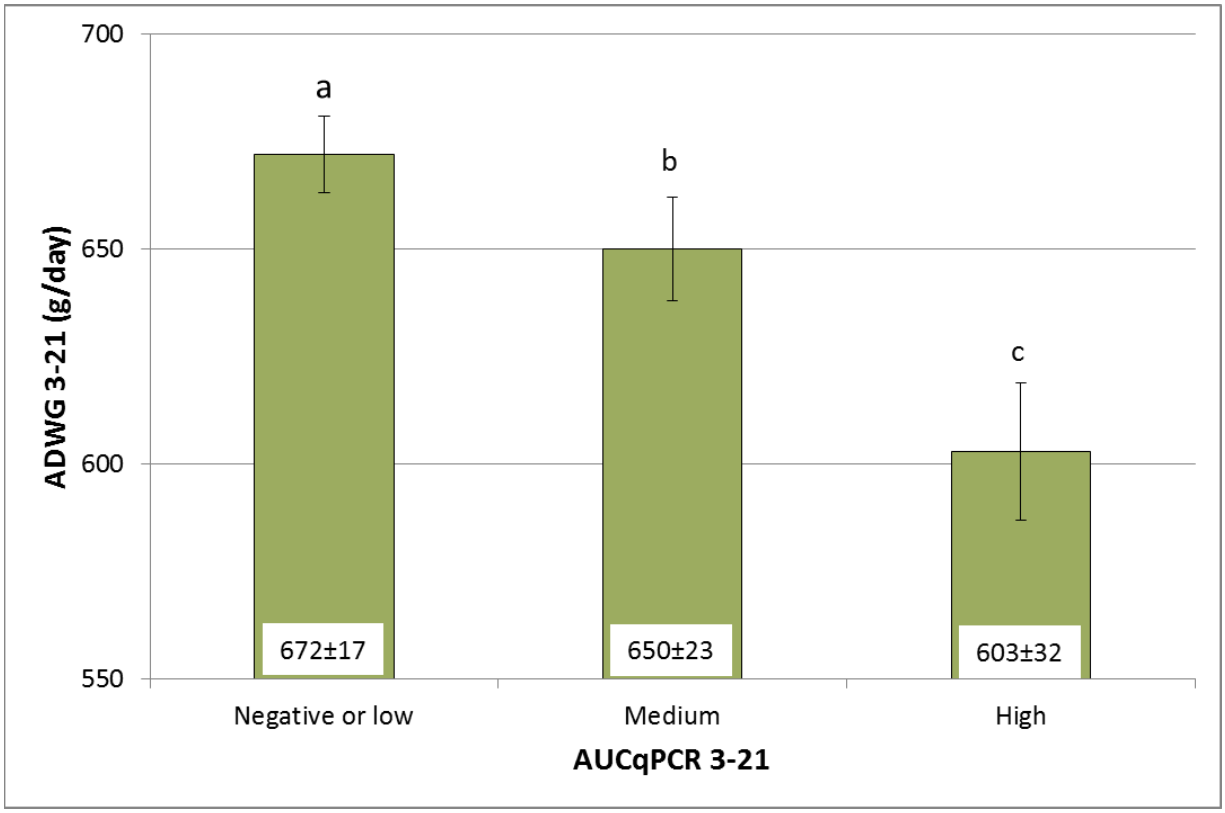
Parameter	Estimate	Standard Error	Significance*
Intercept	589.6	14.5	<0.001
Paternal genetic line:			
PI	45.3	17.0	0.009
PIxLW	0	.	.
DUxLW	13.3	17.9	0.458
AUCqPCR 3-21 (PCV2 genome copies/ml serum):			
$<10^{4.3}$	76.5	17.4	<0.001
$\geq 10^{4.3}$ to $\leq 10^{5.3}$	43.6	18.0	0.017
$>10^{5.3}$	0	.	

