Universitat Autònoma de Barcelona

New insights into the epidemiology of postweaning multisystemic wasting syndrome (PMWS)

Tesi doctoral presentada per Llorenç Grau i Roma 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 (UAB), sota la direcció del Dr. Joaquim Segalés i Coma i del Dr. Lorenzo José Fraile Sauce.

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FACULTAT DE VETERINÀRIA DE LA UAB

JOAQUIM SEGALÉS I COMA, professor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona (UAB), i LORENZO JOSÉ FRAILE SAUCE, investigador del Centre de Recerca en Sanitat Animal (CReSA)

Certifiquen:

Que la memòria titulada "New insights into the epidemiology of postweaning multisystemic wasting syndrome (PMWS)", presentada per Llorenç Grau i Roma per l'obtenció del grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, n'autoritzen la presentació per tal de ser jutjada per la comissió corresponent.

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Dr. Joaquim Segalés i Coma

Dr. Lorenzo José Fraile Sauce

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Havia dit tantes vegades que mai faria una tesi doctoral que la primera cosa que voldria agrair, a la tesi mateixa, és que m'hagi fet adonar que hauria d'anar més en compte alhora d'utilitzar aquest adverbi.

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Tot el que hi ha aquí escrit és molt important per mi i forma part del meu petit món. Però no hi ha res que ho sigui tant com aquells que m'hi van portar: el meu pare i la meva mare. Ells m'ho han donat tot, a mi i als meus germans. Ells s'han volgut estar de moltes coses per tal de fer-nos cada dia una mica millors i de veure'ns feliços. Ells tenen doncs una bona part de la culpa de què aquesta tesi s'hagi pogut començar i acabar. Ja em donaria per satisfet si jo sabés fer de fill la meitat de bé que ells han fet i fan de pares.

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hi dieu tots, hi tornem? Si un cas però, jo, aquesta vegada, m'ho miraria una mica més de lluny. Podria fer de tiet, o de cosí, o cuidar-me del càtering...

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Summary

Postweaning multisystemic wasting syndrome (PMWS) is considered a multifactorial pig disease in which *Porcine circovirus* type 2 (PCV2) is the essential infectious agent. PMWS affects nursery and growing pigs, and causes severe economic losses to the swine industry worldwide. The most representative clinical sign is wasting, and affected pigs usually show a poor response to therapeutic treatments. Due to the ubiquitous nature of PCV2, PMWS diagnosis is one of the most complex diagnoses within the currently known animal diseases. Thus, the individual PMWS diagnosis is established when the suspected pig fulfils the following three conditions: (i) presence of compatible clinical signs; (ii) presence of moderate to severe characteristic microscopic lymphoid lesions; (iii) detection of moderate to high amount of PCV2 within these lesions. The lack of an effective and consistent experimental model to reproduce PMWS makes especially relevant to carry out epidemiological studies within PMWS affected farms. The present thesis aimed to expand the epidemiological knowledge on PCV2 infection and PMWS through the realization of case-control field studies. Mainly, the potential influence of PCV2 genetics, the timing of PCV2 infection, the PCV2 maternal derived humoral immunity and the pig humoral response against PCV2 infection in PMWS presentation were investigated.

In the first study (Study I) 87 open reading frame 2 (ORF2) PCV2 sequences obtained from pigs with different clinical and pathological conditions were analyzed together with 148 PCV2 sequences available at the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) in September 2005. Results further confirmed the existence of two main genogroups and the definition of two PCV2 genotypes (1 and 2) was proposed. The suggested methodology to define PCV2 genotypes is based on the p-distance/frequency distribution of PCV2 sequences together with PCV2 phylogenetic analyses. Genotype 1 was shown to be predominant within pigs coming from PMWS affected farms. Moreover, all

sequences included in genotype 1 came from pigs from PMWS affected farms, while all sequences obtained from non-PMWS affected farms corresponded to genotype 2. Consequently, it was suggested that PCV2 genotype 1 might potentially be more pathogenic than PCV2 genotype 2. In addition, infection of single pigs from PMWS affected farms harbouring both genotypes at the same time was described.

The present thesis was developed within the European Union (EU) project entitled *Control of Porcine Circovirus Diseases (PCVD): Towards Improved Food Quality and Safety* (www.pcvd.org), which was funded by the EU Sixth Framework Programme. Few months after the acceptation of the manuscript presented in the study I, the EU consortium on PCVD (www.pcvd.org) discussed and agreed in supporting the definition of PCV2 genotypes based on the proposed methodology. However, it was considered that the designation of genotype 1 and 2 could cause confusion with the current nomenclature. Thus, the mentioned consortium published the letter here presented as an *addendum* of study I. In this letter it was proposed the nomenclature of PCV2 genotype a (PCV2a) and genotype b (PCV2b), corresponding to genotypes 2 and 1, respectively. In addition, following this definition, a third PCV2 genotype was retrospectively detected in Denmark, being initially denominated PCV2 genotype 3 and subsequently renamed as PCV2 genotype c (PCV2c) in the mentioned letter.

In the second study (Study II) two different real-time quantitative PCR (qPCR) assays were compared on DNA extracted from serum and nasal as well as rectal swabs. The two compared techniques were used routinely in two laboratories from two different countries, Denmark and Spain, both members of the above-mentioned EU consortium. This comparison was made in order to be able to interpret together the work performed in both countries, which is mainly presented in studies III and IV. Results from study II showed a significant linear association between the assays, and a systematic difference of 1.4 $\log_{10}$ copies of

PCV2 per millilitre of sample. This difference indicated that the assay from the Danish laboratory yielded a higher output than the assay from the Spanish laboratory. Moreover, the Danish assay had higher sensitivity than the Spanish one. Results also showed that there was no linear association between the amount of PCV2 DNA and the amount of total DNA, neither in nasal nor in rectal swabs, suggesting that normaliziation of PCV2 DNA load in swab samples to total DNA concentration is not suitable to express the amount of PCV2 present in swab samples.

In studies III and IV, longitudinal case–control studies were performed in PMWS affected farms from Denmark and Spain using similar designs. Fourteen independent batches of 100–154 pigs per batch were monitored from birth to PMWS outbreak occurrence. Pigs displaying PMWS-like signs and matched healthy cohorts were euthanized during the clinical outbreak. PMWS was diagnosed according to internationally accepted criteria and pigs were classified as: (i) PMWS cases, (ii) wasted non-PMWS cases and (iii) healthy pigs. Similar PCV2 infection dynamic patterns were observed in Spain and Denmark, with a delay in PMWS age-presentation in Spain compared to the one in Denmark. Thus, all Spanish PMWS outbreaks occurred at the fattening phase, whereas all Danish clinical outbreaks were observed at nurseries. PMWS diagnoses were confirmed by laboratorial tests in only half of pigs clinically suspected to suffer from PMWS. PCV2 qPCR and serological techniques were applied to analyse longitudinally collected sera. Moreover, PCV2 qPCR was also applied to nasal and rectal swabs. Results from these determinations were presented in study III. Moreover, the evolution of two acute phase proteins (APPs), pig-major acute phase protein (pig-MAP) and haptoglobin (HPT), in serum from studied pigs was also assessed. The corresponding results were presented in study IV. Overall, results showed that PCV2 load increased concomitantly to maternal antibody level waning, reaching the maximum viral load concurrently with the development of clinical signs. Interestingly, the acute phase response (APR) in PMWS affected pigs occurred in parallel to PCV2 viremia, suggesting that PCV2

is the main responsible for the systemic inflammatory status suffered by diseased pigs. As a collective, PMWS affected pigs harboured higher PCV2 loads and higher Pig-MAP and HPT concentrations in sera, shed higher viral loads through both nasal secretions and faeces, and had lower level of maternal antibodies against PCV2 than non-PMWS affected pigs. Moreover, in Spain, PMWS affected pigs showed also higher APR and higher PCV2 prevalence than non-affected ones at the sampling prior to PMWS outbreak. Besides, in Denmark, PMWS affected pigs at the sampling prior to PMWS outbreak showed higher PCV2 loads than non-affected ones. Furthermore, an impaired humoral response was observed in PMWS affected pigs from Spain at 11 weeks of age (prior to the appearence of clinical signs) and at the moment of necropsy, suggesting that this circumstance might be more a cause rather than a consequence of the disease. On the other hand, the lack of sensitivity and/or specificity observed from qPCR and/or serological techniques suggests that those techniques are not able to substitute histopathology plus detection of PCV2 in tissues for the individual PMWS diagnosis. However, results indicated that qPCR might potentially be a reliable technique to diagnose PMWS on a population basis. Additionally, obtained results supported the idea that although APPs are unspecific markers of inflammation, they might be useful indicators of health, becoming a potentially interesting tool to monitor PMWS development in epidemiological studies or in the assessment of the efficacy of PCV2 vaccines in the field.

Resum

La síndrome d'aprimament post-deslletament (SAPD) és considerada com una malaltia porcina d'origen multifactorial en la qual el *Circovirus porcí* tipus 2 (CP2) és l'agent infecciós essencial. La SAPD afecta porcs en fases de transició i engreix, i causa greus pèrdues econòmiques a la indústria porcina arreu del món. El símptoma clínic més representatiu és l'aprimament, i els porcs afectats normalment mostren una pobra resposta als tractaments terapèutics. Com a conseqüència del caràcter ubic del CP2, la SAPD és, d'entre les malalties animals conegudes, una de les patologies amb el diagnòstic més complex. Així, el diagnòstic individual de la SAPD es realitza quan, en un animal sospitós, es compleixen les tres condicions següents: (i) presència de símptomes clínics compatibles; (ii) presència de les lesions microscòpiques característiques en òrgans limfoides en grau de moderat a intens; (iii) detecció de quantitats moderades a elevades de CP2 en aquestes lesions. L'objectiu de la present tesi doctoral era expandir el coneixement en l'epidemiologia de la infecció per CP2 i el SAPD a través de la realització d'estudis de cas-control a nivell de camp. De manera general, es va investigar la potencial influència de la genètica del CP2, el moment d'infecció del CP2, els anticossos contra CP2 derivats de la immunitat maternal i la resposta humoral dels porcs contra el CP2, en la presentació de la SAPD.

En el primer estudi (estudi I) 87 seqüències del marc obert de lectura 2 (MOL2) de CP2 obtingudes de porcs amb diferent condició clínica i patològica van ser analitzades conjuntament amb 148 seqüències de CP2 que estaven disponibles a la base de dades nucleotídica del NCBI (http://www.ncbi.nlm.nih.gov) el setembre del 2005. Els resultats van confirmar l'existència de dos genogrups principals i es va proposar la definició de dos genotipus de CP2 (1 i 2). La metodologia suggerida per definir els genotipus està basada en la distribució de la relació p-distància/freqüència de les seqüències de CP2 conjuntament amb anàlisis filogenètics de CP2. Es va observar que el genotipus 1 era predominant

en porcs de granges afectades per SAPD. A més, totes les seqüències incloses en el genotipus 1 provenien de porcs de granges afectades per la SAPD, mentre que totes les seqüències obtingudes de granges no afectades per la SAPD corresponien al genotipus 2. Conseqüentment, es va suggerir que el genotipus 1 de CP2 podria ser potencialment més patogènic que el genotipus 2. Addicionalment, es va descriure la presència dels dos genotipus en el mateix moment en porcs individuals procedents de granges afectades per la SAPD.

La present tesi doctoral es va desenvolupar dins del marc del projecte de la Unió Europea (UE) titulat Control de les malalties associades al Circovirus porcí (MACP): Cap a la Millora en la Qualitat i Seguretat Alimentàries (www.pcvd.org), la qual era finançada pel 6è programa marc de la UE. Pocs mesos després de l'acceptació de l'article presentat en l'estudi I, el consorci de la UE per les MACP (www.pcvd.org) va discutir i acordar donar suport a la definició de genotipus de CP2 basada en la metodologia proposada. Malgrat això, es va considerar que la designació dels termes genotipus 1 i 2 podria causar confusió amb l'actual nomenclatura. D'aquesta manera, el mencionat consorci va publicar una carta presentada com a *addendum* de l'estudi I. En aquesta carta es va proposar la nomenclatura de genotipus a de CP2 (CP2a) i genotipus b (CP2b), corresponent als genotipus 2 i 1, respectivament. A més, seguint aquesta definició, un tercer genotipus de CP2 va ésser detectat retrospectivament a Dinamarca, essent inicialment denominat genotipus 3 i subseqüentment reanomenat genotipus c de CP2 (CP2c) a través de la carta esmentada.

En el segon estudi (estudi II) es van comparar dues tècniques de PCR quantitativa (qPCR) de PCV2 utilitzant ADN extret de sèrum, hisops nasals i hisops rectals. Les dues tècniques comparades eres utilitzades de manera rutinària en dos laboratoris de dos països diferents, Dinamarca i Espanya, ambdós membres del mencionat consorci europeu. Aquesta comparació es va fer per tal de poder interpretar conjuntament el treball realitzat en ambdós països, el qual es presenta majoritàriament en els estudis III i IV. Els resultats de l'estudi II

mostraven una associació lineal significativa entre les dues tècniques, i un biaix sistemàtic de 1.4 $\log_{10}$ copies of CP2 per mil·lilitre de mostra. Aquesta diferència indicava que la tècnica del laboratori danès generava un resultat sistemàticament més elevat que el generat a través de la tècnica del laboratori espanyol. A més, la tècnica del laboratori danès mostrava major sensibilitat que la tècnica del laboratori espanyol. Els resultats també van mostrar que no hi havia cap associació lineal entre la quantitat d'ADN de CP2 i la quantitat total d'ADN, ni en hisops nassal així com tampoc en hisops rectals, suggerint que la normalització de la quantitat d'ADN de CP2 a través de la quantitat total d'ADN no és adequada per expressar la quantitat de CP2 present en mostres d'hisops.

En els treballs III i IV es van realitzar estudis longitudinals de tipus cas-control en granges afectades per la SAPD de Dinamarca i Espanya tot utilitzant dissenys similars. Catorze lots independents de 100-154 porcs per lot van ser monitoritzats des del seu naixement fins el moment d'aparició de la SAPD. En aquest moment, es va realitzar l'eutanàsia de porcs que mostraven símptomes clínics compatibles amb la SAPD així com d'animals sans. El SAPD va ser diagnosticat atenent els criteris acceptats internacionalment i els porcs van ser classificats com: (i) casos de SAPD, (ii) casos amb retard en el creixement sense SAPD i (iii) porcs sans. Es van observar patrons similars en la dinàmica d'infecció per CP2 tant a Espanya com a Dinamarca, amb un retard en l'edat de presentació de la SAPD a Espanya en comparació a Dinamarca. Així, mentre que a Espanya tots els brots de SAPD es van produir a la fase d'engreix, tots els brots observats a Dinamarca es van produir a la fase de transició. El diagnòstic individual de la SAPD es va confirmar, mitjançant tests laboratorials, en només la meitat dels porcs en els quals hi havia la sospita clínica. Les mostres de sèrum recollides longitudinalment van ser analitzades mitjançant tècniques de serologia i qPCR. Aquesta darrera tècnica es va aplicar també per analitzar les mostres d'hisops nasals i rectals. Els resultats d'aquestes determinacions es van presentar en l'estudi III. A més, també es va determinar l'evolució en sèrum de les concentracions de dues proteïnes de fase aguda (PFA), la proteïna major de fase

aguda del porc (porc-MFA) i l'haptoglobina (HPT). Aquests resultats es van presentar en l'estudi IV. Globalment, els resultats van mostrar que la quantitat de CP2 incrementava concomitantment a la caiguda dels nivells d'anticossos maternals, assolint valors màxims de càrrega vírica en el moment d'aparició dels símptomes clínics. De manera interessant, la reacció de fase aguda (RFA) en porcs afectats per la SAPD s'observava paral·lelament a l'evolució de la virèmia per CP2, suggerint que el CP2 és el principal responsable de l'estat d'inflamació sistèmica que pateixen els porcs afectats per aquesta malaltia. Col·lectivament, els porcs afectats tenien quantitats de CP2 i concentracions de porc-MFA i HPT superiors en sang, excretaven càrregues víriques superiors tant per via nasal com a través de les femtes, i tenien nivells inferiors d'anticossos maternals enfront al CP2 que els porcs que no estaven afectats per la SAPD. A més, en el cas d'Espanya, els porcs afectats per la SAPD mostraven, també en el mostreig anterior al brot de SAPD, una major RFA i una més elevada prevalença de CP2 que els porcs no afectats. Per la seva banda, a Dinamarca, els porcs afectats per la SAPD mostraven, en el mostreig anterior al brot clínic de SAPD, major quantitat de CP2 que els animals no afectats. Addicionalment, es va observar una menor resposta humoral en els porcs afectats per la SAPD provinents d'Espanya a les 11 setmanes de vida (abans de l'aparició dels símptomes clínics) i en el moment de la necròpsia, suggerint que aquesta circumstància era podria associar-se com a causa més que no pas una conseqüència de la malaltia. D'altra banda, la falta de sensibilitat i/o especificitat observades de les tècniques de qPCR i/o de serologia suggereixen que aquestes tècniques no poden substituir la histopatologia més la detecció de CP2 en teixits per l'establiment del diagnòstic individual de la SAPD. Malgrat això, els resultats indicaven que la qPCR podria ser potencialment útil per diagnosticar la SAPD a nivell poblacional. Addicionalment, els resultats obtinguts donaven suport a la idea que, malgrat que les PFA són marcadors inespecífics d'inflamació, aquestes proteïnes podrien ser marcadors útils de salut, esdevenint una eina potencialment útil per monitoritzar el desenvolupament de la SAPD en estudis epidemiològics o per la valoració de l'eficàcia de vacunes de CP2 a nivell de camp.

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List of abbreviations

ATCC:	American Type Culture Collection
CAV:	*Chicken anemia virus*
CT:	congenital tremor
CSFV:	*Classical swine fever virus*
DC:	dentritic cells
dsDNA:	double stranded DNA
ELISA:	enzime-linked immunosorbent assay
GM-CSF:	granulocyte-macrophage colony-stimulating factor
ICTV:	International Committee on Taxonomy of Viruses
IFN:	interferon
IHC:	immunohistochemistry
IL:	interleukin
IFN-γ-SC:	interferon-γ-secreting cells
IPMA:	immunoperoxidase monolayer assay
ISH:	*in situ* hybridisation
Kb:	kilobases
KLH/ICFA:	keyhole limpet hemocyanin in incomplete Freund's adjuvant
LPS:	lipopolysaccharide
mRNA:	messenger RNA
NA:	neutralizing antibodies
NIPC:	natural interferon producing cells
ORF:	open reading frame
PBMC:	peripheral blood mononuclear cells
PCR:	polymerase chain reaction
PCVD:	porcine circovirus diseases
PCVAD:	porcine circovirus associated diseases
PCV1:	*Porcine circovirus* type 1
PCV2:	*Porcine circovirus* type 2
PCV2a:	*Porcine circovirus* type 2 genotype a

PCV2b: *Porcine circovirus* type 2 genotype b

PK-15: porcine kidney-15

PMWS: postweaning multisystemic wasting syndrome

PNP: proliferative and necrotizing pneumonia

PPV: *Porcine parvovirus*

PRDC: porcine respiratory disease complex

PRRSV: *Porcine reproductive and respiratory syndrome virus*

qPCR: quantitative PCR

RF: replicative form

ssDNA: single-stranded DNA

TCID50: tissue culture infectious dose 50%

TNF: tumor necrosis factor

TTV: *Torque teno virus*

I. INTRODUCTION

The beginning is the most important part of the work
Platón

1. HISTORICAL BACKGROUND

In 1974, a new virus was described in Germany as a non-cytopathogenic, picornavirus-like contaminant of the porcine kidney cell line PK-15 (ATCC-CCL-33) (Tischer et al., 1974). Later on, this virus was demonstrated to be a small icosahedral, naked virus, containing a single-stranded (ss), circular DNA genome, and the name *Porcine circovirus* (PCV) was proposed (Tischer et al., 1982). Serological surveys showed that antibodies against PCV were present in high percentages of pigs in Germany (Tischer et al., 1986), Canada (Dulac and Afshar, 1989), Great Britain (Edwards and Sands, 1994) and Northern Ireland (Allan et al., 1994), leading to the conclusion that it was ubiquitous in nature. PCV was considered to be non-pathogenic since it was not able to produce disease in conventional pigs infected under experimental conditions (Tischer et al., 1986; Allan et al., 1995).

In 1991 firstly, and afterwards in 1994, a new mysterious syndrome was described in Saskatchewan and Alberta (Canada) by Dr. Edward Clark and Dr. John Harding (Harding, 1996; Clark, 1997). Affected pigs were characterized by suffering from wasting, respiratory distress, skin pallor and icterus, and the mortality was about 12-15%. Gross lesions consisted of generalized lymphadenopathy in combination with interstitial pneumonia, hepatitis, renomegaly, splenomegaly, gastric ulcers and intestinal wall oedema. Microscopic lesions were mainly lymphocyte depletion and granulomatous infiltration in lymphoid tissues (Harding and Clark, 1997). Due to the affectation of several organs and the age of incidence, the disease was named postweaning multisystemic wasting syndrome (PMWS) (Harding, 1996, 1998; Clark, 1997; Harding and Clark, 1997). The fact that those initially affected farms were free from *Porcine reproductive and respiratory syndrome virus* (PRRSV) infection and from most of known respiratory and enteric pathogens, suggested that it was not caused by a known pathogen. Few months after the initial description, a variant of PCV was demonstrated to be present in abundance within the lesions

observed in lymphoid tissues of a 6-week-old affected pig from California (USA) (Daft et al., 1996). Afterwards, the virus was isolated for the first time from PMWS affected pigs from North America and Canada in 1998 (Allan et al., 1998; Ellis et al., 1998). Phylogenetic and serologic analysis showed that this virus was genetically and antigenically distinct of the previously described PCV (Meehan et al., 1998; Morozov et al., 1998), and it was proposed to use the name PCV type 1 (PCV1) for the PK-15 cell-culture contaminant virus and PCV type 2 (PCV2) for the virus isolated from PMWS affected pigs (Meehan et al., 1998; Allan et al., 1999b). PCV1 and PCV2 are currently recognized by the International Committee on Taxonomy of Viruses (ICTV) as two different species within the genus *circovirus* (www.ictvonline.org).

Shortly after the first descriptions of the disease in Canada and USA, a similar syndrome was reported in Brittany (France), which was called *"Maladie de l'Amaigreissement du Porcelet"* (LeCann et al., 1997). Afterwards, the disease was also described in Spain (Segalés et al., 1997). Today, PMWS has been reported in almost all pig producing countries, showing a worldwide distribution, and it has been estimated to cause significant economic losses to the pig industry (Armstrong and Bishop, 2004).

PMWS is considered an emerging disease that severally hit the pig population from 1990s onwards. However, both PCV2 and PMWS were already present within the swine livestock many years before the first description of the disease. Thus, retrospective serologic polymerase chain reaction (PCR) studies have demonstrated evidences of PCV2 infection in stored sera or tissues from 1962 in Europe (Jacobsen et al., 2009), 1973 in America (Ramírez-Mendoza et al., 2009) and 1989 in Asia (Mori et al., 2000). Besides, characteristic histopathological lesions together with the detection of abundant PCV2 antigen have been demonstrated in paraffin embedded lymphoid tissues taken in 1985 in Germany (Jacobsen et al., 2009), 1986 in Spain and United Kindom (Rodríguez-Arrioja et al., 2003b; Grierson et al., 2004) and in 1989 in Japan (Mori et al., 2000).

2. PORCINE CIRCOVIRUSES (PCVs)

2.1. Taxonomy

Both PCV1 and PCV2 belong to the C*ircoviridae* family, which is one out of the 64 virus families that, according to the ICTV, is not assigned to an order. Viruses belonging to the *Circoviridae* family have small, naked, icosahedral shaped virions, and contain covalently closed, circular, ssDNA genome, which range in size from 1.8 to 2.3 kb (Lukert et al., 1995).

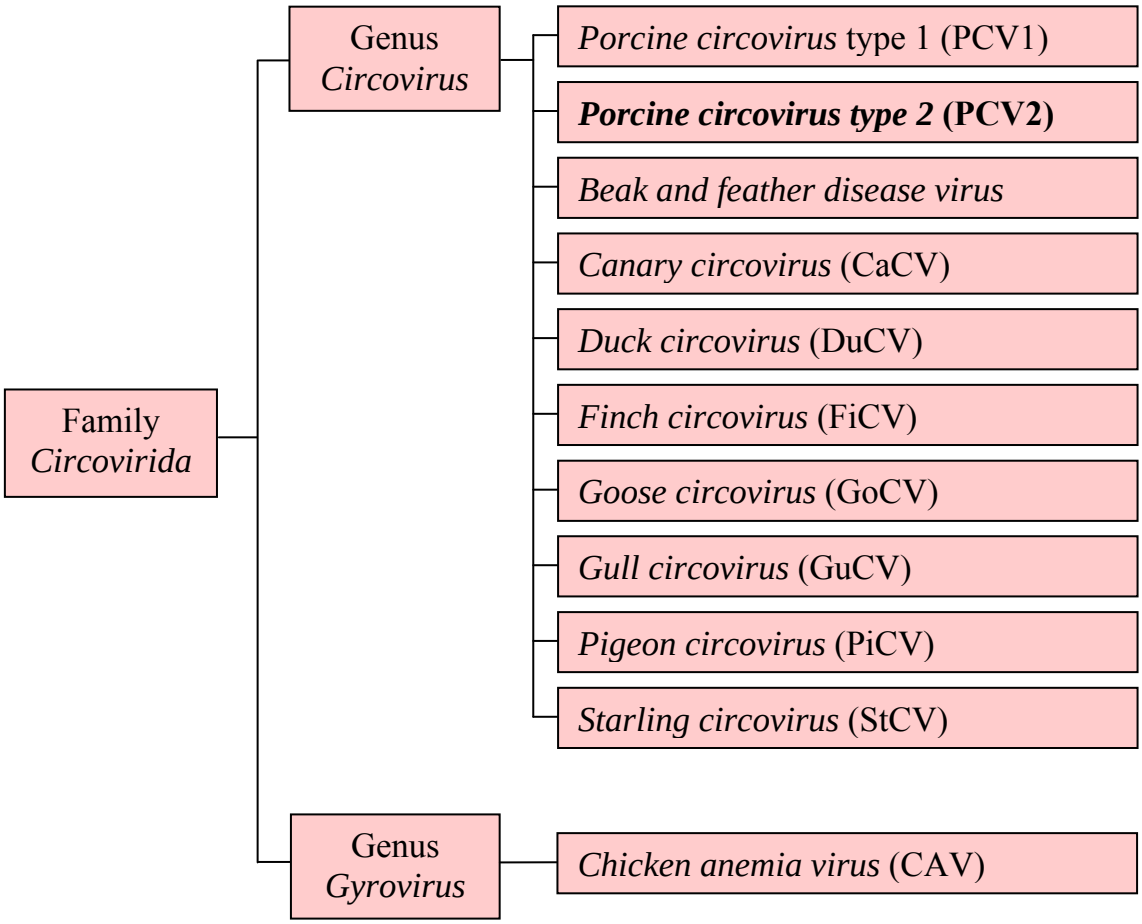


Figure 1. Genera and animal species included within the *Circoviridae* family (www.ictvonline.org).

The classification of the viruses within the *Circoviridae* family have suffered many modifications since its creation, and probably it will suffer more changes in the next future since newly described circoviruses or diseases associated to circovirus-like agents are continuously being reported (Todd, 2004; Davidson

and Silva, 2008). Today, the *Circoviridae* family is divided into two genera on the basis of virion size and genomic organization: the genus *Circovirus* (*Circo* indicates that the viral genome has circular DNA) and the genus *Gyrovirus* (*Gyro* is a derivation from the Greek work *gyrus*, meaning "ring" or "circuit", thus also highlighting the DNA conformation). Circoviruses are host-specific or exhibit a narrow host-range. Genera *Circovirus* contains nowadays 10 different species, infecting several birds species and swine (figure 1). Although it was also suggested the existence of a *Bovine circovirus* infecting cattle (Nayar et al., 1999), later studies did not confirm this finding (Allan et al., 2000c; Ellis et al., 2001). C*hicken anemia virus* (CAV) is the only species included in the genera *Gyrovirus*. CAV shares similar characteristics with two viruses that infect humans, swine and other mammals: *Torque teno virus* (TTV) and *TTV-like mini virus* (TLMV) (Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 2002; Hino and Miyata, 2007).

Circoviruses share genome and replication cycle similarities with members of two families of plant viruses: *Geminiviridae* and *Nanoviridae* (Gronenborn, 2004). Their genome similarities are mainly located in the origin of virus replication and in the genes encoding for replicases. It has been suggested that a predecessor of PCV1 may have originated from a plant *Nanovirus* that infected a vertebrate host and recombined with a vertebrate-infecting RNA virus, most likely a C*alicivirus* (Gibbs and Weiller, 1999; Davidson and Silva, 2008).

2.2. Morphology and genomic organisation

Both PCV1 and PCV2 have identical virion size and morphology. They are simply constituted by a single circular strand of DNA, which is surrounded by a protein-based capside. The capside exhibits icosahedral symmetry, has no envelope, and measures around 17 ± 1.3 nm in diameter (Tischer et al., 1982) (figure 2).

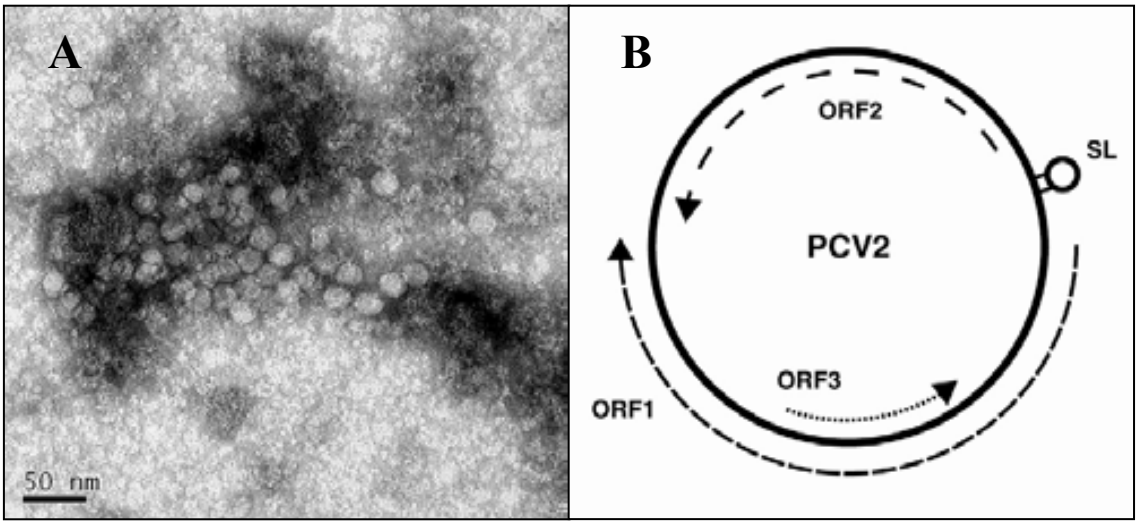


Figure 2. (A) PCV2 viral particles from a supernatant of a cellular culture. Image obtained by negative stain on electron microscopy (kindly provided by Carolina Rodríguez, CReSA). (B) Schematic representation of PCV genome showing the position and the transcription sense of the three open reading frames (ORF) (arrows). The stem-loop (SL) region encompassing the nonanucleotide motif is also shown. Adapted from Meehan et al. (1998).

PCV1 and PCV2 share about 68-76% nucleotide sequence identity and are 1,758-1,760 (Tischer et al., 1982; Fenaux et al., 2004b) and 1,767-1,768 (Hamel et al., 1998; Meehan et al., 1998; Mankertz et al., 2000) nucleotides in length, respectively. There are several small insertions and deletions located throughout their genomes that have suggested to account for the differences in pathogenicity between both viruses (Hamel et al., 1998; Meehan et al., 1998). The ssDNA is ambisense, meaning that it encodes for proteins in both senses. Potentially, up to 11 open reading frames (ORFs) have been suggested for the PCV1 and PCV2 genomes (Hamel et al., 1998; Meehan et al., 1998; Morozov et al., 1998). However, only ORF1 and ORF2 seem to be expressed in both PCV1 and PCV2. The ORF1 of both viruses has around 940 nucleotides in length, is located in the plus strand and encodes two essential initiator replicase proteins: rep and rep' (Mankertz et al., 1998; Cheung, 2003). The ORF2 has about 701 nucleotides in length, is located in the complementary strand and encodes for the capside (Cap) protein (Nawagitgul et al., 2000; Mankertz and Hillenbrand, 2002) (figure 2). The *rep* gene is highly conserved between PCV1 and PCV2, with about 83%

nucleotide sequence identity, whereas the *cap* gene has about 67-70% identity (Mankertz et al., 2004).

Apparently, *rep*, *rep'* and *cap* genes are the only major genes involved in completing the replicative cycle of the virus. However, a protein encoded by the ORF3 has also been identified in PCV2 and it has been suggested playing an important role in viral pathogenesis (Liu et al., 2005, 2006b). These studies showed that ORF3 is dispensable for viral replication and it seemed to be involved in host cell apoptosis (Liu et al., 2005, 2006b; Karuppannan et al., 2009). However, taking into account that others could not confirm these results, this point remains still controversial (Ramamoorthy and Meng, 2008).

2.3. Physico-chemical characteristics

Little work has been performed on investigating the physico-chemical characteristics of PCVs. It is known that PCV1 has a buoyant density of 1.35-1.37 g/ml in CsCl (Tischer et al., 1982; Allan et al., 1994), a sedimentation coefficient of 57S and is not able to hemagglutinate erythrocytes from a wide range of species (Allan et al., 1994). It is probable that these properties also apply to PCV2, taking into account its highly similar morphological and genetic characteristics with PCV1. Due to its simple structure without external envelope, PCVs are very resistant to lipid solving agents. Thus, PCV1 was demonstrated to be resistant to inactivation after exposure to chloroform (Allan et al., 1994), and PCV2 resisted expositions to a number of commercial disinfectants based on alcohol, chlorhexidine, iodine or phenol (Royer et al., 2001; Martin et al., 2008). Besides, PCVs can be inactivated by some commercial desinfectants based on alkali (i.e. sodium hydroxide), oxidazing agents (i.e. sodium hypochlorite) or quaternary ammonium (Royer et al., 2001; Martin et al., 2008). Moreover, both PCV1 and PCV2 also have extreme thermal and chemical resistance. Thus, PCV1 showed resistance to inactivation at pH=3 and at 70ºC for 15 min (Allan et al., 1994), and PCV2 retained infectivity when heated at 75ºC for 15 min but was inactivated by heating at 80ºC during more than 15 min (O'Dea et al., 2008). In

addition, PCV2 showed reductions of its infectivity by 1.6 to 1.25 log $TCID_{50}$ after long-time pasteurization or extreme dry heat treatment (Welch et al., 2006). These findings indicate that recipients of plasma-derived products may potentially content small DNA viruses, despite the implementation of viral inactivation steps (Welch et al., 2006). However, a recent experimental study demonstrated no piglet infection after feeding them with spray-dried porcine plasma infected with PCV2 (Pujols et al., 2008).

2.4. Antigenic properties

As mentioned in the historical background section, first serological surveys suggested that PCV1 was ubiquitous (Tischer et al., 1986; Dulac and Afshar, 1989; Allan et al., 1994; Edwards and Sands, 1994). However, subsequent studies demonstrated that there was a significant serological cross-reactivity between PCV1 and PCV2 antigens, suggesting that rates of PCV1 infection may have been overestimated (Magar et al., 2000b; Rodríguez-Arrioja et al., 2000). This fact was confirmed by later works, some of which indicated very low PCV1 prevalence in swine population (0-5 %) (Mankertz et al., 2000; Calsamiglia et al., 2002; de Boisseson et al., 2004), meanwhile other reported higher prevalence (8-41.4%) (Magar et al., 2000b). In any case, all studies assessing prevalence of both PCV1 and PCV2 agreed that the number of PCV2 positive pigs was always higher than PCV1 positive ones, and that PCV2 antibody titres were higher than PCV1 titres (Magar et al., 2000b; Mankertz et al., 2000; Calsamiglia et al., 2002; de Boisseson et al., 2004). All together, these results indicated that PCV2 was the main PCV species circulating in the swine population, rather than PCV1.

2.5. Porcine circovirus diseases (PCVD)

The clinical and pathological scope of PCV2 has been expanded since 1991. Thus, apart from PMWS, PCV2 has been associated with a number of conditions that are collectively known as porcine circovirus diseases (PCVD) in Europe (Segalés et al., 2005a) or porcine circovirus associated diseases (PCVAD) in North America (Opriessnig et al., 2007). Besides PMWS [now named as *PCV2*-

associated systemic infection in North America (Opriessnig et al., 2007)], porcine dermatitis and nephropathy syndrome (PDNS) (Allan et al., 2000a; Rosell et al., 2000b), reproductive disorders (West et al., 1999; Ladekjaer-Mikkelsen et al., 2001), PCV2-associated enteritis (Jensen et al., 2006), proliferative and necrotizing pneumonia (PNP) (Drolet et al., 2003; Grau-Roma and Segalés, 2007) and porcine respiratory disease complex (PRDC) (Kim et al., 2003a) are conditions currently included within PCVD or PCVAD. Congenital tremor (CT) type AII was also linked to PCV2 (Stevenson et al., 2001), but such association has never been reported again (Kennedy et al., 2003; Ha et al., 2005). Consequently, to date, CT is not considered into the scope of PCVD.

3. PORCINE CIRCOVIRUS TYPE 2 (PCV2) AND POSTWEANING MULTISYSTEMIC WASTING SYNDROME (PMWS)

3.1. Epidemiology of PMWS and PCV2

3.1.1. Susceptible species

Despite few initial studies suggested that PCV infection might occur in non-porcine species, describing infection in humans, cattle and mice (Tischer et al., 1995; Nayar et al., 1999), subsequent serological surveys in cattle, goats, sheep, horses, dogs, cats, mice and humans have shown no evidence of infection (Allan et al., 2000c; Ellis et al., 2000b, 2001; Rodríguez-Arrioja et al., 2003a). On the other hand, some controversial data have also been reported by experimental infections performed in species different from swine. Thus, meanwhile one study did not find evidences of PCV2 replication in mice (Quintana et al., 2002), others reported that PCV2 could replicate and even could be transmitted between mice (Kiupel et al., 2001; Liu et al., 2006a; Cságola et al., 2008). At the same time, experimental infection of sheep and rabbits with PCV2 did not result in seroconversion or presence of lesions (Allan et al., 2000c; Quintana et al., 2002). To date, the most accepted idea is that most of non-porcine species are not susceptible to PCV2 infection and do not play any role in the PCV2 epidemiology, being mice the only possible exception.

Several works have indicated that PCV2 can infect and even cause disease in *Sus scrofa* subspecies other than domestic pigs, reporting several PMWS cases in wild boar (Ellis et al., 2003; Schulze et al., 2003; Vicente et al., 2004; Lipej et al., 2007). Despite the epidemiological importance of wild boar in PCV2 is still not fully understood, it has been suggested that the natural reservoir for PCV2 might be the domestic swine itself, more than the wild boar (Vicente et al., 2004).

3.1.2. Geographical distribution

PCV2 seroprevalence is close to 100% in slaughter age animals and almost no PCV2 antibody free farms have been found across the world in epidemiological studies (Larochelle et al., 2003; Rose et al., 2003; López-Soria et al., 2005). Consequently, PCV2 is considered ubiquitous in the domestic pig population (Allan and Ellis, 2000; Segalés et al., 2005a). To date, PMWS have been diagnosed in countries from the five continents and in most pig producing countries (figure 3). It is noteworthy the special situation of Australia, where PCV2 has been demonstrated to be present in swine livestock but it is considered to be free of PMWS, because the disease has been never reported (Raye et al., 2005; Finlaison et al., 2007).

Figure 3. Political map of the world. Countries where PMWS diagnosis has been reported are highlighted in red. Adapted from Chae 2004.

3.1.3. Transmission Routes

3.1.3.1. Horizontal transmission

It is necessary to distinguish between transmission of PCV2 and transmission of the disease. Regarding the viral agent, the fact that almost all pigs (if not all) have seroconverted against PCV2 at 6 months of age (Larochelle et al., 2003; Sibila et al., 2004) already suggests that the horizontal transmission of PCV2 between pigs is very efficient. This idea is further supported by experimental infections, where control pigs comingled with previously PCV2 infected pigs resulted in transmission of the virus to all contact sentinel pigs (Albina et al., 2001; Bolin et al., 2001; Shibata et al., 2003). Additionally, a recent study indicated that PCV2 transmission between pigs allocated within the same pens (having direct contact) was more efficient than transmission between pigs allocated in different pens (having indirect contact, at 10 cm in distance) (Andraud et al., 2008).

Oro-nasal route is considered the most likely and frequent route of PCV2 infection and transmission and, consequently, most experimental studies on PCV2 infection have used this route (Tomás et al., 2008). PCV2 has been detected in all potential routes of shedding: nasal excretions, tonsillar, bronchial and ocular secretions, faeces, saliva, and urine (Krakowka et al., 2000; Shibata et al., 2003; Segalés et al., 2005b) of both naturally PMWS-affected and non-affected pigs, and even in sow milk and boar semen (Hamel et al., 2000; Larochelle et al., 2000; Shibata et al., 2006). Therefore, this virus can potentially be shed by all excretion and secretion routes. In addition, one recent experimental study has demonstrated the transmission of the virus using uncooked lymphoid tissues, bone marrow and skeletal muscle from PCV2 viremic to naïve pigs by the oral route (Opriessnig et al., 2009c).

Horizontal transmission of PMWS have also been experimentally demonstrated after mingling healthy pigs with pigs suffering from PMWS (Jaros et al., 2006; Kristensen et al., 2006, 2007). Healthy pigs developed clinical signs of PMWS 1-

2 weeks after mingling 7them with PMWS affected ones (Kristensen et al., 2006). Despite transmission of the disease was more frequent between animals that had direct contact (allocated in the same pen), it also occurred between pigs that had indirect contact (allocated in neighbouring pens) and even it was observed in one animal that had no physical contact (allocated across the aisle) with the PMWS affected pigs (Kristensen et al., 2007).

3.1.3.2. Vertical transmission

There are several evidences indicating that PCV2 vertical transmission can occur, understanding vertical transmission as the transmission of an infectious agent from one generation to the next one by infection of the embryo or foetus *in utero*. On one hand, transplacental transmission has been demonstrated twice after experimental intranasal infection of pregnant sows three weeks before the expected date of farrowing (Park et al., 2005; Ha et al., 2008). One of those studies reported abortion and premature farrowing in the inoculated sows (Park et al., 2005), while the other described normal farrowings (Ha et al., 2008). Both were able to detect genome and/or the proteins of PCV2 in aborted foetus or newborn piglets. On the other hand, PCV2 has been isolated or detected to high amounts within characteristic myocarditis lesions from aborted foetus or stillborns in cases of reproductive failure (West et al., 1999; O'Connor et al., 2001; Brunborg et al., 2007).

PCV2 has been detected in semen of naturally and experimentally infected boars, even after the appearance of antibodies in serum (Larochelle et al., 2000; Madson et al., 2009b). PCV2 is frequently detected in seminal plasma and in the sperm and non-sperm cell fractions (Hamel et al., 2000; Kim et al., 2003b). Furthermore, it has been recently demonstrated that PCV2 shed by semen in experimentally infected boars can be infectious (Madson et al., 2009b). This finding was demonstrated by the bioassay method, which consists of the detection of PCV2 seroconversion and viremia in naïve piglets inoculated intraperitoneally with semen from those PCV2 infected boars. However, when

this semen was used to inseminate sows, none of the sows or their offspring got PCV2 infection (Madson et al., 2009b). In addition, another recent experimental infection in PCV2 naïve sows reported reproductive failure as well as foetal infection (Madson et al., 2009a). However, this result was achieved by using semen spiked with PCV2. On the other hand, another study reported PCV2 infection and myocarditis lesions in foetus after artificial insemination using semen infected with PCV2. However, these sows had already been infected with PCV2 two months before the insemination (Rose et al., 2007). Therefore, it remains unknown if the amount of PCV2 naturally shed by boars is enough to transmit the virus to the foetus or the sow.

It is noteworthy that the frequency by which PCV2 is associated to reproductive alterations under field conditions remains controversial. Thus, it has been described from rare (Ladekjaer-Mikkelsen et al., 2001; Maldonado et al., 2005) to a frequent event, with reported PCV2 infection in about 13% of aborted foetuses and stillborn piglets (Kim et al., 2004).

3.1.4. Factors related to PMWS development

Today it is widely recognized that PCV2 is the essential infectious agent for PMWS development (Segalés et al., 2005a; Opriessnig et al., 2007; Ramamoorthy and Meng, 2008). However, the fact that PCV2 can be present in both diseased and healthy pigs and herds indicates the need for additional associated causes or specific circumstances that trigger PMWS development. Different epidemiological studies have investigated potential circumstances that are likely to increase the probability of disease onset or severity (risk factors).

3.1.4.1. Sow infection and immune status

PMWS is usually observed in pigs older than 4 weeks of age (Segalés and Domingo, 2002). There is only one report describing a natural PMWS case in a 3 day-old suckling piglet, which was indeed dually infected with PCV2 and *Porcine epidemic diarrhoea virus* (PEDV) (Hirai et al., 2001). On the other hand,

since almost all sows are PCV2 seropositive, most pigs receive antibodies through the colostrum (Rodríguez-Arrioja et al., 2002; Larochelle et al., 2003). Taken together, these facts suggest a protective effect of maternal immunity against PMWS development. In addition, an experimental infection suggested that the protection against PCV2 infection conferred by maternal antibodies was titre dependent (McKeown et al., 2005). Thus, higher titers were reported to be generally protective for PCV2 infection, but low titres were not (McKeown et al., 2005). Accordingly, a recent meta-analysis on reported experimental PCV2 infections showed that the most successful animal experiment aimed to develop PMWS should include the use of colostrum-deprived pigs (Tomás et al., 2008).

Moreover, a recent study made on 7 different PMWS affected farms reported higher mortality in piglets coming from that had low antibody titres against PCV2 than in piglets from sows with high antibody titres (Calsamiglia et al., 2007). This study also reported that sow PCV2 viremia was significantly related to piglet mortality since more piglets per litter died from viremic than from non-viremic sows. These data supported and further characterised the previously described “litter effect” (Madec et al., 2000). The authors suggested the term “sow effect” to refer the fact that the sow PCV2 status had a significant effect on litter mortality in PMWS affected farms (Calsamiglia et al., 2007).

3.1.4.2. Timing of infection

Case-control studies comparing PMWS affected and non-affected farms showed that the former ones had a higher percentage of pigs with antibodies against PCV2 around 3 months of age. This finding suggests that the PCV2 infection occurs earlier in PMWS affected farms than in non-affected ones. Thus, apparently, the earlier the PCV2 infection, the higher the risk of developing PMWS (Rose et al., 2003; López-Soria et al., 2005).

3.1.4.3. Differences in PCV2 virulence

The fact that PCV2 could be detected in both PMWS and non-PMWS affected farms and pigs raised the question about the possibility that different viral strains may vary in their pathogenicity. Moreover, the finding that PCV2 infection was already present in the swine livestock many years before the emergence of PCVD in the late 1990s indirectly support such hypothesis (Magar et al., 2000b; Rodríguez-Arrioja et al., 2003b; Jacobsen et al., 2009). Nevertheless, first studies on PCV2 sequencing did not find any clear relation between the PCV2 sequence and the occurrence of the disease (Larochelle et al., 2002, 2003; Pogranichniy et al., 2002; de Boisseson et al., 2004; Grierson et al., 2004; Wen et al., 2005). Since all PCV2 obtained sequences had >90% nucleotide identity, the potential hypothesis of difference in virulence between different strains of PCV2 was poorly investigated. However, later on, studies carried out in Canada and United States related the new epizootic outbreaks of PMWS with the introduction of an apparently new variant of PCV2, which had already been described in Europe and Asia (Carman et al., 2006; Cheung et al., 2007b). More recently, one experimental infection (Opriessnig et al., 2006d) and preliminary results from other experimental and *in vitro* works also suggested that differences in pathogenicity may exist (Cheung et al., 2007b; Stevenson et al., 2007). In addition, a retrospective epidemiological study indicated that the spread of PMWS in Great Britain was compatible to the dissemination of a novel agent or novel strain of agent (Woodbine et al., 2007).

Table 1. Different published nomenclatures referring to the two main phylogenetical PCV2 groups.

Author	De Boissesson et al., 2004	Timmusk et al., 2005	Carman et al., 2006	Olvera et al., 2007	Martins Gomes de Castro et al., 2007	Gagnon et al., 2007
Classification	I	SG3	Pattern 321-like	Group 1	Group A	PCV2b
	II	SG1/SG2	Pattern 422-like	Group 2	Group B	PCV2a

Independently of the potential differences of pathogenicity between strains, most phylogenetic works agree that there are two main genogroups within PCV2 (de Boisseson et al., 2004; Carman et al., 2006; Gagnon et al., 2007; Olvera et al., 2007). However, those two groups are named differently depending on the author, which creates certain confusion within the scientific and veterinarian community (table 1).

3.1.4.4. Host-dependent factors: pig genetics and sex

Differences in genetic host susceptibility or resistance in disease have been indicated in pigs for a variety of pathogens including viruses, bacteria and parasites (Duchet-Suchaux et al., 1991; Michaels et al., 1994; Reiner et al., 2002a, b). However, the influence of host genetics on PCV2 infection has only been investigated in few works. Pig breeder and veterinarian field observations have suggested that the susceptibility to PMWS varies depending on boar lines used. Accordingly, a field study reported a high difference in postweaning mortality due to PMWS in piglets derived from boars of a Large White-Duroc cross (up to 26.3 %) compared to piglets of boars from a Large White-Pietrain cross (up to 5.9 %) and piglets derived from pure breed Pietrain boars (up to 2.1 %) (López-Soria et al., 2004). In contrast, another field study comparing the offspring of sows inseminated with Pietrain semen with the ones from sows receiving the semen typically used on the commercial studied farms showed that there were no differences in terms of PCV2 seroconversion, morbidity and mortality (Rose et al., 2005). On the other hand, an experimental infection has reported a higher predisposition to PCV2-induced disease and lesions in Landrace pigs compared to Duroc and Large White (Opriessnig et al., 2006a). Moreover, another recent experimental infection showed that Landrace-PCV2 infected pigs had more severe lymphoid lesions than Pietrain-PCV2 infected ones (Opriessnig et al., 2009b). However, whether those observed differences are due to certain breeds, to certain genetic lines or even to individual pigs carrying some genes that might confer resistance or susceptibility to PMWS development remains unknown. The only work assessing this later hypothesis is very recent

and it is still preliminary, but pointed out that 2 regions of the pig genome could contain genes responsible for higher PMWS susceptibility (Karlskov-Mortensen et al., 2008).

Another host-dependent factor that has been related to the development of the disease is the sex of the pig. Thus, two field works have indicated that castrated male pigs are more susceptible to suffer from the disease than females (Corrégé, 2001; Rodríguez-Arrioja et al., 2002). However, this difference could be due to castration and possible coinfections acquired after this surgical procedure.

3.1.4.5. Coinfections and immune modulation

Field studies have shown that PMWS affected pigs can suffer from a wide spectrum of concomitant infections and/or diseases (table 2). However, no single co-pathogen with PCV2 has been identified as the unique responsible of enhancing the severity and the incidence of PMWS (Segalés et al., 2005a; Opriessnig et al., 2007). This situation raises the question whether the usual presence of coinfections in PMWS affected pigs/farms is because they trigger PMWS or, on the contrary, they are consequence to the immunosupression status due to PMWS (Segalés et al., 2004a; Segalés and Mateu, 2006). Since several works have reported the reproduction of PMWS by using coinfections of PCV2 together with other pathogens, it is accepted that at least some of those pathogens could potentially trigger PMWS development. In fact, only relatively few studies have been able to reproduce PMWS by inoculating PCV2 alone (Albina et al., 2001; Bolin et al., 2001; Harms et al., 2001; Ladekjaer-Mikkelsen et al., 2002; Okuda et al., 2003; Hasslung et al., 2005; Lager et al., 2007). Moreover, the above-mentioned meta-analysis on reported experimental infections concluded that coinfection with another swine pathogen as a triggering factor was one out of the conditions that was mainly included in the most successful infections (Tomás et al., 2008).

Table 2. List of coinfections reported in PMWS field cases.

Agent		Report	Country
Viruses	*Porcine reproductive and respiratory syndrome virus* (PRRSV)	Gagnon et al. 2007	Canada
		Larrochelle et al. 2003	Canada
		Dewey et al. 2006	Canada
		Pallarès et al. 2002	United States
		Pogranichniy et al. 2002	United States
		Dorr et al. 2007	United States
		Calsamiglia et al. 2007	Spain
		Fraile et al. 2009	Spain
		Segalés et al. 2002	Spain
		Quintana et al. 2001	Spain
		Rose et al. 2003	France
		Wellenberg et al. 2004	The Netherlands
		Kim et al. 2002	Korea
		Kawashima et al. 2007	Japan
		Murakami et al. 2006	Japan
	Porcine parvovirus (PPV)	Ellis et al. 2000a	Canada
		Rose et al. 2003	France
		Cao et al. 2005	China
		Kim and Chae 2002	Korea
	Porcine epidemic diarrhoea virus (PEDV)	Hirai et al. 2001	Japan
	Aujeszky's disease virus (ADV)	Rodríguez-Arrioja et al. 1999	Spain
		Quintana et al. 2001	Spain
		Cao et al. 2005	China
	Hepatitis E virus (HEV)	Martín et al. 2007	Spain
	Torque teno virus (TTV)	Gagnon et al. 2007	Canada
		Horlen et al. 2008	United States
		Kekarainen et al. 2007	Spain
	Porcine Teschovirus (PTV)	Takahashi et al. 2008	Japan
	Swine influenza virus (SIV)	Dorr et al. 2007	United States
		Pallarès et al. 2002	United States
Mycoplasmas	*Mycoplasma hyopneumoniae*	Dewey et al., 2006	Canada
		Dorr et al. 2007	United States
		Pallarés et al. 2002	United States
	Mycoplasma hyorhinis	Gagnon et al. 2007	Canada
		Kawashima et al. 2006	Japan
	Mycoplasma suis	Pereyra et al. 2006	Argentina
Bacteria	*Salmonella cholerasuis*	Murakami et al. 2006	Japan
	Escherichia coli	Dewey et al. 2006	Canada
	Haemophilus parasuis	Kim et al. 2002	Korea
Opportunistic pathogens	*Pneumocystis carinii*	Clark et al. 1997	Canada
	Candida albicans	Zlotowski et al. 2006	Brazil
	Aspergillus spp.	Segalés et al. 2003	Spain
	Criptosporidium parvum	Núñez et al. 2003	Spain
	Chlamydia spp.	Carrasco et al. 2000	Spain
	Zygomycetes spp.	Szeredi and Szentirmai 2008	Hungary

Among all coinfections described in the field, experimental coinfections of pigs with PCV2 and *Porcine parvovirus* (PPV) (Allan et al., 1999a; Kennedy et al., 2000; Krakowka et al., 2000; Opriessnig et al., 2004a; Ha et al., 2008), PRRSV

(Allan et al., 2000b; Harms et al., 2001; Rovira et al., 2002) and *Mycoplasma hyopneumoniae* (Opriessnig et al., 2004b) have been shown to enhance the PCV2-associated lesions and to increase the incidence of PMWS. In fact, PRRSV was repeatedly shown to increase the risk of developing PMWS in several epidemiological studies (Pogranichniy et al., 2002; Rose et al., 2003; Wellenberg et al., 2004; Dorr et al., 2007; Kawashima et al., 2007). Also *M. hyopneumoniae* and PPV, although less frequently than PRRSV, have been shown to increase the risk of having disease under field conditions (Rose et al., 2003; Dorr et al., 2007; Kawashima et al., 2007). Obviously, some reported coinfections may be merely concomitant infections, without any relation of synergism with PCV2, and others may be consequence of the immunosupression. Opportunistic pathogens such as *Aspergillus* spp. (Segalés et al., 2003), C*andida albicans* (Zlotowski et al., 2006) or *Chlamydia* spp. (Carrasco et al., 2000) would belong to the latest category.

Several studies have also suggested that immunostimulation may trigger the development of PMWS. This hypothesis was initiated in 2001, when Krakowka et al. reproduced the clinical signs and lesions of PMWS in gnotobiotic piglets (Krakowka et al., 2001). Those animals were infected with PCV2 and received keyhole limpet hemocyanin in incomplete Freud's adjuvant (KLH/ICFA) as immunostimulant; non-stimulated pigs only showed subclinical PCV2 infection (Krakowka et al., 2001). Subsequent works using again KLH/ICFA or an immuno-modulator drug derived of parapoxvirus have support the effect of immunostimulation (Kyriakis et al., 2002; Grasland et al., 2005; Krakowka et al., 2007). However, other studies using also KLH/ICFA (Ladekjaer-Mikkelsen et al., 2002), lipopolysaccharide (LPS) (Fernandes et al., 2007) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Loizel et al., 2005) failed to confirm such effect. Besides, one study reported that non-specific stimulation of the immune system by an oil-adjuvanted *M. hyopneumoniae* vaccine was able to potentiate PCV2 replication and increased the severity of clinical signs during an outbreak of PMWS under field conditions (Kyriakis et al., 2002). Consequently,

a considerable interest and concern about the effect of adjuvanted vaccines as enhancers of PMWS was generated. Thus, the potential PCV2 replication enhancement after application of *M. hyopneumoniae, Actinobacillus pleuropneumoniae*, PRRSV vaccines and even *Classical swine fever virus* (CSFV) vaccines was also shown experimentally (Opriessnig et al., 2003, 2006b; Allan et al., 2007a; Ha et al., 2009). One of those studies indicated that the immunostimulatory effect on PCV2 replication varies depending on the timing of vaccine application, suggesting that the deleterious effect of the tested *M. hyopneumoniae* vaccine could be prevented by applying the vaccine 2-4 weeks before the PCV2 exposure (Opriessnig et al., 2006b). Additionally, some works indicated that oil-based adjuvants have an apparently higher enhancing effect than aqueous or aluminium hydroxide products (Hoogland et al., 2006; Krakowka et al., 2007). However, not all oil-adjuvants used in commercial vaccines seem to be capable of triggering the mechanisms that potentiates PCV2 replication and generate disease (Resendes et al., 2004a).

Overall, the current knowledge indicates that the stimulation and/or activation of the immune system by both infectious and non-infectious factors may contribute to PMWS development. However, controversial data on this issue together with the occasional achievement of the PMWS experimental reproduction using PCV2 alone (Albina et al., 2001; Bolin et al., 2001; Harms et al., 2001; Ladekjaer-Mikkelsen et al., 2002; Okuda et al., 2003; Hasslung et al., 2005; Lager et al., 2007) suggest that none of these factors are crucial elements for PMWS development.

3.1.4.6. Agent X

The systematic difficulties to reproduce PMWS using PCV2 alone, together with the ubiquity of the virus within the pig population around the world, raised the hypothesis that PMWS was caused or triggered by an unknown potential pathogen, which was called “agent X”. In fact, spatiotemporal distribution studies indicated that PMWS dissemination in Denmark and United Kingdom

was compatible with the introduction of a novel agent (Vigre et al., 2005; Woodbine et al., 2007). Nevertheless, another similar study conducted in Sweden indicated the opposite (Wallgren et al., 2004). Moreover, the mentioned British study did not rule out the existence of new strains of already known pathogens as a possible explanation of the PMWS epidemiological presentation in Britain (Woodbine et al., 2007). To date, all the attempts to recover or identify a common new infectious agent associated with PMWS-affected herds and pigs have failed (Segalés et al., 2005a; Opriessnig et al., 2007; Lohse et al., 2008; Ramamoorthy and Meng, 2008). Although this does not indicate that such agent does not exist, the above mentioned reproduction of the disease using PCV2 as the only infectious agent and the recent successful results reported by the first PCV2 commercial vaccines (Fachinger et al., 2008; Horlen et al., 2008; Kixmöller et al., 2008) are strongly supportive of PCV2 as the essential infectious causal agent of PMWS.

3.1.4.7. Management measures

During the first descriptions of the disease in France it was noticed that PMWS affected farms had several management shortcomings. Moreover, the correct implementation of general management measures significantly decreased the percentage of mortality in severely affected farms (Madec et al., 2000). These measures are known as the "Madec's 20-point plan" and were designed to reduce the infection pressure in regards to PCV2 and other pathogens, to improve hygiene and to reduce stress at the different production stages. As a result of these observations, it was suggested that several environmental conditions might be necessary, in association with PCV2, to lead the clinical expression of the disease. A summary of the factors related to facilities, management practices and treatments and vaccinations that have been reported to influence PMWS development are listed in table 3. It is important to notice that some of the factors compiled in this table might be spurious effects, meaning that despite being statistically significant in one study it could not have a real effect to the disease development. Thus, for example, it is difficult to rationally explain how

separated PPV and Erysipelas vaccination of gilts, vaccination of sows against atrophic rhinitis or *Escherichia coli*, or the use of oxytocin injection during farrowing can influence the development of a disease in pigs of several months of age (Rose et al., 2003, 2005; López-Soria et al., 2005).

Table 3. Summary of factors related to facilities, management practices, vaccinations and treatments that have been described to influence the risk of PMWS development.

	Factors increasing the risk of PWMS	Factors decreasing the risk of PMWS
Facilities	- Large number of sows - Large pens at nursery and growing ages - Proximity with other pig farms	- Separate pit for adjacent fattening rooms - Having shower facilities
Management practices	- High level of cross-fostering - Short empty periods at weaning and fattening facilities - Large range in age and weight entering to the nursery - Continuous flow at nursery - Purchase of replacement gilts - Neck injuries due to poorly performed injections	- Sorting pigs by sex at nursery pens - Higher minimum weight at weaning - Housing of sows in collective pens during pregnancy - Visitors requested to avoid pigs for several days before visiting the farm - Semen purchased from an insemination centre
Vaccinations and treatments	- Vaccination of gilts against PRRSV - Vaccination of sows against *E. coli* - Use of separate vaccines against Erysipelas and parvovirus to the gilts versus associated vaccines	- Vaccination of sows against atrophic rhinitis - Regular treatments against external parasites - Using oxytocin injection during farrowing

Information derived from Cook 2001, Rose et al. 2003, 2005 and 2007, López-Soria et al. 2005, Dewey et al. 2006 and Woodbine et al. 2007.

3.1.4.8. Nutrition

A recent *in vitro* study showed that the addition of selenomethionine reduces PCV2 replication in PK-15 cells in a concentration-dependent manner (Pan et al., 2008). The authors speculated that such effect might be due to the enhancement of the activity of glutathione peroxidase. These results are in agreement with

previously reported field experiences, which described partial control of epizootic PMWS by addition of feed additive with anti-oxidant effects (Donadeu et al., 2003). Moreover, the supplementing of food with conjugated linoleic acid tended to amelio0rate the microscopic lesions and to improve the cellular response after PCV2 experimental infection (Bassaganya-Riera et al., 2003). On the other hand, an epidemiological case-control study indicated that the use of spray-dried plasma in the first nursery ration decreased the risk of developing PMWS (Dewey et al., 2006).

3.2. Clinical findings

PMWS most commonly affects pigs from 7 to 15 weeks of age, corresponding to pigs at the late nursery and early fattening phases of the production system (Harding, 1998, 2004; Segalés and Domingo, 2002; Madec et al., 2008). However, the disease has been described in piglets as young as 3-day-old (Hirai et al., 2001) and as old as 6-months of age. Moreover, PMWS has been diagnosed in pigs from almost all types of farms, including farrow-to-finish farms and multi-site operation farms (Segalés and Domingo, 2002).

The acronym PMWS already defines the major clinical sign, which is wasting and/or growth retardation. Moreover, affected pigs can also frequently show other signs such as fever (up to 41ºC), skin pallor, dyspnoea, diarrhoea and gastric ulceration (Harding, 1998, 2004; Segalés and Domingo, 2002; Madec et al., 2008). Signs such as icterus, sudden death, meningitis or pyrexia have also been described but to a lower frequency (Segalés and Domingo, 2002; Harding, 2004). Some of these clinical signs may be caused in part or exacerbated by the previously mentioned concomitant and secondary infections.

The severity and type of disease expression vary considerably between pigs within a group. Thus, while some animals in a pen may be severely affected, the rest of pigs do not display any obvious sign of sickness throughout the weaning and fattening phases. Morbidity and lethality rates are variable depending on the

farm and the batch of animals, ranging most commonly 4-30% and 70-80%, respectively. However, cases with exceptional morbidity ratios, over 50-60%, and cases with sporadic few affected pigs on farms with very good production records have also been described (Segalés and Domingo, 2002).

3.3. Pathological findings

There is a wide range of non-specific gross lesions that can be present in multiple tissues from PMWS affected pigs. Wasting is the most frequent macroscopic feature of affected pigs. Non-collapsed, tan-mottled lungs, sometimes with very marked interstitial oedema, enlargement of lymph nodes and thymus atrophy are also relatively usual findings (Harding and Clark, 1997; Rosell et al., 1999; Darwich et al., 2003b; Harding et al., 2008b). Enlargement of lymph nodes is associated to early clinical phases of PMWS, while normal or even atrophic lymph nodes can be usually seen in more advanced phases of PMWS (Rosell et al., 1999). Additionally, a low proportion of lymph nodes may have the presence of multifocal areas of necrosis that can be visible macroscopically (Segalés and Domingo, 2002). A percentage of PMWS affected animals may also have multifocal white spots in the kidney's cortices and reduction or increase in the liver size, with orange-yellow discoloration in those cases where icterus is present (Rosell et al., 2000a; Kim et al., 2002; Martínez et al., 2006). Bronchopneumonia and gastric ulceration of the pars oesophagea are frequently found in PMWS affected cases, but are not related to the direct effect of PCV2. Bronchopneumonia is associated with bacterial infections while gastric ulceration is of multifactorial origin. However, the lesion in the stomach cause severe haemorrhage and it is the cause of death of a number of pigs with PMWS and also responsible, in part, for the paleness of skin. Pigs with chronic wasting disease may develop cachexia, with marked muscle wasting and serous atrophy of fat (Segalés et al., 2004c).

Microscopic lesions in lymphoid tissues are almost unique (Rosell et al., 1999; Segalés et al., 2004c). A variable degree of lymphocyte depletion is

systematically present, usually combined with a multifocal to diffuse, slight to very intense histiocytic infiltration with the possible presence of giant cells. Thymus atrophy is microscopically characterized by cortical atrophy due to lymphocyte depletion (Darwich et al., 2003b). PCV2 inclusion bodies can be frequently observed in the cytoplasm of histiocytes or dendritic cells (DC) of lymphoid tissues. They appear as basophilic, round shaped and with variable sizes (Rosell et al., 1999; Segalés et al., 2004c). The previously mentioned grossly visible necrosis of lymph nodes corresponds microscopically to multifocal to coalescent areas of coagulative necrosis associated with vascular thrombosis, but without evidence of necrotizing vasculitis (Segalés et al., 2004c; Kim and Chae, 2005).

Among non-lymphoid tissues, the most common affected ones are lungs, liver and kidneys (Rosell et al., 1999; Segalés et al., 2004a, c) (figure 4). Lungs show sub-acute interstitial pneumonia, with alveolar, peribronchial and peribronchiolar mononuclear (lymphocytes and macrophages) infiltrates. Peribronchial fibrosis and bronchiolitis fibrosa occur in advanced cases (Clark, 1997; Segalés et al., 2004c). The interstitial oedema is observed microscopically as distension of pulmonary septa. In some animals, lymphocyte depletion and histiocytic infiltration can also be observed in bronchus-associated lymphoid tissue (BALT).

Hepatic lesions also consist of lymphohistiocytic infiltration, but they are usually slight and focally or multi-focally distributed (Rosell et al., 2000a). A variable number of single hepatocytes showing clear signs of apoptosis can be seen in a proportion of animals (Rosell et al., 1999, 2000a). In a few number of cases, a diffuse cytopathic change and inflammation of hepatic parenchyma can occur, with a marked loss of hepatocytes, increased amount of fibrous tissue in the interstitium, and inflammatory infiltrates through the hepatic tissue (Krakowka et al., 2000; Rosell et al., 2000a). These latter lesions are associated with generalized icterus and macroscopic lesions described in the liver. In fact, four

stages of hepatic damage in PMWS-affected pigs have been established based on intensity and distribution of lesions (Rosell et al., 2000a).

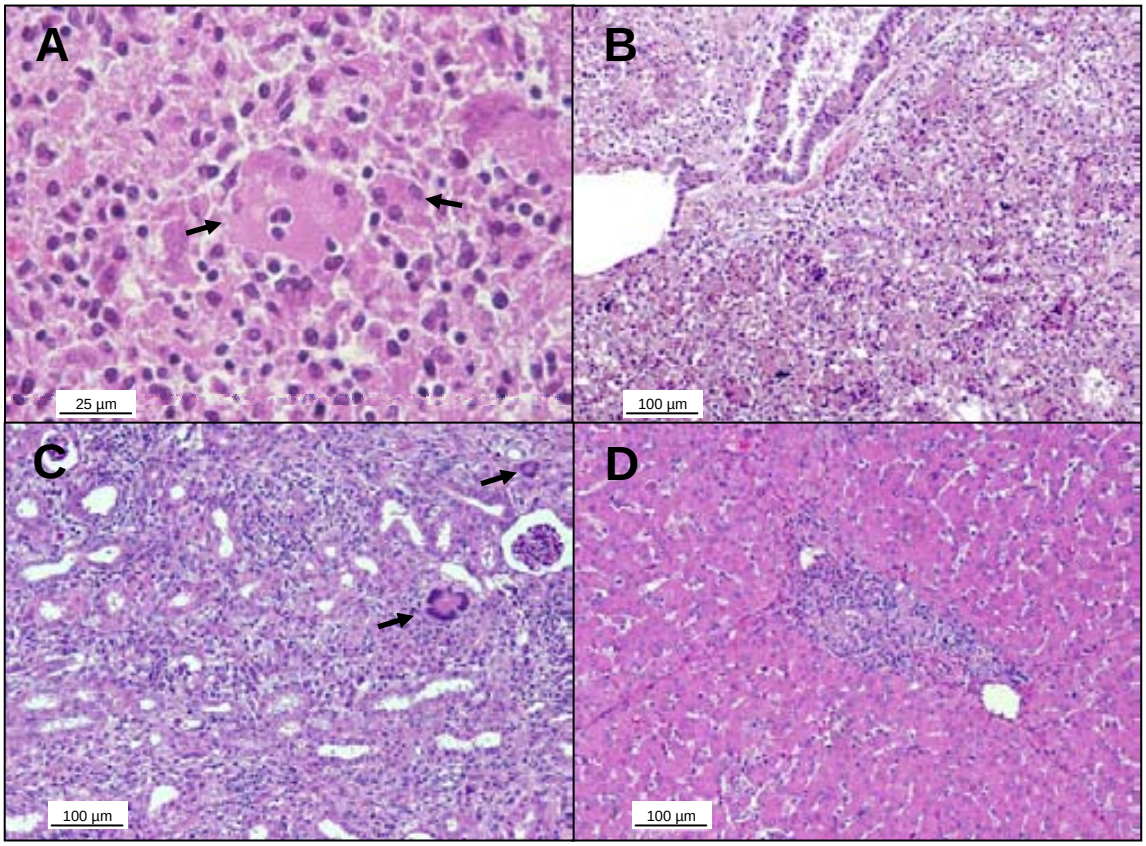


Figure 4. Microscopic inflammatory lesions frequently observed in PMWS affected pigs. Hematoxilin and eosin stains. (A) Granulomatous infiltration, lymph node. (B) Subacute interstitial pneumonia. (C) Interstitial nefritis with granulomatous infiltration. (D) Mononuclear hepatitis. Note the presence of multinuclear giant cells in lymph node and kidney (arrows).

Microscopic lesions observed in the kidneys consist in non-purulent interstitial nephritis. This lesion, at the age that PMWS usually occur, is almost exclusive of this disease (Clark, 1997; Rosell et al., 1999; Segalés et al., 2004c; Sarli et al., 2008).

Foci of lympho-histiocytic inflammatory infiltrates can also be detected in virtually all tissues, although to a lesser intensity and extension than the main affected organs cited above (Segalés and Domingo, 2002).

3.4. Pathogenesis and immune response

3.4.1. Experimental reproduction

Experimental models are essential for researchers to study the pathogenesis of infectious diseases, host immunological responses, as well as to evaluate the efficacy of vaccines against an etiological agent. However, in the case of PMWS, a large number of unsuccessful (understood as lack of clinical PMWS reproduction) experiments have been reported (Allan et al., 2000b, d; Loizel et al., 2005; Ostanello et al., 2005; Opriessnig et al., 2006b, c, d; Fernandes et al., 2007; Fort et al., 2008; Lefebvre et al., 2008b), evidencing the lack of a consistent experimental model to develop the disease.

Early experimental models using piglets inoculated with apparently PCV2 alone developed a mild clinical disease with slight histological lesions characteristic of PMWS (Balasch et al., 1999; Kennedy et al., 2000; Magar et al., 2000a). However, as mentioned before, when PCV2 was combined with another infectious agent, such as PPV (Allan et al., 1999a; Ellis et al., 2000a; Kennedy et al., 2000; Krakowka et al., 2000; Ha et al., 2008), PRRSV (Allan et al., 2000b; Harms et al., 2001; Rovira et al., 2002; Opriessnig et al., 2006c) or *M. hyopneumoniae* (Opriessnig et al., 2004b), severe clinical signs and lesions of PMWS were occasionally reproduced in both conventional and gnotobiotic pigs. It was hypothesized that PCV2 alone was not able to cause disease and needed the help of other infectious agents as triggers. Moreover, modulation of the host immune system with non-infectious factors has also been described as a trigger for PMWS. The effect of immunomodulation has been demonstrated either experimentally using KLH/ICFA (Krakowka et al., 2000, 2007; Grasland et al., 2005) or under natural conditions using a parapoxvirus immunomodulator combined with a vaccine against *M. hyopneumoniae* (Kyriakis et al., 2002). Nevertheless, other attempts using also KLH/ICFA (Ladekjaer-Mikkelsen et al., 2002) and other immunostimulants like LPS (Fernandes et al., 2007), GM-CSF (Loizel et al., 2005) or *M. hyopneumoniae* vaccine (Haruna et al., 2006) failed to confirm such effect. Additionally, previously mentioned commercial inactivated

or attenuated vaccines against other pig pathogens (Opriessnig et al., 2003; Hoogland et al., 2006; Krakowka et al., 2007; Ha et al., 2009) and the use of mitogens such as concavalin A (Yu et al., 2007b; Lefebvre et al., 2008b) have also been suggested as enhancers of PMWS by up-regulating of PCV2 replication. The picture becomes even more complex with the reports indicating that the use of an immunosuppressor such as cyclosporine A was able to increase PCV2 replication in pigs (Krakowka et al., 2002; Meerts et al., 2005b). On the other hand, few but several authors have described the reproduction of the full spectrum of PMWS clinical and pathological signs using PCV2 alone (Albina et al., 2001; Bolin et al., 2001; Ladekjaer-Mikkelsen et al., 2002; Okuda et al., 2003; Hasslung et al., 2005; Lager et al., 2007). All together, indicate that, among all the suggested factors influencing PMWS development, PCV2 seems to be the only one with an essential role.

Due to the difficulty of reproducing the severe clinical expression of PMWS with PCV2 alone and to obtain repeatable results, a meta-analysis on published results of experimental infections was recently performed (Tomás et al., 2008). This study concluded that the highest likelihood to achieve PMWS in an experiment include: (1) colostrum-deprived pigs, (2) age of inoculation below 3 weeks, (3) high doses of PCV2 inoculum (over 10^5 $TCID_{50}$ /pig), (4) PCV2 strain from what is known today as PCV2 genotype b, and (5) coinfection with one of the three above-mentioned swine pathogens as a triggering factor (Tomás et al., 2008). The route of administration was not relevant in such analysis, but it is noteworthy that around 90% of experiments used oro-nasal route, which is believed to be the main natural route of infection (Segalés et al., 2005a).

3.4.2. Evolution of microscopic lesions and PCV2 amounts in lymphoid tissues

A percentage of pigs that suffer from PMWS are able to recover from the disease. Thus, although animals show emaciation in this chronic stage, histopathological examination of them reveals only mild or no lesions compatible

with PMWS together with minimal amounts of PCV2 in lymphoid tissues (Segalés et al., 2004c). Recently, the evolution dynamics of microscopic lesions and amount of PCV2 in lymphoid tissues in experimentally infected pigs have been outlined by Opriessnig et al. (2007) (table 4). However, the evolution of clinical signs was not mentioned in this description, which would have been interesting for its diagnostic implications.