*Fixed at P<0.05

ADWG 3-21: average daily weight gain from 3 to 21 weeks of age

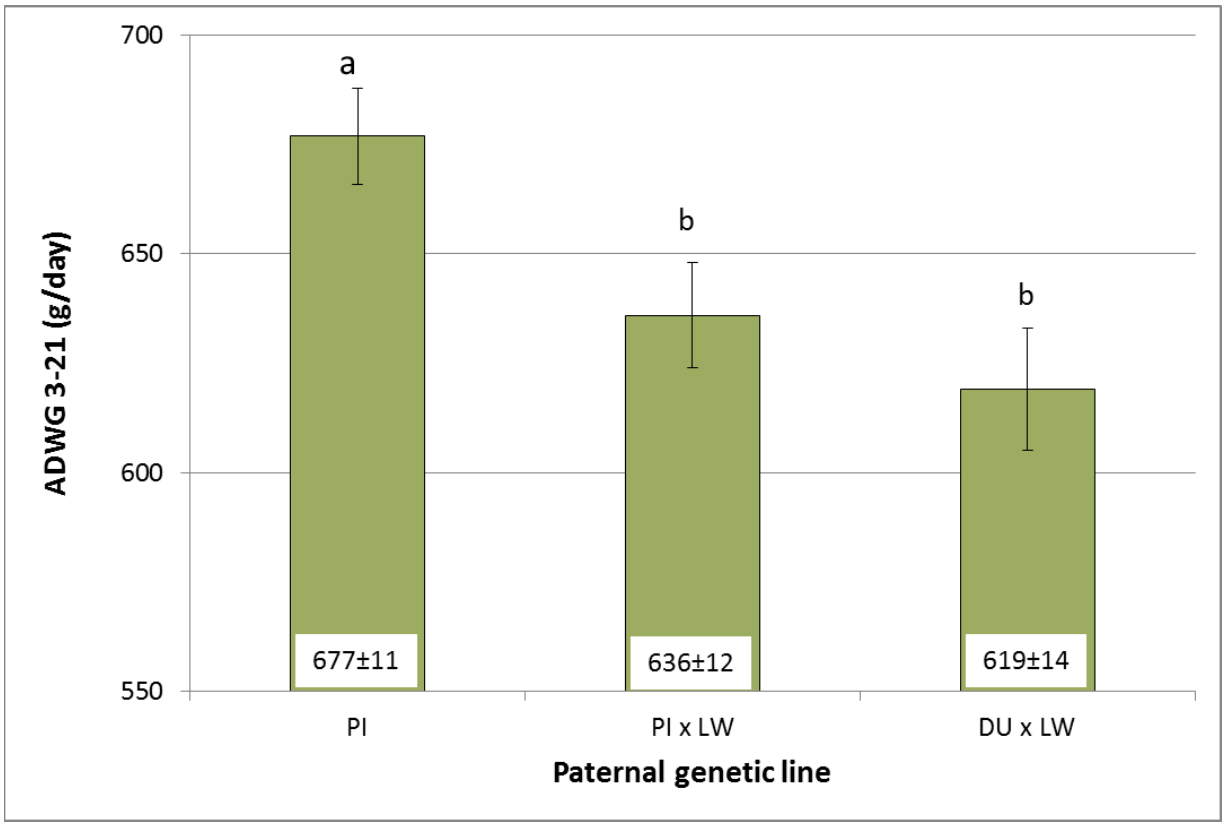
Results on ADWG 3-21 for piglets with different AUCqPCR 3-21 categories are shown in Figure 4. Piglets with negative or low AUCqPCR 3-21 values experienced higher ADWG 3-21 than those with medium (P=0.045) and high (P<0.001) ones; furthermore, piglets with medium AUCqPCR 3-21 values obtained higher ADWG 3-21 than animals with high ones (P=0.014). On the other hand, ADWG 3-21 results for PI, PIxLW and DUxLW are shown in Figure 5. Piglets from PI boar line showed higher ADWG 3-21 than piglets from PIxLW (P<0.001) and DUxLW (P=0.006) boar lines; however, no differences were observed between piglets with PIxLW and DUxLW paternal origin.

Figure 4. ADWG 3-21 records (mean ± typical error) by AUCqPCR 3-21 categories.



"a", "b" and "c" indicate statistical differences between groups with different letters (P<0.05)
AUCqPCR 3-21: area under the curve of real time quantitative PCR at 3, 9, 15 and 21 weeks of age (Negative or low: negative or $<10^{4.3}$ PCV2 genome copies/ml; Medium: $\geq 10^{4.3}$ to $\leq 10^{5.3}$; High: $>10^{5.3}$).
ADWG 3-21: average daily weight gain from 3 to 21 weeks of age

Figure 5. ADWG 3-21 records (mean ± typical error) by paternal genetic background.



"a" and "b" indicate statistical differences between groups with different letters (P<0.05)
ADWG 3-21: average daily weight gain from 3 to 21 weeks of age

6.4 Discussion

The present study was conducted in a batch of animals that suffered from PCV2-SD, where 6.5% mortality was reported and the disease was diagnosed following classical pathologic and viral detection criteria (Segalés, 2012) in 66% of dead animals (data shown in Study III of the present PhD thesis). This work aimed to assess whether the PCV2 systemic infection, in terms of viraemia length and amount, produced a load-dependent effect on ADWG. Animals that died during the study were not included in the present study since the impact of PCV2 is clear in those pigs with a post-mortem PCV2-SD diagnosis, and insufficient

valuable data on viral load and ADWG was available in the remaining dead ones. Therefore, only animals that survived until 21 weeks of age were selected, being a population of animals mainly composed by PCV2-SI affected ones.