Table 4. Relative timing of PCV2-associated lymphoid lesions (depletion, histiocytic-to-granulommatous infiltration, and amount of PCV2-antigen ranging from 0 to 3) based on observations in pigs experimentally infected with PCV2 (Opriessnig et al., 2007).

	Acute (7-14 DPI)	Subacute (14-21 DPI)	Chronic (21-28 DPI)	Resolving (28-49 DPI)
Depletion	0-1	2-3	3	2-3
Inflammation	0-1	1	2-3	2-3
PCV2 antigen	3	3	2-3	0-1

3.4.3. PCV2 replication and its target cells

Since PCV2 does not encode its own polymerase, the replication of PCV2 depends on the cell polymerases present in the nucleus of during the S phase of the cell cycle (Tischer et al., 1987). Once there, PCV2 replicates by a rolling-cycle mechanism, which is similar to the one used by other circular ssDNA viruses, such as plant viruses from the family *Geminiviridae* (Cheung, 2004; Steinfeldt et al., 2006). Thus, a complementary DNA strand is synthesized and the resulting double-stranded DNA (dsDNA) is known as the replicative form (RF) of the virus (Tischer and Buhk, 1988; Mankertz et al., 2004). The helicase and nickase activities of Rep and Rep´, respectively, facilitate the interaction of the host DNA polymerase with the viral genome, synthesizing the complementary DNA strand of PCV2 (Mankertz and Hillenbrand, 2001; Steinfeldt et al., 2001). Viral replication, DNA transcription as well as encapsidation of the virus apparently take place in the nucleus (Tischer et al., 1987; Finsterbusch et al., 2005; Lefebvre et al., 2008b).

The identification of the target cells for PCV2-replication has been the subject for many studies, giving often controversial results. Immunohistochemistry (IHC) and *in situ* hybridisation (ISH) techniques demonstrated large amounts of PCV2 antigen or nucleic acid in the cytoplasm of macrophages and DC in the depleted follicles of lymphoid tissues (Rosell et al., 1999; Allan and Ellis, 2000; Sorden, 2000). Moreover, PCV2 has also been detected in the cytoplasm and, occasionally, in the nucleus of fibroblasts, lymphocytes, pancreatic acinar cells, endothelial cells, other macrophage cell types such as alveolar macrophages and Kupffer cells, and several types of epithelial cells such as renal and respiratory epithelial cells, hepatocytes or enterocytes (Kiupel et al., 1998; McNeilly et al., 1999; Rosell et al., 1999, 2000a; Kennedy et al., 2000; Chianini et al., 2003). However, the presence of nucleic acid and/or antigen in tissues or cell cultures, even in the cell nucleus, does not necessarily imply PCV2 replication. For this reason, new strategies have been developed to investigate PCV2 replication, including IHC using antibodies against ds and ssDNA (Hamberg et al., 2007), ISH with a probe specific for RF (Pérez-Martín et al., 2007), rolling-cycle amplification (Navidad et al., 2008), and the measurement of Cap messenger RNA (mRNA) and viral DNA synthesis by quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR) assays (Yu et al., 2007a, b). The application of some of these techniques *in vivo* indicated that PCV2 replication mainly occurs in lymph nodes, lung, tonsil and liver (Pérez-Martín et al., 2007; Yu et al., 2007a). Despite the replication of PCV2 in macrophages and lymphocytes was ruled out in some initial *in vitro* studies (Gilpin et al., 2003; Vincent et al., 2003), other works indicated the opposite (Meerts et al., 2005a; Pérez-Martín et al., 2007; Yu et al., 2007a; Lefebvre et al., 2008b; Lin et al., 2008) and even one of them have suggested lymphocytes as the primary site of PCV2 replication (Yu et al., 2007a). Pérez-Martín et al. (2007) showed that a low proportion of macrophages and lymphocytes may support PCV2 replication. Moreover, these authors indicated that, apparently, the main cells where PCV2 replicates are of epithelial/endothelial origin, suggesting that PCV2 is epitheliotropic (Pérez-Martín et al., 2007), as it has been described for other circoviruses (Todd, 2004).

Besides, it has been suggested that persistent infected monocytes and DC might be the potential mechanism of PCV2 dissemination throughout the body (Vincent et al., 2003).

The cellular tropism of PCV2 appears to change with the maturation status of the pig. Sánchez et al. (2003) studied the target cells during development from foetal to postnatal life after PCV2 infection. During foetal life, viral antigens were detected mainly in cardiomyocytes and, to a fewer amounts, in hepatocytes and macrophages. However, the number of infected cells decreased with increasing foetal age at PCV2 inoculation. Postnatally, infected cell numbers were similar to those of foetuses inoculated at the third month of gestation and macrophages were the main target cell type in different organs (Sanchez et al., 2003).

3.4.4. Immune response

Stimulation and/or activation of the immune system of PCV2-infected pigs by some viruses or non-infectious factors up-regulate PCV2 replication and increase viral loads in tissues and serum. Therefore, PCV2 infection and immunostimulation were pointed as pivotal events for PMWS development (Allan et al., 1999a; Krakowka et al., 2000, 2001; Harms et al., 2001; Rovira et al., 2002). Conversely, when clinical PMWS is apparent, damage of the immune system is the main feature in affected pigs (Nielsen et al., 2003; Darwich et al., 2004). The characteristic microscopic lesions, the consistent loss of peripheral B and T cells and the association of the disease with opportunistic pathogens (Segalés et al., 2001; Darwich et al., 2002, 2003b; Chianini et al., 2003; Nielsen et al., 2003) that are regularly found in PMWS affected pigs suggest an immunosuppressive status of diseased pigs (Segalés et al., 2004a).

3.4.4.1. Humoral immune response

In experimentally infected pigs, seroconversion against PCV2 occurs between 14 and 28 days post-inoculation (Allan et al., 1999a; Balasch et al., 1999; Pogranichnyy et al., 2000; Krakowka et al., 2001). Despite seroconversion has

been demonstrated in experimentally infected pigs with and without clinical disease, several studies reported that PMWS affected pigs show an apparently delayed antibody production (Bolin et al., 2001; Rovira et al., 2002; Okuda et al., 2003) or produce lower antibody titres against PCV2 compared to subclinically infected ones (Ladekjaer-Mikkelsen et al., 2002; Rovira et al., 2002; Hasslung et al., 2005). Those results suggest an impaired humoral immune response in diseased animals.

Under field conditions, colostral antibodies typically decline during the lactating and nursery periods, followed by an active seroconversion (Rodríguez-Arrioja et al., 2002; Larochelle et al., 2003). This seroconversion usually occurs between 7 and 12 weeks of age, and antibodies may last at least until 28 weeks of age (Rodríguez-Arrioja et al., 2002). As mentioned before, there is also one field study suggesting a protective effect of maternal immunity (Calsamiglia et al., 2007). However, the fact that a variable percentage of growing, finishing or adult pigs without clinical signs of the disease and high PCV2 antibody titres may also be viremic suggests that PCV2 antibodies are not fully protective against the infection (Rodríguez-Arrioja et al., 2002; Larochelle et al., 2003; Sibila et al., 2004).

Studies under experimental and field conditions indicated that PMWS-affected pigs lack or had lower neutralizing antibody (NA) titres than healthy animals (Pogranichnyy et al., 2000; Meerts et al., 2006; Fort et al., 2007). In experimentally infected animals, the evolution of NA paralleled the course of total antibodies, although a slight delay in NA production was seen in some animals (Fort et al., 2007). These findings together with the fact that the increase of NA coincided with the drop in PCV2 load suggested that NA play an important role in the viral clearance and further support the idea that PMWS-affected pigs suffered from an impaired humoral immune response (Fort et al., 2007). However, the inability of some pigs to develop a strong NA response

could be interpreted as either a cause or consequence of the development of the PWMS (Fort et al., 2007).

3.4.4.2. Cellular immune response and haematological parameters

It is still unknown whether the reduction in lymphocytes in PMWS-affected pigs is due to reduced production in the bone marrow and/or thymus, reduced proliferation in secondary lymphoid tissues or increased loss of lymphocytes in the bone marrow, thymus, peripheral blood or secondary lymphoid tissues via virus-induced necrosis or apoptosis (Opriessnig et al., 2007). Among these hypotheses, very controversial results have been reported about the importance of apoptosis. Thus, meanwhile several authors did not find a higher frequency of apoptosis in lymphoid tissues of PMWS affected pigs than in non-diseased pigs (Mandrioli et al., 2004; Resendes et al., 2004b), others suggested the opposite (Shibahara et al., 2000; Kiupel et al., 2005). In addition, the product of ORF3 was suggested to be involved in PCV2-induced apoptosis (Liu et al., 2005, 2006b, 2007). Recently, the same research group performed an experiment using a mutant of PCV2 lacking the expression of ORF3 and reported an attenuation of the PCV2 pathogenicity compared with the wild type virus infection (Karuppannan et al., 2009). Authors hypothesized that the apoptosis induced by the ORF3 might be a factor that helps in the spread of the virus *in vivo*, even though it is not necessary for the replication of the virus (Karuppannan et al., 2009).

Naturally PMWS-affected pigs suffer from an altered cytokine mRNA expression profile in the lymphoid tissues (Darwich et al., 2003b). This cytokine imbalance is characterized by an IL-10 over-expression in the thymus (Darwich et al., 2003b) and peripheral blood mononuclear cells (PBMC) (Sipos et al., 2004), and decreased IL-2, IL-4 and IL-12 expression in several secondary lymphoid organs, suggesting an impairment of the T cell immune response (Darwich et al., 2003b). Accordingly, cytokine profile evaluation of blood samples from PCV2-experimentally inoculated pigs showed an association

between plasma levels of IL-10 and development of PMWS (Hasslung et al., 2005; Stevenson et al., 2006). Moreover, PCV2 has been demonstrated to induce IL-10 secretion in *in vitro* cultured PBMC (Darwich et al., 2003a), leading to down-regulation of other cytokines produced during recall antigen responses (Kekarainen et al., 2008a, b). These data suggest the involvement of IL-10 in the suppressed Th1 responses observed during the course of PMWS (Kekarainen et al., 2008b). On the other hand, PCV2 was demonstrated to block the induction of IFN-α and other cytokines by natural interferon producing cells (NIPC), a DC subpopulation (Vincent et al., 2007). This effect is presumably induced through the presence of oligodeoxynucleotides (ODN) with central cytosine-phosphate-guanine (CpG) motifs within the PCV2 genome, thereby preventing the maturation of both NIPC and other DC subpopulations (Vincent et al., 2007; Kekarainen et al., 2008a). This situation would diminish the ability of these cells to mediate antiviral responses upon the infection and render the host more susceptible to secondary or concomitant microbial infections (Vincent et al., 2007). Thus, the presence of immunomodulatory sequences in the PCV2 genome is a potential mechanism to escape the immune response of the host and it may attribute to the development of clinical disease. However, the interaction between PCV2 and the immune system is highly complex. Thus, regulation of cytokine production appeared to be different depending on the PCV2 viral elements or the cell populations studied (Wikstrom et al., 2007; Kekarainen et al., 2008a). Recently, a subclinical PCV2 experimental infection reported the increase of IL-8 and of IFN-α blood concentration few days after infection, suggesting the capacity of the innate immune response of non-PMWS affected pigs to counteract the inhibitory activity of PCV2 (Fort et al., 2009). Taken together, these results suggest that the balance between the host ability to mount a proper innate antiviral response and the virus ability to dampen it might be determinant for the infection evolution and triggering of the disease (Fort et al., 2009). These authors also observed that the drop in the viral load in serum was concurrent with the appearance of PCV2-specific IFN-γ-secreting cells (IFN-γ-SC) and NA,

suggesting that the viral clearance might be mediated by the development of PCV2-IFN-γ-SC in contribution to the PCV2-specific NA.

The haemogram of pigs with PMWS also shows significant alterations (Segalés et al., 2000; Darwich et al., 2002). In diseased pigs, the number of lymphocytes is significantly decreased, and monocytes and neutrophils are increased, with an inversion of the ratio lymphocyte/neutrophil. However, the total number of leukocytes is not altered. Pigs with PMWS usually have normocytic hypochromic anemia with a slight increase of total number of red blood cells (Segalés et al., 2000; Darwich et al., 2002). In addition, analysis of the acute phase proteins in PMWS affected pigs showed an increase in the serum concentrations of several positive APPs including haptoglobin (HPT), pig-major acute phase protein (pig-MAP), C-reactive protein (CRP) and serum amyloid A (SAA), and a decrease of albumin concentration, which is considered a negative APP (Segalés et al., 2004b; Parra et al., 2006). It is known that the increase in hepatic release of positive APP is the consequence of an increase of proinflammatory cytokines, mainly IL-1, IL-6 and tumor necrosis factor-α (TNF-α), which are in turn primary secreted by monocytes in response to infection stimuli (Petersen et al., 2004; Gruys et al., 2005a). Thus, increases in IL-1, IL-6 and TNF-α have also been demonstrated in cases of PMWS (Sipos et al., 2004; Kim et al., 2006). However, other authors could not detect such increases in proinflammatory cytokines concentrations (Stevenson et al., 2006). Those controversial results might be explained by the fact that, meanwhile APPs serum concentrations can be elevated during days or weeks, the increase of inflammatory cytokines is usually cleared from the circulation within few hours (Petersen et al., 2004; Gruys et al., 2005a).

3.5. Diagnosis

The wide range of possible clinical manifestations of PMWS produces a very extensive and variable differential diagnosis, depending on the dominant clinical signs (Harding and Clark, 1997; Segalés et al., 2005a). Moreover, there are a

number of infectious and non-infectious diseases that can produce wasting, the major clinical sign of affected pigs. Consequently, diseases such as PRRS, Glässer's disease, classical swine fever, Aujeszky's disease, blue eye disease, carbadox/olaquindox toxicity, swine dysentery, porcine colonic spirochetosis, porcine intestinal adenomatosis, postweaning colibacillosis, and eperythrozoonosis should be included in the differential diagnosis list, depending on the dominant clinical signs and the country of origin (Segalés et al., 2005a).

3.5.1. Individual diagnosis

Although wasting and respiratory alterations in a proportion of late nursery and early fattening pigs favour a diagnosis of PMWS, most clinical signs and gross lesions of this disease are non-specific and, therefore, they are not sufficient to diagnose the disease. In addition, the fact that PCV2 is ubiquitous in the swine population makes the diagnostic picture even more complicate. Consequently, PMWS diagnosis in individual pigs is one of the most complex diagnoses among the currently known pig diseases. Today, an individual pig is considered to suffer from PMWS when fulfils the three following criteria (figure 5) (Sorden, 2000; Segalés et al., 2005a):

1) Presence of compatible clinical signs, including wasting or growth retardation.
2) Presence of moderate to severe characteristic histopathological lesions in lymphoid tissues, including lymphocyte depletion and histiocytic infiltration.
3) Detection of moderate to high amounts of PCV2 within the lesions in lymphoid and other tissues of affected pigs.

Several methods have been developed to detect PCV2 in tissues and to correlate its detection with the presence of lesions. Among them, ISH and IHC are the most routinely used tests (McNeilly et al., 1999; Rosell et al., 1999). These methods have shown a strong correlation between the amount of PCV2 nucleic acid or antigen and the severity of microscopic lymphoid lesions (Rosell et al.,

1999; Quintana et al., 2001). Consequently, the fact that PMWS diagnosis requires detection of moderate to high amounts of PCV2 within lymphoid tissues also implies the presence of moderate to severe microscopic lesions in affected pigs.

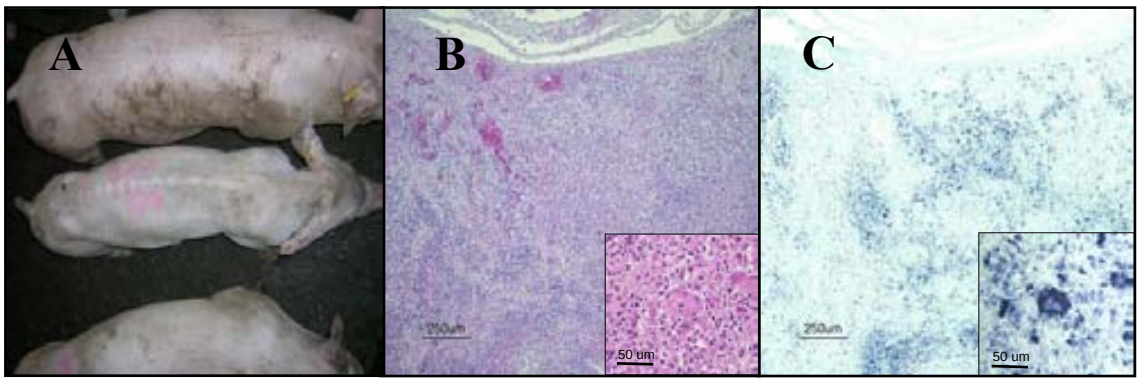


Figure 5. Conditions required to establish the individual PMWS diagnosis: A) Clinical signs; B) Moderate to severe lymphoid depletion and granulomatous infiltration in lymphoid tissues (Haematoxylin and eosin stain); C) Detection of moderate to high amount of *Porcine circovirus* type 2 (PCV2) within microscopic lesions in lymphoid tissues (*In situ* hybridization to detect PCV2, fast green counterstain).

It has been reported that healthy pigs can have a low amount of PCV2 antigen or nucleic acid and slight microscopic lesions (Quintana et al., 2001). In addition, recently infected pigs or pigs in a convalescent phase from PMWS may have very mild or no histological lesions in lymphoid tissues, and low amount of PCV2 antigen or nucleic acid (Quintana et al., 2001). These findings have important implications when selecting the most appropriate submissions for diagnosis. Pigs within the first week of clinical affectation are the preferred ones to accomplish the diagnosis criteria (Segalés et al., 2004c). At the same time, taking into account the fact that subclinical PCV2 infection occurs in almost all farms, interpretation of laboratorial results must be made with caution (Rosell et al., 2000b; McNeilly et al., 2002).

3.5.2. Herd diagnosis

The establishment of PMWS diagnosis at herd level is also controversial, because it is known that farms with very good performance scores can have

individual animals fulfilling the three abovementioned criteria (Jorsal et al., 2006). For this reason, scientists from a European Union (EU) Consortium on PCVD research (UE project No. 513928 from the VI Framework Programme, Priority 5. Food Quality and Safety) have proposed a herd case definition (www.pcvd.org/news.php). According to this proposal, a farm should be considered affected by PMWS when fulfils the following 2 criteria:

1) There is a significant increase in postweaning mortality and wasting rates compared to the historical background in the herd. The increase of mortality is considered significant when is equal to or more than the historic level plus 1.66 times the standard deviation or, alternatively, it can be determined using the chi-square test. If there is no historical data available, the increase of mortality should exceed the national or regional level by 50%.
2) Individual diagnosis of PMWS in at least one out of five necropsied pigs has to be reported.

Moreover, it is also stated that other relevant diagnostic procedures must be carried out to exclude other potential causes of high mortality.

3.5.3. Methods to detect PCV2 antigen or nucleic acid and PCV2 antibodies

As mentioned above, IHC and ISH are the most commonly used techniques to establish a PMWS diagnosis, giving both similar results (McNeilly et al., 1999; Rosell et al., 1999). However, other techniques to detect PCV2 antigen or nucleic acid and its antibodies are reported in the literature. Among them, PCR has been shown as a very sensitive test (Larochelle et al., 1999; Hamel et al., 2000; Kim and Chae, 2001; Calsamiglia et al., 2002) and have the advantage that can be applied to various specimens such as serum, body excretions and secretions and tissues (Quintana et al., 2001; Shibata et al., 2003). However, PCR is unable to differentiate between clinical and subclinical infections (McNeilly et al., 2002). Taking into account that subclinical infections with PCV2 are very common and that PCV2 infection results in clinical disease only under certain circumstances,

non-quantitative PCR methods should not be used to diagnose PMWS (McNeilly et al., 2002; Segalés et al., 2005a). Similarly, the wide number of serological techniques published in the literature, including immunoperoxidase monolayer assay (IPMA) (Rodríguez-Arrioja et al., 2000), indirect immunofluorescence assays (IIF) (Cottrell, 1999; Allan and Ellis, 2000) or enzyme-linked immunosorbent assays (ELISA) (Walker et al., 2000; Truong et al., 2001; Wu et al., 2008) are useful to monitor PCV2 infection but can not be used for diagnostic purposes (Rodríguez-Arrioja et al., 2000; Sibila et al., 2004). In addition, techniques to determine NA have been recently developed and suggested as useful to exclude the diagnosis of PMWS in combination with PCV2 qPCR in sera (Meerts et al., 2005b; Fort et al., 2007). Interestingly, techniques such as qPCR methods, antigen capture ELISA and immuncytochemical analysis of cryostat sections allow the quantification of the virus in tissues (McNeilly et al., 2002). Taking into account the above mentioned correlation between the amount of PCV2 nucleic acid or antigen and the severity of characteristic PMWS microscopic lesions (Rosell et al., 1999; Quintana et al., 2001), these techniques could potentially be used to diagnose PMWS in the absence of histopathological expertise, equipment or paraffin embedded tissue samples (McNeilly et al., 2002). Thus, some authors have suggested threshold values to diagnose PMWS in live animals using qPCR techniques in easy-to-collect samples such as blood or swabs (Brunborg et al., 2004; Olvera et al., 2004; Segalés et al., 2005b; Fort et al., 2007).

3.6. Prevention and control

Prior to the availability of PCV2 commercial vaccines, most efforts to control and prevent the disease were focused on the co-factors and triggers for PMWS developmen. To date, commercial PCV2 vaccines have been shown as excellent tools to prevent and diminish the impact of the disease.

3.6.1. Management measures

The implementation of the "Madec's 20-point plan" was demonstrated to be effective in decreasing the percentage of mortality in severely affected farms (Madec et al., 2000). Briefly, those recommendations include all-in-and-all-out procedures, disinfection, to limit pig-to-pig contact, to avoid mixing of batches and cross-fostering practices, immediate isolation or euthanasia of diseased pigs, to maintain appropriate temperature airflow and space conditions in pens, and to apply recommended de-worming, ectoparasite treatments and vaccination schedules. Thus, this plan includes a wide range of actions, which could be applicable to prevent most swine diseases.

3.6.2. Control of coinfections

Evidences from the field and experimental trials have indicated that diagnosis and control of other infectious agents commonly found in PMWS affected pigs diminish the severity of the coinfection and improve the outcome (Opriessnig et al., 2007). Thus, first attempts to control PMWS through vaccination against PPV in USA on finishing sites with confirmed PPV circulation reported successful results (Halbur, 2001). Experimentally, the vaccination of PCV2 infected sows against PPV together *Erysipelothrix rhusiopathiae* reduced the number of mummified piglets. In addition, piglets born from PCV2-infected/vaccinated sows were less likely to be affected by PMWS than those coming from PCV2-infected/unvaccinated sows (Rose et al., 2007). Besides, other experimental trials could not demonstrate the positive effect of PPV vaccination in reducing the clinical incidence of PMWS (Opriessnig et al., 2004a). Regarding PRRSV, one field study performed in farms with both PRRS and PMWS using a PRRSV vaccine in sows and piglets showed reduction of growers' morbidity and mortality and an improvement in the average daily gain and feed conversion ratio in vaccinated pigs compared to unvaccinated ones (Kritas et al., 2007). Moreover, it has also been indicated that the control of *M. hyopneumoniae* infection by vaccination (Halbur et al., 2006), or the use appropriate antimicrobials (Opriessnig et al., 2006e) can improve the

performance of PCV2 and *M. hyopneumoniae* coinfected pigs. Again, others reported no difference in the incidence of PMWS between *M. hyopneumoniae* vaccinated and sham-vaccinated pigs (Haruna et al., 2006). On the other hand, as have been previously indicated, vaccination of the sows against *E. coli* or atrophic rhinitis theoretically diminishes the risk of PMWS development (Rose et al., 2003; López-Soria et al., 2005). However, there is no clear explanation for the potential effect of such vaccination.

In any case, one must be careful in regards vaccine application, since potential negative consequences might be derived from its immunostimulating effect (Opriessnig et al., 2003, 2006b). The application of *M. hyopneumoniae* vaccines 2 to 4 weeks prior to expect PCV2 exposure was suggested to avoid potential PCV2-replication enhancement (Opriessnig et al., 2006b). In general, producers with PMWS-affected herds should consider determining the approximate timing of PCV2 infection with the objective or re-scheduling the timing of vaccination as a potential plan to minimize their potential immunostimulatory effect (Segalés et al., 2005a).

3.6.3. Changes in the genetic pig-line

Looking at the apparent differences in susceptibility of some genetic pig lines or breeds reported in the literature (López-Soria et al., 2004; Opriessnig et al., 2006a), the change of boar line have shown to be of benefit in severe cases of PMWS.

3.6.4. Semen quality control

Although the presence of PCV2 in semen used for artificial infection (AI) is not proof of causing infection in the sow herd, the demonstration of its infectious capacity indicates that the risk of transmission does exist (Maes et al., 2008; Madson et al., 2009b). Since the best way to prevent disease is the absence of the pathogen, AI centers should strive to use boars that are free of major pathogens, including PCV2. Animals should be quarantined and tested before introduction

into AI centers, strict biosecurity measures should be maintained, and a statistically appropriate number of animals and samples should be tested periodically (Maes et al., 2008).

3.6.5. Improvements in nutrition

Addition of feed additives with anti-oxidant effects (Donadeu et al., 2003), conjugated linoleic acid (Bassaganya-Riera et al., 2003) and the use of spray-dried plasma in the first nursery ration (Dewey et al., 2006) contribute to ameliorate the deleterious effects of PMWS. In addition, the administration of plant phytosterols with immunomodulating activity was suggested to control the productive losses in PRDC and PMWS affected farms (Fraile et al., 2009).

3.6.6. "Serum therapy"

When PCV2 commercial vaccines were still not available, some veterinarians used "serum therapy" to face heavy losses. The principle of this technique was based on collecting sera from pigs that had survived the problem and were therefore assumed to have high antibody titres in their blood. This "hyperimmune" serum was then injected into young piglets in order to confer protection, before the critical period of wasting. Inconsistent results were obtained. Anyhow, the large-scale use of "serum therapy" is to be discouraged because of the biosecurity risks (Madec et al., 2008).

3.6.7. PCV2 vaccines

Several experimental PCV2 vaccines have been described, tested and generally found to be efficacious in different *in vivo* models (Blanchard et al., 2003; Fenaux et al., 2004a; Kamstrup et al., 2004; Wang et al., 2007). These successful results encouraged the private industry to develop commercial PCV2 vaccines.

To date, four vaccines against PCV2 have been introduced in the international market (table 5). One vaccine (CIRCOVAC®) is designed for sows and gilts, meanwhile the other three (Ingelvac CIRCOFLEX®, Suvaxyn® PCV2 One Dose

and Porcilis PCV®[in EU] / Circumvent PCV® [in US]) are indicated for piglets. The use of vaccines in sows and gilts increases PCV2 antibody titres in both serum and colostrum; the transfer of the later one provides protection of piglets against PMWS development (Charreyre et al., 2005; Joisel et al., 2007a, b). In addition, it has been suggested that vaccination of the sow may also contribute to protection during the gestation phase and prevent potential pathogenic effects of PCV2 during this physiological state (Joisel et al., 2007a). Besides, piglet vaccines are applied around 3 weeks of age or later, during the waning of maternal antibodies, eliciting PCV2 total and NA responses and reducing or delaying PCV2 infections at weaning or fattening ages (Fort et al., 2008; Opriessnig et al., 2009a).

Table 5. Characteristics of commercial *Porcine circovirus* type 2 (PCV2) vaccines. Adapted from Opriessnig et al. 2007.

PCV2 vaccine	Company	Antigen	Posology	Licensed for
Ingelvac CIRCOFLEX®	Boehringer Ingelheim	PCV2 ORF2 protein	1 ml IM* Single dose	Piglets (2 weeks and older)
Suvaxyn® PCV2 One Dose	Fort Dodge	Inactivated PCV1-2 chimera	2 ml IM Single dose	Piglets (4 weeks and older)
Porcilis PCV®(EU) / Circumvent PCV® (US)	Intervet-Schering Plough	PCV2 ORF2 protein	2 ml IM 2 doses	Piglets (3 weeks and older)
CIRCOVAC®	Merial	Inactivated PCV2	2 ml IM 2 doses	Female breeding-age pigs

*IM = intramuscularly

Excellent results have been reported with all four vaccines either under experimental or field conditions. During field efficacy studies in Germany and France, the use of the vaccine in sows produced a rise in PCV2 antibody titres in the breeder herds and a concurrent decrease in PMWS rates and mortality ratios in the growing pigs originating from those vaccinated breeding herds (Charreyre et al., 2005; Joisel et al., 2007a). When used in Canada, a reduction of mortality was observed after 6 months of use (Plourde and Machell, 2007).

Experimental studies assessing the performance of PCV2 vaccines for piglets showed a reduction of PCV2 viremia and/or microscopic lesions, even in the presence of maternal antibodies (Fort et al., 2008; Opriessnig et al., 2008a). In addition, PCV2 vaccination with two doses of vaccine reduced PCV2 viremia and microscopic lesions by 97.1% and 81.8%, respectively, meanwhile such reduction was by 78.5 and 78.7% when piglets were vaccinated with only one dose (Opriessnig et al., 2009a). Under field conditions, the application of PCV2 vaccines in 3 or in 6-week-old pigs from PMWS affected farms showed that vaccinated pigs had lower mortality rate and higher average daily gain than unvaccinated pigs (Horlen et al., 2008). Moreover, vaccinated pigs had reduced number of coinfections with PRRSV and *Mycoplasma hyorhinis* than unvaccinated animals (Kixmöller et al., 2008). In addition, PCV2 piglet vaccination was reported to improve the growth performance of pigs affected by PRDC (Fachinger et al., 2008).

On the other hand, autogenous PCV2 vaccines have been sporadically used by some practitioners facing severe losses associated with PMWS. This situation mainly occurred some years ago, when commercial vaccine availability was limitated. These products were prepared from lung or lymphoid tissue homogenates obtained from diseased pigs and inactivated with 2% formaldehyde. Those practitioners generally reported marked reduction of mortality. However, the industry remains concerned about the safety and potential legal ramifications from using killed autogenous viral vaccines (Opriessnig et al., 2007).

Overall, despite the successful results reported by commercial PCV2 vaccines, it is of paramount importance to keep in mind that PCV2 is a multifactorial disease. Consequently, the strategies of control and prevention must be implemented at different level. In case of PMWS suspicion, the first action should be to confirm the PMWS diagnosis. Afterwards, it would be necessary to evaluate the potential risk factors in the farm and to act on those triggers that apparently have more

importance in the specific disease situation. The application of PCV2-vaccines should be considered by an appropriate cost-benefit analysis (Madec et al., 2008).

II. HYPOTHESIS AND OBJECTIVES

Si no saps per on vas, torna per saber d'on vens
Proverbi africà

PMWS is considered a multifactorial disease in which PCV2 is essential but not sufficient. However, the relative importance of the factors related to PMWS development has been controversially reported in the literature. In the present thesis, in order to expand the current epidemiological knowledge on PCV2 infection and PMWS, it was hypothesised that PMWS presentation could be influenced by differences in PCV2 genetics, the timing of PCV2 infection as well as by the PCV2 maternal derived humoral immunity and the pig humoral response against PCV2 infection. In addition, the potential use of PCV2 qPCR, PCV2 serology and APPs serum concentrations to help PMWS diagnosis in live animals was explored. Specific objectives of this thesis were:

1. To assess the potential relationship between PCV2 sequences and different health or disease status of pigs and/or farms.

2. To explore a potential PCV2 genotype definition using the sequences available at the NCBI database (www.ncbi.nlm.nih.gov) and the ones obtained in this thesis.

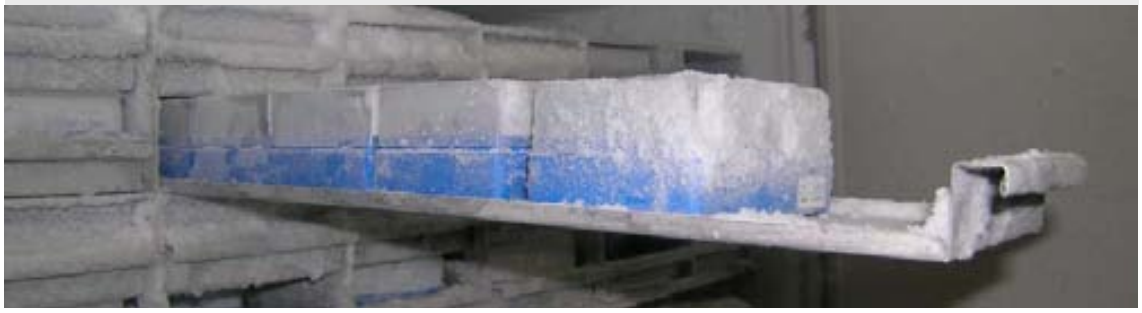
3. To describe and compare the evolution of PCV2 infection, seroconversion and excretion in histopathologically well-characterised pigs from PMWS affected farms using qPCR and serological techniques.

4. To assess the diagnostic performance of PCV2 serology and qPCR techniques on clinically affected animals.

5. To assess the evolution of two APPs (pig-MAP and HPT) in serum samples throughout the productive life of pigs that developed PMWS in comparison to healthy pigs and pigs that developed wasting without fulfilling the PMWS diagnosis.

6. To determine potential associations between variations in the studied APPs concentrations in sera and the moment of infection by different swine pathogens.

III. STUDIES



Epidemiologic studies demonstrate nothing. They suggest, indicate…
Laura Green

Study I

A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence.

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A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence

L. Grau-Roma [a,b,*], E. Crisci [a], M. Sibila [a], S. López-Soria [a], M. Nofrarias [a], M. Cortey [c], L. Fraile [a], A. Olvera [a], J. Segalés [a,b]

[a] *Centre de Recerca en Sanitat animal (CReSA), Esfera Universitat Autònoma de Barcelona (UAB), Bellaterra 08193, Spain*
[b] *Department de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona (UAB), Bellaterra 08193, Spain*
[c] *Bofill i Codina 14, Calella de P, Girona, 17210, Spain*

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Abstract

Porcine circovirus type 2 (PCV2) is the essential infectious agent of postweaning multisystemic wasting syndrome (PMWS). Despite first sequencing studies did not find any association between PCV2 sequences and PMWS occurrence, recent works have suggested the opposite. In the present study, 87 open reading frame 2 (ORF2) sequences obtained from pigs with different clinical conditions and coming from farms with different PMWS status were analyzed. Results further confirmed the existence of two genogroups and the definition of two PCV2 genotypes (1 and 2) is proposed. All sequences included in genotype 1 came from pigs from PMWS affected farms, while all sequences obtained from non-PMWS affected farms corresponded to genotype 2. Moreover, infection of single pigs from PMWS affected farms harbouring both genotypes is described. Present results suggest that PCV2 genotype 1 may potentially be more pathogenic than PCV2 genotype 2.

Keywords: Porcine circovirus type 2 (PCV2); Postweaning multisystemic wasting syndrome (PMWS); Genotype; Phylogeny; Epidemiology; Pathogenicity

1. Introduction

Porcine circovirus type 2 (PCV2) is recognized as the essential infectious agent of postweaning multisystemic wasting syndrome (PMWS), which is considered to have a severe economic impact on swine production (Segalés et al., 2005). The major clinical signs of PMWS are wasting and growth retardation, but can also include pallor of the skin, icterus, respiratory distress and diarrhoea (Harding and Clark, 1998).

PCV2 belongs to the family *circoviridae*, genus *cirvovirus*, and is a small, non-enveloped, single-

* Corresponding author at: Department de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona (UAB), Bellaterra, Spain. Tel.: +34 93 581 32 84; fax: +34 93 581 44 90.
E-mail address: llgrau@cresa.uab.cat (L. Grau-Roma).

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stranded DNA virus containing a circular genome of 1767–1768 nucleotides (Hamel et al., 1998; Meehan et al., 1998; Mankertz et al., 2000). The genome contains three open reading frames (ORF): *ORF1* encodes the replicase (*rep* and *rep′*) proteins involved in virus replication (Mankertz et al., 1998), *ORF2* encodes the *capsid* (*cap*) protein (Nawagitgul et al., 2000) and *ORF3* encodes a protein that is not essential for PCV2 replication with potential apoptotic activities (Liu et al., 2005, 2006). It was proposed that, since *cap* protein is the most variable PCV2 protein, a link between capsid protein variation and pathogenicity of PCV2 could exist (Larochelle et al., 2002; Todd et al., 2002). In addition, *ORF2* has been shown as a good phylogenetic and epidemiologic marker for PCV2, since it was able to reconstruct the same phylogenetic tree as using the whole viral PCV2 genome (Olvera et al., 2007).

The first description of PMWS was in Canada in 1991 (Harding and Clark, 1998) and, since then, it has been described in many parts around the world (Segalés et al., 2005). Retrospective analysis in pig samples demonstrated that PCV2 infection in the livestock occurred many years before the epizootic outbreaks described by mid and late 90s (Magar et al., 2000; Rodriguez-Arrioja et al., 2003). This fact together with presence of PCV2 in both PMWS and non-PMWS affected pigs and farms also suggested the possible existence of differences in pathogenicity between different PCV2 strains. Despite most sequencing studies did not find any relation between PCV2 sequences and the occurrence of the disease (Larochelle et al., 2002, 2003; Pogranichniy et al., 2002; de Boisseson et al., 2004; Grierson et al., 2004; Wen et al., 2005; Martins Gomes de Castro et al., 2007), some recent studies and field observations (Timmusk et al., 2005; Opriessnig et al., 2006; Cheung et al., 2007a,b; Stevenson et al., 2007; Woodbine et al., 2007) have suggested the opposite. Moreover, epidemiological Danish and British studies strongly suggested that the spread of porcine circovirus diseases (PCVDs) is consistent with the introduction of a ‘‘new infectious agent’’ or a ‘‘new strain’’ of a known agent into a naïve population (Vigre et al., 2005; Woodbine et al., 2007).

Based on the controversy of potential different pathogenicity among PCV2 strains, the main objective of this study was to elucidate if *ORF2* PCV2 sequences could be correlated with different health or disease status of farms and/or pigs. Concomitantly, a potential definition of genotypes in PCV2 was explored. Moreover, we intended to determine if multiple sequences can be present in the same animal at the same time, as previously suggested (de Boisseson et al., 2004; Opriessnig et al., 2006; Cheung et al., 2007a).

2. Materials and methods

2.1. Sample collection

Six farms (Nos. 1–6, Table 1) located in North-Eastern Spain with historical records of PMWS were

Table 1
Characteristics of farms and studied animals

Farm	History of PMWS	Clinical PMWS compatible picture	Weeks of age	PMWS confirmation	Number of studied animals (number of animals with available ORF2 PCV2 sequence)				% Mortality[a]
					PMWS	Wasted non-PMWS	Healthy	Sows	
1	Yes	Yes	17–18	Yes	2 (1)	6 (4)	4 (3)	14 (2)	6.0
2	Yes	Yes	18–21	Yes	7 (6)	4 (4)	5 (2)	12 (0)	8.0
3	Yes	Yes	15–17	Yes	6 (5)	6 (3)	4 (1)	12 (0)	10.0
4	Yes	Yes	12–15	Yes	4 (3)	6 (3)	5 (0)	12 (0)	17.4
5	Yes	Yes	11–15	Yes	9 (7)	2 (0)	5 (1)	12 (0)	17.4
6	Yes	Yes	15–17	No	0 (0)	12 (7)	5 (2)	12 (0)	7.0
7	No	No	12	No	0	0	40 (3)	0	4.3
8	No	No	12	No	0	0	40 (3)	0	4.9
Total					28 (22)	36 (21)	108 (15)	74 (2)	

[a] Percentage of mortality during the fattening period (from 8 to 24 weeks of age).

included in a longitudinal case-control study performed during years 2005 and 2006. Actually, farms 4 and 5 corresponded to two batches of pigs from the same farm. The diagnosis of PMWS at farm level was confirmed before the start the study. Diagnostic procedures included a high percentage (>10%) of pigs with wasting and mortality in postweaning (nurseries, fattening and finishing) areas and the individual diagnosis of PMWS (Segalés et al., 2005) in at least one out of five necropsied pigs.

One hundred piglets per farm from 12 to 14 randomly selected sows were ear-tagged at birth and followed up until PMWS outbreak occurrence. Sows were bled at farrowing. When PMWS compatible clinical picture (Segalés and Domingo, 2002) appeared, those animals showing clinical signs were bled, euthanized and necropsied (n = 8–12 per farm). Moreover, one healthy age matched pig per every two diseased pigs was also euthanized and sampled in the same manner with a maximum of 5 per farm (n = 4–5 per farm). Sections of lymph nodes (tracheobronchial, mesenteric, superficial inguinal and submandibular) and tonsil were collected and fixed by immersion in neutral-buffered 10% formalin.

On the other hand, in 2006, 80 pigs from 2 different farms (Nos. 7 and 8) located also in North-Eastern of Spain and without history of PMWS were bled at 3 months, which corresponds to an age where PCV2 viremia is usually present (Larochelle et al., 2003; Shibata et al., 2003).

All treatments, housing, husbandry and slaughtering conditions were conformed to the European Union Guidelines and Good Clinical Practices.

2.2. Histopathology

Formalin-fixed paraffin-embedded blocks containing the above-mentioned lymphoid tissues were prepared. Two consecutive 4 μm thick sections corresponding to each pig were cut from each block. One section was processed for histopathology, while the other was processed for PCV2 nucleic acid detection by *in situ* hybridization (ISH) (Rosell et al., 1999). PMWS was diagnosed when pigs fulfilled international accepted criteria (Segalés et al., 2005). Pigs were finally classified into three different categories: (i) *PMWS cases*: pigs showing clinical wasting, moderate to severe PMWS characteristic histopathological lymphoid lesions and moderate to high amount of virus within the lesions; (ii) *wasted non-PMWS cases*: pigs showing clinical wasting but without or slight PMWS characteristic histopathological lymphoid lesions and none or low amount of PCV2 genome within lymphoid tissues; and (iii) *healthy pigs*: pigs showing good clinical condition, which presented none or slight PMWS characteristic histopathological lymphoid lesions and none or low amount of PCV2 genome within lymphoid tissues.

2.3. Screening of serum samples by polymerase chain reaction (PCR) and sequencing

DNA from serum was extracted using a commercial kit (Nucleospin® Blood, Macherey Nagel) and tested using a previously described PCV2 PCR (Quintana et al., 2001). From samples positive by PCV2 PCR, whole ORF2 was amplified using specific primers (capFw 5′-CTTTTTTATCACTTCG TAATG-3′ and capRw 5′-CGCACTTCTTTCGTTTTC-3′) under previously reported conditions (Fort et al., 2007). Amplicon products from ORF2 PCV2 PCR positive samples were purified (MiniElute®, Qiagen) and both strands were sequenced at least twice, using the same above-mentioned specific primers. Cycle sequencing was carried out with BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) following the manufacturers' instructions.

When sequences with multiple peaks at the same position were observed in the chromatogram, PCR products were cloned and sequenced in order to elucidate possible multiple sequences. The ORF2 PCV2 gene was amplified as already mentioned (Fort et al., 2007) with a final extension of 72 °C for 20 min and was cloned into the pGEMT® vector system (Promega), transformed in *Escherichia coli* TOP10 competent cells and screened following manufacturers' instructions. Positive colonies were detected with the mentioned ORF2 PCR with the unique variation of the first denaturation, being at 96 °C for 10 min. Plasmid DNA was extracted by QIAprep spin Kit according the manufacturers' instructions and sequenced using universal M13 primers.

2.4. Phylogenetic analysis of ORF2 PCV2 sequences

Sequence data was compiled and analyzed using Sequence Analysis (Applied Biosystems, Foster City, USA) and Fingerprinting II software (Informatix™ Software, 2000). Sequences were aligned using Crustal W method. Phylogenetic relationships among sequences were analyzed as described in Olvera et al. (2007) using parsimony and nucleotide distance methods. Firstly, the heuristic search option of PAUP 4.0.b (Swofford, 1998), considering a single stepwise addition procedure, and a tree bisection-reconnection (TBR = 100) branch swapping algorithm, was used for unweighted maximum parsimony analysis (MP). A majority rule consensus tree was then generated from the 100 most parsimonious trees found in each of the 1000 bootstrap replicates of the analysis. Secondly, we computed a nucleotide distance matrix between sequences to infer phylogenies by a neighbor-joining (NJ) and a maximum likelihood (ML) trees using, respectively MEGA 3.1 (Kumar et al., 2001) and TreePuzzle 5.0 (Schmidt et al., 2002). Confidence in the NJ tree was estimated by 1000 bootstrap replicates. The tree search quartet-puzzling algorithm directly assigned estimations of support to each internal branch of the ML tree. Trees were rooted with two ORF2 PCV1 sequences (accession numbers AY660574 and AY193712).

2.5. Genotype definition

Data from the pairwise comparison of the obtained ORF2 PCV2 sequences together with 148 ORF2 PCV2 sequences present at the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) in September 2005 (Olvera et al., 2007) and two PCV1 ORF2 sequences (for rooting purposes) (accession numbers AY660574 and AY193712) were used to construct a matrix of *p*-distance values. *p*-Distance is the proportion of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared (Kumar et al., 2001). Afterwards, *p*-distance/frequency histogram was constructed in order to determine possible cut-off values to distinguish different PCV2 genotypes (Biagini et al., 1999; Rogers and Harpending, 1992).

2.6. Phylogenetic analysis among populations (farms)

Patterns of nucleotide diversity distribution among farms were estimated by a hierarchical nested analysis of molecular variance (AMOVA) (Excoffier et al., 1992) of the frequency distribution of sequences and their pairwise divergence at three hierarchical levels: within farms (φ_{st}), among farms within groups (φ_{sc}) and among groups (φ_{ct}). Those analyses were performed using Arlequin software (Version 3.1) and considered two groups of farms: (i) PMWS affected farms (Nos. 1–6) and (ii) non-PMWS affected farms (Nos. 7 and 8). Moreover, phylogenetic relationships among populations were assessed by computing a NJ population tree from the distance matrix of nucleotide divergence among farms.

2.7. Nucleotide sequence accession numbers

The ORF2 PCV2 sequences reported in this work have been deposited at GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers EF647642-EF647728.

3. Results

3.1. Clinical picture and PMWS diagnosis

Clinical picture compatible with PMWS appeared in farms Nos. 1–6 between 11 and 21 weeks of age, depending on the farm (Table 1). Acute clinical signs occurred during a period of about 3 weeks. Farms 1–5 suffered from PMWS based on clinical signs, histopathological lesions in lymphoid tissues and PCV2 detection within lesions. In farm 6, PMWS was suspected based on the occurrence of wasting in the growing phase, but pathological studies could not finally confirm the diagnosis of the disease. No clinical signs compatible to PMWS were observed in farms 7 and 8 and no pathological studies were carried out. Mortality rates of the fattening period in each studied farm are given in Table 1.

Table 2
Animals showing more than one ORF2 PCV2 sequence at the same time

Pig reference[a]	Clinical status	Number of different sequences in the same pig (number of clones sequenced)	Number of different sequences within genotype 1 (number of sequenced clones)	Number of different sequences within genotype 2 (number of sequenced clones)	Sequence identity between different sequences
1–17	Wasted non-PMWS	3(4)	3(4)	0(0)	98.8–99.2
2–26	Wasted non-PMWS	3(3)	2(2)	1(1)	92.9–99.7
4–87	Wasted non-PMWS	3(4)	3(4)	0(0)	99.7–99.8
2–75	PMWS	6(7)	5(6)	1(1)	91.3–99.8
3–85	PMWS	3(5)	3(5)	0(0)	99.4–99.8
4–88	PMWS	3(5)	3(5)	0(0)	99.1–99.8
1–50	Healthy	5(6)	1(2)	4(4)	92.5–99.8
Total		26(34)	20(28)	6(6)	90.2–99.8

[a] Number of farm-number of pig.

3.2. PCV2 PCR and ORF2 sequencing

Prevalence of PCV2 by PCR in pig sera from PMWS affected farms calculated based on necropsied animals ranged from 40 to 93.8%. The highest prevalence in those farms was found in PMWS affected pigs (median 85.7%; max. 100%, min. 66.7%), followed by wasted non-PMWS affected pigs (median 83.3%; 100–33.3%), healthy pigs (median 60.0%; 75.0–20.0%) and sows (median 0%; 14.3–0%). On the other hand, the prevalence was 10% in both non-affected PMWS studied farms.

A total of 87 ORF2 PCV2 sequences coming from 60 animals (58 pigs and 2 sows) were obtained (Table 1). Seven out of 60 (11.7%) serum samples tested corresponded to pigs that yielded more than one sequence; all of them from PMWS affected farms. Thirty-four clones containing the ORF2 PCV2 sequence were obtained and sequenced from those seven serums containing multiple sequences. Results confirmed that those seven animals had more than one sequence at the same time. From these 34 sequences, 26 were different in at least 1 nucleotide. Three out of the seven animals harboured ORF2 PCV2 sequences showing low percentage of identity (91–93%) (Table 2).

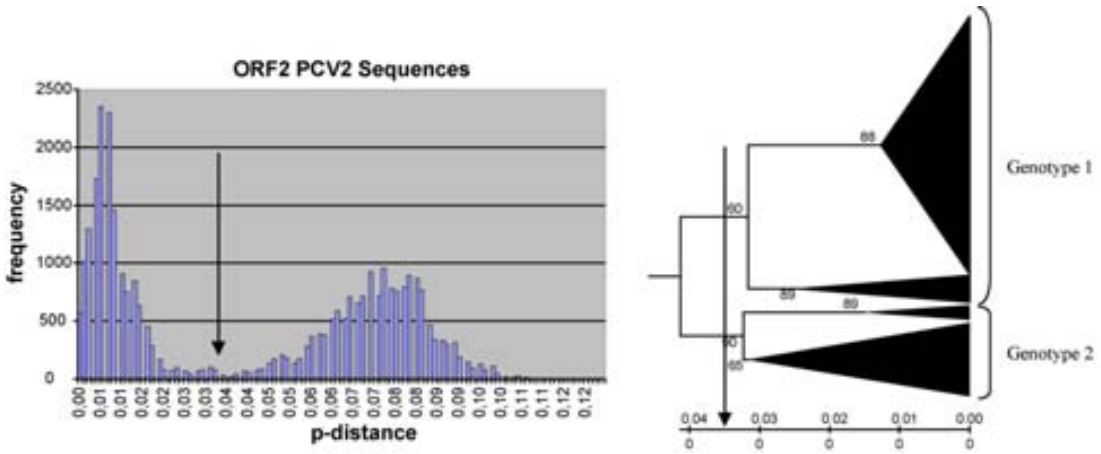

Fig. 1. Plot: frequency distribution of pairwise distances between ORF2 PCV2. Tree: collapsed PCV1 rooted NJ tree deduced from the comparison of 235 PCV2 ORF2 sequences. Vertical arrows indicate the cut-off value to distinguish both genotypes. Sequences belonging to distinct genotypes show genetic distances >0.035.

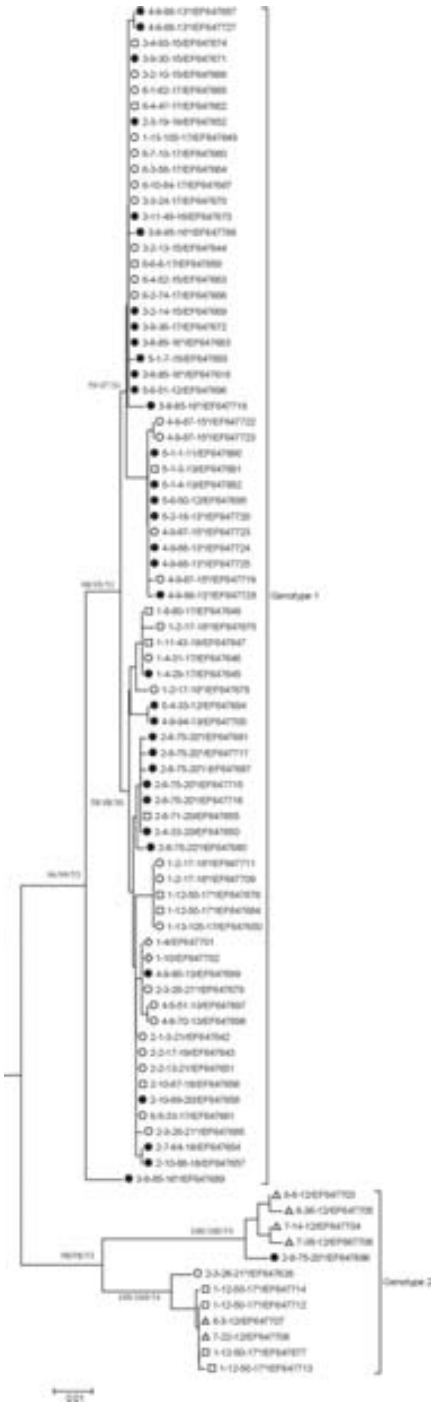

Fig. 2. Phylogenetic tree based on the NJ method for 87 ORF2 PCV2 sequences and PCV1 rooted using 1000 bootstraps. Numbers along the branches refer to the percentages of confidence in the NJ, MP and ML analyses, respectively. Minor branches values are hidden. References means: number of farm-number of sow-number of piglet-weeks of age. (●) PMWS case, (○) wasted non-PMWS case, (□) healthy from PMWS affected farm, (△) healthy from non-PMWS affected farm, and (◇) sow. *Sequences obtained from different clones of same animal.

3.3. Genotype definition

The *p*-distance/frequency histogram obtained from the 235 ORF2 PCV2 sequences was clearly bimodal; one mode corresponded to the number of differences between phylogenetic groups, and the other to differences among sequences within groups (Fig. 1). Considering the possible different cut-off values tested for the definition of genotypes, only one gave results that matched phylogenetic results. That value was finally established at 3.5%, since it was located in a fairly equidistant position between both peaks and agreed with the distance observed between both groups 1 and 2 in the NJ phylogenetic tree. Thus, two ORF2 PCV2 sequences could be assigned to different genotypes (1 or 2) when the genetic distance between them was >3.5%. On the other hand, the branch length observed in the collapsed PCV1 rooted NJ tree (Fig. 1) was higher in genotype 2 than in genotype 1, indicating major variability inside genotype 2.

According to this definition, sequences included in groups 1 and 2 (Olvera et al., 2007) would constitute genotypes 1 and 2, respectively. It is worthy to say that a similar *p*-distance/frequency histogram is obtained using the 148 whole PCV2 sequences present in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) in September 2005 (Olvera et al., 2007). In that case, the cut-off value to differentiate PCV2 genotypes was established at 2.0% (data not shown).

3.4. Phylogenetic analysis of ORF2 sequences

All three algorithms for ML, MP and NJ methods reported congruent results, and the groupings were supported by high confidence values. NJ tree with 1000 bootstrap is shown in Fig. 2. The 87 sequences obtained could be divided into two main clusters supported by high confidence values that matched the two genotypes defined above: genotype 1 ($n = 75$) and genotype 2 ($n = 12$), equivalent to groups 1 and 2 previously reported by Olvera et al. (2007). Interestingly, PCV2 sequences detected in animals coming

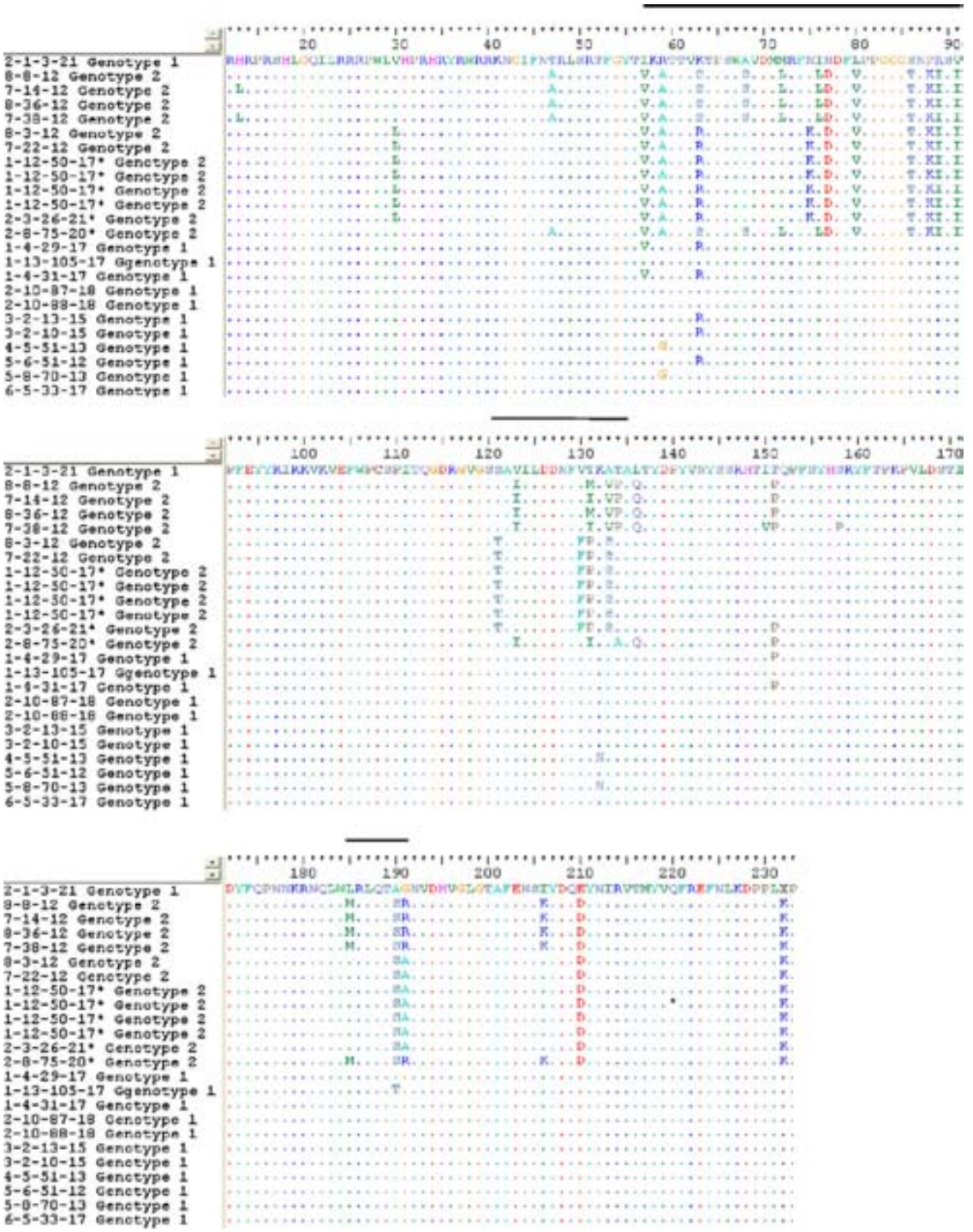

Fig. 3. Amino acid sequences of capsid protein. All 12 obtained sequences from genotype 2 as well as 12 randomly selected sequences from genotype 1 are represented. Heterogenic regions are marked in black lines. References means: number of farm-number of sow-number of piglet-weeks of age. *Sequences obtained from different clones of same animal.

Table 3
Hierarchical nested analysis of molecular variance (AMOVA) results

Source of variation	d.f.	Sum of squares	Variance components	% of variation	Related φ-statistics	p-Value
Within farms	81	440.6	5.43	20.50	$\varphi_{st} = 0.79494$	$\ll 0.001$
Among farms within groups	6	128.3	1.34	5.08	$\varphi_{sc} = 0.19840$	$\ll 0.001$
Between groups	1	231.5	19.74	74.42	$\varphi_{ct} = 0.74418$	$\ll 0.001$
Total	88	800.3	26.52	100		

from PMWS affected farms were mainly included within genotype 1, corresponding more specifically to cluster 1A of Olvera et al. (2007) classification, and were randomly distributed among this group. On the other hand, genotype 1 was never found in non-PMWS affected farms. All sequences obtained from healthy pigs from non-PMWS farms ($n = 6$) were included within genotype 2, corresponding to clusters 2C and 2D of Olvera et al. (2007) classification. The other six sequences included within genotype 2 corresponded to sequences obtained from three animals (one PMWS case, one wasted non-PMWS and one healthy pigs) from PMWS affected farms. At the same time, these three animals also harboured at least one sequence included in genotype 1. Therefore, none of the PCV2 infected pigs coming from PMWS affected farms contained only ORF2 PCV2 sequences genotype 2, since all of them were always co-infected with PCV2 genotype 1.

Two ORF2 PCV2 sequences obtained from two sows from the same PMWS affected farm (No. 1) were also included in genotype 1 and had a sequence identity of 100%. Moreover, from one of those sows, two ORF2 PCV2 sequences were obtained from two of its offspring (one PMWS and one wasted non-PMWS affected pigs). Interestingly, sequences obtained from both pigs had a 100% of identity between them and were different from that obtained from its mother (sequence identity: 99.4%, having four nucleotide differences, resulting in four amino acid predicted differences).

The lowest nucleotide and amino acid homology observed between all ORF2 PCV2 studied sequences was 90.2 and 86.5%, respectively. Three different regions with high heterogeneity were observed in amino acid positions 57–91, 121–136 and 185–191 and the amino acid alignment showed the existence of specific patterns to each group. Thus, up to 15 amino acid substitutions were observed in all the 12 sequences present in genotype 2, all of them fairly conserved within genotype 1. Eleven out of 15 positions suffered always the same substitution, and 8 out of those 11 positions were located within the first described heterogenic region (residues 57–91). The other three constant substitutions were located at positions 190, 210 and 232 (Fig. 3).

3.5. Phylogenetic analysis among populations (farms)

The high level of population structuring observed in the AMOVA analyses ($\varphi_{st} = 0.79494$, $p \ll 0.001$) indicates that the pattern of population relationships is related with the presence or absence of PMWS at farm level ($\varphi_{ct} = 0.74418$, $p \ll 0.001$). In addition, the low level of variation detected among farms within groups ($\varphi_{sc} = 0.19840$, $p \ll 0.001$) emphasizes the uniformity of the groups described (Table 3).

Additional support to the differences detected among farms is provided by the population tree (Fig. 4), where clustering also followed the presence or absence of PMWS disease. Moreover, farms 2–6 were more closely related between them than with farm No. 1. Despite PMWS could not be diagnosed in the batch of studied animals from farm No. 6, that farm was grouped with PMWS affected, being in agreement with the clinical picture observed and the previously diagnosed history of PMWS.

4. Discussion

In the present study, we characterized and compared 87 ORF2 PCV2 sequences obtained from 60 animals from 7 different farms with the main aim to elucidate if ORF2 PCV2 sequences could be correlated with different health/disease status of PCV2 infected pigs and/or farms. Present results

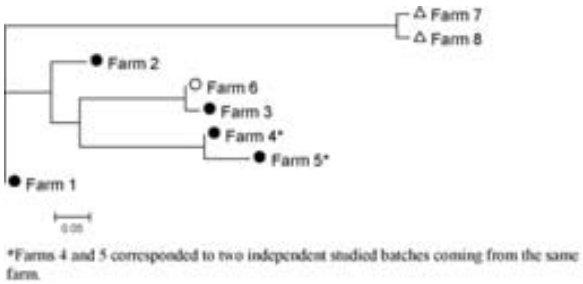

Fig. 4. Population tree (NJ) clustering eight farms according to the distance matrix of nucleotide divergence. (●) History of PMWS with PMWS diagnosed in the studied batch, (○) history of PMWS without PWMS diagnosis in studied batch, and (△) non-PMWS affected farm.

demonstrate the existence of two different genotypes within PCV2 sequences: genotypes 1 and 2. While pigs from PMWS affected farms harboured PCV2 genotype 1 always (with or without sequences from genotype 2), pigs from non-PMWS affected farms had exclusively sequences from genotype 2. Moreover, we examined if multiple PCV2 different sequences can be present in the same animal at the same time, which was demonstrated in 7 out the 60 PCV2 infected studied pigs.

Globally, in the seven studied farms, we observed a higher prevalence of PCV2 in pigs coming from PMWS affected farms than in pigs coming from non-PMWS affected farms, being in agreement with previous reports (Larochelle et al., 2003; Shibata et al., 2003; Sibila et al., 2004). At the same time, within PMWS affected farms, the prevalence of PCV2 in PMWS affected pigs was also higher than in healthy pigs (Liu et al., 2000; Larochelle et al., 2003).

It is known that the highest amount of virus in PCV2 infected pigs is found in lymphoid tissues (Mankertz et al., 2000). However, we decided to perform the present study on serum samples based on previous works that indicated blood as the most suitable sample for PCV2 detection by PCR without the need of euthanizing the animal (Shibata et al., 2003). Despite we sacrificed either diseased and healthy pigs from PMWS affected farms, the use of serum samples allowed us to analyze a high number of healthy animals from non-PMWS affected farms without euthanizing them.

The mandate of the International Committee on Taxonomy of Viruses (ICTV) does not include any consideration below the species level and there is no formally accepted definition for any taxa below it; hence, this has been left to the initiative of specialty groups (Fauquet and Stanley, 2005). In the present work, an effort was made to evaluate the presence of distinct PCV2 genotypes and to unify the variable terminology that is nowadays reported in the literature. Obtained results demonstrate the existence of two clearly distinct PCV2 genotypes: genotypes 1 and 2 supported by molecular and biological features. Both genotypes corresponded to two different groups that were already described in different studies but usually using different nomenclature. Thus, genotypes 1 and 2 correspond, respectively to groups 1 and 2 of Olvera et al. (2007), patterns 321 and 422 reported by Carman et al. (2006), I and II reported by de Boisseson et al. (2004), SG3 and SG1/SG2 reported by Timmusk et al. (2005), and A and B reported by Martins Gomes de Castro et al. (2007).

Our results support the hypothesis of differences in pathogenicity between PCV2 genotypes, since differences between ORF2 PCV2 sequences obtained from animals from PMWS affected farms (mainly from genotype 1) and from animals from non-PMWS affected farms (all of them from genotype 2) were observed. Therefore, present results suggest that viruses from genotype 1 may potentially be more pathogenic than those from genotype 2 (Timmusk et al., 2005; Cheung et al., 2007a,b; Stevenson et al., 2007). In addition, the major variability found inside genotype 2 using the data from NCBI until September 2005 suggest that this genotype is older than genotype 1 (Carman et al., 2006; Cheung et al., 2007a). Although in favour, if this explains the emergence of PMWS by late 90s in many European and Asiatic countries as well as the recent re-emergence of the disease in North-America remains to be elucidated. On the other hand, present data confirmed that within PMWS affected farms there is no association between

the PCV2 sequences contained in individual pigs and its clinical status, being in agreement with some previous case-control studies (Larochelle et al., 2002; Pogranichniy et al., 2002).

There are different possible explanations because some previous works were not able to find such association between PMWS affected and non-PMWS affected farms (de Boisseson et al., 2004; Martins Gomes de Castro et al., 2007): on one hand, the presence of multiple sequences in the same animal can produce the sequencing of only one of the virus present in the pig. Moreover, obtaining PCV2 from non-PMWS affected farms requires exhaustive sampling due to the low viral load in serum of the animals (Olvera et al., 2004). On the other hand, it is very difficult to establish a farm as a non-PMWS affected due to the ubiquitous presence of PCV2 and that low number of cases of PMWS can be unnoticed (for example, one of the sampled animals in de Boisseson et al. (2004) initially considered as a non-PMWS farm was finally diagnosed as PMWS). Moreover, the lack of confident information about the farm sanitary status in the public available GenBank data makes practically impossible to compare both populations using GenBank data (Olvera et al., 2007).

Since the first description of the disease (Harding and Clark, 1998), many experimental infections have been performed and just a few of them have been successful in reproducing the disease using either PCV2 genotype 1 (Albina et al., 2001; Grasland et al., 2005; Wang et al., 2007) and genotype 2 (Allan et al., 1999, 2003; Krakowka et al., 2000; Harms et al., 2001). Therefore, both PCV2 genotypes seem to be able to reproduce PMWS under the appropriate circumstances. However, one recent experimental infection with PCV2 from both groups described more severe PMWS clinical signs and lesions in pigs infected with PCV2 genotype 1 than those infected with PCV2 genotype 2 (Cheung et al., 2007b). On the other hand, using PCV2 genotype 2, Opriessnig et al. (2006) were not able to reproduce PMWS, but they demonstrated differences in pathogenicity even within genotype 2, further supporting such variability among PCV2 sequences.

Population phylogenetic analysis showed significant differences between ORF2 PCV2 sequences from PMWS affected and non-affected farms. Those findings were strongly supported by the population tree, where non-PMWS affected farms were closed to each other and separately from PMWS affected farms. These two groups fitted perfectly the two genotypes obtained in the nucleotide sequence analysis. Moreover, the low variation observed in the population phylogenetic tree between PMWS affected farms could apparently be explained by the geographic location of breeding and/or weaning facilities, being in agreement with previous reports suggesting that minor genetic differences among PCV2 sequences could be accounted by their geographic origin (Fenaux et al., 2000; Meehan et al., 2001). Thus, sequences obtained from farm 1 (with breeding and weaning facilities separated approximately by 180 km from the rest of the studied farms, data not shown) had less similitude with those obtained from farms 2 to 6 (located all five in a radium of 40 km). On the contrary, there was no apparent relation with the geographic distribution of fattening facilities. On the other hand, data from one sow and its two offspring showed that ORF2 PCV2 sequences obtained from both pigs were identical between them and different from that obtained from the mother. Considering the possibility here demonstrated that different sequences can be present in the same animal at the same time, we can not rule out that both pigs would have got PCV2 from its sow. Moreover, the fact that both ORF2 PCV2 sequences obtained from two different sows located in the same farm and in the same room were identical could be explained easily by horizontal transmission of PCV2 (Albina et al., 2001; Sibila et al., 2004).

Previous reports suggested that one animal can contain different PCV2 sequences at the same time (de Boisseson et al., 2004; Opriessnig et al., 2006) but this is the first work that has exhaustively studied this issue. Present results demonstrate that this is a relatively frequent finding in PMWS affected farms and that occurs in PMWS affected as well as in healthy animals. Interestingly, three animals (one PMWS, one wasted non-PMWS and one healthy) harboured sequences from genotypes 1 and 2 at the same time. Curiously, clones obtained from the PMWS affected pig contained predominantly sequences from genotype 1 while clones obtained from the healthy pig contained sequences predominantly from genotype 2. The biological importance of this fact is nowadays unknown, but it may reinforce the hypothesis that sequences from genotype 1 could be more pathogenic than those from genotype 2.

The amino acid alignment identified three major heterogenic regions between different ORF2 PCV2 sequences in regions 57–91, 121–136 and 185–191, similarly to those already described (Larochelle et al., 2002) and corresponding to two out of the three antigenic domains described by Mahe et al. (2000) (65–87, 113–139 and 193–207). Larochelle et al. (2002) pointed out the possible implication of these immunoreactive regions of the capsid protein of PCV2 as potential candidate regions involved in the emergence of PCV2 variants, data supported also by other recent reports (Wen et al., 2005). Specifically, up to 8 amino acid positions located in positions 57–91 were constantly different between sequences included in genotype 2 in respect those included in genotype 1. These data suggest that specific primers within this region could be designed to differentiate both genotypes by PCR techniques instead of using sequencing, resulting in a less expensive and time consuming diagnostic method.

In summary, the present work contributes to the understanding of PCV2 epidemiology, including the establishment of a genotype definition for PCV2 that should help unifying different nomenclatures and classifications used. Taking into account the present results and other recent data from USA and Canada (Carman et al., 2006; Cheung et al., 2007a) it seems evident that nowadays PCV2 sequences included in genotype 1 are more related to PMWS occurrence than PCV2 sequences included in genotype 2. Further *in vivo* and *in vitro* studies are needed to confirm the suggested differences in pathogenicity between genotypes and to establish relationship between PCV2 genotypes and PMWS occurrence.

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Addendum of study I

PCV-2 genotype definition and nomenclature.

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PCV-2 genotype definition and nomenclature

SIR, – To date, at least three different phylogenetic groups of porcine circovirus type 2 (PCV-2) have been recognised (Gagnon and others 2007, Dupont and others 2008, Grau-Roma and others 2008, Timmusk and others 2008). However, the mandate of the International Committee on Taxonomy of Viruses (ICTV) does not include any consideration below the species level; therefore, a definition for any taxon below such level has been left to the initiative of speciality groups (Fauquet and Stanley 2005). The objectives of the present letter are to reinforce the usefulness of the published guidelines to define PCV-2 genotypes and to propose a unified nomenclature for PCV-2 genotypes, replacing previous existing ones in the literature.

Grau-Roma and others (2008) addressed a non-arbitrary definition for PCV-2 genotypes. The methodology is based on the proportion of nucleotide sites at which two sequences being compared are different (p distance); this value is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared (Kumar and others 2001). Subsequently, the construction of a p distance/frequency histogram (Fig 1) allows the determination of potential cut-off values to distinguish different genotypes (Rogers and Harpending 1992, Biagini and others 1999). This system can be used either on the whole sequence of the virus or on partial sequences. Since phylogenetic trees based on PCV-2 open reading frame 2 (ORF2) are similar to those based on the whole PCV-2 genome (Olvera and others 2007), we suggest using the ORF2 gene (which can be sequenced based on a single PCR) to perform genotyping for PCV-2. Using this methodology, ORF2 PCV-2 sequences are assigned to different genotypes when the genetic distance between them is 0·035. This cut-off is located in a fairly equidistant position between both peaks (Fig 1) and agrees with the distance observed between viral sequence groups in the phylogenetic trees. Using these criteria with sequences available at the GenBank database (www.ncbi.nlm.nih.gov), three PCV-2 genotypes have been defined.

Two of these PCV-2 genotypes were already known, as they fit with the major branches of the phylogenetic trees derived from different methods. However, their nomenclature has become 'research group-dependent'. Thus, genotypes '1' and '2' from Grau-Roma and others (2008) correspond, respectively, to groups '1' and '2' (Olvera and others 2007), 'I' and 'II' (De Boisséson and others 2004), 'SG3' and 'SG1/SG2' (Timmusk and others 2008), 'A' and 'B' (Martins Gomes de Castro and others 2007), 'b' and 'a' (Gagnon and others 2007) and restriction fragment length polymorphism patterns '321' and '422' (Carman and others 2008). Therefore, in order to counteract the current scientific confusion on genotype names, the members of the EU consortium on porcine circovirus diseases (www.pcvd.org) propose a standardised nomenclature for PCV-2 genotypes. This proposal aims to avoid confusion with the designation of the two existing species of porcine circoviruses (types 1 and 2) and recommends reconciliation with an existing nomenclature system (Gagnon and others 2007).

The consortium proposes naming the three PCV-2 genotypes as PCV-2a, PCV-2b and PCV-2c. The first sequences recorded in GenBank will be regarded as the prototype viruses: accession number AF055392 (Stoon-1010) for PCV-2a, accession number AF055394 (48285) for PCV-2b and accession number EU148503 for PCV-2c. PCV-2a and PCV-2b genotype viruses have been recovered from postweaning multisystemic and wasting syndrome (PMWS) cases worldwide. Although retrospective studies have demonstrated that PCV-2a genotype viruses predominated on pig farms with and without PMWS in some countries from 1997 until 2003 (Allan and others 2007), other data indicate that PCV-2b strains have been more common in outbreaks of PMWS from 2004 onwards in North America (Gagnon and others 2007) and also in PMWS epizootics in some European countries (Dupont and others 2008, Timmusk and others 2008). To date, only three sequences fit into the PCV-2c genotype; these were from viruses recovered from Denmark during the 1980s (Dupont and others 2008), when PMWS was not present or at least was undetected.