A new approach to measure how PCV2 infection may affect piglets' growth has been successfully used in the present study, using the AUC of the viral load experienced by animals during its productive life. This method takes into account duration and load of the PCV2 infection using the AUC of the viral load experienced by animals during its productive life. However, the measure of viral load as an estimator of PCVD has been previously used in many studies. In fact, one of the criteria that must be fulfilled for PCV2-SD individual diagnosis is presence of a moderate to high amount of PCV2 in lymphoid lesions (Segalés, 2012). Furthermore, the interest on using pooled serum samples to quantify viral loads in PCV2-SD affected farms has been raised (Cortey et al., 2011b). The studies that have explored the use of qPCR as a predictor of PCV2-SD have reported thresholds of qPCR in serum indicative of PCV2-SD diagnosis ranging from $10^{4.7}$ to $10^{7.43}$ viral copies/mL, existing an inter-laboratory bias due to variations of techniques (Segalés, 2012). In our laboratory, thresholds ranging from $10^{6.21}$ to 10^{7} or higher have been found as discriminatory of PCV2-SD (Olvera et al., 2004; Segalés et al., 2005b; Segalés, 2012). From a practical point of view, animals with qPCR values higher than these thresholds for PCV2-SD diagnosis would correspond to the "high" AUCqPCR 3-21 category of the present study. However, it is difficult to affirm if specific pigs under study suffered only PCV2-SI or PCV2-SD during their productive life since: a) PCV2 load was assessed every 6 weeks, so a peak of viral load could occur in-

between, and more importantly b) there is not a reliable *in vivo* technique for individual PCV2-SD diagnosis (Grau-Roma et al., 2009).

Nevertheless, besides if specific studied animals suffered from PCV2-SD or PCV2-SI, the relevance of the obtained results resides in the existence of different ADWG 3-21 among the three studied AUCqPCR 3-21 ranges. Furthermore, the coefficient of variation (CV) of ADWG 3-21 increased with the level of PCV2 load suffered by piglets, being of 11, 13 and 19% in “negative or low”, “medium” and “high” AUCqPCR 3-21, respectively. This situation would explain the effect of PCV2 vaccination, where there is a reduction of the prevalence of infected animals and their viral load and improvement of ADWG and production of more homogeneous batches (Kixmöller et al., 2008; Segalés et al., 2009; Pejsak et al., 2010), even in farms without PCV2-SD (Kurmann et al., 2011; Fraile et al., 2012a).

On the other hand, different ADWG for different genetic lines is something that should be expected since they have different growth rates and conformations. In this sense, piglets coming from PI boars experienced a higher growth than piglets from PIxLW and DUxLW. However, it is interesting to note that higher ADWG 3-21 scores should be *a priori* expected for Duroc and Large White breeds than Pietrain (Whittemore, 1998). The existence of genetic susceptibility on PCV2-SD could have an influence on these better results for piglets from PI boars, since lower mortality and higher body weight dynamics than PIxLW and DUxLW have been already observed in Study III of the present PhD thesis using a higher population of animals. Furthermore, an experimental study also reported that PI pigs developed less severe PCV2-SD-like lesions than Landrace ones (Opriessnig et al., 2009c).

Finally, the following studied variables did not significantly influence ADWG 3-21 in the present study: farm, sex, PCV2 IPMA antibody titres at 3 weeks of age and sow parity. A higher ADWG was expected in males than females; however it was not observed in this study. In fact ADWG 3-21 was very similar between them, showing a mean±standard deviation of 645±109 and 646±75 g/day in males and females, respectively. Regarding PCV2 antibodies, it has been shown that they can protect against PCV2 infection and can be titre-dependent protective against PCV2-SD expression (McKeown et al., 2005). However, PCV2 antibody titre at 3 weeks of age was neither significant, most probably because the real effect on ADWG 3-21 is not related to MDA but to PCV2 viraemia. It must be indicated that these results are in controversy with those from Harding et al. (2011), which suggested that MDA, not viraemia, was associated with ADWG. Finally, the lack of farm and sow parity effects on ADWG 3-21 is not surprising, since there were similar conditions in Farms 1 and 2 and sow parity is a general variable where multiple factors are merged, so it would not necessarily have an effect on ADWG.

The amount of PCV2 in serum experienced by a pig from weaning to slaughter age in PCV2-SD affected farms had a load dependent effect on ADWG under the conditions of the present study, concluding that the higher the PCV2 load the lower the ADWG.