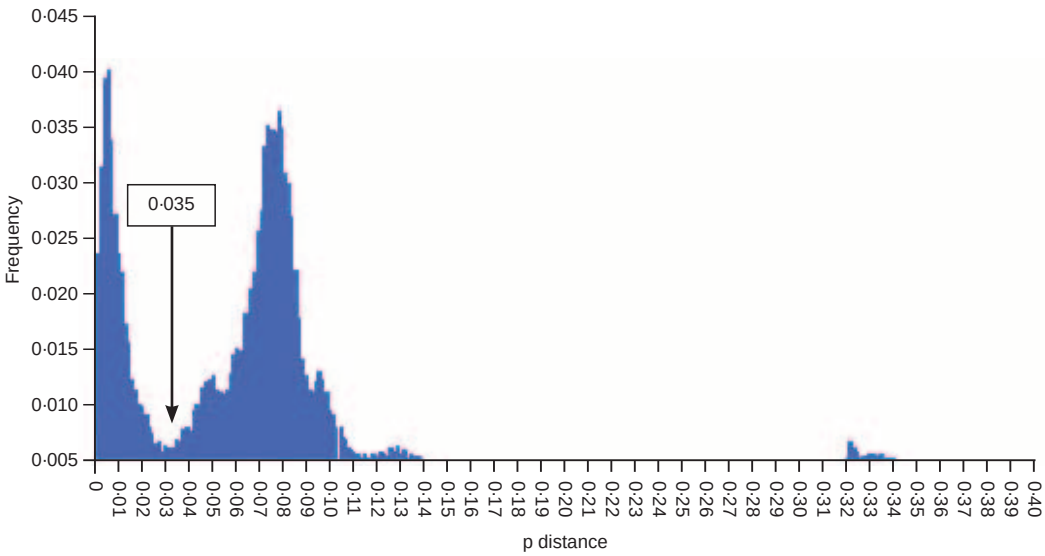

FIG 1: P distance/frequency histogram showing the frequency distribution of pairwise distances among 196 open reading frame 2 (ORF2) porcine circovirus (PCV) type 2 sequences. One ORF2 PCV type 1 sequence was also included for rooting purposes

J. Segalés, A. Olvera, L. Grau-Roma, *Centre de Recerca en Sanitat Animal – Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
C. Charreyre, *Merial, 69007 Lyon, France*
H. Nauwynck, *Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium*
L. Larsen, K. Dupont, *Department of Veterinary Diagnostics and Research, Technical University of Denmark, 1790 Copenhagen, Denmark*
K. McCullough, *Institute of Virology and Immunoprophylaxis, CH-3147 Mittelhäusern, Switzerland*
J. Ellis, *Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon 57N 5B4, Canada*
S. Krakowka, *Department of Veterinary Biosciences, College of Veterinary Medicine, Ohio State University, Columbus 43210, USA*
A. Mankertz, *Robert Koch-Institut, Division of Viral Infections (FG12), Nordufer 20, 13353 Berlin, Germany*
M. Fredholm, *Department of Animal and Veterinary Basic Sciences, Division of Genetics and Bioinformatics, Faculty of Life Sciences, University of Copenhagen, Copenhagen, Denmark*
C. Fossum, S. Timmusk, *Department of Biomedicine and Veterinary Public Health, Section for Immunology, Biomedical Centre, Swedish University of Agricultural Sciences, PO Box 588, SE 751 23 Uppsala, Sweden*
N. Stockhofe-Zurwieden, *Animal Sciences Group, Wageningen UR Infectious Diseases, Edelhertweg 15, PO Box 65, 8200 AB Lelystad, The Netherlands*
V. Beattie, *Devenish Nutrition, 96 Duncrue Street, Belfast BT3 9AR*
D. Armstrong, *BPEX, PO Box 44, Winterhill House, Snowdon Drive, Milton Keynes MK6 1AX*

B. Grassland, *Agence Française de Sécurité Sanitaire des Aliments, BP 53, F-22440 Ploufragan, France*
P. Baekbo, *Section for Veterinary Research and Development, Danish Pig Production, Vinkelvej 11, DK-8620 Kjellerup, Denmark*
G. Allan, *Virology Laboratory, Veterinary Sciences Department, Queen's University of Belfast, Belfast BT4 3SD*

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Study II

Inter-laboratory and inter-assay comparison on two real-time PCR techniques for quantification of PCV2 nucleic acid extracted from field samples.

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Short communication

Inter-laboratory and inter-assay comparison on two real-time PCR techniques for quantification of PCV2 nucleic acid extracted from field samples

C.K. Hjulsager [a,1,*], L. Grau-Roma [b,c,1], M. Sibila [b], C. Enøe [a], L. Larsen [a], J. Segalés [b,c]

[a] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1790 Copenhagen V, Denmark
[b] Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Barcelona, Spain
[c] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

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Abstract

Several real-time PCR assays for quantification of PCV2 DNA (qPCR) have been described in the literature, and different in-house assays are being used by laboratories around the world. A general threshold of 10^7 copies of PCV2 per millilitre serum for postweaning multisystemic wasting syndrome (PMWS) diagnosis has been suggested. However, neither inter-laboratory nor inter-assay comparisons have been published so far. In the present study, two different qPCR probe assays used routinely in two laboratories were compared on DNA extracted from serum, nasal and rectal swabs. Results showed a significant linear association between the assays ($p < 0.0001$), and a systematic difference of 1.4 $\log_{10}$ copies of PCV2 per millilitre of sample ($p < 0.0001$). This difference indicated that the assay from laboratory 1 yielded a higher output than the one from laboratory 2. Results also showed that there was no linear association between the amount of PCV2 DNA and the amount of total DNA, neither in nasal ($p = 0.86$) nor in rectal ($p = 0.78$) swabs, suggesting that normalizing of PCV2 DNA load in swab samples to total DNA concentration is not suitable. The present exploratory study highlights the need for the performance of ring trials on qPCV2 protocols between laboratories. Meanwhile, the proposed thresholds for PMWS diagnosis should only be considered reliable for each particular laboratory and each particular assay.

Keywords: Porcine circovirus type 2 (PCV2); Postweaning multisystemic wasting syndrome (PMWS); Quantitative real-time PCR; Inter-laboratory comparison

* Corresponding author. Tel.: +45 72346626; fax: +45 72346340.
E-mail address: ckh@vet.dtu.dk (C.K. Hjulsager).
[1] These authors contributed equally to the authorship of this work.

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1. Introduction

Porcine circovirus type 2 (PCV2) belongs to the Circoviridae family, genus *circovirus*, and is a small, non-enveloped, single-stranded ambisense circular DNA virus (Meehan et al., 1998). PCV2 is considered the essential infectious agent of postweaning multisystemic wasting syndrome (PMWS), a global disease of great economic impact (Segalés et al., 2005). Most if not all pigs become infected during their life, but only a proportion of them develop PMWS (Segalés et al., 2005). Therefore, the exclusive detection of PCV2 antibodies and/or viral DNA in serum or other samples is non-diagnostic for PMWS, indicating only current or past infection with PCV2 (Krakowka et al., 2005). Hence, diagnosis of PMWS requires clinical signs compatible with the disease (mainly wasting and respiratory distress), presence of characteristic PMWS microscopic lesions in lymphoid tissues (lymphoid depletion and granulomatous infiltration) and detection of moderate to high amounts of PCV2 within those lesions (Segalés et al., 2005).

Several quantitative PCR (qPCR) methods for detection of PCV2 nucleic acid have been developed in different laboratories, targeting either ORF1 (Ladekjaer-Mikkelsen et al., 2002) or ORF2 genes (Brunborg et al., 2004; Olvera et al., 2004). A threshold of 10^7 PCV2 DNA copies per millilitre of serum has been suggested to establish a PMWS diagnosis (Olvera et al., 2004). However, it is well known that many factors may influence the performance of PCR assays and that the results using different assays may vary, even when identical template DNA is tested (Bustin, 2002).

Although published qPCR methods to detect and quantify PCV2 have been evaluated separately, to date, no inter-laboratory or inter-assay comparisons have been published. The present study compares the performance of two different PCV2 qPCR assays used routinely in two different laboratories: one located in Denmark (laboratory 1), and another located in Spain (laboratory 2). Comparison was performed on the same DNA samples extracted from serum and nasal and rectal swabs collected from pigs from PMWS affected farms. The usefulness of normalizing PCV2 DNA load in swab samples to the total DNA present in each sample, in order to avoid possible PCV2 load variation due to sampling procedure, was also evaluated.

2. Material and methods

Ninety-nine samples randomly collected from pigs of different ages (1–21 weeks of age) from PMWS affected farms located in Spain and Denmark were studied. Specifically, 33 serum samples, and 33 nasal and 33 rectal swabs were analysed. For each type of sample, 16 out of 33 were obtained from Danish and 17 out of 33 from Spanish pigs. Danish (Kirudan A/S, Denmark) and Spanish (Collection swab, Eurotubo, Rubí, Spain) obtained swabs were placed in tubes containing 2 and 1 millilitre of phosphate-buffered saline (PBS) solution, respectively. Pigs were bled at the cranial cava vein and 5–10 millilitre of blood was collected in vacuum glass tubes (Vacutainer®, Becton-Dickinson, Meylan Cedex, France). Blood samples were allowed clotting at 4 °C or room temperature and then centrifuged at 200 × *g* for 10 min at 4 °C. All samples were kept at −80 °C until their use. DNA from Danish samples was extracted in laboratory 1 and DNA from Spanish samples was extracted in laboratory 2. In laboratory 1, DNA was extracted from 200 μl of serum, nasal swab (QIAamp DNA Mini Kit, Qiagen® GmbH, Germany) or rectal swab PBS solutions (QIAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany), and eluted in 200 μl elution buffer. In laboratory 2, DNA was extracted from 200 μl of serum or 300 μl of nasal swab PBS solution (Nucleospin® Blood, Macherey-Nagel, GmbH & Co. KG, Düren, Germany), and eluted in 100 μl buffer, whereas DNA was extracted from 300 μl of rectal swab PBS solution (QIAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany) and eluted in 200 μl buffer. Extracted DNA was divided into two tubes, one for analysis in each laboratory. Samples were sent between laboratories on dry ice. Thus, both laboratories analysed all 99 samples using their own qPCR assay described below.

The assay of laboratory 1 used Primer Probe Energy Transfer chemistry and the primers and PCV2 probe targeting ORF 1 described previously (Ladekjaer-Mikkelsen et al., 2002). PCR reactions containing 3 μl DNA, 1× PCR Gold Buffer (Applied Biosystems), 2.6 mM $MgCl_2$ (Applied Biosystems), 0.2 mM (each) dNTP mix, 100 nM forward primer, 300 nM reverse primer, 500 nM PCV2 probe, and 0.05 units/μl AmpliTaq Gold DNA Polymerase (Applied Biosystems) in 25 μl. PCR reactions were run on Rotorgene

3000 PCR machines (Corbett Research). PCR cycling was: 95 °C 10 min; 45 cycles of 95 °C 15 s, 60 °C 40 s, 75 °C 20 s; 75 °C 5 min; 95 °C 30 s; 45 °C 1 min; followed by melt curve analysis: ramping from 45 to 99 °C, rising by 1 °C per step. Fluorescence data were collected at the annealing step of the PCR amplification and at each step during the melt curve analysis. Samples were analysed in duplicate, including in each separate PCR run: negative PCR controls (Nuclease-free water, Amresco) and standards representing 10^7, 10^6, 10^4, and 10^2 plasmid copies of PCV2 per reaction, respectively. Standards were prepared as a dilution series in 100 ng/μl yeast tRNA (Ambion) of a PCR-product cloned into pCR2.1-TOPO vector (Invitrogen), the DNA concentration of which was determined spectrophotometrically (ND-1000 v.3.1, NanoDrop Technologies, Inc., USA). Only replicates having a standard deviation (S.D.) less than 0.30 were considered. The correlation coefficient (R^2) of standard curves was always >0.99 and the PCR assays showed a PCR efficiency of 90–100% measured from the standards. The quantification range of the assay was 3.3×10^4–3.3×10^9 copies of PCV2 per millilitre of sample material.

Laboratory 2 used a previously described TaqMan PCR method using primers and probe targeting specifically the PCV2 ORF2 gene (Olvera et al., 2004) with the only exception that an internal control was not included.

Intra-assay and inter-assay variability (Martell et al., 1999) was established by calculating the coefficient of variation (CV) percentage of the cycle threshold (Ct) values of each PCV2 standard dilution of the different independent PCR runs used in each laboratory (five runs in laboratory 1 and six runs in laboratory 2).

PCR results from both assays were expressed as the number of PCV2 copies per millilitre of serum, or nasal swab/rectal swab suspensions. Data were $\log_{10}$ transformed prior to statistical analysis, because of the highly skewed distribution of PCV2 copies per millilitre of sample. Descriptive statistics, agreement test, paired *t*-test and linear regression were conducted using SAS, Version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Agreement (Bland and Altman, 1986) and linear regression plots were constructed by SPSS, Version 15.0.1 (SPSS, Inc., Headquarters, USA) to further illustrate the agreement of the two assays and to assess bias. Moreover, to investigate the PCV2 DNA concentration measurements of the plasmid standards as a source of inter-assay variability, the DNA concentration of seven randomly selected DNA samples was determined by spectrophotometry in laboratory 1 (ND-1000 v.3.1, NanoDrop Technologies, Inc., USA) and laboratory 2 (BioPhotometer Eppendorf®, Hamburg, Germany).

Possible mismatches between the primers and probes used and PCV2 sequences from NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) were assessed by comparison with the previously described alignment focusing on marker positions, the nucleotide changes characteristic of each clade described in Olvera et al. (2007).

In order to evaluate the usefulness of normalizing PCV2 DNA load in swab samples by the total DNA present in each sample, a second set of rectal and nasal swabs was analysed in laboratory 2. Nasal ($n = 625$) and rectal ($n = 625$) swab samples collected from 120 pigs aged between 1 and 21 weeks and coming from Spanish PMWS affected farms, were processed and analysed by PCV2 qPCR as described above for laboratory 2. In addition, total DNA was assessed from all the 1250 DNA extracts by fluorometry (Fluoroscan Ascent1 FL, Thermo Labsystem, Helsinki, Finland), using Picogreen staining (PicoGreen1 dsDNA Quantitation Reagent Kit, Molecular Probes, Eugene, USA). Thus, the viral concentrations in swabs were also expressed as the mean $\log_{10}$ viral DNA copy number per total amount of DNA present in the sample. Regression analyses were performed to assess whether there was a linear association between the results expressed as $\log_{10}$ viral DNA copy numbers per millilitre of PBS and $\log_{10}$ viral DNA copy numbers per microgram of total DNA, as well as to compare the association between the total amount of DNA and copies of PCV2 per millilitre present in each sample.

3. Results

Mean and S.D. of the quantification of PCV2 DNA in the three different types of samples by both assays are summarized in Table 1. Obtained results comprised a wide spectrum of PCV2 loads, from negative samples to 10^{11} copies PCV2 per millilitre of sample. Considering the 62 samples that were positive by both tests, a significant association between the assays was observed by linear regression ($p < 0.0001$), with a

Table 1
Log_{10} copies of PCV2 per milliliter of sample by laboratories 1 and 2 qPCR assays

	Number of samples (sera; nasal swabs; rectal swabs)	Mean ± S.D. in laboratory 2 assay (sera; nasal swabs; rectal swabs)	Mean ± S.D. in laboratory 1 assay (sera; nasal swabs; rectal swabs)
Positive results in both assays	62 (23; 20; 19)	6.3 ± 1.8 (6.2 ± 1.8; 6.9 ± 1.9; 5.9 ± 1.7)	7.7 ± 1,9 (7.7 ± 1.8; 8.4 ± 1.9; 7.0 ± 1.8)
Positive results by laboratory 1 and negative by laboratory 2	25 (6; 9; 10)	Negative	4.6 ± 0.7 (4.6 ± 0.9; 4.7 ± 0.8; 4.7 ± 0.6)
Negative results by both laboratories	12 (4; 4; 4)	Negative	Negative

slope close to one (0.979) and an intercept of 1.505 (Fig. 1). The mean (±S.D.) difference between the assays was $1.4 \pm 0.6\ \log_{10}$ copies of PCV2 per milliliter, which was significant in the paired *t*-test ($p < 0.0001$). This difference indicated that the assay from laboratory 1 generally yielded a higher output than the one from laboratory 2. Similar results were obtained when linear regression and paired *t*-tests were performed for serum, nasal and rectal swabs separately (data not shown).

In order to further characterise the significant difference and compare results from the two assays, an agreement plot of the differences in PCV2 copy number between the assays against their mean was constructed (Fig. 2). Normality plot of the differences between the assays showed an apparently normal distribution (figure not shown). However, a Shapiro-wilk test for normality showed that the differences were not normally distributed ($p = 0.0107$, skewness = −0.15, kurtosis = 1.96), probably due to a high peakness of the distribution. When a linear regression of the differences in PCV2 copy number between the assays and their mean was assessed (Fig. 2), a non-significant slope was found ($p = 0.405$). Moreover, when a linear regression of the absolute values of the centred difference between the assays and their mean was assessed, a non-significant slope was found ($p = 0.3195$). Both regression analyses indicated that the difference between the values of the two assays is independent of the virus count. The mean difference of $1.4\ \log_{10}$ copies of PCV2 per milliliter, with limits of agreement being $0.2–2.6\ \log_{10}$ copies of PCV2 per milliliter (Fig. 2), showed that the results from laboratory 1 were systematically higher than those from laboratory 2.

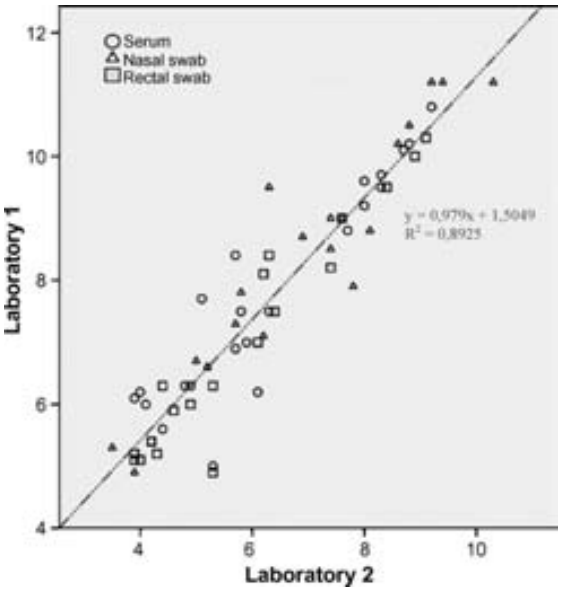

Fig. 1. Linear regression of 62 positive samples by both laboratories. All the results are expressed as $\log_{10}$ copies of PCV2 per millilitre of sample.

Twenty-five out of the 99 samples analysed were positive for PCV2 in laboratory 1 but negative in laboratory 2 (Table 1). All these samples contained levels of PCV2 DNA which, taking into account the systematic difference between the assays, would be below or close to the limit of detection of the assay used in laboratory 2 (10^4 copies of PCV2 per millilitre). Twelve samples gave negative results in both laboratories. Since negative results by qPCR mean that the amount of PCV2 is either zero or below the detection limit of the respective technique, negative samples were excluded from the statistical analysis described above.

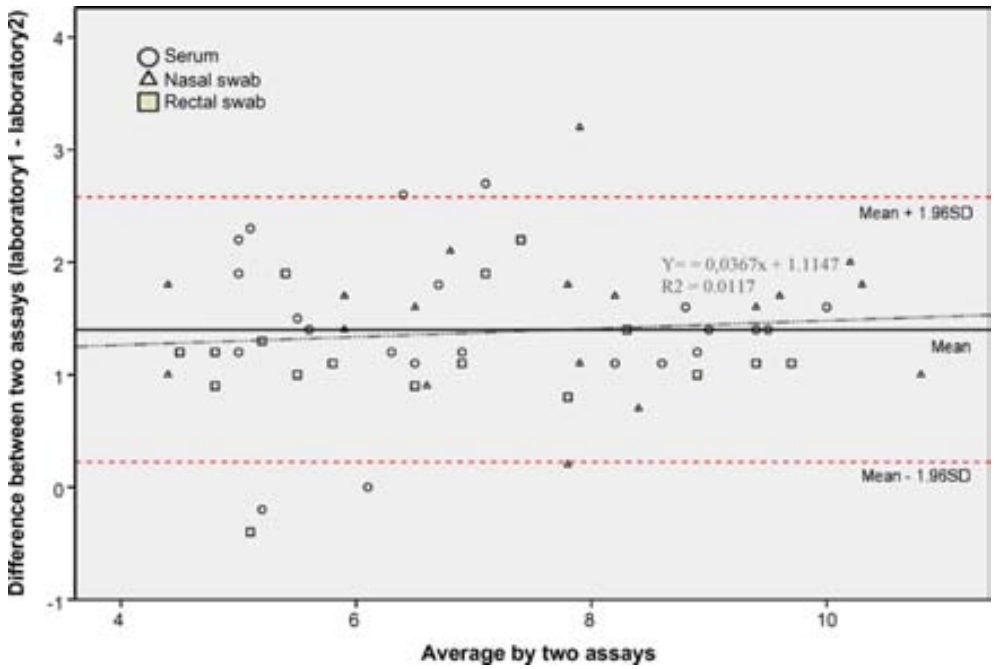

Fig. 2. Agreement plot including the 62 positive samples by both assays. All the results are expressed as $\log_{10}$ copies of PCV2 per milliliter of sample.

The average intra-assay CV was 0.79% for laboratory 1 and 0.67% for laboratory 2. The average inter-assay CV was 1.73% in laboratory 1 (between five PCR runs used to obtain present results) and 1.36% in laboratory 2 (between the six PCR runs used).

Significant linear association ($p < 0.0001$) with a high coefficient of correlation ($R^2 = 0.998$) was found between total DNA concentration measurements of the seven DNA samples analysed by both laboratories. Moreover, no bias was observed between measurements from both laboratories when agreement plot was constructed (data not shown).

One of the targeting nucleotides of the forward primer used in laboratory 1 contained a described marker position at genome position 737. The primers and probes used in laboratory 2 all annealed outside described marker positions.

In the second set of studied samples, 237 and 147 out of 625 nasal and 625 rectal swabs, respectively, gave positive PCV2 qPCR results. Total DNA mean ± S.D. in nasal and rectal swab DNA extracts was 7.9 ± 17.2 and 0.7 ± 0.13 μg DNA per milliliter of sample material (PBS), respectively. A significant association between the expression of copies of PCV2 per millilitre of PBS and copies of PCV2 per microgram of total DNA, was observed by linear regression for both nasal ($p < 0.0001$) and rectal swabs ($p < 0.0001$). However, linear regression of copies of PCV2 per millilitre of PBS versus total amount of DNA per millilitre of PBS, from the studied swabs with positive PCV2 qPCR, showed that there was no association between parameters, neither when all swabs were considered together ($p = 0.32$) nor separately (nasal swabs: $p = 0.86$, rectal swabs: $p = 0.78$). Therefore, although a significant correlation between viral DNA copy numbers per millilitre of PBS and per microgram of total DNA was observed, no association between the viral load and the total DNA concentration of samples was detected.

4. Discussion

PMWS diagnosis requires the necropsy of suspicious animals and the access to histopathological expertise, which is not available in all diagnostic laboratories worldwide. On the other hand, qPCR is a very attractive methodology for laboratory diagnosis

of infectious diseases, because it could be carried out on live animals on easily accessible material such as serum samples and/or faecal and rectal swabs, and could provide rapid results and precise quantification in an easy-to-use platform (Bustin, 2002). However, the lack of harmonization among PCV2 qPCR methods raises the question whether results obtained by different laboratories are comparable and thereby whether a theoretical general threshold is valid for all methods.

The inter-laboratory assay comparison performed in this study showed a close relative association between viral loads measured by both laboratories in all three types of samples studied. However, a bias of 1.4 $\log_{10}$ copies PCV2 per millilitre was observed, with laboratory 1 constantly yielding a higher output than laboratory 2. The laboratory 1 assay was shown to have a lower detection level than the one used in laboratory 2, being able to detect PCV2 loads of 3–4 $\log_{10}$ copies of PCV2 per millilitre in samples that gave negative results in the laboratory 2 assay. Taking the 1.4 $\log_{10}$ copies PCV2 per millilitre bias into account, results of 3–4 $\log_{10}$ copies of PCV2 per millilitre in laboratory 1 corresponds to results of 2–3 $\log_{10}$ copies of PCV2 per millilitre in laboratory 2. This is below the detection limit of the assay used in laboratory 2, explaining why more samples are considered negative by laboratory 2 than by laboratory 1. There are many possible factors that could produce variation in the results between assays and laboratories (Bustin, 2002). In the present exploratory study, comparison was performed on exchanged aliquots of DNA extracts, ruling out any cause related to pre-PCR procedures. Also, differences in the actual concentration of plasmid DNA in the standards, which potentially could introduce a systematic bias in the results as the one observed, were excluded. Two different qPCR platforms (ABI PRISM® 7000 and Rotorgene® 3000) were used, but previous studies reported similar accuracy and performance for different platforms using the same assay (Nitsche et al., 1999). Variation of assays may also rely on the nature of the design of the assays, such as length of amplicon or primer and probe design to conserved regions. However, both assays evaluated in this study seem to meet the criteria for well-designed qPCR assays: amplicon lengths were below 200 bp as generally recommended (Qvarnstrom et al., 2005). It is known that even few or only one mismatch in the primers can reduce the PCR efficiency, and if in fluorescent probes, may reduce or eliminate the strength of the fluorescence signal (Ratcliff et al., 2007). Although PCV2 ORF1 is known to be more conserved than ORF2 PCV2 (de Boisseson et al., 2004), primers and probes used in the assay targeting ORF2 performed by laboratory 2, did not contain any of the marker positions previously identified (Olvera et al., 2007), whereas one of the primers used in laboratory 1 did contain one of the described marker positions. This suggests that the higher heterogeneity of ORF2 could not explain the differences observed between the laboratories. Finally, other potential factors might influence the difference in qPCR performance observed in the present study. On one hand, the studied assays used different primers and probes chemistry. On the other hand, different PCV2 DNA regions (ORF1 and ORF2) may have different topology, offering different accessibility to the DNA polymerase.

Results showed a significant linear association between $\log_{10}$ viral DNA copy numbers per millilitre of PBS and $\log_{10}$ and viral DNA copy numbers per microgram of total DNA, indicating that such normalization would not substantially change the relative results obtained between different swabs collected in the same way. This is due to the fact that the mean and the S.D. of total DNA present in nasal and rectal swabs was relatively low compared to the amount of PCV2. However, results also showed that there was no association between the amounts of total and PCV2 DNA present in nasal and rectal swabs. This suggests that normalizing PCV2 DNA load in swab samples to the total DNA concentration is not suitable for the expression of the amount of PCV2 present in swab samples. Thus, if swabs are processed similarly within a study, expression as copies of PCV2 per milliliter or copies of PCV2 per swab would be more adequate for relative comparison of results. It may always be associated with a certain degree of error if results from swab samples collected in different ways are being compared.

The present exploratory study provides the basis for the need of performing ring trials on PCV2 qPCR protocols between laboratories, towards a harmonization of the methodology and towards the full understanding of possible different outputs between laboratories. Faced with the lack of ring trials and standardization procedures, proposed thresholds for

PMWS diagnosis (Brunborg et al., 2004; Olvera et al., 2004; Segalés et al., 2005) should be interpreted with caution, being only considered reliable for each particular laboratory and each particular assay.

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Study III

Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark.

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Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark

Llorenç Grau-Roma [a,b,1,*], Charlotte K. Hjulsager [c,1], Marina Sibila [a], Charlotte S. Kristensen [d], Sergio López-Soria [a], Claes Enøe [c], Jordi Casal [a,b], Anette Bøtner [e], Miquel Nofrarías [a], Vivi Bille-Hansen [c], Lorenzo Fraile [a,f], Poul Baekbo [d], Joaquim Segalés [a,b], Lars E. Larsen [c]

[a] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Departament de Sanitat i d'Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[c] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1790 Copenhagen V, Denmark
[d] Danish Pig Production, Vinkelvej 11, DK-8620 Kjellerup, Denmark
[e] National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark
[f] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain

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ABSTRACT

Longitudinal case–control studies were performed in post-weaning multisystemic wasting syndrome (PMWS) affected farms from Denmark and Spain using similar designs. Fourteen independent batches of 100–154 pigs per batch were monitored from birth to PMWS outbreak occurrence. Pigs displaying PMWS-like signs and matched healthy cohorts were euthanized during the clinical outbreak. PMWS was diagnosed according to internationally accepted criteria and pigs were classified as: (i) PMWS cases, (ii) wasted non-PMWS cases and (iii) healthy pigs. Porcine circovirus type 2 (PCV2) quantitative PCR (qPCR) and serology techniques were applied to analyse longitudinally collected sera and/or nasal and rectal swabs. Results showed that PCV2 load increased in parallel to waning maternal antibody levels, reaching the maximum viral load concurrent with development of clinical signs. PMWS affected pigs had higher PCV2 prevalence and/or viral load than healthy pigs in all collected samples at necropsy ($p < 0.0001$–0.05) and even in sera and nasal swabs at the sampling prior to PMWS outbreak ($p < 0.01$–0.05). Danish farms had a higher PCV2 prevalence in young piglets as well as an earlier PMWS presentation compared to Spanish farms. PMWS diagnoses were confirmed by laboratory tests in only half of pigs clinically suspected to suffer from PMWS. Positive and significant correlations were found among PCV2 viral loads present in sera, nasal swabs, rectal swabs and lymphoid tissues ($R = 0.289$–0.827, $p < 0.0001$–0.01), which indicates that nasal and rectal swabs were suitable indicators of PCV2 excretion. Sensitivity and/or specificity values observed from both tests used separately or combined suggested that qPCR and/or serology tests are not apparently able to substitute histopathology plus detection of PCV2 in tissues for the individual PMWS diagnosis within PMWS affected farms. However, qPCR appears to be a potential reliable technique to diagnose PMWS on a population basis.

* Corresponding author at: Departament de Sanitat i d'Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel.: +34 93 581 32 84; fax: +34 93 581 44 90.
E-mail address: llgrau@cresa.uab.cat (L. Grau-Roma).
[1] They contributed equally to the authorship of this work.

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1. Introduction

Porcine circovirus type 2 (PCV2) is considered the essential infectious agent of post-weaning multisystemic wasting syndrome (PMWS), which causes significant economic losses to the swine industry worldwide (Armstrong and Bishop, 2004). First PMWS description in Spain and Denmark were in 1997 (Segalés et al., 1997) and at the end of 2001 (Hassing et al., 2002), respectively. Afterwards, the periods of maximum number of PMWS diagnoses emitted by the two major diagnostic laboratories in both countries were 1999–2003 in Spain and 2002–2005 in Denmark (Vigre et al., 2005; Segalés et al., 2007).

PMWS most commonly affects 2–4-month-old pigs, although the disease has been also described in younger and older animals (Segalés et al., 2005a). The internationally accepted criteria to diagnose PMWS include the presence of compatible clinical signs (mainly wasting and respiratory distress), moderate to severe lymphocyte depletion with granulomatous inflammation in lymphoid tissues, and detection of moderate to high amount of PCV2 within these lesions (Segalés et al., 2005a). However, PCV2 is an ubiquitous virus that has been detected in both PMWS affected and non-affected pigs and farms by pathogen detection as well as serological methods (Larochelle et al., 2003; Rose et al., 2003; Sibila et al., 2004). Therefore, the sole detection of PCV2 antibodies in serum and/or viral DNA or antigen does not constitute PMWS diagnosis, since it only indicates evidence of PCV2 infection (Krakowka et al., 2005; Caprioli et al., 2006). However, serological and DNA detection techniques (PCR) have been widely used to monitor PCV2 infection dynamics in both PMWS affected and non-affected farms.

Most PMWS epidemiological field studies based on the above-mentioned laboratory techniques have been focused on the moment of disease outbreak. These studies have shown higher PCV2 viral load in serum, lymphoid tissues and potential shedding sites in PMWS affected pigs compared to non-affected pigs (Brunborg et al., 2004; Olvera et al., 2004; Sibila et al., 2004; Segalés et al., 2005b). Moreover, a viral load threshold to discriminate PMWS affected and non-affected pigs has been suggested (Brunborg et al., 2004; Olvera et al., 2004). In contrast, only few studies have focused on PCV2 infection dynamics, based on cross-sectional (Larochelle et al., 2003; Rose et al., 2003; Sibila et al., 2004; López-Soria et al., 2005) and longitudinal studies (Rodríguez-Arrioja et al., 2002; McIntosh et al., 2006; Carasova et al., 2007). These studies have been mainly based on serology and non-quantitative PCR techniques, and only one used quantitative PCR (qPCR) technique (Carasova et al., 2007). Overall, those studies demonstrated high levels of maternal antibodies in lactating piglets, which decreased gradually during the nursery period. PCV2 viremia usually occurred when maternal derived antibody levels waned, and decreased progressively after the increase of antibody titres (Rodríguez-Arrioja et al., 2002; Larochelle et al., 2003; Sibila et al., 2004).

Some epidemiological studies have been designed as case–control studies. Overall, higher percentages of pigs with PCV2 in serum and in nasal cavities were detected using PCR in PMWS affected farms (Sibila et al., 2004). Moreover, the earlier the PCV2 infection, the higher the risk of developing PMWS (Rose et al., 2003; López-Soria et al., 2005). However, those studies did not describe in detail the infection dynamics of PCV2 infection in PMWS affected and non-affected farms.

The aim of the present study was to describe the evolution of PCV2 infection, excretion and seroconversion in PMWS, wasted non-PMWS and healthy pigs from PMWS affected farms in two different countries, Denmark and Spain. The study period went from the first week of age until the moment of disease development.

2. Materials and methods

2.1. Study design

Two longitudinal case–control studies in PMWS affected farms, one in Spain and one in Denmark, were performed in parallel during 2005 and 2006. The diagnosis of PMWS at the farm level was confirmed according to the EU definition (www.pcvd.org/news.php) before the start of the study. Diagnostic procedures included a prevalence of pigs with wasting and mortality in nurseries plus fattening/finishing areas higher than 10%, as well as the individual diagnostic case definition fulfilment of PMWS (Segalés et al., 2005a) in at least 1 out of 5 necropsied pigs.

Production systems slightly varied between Spain and Denmark. Piglets were nursed at farrowing facilities from birth until 3 (Spain) and 4 (Denmark) weeks of age. After weaning, piglets were moved into the nursery until 8–9 (Spain) or 11–13 (Denmark), weeks of age. Finally, animals were placed in fattening areas until slaughter age.

Both studies were carried out using similar designs, and were performed on 14 independent batches of 100–154 animals from each of 8 Danish (8 batches) and 3 Spanish (6 batches) farms. Ten to 21 sows were selected in order to reach 100 piglets per batch. Number of studied farms, batches and animals, as well as specific differences between Spanish and Danish protocols, are displayed in Table 1. Studied piglets were ear-tagged at 1 week of age. Nasal and rectal swabs (Collection swab, Eurotubo, Spain; and Kirudan A/S, Denmark) as well as blood samples (Vacutainer®, Becton-Dickinson, Meylan Cedex, France) were serially collected from those piglets at established weeks of age (Table 1) and when the PMWS compatible clinical picture appeared (necropsy) (Segalés et al., 2005a). The animals were evaluated clinically at the time of samplings and the herds were visited regularly by the corresponding responsible veterinarians, in order to alert the researchers of any potential apparition of clinical signs compatible to PMWS in the studied pigs. Nasal samples were collected by swabbing deeply into the turbinates (both nostrils). Rectal swabs were obtained by introducing the swab into the rectum and swabbing the surface of the colo-rectal region. All swabs were placed in tubes containing 1 (Spain) or 2 (Denmark) ml of phosphate-buffered saline (PBS) solution. Pigs were bled at the anterior vena cava. Blood from sows were also collected at 1st sampling day by puncture in the tail

Table 1
Experimental design of the study in Denmark and Spain.

Country	No. of farms	No. of batches	Pigs per batch	Sows per batch	Cross-fostering	Age of sampling prior to necropsy (weeks)	No. of healthy pigs necropsied per batch	No. of PMWS-like affected pigs necropsied	Tissues collected[a]
Denmark	8	8	109–154	10	Yes	1, 4, 6, 9	2–5	4–15[b]	MLN, SILN
Spain	3	6	100–105	10–14[c]	No	1, 3, 7, 11	3–5	8–19	MLN, SILN, SLN, TBLN, TONSIL

[a] MLN = Mesenteric lymph node, SILN = superficial inguinal lymph node, SLN = sub-mandibular lymph node, TBLN = tracheo-bronchial lymph node.
[b] This range comprised 7 out of 8 studied Danish batches, but in one batch (DK-3) this number was 38.
[c] In 2 out of 6 Spanish studied batches (SP-3a and SP-3b) only males were followed up. Consequently, up to 21 sows were needed to select 100 pigs in those two batches.

Table 2
Characteristics of farms and studied animals. Sanitary status describes which other pathogens were known to be present or absent in the sow-farm by serological determinations at the time the study was performed. Aujeszky disease virus (ADV), porcine parvovirus (PPV), porcine reproductive and respiratory virus (PRRSV), swine influenza virus (SIV), *Actinobacillus pleuropneumoniae* (Ap), *Lawsonia intracellularis* (law), *Mycoplasma hyopneumoniae* (Myc), *Salmonella* spp. (salm), toxigenic *Pasteurella multocida* (PMT).

Farm	Health status	Production system	Number of sows	Batch	Weeks of age at necropsy	Number of necropsied pigs during PMWS outbreak			% Mortality[a]	
						PMWS	Wasted non- PMWS	Healthy	Nurseries	Fattening
SP-1	PRRSV+, ADV+, Myc+, PPV+, SIV+, Salm+	3 sites	2400	SP-1	18–21	3	8	5	1.9	8.0
SP-2	PRRSV+, ADV−, Myc+, PPV+, SIV+, Salm+	2 sites[b]	600	SP-2a	15–17	6	5	5	4.8	10.0
				SP-2b	13–15	3	7	5	2.9	17.4
				SP-2c	11–15	6	5	5	7.7	17.4
SP-3	PRRSV−, ADV−, Myc−, PPV+, SIV+, Salm+	2 sites	950	SP-3a	12–15	7	9	5	6.0	16.8
				SP-3b	12–15	10	9	5	1.9	16.8
Total number of studied animals in Spanish study						35	43	30		
DK-1	PRRSV+, Myc+, PPV+, SIV+, law+, Ap12+	1 site	570	DK-1	9–10	2	2	2	11.0	5.0
DK-2	PRRSV+, Myc+, PPV+, SIV+, law+, Ap6+	2 sites	700	DK-2	10–13	5	3	3	6.0	[c]
DK-3	PRRSV+, Myc+, PMT+, PPV+, SIV+, law+, Ap6+	3 sites	240	DK-3	10–13	18	20	5	22.0	4.5
DK-4	PRRSV+, Myc+, PPV+, SIV+, law+, Ap2+, Ap6+, PMT+	1 site	300	DK-4	10–13	9	3	5	15.0	7.0
DK-5	PRRSV+, Myc+, PPV+, SIV+, law+, Ap6+, Ap12+	2 site	570	DK-5	10–11	2	2	2	7.2	[c]
DK-6	PRRSV+, Myc+, PMT+, PPV+, SIV+, law+, Ap6+	2 sites	900	DK-6	10–13	7	8	4	5.4	[c]
DK-7	PRRSV+, Myc+, Ap2+, Ap12+, PPV+, SIV+, law+	1 site	400	DK-7	8–10	3	1	3	12.0	5.0
DK-8	PRRSV−, Myc−, PPV+, SIV+, law+	1 site	550	DK-8	9	5	1	3	6.0	[c]
Total number of studied animals in Danish study						51	40	27		

[a] Percentages of mortality are referred to the total number of pigs present in each phase.
[b] All studied 2 site farms had sites I and II located in the same farm and site III located separately.
[c] Not recorded data.

vessels. Once in the laboratory, blood samples were allowed to clot at 4 °C and then centrifuged at 1500 × *g* for 10 min at 4 °C. All samples were frozen at −80 °C until testing. When the PMWS compatible clinical picture appeared at the studied farms, healthy animals and pigs displaying PMWS-like signs were selected, euthanized and necropsied. Necropsied animals were also bled and nasal and rectal swabs taken. Piglets suffering natural death and displaying clinical signs compatible with PMWS were also selected. At necropsy, sections of lymphoid tissues were collected (Table 1) and fixed by immersion in neutral-buffered 10% formalin to assess the pathological status of both clinically healthy and diseased animals.

Characteristics of studied farms, batches and necropsied animals are displayed in Table 2. All treatments, housing, husbandry and slaughtering conditions were conformed to the European Union Guidelines and Good Clinical Practices.

2.2. Histopathology

Formalin fixed tissues were dehydrated and embedded in paraffin blocks. Two consecutive 4 μm thick sections containing collected lymphoid tissues from each pig were cut from each block. One section was processed for haematoxylin and eosin stain, while the other was for PCV2 nucleic acid detection by *in situ* hybridization (ISH) (Rosell et al., 1999) for Spanish cases, and for immunohistochemistry (IHC) for PCV2 antigen detection (Jensen et al., 2006) for Danish cases. Four lymph nodes and tonsil (Spain) and two lymph nodes (Denmark) per pig were evaluated in a blinded fashion, by the same pathologist for three different parameters using a criteria based on previously described scoring systems (Krakowka et al., 2005; Opriessnig et al., 2006) from 0 to 4, as summarised in Table 3. The score was calculated in each lymphoid tissue as: score = (lymphoid depletion + granulomatous infiltration)/2 + PCV2 amount. PCV2 amount corresponded to the

Table 3
Histopathological scoring system used to evaluate the presence of PMWS-like lesions in studied lymphoid tissues of each necropsied pig.

Evaluated parameter	Scoring value				
	0	1	2	3	4
Lymphocyte depletion	Absence	Lack of lymphoid follicles in <25% of the tissue section	Lack of lymphoid follicles in 25 to 75% of the tissue section	Lack of lymphoid follicles in >75% of the tissue	Lack of lymphoid follicles
Granulomatous infiltration	Absence	Mild and focal or multifocal granulomatous infiltration in <25 of the lymphoid follicles	Multifocal granulomatous infiltration in 25 to 75% of the lymphoid follicles	Granulomatous infiltration in >75% of the follicles	Sections with diffuse granulomatous infiltration
ISH/IHC labelling	No labelling	Low and focal or multifocal labelling in <25% of the follicles	Labelling located exclusively at follicles (from 25% to 100% of the follicles) or labelling between 25% and 50% tissue section	Labelling at follicles and parafollicular areas in >50% tissue section	Diffuse labelling

level of PCV2 DNA staining (ISH) or PCV2 antigen (IHC) detected in the lymphoid tissue (Table 3). Afterwards, a global average histopathological score per pig was calculated by summing the individual scores of all five or two lymphoid tissues and divided by five or two, respectively. Global average scores ranged from 0 to 8. A pig was diagnosed as PMWS when it suffered clinical signs compatible with PMWS (mainly wasting) and the global average score was superior to 4. All the pigs classified as PMWS cases had to have an amount of PCV2 antigen/nucleic acid of 2 or more in at least one studied lymphoid tissue. Based on this global scoring system and the clinical condition, necropsied pigs were classified into three different categories (Grau-Roma et al., 2008): (i) PMWS cases: pigs showing clinical wasting and a global scoring >4, corresponding to pigs with moderate to severe lymphoid lesions and moderate to high amount of PCV2 antigen/nucleic acid; (ii) Wasted non-PMWS cases: pigs showing clinical wasting but a global scoring ≤4, corresponding to no or slight PMWS characteristic histopathological lesions and no or low amount of PCV2 antigen/nucleic acid within lymphoid tissues; (iii) Healthy pigs: pigs showing good clinical condition, which presented low global scoring averages (≤4), corresponding to no or slight PMWS characteristic histopathological lesions and no or low amount of PCV2 antigen/nuleic acid within lymphoid tissues.

2.3. PCV2 serology

Collected sera were analyzed by serology to detect individual antibody titers to PCV2. An immunoperoxidase monolayer assay (IPMA) (Rodríguez-Arrioja et al., 2000) was applied on Spanish samples using serial three-fold dilution of sera from 1:20 to 1:4860. An in-house Enzyme Linked Immunosorbent Assay (ELISA) technique (EnØe et al., 2006) was used to analyze the Danish samples. All serologic titers were expressed as reciprocal dilutions and coded titers corresponding to $\log_{10}$ values in order to facilitate statistical analysis and graphic representations. Thus, the equivalence used between serologic titers and coded titers in IPMA was: 0 = seronegative, 1.3 = 1:20, 1.8 = 1:60, 2.3 = 1:180, 2.7 = 1:540, 3.2 = 1:1620 and 3.7 = 1:4860. The equivalence in ELISA test was: 0 = seronegative, 1.0 = 1:10, 1.7 = 1:50, 2.4 = 1:250, 3.1 = 1:1250, 3.8 = 1:6250 and 4.5 = 1:31250.

In order to compare the two serological methods, a set of 94 serum samples were analyzed in both laboratories. A positive correlation was detected between IPMA and ELISA titres ($R = 0.84$, $p < 0.001$), and a good agreement was determined by the kappa statistic ($k = 0.688$), showing that both tests gave congruent results. It is worthy mentioning that all samples that gave negative results by IPMA were also negative by ELISA test; 6 out of 94 samples gave positive results by IPMA (low titres 1:20–1:60) and negative by ELISA.

2.4. DNA extraction and PCV2 quantitative PCR (qPCR)

DNA from Spanish samples was extracted from 200 μl of serum or 300 μl of nasal PBS solution (Nucleospin® Blood, Macherey-Nagel, GmbH & Co KG, Düren, Germany) or from 300 μl of rectal PBS solution (QIAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany), according to the manufacturers instructions. DNA from serum and nasal swab samples was eluted in 100 μl of elution buffer, and in 200 μl for rectal swab samples. DNA from Danish samples was extracted from 200 μl of serum or 200 μl nasal PBS solution (QIAamp DNA Mini Kit, Qiagen® GmbH, Germany) or 200 μl of rectal PBS solution (QIAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany), according to the instructions of the manufacturer. DNA was eluted with 200 μl elution buffer.

PCV2 DNA was quantified using two previously described real-time qPCR techniques on Spanish (Olvera et al., 2004) and Danish (Hjulsager et al., 2009) samples, respectively. The performance of both qPCRs was compared in a previous study based on field samples (Hjulsager et al., 2009). This latter work showed that the two qPCRs gave comparable results showing a highly significant linear association between results of the two assays. However, the method performed by the Danish laboratory had higher sensitivity and yielded systematically higher PCV2 load values than the one used in the Spanish laboratory (corresponding to a mean difference of 1.4 $\log_{10}$ copies of PCV2 per ml of sample).

2.5. Statistical analyses

Statsdirect (version 2.6.6, CamCode, Ashwell, UK) and SPSS 15.0 (SPSS Inc. Chicago, USA) were used for statistical analyses. Analysis of variance with Bonferroni multiple

comparisons (for normally distributed variables) and Kruskal–Wallis and Mann–Whitney tests (for non-normally distributed variables) were used to compare IPMA titres, ELISA titres, lesional scores, antigen or DNA staining levels in lymphoid tissues, and viral loads in sera, nasal swabs and rectal swabs between the three categories of pigs studied (PMWS cases, wasted non-PMWS cases and healthy) in both countries. Chi-square test was used to compare the proportions of positive qPCR results between the three categories of pigs studied; when statistically significant different proportions were detected, the Fischer's exact test was applied to compare in pairs the different categories of pigs. The Fischer's exact test was also applied to compare the proportion of viremic and non-viremic sows that had at least one PMWS case in their litter. Linear associations between viral loads detected in the different samples, between lesions characteristic of PMWS and amount of PCV2 in lymphoid tissues, and between total antibody titres detected in sows and their piglets at 1 week of age, were determined by Pearson's (normally distributed variables) and Spearman's (non-normally distributed variables) correlations. To determine the diagnostic performance of serology and qPCR techniques on clinically affected animals, a Receiver Operating Characteristic (ROC) analysis was carried out using Statsdirect. For this, only wasted pigs were considered, which perfectly fits a practical situation where a diagnosis of clinically suspected animals under field conditions is required. Thus, PMWS cases were considered as diseased, whereas wasted non-PMWS cases were considered as non-diseased animals. The probability of making a correct diagnosis was assessed through calculation of positive predictive value (PV+) and negative predictive value (PV−). The level of significance for all analyses (α) was set to $p < 0.05$.

3. Results

3.1. Studied animals and histopathology

A total of 108 and 118 animals were necropsied and pathologically characterized in Spain and Denmark, respectively (Table 2). All Spanish PMWS outbreaks occurred at the beginning of the fattening phase, whereas all Danish clinical outbreaks were observed at nurseries. Mean ± SD of weeks of age at necropsy were 10.6 ± 1.8 (PMWS: 10.6 ± 1.7, healthy: 10.8 ± 2.0, wasted non-PMWS: 10.3 ± 1.9) and 14.7 ± 2.5 (PMWS: 14.0 ± 2.1, healthy: 14.7 ± 2.5, wasted non-PMWS: 15.2 ± 2.7) in Denmark and Spain, respectively. Accordingly, highest mortality ratios were observed at nurseries (Denmark) and fattening facilities (Spain).

After histopathological examination, 35 out of 78 (45%) and 51 out of 91 (56%) pigs showing wasting were diagnosed as PMWS cases in Spain and Denmark, respectively. PMWS cases had higher amount of PCV2 in tissues (antigen or DNA staining), as well as higher histopathological scores for lymphoid depletion and granulomatous infiltration compared to wasted non-PMWS cases and healthy pigs from both countries ($p < 0.0001$). Moreover, wasted non-PMWS pigs had higher global histopathological average scores ($p < 0.0001$), as well as higher lymphoid depletion ($p < 0.02$) and granulomatous infiltration ($p < 0.01$) scores, than healthy pigs from both countries. The PCV2 amount in lesions of wasted non-PMWS pigs was higher than in healthy pigs from Spain ($p < 0.01$) but not from Denmark ($p = 0.5$). Surprisingly, 1 (Spain) and 2 (Denmark) pigs with good clinical condition (apparently healthy) had a global average score >4, showing both the lesional score and the score for PCV2 amount, each above 2. These three animals were finally not classified into the category of healthy pigs and were consequently excluded from the statistical analyses due to the low number of cases with this situation.

3.2. PCV2 qPCR

Percentage of pigs with PCV2 qPCR positive results at each sampling point from both countries are displayed in Table 4. In Spain, results showed only sporadic positive values before 11 weeks of age. During the last two samplings, the number of qPCR positive pigs increased progressively over time in all the three types of material studied (serum and nasal and rectal swabs), reaching the maximum levels at the moment of PMWS outbreak. In Danish farms, 18%, 69% and 23% of sera, nasal swabs and rectal swabs, respectively, gave positive results by PCV2 qPCR technique at the first week of age, increasing also progressively to the moment of occurrence of clinical disease. In Spain, a higher proportion of qPCR PCV2 positive pigs in sera were observed among PMWS cases compared to healthy animals at 11 weeks of age ($p < 0.015$), as well as in sera ($p < 0.0001$), nasal ($p < 0.05$) and rectal swabs ($p < 0.01$) at necropsy. Contrary, no statistical significant differences were found between the prevalence of PCV2 among the three categories (PMWS, healthy and wasted non-PMWS) of studied Danish pigs. At necropsy, PCV2 prevalence in PMWS affected pigs was 100% in all 3 types of samples from both countries, with the exception that rectal swabs from PMWS Spanish cases had a prevalence of only 68% (23 out of 34).

Means of PCV2 load by qPCR in positive samples (Fig. 1) showed significant differences between PMWS cases and healthy pigs at necropsy in serum, nasal and rectal swabs from both countries ($p < 0.001$). Furthermore, it was possible to distinguish PMWS cases from healthy pigs in Danish farms at the sampling prior at the necropsy on sera ($p < 0.01$) and nasal swabs ($p < 0.05$), but not in Spanish samples, even though a tendency was observed for serum samples ($p = 0.08$).

Positive and significant correlations were found in both countries among PCV2 viral loads in sera and nasal swabs (Spain: $R = 0.693$, $p < 0.0001$; Denmark: $R = 0.663$; $p < 0.001$), in sera and rectal swabs (Spain: $R = 0.608$, $p < 0.0001$; Denmark: $R = 0.720$, $p < 0.0001$) and in nasal and rectal swabs (Spain: $R = 0.593$; $p < 0.0001$; Denmark: $R = 0.736$, $p < 0.0001$) considering longitudinally collected samples. Moreover, positive and significant correlations were also found between PCV2 amount detected in tissue by ISH/IHC and viral loads detected by qPCR in sera (Spain: $R = 0.625$, $p < 0.0001$; Denmark: $R = 0.827$, $p < 0.0001$), in nasal swabs (Spain: $R = 0.573$, $p < 0.0001$; Denmark: $R = 0.779$, $p < 0.0001$) and in rectal swabs (Spain:

Table 4
Percentage of PCV2 qPCR positive serum, nasal and rectal samples at the different samplings. When statistical significant differences between studied groups were detected, percentage of qPCR positive animals per group are given in parentheses (PMWS, wasted non-PMWS and healthy pig, respectively) and differences indicated by superscript letters (a–c).

		Samplings (weeks of age in Spain–Denmark)				
		1st (1)	2nd (3–4)	3th (6–7)	4th (9–11)	5th*
Spanish farms	Sera	0	0.9	0.9	40.7 (54^{a}; 46^{b}; 21^{b})	76.0 (100^{a}; 83^{b}; 41^{c})
	Nasal	0.9	2.8	31.5	70.4	87.0 (100^{a}; 86^{b}; 76^{b})
	Rectal	8.4	1.9	10.3	45.8	50.5 (66^{a}; 51^{a}; 28^{b})
Danish farms	Sera	18.4	16.0	41.0	93.4	94.8
	Nasal	69.3	87.5	96.4	96.4	100
	Rectal	22.8	35.1	50.9	96.3	98.5

* 5th sampling correspond to necropsy moment in both countries.

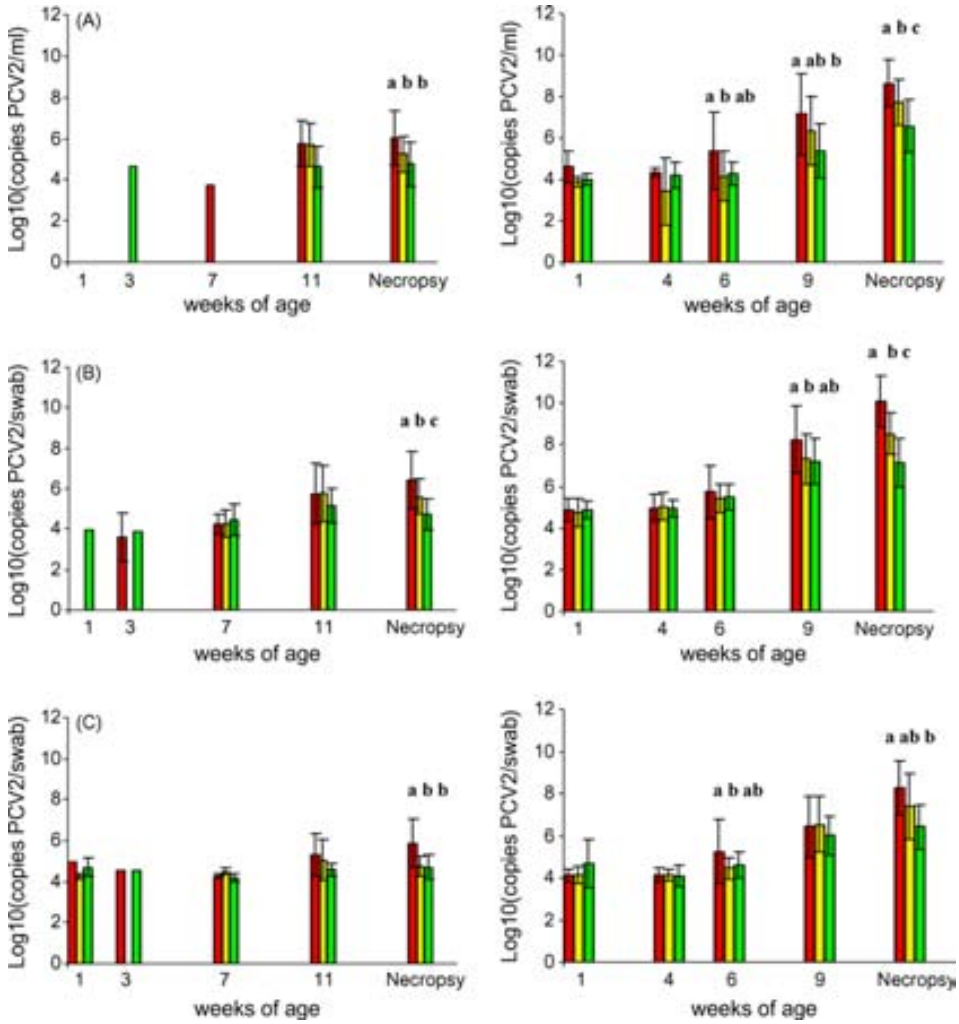

Fig. 1. Mean of positive $\log_{10}$(copies of PCV2 per ml of serum or per ml of swab material) in serum (A), nasal swabs (B) and rectal swabs (C) by qPCR in PMWS (▬), wasted non-PMWS (▬) and healthy (▬) pigs at the different samplings from Spanish (left) and Danish (right) farms. Letters (a)–(c) indicates statistical significant differences between groups ($p < 0.05$).

$R = 0.289$, $p < 0.01$; Denmark: $R = 0.507$, $p < 0.0001$) collected at the moment of necropsy from both countries.

One healthy and 2 PMWS Danish cases were qPCR positive in sera at all sampling times (from 1 to 10–11 weeks of age). These animals were positive in nasal swabs and negative in rectal swabs at the first week of age and intermittently positive in both swabs afterwards. Moreover, the only Spanish pig that was PCV2 positive by qPCR

Table 5
Number of qPCR PCV2 positive sows in sera, mean of viral load of positive sows, and number of PMWS cases from viremic and non-viremic sows per studied batch.

Country	Batch	Number of PCV2 qPCR positive sows/number studied sows	Viral load of positive sows (log_{10} copies PCV2/ml) (mean ± standard deviation)	Number of PMWS cases	
				From PCV2 positive sows	From PCV2 negative sows
Spain	SP-1a	0/12	–	0	3
	SP-2a	1/12	5.35	0	6
	SP-2b	0/12	–	0	3
	SP-2c	0/12	–	0	6
	SP-3a	0/20	–	0	7
	SP-3b	1/19	5.13	3	7
	Total	2/87	5.24 ± 0.16	3	32
Denmark	DK-1a	2/8	4.16 ± 0.6	2	0
	DK-2a	1/10	3.84	0	5
	DK-3a	0/10	–	0	18
	DK-4a	6/10	3.79 ± 0.31	9	0
	DK-5a	6/10	3.87 ± 0.12	1	1
	DK-6a	2/10	3.97 ± 0.34	2	5
	DK-7a	1/4	3.79	1	2
	DK-8a	2/6	3.71 ± 0.19	2	2
	Total	20/68	3.86 ± 0.27	17	33

in sera at 7 weeks of age was a PMWS case which was also PCV2 qPCR positive in sera, nasal and rectal swabs until necropsy (at 12 weeks of age).

Positive PCV2 qPCR results were found in 2 out of the 87 (2.3%) and in 20 out of 68 (29.4%) studied sow's sera from Spain and Denmark, respectively (Table 5). In Denmark, 9 out of 20 sows with low but detectable viremia (45.0%) and 18 out of 48 sows with no detectable viremia (37.5%) had one or more PMWS affected pig in their litter ($p = 0.596$). Thus, there was no indication of any association between sow-viremia and bearing of PMWS cases in the Danish study. Due to the low number of PCV2 positive sows in the Spanish data-set, a similar analysis based on Spanish data was not possible.

3.3. PCV2 serology

PCV2 serology results from both countries are displayed in Fig. 2. PCV2 maternal antibodies were present at the first week of age in all pigs from both countries. In Spain, healthy pigs had significantly higher titres than PMWS and wasted non-PMWS cases at 1 week of age ($p < 0.05$). However, no statistical significant differences were found between antibody titres detected at 3 and 7 weeks of age. Moreover, Spanish PMWS cases had lower antibody titres than non-PMWS affected pigs (healthy and wasted non-PMWS cases) at 11 weeks of age ($p < 0.05$) and at necropsy ($p < 0.05$). In Denmark, healthy pigs had statistically significant higher PCV2 maternal antibody titres than PMWS cases during the first three samplings ($p < 0.01$). No statistical significant differences were obtained in Danish pigs after seroconversion.

For the Spanish study, a significant and positive correlation was found between sow PCV2 antibody titres and PCV2 antibody titres of their piglets at 1 week of age ($R = 0.66$, $p < 0.0001$). No correlation was found for antibody titers between Danish sow and piglets ($R = 0.2$, $p = 0.2$).

3.4. Diagnostic performance of qPCR and serological tests

Serology and qPCR results from samples coming from pigs with clinical signs compatible with PMWS at necropsy were used in a ROC analysis to evaluate the diagnostic performance of those techniques for live animals and the optimal conditions under which those techniques should be applied. Optimal cut-off values for each studied sample material and technique applied, together with their sensitivity, specificity, PV+ and PV− values, are displayed in Table 6. Optimal cut-offs were calculated maximizing both sensitivity and specificity values.

None of the tests on the studied samples were specific and sensitive enough to diagnose PMWS at the level of individual pigs in any of the two countries. Consequently, none of the tests on the studied samples gave PV+ or PV− values higher than 90% in both countries. On the other hand, taking into account the previously described bias between the two qPCR assays of approximately 1.4 log copies PCV2/ml (Hjulsager et al., 2009), qPCR on sera gave similar optimal cut-off for both qPCRs (6.21 in Spain and 7.43 in Denmark). This fact led us to further explore the diagnostic performance of a combination of qPCR in sera and serology tests for the Danish and the Spanish data-sets. Results showed that the serial use of qPCR from sera and serology gave adequate results in terms of specificity in both countries, but with an evident lack of sensitivity. Thus, when Spanish qPCR serum load $\geq$6.21 and IPMA titres $\leq$1:1620 were simultaneously considered to confirm or discard a PMWS case, specificity and PV+ were 100%, but sensitivity was 42.9% and PV− was 68.3%. Regarding Danish tests, when qPCR values in sera were $\geq$7.43 and ELISA titres were 0, specificity was 93.3%, PV+ 83.3%, sensitivity 21.7% and PV− 43.8%.

4. Discussion

The lack of an effective and consistent experimental model to reproduce PMWS makes especially relevant to

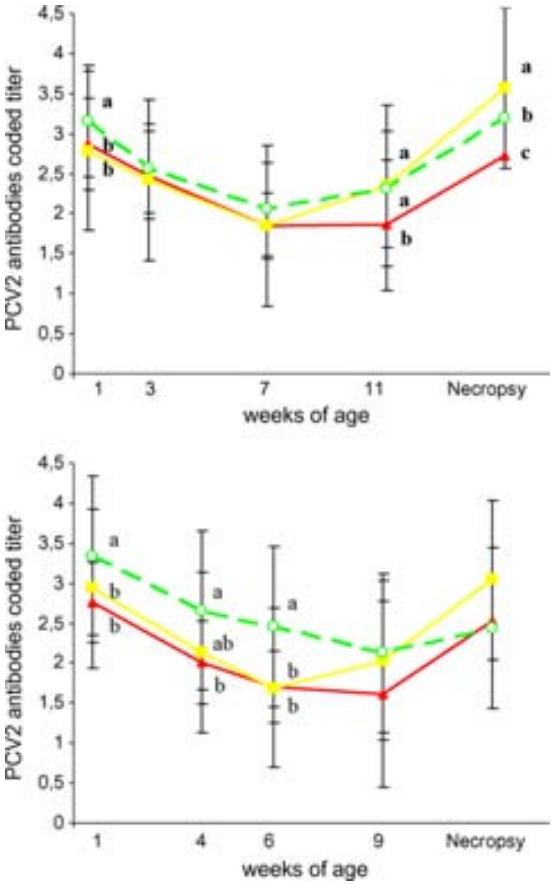

Fig. 2. Mean ± SD coded titres for porcine circovirus type 2 (PCV2) total antibodies in PMWS (▲), wasted non-PMWS (■) and healthy (○) pigs at the different samplings from Spanish (above) and Danish (below) farms. Different letters (a–c) in each sampling indicate significant differences between groups ($p < 0.05$).

carry out epidemiological studies within PMWS affected farms. Up to date, few longitudinal epidemiological data are available on PCV2 infection dynamics in PMWS affected farms (Rodríguez-Arrioja et al., 2002; McIntosh et al., 2006; Carasova et al., 2007). In the present work, serology and qPCR techniques were used to describe the evolution of PCV2 infection and excretion in histopathologically well-characterized pigs (PMWS cases, wasted non-PMWS cases and healthy pigs) from two different countries: Spain and Denmark. This is the first collaborative multi-centre epidemiological study on PMWS, which has been carried-out on different farms and in two different countries.

Overall, similar PCV2 dynamic patterns were observed between countries. Serological analyses showed maternal antibody titres in all piglets at 1 week of age, which decreased gradually and reached a minimum at approximately 6–7 weeks of age. PCV2 viral load in serum increased concurrently to waning of maternal antibody levels, reaching the highest level at PMWS outbreak in all the three studied types of samples (serum, and nasal and rectal swabs) in both countries. As expected (Brunborg et al., 2004; Olvera et al., 2004), PCV2 load was higher in PMWS affected than in healthy pigs at the moment of necropsy. Interestingly, even at the sampling previous to the clinical outcome (9 and 11 weeks of age for Denmark and Spain, respectively), PMWS cases had significant higher PCV2 load (Denmark) or PCV2 prevalence (Spain) than healthy pigs, suggesting that PMWS affected pigs suffered from a higher viral load and/or longer viremia than healthy ones.

A previous comparison of both qPCR showed that despite the results obtained by both techniques had a good correlation, the Danish assay was more sensitive and yielded systematically higher PCV2 viral loads than the Spanish assay (Hjulsager et al., 2009). However, these differences in qPCR performance cannot explain all the variation between the findings in Spain and Denmark. Additional variation in results potentially could be due to the employment of different extraction methods, which could also affect the sensitivity of PCV2 detection (Fahle and Fischer, 2000; Cler et al., 2006).

PMWS was described as enzootic and epizootic presentations during the studied period in Spain and Denmark, respectively (Segalés et al., 2007). Interestingly, the high percentage of nasal Danish swabs positive by qPCR at younger ages agrees with a previous work performed during the years of maximum PMWS affectation (epizootic presentation) in Spain, which was based on conventional non-quantitative PCR on nasal swabs (Sibila et al., 2004). Furthermore, present results showed a substantial delay in PMWS age-presentation in Spain compared to Denmark. These data suggested the existence of a relation between early infection and age at disease presentation. The apparent higher PCV2 prevalence observed in young Danish piglets and sows suggested that an earlier and

Table 6
Optimal cut-off values, sensitivity, specificity, positive predictive value (PV+) and negative predictive value (PV−) obtained from ROC analyses from serological and quantitative PCR (qPCR) techniques in each of studied samples. qPCR results are expressed as $\log_{10}$ copies of PCV2 per ml sample.

Country	Sample	Test	Optimal cut-off	Sensitivity ($CI_{95\%}$)	Specificity ($CI_{95\%}$)	PV+ ($CI_{95\%}$)	PV− ($CI_{95\%}$)
Spain	Nasal swab	qPCR	≥5.9	68.6 (31.4–66.0)	74.4 (77.8–97.4)	68.6 (52.5–86.9)	74.4 (67.5–90.4)
	Rectal swab	qPCR	≥5.9	41.2 (24.7–59.3)	97.7 (87.7–99.9)	93.3 (68.1–99.8)	67.7 (55.3–80.2)
	Sera	qPCR	≥6.21	48.6 (31.4–66.0)	90.7 (77.8–97.4)	81.0 (58.1–94.6)	68.4 (55.5–81.4)
		IPMA	≤1:1620	73.5 (55.6–87.1)	88.4 (74.9–96.1)	83.3 (65.3–94.4)	80.9 (68.5–93.2)
Denmark	Nasal swab	qPCR	≥9.2	86.2 (68.3–96.1)	81.3 (54.4–96.0)	89.3 (71.8–97.7)	76.4 (50.1–93.2)
	Rectal swab	qPCR	≥8.1	50.5 (31.3–68.7)	82.4 (56.6–96.2)	83.3 (58.6–96.4)	48.3 (28.4–68.2)
	Sera	qPCR	≥7.43	91.3 (72.0–98.9)	46.7 (21.3–73.4)	72.4 (54.4–90.4)	77.8 (40.0–97.2)
		ELISA	0	92.9 (76.5–99.1)	27.8 (9.7–53.5)	66.7 (50.6–82.7)	71.4 (29.0–96.3)

higher PCV2 infection pressure was present in Danish farms compared to Spanish ones, which might be the reason for an earlier PMWS presentation and reflects the epidemiological disease presentation in both countries during the studied period.

The positive and significant correlation found between amounts of PCV2 detected in lymphoid tissues, sera and swabs suggests that nasal and rectal swabs are suitable indicators of the level of PCV2 excretion. However, it has to be taken into account that swab samples may be associated with a certain degree of error if results from swab samples collected in different ways are being compared, which is not the case in the present study. Significant differences in viral loads in rectal and nasal swabs between PMWS affected and healthy animals support the idea that pigs suffering from PMWS excrete higher levels of PCV2 by nasal and rectal routes when showing the disease (Segalés et al., 2005b). These differences were also observed at the sampling prior to the clinical manifestation, when higher viral load in PMWS affected pigs was detected in Danish nasal swabs. Results also showed that a higher prevalence of PCV2 was present in the nasal cavity than in faeces during the PMWS outbreak, further supporting the oro-nasal route as the most important route of PCV2 infection and transmission (Segalés et al., 2005a).

The results of the present study indicated that the major spread of PCV2 occurred between 4 and 6 weeks of age in Danish farms and between 7 and 11 weeks of age in Spanish farms, which corresponded to the nursery phase (Denmark) and/or at the beginning of fattening (Spain). However, the fact that some sows were viremic during the lactating period together with PCV2 detection in piglets demonstrated that PCV2 infection was already present at pre-weaning ages and support the possible transmission of virus from sows to nursing piglets (Larochelle et al., 2003; Sibila et al., 2004) and also points out a potential vertical transmission (Pensaert et al., 2004; Shibata et al., 2006). In fact, PCV2 DNA was repeatedly found in sera of pigs from 7 to 70 days of life, further supporting the idea that some animals can be persistently infected under field conditions, even in the presence of high levels of PCV2 specific antibodies (Rodríguez-Arrioja et al., 2002; McIntosh et al., 2006). Those viremic pigs were demonstrated to intermittently excrete PCV2 in nasal excretions and faeces, which could potentially cause infection to their littermates and contribute to virus dissemination in weaning and/or fattening facilities. On the other hand, no difference between PMWS occurrence in piglets from viremic and non-viremic Danish sows was found, which is in contradiction to a previously published field study (Calsamiglia et al., 2007), probably due to cross-fostering practices which were allowed in Danish farms, in contrast to the above-mentioned study.

PMWS cases had lower maternal antibody titres than healthy ones in both studied countries, supporting previous studies indicating that total antibody titres may confer a certain protection against PCV2 infection and clinical disease (Rodríguez-Arrioja et al., 2002; López-Soria et al., 2005; Calsamiglia et al., 2007). Maternal antibodies at the first week of age were correlated with sow antibody levels in the Spanish study, but not in the Danish one, probably as a consequence of cross-fostering practice in the Danish study. Antibody titres after infection in Spanish pigs showed that non-PMWS affected animals (both healthy and wasted non-PMWS affected pigs) had a higher antibody response, being able to produce higher antibody titres than PMWS affected animals. The fact that Spanish wasted non-PMWS and healthy pigs were as a mean 2 and 1 week, respectively, older than PMWS could contribute in explaining these differences at the moment of necropsy, but not at 11 weeks of age. It is possible that wasted non-PMWS affected animals, although infected with higher PCV2 loads than healthy pigs, were able to overcome or limit the disease expression by the development of PCV2 antibodies, among other potential factors. However, these differences were not observed in Danish farms. Taken together, these findings suggest that total antibodies are not the only factor involved in avoiding PMWS development (Meerts et al., 2006; Fort et al., 2007). Other factors such as levels of neutralizing antibodies or cellular immunity, although they were not evaluated in the present work, were previously demonstrated to play an important role in PMWS development (Segalés et al., 2004; Fort et al., 2007).

Histopathological results showed that only half of pigs suspected to suffer from PMWS in an affected farm during the acute outbreak corresponded to true PMWS affected animals, according to the internationally accepted individual case definition (Segalés et al., 2005a). There is a wide range of causes apart from PMWS that can produce wasting in pigs (Harding, 1997), which makes the group of wasted non-PMWS affected pigs difficult to interpret collectively from a diagnostic point of view. Severity of PMWS-like lesions and amount of PCV2 in lymphoid tissues were evaluated by the above-described average histopathological score, which is based on scores for each histopathological finding, each varying from 0 to 4. Thus, wasted non-PMWS pigs with a global average score close to 0 might correspond to animals suffering from wasting due to PMWS-unrelated causes such as other infectious or non-infectious diseases and/or management conditions. On the contrary, wasted non-PMWS pigs with global scoring close to 4 might correspond to convalescent pigs or to pigs that were able to overcome or limit the disease expression (Segalés, 2002; Krakowka et al., 2005). The fact that wasted non-PMWS affected pigs had higher global histopathological scores than healthy animals, suggests that PCV2 was involved to some degree in the clinical signs that those pigs displayed.

Most healthy pigs had practically no PMWS-like lesions and none to low amounts of PCV2 in lymphoid tissues. However, approximately one third of Spanish and Danish healthy pigs had global histopathological scores $\geq$2. This finding indicated that characteristic mild PMWS microscopic lesions can be present in subclinically PCV2 infected pigs in the field, which has been previously observed in both experimental (Allan et al., 1999; Grasland et al., 2005; Krakowka et al., 2007) and field conditions (Quintana et al., 2001). Surprisingly, 3 out of the 57 euthanized clinically healthy animals (one from Spain and two from Denmark) had moderate to severe lesions and moderate to high amount of PCV2 in lymphoid tissues. These three animals

had also high amount of PCV2 in sera, nasal and rectal swabs. One possible explanation for this situation would be that these animals were in the initial phase of PMWS development (Segalés, 2002), thus displaying minimal or no signs of disease yet. On the other hand, those three animals had moderate to high PCV2 antibody titres, which alternatively suggests that antibody response was maybe enough to counteract the potential detrimental effect of the infection (overt clinical disease). It has to be taken into account, however, that the criterion to select healthy animals was the clinical evaluation at the moment of PMWS outbreak, selecting the best pigs from a reduced group comprised by 100 pigs ear-tagged at 1 week of age. Therefore, pre-clinical signs such as initial decrease of growing or loose of weight could have been unnoticed. For obvious reasons, it is not possible to assess if these 3 animals would have been clinically affected by the disease subsequently.

Diagnostic performance of both qPCR and serology tests used in Denmark and Spain was assessed to evaluate if they can be useful to diagnose PMWS in live animals. This was especially relevant in the present work because most of samples were obtained from live animals, fitting perfectly a practical situation in which veterinarians may be able to diagnose PMWS without having to euthanize suspected animals. Taking into account the bias among the qPCR methods used (Hjulsager et al., 2009), sera was the only type of sample that gave an optimal cut-off value similar in both countries. Those values were close to the threshold of 10^7 PCV2 copies per ml of sera previously proposed (Brunborg et al., 2004; Olvera et al., 2004; Fort et al., 2007). Differences in sampling procedure and material used could explained the different optimal cut-offs values obtained by nasal and rectal swabs in both countries. Overall, our results indicated that PCV2 qPCR (mainly from sera and nasal swabs) can give valuable information related to PMWS status in a group of pigs, being able to differentiate a population of PMWS affected from one of non-PMWS affected pigs during a PMWS outbreak and even some days before. However, a desirable probability of making a correct individual diagnosis higher than 90% was far from being achieved. The combination of the qPCR technique and a serology test improved specificity and PV+, indicating that such combination could be used to potentially confirm PMWS in live PMWS-suspected animals, but with a high percentage of false negatives due to their low combined sensitivity and PV−. Therefore, the lack of sensitivity and/or specificity values observed from both tests used separately or combined suggested that qPCR and/or serology tests are not able to substitute histopathology plus detection of PCV2 in tissues for the individual PMWS diagnosis within PMWS affected farms.

The present report is the first descriptive infection dynamics study performed in multiple PMWS affected farms from different countries. This work describes the evolution of PCV2 infection, excretion and PCV2 antibodies in histopathologically well-characterized pigs. Vaccines against PCV2 have been recently developed and are under commercial use in different parts of the world. Understanding PCV2 infection dynamics in the field is considered crucial for a proper design of vaccine strategies and its corresponding monitoring.