CHAPTER 7

GENERAL DISCUSSION

It has been estimated that circoviruses are present in nature since 500 years ago and PCV2 has been originated within approximately the last 100 years (Firth et al., 2009). The fact that PCV2 possesses a long persistence in the host, a high resistance in the environment and an easy transmissibility, together with the increased trade of animals and their products by the international commerce, have facilitated PCV2 to become a ubiquitous virus for probably more than 50 years (Grau-Roma et al., 2011; Rose et al., 2012). Despite this situation, the impact of PCVDs in the pig industry is a relatively recent event, where the first known PCV2-SD case was retrospectively dated in 1985 (Jacobsen et al., 2009), while the PCV2-SD main epidemic outbreaks in a global scenario occurred between 1998 and 2007.

At the beginning of this PhD Thesis, in the year 2002, there were lots of missing pieces in the puzzle of PCV2 and PCVDs. In fact, one of the most controversial matters was the causality of PCV2-SD (named initially as PMWS). At that moment two currents of thought coexisted; the one that linked the effects of this new disease to PCV2 and the other that considered that the true cause of the problem was an unknown cause named “agent X“ (Rose et al., 2012). This situation was motivated by the ubiquitous nature of PCV2 despite only some farms experienced the clinical disease.

The studies included in the present PhD Thesis have tried to bring some light to this significant question. In this sense, the first study consisted in a large epidemiological study that aimed to assess the prevalence and correlation of PCV2 and four other viruses (PRRSV, PPV, SIV and ADV) in different pig populations (sows, piglets and boars) of the Spanish swine livestock. The obtained results revealed PCV2

serological ubiquity in sows, fattening pigs and boars, in accordance with the minimal studies performed in our territory (Segalés et al., 2003a,b; Sibila et al., 2004). To our knowledge, this study was the most complete one on this topic in our country, and provided an update on the epidemiological situation not only of PCV2, but also of PRRSV, PPV, SIV and ADV. It is true, however, that since then the epidemiological picture of some of these pathogens may have changed significantly. Special mention should be paid to ADV, since the number of regions free of this virus has steadily increased due to the eradication program in Spain (RD 360/2009).

Once it was corroborated the ubiquitous presence of PCV2, the reason why certain farms experienced clinical outcome and not others remained still unknown. This situation led to categorize PCV2-SD as a multifactorial disease from the very beginning. Therefore, the second study aimed to assess the risk factors that triggered the clinical disease. For this purpose a case-control study was performed in Spanish pig farms. This study was performed in collaboration with the *Agence Française de Securite Sanitaire des Aliments* (AFSSA, nowadays ANSES), which also performed a similar study in France (Rose et al., 2003a). A total of 211 variables including facilities, management, vaccination schedules, biosecurity and serological information were included in this study. The most relevant conclusion obtained was that farms where animals become infected earlier with PCV2 had a higher risk of developing PCV2-SD than farms experiencing a later infection. In fact, both studies (the one performed in France and the one presented in this PhD Thesis) coincided that the percentage of seropositive fattening pigs against PCV2 was higher in those farms with PCV2-SD.

For the third study, it was planned to focus on a specific risk factor. The risk factor to be assessed was selected by convenience following the pig producing sector suspicion that the pig genetic background had an effect on PCV2-SD expression. This objective was approached through a prospective field study. Such work indicated that piglets coming from Pietrain boars were less susceptible to PCV2-SD, followed by piglets coming from Large White x Pietrain boars and, finally, with the worst results, those piglets coming from Large White x Duroc boars. The results obtained from this study had an important impact in the pig industry, since some companies, not only from Spain but also from abroad, included Pietrain breed in the commercial pig farms.

As more information on PCV2 was obtained by the scientific community, the virus was associated with a longer list of pathological conditions which were collectively named as PCVDs (Segalés, 2012). The two main PCVDs in terms of prevalence and economic impact are PCV2-SD and PCV2-SI, both of them affecting pigs during the nursery and fattening ages. One of the common manifestations of these two diseases is the reduction of the ADWG. Therefore, the fourth and last study aimed to assess the effect of serum PCV2 load from weaning to slaughter age on ADWG in two farms affected by PCV2-SD. This study was performed retrospectively using data collected from the third work presented in this PhD Thesis. The most interesting result from this study consisted of finding three populations of pigs with different serum PCV2 load during their productive life that experienced different ADWG. Specifically, the estimated ADWG was 76.5 and 43.6 g/day lower for pigs with “high” and “medium” serum PCV2 loads, respectively, than for those with “negative or low” viral load in serum. This scenario would imply a mean loss of 13.1 € and 7.5 €/pig at 21 weeks of age for pigs with “high”