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Study IV

Pig-major acute phase protein and haptoglobin serum concentrations correlate with PCV2 viremia and the clinical course of postweaning multisystemic wasting syndrome.

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Pig-major acute phase protein and haptoglobin serum concentrations correlate with PCV2 viremia and the clinical course of postweaning multisystemic wasting syndrome

L. Grau-Roma [a,b,*], P.M.H. Heegaard [c], C.K. Hjulsager [c], M. Sibila [a], C.S. Kristensen [d], A. Allepuz [a,b], M. Piñeiro [e], L.E. Larsen [c], J. Segalés [a,b], L. Fraile [a,f]

[a] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[c] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1790 Copenhagen V, Denmark
[d] Danish Pig Production, Vinkelvej 11, DK-8620 Kjellerup, Denmark
[e] PigCHAMP Pro Europa S.A., Gremio de los Segovianos, 13, 40195 Hontoria, Segovia, Spain
[f] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain

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ABSTRACT

The aim of the present longitudinal study was to assess the evolution of two acute phase proteins (APPs), pig-major acute phase protein (pig-MAP) and haptoglobin (HPT), in serum from pigs that developed postweaning multisystemic wasting syndrome (PMWS) in comparison to healthy and wasted non-PMWS affected pigs. In addition, evidence of infection with other pathogens and its relation with variations in APPs concentrations was also assessed. Fourteen independent batches of 100–154 pigs were monitored from birth to PMWS outbreak occurrence in 11 PMWS affected farms. Pigs displaying PMWS-like signs and age-matched healthy controls were euthanized during the clinical outbreak. PMWS was diagnosed according to internationally accepted criteria and pigs were classified as: (i) PMWS cases, (ii) wasted non-PMWS cases and (iii) healthy pigs. At the moment of PMWS occurrence, pig-MAP and HPT concentration in PMWS affected pigs were higher than in healthy ones ($p < 0.0001$). No differences in APPs serum concentrations between subclinically PCV2-infected pigs and healthy non-PCV2-infected pigs (based on quantitative PCR on serum results) were detected. Results showed a significant correlation between PCV2 loads and both pig-MAP ($R = 0.487–0.602$, $p < 0.0001$) and HPT ($R = 0.326–0.550$, $p < 0.05–0.0001$) concentrations in serum of PMWS affected pigs, indicating that the acute phase response in PMWS affected pigs occurred concomitantly to PCV2 viremia. No other pathogen, apart from PCV2, was consistently related with variations in APPs concentrations. A ROC analysis, made to determine the capacity of discrimination of both APPs between PMWS affected and non-affected pigs, showed higher sensitivity and specificity values using pig-MAP compared to HPT. These results suggest that pig-MAP might be a better indicator of PMWS status than HPT. Moreover, the fact that APR occurred some weeks before the start of clinical signs suggests that APPs could provide valuable prognostic information for PMWS development.

1. Introduction

The acute phase response (APR) is an innate, non-specific immune response which occurs after many different stimuli such as infections, tissue damage,

* Corresponding author at: Departament de Sanitat i d'Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel.: +34 93 581 32 84; fax: +34 93 581 44 90.
E-mail address: llgrau@cresa.uab.cat (L. Grau-Roma).

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neoplastic growth or immunological disorders (Baumann and Gauldie, 1994; Gruys et al., 2005a). This complex reaction is mediated by pro-inflammatory cytokines and involves both local and systemic reactions, including fever, increase in muscle protein catabolism, alterations in sleep and appetite patterns, and changes in the concentration of a group of plasma proteins which are called acute phase proteins (APPs) (Gruys et al., 2005a). These proteins are mainly synthesised in the liver and are classified according to the direction of change (positive APPs if their concentration increases, negative if it decreases), and according to the extent to which their concentrations change (minor, intermediate or major) during the APR (Petersen et al., 2004; Gruys et al., 2005a).

APPs have been described as useful for assessing health in humans and animals (Petersen et al., 2004; Gruys et al., 2005b). In pigs, APPs concentrations have been demonstrated to be increased after experimental infections with several bacteria, viruses or parasites, as for instance *Streptococcus suis* (Sorensen et al., 2006), porcine reproductive and respiratory syndrome virus (PRRSV) (Asai et al., 1999) and *Toxoplasma gondii* (Jungersen et al., 1999). Moreover, increases in APPs concentrations have also been detected in pigs affected by tail- and ear-biting or after stress induced by transport conditions or changes in the pattern of food administration (Parra et al., 2006; Piñeiro et al., 2007; Salamano et al., 2008). Specifically, haptoglobin (HPT) and pig-major acute phase protein (pig-MAP) have been suggested as the most robust APPs as indicators of infection in pigs (Sorensen et al., 2006).

Porcine circovirus type 2 (PCV2) is considered the essential infectious agent for postweaning multisystemic wasting syndrome (PMWS) development, but it is also known that other triggering factors are necessary for the full expression of the clinical disease (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés et al., 2005). The internationally accepted criteria for diagnosis of PMWS include the presence of compatible clinical signs (mainly wasting and respiratory distress), moderate to severe lymphocyte depletion with granulomatous inflammation in lymphoid tissues, and detection of moderate to high amounts of PCV2 within these lesions (Sorden, 2000; Segalés et al., 2005). Although it is known that APPs serum concentrations are increased in PMWS affected pigs (Segalés et al., 2004; Parra et al., 2006), few field data are available in the literature. Moreover, the longitudinal evolution of APPs in animals that finally develop the disease has not been assessed.

The aim of this study was to assess the evolution of two APPs, pig-MAP and HPT, in serum throughout the productive life of pigs that developed PMWS in comparison to healthy pigs and pigs that developed wasting without fulfilling PMWS diagnosis. Moreover, the infection dynamics of PCV2 and several other pathogens was monitored by quantitative PCR, nested PCR and/or serological techniques to determine potential associations between studied APPs concentrations and the moment of infection for each pathogen.

2. Materials and methods

2.1. Study design

Two longitudinal case–control studies in PMWS affected farms, one in Spain and one in Denmark, were performed as previously described (Grau-Roma et al., 2009). Briefly, both studies were carried out using similar designs, and were performed on 14 independent farm batches (6 in Spain and 8 in Denmark) of 100–154 animals per batch, coming from 11 different farms (3 from Spain and 8 from Denmark). Studied piglets were ear-tagged at 1 week of age and monitored until the occurrence of the PMWS outbreak. Nasal and rectal swabs as well as blood samples were serially collected from those piglets at established weeks of age (1, 3, 7 and 11 in Spain, and 1, 4, 6 and 9 in Denmark) and at the time of PMWS outbreak occurrence (necropsy). When the PMWS compatible clinical picture (Segalés, 2002) appeared at the studied farms, pigs displaying PMWS-like signs and healthy age-matched pigs were selected, euthanized and necropsied. At necropsy, sections of lymphoid tissues were collected and fixed by immersion in neutral-buffered 10% formalin to assess the pathological status of both clinically healthy and diseased animals.

All treatments, housing, husbandry and slaughtering conditions followed the European Union Guidelines and Good Clinical Practices.

2.2. Histopathology

Formalin fixed tissues were dehydrated and embedded in paraffin blocks. Two consecutive 4-μm thick sections containing collected lymphoid tissues from each pig were cut from each block. One section was processed for haematoxylin and eosin stain, while the other was used for PCV2 nucleic acid detection by *in situ* hybridization (ISH) (Rosell et al., 1999) for Spanish cases, and for immunohistochemistry (IHC) for PCV2 antigen detection (Jensen et al., 2006) for Danish cases. Pathological evaluation and PMWS diagnosis was carried out using a previously described scoring system evaluating the PCV2 amount and the intensity of lymphoid depletion and granulomatous infiltration present in lymphoid tissues (Grau-Roma et al., 2009). Three different categories of pigs were established: (i) PMWS cases: corresponding to pigs showing clinical wasting and with moderate to severe lymphoid lesions and moderate to high amount of PCV2 antigen/nucleic acid; (ii) wasted non-PMWS cases: corresponding to pigs showing clinical wasting but without or with slight PMWS characteristic histopathological lesions and no or low amount of PCV2 antigen/nucleic acid within lymphoid tissues; (iii) healthy pigs: corresponding to pigs showing good clinical condition, without or with slight PMWS characteristic histopathological lesions and no or low amount of PCV2 antigen/nucleic acid within lymphoid tissues.

2.3. Acute phase proteins determination in serum

Two APPs, HPT and pig-MAP, were measured in serum of studied pigs. Both APPs were determined in all

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longitudinally collected sera from Spanish necropsied pigs (107 pigs coming from 3 different farms). For the Danish pigs, serum samples collected at 1, 6 or 9 weeks of age and at necropsy from only those pigs with available serum at the moment of necropsy were analysed (53 pigs coming from 7 different batches).

Pig-MAP concentration was determined by a sandwich enzyme-linked immunosorbent assay (ELISA), using a commercial kit based on two anti-pig-MAP-specific monoclonal antibodies (PigCHAMP Pro Europa S.A.), according to the manufacturers' instructions. This kit was based on an ELISA previously developed and validated (Piñeiro et al., 2009b). HPT concentration was determined by a sandwich ELISA as previously described (Sorensen et al., 2006). Pig-MAP and HPT results were expressed as mg per millilitre of serum (mg/ml).

2.4. Quantitative PCV2 PCR

PCV2 real-time quantitative PCR (qPCR) was performed on all longitudinally collected serum samples. DNA was extracted from 200 μl of serum from Spanish (Nucleospin® Blood, Macherey-Nagel, GmbH & Co KG, Düren, Germany) and Danish samples (QIAamp DNA Mini Kit, Qiagen® GmbH, Germany), according to the manufacturers' instructions. DNA was eluted in 100 μl and 200 μl of elution buffer in Spain and Denmark, respectively.

PCV2 DNA was quantified using two previously described qPCR techniques on Spanish (Olvera et al., 2004) and Danish (Hjulsager et al., 2009) samples, respectively. The performance of both qPCRs was compared in a previous study based on field samples (Hjulsager et al., 2009). This latter work showed a highly significant linear association between results of the two qPCR assays. However, the method performed by the Danish laboratory had higher sensitivity and yielded systematically higher PCV2 load values than the one used in the Spanish laboratory. All PCV2 qPCR results were expressed as $\log_{10}$ (number of PCV2 copies per ml of sera).

2.5. Serology

Serological analyses were performed on all longitudinally collected serum samples. In Spain, ELISAs were used to detect antibodies against porcine reproductive and respiratory syndrome virus (PRRSV) (HerdCheck PRRS virus antibody test, IDEXX, Inc., USA), Aujeszky's disease virus (HerdCheck anti-PRV gpI, IDEXX, Inc., USA), porcine parvovirus (PPV) (Ingezim PPV, Ingenasa, Spain), swine influenza virus (SIV) (Civtest Suis Influenza, Laboratorios Hipra, Spain), *Mycoplasma hyopneumoniae* (Civtest Suis *Mycoplasma hyopneumoniae*, Laboratorios Hipra, Spain), and *Salmonella* spp. (Salmonella covalent mix-ELISA, Svanovir™, Svanova, Sweden). In Denmark, assays used were previously described immunoperoxidase monolayer assays (IPMAs) to detect antibodies against European and American PRRSV strains (Sorensen et al., 1998), ELISAs to detect antibodies against PPV (Madsen et al., 1997) and *Lawsonia intracellularis* (Boesen et al., 2005), and haemagglutination inhibition (HI) test to detect antibodies against H1N1 and H3N2 subtypes of SIV, which was essentially carried out according to the OIE manual (OiE manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008). A seroconversion against studied pathogens was considered to be present when an increase in the antibody titres in two consecutive samples or when a positive result preceded by a previous negative result was detected.

2.6. Mycoplasma hyopneumoniae nested PCR

It is known that the appearance of antibodies to *M. hyopneumoniae* can be delayed as much as 8 weeks after infection (Sitjar et al., 1996). Thus, in order to avoid underestimating its potential prevalence in studied animals, a nested PCR (nPCR) assay to detect *M. hyopneumoniae* was performed in the Spanish nasal swabs collected at the moment of necropsy (Calsamiglia et al., 1999). For 1 out of 107 Spanish pigs the nasal swab sample was lacking.

2.7. Statistical analyses

For statistical evaluation, the SAS 9.1.3 software was used (SAS institute Inc., Cary, NC, USA). A repeated measurement analysis of variance according to the general linear model procedure (PROC MIXED) and LSMEANS follow-up test was used to evaluate APPs mean concentrations differences between: (i) PMWS cases, wasted non-PMWS and healthy pigs at each sampling time and within them at different samplings points; (ii) healthy PCV2 subclinically infected pigs (PCV2 qPCR positive) and healthy non-infected pigs (PCV2 qPCR negative) at different sampling points; (iii) seroconverted and non-seroconverted pigs against studied pathogens at each sampling; and (iv) *M. hyopneumoniae* infected (nPCR positive) and non-infected (nPCR negative) at necropsy. Constant correlation was assumed between repeated measurements. Post hoc multiple comparisons were addressed using the Tukey's test. PCV2 qPCR data were $\log_{10}$ transformed prior to graphic representation, and negative results were coded as 0. A Pearson's Chi-square test was used to compare the proportions of seroconverted animals to each studied pathogen at the moment of necropsy between the three categories of pigs studied; same comparison was also established using *M. hyopneumoniae* nPCR results. The level of significance for all analyses (α) was set to $p < 0.05$.

To determine the capacity of discrimination of both pig-MAP and HPT between PMWS affected and healthy pigs, a Receiver Operating Characteristic (ROC) analysis from samples collected at necropsy was carried out using Statsdirect (version 2.6.6, CamCode, Ashwell, UK). Moreover, ROC analysis was also performed to determine the diagnostic performance of both APPs on clinically affected animals. For this later analysis, only wasted pigs were considered, considering PMWS as "diseased" and wasted non-PMWS as "non-diseased" animals. Taking into account that APPs concentration was determined by the same laboratorial tests in both studies, all samples collected at necropsy coming from Spain and Denmark were analysed together.

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Table 1
Number of pigs within each pathological category from which haptoglobin and pig-MAP concentrations were determined in each country.

	PMWS	Healthy	Wasted non-PMWS	Total
Spain	35	29	43	107
Denmark	21	17	15	53
Total	56	46	58	160

3. Results

3.1. Studied animals, histopathology and acute phase proteins

The number of pigs from which APPs concentrations were analysed and their pathological classification (PMWS, healthy and wasted non-PMWS) are detailed in Table 1. Mean ± standard deviation (S.D.) of weeks of age at necropsy for PMWS, healthy and wasted non-PMWS pigs were 14.0 ± 2.1, 14.7 ± 2.5, 15.2 ± 2.7, respectively in Spain, and 11.2 ± 1.5, 10.0 ± 3.5, 10.9 ± 1.9, respectively, in Denmark.

The longitudinal evolution of pig-MAP serum concentrations is displayed in Fig. 1. Pig-MAP concentration in PMWS affected pigs was higher than in non-PMWS affected ones (healthy and wasted non-PMWS) both in Spain ($p < 0.0001$) and Denmark ($p < 0.001$) at the moment of necropsy. Although no statistically significant differences were found between healthy and wasted non-PMWS pigs, the mean pig-MAP serum concentration at the moment of necropsy in wasted non-PMWS was higher than in healthy ones (Spain: $p = 0.0715$; Denmark: $p = 0.1472$). Moreover, the pig-MAP concentration in PMWS pigs at the sampling prior to necropsy in Spain was higher than its concentration in non-PMWS affected pigs (healthy and wasted non-PMWS) ($p < 0.01$). Accordingly, in the Spanish pigs, the increase of pig-MAP

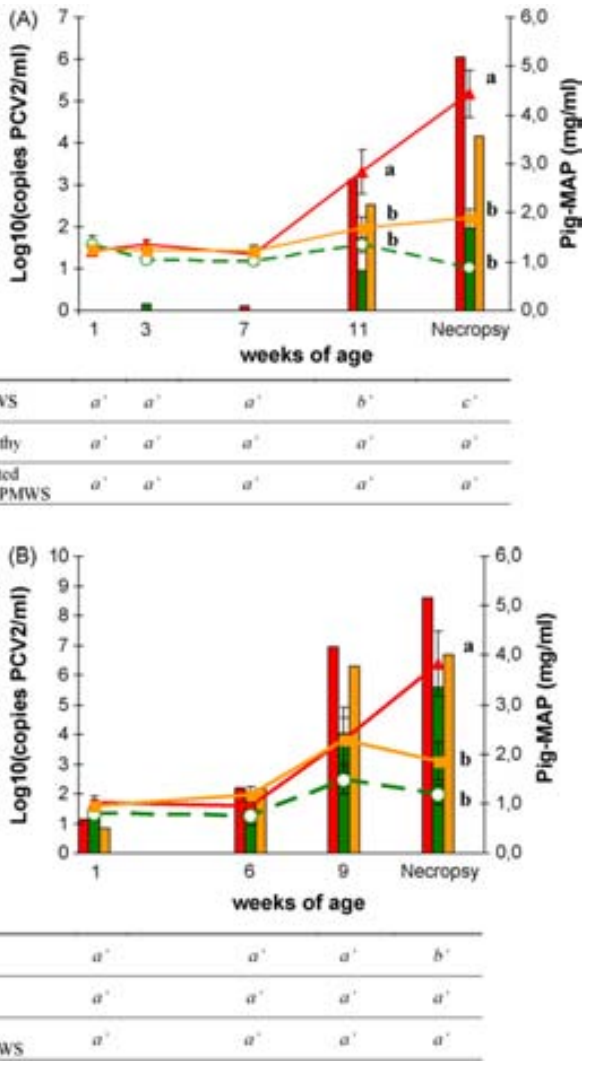

Fig. 1. Pig-MAP concentrations (mg/ml) (lines) and PCV2 loads (Log_{10}[copies of PCV2/ml of sera]) (columns) in PMWS (,), healthy (,) and wasted non-PMWS (,) pigs at the different sampling points from Spanish (A) and Danish (B) farms. **a**, **b** indicate statistical significant differences in APP concentration between animal groups in a given sampling time. a',b',c' indicate statistical significant differences in APP concentration within each animal group along the study period ($p < 0.05$). Error bars represent standard error of the mean.

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concentration in PMWS affected pigs was already significant at the sampling prior to necropsy, compared to basal pig-MAP concentrations detected at weeks 1–7. In Denmark, a significant increase in pig-MAP concentration was only detected at necropsy in PMWS pigs. No statistical significant changes in pig-MAP concentration were found among all samplings in healthy and in wasted non-PMWS pigs from both countries.

The longitudinal evolution of HPT serum concentrations is displayed in Fig. 2. HPT concentration at the moment of necropsy was higher in PMWS than in healthy pigs both in Spain ($p < 0.0001$) and Denmark ($p < 0.01$). On the other hand, wasted non-PMWS could not be distinguished from the other two groups in Denmark, but had significantly lower HPT concentration than PMWS ($p < 0.001$) in Spain. Regarding the longitudinal evolution of HPT concentration within each group, HPT increased progressively in both PMWS and wasted non-PMWS pigs in Spain from 11 weeks of age ($p < 0.01$) to the necropsy moment ($p < 0.0001$). In Denmark, only PMWS affected pigs gave significant increase in HPT concentrations with respect to the basal concentrations detected at the 1st week of age ($p < 0.01$). No statistically significant variation was found among the HPT concentrations of healthy pigs neither in Spain nor Denmark.

Pig-MAP concentrations obtained at the moment of necropsy were statistically correlated with the global average scoring (histopathology plus PCV2 detection in lymphoid tissues) in Spain ($R = 0.622$; $p < 0.0001$) and also in Denmark ($R = 0.524$; $p < 0.0001$). On the other hand, significant correlation between HPT concentration at the moment of necropsy and the global average scoring was

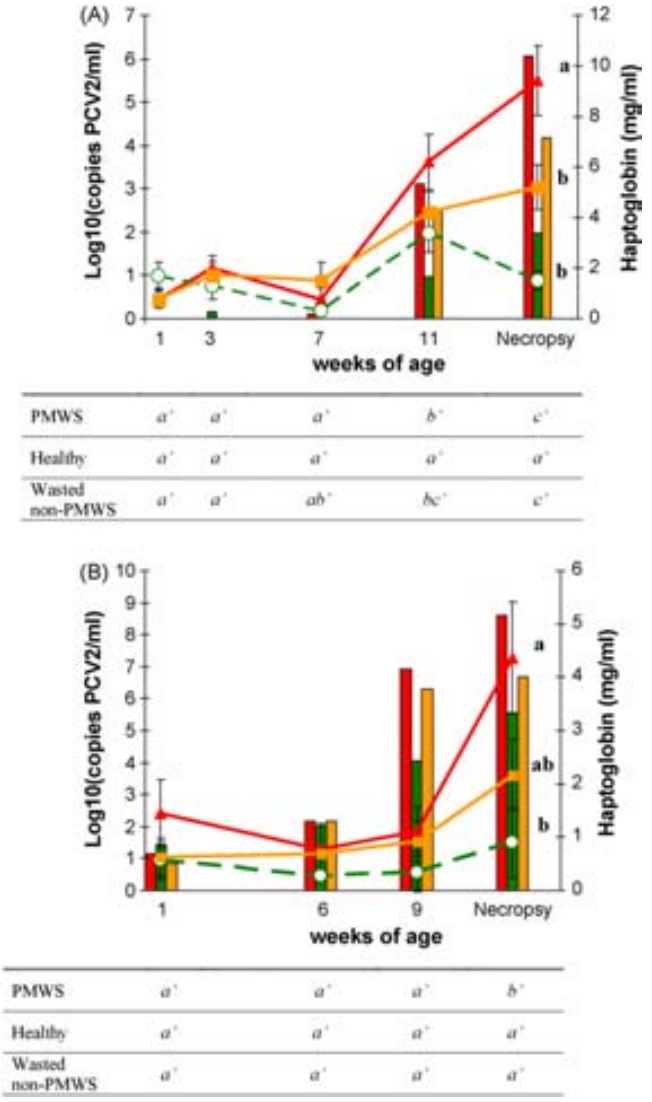

Fig. 2. Haptoglobin concentrations (mg/ml) (lines) and PCV2 loads (Log_{10}[copies of PCV2/ml of sera]) (columns) in PMWS (,), healthy (,) and wasted non-PMWS (,) pigs at the different samplings from Spanish (A) and Danish (B) farms. **a**, **b** indicate statistical significant differences in APP concentration between animal groups in a given sampling time. a', b', c' indicate statistical significant differences in APPs concentration within each animal group along the studied period ($p < 0.05$). Error bars represent standard error of the mean.

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detected in Spain ($R = 0.443$; $p < 0.0001$), but only a tendency was observed in Denmark ($R = 0.254$; $p = 0.066$). Moreover, pig-MAP and HPT concentrations were significantly correlated (Spain: $R = 0.462$, $p < 0.0001$; Denmark: $R = 0.576$, $p < 0.0001$).

3.2. PCV2 qPCR

PCV2 loads in sera are schematically displayed together with APPs results in Figs. 1 and 2. PCV2 loads increased progressively from the 1st week of age until necropsy in Denmark and during the last two samplings in Spain. Maximum PCV2 loads were observed at the moment of disease outbreak (necropsy) in both countries.

Significant correlations were observed between pig-MAP serum concentrations and PCV2 loads in serum (Spain: $R = 0.278$, $p < 0.0001$; Denmark: $R = 0.47$, $p < 0.001$). Considering the three groups of pigs separately (Fig. 3), PMWS affected pigs corresponded to the group with higher correlation between PCV2 load and pig-MAP (Spain: $R = 0.487$, $p < 0.0001$; Denmark: $R = 0.602$, $p < 0.0001$), followed by the correlations observed in wasted non-PMWS pigs (Spain: $R = 0.218$, $p < 0.01$; Denmark: $R = 0.437$, $p < 0.01$). On the contrary, no significant correlation was found between pig-MAP concentrations and PCV2 loads when only healthy pigs were considered (Spain: $R = -0.152$, $p = 0.07$; Denmark: $R = 0.2$, $p = 0.194$) (Fig. 3).

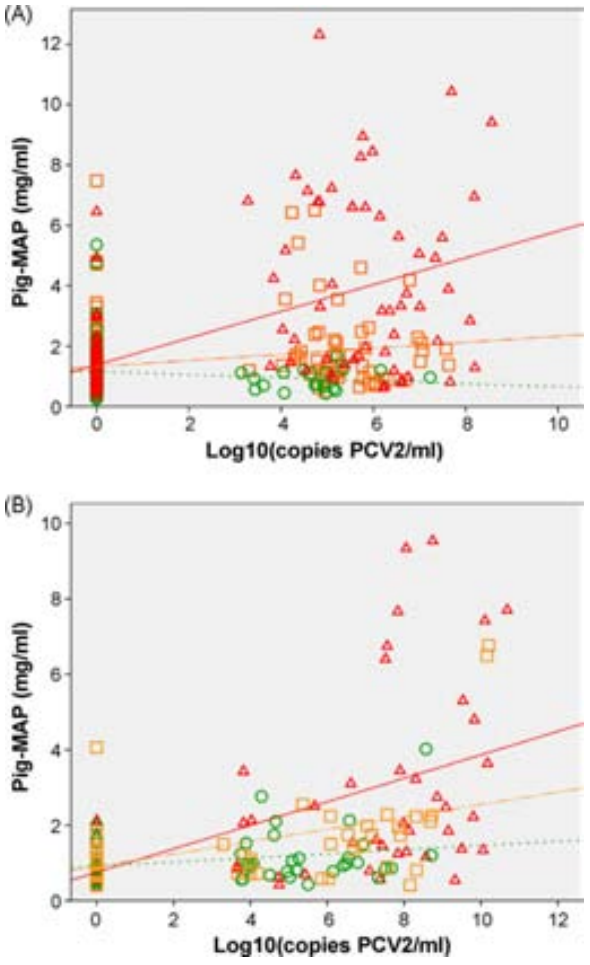

Fig. 3. Correlation between pig-MAP concentration and PCV2 load in each studied category of pigs: PMWS (△, ▬), wasted non-PMWS (□, ▬) and Healthy (○, ▬) in Spain (A) and Denmark (B).

Significant correlations were also found between HPT serum concentrations and PCV2 loads in serum (Spain: $R = 0.443$, $p < 0.0001$; Denmark: $R = 0.326$, $p < 0.001$). Considering the three groups of pigs separately, the group of pigs with PMWS had again the highest correlation (Spain: $R = 0.550$, $p < 0.0001$; Denmark: $R = 0.326$, $p < 0.05$), followed by the correlations observed in wasted non-PMWS pigs (Spain: $R = 0.437$, $p < 0.0001$; Denmark: $R = 0.295$, $p = 0.057$). The lowest correlation between HPT concentration and PCV2 load was found in healthy pigs, being significant in Spain ($R = 0.186$, $p < 0.05$) but not in Denmark ($R = 0.273$, $p = 0.73$).

Taking into account that more than half of healthy Spanish pigs gave negative results at all samplings by PCV2 qPCR, mean APPs concentrations in healthy PCV2-infected and non-infected pigs were also compared. No statistical differences were seen between both groups of pigs neither in pig-MAP nor HPT concentrations. Such comparison was not done in any other group of pigs due to the low number of negative PCV2 qPCR results.

3.3. Serology and PCR against different swine pathogens

The number of pigs that had seroconverted against studied pathogens and PCV2 qPCR results at the moment of necropsy in both countries, and *M. hyopneumoniae* nPCR in Spain, are displayed in Table 2.

No serological evidence of infection with ADV was detected. Evidence of *M. hyopneumoniae*, PPV or PRRSV infections in Spain and SIV infection in Denmark were found in less than 10% of analysed pigs. Prevalence of SIV and *Salmonella* spp. in Spain and PPV, PRRSVe, PRRSVu and *L. intracellularis* in Denmark was detected in 25–55% of the studied pigs.

No differences in the proportions of infected and/or seroconverted animals to studied pathogens between PMWS, wasted non-PMWS and healthy pigs were observed. Moreover, no significant differences in APPs concentrations between seroconverted and non-seroconverted pigs to studied pathogens were detected. However, the five pigs that gave positive nPCR results for *M. hyopneumoniae* in Spain had higher pig-MAP mean serum concentrations (mean ± S.D. = 4.47 ± 4.49) than the 101 pigs that gave negative nPCR (2.35 ± 2.13) ($p < 0.05$).

3.4. ROC analysis

Optimal cut-off values for both studied APPs maximizing sensitivity (Sn) and specificity (Sp) to discriminate diseased and non-diseased pigs are displayed in Table 3.

Sn and Sp values were higher when comparing PMWS versus healthy pigs than comparing PMWS versus wasted non-PMWS animals. Moreover, Sn and Sp values obtained using pig-MAP concentrations were always higher than when HPT was used. Further exploration of the data showed that, when comparing PMWS and healthy pigs, Sp

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Table 2
Number of seroconverted pigs against studied pathogens, and nPCR and/or qPCR results at the moment of necropsy in Spain (A) and Denmark (B). Results are indicated as the number of seropositive (serology) or positive (qPCR and nPCR) pigs at euthanasia during the PMWS outbreak with respect to the total of studied pigs. Percentage is given between brackets.

A.

	Serology						nPCR	qPCR
	PPV[a]	PRRSV	SIV	ADV	Salm	Myco	Myco	PCV2
PMWS	2/35 (6)	1/35 (3)	16/35 (46)	0/35 (0)	8/35 (23)	0/35 (0)	3/35 (9)	35/35 (100)
Healthy	3/29 (10)	0/29 (0)	17/29 (59)	0/29 (0)	9/29 (31)	0/29 (0)	1/29 (3)	12/29 (41)
Wasted non-PMWS	2/43 (5)	1/43 (2)	26/43 (60)	0/43 (0)	19/43 (44)	0/43 (0)	1/43 (2)	34/43 (79)
Total	7/107 (7)	2/107 (2)	59/107 (55)	0/107 (0)	36/107 (34)	0/107 (0)	5/107 (5)	81/107 (76)

B.

	Serology						qPCR
	PPV	PRRSVe	PRRSVu	SIV_H1N1	SIV_H3N2	Law	PCV2
PMWS	7/21 (33)	9/21 (43)	10/21 (48)	1/21 (5)	3/21 (14)	5/21 (24)	21/21 (100)
Healthy	11/17 (65)	7/17 (41)	10/17 (59)	3/17 (18)	1/17 (6)	5/17 (29)	15/17 (88)
Wasted non-PMWS	4/15 (27)	8/15 (53)	9/15 (60)	0/15 (0)	0/15 (0)	3/15 (20)	13/15 (87)
Total	22/53 (42)	24/53 (45)	29/53 (55)	4/53 (8)	4/53 (8)	13/53 (25)	49/53 (93)

[a] Porcine parvovirus (PPV), Porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky disease virus (ADV), Swine influenza virus (SIV), *Salmonella* spp. (Salm), *Mycoplasma hyopneumoniae* (Myco), Porcine circovirus type 2 (PCV2), European and American PRRSV strains (PRRSVe, PRRSVu), *Lawsonia intracellularis* (Law).

Table 3
Optimal cut-off, sensitivity and specificity values of ROC analyses for pig-MAP and Haptoglobin serum concentrations considering PMWS pigs as "diseased" and healthy or wasted non-PMWS as "non-diseased animals".

APPs	PMWS vs. healthy			PMWS vs. wasted non-PMWS		
	Optimal cut-off	Sensitivity ($CI_{95\%}$)	Specificity ($CI_{95\%}$)	Optimal cut-off	Sensitivity ($CI_{95\%}$)	Specificity ($CI_{95\%}$)
Pig-MAP	≥1.3	85.7 (73.8–93.6)	87.0 (73.7–95.0)	≥2.7	60.7 (46.7–73.5)	87.7 (76.3–94.9)
Haptoglobin	≥2.7	67.9 (54.0–79.7)	84.8 (71.1–93.7)	≥2.7	67.9 (54.0–79.7)	55.2 (41.5–68.3)

values were slightly improved when the presence of PCV2 (detected by qPCR) was considered together with the APPs concentration. Thus, a potential pre-mortem diagnosis of PMWS could be established only when APP concentrations were above optimal threshold and qPCR gave a positive result. Following this condition, pig-MAP and HPT maintained their Sn at 85.7% (73.8–93.6) and 67.9% (54.0–79.7), respectively, and Sp increased to 93.5% (82.1–98.6) in both cases.

4. Discussion

The present study represents the first longitudinal determination of pig-MAP and HPT concentrations in pigs that subsequently developed PMWS. Both APPs increased in parallel to the increase of PCV2 load in sera and reached the maximum values at the moment of disease manifestation. Pig-MAP and HPT concentrations were higher in PMWS affected pigs than in healthy ones, which is in agreement with previous reports (Segalés et al., 2004; Parra et al., 2006). This situation is probably a reflection of the systemic inflammatory status suffered by PMWS affected pigs (Rosell et al., 1999).

It is known that APPs serum concentrations might vary with the age (Piñeiro et al., 2009a). However, in the present study, samples from all pigs were collected at previously established weeks of age to accomplish a longitudinal study (Grau-Roma et al., 2009). Moreover, at the moment of necropsy, pigs displaying PMWS-like signs and healthy pigs were euthanized in an age-matched way. Therefore, the differences observed between the three studied groups of pigs should not be influenced by a potential age-effect.

The higher correlation between PCV2 load and both APPs detected in PMWS affected pigs, together with the absence or weak correlation detected in healthy pigs, indicates that the APR in PMWS affected pigs occurs concurrently with PCV2 infection, further supporting the idea that PCV2 is the essential infectious agent for PMWS development (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés et al., 2005). Based on qPCR PCV2 results, an important percentage of healthy and wasted non-PMWS pigs were also infected by PCV2. However, significant increase in APPs concentrations was only clearly observed in PMWS affected animals. It has been reported that infections with pathogens like *S. suis* produce an increase of APPs in both clinically and subclinically infected pigs (Sorensen et al., 2006). However, present data showed that, in accordance with previous results (Segalés et al., 2004), no differences between subclinically PCV2-infected pigs (healthy PCV2 qPCR positive pigs) and healthy non-PCV2-infected pigs (healthy PCV2 qPCR negative pigs) were detected. These findings suggest that sole infection with PCV2 does not imply a systemic inflammatory status causing a detectable APR; only when the disease develops, HPT and pig-MAP concentrations increase significantly. Taking into account that PMWS affected pigs have higher serum viral loads than non-affected pigs (Brunborg et al., 2004; Olvera et al., 2004; Grau-Roma et al., 2009), a certain

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level of serum PCV2 load might be associated with the increase of HPT and pig-MAP serum concentrations. Therefore, it is likely that when the animal limits the PCV2 replication to certain levels and/or the virus is not able to overcome the defences of the host, the APR becomes much less evident or even non-detectable using HPT and pig-MAP determinations in sera.

It is difficult to collectively interpret the results of the pigs suffering wasting but without a PMWS diagnosis (wasted non-PMWS animals). This group of animals might include pigs suffering from wasting due to PMWS-unrelated causes, such as other infectious or non-infectious diseases and/or management conditions (Harding, 1997), but might also include PMWS convalescent pigs or pigs that were able to overcome or limit the disease expression (Segalés, 2002; Krakowka et al., 2005). The obtained pig-MAP concentration profile in wasted non-PMWS pigs was similar to the one obtained in healthy pigs. However, an increase in HPT concentration was detected at the two last samplings in Spain. Moreover, in Denmark, HPT concentrations at the moment of necropsy in wasted non-PMWS animals could not be distinguished from those in PMWS affected pigs. These results, together with the wider APPs concentration variation observed in wasted non-PMWS pigs compared to healthy ones, suggest that at least a proportion of wasted non-PMWS animals mounted an APR around the PMWS outbreak. On the other hand, this group of pigs had lower PCV2 serum loads than PMWS-affected ones and higher than healthy pigs during PMWS outbreak (Grau-Roma et al., 2009). Moreover, a correlation between PCV2 viral load and APPs concentration was observed. Taken together, these findings suggest that the APR detected in the groups of wasted non-PMWS affected pigs might also be associated with PCV2 infection.

The fact that an increase of both pig-MAP and HPT proteins was seen in Spanish pigs prior to the PMWS outbreak suggests that the detectable APR occurred some weeks before the start of clinical signs. Therefore, those APPs could provide valuable prognostic information if proper timing of sampling is assured (Murata et al., 2004). These increased values before the PMWS outbreak were not detected in the Danish animals, probably due to the low number of samples analysed at week 9 in each group.

Despite it is known that APPs are generic markers of inflammation and therefore are not specific of any disease (Petersen et al., 2004; Gruys et al., 2005a), the previously described potential use of APP as markers of health (Gruys et al., 2005b; Parra et al., 2006) prompted us to evaluate their diagnostic performance for PMWS. Both HPT and pig-MAP serum concentrations were significantly correlated. Nevertheless, pig-MAP gave a better diagnostic value of PMWS, since this protein displayed higher Sp and Sn values than HPT in the ROC analysis. Moreover, pig-MAP was able to differentiate PMWS from non-PMWS affected pigs (comprising wasted non-PMWS and healthy pigs) even at the sampling prior to PMWS development (at least in Spain). The previously described individual higher variation in HPT concentrations (Piñeiro et al., 2009a) could partially explain the lower diagnostic value of HPT. In any case, as previously indicated, the APPs values of single reactants are not sensitive enough to detect a special subject in a livestock population (Gruys et al., 2005a). It has been suggested that the acute phase signal situation obtained for an individual animal can be enhanced when the values of positive APPs are combined with negative APPs as an index. Such an index could enhance Sn and Sp remarkably in comparison to single APPs determination in the search for unhealthy subjects among a population of apparently normal animals (Gruys et al., 2005a).

PMWS is considered a multifactorial disease in which the occurrence of PCV2 infection is necessary but not sufficient (Harding, 2004; Ghebremariam and Gruys, 2005; Segalés et al., 2005). Present results showed that, among all studied pathogens, PCV2 was the only one systematically present in PMWS affected farms and pigs. This fact suggests that none of the other studied pathogens are necessary for the PMWS development under field conditions. Moreover, no significant differences in APP serum concentrations between those pigs showing evidences of infection for pathogens different from PCV2 at the moment of clinical PMWS outbreak were detected, with the only exception of *M. hyopneumoniae* in Spain. Specifically, the 5 pigs that gave positive PCR results for *M. hyopneumoniae* had higher pig-MAP concentration than the 101 pigs with negative results. In fact, the two pigs with higher pig-MAP concentration (10 and 9 mg/ml) corresponded to 2 out of the 5 *M. hyopneumoniae* nPCR positive, suggesting that PCV2 co-infection with other pathogens could increase the APR, aggravating the severity of the disease (Ellis et al., 2004). The potential influence of other not studied pathogens on the observed clinical signs and, therefore, on the APR of the PMWS affected animals, cannot be ruled out.

In conclusion, the present results indicate that the acute phase response in PMWS affected pigs occurred concomitantly to PCV2 viremia. In addition, present work supports the hypothesis that the increase of APPs levels depends on the development of PMWS and not on infection with PCV2 alone, as previously suggested (Segalés et al., 2004).

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IV. DISCUSSION

Insanity: doing the same thing over and over again and expecting different results
Albert Einstein

The use of nucleotide sequencing techniques allowed differentiating the variant of PCV detected in the first cases of PMWS (PCV2), from the contaminant PCV in PK-15 cell culture (PCV1) (Meehan et al., 1998; Morozov et al., 1998). Both PCV1 and PCV2 exhibit <80% sequence identity (Meehan et al., 1998; Morozov et al., 1998) and are today recognized as two different species by ICTV (www.ictvonline.org). Later on, sequencing works indicated a high sequence identity among PCV2 sequences (>90%) without any apparent relation between PCV2 sequences and the occurrence of the disease (Larochelle et al., 2002, 2003; de Boisseson et al., 2004; Grierson et al., 2004). However, recent data suggesting the opposite encouraged the scientific community to further explore such association (Timmusk et al., 2005; Carman et al., 2006; Opriessnig et al., 2006d; Cheung et al., 2007a, b). The analysis of PCV2 sequences present at the NCBI nucleotide database in September 2005 with and without the obtained 87 ORF2 PCV2 sequences in the study I confirmed that PCV2 sequences were distributed in two main phylogenetic groups (de Boisseson et al., 2004; Martins Gomes de Castro et al., 2007; Olvera et al., 2007), which we referred as PCV2 genotype 1 and PCV2 genotype 2. On one hand, the assignation of the numbers 1 and 2 was made to maintain the same nomenclature used by our previous research (Olvera et al., 2007). On the other hand, the term genotype was proposed in homology with the nomenclature used for other DNA or RNA viruses infecting swine such as TTV (Biagini et al., 1999), *Hepatitis E virus* (VHE) (Zhai et al., 2006) or *Japanese encephalitis virus* (JEV) (Nitatpattana et al., 2008). Few months after the acceptation of the manuscript presented in the study I, the EU consortium on PCVD (www.pcvd.org) discussed and agreed in supporting the definition of PCV2 genotypes based on the proposed methodology. However, after an intense debate, it was considered that the designation of genotype 1 and 2 could cause some confusion with the current nomenclature that already differentiates both species by means of using Arabic numbers, PCV1 and PCV2. Thus, the mentioned consortium proposed the nomenclature PCV2 genotype a (PCV2a), which corresponds to genotype 2 from study I, and PCV2 genotype b (PCV2b), which corresponds to genotype 1. The decision of giving “a” and “b” to

genotypes “2” and “1”, respectively, and not doing it on the other way around, was based mainly on two circumstances: (i) phylogenetical data from study I showed a major variability inside PCV2a (PCV2 genotype 2) suggesting that PCV2a might be older than PCV2b (PCV2 genotype 1). This hypothesis was supported by a retrospective Danish study that, using the proposed criteria to distinguish PCV2 genotypes, recovered only PCV2a sequences from two stored samples from 1990s and only PCV2b sequences from samples taken during 2000s (Dupont et al., 2008); (ii) Concomitantly to the publication of study I, researchers from USA and Canada also started to use the terminology genotype to differentiate the two main phylogenetical groups, but most of them used the terms PCV2a (for genotype 2) and PCV2b (for genotype 1) (Gagnon et al., 2007, 2008; Horlen et al., 2007; Carman et al., 2008; Opriessnig et al., 2008b). Therefore, it was decided to publish the letter included as addendum of study I of this thesis, in order to unify and standardise the different nomenclatures and classifications used in the literature. Interestingly, following the proposed genotype definition, the above-mentioned retrospective Danish study identified a new genotype in samples stored from 1980, 1987 and 1990, which was initially denominated PCV2 genotype 3 (Dupont et al., 2008) and was subsequently renamed as PCV2c through the letter emitted by the EU consortium. To date, PCV2c has not been found in any other country yet.

Both genotypes, PCV2a and PCV2b, are different in >2% of their whole genome, which corresponds to 35 or more nucleotide differences. Despite these numbers are relatively low, evidences from other ssDNA viruses indicate that a small number of changes may result in an increase of virulence. As an example, substitutions in 1 or 2 aminoacidic positions of *Feline parvovirus* (FePV) allowed a shift from cats to dogs and resulted in a virus that was highly pathogenic to dogs (Chang et al., 1992). Moreover, a single amino acid mutation in the capsid protein of another member of *Circoviridae* family, CAV, was identified as the major determinant of pathogenicity (Yamaguchi et al., 2001). Results obtained from study I showed that PCV2b sequences predominated in the

studied PMWS affected farms and that all sequences obtained from non-PMWS affected farms corresponded to PCV2a. These results led us to suggest that PCV2b might potentially be more pathogenic than PCV2a. A similar situation was detected in parallel in USA and Switzerland, where PCV2b was shown to predominate within PMWS farms, being detected in all diseased pigs (Cheung et al., 2007b; Horlen et al., 2007; Wiederkehr et al., 2009). Interestingly, only PCV2a sequences were recovered from the non-PMWS affected farms in Kansas (Horlen et al., 2007) and from healthy PCV2-PCR positive pigs sampled after 2003 in Switzerland (Wiederkehr et al., 2009). Those studies suggested that PCV2b was a major contributing factor for PMWS disease epidemic manifestation in the respective countries. Conversely, previous (Larochelle et al., 2002, 2003; de Boisseson et al., 2004) and also recent studies (Allan et al., 2007; Dupont et al., 2008) reported the existence of PCV2b in both PMWS-affected and non-PMWS affected farms. Additionally, several of the epidemiological works performed concomitantly or later than study I of this thesis suggested a shift in PCV2 genotypes from PCV2a to PCV2b. Several evidences pointed out to such shift. First, PCV2b was only sporadically detected in Canada and USA prior to severe PMWS outbreaks at the end of 2004 and it was apparently predominant in PMWS affected farms and pigs from 2004 onwards (Cheung et al., 2007b; Gagnon et al., 2007; Horlen et al., 2007; Carman et al., 2008). Secondly, although PCV2b already existed before in Europe and Asia (Mankertz et al., 2000; de Boisseson et al., 2004; Wen et al., 2005), most recent studies performed in Sweden (Timmusk et al., 2008), Switzerland (Wiederkehr et al., 2009), Denmark (Dupont et al., 2008), Ireland and Northern-Ireland (Allan et al., 2007) and Japan (Takahagi et al., 2008), indicated that PCV2b is currently predominant in the field. PCV2b is also apparently the predominant one in Brazil (Chiarelli-Neto et al., 2009). Thirdly, longitudinal data from Denmark also showed an apparent change from PCV2c to PCV2a during 80s-90s, and from PCV2a to PCV2b during 90-00s (Dupont et al., 2008). Last shift was suggested to be related with the outbreak of PMWS in this country (Dupont et al., 2008). Dupont et al. (2008) also found evidences of this shift when analysing all PCV2

sequences available at the NCBI database in February 2007. Thus, PCV2b apparently became predominant with time in several countries, and this global shift was suggested to occur on 2003 or before. Interestingly, all sequences uploaded from Australia, where PMWS has never been reported yet, corresponded to PCV2a (Dupont et al., 2008). All together, evidences coming from several countries suggest a real shift from PCV2a to PCV2b and an association between PCV2b with increased severity of PMWS clinical presentation. Although reasons of this association are unclear, one possibility is that the PCV2b genotype possesses greater virulence or, perhaps, that PCV2b virus can escape existing herd immunity stimulated by previously circulating PCV2a strains. In fact, 2 out of the 3 major ORF2 heterogenic regions described in the study I corresponded to 2 of the antigenic domains described by Mahé et al. (2000). Very recently, antigenic differences at the capsid protein level among different PCV2 strains of the two main genotypes have been described (Lefebvre et al., 2008a; Shang et al., 2009). Thus, Lefebvre et al. (2008) showed that 4 out of the 16 studied monoclonal antibodies did not react or had reduced affinity with PCV2b strains compared with PCV2a strains by using IPMA or neutralization assays. These authors speculated that some of the mutations described in study I might be the responsible of antigenic differences. In addition, despite these apparent antigenic differences, recent data have shown that commercial PCV2 vaccines (all of them based on PCV2a) apparently protect against both PCV2a and PCV2b (Fort et al., 2008). Moreover, the immunologic response of pigs after infection with a specific genotype apparently confers protection against infection with the other one (Opriessnig et al., 2008b).

In spite of the controversial data, the above-mentioned shift appears, to date, as one of the few reasonable explanations for the epidemic outbreak of PMWS around the world during 1990s and 2000s. Weather or not PCV2b and PCV2a differ in pathogenicity still remains to be experimentally proved. Thus, meanwhile some experimental infections support the potential higher pathogenicity of PCV2b isolates (Lager et al., 2007; Fort et al., 2008), others

stated the opposite (Opriessnig et al., 2008b). In addition, a recent meta-analysis on experimental PCV2 infections also supported that the use of PCV2b isolates is apparently more successful to reproduce PMWS (Tomás et al., 2008). However, it has to be kept in mind that the disease has been reproduced using either PCV2b or PCV2a, pointing out that the potential difference in virulence between both genotypes might be only one more among the numerous factors that are involved in the development of this multifactorial disease. Further successful experimental infections using several PCV2 isolates representative of both genotypes should help in clarify the role of genotypes in disease development and severity. On the other hand, more extensive retrospective studies assessing PCV2 sequences before, during and after PMWS outbreaks should be performed in the different deemed countries to clarify the importance of the PCV2 genotype shift on PMWS clinical presentation.

In the study I it was demonstrated that multiple PCV2 sequences and even genotypes could be present in the same animal at the same time. Concomitantly to the present study, others have designed PCR techniques to differentiate both genotypes (Horlen et al., 2007; Gagnon et al., 2008; Hesse et al., 2008). The application of these PCR techniques confirmed that coinfection with both genotypes is a relatively frequent event in the field (Horlen et al., 2007; Gagnon et al., 2008; Hesse et al., 2008). The presence of the two viruses within a single animal raised the possibility that the two PCV2 genotypes may undergo recombination. In fact, evidences of natural recombination among PCV2 have been already previously reported (Ma et al., 2007; Olvera et al., 2007). Recently, two independent studies have shown that such recombination between genotypes can definitely occur, describing several naturally occurring chimerical genomes (Hesse et al., 2008; Cheung, 2009). Moreover, Cheung (2009) was able to recover infectious viruses after transfection of tissue culture cells with described chimerical genomes. Nevertheless, the biological significance of PCV2 recombination remains unknown. Although a recent experimental infection suggests that dual heterologous PCV2a/PCV2b infections enhance PCV2

replication and PMWS expression under experimental conditions (Harding et al., 2008a), more evidences are needed to support this hypothesis. It is worthy mentioning that, in study I of this thesis, it was not possible to identify potential recombination such as the one described by Hesse et al. (2008) because the analysis was based on ORF2 PCV2 sequences. This situation shows that, despite ORF2 is a good phylogenetic marker for PCV2 (Olvera et al., 2007), the incorporation of whole virus genomes is necessary for the full understanding of PCV2 diversity and evolution (Hesse et al., 2008).

The rest of this thesis work was focused on characterizing the longitudinal PCV2 infection, excretion and seroconversion dynamics as well as on describing the longitudinal evolution of APPs serum concentrations in pigs from birth until the PMWS outbreak occurrence. Most of previous epidemiological studies were mainly focused on comparing PMWS affected and non-PMWS affected farms (Larochelle et al., 2003; Rose et al., 2003; de Boisseson et al., 2004; Sibila et al., 2004; López-Soria et al., 2005; Vigre et al., 2005). In contraposition, studies III and IV of the present thesis were focused at individual level. Thus, comparisons were established between pigs with different health status, all coming from PMWS affected farms.

Most of the farms included in study I were also used for studies III and IV; also, samples used in study II came from some of those farms (annex). Longitudinal studies within PMWS affected farms were performed in Denmark and Spain. It would have been ideal to process and analyse all samples from both countries using the same laboratorial equipments and techniques and by the same personnel. However, different research teams were in charge of collecting and analyzing the samples in each country. Therefore, before studying PCV2 infection dynamics, the performance of the two PCV2 qPCR and the two serological methods used was compared. Taking into account that no PCV2 qPCR comparison was published in the literature, results obtained from the comparison between Spanish and Danish techniques were presented in the study

II. This study showed a good association between Spanish and Danish PCV2 qPCR methods, allowing the comparison of results obtained by both techniques. However, this comparison had to be made with caution, since the Danish technique had higher sensitivity and yielded systematically higher results than the Spanish one. Although several potential explanations were evaluated, it was not possible to elucidate the specific reason for such a systematic difference. Among them, the existence of potential mismatches between primers and probes used and PCV2 sequences present in studied samples were indirectly evaluated through comparison with previously described marker positions (Olvera et al., 2007). However, it has to be taken into account that the mentioned marker positions corresponded to the nucleotide changes characteristic of the PCV2 clusters and groups described in this previous work (Olvera et al., 2007), but did not show all existent nucleotide differences between PCV2 sequences. Table 1 shows the number of mismatches observed when comparing the Spanish and Danish sets of primers and probes with the prototypes of the three PCV2 genotypes proposed in the letter presented as *addendum* of study I:

Table 1. Number of mismatches observed when aligning the Spanish and Danish sets of primers and probes with the genotype prototypes of PCV2a, PCV2b and PCV2c.

Technique	Primers and probes	PCV2a (AF055392)	PCV2b (AF055394)	PCV2c (EU148503)
Spanish	Forward	0	1	5
	Reverse	0	2	0
	Probe	0	0	3
Danish	Forward	0	0	0
	Reverse	0	0	0
	Probe	0	0	0

Additionally, ORF2 PCV2 sequences from 8 out of the 17 serum Spanish samples used in study II were also investigated in study I. All the 8 sequences corresponded to PCV2b, and their comparison with the set of Spanish primers and probe showed the same mismatches than the ones described in table 1. Obviously, it was not possible to compare these sequences with the set of Danish

primers and probe because they targeted ORF1 gene. Moreover, PCV2 sequences from Danish samples used in the comparison were not available. Nevertheless, it is noteworthy that, as mentioned above, a previous Danish study on PCV2 sequences showed that PCV2b predominated in the field in Denmark during 2003-2004 (Dupont et al., 2008). This fact suggests that the mismatches described in table 1 might also occur between Spanish primers and the PCV2 sequences present in Danish samples. It has been previously demonstrated in another PCV2 qPCR test that a total of five or six mismatches in primers and probe resulted in an underestimation of copy number between one and two $\log_{10}$ units, respectively (Brunborg et al., 2004). Therefore, the higher heterogeneity between PCV2b sequences and the set of Spanish primers and probe might theoretically contribute in diminishing the qPCR efficiency and might explain, at least partially, the bias observed between results obtained by both techniques. Therefore, to confirm the presence of mismatches in the Spanish technique and its absence in the Danish one, the whole PCV2 sequences of the strains being detected in the Danish and Spanish pigs should have been assessed. Moreover, the use of primers and probe with various modifications should allow quantifying the potential loose of efficacy due to the presence of mismatches (Brunborg et al., 2004).

Literature shows that differences in sensitivity or biases between outputs are frequent findings when comparing qPCR techniques (Caliendo et al., 2001; Wang et al., 2005; Braun et al., 2007; Perandin et al., 2007). Thus, a recent comparison of two different *Epstein-Barr virus* (EBV) qPCR also reported a systematic bias between the outputs of both techniques (Perandin et al., 2007). Similarly, reasons for this bias could not be elucidated, and authors speculated that it might be due to differences in PCR efficiency (Perandin et al., 2007). One strategy that should help in reducing variability between different qPCR is the use of common calibration materials (Perandin et al., 2007; Hayden et al., 2008). This should also be taken into account for future collaborative multi-centre studies.

Study II also evaluated the usefulness of normalizing PCV2 DNA load in swabs samples to the total DNA present in each sample. This normalization was previously used in other works assuming that it allowed minimizing potential PCV2 load variation due to sampling procedure (Segalés et al., 2005b; Fort et al., 2008). However, results from study II suggested that such normalization was not suitable, since there was no association between the amounts of total DNA and PCV2 DNA present in swabs. Consequently, PCV2 qPCR results reported in the study III were expressed as copies of PCV2 per swab. Although this expression is susceptible to certain variability due to sampling procedure, it was assumed that this variability would be low when all swab samples were collected by the same persons, using the same types of swabs and following the same procedure. The significant correlations found between PCV2 amounts detected in lymphoid tissues, sera and swab samples presented in study III supported this assumption, and pointed out the use of swab samples as suitable indicators of PCV2 shedding. In fact, this expression have already been used in longitudinal studies of other pathogens such as *Ehrlichia canis* in dogs (Baneth et al., 2009) or *Chlamydia trachomatis* in humans (Burton et al., 2005). Nevertheless, it is noteworthy that the results reported by using total DNA normalization should also be considered with confidence, since study II also showed that this normalization did not substantially change the relative results obtained between swabs, at least when all of them were collected in the same way (Segalés et al., 2005b; Fort et al., 2008).

In the study I, histopathological lesions of lymphoid tissues were semi-quantitatively assessed (Rosell et al., 1999). This assessment categorises the characteristic lesions as slight, moderate and severe, and the amount of PCV2 as low, moderate or severe. On the other hand, initially, a different pathologist did a similar evaluation of histopathological lesions and amount of PCV2 from samples collected in Denmark. It is known that a certain degree of subjectivity might always be present in the diagnosis when samples are not assessed contemporaneously or when are assessed by different pathologists (Walker, 2006). Moreover, it has been indicated that scoring systems are useful to

overcome variations due to the histopathological assessment (Walker, 2006). Taking these considerations in mind, a scoring system based on previously published systems (Krakowka et al., 2005; Opriessnig et al., 2006d) was developed and it was used to re-evaluate the lymphoid tissues collected from all pigs coming from both countries. This re-evaluation was performed blindly and by the same person, which guaranteed the application of the same criteria in the classification of the animals from both countries. It is worthy mentioning that, as a consequence of the implementation of this system, some animals that were diagnosed as PMWS cases in study I were re-classified as wasted non-PMWS. Those variations were mainly due to the slightly more demanding criteria used when cases were re-evaluated. In addition, the fact that ISH and IHC were used in Spain and Denmark, respectively, should not significantly influence the results, since both techniques give similar results when used in studies of PCV2 distribution and semi-quantitation (McNeilly et al., 1999; Rosell et al., 1999).
Another relevant difference between the Spanish and the Danish studies was the moment of sample collection. Both studies were designed to have at least 5 longitudinal samples per pig, but they were collected at different intervals. The main explanation for this difference was the age of PMWS presentation in each country. The surveys made before the starting of the study already indicated that the disease presentation was mainly at nursery in the Danish farms, while it was at the beginning of fattening in the Spanish ones. Consequently, sampling points were adapted to the expected age of disease presentation.

Finally, it was not possible to homogenise other differences between the Spanish and the Danish studies such as the ones due to husbandry and management practices, some of which are detailed in study III. Overall, despite the efforts of homogenization, all the mentioned differences between both studies induced to present the data separately and to compare obtained results carefully.

PCV2 infection dynamics was similar in both countries and agreed with previous descriptions based on serology and/or non-quantitative PCR techniques

(Rodríguez-Arrioja et al., 2002; Larochelle et al., 2003; Sibila et al., 2004). PCV2 viral loads and prevalence increased progressively with the maternal antibody waning and, in PMWS affected pigs, reached maximum values at the moment of PMWS presentation. Interestingly, the evolution of PCV2 viremia was significantly correlated with the evolution of HPT and Pig-MAP serum concentrations in PMWS affected pigs. However, such a significant correlation was much weaker or did not exist in wasted non-PMWS and healthy pigs. All together, these findings support the idea that PCV2 is the main responsible of the systemic inflammatory status suffered by PMWS affected pigs (Rosell et al., 1999). Moreover, the fact that there was no apparent difference between healthy PCV2-infected and healthy non-PCV2-infected pigs indicated that the sole infection of PCV2 is not sufficient to develop such a systemic inflammatory status. From a practical point of view, data from studies III and IV should help in establishing the optimum application timing of recently available commercial piglet PCV2 vaccines. On one hand, piglet PCV2 vaccination should be performed some weeks prior to the PCV2 spread and PMWS presentation. On the other hand, the levels of maternal immunity should be as lower as possible at the time of vaccine application, in order to avoid potential interference between the PCV2 vaccine and the antibodies derived from maternal immunity (Fort et al., 2008; Opriessnig et al., 2008a; Pérez-Martín, 2009). Thus, theoretically, an apparently ideal moment to vaccinate piglets in the studied Danish and Spanish farms would be between 4-6 and 3-7 weeks of age, respectively.

The high PCV2 qPCR prevalence observed at nasal cavities supports the idea that the oro-nasal route is the most important route of PCV2 infection and transmission (Segalés et al., 2005a). Whether the detection of PCV2 DNA in nasal cavity corresponds to PCV2 replication or to PCV2 inhaled or excreted through respiratory airways is still unknown. However, all three possibilities may apply, since it is known that PCV2 replicates in respiratory epithelial cells (Pérez-Martín et al., 2007) and that high amount of PCV2 can be found in lungs

of pigs affected by PMWS and PNP, pathological condition considered a PCVD (Rosell et al., 1999; Grau-Roma and Segalés, 2007).

The low percentage of PCV2 positive pigs at first weeks of age together with the percentage of PCV2 infected sows one week post-farrowing suggest that, apparently, piglets would become infected from their sows either through horizontal (Albina et al., 2001; Andraud et al., 2008) and/or vertical routes (Park et al., 2005; Ha et al., 2008). Moreover, after maternal antibody waning, PCV2 might easily be horizontally transmitted from infected piglets to their pen-mates through viral shedding in faeces and/or nasal secretions. Accordingly, the most significant increase of PCV2 prevalence was observed at the late phase in nurseries or at the beginning of fattening.

PMWS-affected pigs harboured higher PCV2 loads and APPs concentrations in sera, and shed higher viral loads through faeces and nasal secretions than healthy pigs. These findings are in agreement with previous reports assessing PCV2 viral load in sera (Brunborg et al., 2004; Olvera et al., 2004), PCV2 loads in swabs secretions (Segalés et al., 2005b), and HPT and Pig-MAP in serum concentrations (Segalés et al., 2004b; Parra et al., 2006) of PMWS affected and non-affected pigs. These observations suggested the existence of a possible mechanism that might allow the virus to replicate to a higher extent in a limited number of pigs (Meerts et al., 2006), and thus triggering a more severe acute phase response. This mechanism might be related with the pronounced immunosuppressive status of PMWS affected pigs, which affects both cellular and humoral immune responses (Nielsen et al., 2003; Darwich et al., 2004; Sanchez et al., 2004; Segalés et al., 2004a; Meerts et al., 2006). However, until today, it is not clear whether these changes are important in the induction of PMWS or are merely the consequence of it. In the study III of this thesis, evidences of impaired humoral immune response were seen in PMWS affected pigs from Spain. The fact that those pigs that developed PMWS had lower antibody titres than the healthy ones at 11 weeks of age, prior to the observation

of clinical signs, suggested that these changes are important in the induction of PMWS more that being merely a consequence of the disease. These results were further confirmed by a later study assessing the antibody dynamics against both Cap and Rep proteins in the same set of Spanish pigs (Pérez-Martín et al., 2008). Conversely, such an impaired humoral response was not observed in Denmark, indicating that total antibodies are not the only factor involved in avoiding PMWS development (Meerts et al., 2006; Fort et al., 2007).

It would have been interesting to assess the presence of NA in all studied pigs to further characterize the humoral response. It would be expectable that the group of PMWS affected pigs lacked or had lower NA titres after seroconversion in both countries, since it has been demonstrated that the absence or low levels of PCV2 NA is a recurring phenomenon in pigs with high amounts of PCV2 (Meerts et al., 2006; Fort et al., 2007). It is possible that a limited number of pigs are unable to recognize or produce antibodies against certain epitopes involved in the neutralization of the virus. In fact, this situation has been already described in other diseases such as Aujeszky's disease in swine (Jacobs and Kimman, 1994), Marek's disease in chickens (Wakenell et al., 1996) or influenza in rabbits (Lambkin and Dimmock, 1995). To our knowledge, there is only one report giving information on longitudinal evolution of PCV2 NA in field cases of PMWS (Meerts et al., 2006) and there is still no published longitudinal determination of PCV2 NA from birth to the moment of disease development. This information should help in understanding the role of NA in the pathogenesis of the disease as well as to characterize humoral maternal immunity that suckling piglets receive through colostrum intake.

Low levels of sow antibodies against PCV2, sow PCV2 viremia and early PCV2 infection in pigs have been suggested to increase the risk of PMWS development (Rose et al., 2003; López-Soria et al., 2005; Calsamiglia et al., 2007). The qPCR and serology results presented in study III of this thesis gave further insights in the potential importance of some of these factors. On one hand, the fact that pigs

that developed PMWS had lower maternal antibody titres than animals that did not suffered from the disease supports the idea that antibody titres confer certain protection against PMWS development (Rodríguez-Arrioja et al., 2002; McKeown et al., 2005). Indirectly, this result agrees with the importance of high levels of sow antibodies against PCV2 to prevent PMWS development (Calsamiglia et al., 2007). On the other hand, the relation between viremic sows and development of the disease could not be assessed in Denmark because it was not possible to avoid cross-fostering practices, and in Spain because of the low number of viremic sows. Finally, no apparent differences in the timing of PCV2 infection in pigs were observed. Thus, at early ages, the percentage of positive PCV2 was similar in all the three groups of pigs. Before PMWS outbreak, differences in PCV2 prevalence among groups were seen only at 11 weeks of age in Spain, being higher in PMWS affected pigs than in the other two groups. However, when PMWS affected farms are compared with non-affected ones, the earlier the PCV2 infection, the higher the risk of developing PMWS (Rose et al., 2003; López-Soria et al., 2005). Then, based on results of the present longitudinal study, it seems that a potential difference in the timing of PCV2 infection does not occur at individual level among pigs of different pathological conditions coming from PMWS affected farms.

Overall, serological and qPCR results obtained in studyies of this thesis suggested that measures directed to increase PCV2 maternal immunity and piglet's humoral response and to diminish PCV2 load at weaning and/or fattening phases should help in ameliorating the impact of PMWS. These measures would include strategies described at Madec's 20-point plan (Madec et al., 2000) and PCV2 vaccination. In fact, data from this work indirectly contribute to understand the recently reported successful results obtained by PCV2 sow and piglet vaccines. On one hand, sow and gilt vaccination causes an increase of PCV2 antibody titres in both serum and colostrum (Joisel et al., 2007a; Plourde and Machell, 2007). On the other hand, piglet vaccination reported reduction of

PCV2 viremia together with reduction of clinical signs and microscopic lesions (Fort et al., 2008; Opriessnig et al., 2008a).

The evaluation of the potential diagnostic ability of PCV2 qPCR and serological techniques in live animals showed that they are apparently not specific and/or sensitive enough to substitute histopathology plus detection of PCV2 within lymphoid tissues for individual PMWS diagnosis. However, taking into account the described evolution of characteristic lesions and PCV2 amounts in lymphoid tissues during the infection (Opriessnig et al., 2007), it might be questioned whether current PMWS diagnosis is considering all pigs affected by the disease. In that sense, the group of pigs suffering from wasting without fulfilling PMWS individual case definition showed higher PCV2 loads and a higher APR than healthy ones during the PMWS outbreak. Therefore, it is feasible that clinical signs displayed by this group of pigs might be due, at least in a proportion of them, consequence of PCV2 infection. Nevertheless, histopathology plus detection of PCV2 within lymphoid tissues is the accepted international criteria and, to date, the apparently most accurate approach to diagnose the disease (Sorden, 2000; Segalés et al., 2005a). However, study III showed that PCV2 qPCR could provide certain valuable diagnostic and even prognostic information, at least on a population basis. It is noteworthy that, after the publication of study III, the diagnostic performance of another PCV2 qPCR assay on different tissues was reported using 30 pigs of different clinical status (Harding et al., 2008b). Surprisingly, the use of qPCR on gluteal muscle samples yielded the highest diagnostic performance capacity, showing 100% sensitivity and specificity at the optimum PCV2 qPCR cut-off concentration (Harding et al., 2008b). Moreover, in this work, the PCV2 qPCR threshold proposed for PMWS diagnosis on serum samples was 4.7 $\log_{10}$ copies PCV2 per ml serum, which is much lower than the ones obtained in study III and also than the ones suggested previously (Brunborg et al., 2004; Olvera et al., 2004; Fort et al., 2007). This difference might be partially due to the use of a different PCV2 qPCR technique, to the low number of studied pigs and, mainly, to the criteria used to classify the animals. Thus,

these authors classified the pigs only based on clinical signs, distinguishing only wasted and healthy pigs (Harding et al., 2008b).

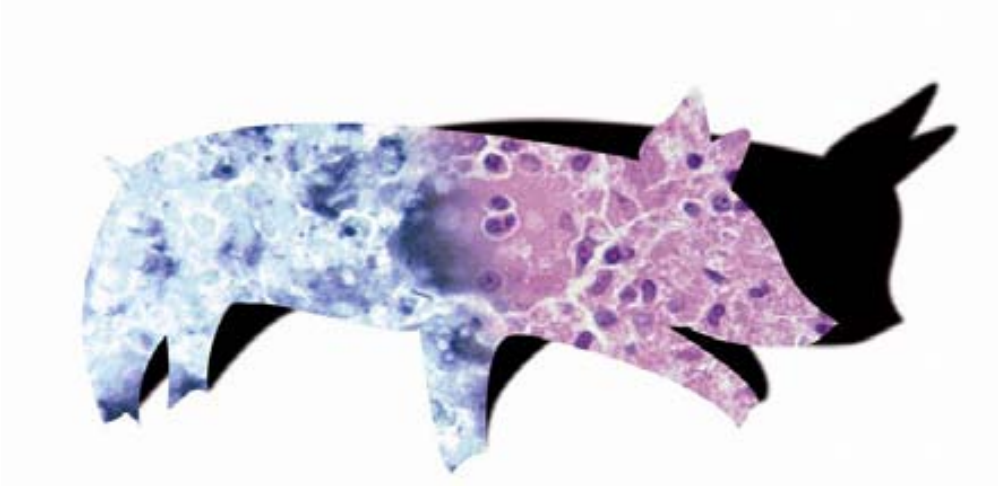
On the other hand, although APPs are unspecific markers of inflammation (Petersen et al., 2004; Gruys et al., 2005a), results obtained from study IV supported the idea that APPs concentrations might be useful markers of health (Gruys et al., 2005b; Parra et al., 2006). Thus, the determination of APPs sera concentrations appears as an interesting tool to monitor PMWS development in epidemiological studies or in the assessment of the efficacy of PCV2 vaccines in the field. Nevertheless, it has to be kept in mind that APPs concentrations might increase because of a wide range of infections (Asai et al., 1999; Jungersen et al., 1999; Petersen et al., 2004; Parra et al., 2006; Sorensen et al., 2006).

The present thesis was developed within the framework of the EU project entitled *Control of Porcine Circovirus Diseases (PCVD): Towards Improved Food Quality and Safety* (www.pcvd.org), which was funded by the EU Sixth Framework Programme. One of the objectives of this project was the identification of pathogens different from PCV2 as common triggers of PMWS development. However, this objective could not be totally reached by the works included in this thesis. Nevertheless, study IV showed that there were no differences in the proportion of infected and/or seroconverted animals against studied pathogens, apart from PCV2, between PMWS, wasted non-PMWS and healthy pigs. Moreover, the observed low prevalence in Spanish pigs for PPV, PRRSV and *M. hyopneumoniae*, the three pathogens that have been more repeatedly related to PMWS triggering (Allan et al., 1999a; Rovira et al., 2002; Opriessnig et al., 2004b), suggests that none of them are essential for PMWS development. A Cox proportional hazards model is currently being carried out to elucidate whether the time of infection of studied pathogens and the maternal immunity against them may influence the risk of developing PMWS.

Finally, it would have also been interesting to assess the influence of sex in disease development (Corrégé, 2001; Rodríguez-Arrioja et al., 2002). However,

on one hand, sex was not recorded in the Danish pigs. On the other hand, all the Spanish batches that suffer from PMWS outbreak performed castration in males, being impossible to appreciate differences between non-castrated males and females. Nevertheless, it is noteworthy that, in Spain, 198 females and 418 castrated males were monitored in the 6 batches presented in studies III and IV. Among them, only 5 out the 35 PMWS cases diagnosed in Spain were females (2.5% of studied females) and 30 out of 35 were castrated males (7.2% of studied males) ($p<0.05$). These data further support the idea that castrated males are more susceptible to suffer from PMWS but do not clarify whether it is due to sex susceptibility or to the effect of castration (Corrégé, 2001; Rodríguez-Arrioja et al., 2002).

V. CONCLUSIONS



Science is the great antidote to the poison of enthusiasm and superstition
Adam Smith

1. A methodology to define PCV2 genotype has been proposed. This methodology is based on the p-distance/frequency distribution of PCV2 sequences together with PCV2 phylogenetic analyses. Using this methodology, up to 3 PCV2 genotypes have been identified around the world, being named as PCV2a, PCV2b and PCV2c.

2. Two different PCV2 genotypes (PCV2a and PCV2b) were detected in studied farms during years 2005 and 2006. PCV2b was found to be more prevalent within PMWS affected farms; in contrast, only PCV2a was recovered from non-PMWS affected farms. Consequently, PCV2b is suggested to be potentially more pathogenic than PCV2a.

3. Coinfection with PCV2a and PCV2b in the same farm and even in the same pig was demonstrated to occur in PMWS affected farms.

4. PCV2 qPCR and serological techniques used in Danish and Spanish laboratories showed good associations, thus giving comparable results. However, the Danish laboratory gave systematically higher PCV2 load than the Spanish one.

5. No association between the amounts of total and PCV2 DNA present in nasal and rectal swabs was detected, indicating that this normalization of PCV2 DNA over total DNA is not suitable to express the amount of PCV2 present in swab samples.

6. PMWS affected pigs, as a collective, harbour higher PCV2 loads, had higher Pig-MAP and HPT concentrations in sera, shed higher viral loads either through nasal secretions and faeces and had lower level of maternal antibodies against PCV2 than non-PMWS affected pigs.

7. The positive and significant correlation found between amounts of PCV2 detected in lymphoid tissues, sera and swabs suggests that nasal and rectal swabs are useful indicators of PCV2 excretion

8. Similar PCV2 infection dynamic patterns were observed in Spain and Denmark, with a delay in PMWS age-presentation in Spain compared to the one in Denmark.

9. The observation of an impaired humoral immune response in PMWS affected pigs from Spain prior to the appearence of clinical signs suggests that it is an important factor for the occurrence of PMWS.

10. No apparent differences in PCV2 infection timing occurred at individual level between pigs of different pathological conditions within PMWS affected farms.

11. The lack of sensitivity and/or specificity values observed from PCV2 qPCR and serology tests either used separately or combined suggested that these techniques are not able to substitute histopathology plus detection of PCV2 in tissues for the individual PMWS diagnosis.

12. The APR in PMWS affected pigs occurred concomitantly to PCV2 viremia, suggesting that PCV2 is the main responsible of the systemic inflammatory status suffered by diseased pigs.

13. Detectable APR occurred some weeks before the starting of clinical signs. Therefore, APPs may provide valuable prognostic information for PMWS development.

VI. ANNEX

Facts are stubborn things, but statistics are more pliable
Mark Twain

Several batches and farms were included in the longitudinal case-control studies performed in Denmark and Spain. Although PMWS was previously diagnosed in all of them, PMWS cases were not diagnosed in all monitored batches. Among studied batches, only those in which at least one out of the longitudinally monitored pigs fulfilled the PMWS diagnosis were included in studies III and IV. Moreover, the samples used in study II came also from some of the studied farms. Table 1 details the total number of studied batches and farms and their equivalences with references given in studies I, III and IV. Although farm or batch references were not given in study II, table also shows the origin of samples used in that study.

Table 1. Total number of batches and farms included in the longitudinal case-control studies.

Country	Studied batches	Sow farm	PMWS confirmation	Study I	Study II	Study III	Study IV*
Spain	1	Sp1	No	-		-	-
	2	Sp2	Yes/No**	Farm 1	X	-	-
	3	Sp3	No	-		-	-
	4	Sp3	Yes	Farm 2	X	Sp-1a	Sp-1a
	5	Sp4	No	Farm 6	X	-	-
	6	Sp5	Yes	Farm 3	X	Sp-2a	Sp-2a
	7	Sp6	No	-		-	-
	8	Sp5	Yes	Farm 4	X	Sp-2b	Sp-2b
	9	Sp5	Yes	Farm 5	X	Sp-2c	Sp-2c
	10	Sp7	No	-		Sp-3a	Sp-3a
	11	Sp7	No	-		Sp-4c	Sp-4c
Denmark	1	Dk1	Yes	-		Dk-1a	Dk-1a
	2	Dk2	No	-		-	-
	3	Dk3	Yes	-		Dk-2a	Dk-2a
	4	Dk4	Yes	-		Dk-3a	-
	5	Dk5	Yes	-	X	Dk-4a	Dk-4a
	6	Dk6	No	-		-	-
	7	Dk7	Yes	-		Dk-5a	Dk-5a
	8	Dk8	Yes	-		Dk-6a	Dk-6a
	9	Dk9	Yes	-		Dk-7a	Dk-7a
	10	Dk10	Yes	-		Dk-8a	Dk-8a
	11	Dk11	No	-		-	-
	12	Dk12	No	-		-	-

* When APPs concentrations were assessed, not all pigs from studied Danish farms had available sera. Only those pigs with available sera at the moment of necropsy were included in study IV. Consequently, one out of the Danish farms was not included in study IV.

**The only 2 animals from this farm that were classified as PMWS in study I had global average scores of 3.9 and 4, respectively. Consequently, both were classified as wasted non-PMWS in studies III and IV and this farm was therefore not included in these studies.

VII. REFERENCES

Abans tenia alguns dubtes. Ara no sé.
Anònim, missatge pintat al carrer

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