and “medium” serum PCV2 load, respectively, compared to those with “negative or low” load (assuming a price of 1.361 €/Kg live pig and a postweaning period of 18 weeks, http://www.3tres3.com/cotizaciones-de-porcino/espana-lleida_2, visited on May 18th, 2014). Furthermore, the coefficient of variation (CV) of ADWG from weaning to slaughter increased with the PCV2 load in serum suffered by piglets, being of 11, 13 and 19% in “negative or low”, “medium” and “high” load, respectively. Besides the cost of growth retardation and heterogeneity, other extra losses not evaluated in the present study could also apply due to mortality, use of veterinary medicines, higher feed consumption, longer wean to slaughter period and batch heterogeneity (higher number of shipments to slaughterhouse and/or slaughterhouse penalization). These results support the idea of a gradual clinical (and economical) effect of PCV2 in pig populations, what would explain the benefits of PCV2 vaccination even in farms without PCV2-SD, but suffering from PCV2-SI (Kurmann et al., 2011; Fraile et al., 2012a).

As a whole, all the published knowledge on PCV2 from the first description of PCV2-SD in 1996 until today, including the herein presented studies, have contributed to change the current perception about this virus. In fact, one of the most revolutionary events has been the appearance of vaccines against PCV2, not only for their excellent results to counteract PCV2-SD (Kixmöller et al., 2008; Segalés et al., 2009; Pejsak et al., 2010; Martelli et al., 2011), but also for dissipating all doubts about the causality role of PCV2 in this, initially, “mysterious” disease. Furthermore, the fact that vaccination increased the global ADWG and homogenised the weight of the pig batches at slaughter, evidenced the effect of PCV2 in apparently normal pigs. Such scenario revealed the existence of a not yet known pathological situation at the

beginning of PCV2 vaccines use, the PCV2-SI. Nowadays, PCV2 is linked to a relevant number of pathological conditions (Segalés at al., 2012), where the most relevant ones are PCV2-SD and PCV2-SI.

However, the pathogenesis of PCVD is still a not completely solved puzzle. In this sense, the complexity of PCV2-SD becomes evident when Koch's postulates are not consistently demonstrated. Nowadays, it is known that the occurrence of PCVDs in a pig population may depend on a number of factors, such as the virus genotype, the transmission route, the viral load, the moment of infection, the presence of certain co-infections, the management, and the host and its immune system (Rose et al., 2012). Additionally, this high number of factors may even have multiple connections among them. Nevertheless, up to date, infection with PCV2b, early infection dynamics, low antibody titres to PCV2 at the time of infection and co-infection with PRRSV, PPV and/or *Micoplasma hyopneumoniae* are considered the most relevant factors related to PCV2-SD development, as it has been pointed out in Study II of the present PhD Thesis and other scientific studies (Rose et al., 2003a, Tomás et al., 2008, Rose et al., 2012).

All the factors involved in PCV2-SD development may be summarised in three groups, corresponding to the elements of the epidemiological triad: factors associated to the virus, to the animals and to the environment. Furthermore, from a practical point of view, the environmental factors can be categorised by their effects on the exposure to PCV2, the infection pressure and the immune system response. A list of PCV2-SD triggering / protective factors for a given farm is shown in Figure 6 following these classification criteria. It must be clarified that this figure includes a collection of results obtained from

different studies, where in some cases there is not *a priori* biological association and could be just spurious effects.

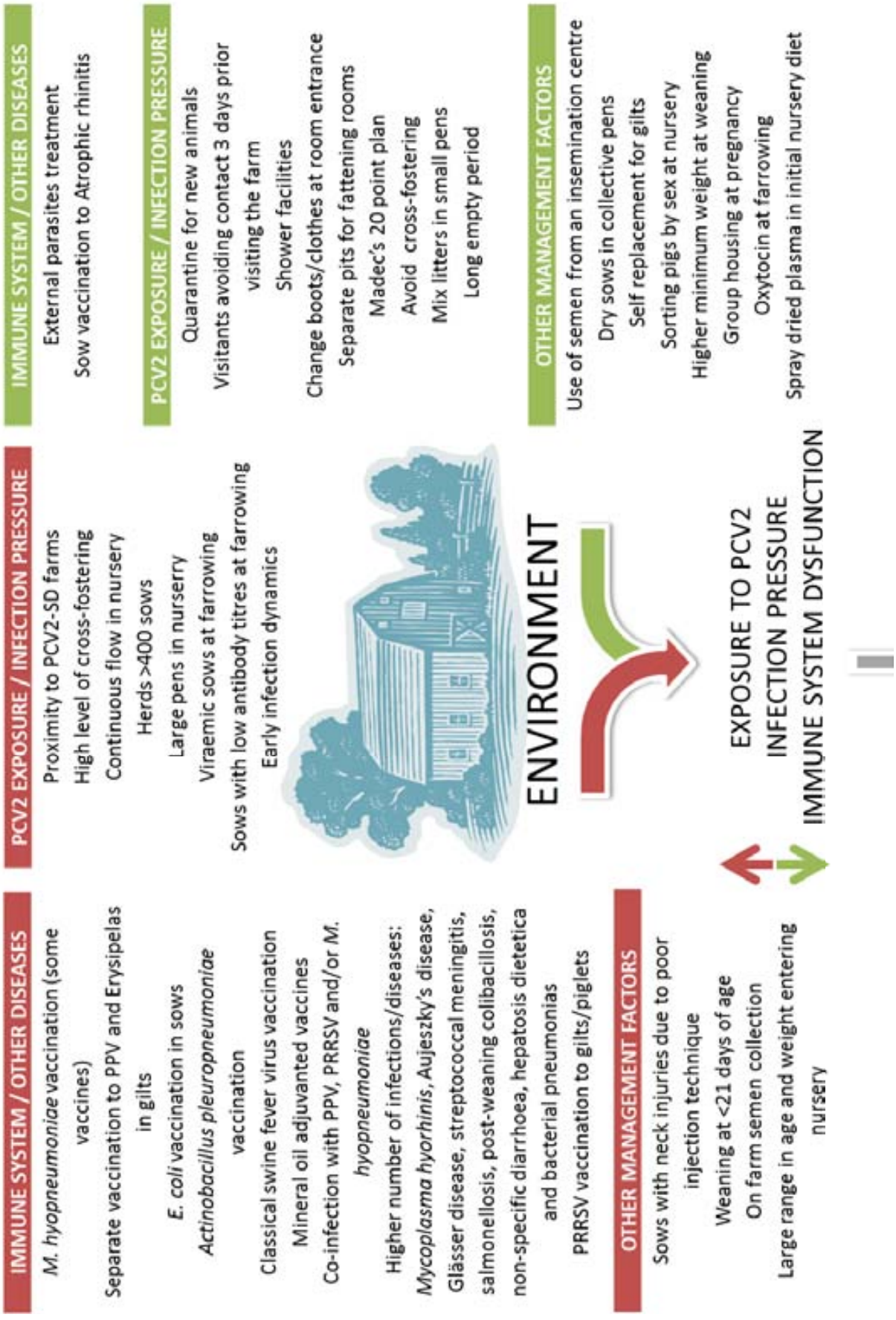
Therefore, in a farm affected by PCV2-SD it is likely to find, at the same time, the following "type" of pigs regarding PCV2 infection (Segalés, 2012; Alarcón et al., 2013b):

- pigs that suffer from PCV2-SD (compatible clinical picture, existence of moderate to severe lymphoid microscopic lesions and detection of moderate to high amount of PCV2 genome within these lesions).
- pigs with PCV2-SI (a pig systemically infected with a low load of PCV2 that shows, none or mild lymphoid lesions and no apparent clinical signs, but has growth retardation).
- non-PCVD affected pigs (a pig that may be infected or not with PCV2 but looks healthy and shows normal growth).

However, it is not possible to assess the proportion of each type of pigs in a farm through individual analysis since PCV2-SD is diagnosed post-mortem (Sorden, 2000; Segalés, 2002). Moreover, a pig with low PCV2 load measured in serum by qPCR could be a PCV2-SI, but the interpretation of this scenario is not easy. Such pig can also represent an animal before PCV2-SD expression or a chronically, convalescent PCV2-SD affected pig. Moreover, it cannot be ruled out that a pig with low PCV2 load and displaying clinical signs of wasting can also be infected subclinically by PCV2 but affected clinically by another condition. However, a PCV2-SD diagnosis approach at a population level can be done by measuring PCV2 load in serum pools (Cortey et al., 2011b), clinical evaluation and diagnosis of other concomitant diseases at the moment of clinical outbreak.

How the infection by PCV2 is driven towards developing its associated diseases in a certain pig population and the severity of affectation might be explained by the “population pathogenesis” concept. Figure 7 summarizes the different elements that can affect PCV2 transmission in a given farm, including the risk factors that may play a role on the expression of different PCVDs. In this sense, a recent study aimed to classify farms by their PCV2-SD severity affectation (Alarcón et al., 2011). Farms were classified as none or slightly, moderately or highly affected by PCV2-SD according to a score ranging from 0 to 10. This score takes into account morbidity in nursery and grower phases, post-weaning mortality and percentage of PCV2 PCR positive animals. The same classification has been used to assess the cost of PCV2-SD and PCV2-SI (Alarcón et al., 2013a) as well as the benefits of PCV2 vaccination (Velasova et al., 2013) and certain control measures (Alarcón et al., 2013b) on these two diseases. As these authors suggested, this farm classification method could be also useful to assess risk factors of PCV2-SD and PCV2-SI.

Figure 6. PCV2-SD triggering (red) / protective (green) factors that can be



present in a farm classified by each element of the epidemiological triad.

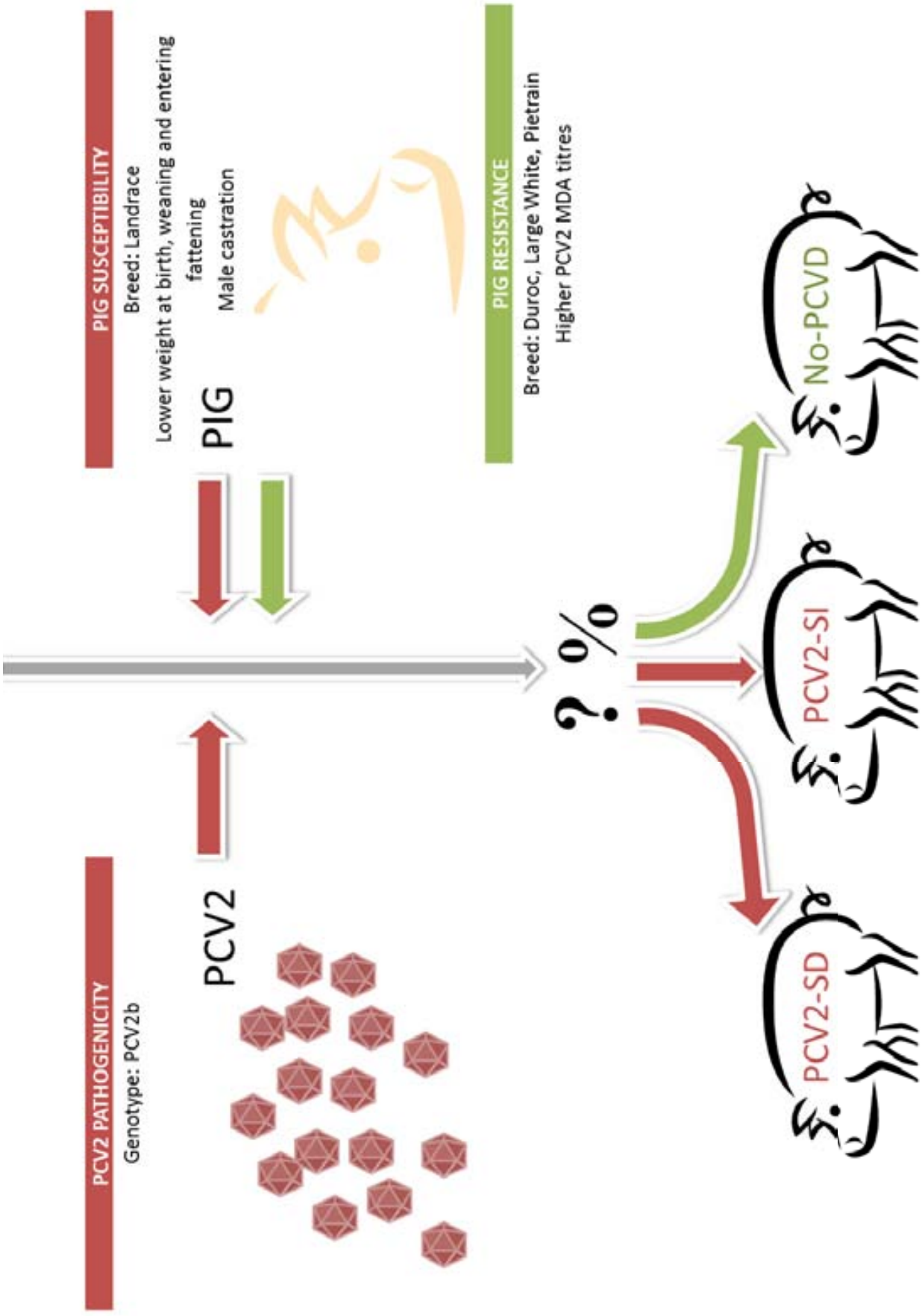
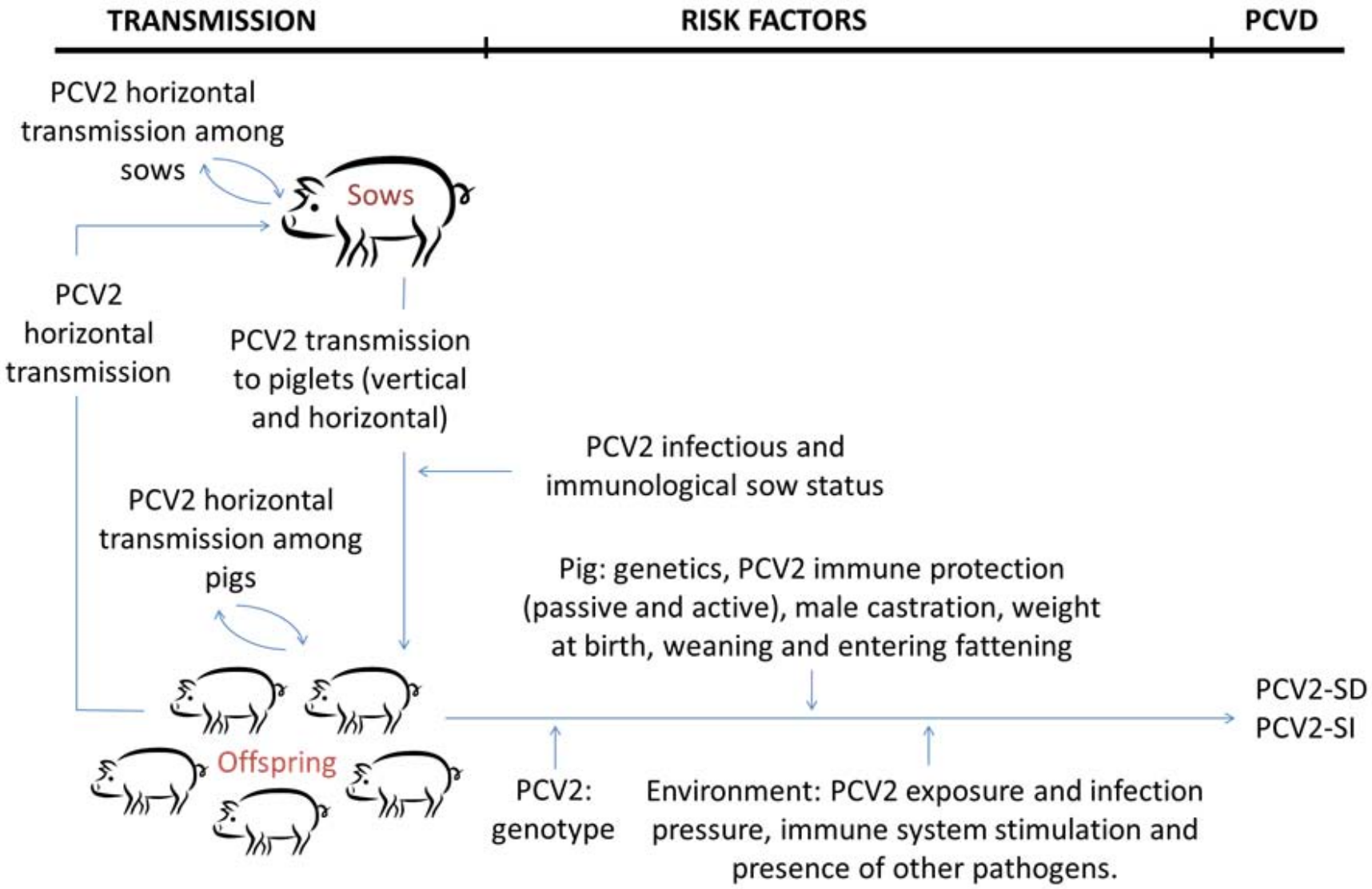


Figure 7. PCV2 transmission in a pig farm and risk factors that may play a role on PCV2-SD and PCV2-SI expression.



CHAPTER 8

CONCLUSIONS

1. In the early-mid 2000s in Spain, PCV2 and PPV showed evidence of ubiquitous distribution in pigs; PRRSV and SIV were also widespread. Seroprevalence against ADV wild virus decreased over time. Boar studs had lower seroprevalences than sow and fattening herds.

2. Early infection by PCV2, measured by evidence of seroconversion in a case-control study, is a predisposing factor for PCV2-SD occurrence.

3. The genetic background is a risk factor for PCV2-SD development. Piglets from pure Pietrain boars showed the best clinical performance followed by piglets from Large White x Pietrain boars. Piglets from Large White x Duroc boars were the most affected by PCV2-SD.

4. ADWG variation among pigs in PCV2-SD affected farms is partly explained by serum PCV2 load from weaning to slaughter age. Three subpopulations of pigs with different serum PCV2 loads from weaning to slaughter age have been identified. These subpopulations experienced significantly different ADWG, in which the higher the PCV2 load the lower the ADWG.

CHAPTER 9

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